Redox Potential Regulates Binding of Universal Minicircle Sequence Binding Protein at the Kinetoplast DNA Replication Origin

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Kinetoplast DNA, the mitochondrial DNA of the trypanosomatid Crithidia fasciculata, is a remarkable structure containing 5,000 topologically linked DNA minicircles. Their replication is initiated at two conserved sequences, a dodecamer, known as the universal minicircle sequence (UMS), and a hexamer, which are located at the replication origins of the minicircle L- and H-strands, respectively. A UMS-binding protein (UMSBP), binds specifically the conserved origin sequences in their single stranded conformation. The five CCHC-type zinc knuckle motifs, predicted in UMSBP, fold into zinc-dependent structures capable of binding a single-stranded nucleic acid ligand. Zinc knuckles that are involved in the binding of DNA differ from those mediating protein-protein interactions that lead to the dimerization of UMSBP. Both UMSBP DNA binding and its dimerization are sensitive to redox potential. Oxidation of UMSBP results in the protein dimerization, mediated through its N-terminal domain, with a concomitant inhibition of its DNA-binding activity. UMSBP reduction yields monomers that are active in the binding of DNA through the protein C-terminal region. C. fasciculata trypanothione-dependent tryaredoxin activates the binding of UMSBP to UMS DNA in vitro. The possibility that UMSBP binding at the minicircle replication origin is regulated in vivo by a redox potential-based mechanism is discussed.

Kinetoplast DNA (kDNA) is a unique extrachromosomal DNA, found in the single mitochondrion of parasitic flagellated protozoa of the family Trypanosomatidae. In the species Crithidia fasciculata, the kDNA network consists of 5,000 duplex DNA minicircles of 2.5 kbp and 50 maxicircles of 37 kbp that are interlocked topologically to form a DNA network. Maxicircles contain mitochondrial genes, encoding mitochondrial proteins and rRNA. Minicircles encode guide RNAs that function in the process of mRNA editing (19, 49, 53). Minicircles in most trypanosomatid species are heterogeneous in sequence. However, two short sequences that are associated with the process of replication initiation are located 70 to 100 nucleotides apart on the minicircle molecule: the dodecameric sequence GGGGTTGGTGTA, designated the universal minicircle sequence (UMS), and the hexameric sequence ACG CCC. These sequences have been mapped to the sites of the proposed replication origins of the minicircle L-strand and H-strand, respectively (for reviews, see references 36, 41, 47, and 48).

Several of the proteins involved in the replication of the kDNA network have been identified, including the origin-binding protein, designated the UMS-binding protein (UMSBP) (2–4, 61, 62), DNA polymerases (23, 57–59), primase (30), topoisomerase II (31, 52), structure-specific endonuclease 1 (SSE1) (13, 14), RNase H (9, 12, 42), RNA polymerase (20), and structural proteins (73, 74). UMSBP has been purified from C. fasciculata, and its encoding gene and genomic locus have been cloned and analyzed (2, 61–63). The protein binds specifically to the two sequences conserved at the minicircle replication origins on the kDNA minicircle H-strand: the UMS dodecamer and a 14-mer sequence, containing the core hexamer, in their single-stranded conformation (2, 3, 6, 61, 62). The 116-amino-acid long protein contains five tandemly arranged zinc knuckle motifs. This motif forms a compact zinc finger, which contains the core sequence Cys2-Cys-X3-His-X2-Cys (where X represents any amino acid). Proteins bearing this motif, such as the human immunodeficiency virus type 1 nucleocapsid protein (22, 64) and several eukaryotic proteins such as the cellular nucleic-acid binding protein (40), bind single-stranded nucleic acids.

The correct folding of the zinc knuckle motif depends on the presence of a metal ion, which serves as a core element inside the folded structure. The metal ion is held in the knuckle via coordinative bonds formed by the three cysteines and the single histidine residue. It has been found that redox potential may affect the capacity of the cysteine residues to bind the zinc ion in the knuckle (8, 24, 70). Regulation through redox potential has been reported for several zinc finger proteins (for a review, see reference 7), including transcription factors and replication proteins such as replication protein A (65, 75), activator protein 1 (71), and glucocorticoid receptor (21). This type of regulation may influence both the DNA-binding properties of the protein and its protein-protein interactions. A few enzymes involved in redox regulation of proteins have been identified, such as thioredoxin (21) and redox factor 1 (71). Recently, the enzyme thioredoxin, known to regulate the redox state of proteins, was localized to the mitochondrial membrane in the rat (44). Redox modulation of the activity of mitochondrial topoisomerase I was recently reported (27). The enzy-
matic machinery that regulates redox state in trypanosomatids differs from that found in other eukaryotes, in both its main thiol substrate and its enzymes. Most of the glutathione in trypanosomatids is converted to an N',N'-bis(glutathionyl) spermidine adduct, known as trypanothione [T(SH)₂], which is the main low-molecular-weight thiol in the genera 

Cricidithia, Trypanosoma, and Leishmania (15, 16). T(SH)₂ reduces a trypanothione-dependent member of the thioredoxin family, the tryperoxadin (TXN), and reduced TXN, in turn, reduces a peroxyredoxin, called TXN peroxidase (reviewed in references 17, 28, 37, and 52). This unique metabolic pathway has been studied in C. fasciculata, and two tryperodoxins, CfTXN I and CfTXN II (33–35, 38), have been found in this trypanosomatid. Classical thioredoxins have also been found in Trypanosoma brucei and Leishmania major (43).

