Endonuclease VII has two DNA-binding sites each composed from one N- and one C-terminus provided by different subunits of the protein dimer

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Endonuclease VII (endo VII) is a Holliday structure-resolving enzyme of bacteriophage T4. Its activity depends on dimerization, DNA binding and hydrolysis of two phosphodiester bonds flanking the Holliday junction. We analysed the DNA-binding activity of truncated monomeric and covalently linked dimeric endo VII proteins. We show that both ends of endo VII are involved in DNA binding. In particular, the C-terminus of one subunit interacts with the N-terminus of the other subunit, constituting one DNA-binding site; the other two termini form the second binding site of the dimer. One binding site is sufficient to bind cruciform DNA. The concerted mechanism involving termini from different subunits ensures that only dimers bind to Holliday structures, thus providing two catalytic centres which introduce two cleavages in opposite strands. This is a precondition for precise resolution of Holliday structures.

Keywords: cruciform DNA/DNA–protein interaction/
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Introduction

Holliday structures are central intermediates in genetic recombination which physically link DNA molecules (Holliday, 1964). These links require precise resolution before chromosomes are partitioned into separate compartments such as daughter cells or newly synthesized virus shells.

Endonuclease VII (endo VII) was the first enzyme shown to react specifically with Holliday structures *in vitro*, resolving them into genetically sensible products (Mizuuchi *et al.*, 1982). The enzyme is the product of gene 49 of phage T4 and is expressed early and late during the infectious cycle (Barth *et al.*, 1988). The enzyme is essential for packaging the newly synthesized DNA into newly synthesized head structures by removing any branch that was left from earlier replication and recombination events. By this means, endo VII exerts control on the maturation process of progeny phage (reviewed in Kemper, 1997).

Endo VII belongs to the growing group of DNA junction-resolving enzymes (operationally named X-solvases) apparently ubiquitous in prokaryotes and eukaryotes. The best studied X-solvases to date are, besides endo VII, endonuclease I from phage T7, RuvC from *Escherichia coli* and cruciform-cutting endonuclease 1 (CCE1) from

Saccharomyces cerevisiae. The enzymes resolve Holliday structures by introducing staggered nicks symmetrically located in opposite strands across the junction, releasing two unbranched, duplex DNA molecules with ligatable nicks. Despite their common abilities to resolve Holliday structures, their primary amino acid sequences lack significant homologies (reviewed in White *et al.*, 1997).

In contrast to the high specificity for four-way DNA junctions as shown for RuvC and CCE1 (Dunderdale *et al.*, 1994; Kupfer and Kemper, 1996; White and Lilley, 1996), endo VII shows a broad profile of substrate specificities. The enzyme recognizes and cleaves many substrates, including three-way junctions, single-strand overhangs, heteroduplex loops, base mismatches, bulky adducts and curved DNA, suggesting the importance of endo VII for repair events in general (Kemper, 1997).

Endo VII consists of 157 amino acids with a molecular mass of ~18 kDa and a strong tendency to form dimers in solution (Kosak and Kemper, 1990). It requires Mg²⁺ or Mn²⁺ ions for its enzymatic activity (Kemper and Garabett, 1981; Pottmeyer and Kemper, 1992). The Holliday structure-resolving activity of endo VII is dissectable into three subactivities, which are in the order of execution: (i) dimerization of the protein; (ii) binding of the dimer to DNA; and (iii) hydrolysis of phosphodiester bonds. This sequence of events was proven by demonstrating that endo VII binds as a dimer to cruciform DNA which was used as a Holliday structure analogue (Pöhler *et al.*, 1996). Furthermore, when a mutation in endo VII (W87R) was selected which prevented formation of dimers, the monomeric protein was unable to bind cruciform DNA, indicating that dimer formation is a prerequisite for DNA binding. The dimerization domain of endo VII is contained in a section comprising amino acids 55–105, as shown by measuring the dimerization abilities of N- and C-terminally truncated peptides *in vitro* (Birkenbihl and Kemper, 1998). Two of the five α -helices predicted by computer analyses to exist in endo VII are located in this region, and helices H2 and H3 were suggested to form the interface between the subunits by hydrophobic interaction.

