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Drosophila DSP1 and Rat HMGB1 Have Equivalent DNA Binding Properties and Share a Similar Secondary Fold

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The protein DSP1 belongs to the group of HMG-box proteins, which share the common structural feature of the HMG-box. This approximately 80 amino acid long motif binds DNA via the minor groove. DSP1 was discovered as a transcriptional co-repressor of Dorsal in *Drosophila melanogaster* and then was shown to participate to the remodeling of chromatin. By means of sequence alignment and gene organization, DSP1 was classified as the fly homologue of the vertebrate proteins HMGB1/2. DSP1 contains two HMG boxes flanked by two glutamine-rich domains at the N-terminus. In addition, the HMG domain of DSP1 displays two differences in its primary sequence as compared to the vertebrate HMGB1: a shorter acidic tail and a linker between the two boxes longer by 6 amino acids. By comparing several functional parameters of DSP1 with those of HMGB1, the present study establishes the functional equivalence of both proteins in terms of DNA recognition. The major structural difference between the two proteins, the glutamine-rich N-terminal tail of DSP1, which does not exist in HMGB1, did not interfere with any of the studied DNA-binding properties of the proteins.

Key words: cisplatin, drosophila, DSP1, EMSA, HMGB1/2.

The HMG-box is a motif found in many DNA-binding proteins. Structural analysis have shown that it consists of approximately 80 amino acid residues, forming three α-helices that finally fold into the L-shaped structure typical of HMG-boxes (*1–4*). HMG-boxes bind double-stranded DNA via the minor groove, thereby bending the DNA-helix towards the major groove (*5*, *6*). HMGB-like proteins are found in a wide variety of eukaryotic organisms and show structural variability outside their HMG domain. For example, the yeast NHP6A/B proteins do not contain an acidic tail whereas insect proteins display a shorter acidic tail than vertebrate or some plant HMGB like proteins (*7–9*). In addition, the length of the linker between the two HMG boxes is flexible, and the basic region linking the acidic and the HMG domains is relatively variable (*10–13*). However, all HMG-like proteins share several DNA-binding properties. They bind preferentially to distorted DNA structures such as four way DNA junctions, cisplatin-modified DNA and DNA minicircles. They also constrain negative supercoils in plasmid DNA.

DSP1 was first discovered as a co-repressor of the transcription factor Dorsal and then was shown to act as a chromatin remodeling factor (*14*, *15*). It is worth noting that a recurring function proposed for HMGB1 is the enhancement of the binding of various transcription factors such as Hox protein (*16*), p53 (*17*), or steroid hormone receptors (*18*). Impaired glucocorticoid receptor binding has been provided as an explanation for the pleiotropic effects on glucose metabolism observed in HMGB1-knock-out mice (*19*). These observations suggest that HMGB1, like DSP1, might be involved in the formation of nucleoprotein complexes that remodel chromatin. By comparing the sequence of DSP1 and vertebrate HMGB1, DSP1 is the closest Drosophila homologue of the vertebrate proteins HMGB1/2. DSP1 displays two HMG boxes (boxes A and B) and an acidic tail. The two A domains share a high degree of identity (69.3% identity) while the B domains are more divergent (46.2% identity). Furthermore, the gene of DSP1 has been shown to be orthologously related to the HMGB1/2 genes (*20*). Outside these similarities, DSP1 exhibits some differences. It contains six additional residues between the two HMG domains and a relatively short C-terminal acidic tail as compared to HMGB1. In addition the HMG domain of DSP1 is flanked by two glutamine rich domains at the N-terminus. The regions flanking the HMG domains have been shown to modulate the general affinity of the protein for DNA (*21–23*). The length of the linker region between the two HMG boxes has also been shown to be important for its binding with DNA (*22*).

The present work compares the biochemical and biophysical properties of DSP1 and DSP1^{-162}, a DSP1 deletion mutant missing the N-terminal glutamine rich tail, with the vertebrate HMGB1 protein *in vitro*. No significant differences have been observed, suggesting that the differences in the primary structures of DSP1 and HMGB1 do not alter the intrinsic properties of the HMG domains.