In this study we show that UMSBP binds the conserved UMS sequence at the replication origin of kDNA minicircles as a monomer in a zinc-dependent manner. Zinc knuckles, located at the C-terminal domain of the protein, are involved in DNA binding, while the N-terminal zinc knuckle is involved in protein-protein interactions that lead to UMSBP dimerization. Moreover, we show that both UMSBP DNA-binding activity and its oligomerization are sensitive to redox potential. In vitro coupling of the trypanothione-dependent tryperoxadin reaction to the UMSBP DNA-binding reaction, reconstituted from pure C. fasciculata proteins, revealed the activation of UMSBP binding to the minicircle origin region and suggested that redox potential may regulate UMSBP function at the kDNA replication origin.

**MATERIALS AND METHODS**

Oligonucleotides, enzymes, and reagents. Primers were prepared by Genset SA. Superdex 75 and protein markers for gel filtration were obtained from Amersham. Dithiotreitol (DTT) was purchased from Boehringer Mannheim; NADPH, dimethyl pimelmidate (DMP), and superoxide dismutase were a generous gift of L. Flohe from the Department of Biochemistry, Technical University of Braunschweig, Germany. Restriction endonucleases, micrococcal nuclease, and polyornucleotide kinase were purchased from MBI Fermentas.

Preparation of UMSBP. The C. fasciculata UMSBP gene open reading frame (2), cloned into a pGEX1 vector (Amersham) (1), was PCR amplified (Turbo Pfu polymerase; Stratagene) with sense primer UU (5'-GTGGCAATGGCCGCT GCTGTCACGTGCTAC-3') and antisense primer UL (5'-GGCCGCAAGCGCCACCTG-3') or F3 (5'-GGC TTTCAGGACACTG-3'), respectively. The C-terminal deletions (d3 and d5-δ) were constructed using either sense primer F4 (5'-GCGCGTCAAGGTCGCT GGCGTCAAGGTCGCT-3') or F3r (5'-GGC GGTTGTGGTGTTGTTGTTGTT-3'), respectively, and antisense primer L6 (5'-GAGTTGGCAGCGGACACTG-3').

Electrophoretic mobility shift assay (EMSA). Analyses were carried out as described previously (61, 62). Samples of UMSBP, as indicated, were incubated in the 20-μl binding-reaction mixture, containing 25 mM Tris·Cl (pH 7.5), 2 mM MgCl₂, 200 (vol/vol) glycerol, 2 μg of bovine serum albumin, 0.5 μg of poly(dI-dC)-poly(dI-dC), and 12.5 fmol of 32P-labeled UMS DNA (5'-GGGCGGTGGTGTTGTTGTTGTT-3'). Unless otherwise stated, the reaction mixture contained 1 mM DTT. Pretreatments of UMSBP were conducted as indicated below. Reaction mixtures were incubated at 30°C for 30 min or on ice for 60 min, and their products were loaded onto an 8% native polyacrylamide gel (1:29, bisacrylamide/acrylamide) in TAE buffer (6.7 mM Tris acetate, 3.3 mM sodium borate, 1 mM EDTA [pH 7.5]). Electrophoresis was conducted at 2 to 4°C and 16 V/cm for 1.5 h. Protein-DNA complexes were quantified by exposing the dried gels to an imaging plate and analyzing them by phosphorimager analysis. One unit of UMSBP activity is defined as the amount of protein required for the binding of 1 fmol of the UMS DNA (5'-GGGCGGTGGTGTTGTTGTTGTT-3') ligand under the standard binding assay conditions (61, 62).

TXN assay. The TXN reaction was conducted by published procedures (see, e.g., reference 17), with the modifications indicated below. C. fasciculata UMSBP (92 μg/ml) was dialyzed extensively against 50 mM potassium phosphate buffer (pH 8) and subsequently oxidized by incubation for 30 min on ice in the presence of 0.5 mM diamin. The protein was then diluted in the binding-reaction mixture, with no DTT, prior to its introduction at a final concentration of 30 ng/ml into the TXN reaction mixture. The reaction was conducted under the standard UMSBP-binding assay conditions in the presence of UMSBP (0.6 ng) and 12.5 fmol of 32P-labeled UMS DNA, as described above, with the following modifications: DTT was omitted and the reaction mixture was supplemented with a trypanosomal TXN system, which included either CtxTN I or CtxTN II, at the indicated concentrations, and 20 mM Tris·Cl (pH 7.5). For the coupling of a T(SH)₂ reductase (TR) system, the reaction mixture was supplemented with 1 unit of TR per ml and 150 μM NADPH. Reactions started by the addition of TXN, proceeded for 10 min at 30°C, and their products were analyzed by EMSA and quantified by phosphorimager analysis, as described above.