A zinc-binding domain comprising amino acids 23–61 was suggested to be involved in DNA binding (Tomaschewski and Rueger, 1987). Consequently, when the protein was either depleted of zinc or when one or two of the four zinc-coordinating cysteine residues were mutated, DNA binding was abolished (Giraud-Panis *et al.*, 1995). However, the zinc-binding domain is not sufficient because proteins containing an intact zinc-binding domain but a C-terminus truncated by >3 amino acids lost their DNA-binding abilities completely (Golz *et al.*, 1997).

Here we report further results describing the mode of DNA binding of endo VII to cruciform DNA. We present evidence that the N-terminus of endo VII is also required

for DNA binding and that two separable DNA-binding sites are formed by cross interactions between the four termini present in one endo VII dimer. The two binding sites are formed by interaction of one N- and one C-terminus each, which are derived from different subunits of the dimer. These results fit a model of two binding sites positioning two active centres to two arms of the 2-fold symmetrical Holliday structure.

Results

Proteins used in this study

For generation of the monomeric endo VII proteins (M-proteins), PCR products of T4 wild-type gene 49 were digested with the restriction endonuclease *Bam*HI and ligated into the *Bam*HI site of the expression vector pET11a-His. Plasmids carrying the wild-type gene were unstable due to the toxicity of its gene product, and clones with inactivating spontaneous mutations in the gene appeared frequently. Two of these clones carried missense mutations in codon 43 (EVII-H43R) and codon 87 (EVII-W87R), respectively. In contrast to reports claiming that His43 is essential for activity (Giraud-Panis and Lilley, 1996), EVII-H43R had ~10-fold reduced activity but the same specificity as the wild-type enzyme (WT; Figure 4A, lanes 1–7). Protein EVII-W87R was deficient in dimerization, and binding and cleavage of DNA (Birkenbihl and Kemper, 1998). EVII-H43R, EVII-W87R and WT were used as controls throughout the experiments described below.

Covalently linked endo VII dimers were made by expressing two endo VII sequences cloned in tandem. The constructs were created in a two-step procedure using mutant gene 49 encoding EVII-H43R as template for PCR (see Materials and methods). The full-length covalent dimer protein (CD-protein) contained, in order, an N-terminal His tag for easy purification, a thrombin cleavage site for removal of the His tag, the first copy of EVII-H43R, a linker of 11 amino acids (TSGTGGGGGIL) and the second copy of EVII-H43R. Deletions at the N-terminus of the N-terminal subunit and at the C-terminus of the C-terminal subunit were introduced using appropriate primers for PCR. The proteins used in this study are listed in Figure 1.

All proteins were expressed with good efficiency. The proteins with His tags were purified by affinity chromatography using Ni-NTA spin columns. These were all of the covalently linked dimers, EVII-H43R, EVII-W87R and EVIIpep(1–120). For some experiments, the His tags were removed by protease digestion. This was possible without leaving any additional amino acids at the N-terminus of the respective protein. The untagged proteins were purified as described before (Golz *et al.*, 1995). These were WT and the truncated proteins EVIIpep(11–157), EVIIpep(21–157) and EVIIpep(1–147).