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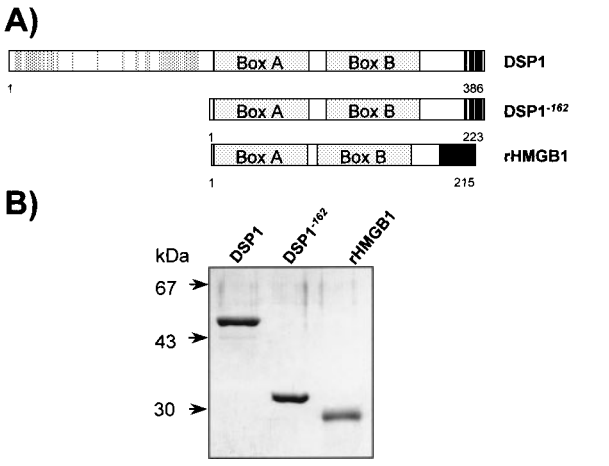

Fig. 1. **Proteins.** (A) Schematic representation of the recombinant proteins used in the present study (proportionally scaled in length): DSP1 is the wild-type protein, which contains a 162 amino acid N-terminal glutamine-rich tail (gray bars). The localization of the HMG-boxes A and B were assigned by sequence alignment with rHMGB1 (*4*). The acidic amino-acids in the C-terminus are marked by black bars. DSP1^{-162} was derived from DSP1 by truncation of the first 162 amino acids. B) 10% SDS-PAGE of the purified recombinant proteins DSP1, DSP1^{-162} and rHMGB1 stained with Coomassie Brilliant Blue.

MATERIALS AND METHODS

Cloning—The full-length DSP1 coding sequence (386 amino acids; Fig. 1) (*20*) was amplified from *Drosophila melanogaster* cDNA using the following primers: 5′-ATG GAA CAC TTT CAT CAA-3′ and 5′-CGA AAC CTC TAA AGC GGG-3′ and taq-polymerase as recommended by the supplier (Promega). The PCR-product was inserted into pMal-c2™ (New England Biolabs) at the *Xmn*I site. This construct was used to transform *E. coli* DH5α strain.

The construct DSP1^{-162} contained the open reading frame starting at methionine 163 (Fig. 1A), acting as the start codon for the expression of the protein. PCR-amplification of the plasmid pMal/DSP1 was performed with the primers 5′-GGC AGT CAC ATA TGA GCA GAG TCA AGG CCG ATG CC-3′ and 5′-CCG TGA CTC TAG ACT TAT TGG TTC TCG TCA TCA TC-3′. The PCR product was cloned into the vector pCR2.1 (Invitrogen). The construct was digested completely with *Nde*I (Eurogentec) and partially with *Eco*RI (Eurogentec), and the fragment of approximately 700 bp was isolated and inserted into the expression vector pET 5a (Novagen). This construct was used to transform the *E. coli* BL21 (DE3) strain.

Recombinant Protein Purification—Strains were grown up to $OD_{600\,nm}$ ≈0.8 at 37°C in 500 ml of LB-medium, containing 100 μg/ml ampicillin. After the addition of 0.3 mM IPTG, incubation was continued for 2 h at 37°C. The bacteria were collected by centrifugation for 15 min at 5,000 ×g, 4°C. Pellets were rapidly frozen at –70°C. Cell lysis was performed by sonication in 60 ml buffer (20 mM Tris/HCl, pH 7.4; 200 mM NaCl; 1 mM EDTA; 1 mM DTT; 0.5 mM PMSF; protease inhibitors, Sigma). The crude extract was obtained by centrifugation at 10,000 ×*g* at 4°C for 10 min. $(NH_4)_2SO_4$ was added to a final concentration of 20%. The solution was stirred gently for 20 min on ice and centrifuged as above. $(NH_4)_2SO_4$ was added to the supernatant to a concentration of 80% and the sample stirred and centrifuged as described above. The pellet was then resuspended in 60 ml ice-cold low salt phosphate buffer (20 mM sodium phosphate, pH 7.0), containing PMSF and protease inhibitors (Sigma).