Analysis of proteins by SDS-PAGE. Protein samples in loading buffer, containing 50 mM Tris·Cl (pH 6.85), 4% (wt/vol) sodium dodecyl sulfate (SDS), 3.5% (vol/vol) β-mercaptoethanol, 10% (vol/vol) glycerol, and 10 mM EDTA, were boiled for 10 min and loaded onto a 16.5% Tris-Tricine SDS-polyacrylamide gel (45), along with protein size markers ("Rainbow" prestained low-molecular-weight marker; Amersham). The upper electrophoresis buffer was 0.1 M Tris·Tricine (pH 8.25) containing 0.1% SDS; the lower buffer was 0.2 M Tris·Cl (pH 8.9). Gels were silver stained (69) or blotted onto nitrocellulose membranes for Western blot analysis, as described below. When nonreducing SDS-polyacrylamide gel electrophoresis (PAGE) analyses were conducted, β-mercaptoethanol was omitted.

Gel filtration. Gel filtration chromatography was conducted using a 100- by 1.6-cm Superdex 75 column (Amersham) in an AKTA purifier FPLC instrument (Amersham-Pharmacia). The running buffer was 25 mM Tris·Cl (pH 7.5)–150 mM NaCl. Protein elution was monitored using a UV detector to track the absorbance at 220 nm. Results were analyzed using Unicorn version 4.11 software.
Cross-linking of UMSBP and UMS DNA-UMSBP complexes. A protein sample (1 μg) was incubated for 1 h at 25°C with 10 mM imidazole dimethyl pimelimidate·2HCl cross-linker (Sigma) in a 10-μl reaction mixture containing 50 mM potassium phosphate buffer (pH 8.0), 2 mM MgCl₂, 5 mM CaCl₂, and 20% (vol/vol) glycerol. The reaction was stopped by adjusting the solution to a final concentration of 100 mM Tris·Cl (pH 8.0), and its products were analyzed by SDS-PAGE. When cross-linking of nucleoprotein complexes was conducted, the reaction was supplemented with 1.0 ng of ³²P-labeled UMS DNA. Radiolabeled DNA was detected by phosphomager analysis, and protein bands were detected by Western blotting and an enhanced chemiluminescence (ECL) procedure using anti UMSBP antibodies as described below. In samples treated with micrococcal nuclease (MBI Fermentas), 50 U of the nuclease was added and the reaction mixture was incubated for 15 min at 30°C. The reaction was stopped by addition of EGTA to final concentration of 12.5 mM. The bound UMS DNA was relabeled at the newly generated 5’ termini, using 10 U of polynucleotide kinase (MBI Fermentas) and 15 μCi of [γ-³²P]ATP (3,000 Ci/mmol; Amersham) in a 25-μl reaction mixture as specified by the manufacturer. The reaction mixtures were incubated for 1 h at 37°C. Free [γ-³²P]ATP was removed using a Sephadex G-50 column.

Western blot analysis. Protein samples were analyzed by SDS-PAGE as described above. Protein bands were transferred onto a Protran BA85 cellulose nitrate membrane (Schleicher & Schuell). The membranes were blocked by incubation for 30 min in 5% dry skim milk (Difco) in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄ [pH 7.4]) containing 0.1% (vol/vol) Tween 20 and probed for 90 min with anti-UMSBP antibodies that was raised in rabbit and were affinity purified. The membranes were probed for 45 min with a 1:13,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies and subjected to ECL as recommended by the manufacturer (Amersham-Pharmacia).

Surface plasmon resonance (SPR) analysis. Surface plasmon resonance SPR studies were conducted using a BIAcore 3000 at the BIAcore unit, the Hebrew University of Jerusalem. A 3'-biointylated UMS DNA (5'-GGGGTTGGTGTA GTAT-3') had been immobilized to an SA sensor chip (BIAcore), as recommended by the manufacturer. The DNA-binding activity of the proteins was measured by injection of 0.025 to 2 μM wild-type or mutated UMSBP into the DNA-bound flow channel, using an empty flow channel as the background. Kinetic analysis was performed by automated injection of various protein concentrations (30 μM/min; 3-min association time and 3-min dissociation time) in 10 mM HEPES (pH 7.0)–150 mM NaCl–5 mM DTT–2 mM MgCl₂. No mass transfer was detectable under these conditions. Binding constants were calculated with the BIAevaluation 3.1 program, using the Langmuir 1:1 binding model.

Atomic absorption spectrophotometry. Analyses of zinc and other metal ions in UMSBP were conducted with a 300/400/400 Zeeman instrument as recommended by the manufacturer (Varian). Zn, Mg, Mn, Cu, and Fe were analyzed at the specific wavelengths and using the corresponding standard solutions in triple-distilled water.