Both termini of endo VII are required for binding cruciform DNA

Earlier studies had revealed that the C-terminus of endo VII is essential for cruciform DNA binding (Golz *et al.*, 1997). To investigate the role of N-terminal amino acids in DNA binding, truncated endo VII peptides were made with 10 [EVIIpep(11–157)] or 20 amino acids [EVII-

pep(21–157)] deleted from their N-termini (Figure 1). These proteins were subjected to DNA-binding assays using cruciform DNA CF01 as substrate. Binding was monitored by electrophoretic mobility shift assays (EMSA), in which the proteins were incubated with the substrate in the absence of Mg²⁺ ions to prevent cutting of the substrate. All truncated M-proteins turned out to be deficient in DNA binding (Figure 3A). While peptide EVIIpep(11–157) still retained residual binding activity, peptide EVII(21–157) completely lacked DNA-binding activity. C-terminal truncated peptides with deletions of 10 [EVIIpep(1–147)] and 37 amino acids [EVIIpep(1–120)], respectively, also did not bind the DNA. While controls WT and EVII-H43R bound well, the dimerization-deficient mutant EVII-W87R exhibited only a very low binding activity due to residual dimerization.

To exclude the possibility that the observed deficiency in DNA binding of the truncated M-proteins was in fact a consequence of a primary deficiency in dimerization, the proteins were tested for their ability to form homodimers in solution by glutaraldehyde (GA) cross-linking. As shown in Figure 2A, all proteins, except the dimerization-deficient EVII-W87R, formed dimers with high efficiencies. WT and EVII-H43R were completely dimeric, while EVII-W87R formed <5% dimers, in full agreement with the results reported earlier (Birkenbihl and Kemper, 1998).

The M-proteins were also subjected to DNA cleavage tests again using cruciform DNA CF01 as substrate. Figure 4A shows the outcome of the DNA cleavage assay. The proteins which bound the DNA also cleaved it. All truncated proteins, irrespective of which end was deleted, and the mutant protein EVII-W87R, which did not bind cruciform DNA, were also deficient in cleavage. These experiments demonstrate that both termini of endo VII are essential for binding of cruciform DNA.

N- and C-termini of different subunits are in close proximity in the dimer

Since endo VII dimers comprise, in total, two N-termini and two C-termini, the question arose whether the termini which are required for one DNA-binding site were provided from the same subunit or from different subunits of the dimer.

To address this question, the C-terminus of one subunit was linked covalently to the N-terminus of the second subunit of EVII-H43R by a linker of 11 amino acids. The resulting CD-protein EVII-H43R(1-CD-157) was tested for binding and cleavage of cruciform DNA as described above. The protein was found to be as active in binding (Figure 3B, lanes 2–4) and cleavage (Figure 4, lanes 22–24) as the 'parental' M-protein EVII-H43R. As shown by GA cross-linking, the CD-protein did not interact with other dimers (Figure 2B, lanes 2 and 3). This experiment demonstrated that the linker did not interfere with the enzymatic activity of the protein, indicating that the three-dimensional structure was not severely affected. Thus the results suggest that in a symmetric WT dimer, the N- and C-termini of different subunits are in close proximity to each other.

One C-terminus and one N-terminus, each originating from a different subunit, are sufficient for binding cruciform DNA

To determine whether the termini of different or the same subunits interact for DNA binding and whether one

Fig. 1. Endo VII derivatives used in this study. The proteins are represented by lines drawn below the complete sequence of gene 49. The calculated molecular weights of the proteins are indicated in kDa to the right. Proteins with an N-terminal his tag are labelled with an asterisk. The locations of the point mutations H43R and W87R are indicated. The terms at the left side of the figure denote the individual proteins. **(A)** Monomeric proteins. Locations of five predicted α -helices in the endo VII secondary structure, labelled H1–H5, are symbolized by boxes on a line below the complete sequence of endo VII. The names of truncated endo VII peptides (pep) contain the numbers of their respective terminal amino acids and in the following figures will be referred to with these numbers. **(B)** Dimeric proteins. Endo VII dimers covalently linked in a head-to-tail fashion are represented by bold lines drawn below a scale of two endo VII subunits calibrated in amino acids. Thin lines connecting the monomers indicate the linker sequence TSGTGGGGIL. Names of the covalently linked dimers (CD) contain the first amino acid of the N-terminal subunit and the last amino acid of the C-terminal subunit to indicate the sites of truncations, and will be referred to in the following figures with these numbers.