Both MBP-DSP1 and DSP1^{-162}–containing samples were enriched in proteins by chromatography on an SP-Sepharose gravity column (HiLoad, Pharmacia) at 4°C using a linear NaCl-gradient (200–800 mM). Fractions containing the recombinant proteins were pooled. The MBP-DSP1 fusion protein was incubated with Factor X_A (New England Biolabs) at 25°C for 4 h under the conditions recommended by the supplier. After completion, DSP1 was separated from the MBP moiety by chromatography on a Uno S (BioRad) column. This chromatographic step was also used for the purification of DSP1^{-162}. Elution was performed using a linear NaCl-gradient (200–800 mM); DSP1 eluted at 400 mM NaCl.

DSP1 and DSP1^{-162} were finally purified on a Mono Q FPLC (Pharmacia) column using a NaCl-gradient (200–600 mM, 0.5 ml/min). In both cases, the pure proteins eluted between 250 and 300 mM. Protein concentration was determined by UV absorption at 280 nm (DSP1 protein: 1 A_{280} = 1.03 mg/ml; DSP1^{-162} protein: 1 A_{280} = 0.98 mg/ml) and the quality of the proteins was checked by SDS–PAGE (*24*).

The rat rHMGB1 protein was purified as described elsewhere (*25*).

Circularization Assay—A 122 bp DNA fragment was obtained by PCR amplification (upper primer: 5′-GGT CTT TAG GAT CCA CGC GGA AGT CAG CGC CCT GC-3′; lower primer: 5′-CGT GCG CGG GAT CCT CGT TAA TAC AGA TGT AGG-3′) of the plasmid pBR322. The PCR-product was digested with *Bam*HI and dephosphorylated using calf intestinal alkaline phosphatase. The resulting 100-bp DNA had compatible sticky ends. It was purified by electrophoresis on a 10% (w/v) TBE-PAGE gel (89 mM Tris/borate, pH 8.5, 2 mM EDTA) followed by electroelution and 5′-labelled using [γ-^{32}P]ATP and T4-kinase according to the manufacturer's protocol (New England Biolabs).

Approximately 1 nM DNA was mixed with different concentrations of proteins in a total volume of 10 μl ligase buffer (New England Biolabs). The samples were incubated for 30 min on ice. After the addition of 0.3 unit of T4 DNA ligase, incubation was continued for 30 min at 30°C. T4 DNA ligase was inactivated by incubating the samples for 10 min at 65°C.

At this point, some samples were incubated with 1 unit of exonuclease III (Life Technology Research Laboratories) at 37°C for 30 min; the enzyme was inactivated at 65°C for 10 min. For deproteinization, samples were incubated with 0.1% (w/v) SDS and 1 unit of proteinase K (Merck) in 20 mM Tris, 1 mM EDTA, 3% ficoll. After 1 hr incubation at 37°C, samples were analyzed by native PAGE on a 6% gel. After electrophoresis, the gels were dried and exposed on a phospho-imager plate (Phospho Imager; Molecular Dynamics) to visualize the radioactivity.

Supercoiling Assay—Native supercoiled plasmid pBR322 was purified using a standard cell lysis technique followed by purification in a CsCl gradient (*26*). Plasmid

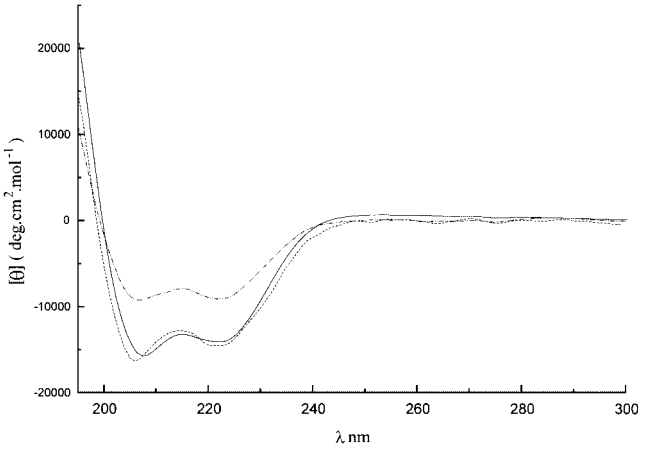

Fig. 2. **CD SPECTRA.** Far-UV CD spectra of HMGB1, DSP1 and DSP1^{-162}. The spectra were recorded at 20°C with measurements every 0.5 nm. Solid line, HMGB1; dotted line, DSP1^{-162}; two-dotted line, SP1.