RESULTS

UMSBP binding to UMS is zinc dependent. UMSBP contains five putative CCHC, retrovirus-type zinc knuckle domains. Correct folding of such domains has been previously shown to be dependent on the binding of zinc ions (18, 54). The intrinsic association of zinc ions with UMSBP has been determined using an atomic absorption spectrograph. A significant signal above the background level was obtained, which was specific to zinc ions, while the signals obtained in the analyses conducted for detecting other metal ions (e.g., Mn, Mg, Cu, and Fe ions) yielded no signal above background levels (data not shown). To explore the possibility that UMSBP interacts with DNA via a zinc-dependent mechanism, we used EMSA to analyze the binding of a ³²P-labeled UMS ligand by UMSBP, which was pretreated with either the zinc chelator 1,10-phenanthroline or its nonchelating close analogue 1,7-phenanthroline. Figure 1 shows that whereas the formation of specific protein-DNA complexes is inhibited in the presence of 1,10-phenanthroline, yielding 55% inhibition at 50 nM and complete inhibition at 150 nM zinc chelator, its closely related 1,7-phenanthroline nonchelating analogue has no effect on the binding of UMSBP to UMS DNA even at concentrations more than an order of magnitude higher. Moreover, the DNA-binding activity of the o-phenanthroline-treated UMSBP could be partially restored by its incubation in the presence of zinc ions (Fig. 1). These observations indicate that UMSBP-DNA interactions are zinc dependent, implying the requirement for the specific zinc knuckle topology for protein-DNA interactions.

Next, we studied the effect of chelating the zinc ions with 1,10-phenanthroline on the structure of UMSBP by gel filtration chromatography using an FPLC Superdex-75 column. Analysis of the untreated native protein yielded a single peak with a retention time of 232.76 ± 0.13 min. Treatment of the native protein with 1,10-phenanthroline prior to the gel filtration analysis resulted in a decrease in its retention time to 229.98 ± 0.13 min. The corresponding Stokes radii, calculated based on analyses of UMSBP and protein markers (Fig. 2), were 18.9 and 18.3 Å (Δ = 0.6 ± 0.0034 Å). We suggest that this effect on the pattern of the UMSBP chromatogram may reflect the distortion of the compact folded structure of the zinc knuckle domains in the protein as a result of the removal of the metal ions.

Overall, these observations indicate that zinc ions are intrinsically present in UMSBP and that the ability of the protein to bind DNA is zinc dependent, probably through the formation of zinc knuckles, as predicted from its amino acid sequence.

UMSBP binds UMS DNA as a monomer. UMSBP was purified from C. fasciculata cell extracts as a protein dimer (61, 62). Stoichiometry analysis, based on the ratio of radioactively labeled UMSBP and UMS DNA in nucleoprotein complexes

FIG. 1. UMSBP binding to DNA is zinc dependent. UMSBP was preincubated in the reaction mixture under the standard binding-reaction conditions, as described in Materials and Methods, with increasing concentrations of either 1,10-o-phenanthroline or the nonchelating form, 1,7-phenanthroline. Lanes: a, no UMSBP was added; b, no 1,10-o-phenanthroline was added; c to f, 0.075, 0.1, 0.125, and 0.15 μM 1,10-o-phenanthroline, was added; g to i, 0.275, 1.325, and 2.75 μM 1,7-phenanthroline was added; j, no 1,10-o-phenanthroline was present; k, UMSBP was preincubated with 0.2 μM 1,10-o-phenanthroline; l, the preincubation with 0.2 μM 1,10-o-phenanthroline was followed by treatment with 2.0 μM ZnCl₂. EMSA and its quantification were as described in Materials and Methods.
The column radii for the 1,10-o-phenanthroline-treated UMSBP was analyzed by gel filtration chromatography either in its native form or in the presence of 5 mM 1,10-o-phenanthroline, using a 100- by 1.6-cm Amersham Superdex 75 column, as described in Materials and Methods. Protein markers and their Stokes radii were as follows: RNase A, 16.4 Å; chymotrypsinogen A, 20.9 Å; ovalbumin, 30.5 Å; and albumin, 35.5 Å. The arrows mark the calculated Stokes radii for the 1,10-o-phenanthroline-treated and untreated UMSBP. The column V₀ and Vᵣ were determined by using Blue Dextran and B12, respectively. All proteins were detected by measuring the absorbance at 220 nm. UMSBP Stokes radii were interpolated from the linear plot of known Stokes radii against the −log Kᵣ/2 of the protein markers. The difference in the retention time between o-phenanthroline-treated and untreated UMSBP was 2.78 ± 0.13 min, reflecting a 0.6-Å difference in the calculated Stokes radii, and the standard deviation measured in these analyses of UMSBP and protein markers yielded an error of ±0.0034 Å.

under native gel electrophoresis conditions, indicated that two protein monomers bind a single DNA molecule (61). However, structural studies have shown that the CCHC-type zinc knuckle in the nucleocapsid protein of retroviruses can bind the nucleic acid ligand as a monomer (11, 46). To study the nature of UMSBP binding, we used a direct in vitro cross-linking approach. UMSBP was incubated with 32P-labeled UMS DNA in the presence of the imidoester cross-linker DMP. The reaction products were analyzed by SDS-PAGE under denaturing and reducing conditions. Alternatively, prior to their analysis in the SDS-PAGE gel, the cross-linked protein-DNA complexes were treated with micrococcal endonuclease and the newly generated 5' termini in the bound DNA were relabeled in a polynucleotide kinase reaction (Fig. 3). 32P-labeled UMS DNA bands were detected by phosphor-imaging, and the protein bands, representing UMSBP monomers, dimers, and multimers, were detected by Western blot analysis using anti-UMSBP antibodies. Figure 3 shows that the electrophoretic mobility of bound UMS DNA was slightly lower than that of free UMSBP monomers (Fig. 3, lane c). This is probably due to the contribution of the bound DNA ligand, since the subsequent digestion of the unprotected sequences in the bound ligand yielded, following its relabeling, a radioactively labeled protein-DNA complex with electrophoretic mobility that was virtually identical to that of the UMSBP monomer (lane d). Neither the bands representing UMSBP dimers nor those representing other protein oligomers displayed any [32P]DNA signal, indicating the generation of nucleoprotein complexes exclusively with the monomeric form of UMSBP. No [32P]DNA signal could be detected in the gel when UMSBP was omitted from the binding-reaction mixture. These results suggest that UMSBP is capable of binding UMS DNA in its monomeric form.