N- and one C-terminus were sufficient for DNA binding, we constructed CD-proteins with truncations at their free N- and C-termini. One construct had a 20 amino acid deletion at the N-terminus of the N-terminal subunit [EVII-H43R(21-CD-157)], a second construct had a 37 amino acid deletion at the C-terminus of the C-terminal subunit [EVII-H43R(1-CD-120)] and a third contained both deletions [EVII-H43R(21-CD-120)]. The constructs are listed in Figure 1B.

When analysed by EMSA using cruciform DNA CF01 as substrate, the three truncated CD-proteins were found to bind the DNA with nearly the same efficiencies (Figure 3B, lanes 6–13). It made no difference whether the C-, the N- or both termini were deleted. None of the constructs, however, reached the binding efficiency of the dimer made from full-length subunits.

To exclude the possibility that the CD-proteins aggregate intermolecularly before they bind to DNA, the proteins were analysed for multimer formation by GA cross-linking. As demonstrated in Figure 2B, lanes 4–9, the proteins did not form aggregates and migrated side by side with the non-cross-linked samples. This indicates that the dimerization domains were occupied intramolecularly

in the covalently linked dimers, excluding subunit exchange with other dimers.

The specificity of DNA binding by the CD-proteins was confirmed by analysing their DNA cleavage abilities using CF01 as substrate. All three proteins cleaved the DNA at the same four positions as WT protein and EVII-H43R (Figure 4B, lanes 22–33). Some alterations in the relative cleavage efficiencies at individual cleavage sites were observed with the different proteins. This may be caused by slight changes in the protein conformation induced by the truncations.

In the constructs described above, the ends of two subunits were brought together via covalent linkage of the C-terminus of one subunit to the N-terminus of the other. It should also be possible to combine two different truncated monomeric peptides via natural dimerization. It was shown that endo VII dimers exchange subunits with a high turnover rate (Pöhler *et al.*, 1996; Birkenbihl and Kemper, 1998). Since in an equimolar mixture of two different monomers the constituents combine freely, a relative distribution of 1:2:1 is anticipated for homodimers containing peptide 1 to heterodimers containing peptides 1 and 2 and to homodimers containing peptide 2. Thus,

Fig. 2. Cross-linking of endo VII proteins using GA. Cross-linked (+) and non-cross-linked (-) samples are shown side by side on a 15% (A) and a 12% (B) SDS-containing polyacrylamide gel. Results from parallel experiments were combined. The positions of monomers (Mon.) and dimers (Dim.) in the gels are indicated. Lanes marked with M contain marker proteins indicated by their molecular weights (kDa) to the left. Abbreviated protein names with numbering as indicated in Figure 1 were used. (A) Cross-linking of monomeric proteins. Full-length proteins WT and EVII-H43R (lanes 2–5) served as positive controls. Dimerization-deficient mutant protein EVII-W87R (lanes 6 and 7) served as negative control. Lanes 9–16 contain the truncated peptides. (B) Cross-linking of covalently linked dimeric proteins. Covalently linked dimers consisting of full-length subunits (lanes 2 and 3) or truncated subunits (lanes 4–9) were analysed.

50% of the protein should yield active dimers with one active DNA-binding site containing one pair of different termini. To verify this prediction, the C-terminally truncated M-peptide EVIIpep(1–147) was co-incubated with the N-terminally truncated M-peptide EVIIpep(21–157) and the mixture analysed for its ability to cleave cruciform DNA CF01. As demonstrated, the mixture indeed regained activity, indicating that heterodimers had formed and restored one DNA-binding site (Figure 4A, lanes 20 and 21). Alone, neither of the peptides was able to bind or cleave the DNA (Figure 4A, lanes 8–10 and 17–19).