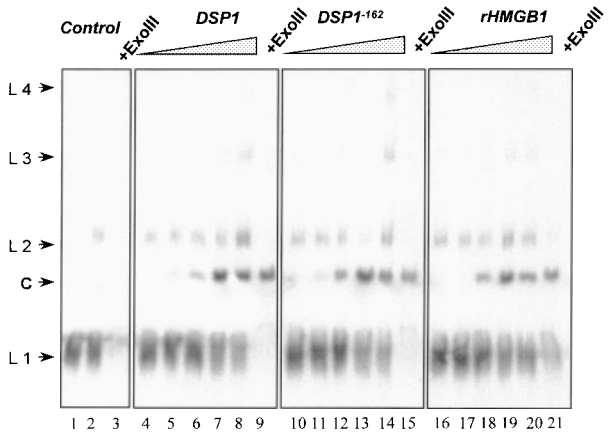

Fig. 3. **Ligase mediated circularization assay.** A 100 bp dsDNA (0.36 nM) fragment was circularized by incubation with increasing concentrations of DSP1, DSP1^{-162} and rHMGB1. The protein concentrations used were 1, 3, 9, 27, and 81 nM. A final sample, circularized with 81 nM of protein, was additionally digested with exonuclease III (lanes 9, 15, 21). (C) circular DNA, (L1) 100 bp monomer, (L2) linear dimer. Control lane 1 contained the untreated 100 bp DNA oligomer; lane 2 was loaded with a sample that was incubated with the ligase in absence of sample proteins; lane 3 contained the 100 bp DNA oligomer treated with ligase and exonuclease III.

DNA (250 ng) was incubated in a final volume of 20 μl in topoisomerase-buffer [50 mM Tris/HCl, pH 7.4; 50 mM NaCl; 1 mM DTT; 0.1 mM EDTA, 15% (v/v) glycerol] with different dilutions of the proteins of interest at 37°C for 15 min. The incubation was continued for 1 h after the addition of 0.2 units of topoisomerase I (Promega).

The reaction was stopped by the addition of SDS to a final concentration of 1% (w/v) followed by 5 min incubation at 37°C. The samples were then loaded onto a 1% (w/v) agarose TBE gel, and the DNA was visualized by ethidium bromide staining.

Platination of Oligonucleotides—A 18-mer intrastrand crosslink was produced by overnight incubation of the single-stranded oligonucleotide (5′-TCC TCT CAG GAT CTT CTC-3′; final concentration 32 μM) with *cis*-platinum [*cis*-diammine-dichloroplatinum(II)] (final concentration: 29 μM) in 10 mM $NaClO_4$ at 37°C. The DNA adduct was purified by HPLC (Waters) on a MonoQ column (Pharmacia) eluted with a linear gradient of NaCl in 10 mM NaOH. After radioactive labeling with T4-kinase (Eurogentec), the oligonucleotide was hybridized with the complementary strand (5′-GAG AAG ATC CTG AGA GGA-3′) in hybridization buffer (10 mM Tris/HCl, pH 7.9; 150 mM NaCl; 10 mM $MgCl_2$; 1 mM DTT; 0.1 mg/ml BSA). In order to create a 35-mer intrastrand crosslink, the modified 18 mer upper strand oligonucleodide was hybridized with a 35-mer lower strand oligonucleodide (5′-CTC TAG AAG CTT CGC CGG AGA AGA TCC TGA GAG GA-3′). Following hybridization, the oligonucleotide was filled by the DNA polymerase Klenow fragment.