Redox potential significantly affects the binding of UMS DNA by UMSBP. It has been previously reported that zinc ions are held in the CCHC-type zinc knuckle via coordinate bonds, formed by its three cysteine and one histidine residues. Oxidation of cysteines in the zinc finger domain may affect the binding of zinc, thus changing the conformation of the protein. Such changes may result in the formation of intran- or interknuckle S-S bonds and the ejection of the zinc ion (8, 24, 70), affecting the protein-DNA-binding activity and protein-protein interactions. It has also been shown that redox potential is regulating the DNA-binding activity of several zinc finger proteins, such as replication protein A (RPA), Sp1, and glucocorticoid receptor (39, 55). To test whether redox potential regulates the DNA-binding activity of UMSBP, the effect of either a reducing agent (DTT) or an oxidizing agent (diamide) on the binding of UMSBP to UMS was studied using EMSA (Fig. 4). In the absence of DTT, almost no DNA-binding activity (<<5.5% of the untreated control) could be detected. Raising the concentration of DTT resulted in an increase in the generation of protein-DNA complexes, until saturation was reached at about 20 mM DTT (Fig. 4A). On the other hand, increasing the concentration of the oxidizing agent dia-
mide (in the presence of a constant level of 0.6 mM DTT) resulted in a concomitant decrease in the generation of UMSBP-UMS nucleoprotein complexes (Fig. 4B). Thus, the redox state of UMSBP is found here to critically affect its DNA-binding activity.

It has been previously reported that reversible oxidation of thiol groups in zinc-coordinated cysteines within zinc fingers may mediate the binding of transcription factors as well as replication proteins onto the DNA (39, 66, 70). Considering the observations presented here that binding of DNA by UMSBP was sensitive to oxidation of thiol groups by diamide and their reduction by DTT (Fig. 4), we have examined the sensitivity of the UMSBP DNA-binding reaction to NEM, an agent that alkylates thiol groups. Figure 5 demonstrates the effect of pretreatment of UMSBP with NEM on the binding of 32P-labeled UMS DNA ligand, either in the absence or in the presence of a 10-fold molar excess of DTT in the binding reaction. NEM completely (>/99.5%) inhibited the generation of UMSBP-UMS complexes, while addition of DTT following the NEM treatment partially reversed this inhibition. Interestingly, addition of DTT following the NEM treatment resulted in the formation of two distinct protein-DNA complexes. The first corresponded in its electrophoretic mobility to the nucleoprotein complex formed with the untreated UMSBP, whereas the second displayed a lower electrophoretic mobility, which may reflect an additional mass due to the addition of alkyl groups to the protein or other changes in the nucleoprotein structure.

CfTXN I and CfTXN II activate UMSBP binding to DNA. Thioredoxin is involved in vivo in the regulation of the redox state and DNA-binding activity of proteins (see, e.g., references 21 and 72). A T(SH)2-dependent member of the thiore-
doxin family. TXN, was found in various trypanosomatid species (reviewed in references 17, 28, 37, and 52) including C. fasciculata, for which the presence of two TXNs, CfTXN I and CfTXN II, has been reported (33–35, 38). To test the physiological significance of the regulation of UMSBP binding to DNA through the protein redox state, we have coupled, in vitro, the UMSBP-binding reaction to the reactions catalyzed by TXN and TR. This coupled reaction, reconstituted from pure trypanosomal proteins, was based on the TXN reaction system (TXN I or TXN II) that uses pure TXN and T(SH)2 and the TR reaction that uses pure TR and NADPH, coupled to a specific DNA-binding reaction that consists of pure (preoxidized) UMSBP and 32P-labeled UMS DNA ligand. The generation of nucleoprotein complexes was analyzed using the EMSA. The results presented in Fig. 6 show clearly the TXN-dependent binding of UMSBP to UMS DNA, similar to the effect observed with the reduction of thiol groups by DTT shown above (Fig. 4). The effect of the TXN reaction on the binding of UMSBP to UMS observed in the in vitro-reconstituted reaction suggests the potential physiological role in vivo for UMSBP redox state control in the regulation of UMSBP binding at the minicircle replication origin.