Discussion

Here we report experiments analysing the DNA-binding activity of endo VII with respect to participating regions of the protein and their mode of interaction. The results show: (i) both ends of the endo VII polypeptide chain are involved in DNA binding; (ii) N- and C-termini from different subunits are in close proximity in the active protein; (iii) one N- and one C-terminus, each originating from a different subunit, form one DNA-binding site; and (iv) the endo VII dimer has two DNA-binding sites, either one of which is sufficient for specific binding to cruciform DNA.

It was found recently that the C-terminus of endo VII is essential for DNA binding, and removal of more than three amino acids from the C-terminus abolished the binding to cruciform DNA completely (Golz *et al.*, 1997). This was confirmed in this study by using peptides lacking 10 or 37 C-terminal amino acids which were also completely inactive in DNA binding. Here we describe that the N-terminus is also essential for DNA binding. In particular, we found that endo VII peptides lacking 20

Fig. 3. Binding of endo VII proteins to cruciform DNA CF01 by EMSA. Samples of 100 pM radioactively labelled cruciform DNA CF01 were incubated with increasing amounts (5, 25 and 125 nM, as calculated for dimers) of endo VII derivatives and separated on native 8% polyacrylamide gels. Bands were visualized by autoradiography of dried gels. Protein names are the same as in Figure 2. (A) EMSA of monomeric proteins. Lanes 1, 11 and 18 contain mock-treated substrate DNA (S). (B) EMSA of covalently linked dimeric proteins. Lane 1 contains mock-treated substrate DNA.

amino acids at the N-terminus were completely inactive, while deletions of 10 amino acids showed some marginal binding to DNA. The zinc-binding domain, comprising amino acids 23–61, which is also essential for DNA

Fig. 4. Cleavage of cruciform DNA CF01 by endo VII proteins. **(A)** Samples of 100 pM radioactively labelled cruciform DNA CF01 were incubated with increasing amounts (5, 25 and 125 nM, as calculated for dimers) of endo VII derivatives and the reaction products separated on a 12.5% denaturing polyacrylamide gel. Bands were visualized by autoradiography. Lanes 1–21 contain the reaction products of non-linked proteins. Lanes 20 (25 nM) and 21 (125 nM) contain the reaction products of equimolar mixtures of peptides EVIIpep(21–157) and EVIIpep(1–147). The reaction products of covalently linked dimers are shown in lanes 22–33. Lane 4 contains mock-treated substrate DNA (S). Names of proteins are as in Figure 2. **(B)** Cruciform DNA CF01. Shown is the sequence of CF01, which is assembled from four oligonucleotides by hybridization. Strand 4 was labelled at its 5' end with ^{32}P (*). The arrows point to the four cleavage sites introduced in strand 4 by wild-type endo VII.

binding (Giraud-Panis *et al.*, 1995), was still present in these peptides.

A loss of binding activity was always accompanied by a loss of cleavage activity of the peptides (Figure 4A). Since it was shown recently that dimerization of endo VII must precede DNA binding (Birkenbihl and Kemper, 1998), we ensured that the deficiency in DNA binding of these peptides was not simply a consequence of the loss of their dimerization proficiency. Using GA cross-linking, all truncated peptides were shown to dimerize efficiently (Figure 2A).

The N- and the C-terminus of endo VII are rich in positively charged amino acids, which may be involved in the binding of DNA. Secondary structure prediction by computer analysis (Rost and Sander, 1993, 1994) suggested five α -helices in endo VII (Figure 1A). Helices H2 and H3 are most likely involved in dimer formation (Birkenbihl and Kemper, 1998). Helical wheel analyses revealed that helix H1 at the N-terminus (amino acids 9–18) and helix H5 at the C-terminus (amino acids 139–155) both have a positively charged and a hydrophobic face, a feature more pronounced in H5. We propose that the DNA–protein interface is stabilized by the positively charged faces

of these helices interacting with the negatively charged backbone of the DNA. This resembles to some extent the situation found in restriction enzymes, e.g. *EcoRI* (McClarín *et al.*, 1986) and the resolvase RuvC (Ariyoshi *et al.*, 1994), in which positively charged amino acids are located in the DNA-binding clefts with their side chains mostly directed to the centre of the clefts, where they are likely to interact with the DNA backbone. Proof of the proposal is expected from co-crystallization of endo VII with cruciform DNA.