Electrophoretic Mobility Shift Assay (EMSA)—^{32}P-labelled oligonucleotides (1 or 0.5 nM) were incubated for 30 min with increasing amounts of recombinant protein in a final volume of 10 μl EMSA buffer [10 mM Tris/HCl, pH 7.5; 100 mM NaCl; 1 mM DTT; 0.05% NP40 (w/v); 0.1mg/ml BSA; 3% (w/v) Ficoll 400] on ice. The samples were loaded onto a 6% polyacrylamide TBE gel and electrophoresis was performed using TBE buffer at 7.5 V per cm. The results were analyzed by phosphorimaging and quantification by ImageQuant software (Molecular Dynamics).

Circular Dichroism (CD)—CD studies were performed in 1 mm length pathway quartz cells on a CD6 Jobin Yvon Dichrograph. The spectra were smoothed by means of standard software. The spectra are presented as molar ellipticity expressed in deg·cm^2·dmol^{-1}, where the concentration refers to amino acid residues, which for proteins DSP1^{-162}, DSP1 and rHMGB were 1.4 mM, 1.2 mM, and 1.1 mM, respectively (buffer: 10 mM NaP pH 7.5, 100 mM NaF, 0.5 mM DTT). Secondary structure analysis was carried out using Johnson's CDsstr software (*27*).

RESULTS

Protein Purification—Both proteins, DSP1 and DSP1^{-162}, were purified using almost the same protocol. The finally purified proteins were pure on SDS–PAGE stained with Coomassie Brilliant Blue, and were stable after several cycles of freezing/thawing (Fig. 1B).

Circular Dichroism—CD spectroscopy was used to detect structural differences between the drosophila and rat proteins. DSP1^{-162} and rHMGB1 (HMGB1 protein from rat) exhibit the same CD spectrum (Fig. 2). Full-length DSP1 has the same type of CD spectrum as the former proteins, but with lower ellipticity values. On the one hand, by deconvolution of the CD spectra, we observed that DSP1, DSP1^{-162} and rHMGB1 have the same content of amino acids involved in α-helices, 89, 94, and 90, respectively. On the other hand, the CD spectra of DSP1 is characterized by a significant increase in amino-acids implicated in β-sheets, 58 *versus* 11 and 6 for DSP1^{-162} and rHMGB1, respectively.

Circularization of Linear DNA—Due to its molecular rigidity, a 100 bp double-stranded (ds) DNA cannot self-ligate into a circular form. This is only possible when the bending or flexibility of the DNA is increased, for example as a result of the binding of a protein (*28*, *29*). To dem-

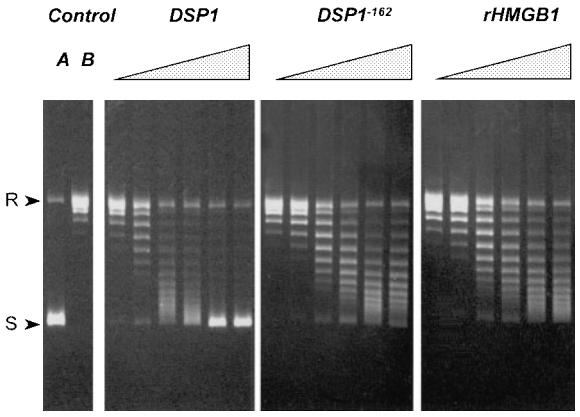

Fig. 4. **Supercoil protection assay.** The plasmid pBR322 in the native supercoiled state (Control, lane A) was relaxed by 1 hr of incubation with 0.2 U of wheat germ topoisomerase (Control, lane B). Increasing amounts of DSP1, DSP1^{-162} and rHMGB1 (molar ratios protein/bp were 436, 109, 54.5, 36.3, 27.3, and 21.8 for each protein), increasingly protected the supercoiled plasmid from topoisomerase I-induced relaxation. (S) highly supercoiled plasmid DNA; (R) relaxed plasmid DNA.

onstrate the ability of HMG-proteins to bend linear DNA, we performed a ligase-mediated circularization assay using such a 100 bp long dsDNA fragment. Mini-circles were detected as an extra band in native PAGE that was retarded compared to its linear counterpart and was resistant to exonuclease III digestion (Fig. 3).

rHMGB1, DSP1^{-162} and DSP1 proteins induced circularization of the 100 bp DNA at a minimal protein concentration of 3 nM (an approximately 10-fold molar excess of protein over DNA). Further increases up to 27 nM protein increased the relative amount of circular DNA. At 81 nM, on the other hand, the amount of linear oligomerized products of the 100-bp DNA increased.