**The N-terminal zinc knuckle of UMSBP is not involved in DNA binding.** Zinc fingers are known to participate in both DNA binding and protein-protein interactions (for a review, see reference 7). To further analyze the involvement of UMSBP zinc finger domains in protein-DNA interactions, we constructed a series of truncation mutants of the protein (Fig. 7). Zinc knuckles located at either the N-terminal or the C-terminal domain of the protein were deleted, and the capacity of the truncated proteins to bind UMS DNA was assayed using EMSA (not shown), as well as by the SPR BIACore technology (Fig. 7), measuring the equilibrium binding constant of the reactions as well as their kinetics parameters. Truncation of the N-terminal knuckle (d1, Fig. 7) had only a limited effect on the binding of UMSBP to UMS DNA. The reaction equilibrium binding constant was only about threefold higher with the mutated protein than that measured with the wild-type UMSBP. A very similar association rate and only an approximately twofold higher dissociation rate, measured with this truncated protein, may suggest some effect of the first N-terminal knuckle on the stability of the nucleoprotein complex. Truncation of both N-terminal knuckles 1 and 2 (d1-2, Fig. 7) resulted in a further increase (~58-fold relative to the wild-type protein) in the reaction Kd, with a decrease of ~9.5-fold in its association rate and an increase of ~6-fold in its dissociation rate, relative to the nonmutated protein. EMSAs have revealed the efficient generation of nucleoprotein complexes with both d1 (see Fig. 10) and d1-2 (data not shown) N-terminally truncated UMSBP. On the other hand, truncation of knuckle 5 (d5, Fig. 7) or both knuckles 4 and 5 (d4-5, Fig. 7) at the UMSBP C-terminal domain had a remarkable effect on the capacity of UMSBP to bind the UMS DNA, resulting in no detectable nucleoprotein complexes by EMSA and no measur-
Binding-reaction parameters were obtained from SPR analyses as described in Materials and Methods. Calculated amino acid residues at positions 65 to 116, encompassing the last two C-terminal knuckles. Numbering is as previously described (11). The indicated 51, encompassing the

C-terminal domain of the protein play a major role in the binding of

the nucleic acid by UMSBP. Preliminary observations revealed

that mutating the single tyrosine residue in zinc

finger sequence, per se, was sufficient to pre-

vent the dimerization of UMSBP in the presence of diamide

(3F 1, Fig. 7). As described above, while truncation of the

N-terminal zinc finger sequence, per se, was sufficient to pre-

vent the dimerization of UMSBP in the presence of diamide

(Fig. 9), it had only a limited effect on protein binding to DNA

(Fig. 7). Figure 10 shows the results of binding experiments in

which radiolabeled UMS DNA was incubated with either the

UMSBP mutant protein (d1, Fig. 7), in which the N-terminal

domain was truncated, interrupting the

first zinc

finger domain (ZF 1, Fig. 7). As described above, while truncation of the

N-terminal zinc finger sequence, per se, was sufficient to pre-

vent the dimerization of UMSBP in the presence of diamide

(Fig. 9), it had only a limited effect on protein binding to DNA

(Fig. 7). Figure 10 shows the results of binding experiments in

which radiolabeled UMS DNA was incubated with either the

S-S bonds; however, the nature of these changes has yet to be explored.

DNA binding of N-terminal deleted UMSBP is insensitive to

the redox potential. Since both UMSBP dimerization and its

capacity to bind the nucleic acid are affected by the redox

potential, an interesting mechanistic question was whether

redox directly affects the C-terminal DNA-binding domain

or, alternatively, acts indirectly through its effect on the pro-

tein dimerization. To address this question, we have used a

UMSBP mutant protein (d1, Fig. 7), in which the N-terminal

domain was truncated, interrupting the first zinc finger domain

(ZF 1, Fig. 7). As described above, while truncation of the

N-terminal zinc finger sequence, per se, was sufficient to pre-

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which radiolabeled UMS DNA was incubated with either the

Multimerization of UMSBP is affected by the redox potential. UMSBP

was purified from C. fasciculata as a homodimer (61, 62). Furthermore, as shown above (Fig. 3), treatment of UMSBP with a chemical cross-linker resulted in the oligomerization of the protein. Considering the effect of the redox potential on the binding of UMS by a UMSBP monomer reported here (Fig. 3), we tested the possibility that multimerization of UMSBP is mediated through the redox potential of the protein. As shown by SDS-PAGE analysis under nonreducing conditions (Fig. 8), treatment of UMSBP with low levels of the oxidizing agent diamide resulted in the generation of UMSBP dimers, while a further increase in the diamide concentration generated higher UMSBP oligomers such as trimers and tetramers.

Next, we identified the domain in UMSBP involved in the

multimerization of the protein. Analysis of two of the truncation

mutants described above (Fig. 7), d1 and d4-5, shows that

deletion of the N-terminal zinc knuckle (zinc finger 1) per se

was sufficient to prevent UMSBP dimerization in the presence of diamide whereas deletion of the C-terminal knuckle (zinc finger 5) had no inhibitory effect on the protein oligomerization (Fig. 9). In fact, truncation of the C-terminal domain even enhanced protein multimerization compared to the full-length protein (Fig. 9). We noticed slight changes in the electrophoretic mobility of the protein in the presence of diamide. These changes may be due to the formation of intramolecular

| ZF 1 2 3 4 5 | $k_{on}$ (1/Ms) $k_{off}$ (1/s) $K_D$ (M) |
|---|---|---|
| wt | | |
| d1 | | |
| d1-2 | | |
| d5 | | |
| d4-5 | | |