The requirement for both termini for DNA binding suggested that the four ends of an endo VII dimer interact to establish binding. Three possible mechanisms of interaction were imaginable: (i) the termini of each subunit interact intramolecularly forming two DNA-binding sites; (ii) the N-terminus of one subunit interacts with the C-terminus of the other subunit and vice versa, again forming two DNA-binding sites; or (iii) the four termini interact in a concerted fashion forming one DNA-binding site. The first possibility fits the principle of independently functioning endo VII subunits. The latter two possibilities depend on dimerization of the protein and explain why proteins deficient in dimerization do not bind DNA. They

Fig. 5. Interaction of N- and C-termini in the endo VII dimer. The experiments revealed that only those endo VII dimers were active which contained an intact N-terminus in one subunit together with an intact C-terminus in the second subunit. In the sketches, different possibilities of termini interaction are illustrated followed by the respective peptide and the experimental result (+, proficient in binding and cleavage; -, no activity). Endo VII subunits are drawn in bold lines, and intact N- and C-termini are marked by the letters N and C. The dimerization domain is boxed. **(A)** Non-covalently linked endo VII dimers are listed in two columns showing the potential intramolecular and the intermolecular interaction of termini. Proteins with both termini intact were found to be active (line 1), which satisfies the intra- as well as the intermolecular interaction model. Homodimers lacking the N- or the C-termini were inactive (lines 2 and 3), which again satisfies both models. In contrast, heterodimers made of one subunit with an intact C-terminus and a second subunit with an intact N-terminus were active (line 4). This is only possible if an intermolecular interaction modus for the termini is used. Here the N- and C-terminus can interact. **(B)** For the covalently linked proteins used in this study, only the intermolecular modus with respect to the termini is shown. The endo VII subunits are linked by an 11 amino acid linker (curved thin line connecting one N- with one C-terminus). All four proteins shown were found to be active, which is consistent with the fact that in all four proteins the termini next to the linker remained intact, providing an active DNA-binding site regardless of the combinations of termini at their free ends. This is only compatible with the model using intermolecular interactions of endo VII termini to establish DNA binding.

are not compatible with the model of independently functioning subunits.

To decide among the three possibilities, covalently linked dimers of endo VII were constructed. The C-terminus of one subunit was connected to the N-terminus of the other subunit by a linker of 11 amino acids. Truncated derivatives of this covalently linked dimer were also made with an N-terminal deletion, a C-terminal deletion, and a dimer containing both deletions. Each of the four covalently linked proteins was found to be active in DNA binding (Figure 3B). The specificity of binding was proven by the conserved cleavage pattern in cruciform DNA typical for endo VII WT (Figure 4A). Since none of the CD-peptides formed multimers, as shown by GA cross-linking, the possibility of intermolecular complementation was excluded (Figure 2B).

In all truncated CD-proteins, the only intact pair of an N- and a C-terminus was the one containing the linker, indicating that a DNA-binding site is created exclusively by one N-terminus and one C-terminus provided they originate from different subunits. This was confirmed by functional complementation of EVII_{pep}(21-157) with EVII_{pep}(1-147). In the resulting active heterodimer, one subunit provided the N- and the other subunit the C-terminus. This mode of interaction of different termini is consistent with the finding that a monoclonal antibody bound exclusively endo VII protein with both termini intact (Christoph *et al.*, 1998). The requirement for termini from different subunits explains why endo VII monomers do not bind to cruciform DNA, as shown with the dimerization-deficient protein EVII-W87R. The observation that removal of two termini of a dimer still resulted in DNA binding-proficient proteins rules out the possibility that all four termini are involved in the formation of

one DNA-binding site. The interaction of the termini is illustrated further in Figure 5.