Supercoil Protection of Plasmid-DNA—The binding of HMG-box proteins to double-stranded DNA changes its helicity by unwinding the double helix. Therefore, these proteins are able to introduce or protect negative supercoils in circular plasmid DNA (*30*, *31*). In the assay presented, the degree of supercoil protection of the plasmid pBR322 was monitored as a function of the molar protein/DNA ratio for DSP1, DSP1^{-162} and rHMGB1.

All three proteins were able to protect the supercoiled state of pBR 322 as can be seen by the increasing gel mobility of the plasmid when incubated with HMG proteins (Fig. 4). Full length DSP1 can protect supercoils very efficiently, with full protection achieved with a ratio of 1 protein for 36 bp of plasmid. DSP1^{-162} and rHMGB1 are less efficient. Nonetheless, both proteins are indistinguishable in their protection of the plasmid supercoiled state.

Specific Binding to Intrastrand cisPt Modified DNA—A striking feature of HMG-box proteins is their specific binding to intrastrand *cis*Pt modified DNA (*32*, *33*). Gel retardation assays were performed with an 18-mer probe containing a single intrastrand *cis*Pt cross-link (intraPt18) at the d(GpG) site located in the center of the oligonucleotide (*34*). All three proteins recognized this cisplatinum modified DNA with equivalent tightness (50% of the total DNA amount was bound at approx. 1–3 nM protein concentration, Fig. 5). Binding was equally similar when a longer probe was used (*35* bp instead of *18*). In that instance, several complexes are formed suggesting the binding of several proteins on the same DNA molecule. Again, full length and truncated DSP1 proteins have behaviors identical to that of rHMGB1.

DISCUSSION

Based on sequence alignment and a comparison of gene organization, the *Drosophila melanogaster* protein DSP1 was proposed as the fly homologue of the mammalian protein HMGB1. *In vivo*, DSP1 acts as a transcriptional co-factor involved in the dorso/ventral and antero-posterior patterning of Drosophila embryos (*14*, *15*, *35*). HMGB1 has been reported to be involved in transcriptional regulation (*19*), although the detailed mechanism remains unknown. So far, the close similarity of these proteins is solely defined at the sequence level. The present study shows that this close similarity holds for a wide variety of properties including secondary folding and protein–DNA interactions.

Compared to HMGB1/2, DSP1 carries an additional N-terminal tail of approximately 162 amino acids that includes two clusters of glutamine residues (Fig. 1A). In order to study possible influences of this tail on DNA-binding properties, a truncated protein lacking these 162 N-terminal residues (DSP1^{-162}) was produced.

CD Spectra—FAR-UV CD spectroscopy was used to visualize the global secondary structure of DSP1, DSP1^{-162} and rHMGB1 (Fig. 2). The DSP1^{-162} CD spectra were nearly identical to those of HMGB1 strongly suggesting a similar secondary folding of the two proteins. Analysis of the CD spectrum of full length DSP1 shows that the number of residues implicated in α-helices (89) is not modified by the presence of the glutamine rich tail. The most plausible explanation for this result is that the N-terminal tail does not contain a significant number of residues involved in α-helices and that it does not have a great influence on the folding of the HMG boxes. Moreover, the greater number of amino acids forming β-sheets in full length DSP1 than in DSP1^{-162} or in rHMGB1, are likely to reside in the N-terminus, and more precisely in the glutamine rich regions. Indeed, studies of proteins and polypeptides having some glutamine rich regions show that these glutamine rich domains fold into β-sheets (*36*).