FIG. 7. UMSBP C-terminal zinc knuckles are involved in DNA binding. A series of truncated mutants of UMSBP were constructed by PCR as described in Materials and Methods. A schematic representation of UMSBP truncation mutants is presented. Deleted amino acid sequences are as follows: in d1, amino acid residues at position 1 to 29, including the N-terminal zinc knuckle; in d1-2, amino acid residues at positions 1 to 51, encompassing the first two N-terminal knuckles; in d5, amino acid residues at positions 90 to 116, including the C-terminal knuckle; and in d4-5, amino acid residues at positions 65 to 116, encompassing the last two C-terminal knuckles. Numbering is as previously described (11). The indicated binding-reaction parameters were obtained from SPR analyses as described in Materials and Methods. Calculated $\chi^2$ values were as follows: wt, 4.44; d1, 2.09; d1-2, 6.58. wt, wild type.

FIG. 8. The redox potential controls UMSBP oligomerization. UMSBP was incubated for 30 min at 25°C with increasing concentrations of diamide and then subjected to SDS-PAGE analysis under nonreducing conditions and silver staining as described in Materials and Methods. Lanes: a to g, reactions were carried out in the presence of 10.00, 5.00, 1.00, 0.50, 0.10, 0.05, and 0.01 mM diamide; h, no diamide was present in the reaction mixture; i, reaction mixture contained 5 mM DTT.
Based on these data, we suggest that the zinc of the C-terminal DNA-binding domain to bind the nucleic acid that this effect results in the changes observed in the capacity directly affects the N-terminal domain of UMSBP and mutant (Fig. 10). These observations indicate that redox potential-mediated regulation of protein-DNA interactions. The observations presented here support the possibility that redox potential controls both DNA binding and dimerization of UMSBP and hence may participate in a mechanism that regulates its function at the replication origin. Furthermore, the data presented here (Fig. 9 and 10) indicate that changes in redox potential affect the protein N-terminal zinc knuckle domain but do not have a direct effect on the capacity of its C-terminal zinc knuckles to interact with DNA. Based on these observations, we suggest that zinc knuckles at the protein C-terminal domain, which are essential for sequence-specific DNA binding, are not involved in the redox potential-mediated regulation of protein-DNA interactions whereas the N-terminal zinc finger domain, which is not involved directly in DNA binding, functions as a regulatory element in the redox potential-dependent binding of UMSBP to DNA.

Our previous analysis of doubly labeled UMSBP-UMS DNA complexes under native conditions suggested a 2:1 stoichiometry of bound UMSBP monomers per UMS site (61). We demonstrate here, by analysis of chemically cross-linked protein-DNA complexes in SDS-PAGE under denaturing and reducing conditions that UMSBP is bound in the nucleoprotein complex as a monomer. Binding of a single site in the protein is also in agreement with our previous observations that when reacted with either of its binding sequences, UMSBP could not bind two such binding sites simultaneously (3, 61). If, indeed, UMSBP was capable of DNA binding as a dimer, one had to present in UMSBP that was purified to apparent homogeneity from C. fasciculata and to be essential for its binding to UMS DNA. These data imply that the CCHC knuckles, predicted in silico from the UMSBP coding sequence, may indeed fold into zinc knuckle conformation in the protein. It was further found that the zinc knuckles involved in DNA binding are distinct from those that participate in oligomerization of the protein. While UMSBP C-terminal zinc knuckles are essential for DNA-binding activity, the sequence of the N-terminal Zn knuckle is involved in oligomerization through protein-protein interactions. The observations presented here support the possibility that redox potential controls both DNA binding and dimerization of UMSBP and hence may participate in a mechanism that regulates its function at the replication origin. Furthermore, the data presented here (Fig. 9 and 10) indicate that changes in redox potential affect the protein N-terminal zinc knuckle domain but do not have a direct effect on the capacity of its C-terminal zinc knuckles to interact with DNA. Based on these observations, we suggest that zinc knuckles at the protein C-terminal domain, which are essential for sequence-specific DNA binding, are not involved in the redox potential-mediated regulation of protein-DNA interactions whereas the N-terminal zinc finger domain, which is not involved directly in DNA binding, functions as a regulatory element in the redox potential-dependent binding of UMSBP to DNA.

FIG. 9. The N-terminal zinc-finger is involved in UMSBP oligomerization. The multimerization was conducted as described in the legend to Fig. 8, in the absence of an oxidizing agent (lanes a, c, and e) or in the presence (lanes b, d, and f) of 0.5 mM diamide. Lanes: a and b, full-length (wild-type [wt]) UMSBP; c and d, N-terminal knuckle truncation mutant (d1); e and f, C-terminal knuckle truncation mutant (d4-5). Mutants are as described in the legend to Fig. 8. The arrows point on the monomers (mono), dimers (dim), and trimers (trim) of the wild-type and mutated UMSBP.

d1 UMSBP mutant or wild-type UMSBP in the presence of diamide and the reaction products analyzed by EMSA. It demonstrates that whereas diamide inhibited the binding of the wild-type UMSBP to DNA, it had no detectable effect on the DNA-binding capacity of the N-terminally truncated UMSBP mutant (Fig. 10). These observations indicate that redox potential directly affects the N-terminal domain of UMSBP and that this effect results in the changes observed in the capacity of the C-terminal DNA-binding domain to bind the nucleic acid. Based on the data presented here, we suggest that the zinc finger motifs at the protein C-terminal domain, which are essential for the sequence-specific DNA-binding activity of the protein, are not involved in the redox potential-mediated regulation of protein-DNA interactions. Instead, the N-terminal zinc finger domain, which is not involved directly in DNA binding, functions as a regulatory element in UMSBP redox potential-dependent DNA-binding activity.