It is reasonable to assume that the endo VII dimer adopts a symmetrical quaternary structure like other dimeric nucleases with specificity for symmetrical target sites, e.g. restriction enzymes or RuvC. Together with the fact that four ends exist per dimer but only two ends are required for DNA binding, this leads to the conclusion that endo VII contains two identical DNA-binding sites per dimer. The two binding sites act independently with high target specificity, as indicated by the conserved cleavage pattern obtained by proteins with only one DNA-binding site. From our experiments, we cannot determine whether proteins with one DNA-binding site can still resolve cruciform DNA in the proper way by cleaving two strands or whether they cleave one strand only. This can be tested by using a four-way junction stabilized by a supercoil as previously described (Giraud-Panis and Lilley, 1997).

It has been shown recently that endo VII dimers with only one active catalytic centre bound cruciform DNA specifically but cleaved only one strand. This was taken as evidence for two completely independently acting subunits (Giraud-Panis and Lilley, 1997). At least for DNA binding, this hypothesis is wrong. As shown here, the activity of endo VII depends on the interaction of termini from different subunits. A mechanism like this guarantees that only dimers bind to four-way DNA junctions, thus ensuring that two catalytic centres are positioned for the cleavage of two opposite strands flanking the junction. This is of crucial importance for proper resolution of Holliday junctions because two single strand nicks must be generated within the lifetime of the enzyme-DNA complex; otherwise the DNA is left with lesions that are potentially dangerous to the cell.

Materials and methods

Plasmids and bacterial strains

Vector pET11a-his was constructed in the laboratory from vector pET11a (Studier *et al.*, 1991). Six histidine codons and a *Bam*HI restriction site were inserted into the *Nde*I site (bp 359) of pET11a, leaving the start codon in the *Nde*I site intact. The new plasmid was cut with *Bam*HI and religated for removal of the DNA between the newly inserted *Bam*HI site and the original *Bam*HI site of pET11a (bp 319). This gave pET11a-His. *Escherichia coli* DH5 α (Hanahan, 1983) was used for plasmid cloning and propagation of template DNA for sequencing, and *E. coli* BL21(DE3) (Studier *et al.*, 1991) was used for overexpression of endo VII proteins.

PCR primers for cloning of His-tagged proteins

For cloning of full-length His-tagged derivatives of gene 49, two sense primers were used. One encoded a thrombin cleavage site (RB24) and the other a cleavage site for factor X_a protease in front of gene 49 (RB11). The sequence of RB24 was: CTA CCG **GGA TCC CTG GTT CCG CGT ATG TTA TTG ACT GGC AAA TTA TAC**. The *Bam*HI cloning site is indicated in bold, the thrombin site in italics and the homology with gene 49 is underlined. Note that the thrombin cleavage site is formed in part by gene 49 sequence. The sequence of RB11 was the same, except that the first four codons of the thrombin site were replaced by the codons of the cleavage site for factor X_a: ATC GAG GGT AGA. The antisense primer for full-length clones was RB29: CCT AAC **GGA TCC GGT ACC TCA TCA TTT TAA ACT CTT TCT AAG CTG CTT**. The *Bam*HI site and a neighbouring *Kpn*I site are in bold, two stop codons are in italics and the homology with gene 49 is underlined. For introduction of terminal deletions in gene 49, primers were used with the same functional parts but homologies to gene 49 differed depending on the site of deletion.

Cloning of T4 gene 49 derivatives

PCRs were performed using the conditions described earlier (Golz *et al.*, 1997). For the monomeric proteins, the T4 wild-type gene 49 was used. The PCR products were purified over spin columns (Qiagen) and, after cleavage with *Bam*HI, ligated into the *Bam*HI site of dephosphorylated vector pET11a-His for tagged proteins or into the *Nde*I site of pET11a for non-tagged proteins. Clones obtained after transformation into *E. coli* DH5 α were sequenced by dideoxynucleotide sequencing (Sanger *et al.*, 1977) using the T7 polymerase sequencing kit from Pharmacia Biotech.