Comparison of DSP1 and DSP1^{-162} Behaviors on DNA—The DNA-binding properties of DSP1 and DSP1^{-162} were found to be remarkably similar. DSP1 and DSP1^{-162} are both able to bend DNA, as shown in the ligase-mediated circularization assay (Fig. 3). Both proteins are also able to protect negative supercoils of a plasmid from topoisomerase activity. Full length DSP1 is, in fact, more efficient in that protection. It can be suggested that there will be better binding of the protein to the DNA when the tail is present, however, this hypothesis was refuted by subsequent analysis of DNA binding. Therefore, the most plausible explanation is a decrease in topoisomerase access to DNA, probably due to greater steric hindrance caused by the presence of the N-terminal tail.

A striking feature of the HMG-box is its specific binding to a double-stranded DNA containing an intrastrand

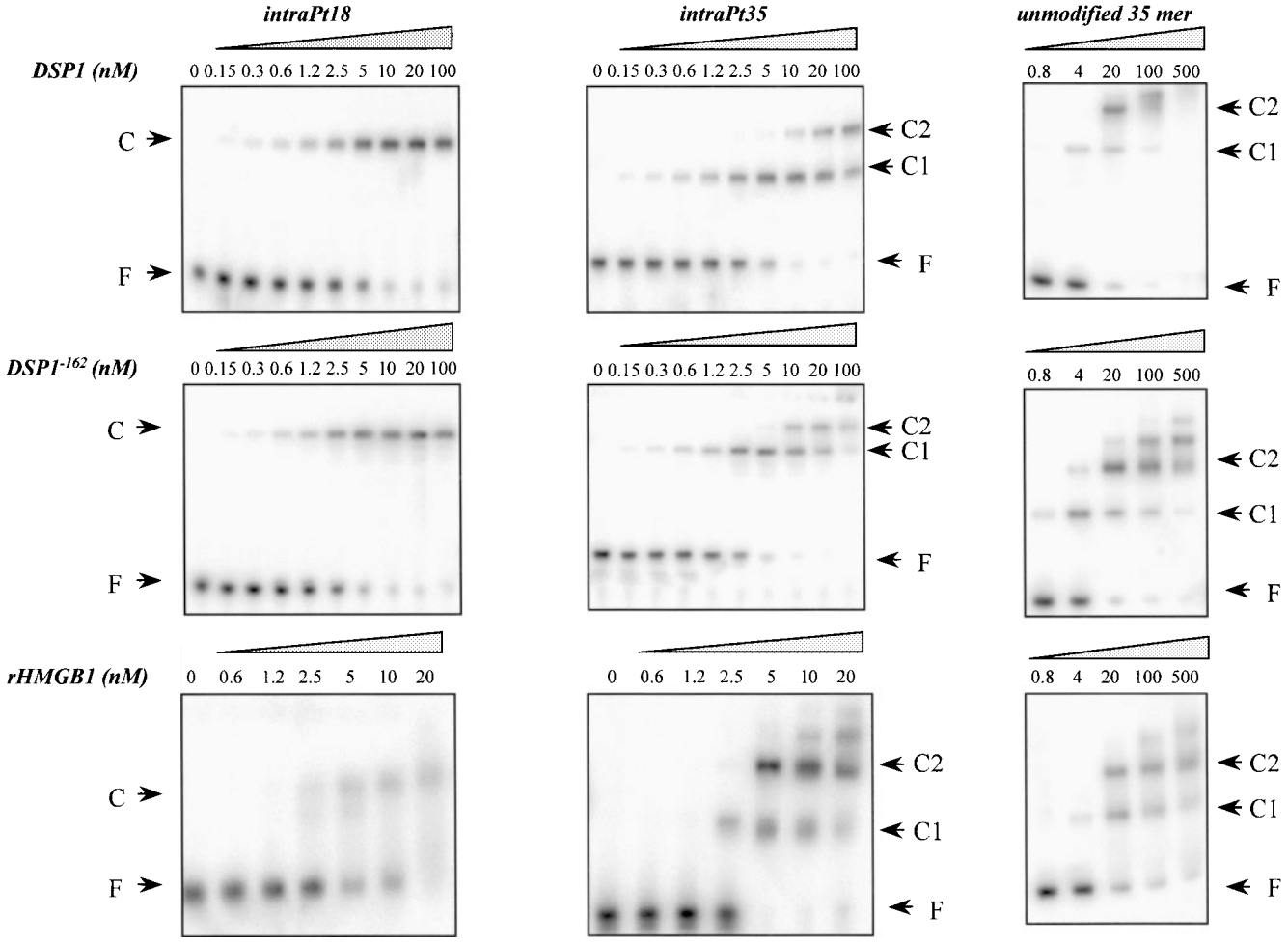

Fig. 5. **Binding to *cis*Pt-modified DNA and unmodified DNA.** intraPt35, intraPt18 and unmodified 35mer were incubated for 30 min with increasing concentrations of DSP1, DSP1^{-162} or rHMGB1 on ice, and the samples were analyzed by PAGE. Unbound DNA (F); protein–DNA complex (C, C1, and C2).

cisplatinum cross-link (*32–34*). In the present study, we analysed the binding of the two proteins to an 18 bp oligomer, modified at a central GpG site. Both DSP1 and DSP1^{-162} showed almost the same behavior. This suggests that the N-terminal tail is not involved in DSP1-DNA interactions. As we have shown (*37*), this is again the case for the effect of DSP1 on Dorsal binding. Indeed, *in vitro* DSP1 as well as rHMGB1 enhance the Dorsal affinity for its binding sites in a similar manner, suggesting that the N-terminal tail, absent in rHMGB1, is also dispensable for this function. Moreover, it appears that the N-terminal tail does not intervene in protein-protein interactions between DSP1 and Dorsal, which are mediated by the HMG boxes (*37*, *38*). However, this domain might be involved in other protein-protein interactions. Glutamine-rich domains are found in numerous transcription factors and have been shown to be involved in transcriptional activation (*39*, *40*). In the case of DSP1, it has been shown that the interaction with the TATA-binding protein is influenced by the glutamine-rich amino-terminal domain (*41*).