Based on these results, we suggest that the redox effect observed here on both UMSBP dimerization (Fig. 9) and its capacity to bind the nucleic acid (Fig. 4, 6, and 7) imply that the protein dimerization, via thiol groups oxidation, may participate in a regulatory mechanism that controls UMSBP binding to the minicircle replication origin.

DISCUSSION

UMSBP has been suggested to play a key role in the replication of kDNA minicircles in trypanosomatids. The protein binds specifically to two origin-associated sequences: a conserved 12-mer UMS and the 14-mer sequence containing the conserved hexamer. One set of the two conserved sequences, which were previously implicated in minicircle replication initiation, is found in each of the two copies of the origin region in C. fasciculata. In this study, we analyzed the DNA-binding characteristics and oligomerization of UMSBP. The protein sequence predicts five potential tandemly arranged, CCHC retrovirus-type zinc knuckles. Zinc ions were found to be

FIG. 10. DNA binding of an N-terminally truncated UMSBP is insensitive to oxidation. 32P-labeled UMS DNA (12.5 fmol) was incubated under the standard binding reaction conditions with 100 fmol of d1 UMSBP mutant (lanes b to f) or 40 fmol of wild-type UMSBP (lanes g and h) in the absence (lanes b and g) or the presence (lanes c to f and h) of diamide, as indicated below. Reaction products were analyzed by EMSA and quantified by phosphorimaging as described in Materials and Methods. Lanes: a, no protein added; b to f, 0.00, 0.25, 0.50, 0.75, and 1.0 mM diamide; g, no diamide added; h, 1.0 mM diamide.
assume that UMSBP dimers different from the ones described here were generated, since the redox potential-dependent UMSBP dimers have no DNA-binding activity.

Traditionally, zinc fingers have been associated with binding of nucleic acids. However, a few zinc finger families, such as the GATA, LIM, and RING domains, have also been implicated in protein-protein interactions (for a review, see reference 7). The human immunodeficiency virus type 1 nucleocapsid protein, bearing two CCHC-type zinc knuckles similar to those of UMSBP, interacts with both RNA and the Vpr protein via its C-terminal zinc knuckle domain (5, 11, 50). In UMSBP, different domains are involved in protein-protein interactions and in the binding of the nucleic acid. The N-terminal zinc knuckle, which was found to be involved in UMSBP dimerization, differs from the other knuckles in the protein in its amino acid composition, as well as in its flanking amino acid residues. It may be significant that the UMSBP N-terminal zinc knuckle differs from the rest of the zinc knuckles in the protein in having a methionine residue within the knuckle. Mass spectrometry analysis under denaturing, nonreductive conditions revealed that this methionine residue was oxidized (data not shown). The presence of oxidized methionine residues within zinc fingers that are regulated by redox potential has been previously reported (10, 51).

As an origin-binding protein, UMSBP is expected to act during the initiation of kDNA replication in the recruitment of other replication proteins, such as helicase and primase, to the replication origin through protein-protein interactions. Preliminary observations indicate that such interactions between UMSBP and other replication proteins may indeed take place in Chlamydomonas reinhardtii chloroplast DNA replication and redox regulation of RPA (65, 75) and mitochondrial topoisomerase I (27) activities suggest that redox potential may be critical for the control of DNA replication in the nucleus as well as in other cell organelles (29). A replication initiation control mechanism, based on the conversion of inactive dimers into active monomers, was reported in the case of TrfA protein that controls the replication of the broad-host-range plasmid RK2 (26, 60). The protein is active as a monomer and inactive as a dimer, and the monomer-dimer ratio in the cell is controlled by the ClpX protein (25). Whether a similar redox potential-controlled mechanism regulates the action of UMSBP at the kDNA replication origin and hence controls kDNA replication initiation has yet to be explored.

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REFERENCES


43. Tzafiti, Y., and J. Shlomai. 1998. Genomic organization and expression of the


AUTHOR’S CORRECTION

Redox Potential Regulates Binding of Universal Minicircle Sequence Binding Protein at the Kinetoplast DNA Replication Origin

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Volume 3, no. 2, p. 277–287, 2004. Page 284, Figure 10: In Fig. 10, we presented a result of an experiment with an N-terminally truncated universal minicircle sequence-binding protein (UMSBP) showing that binding of the mutated protein to UMS DNA was insensitive to oxidation. This observation could suggest that the N-terminal domain could serve in the redox-mediated regulation of UMSBP binding to DNA. However, we have recently found, reproducibly, that the UMSBP mutant lacking its N-terminal domain was redox sensitive, similar to the wild-type protein. This correction is limited to only this specific point in the article and does not alter the overall conclusions of our study.