The covalently linked, head-to-tail dimers of endo VII were derived by a two-step cloning procedure using the mutant gene 49 (H43R) as template for PCR. First, one copy of the gene without a stop codon but with a unique *Kpn*I site at its 3' end was cloned into pET11a-His as described above. A second copy of gene 49 with a linker sequence attached to the 5' end was then inserted into the *Kpn*I site. The final proteins had the following amino acid sequence (one letter code): M (start)-6 $\times$ H (tag)-GS (*Bam*HI cloning site)-LVPR-(thrombin cleavage site)-endo VII sequence-TSGTGGGGGIL (linker)-endo VII sequence.

Protein purifications

Expression and purification of proteins and removal of the 6 $\times$ His tag was performed as described earlier (Birkenbihl and Kemper, 1998). Purifications of non-tagged endo VII proteins were carried out as described in Golz *et al.* (1997).

Cross-linking of endo VII peptides with glutaraldehyde

Samples of 1 μ g of protein were diluted with cross-linking buffer (50 mM NaH₂PO₄, 100 mM NaCl, 0.1% 2-mercaptoethanol, 10% glycerol; pH 7.3) to a final volume of 23.5 μ l. The cross-linking reaction was started at room temperature by addition of 1.5 μ l of 2.5% GA (final concentration 0.15%) and stopped after 2 min with 1.5 μ l of 2 M glycine pH 7.3 (final concentration 113 mM). After 10 min at room temperature, 13 μ l of 3 $\times$ sample buffer (150 mM Tris pH 6.8, 6 mM EDTA, 3% SDS, 3% 2-mercaptoethanol, 24% glycerol, 0.05% bromophenol blue) were added and the probes boiled for 5 min. Aliquots of 15 μ l containing ~400 ng of protein were loaded onto 15% SDS-polyacrylamide gels (12% gels for assays with covalently linked endo VII dimers) with a 5% stacking gel and run in SDS-PAGE buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). The protein bands were visualized by silver staining using standard procedures.

Electrophoretic mobility shift assay

DNA-binding reactions were performed in 10 μ l of binding buffer [50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1 mM dithiothreitol (DTT)].

Varying amounts of protein were incubated with 1 fmol (100 pM final concentration) of radioactively labelled cruciform DNA CF01 (Golz *et al.*, 1995; Figure 4B) at 16°C. After 15 min, 2.5 μ l of loading buffer [40 mM Tris-HCl pH 7.5, 4 mM EDTA, 25% glycerol, 0.05% bromophenol blue and 400 μ g/ml bovine serum albumin (BSA)] were added to each sample. The mixtures were loaded immediately onto an 8% native polyacrylamide gel and run at 4°C for 2.5 h at 7 V/cm in electrophoresis buffer (6.7 mM Tris-HCl pH 8.1, 3.3 mM sodium acetate and 2 mM EDTA). Bands were visualized by autoradiography of the dried gels.

Cleavage of DNA substrate CF01 by endo VII proteins

One fmol of radioactively labelled cruciform DNA CF01 (final concentration 100 pM) was incubated with different amounts of protein in 10 μ l of cleavage buffer (20 mM Tris-acetate pH 7.4, 10 mM magnesium acetate, 1 mM DTT, 1 μ g/ml BSA) for 15 min at 37°C. After ethanol precipitation, samples were resolved in loading buffer (90% vol. formamide, 4.5 mM Tris-borate pH 8.0, 1 mM EDTA, 0.1% bromophenol blue) and loaded on a 12.5% denaturing polyacrylamide gel containing 8 M urea. Electrophoresis buffer was 1 $\times$ TBE. Reaction products were visualized by autoradiography.

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