Although no well-defined role for the glutamine tail has been reported yet, we have observed that this region of DSP1 may be responsible for protein aggregation. Several facts support this hypothesis. In our hands, the full-length DSP1 protein, but not DSP1^{-162}, is lost in significant quantities during the purification procedures. Furthermore, in EMSA-experiments, DSP1-DNA complexes, but not DSP1^{-162} complexes, tend to disappear in the absence of detergent. Both facts could be explained by the formation of protein aggregates. It is known that long glutamine repeats can play a role in the polymerization of proteins, as has been shown in the case of the trinucleotide-expansion diseases (*42*, *43*). However, in that case, the minimum number of repeated glutamine residues needed to obtain insoluble protein-polymers is higher (45 instead of the 30 in DSP1).

Comparison between rHMGB1 and DSP1^{-162}—The comparison of the properties of DSP1^{-162} and rHMGB1 underline once more the functional similarity between the two proteins. No differences could be seen between DSP1^{-162} and rHMGB1 in terms of their DNA binding and bending properties. Both proteins also gave similar results as far as their abilities to protect negative supercoils of circular plasmid DNA were concerned. For specific fixation, DSP1^{-162} and rHMGB1 were equally efficient in binding to a double-stranded DNA bearing an intra-strand *cis*Pt cross-link.

In summary, we have demonstrated *in vitro* the similarity of the DNA-binding proteins rHMGB1 and DSP1. These constitutes with the high sequence similarity and the orthologous relation between their genes, a third line of evidence for the homology between the two proteins. Such similar behavior has also been reported for the interaction with the Rel homology domain (RHD) of transcription factors (*37*). In this study, a specific DNA fragment corresponding to the Dorsal dl2 binding site was used to compare the effects of DSP1 and rHMGB1 on the binding affinity of RHD domains. DSP1 and rHMGB1 were shown to increase RHD binding affinity in an indistinguishable manner. Several questions still remain to be answered such as what is the role of the DSP1 N-terminal glutamine-rich region: does it confer a specialized role to DSP1?

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