DNA Binding Exerted by a Bacterial Gene Regulator with an Extensive Coiled-coil Domain*

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Although quite common in the eukaryotic cell, bacterial proteins with an extensive coiled-coil domain are still relatively rare. One of the few thus far documented examples, TlpA from Salmonella typhimurium, is characterized by a remarkably long (250 amino acids) α-helical coiled-coil domain. Herein, we demonstrate that TlpA is a novel, sequence-specific DNA-binding protein. Several tlpa deletion mutants have been constructed, and their corresponding protein products were purified and tested for DNA binding. Two of the mutant proteins were shown to be deficient in DNA binding. Both mutants were analyzed by circular dichroism and electron microscopy, supporting the notion that mutant proteins were largely intact despite lacking the amino acid residues necessary for DNA binding. In vivo studies with transcriptional tlpa-lacZ fusions demonstrated that TlpA acts as a repressor. Using the repressor phenotype as a readout, the chain exchange previously described in vitro could also be confirmed in vivo. We believe the coiled-coil domain acts not only as a dimerization interface but could also serve a role as a flexible modulator of the protein-DNA interaction.

The α-helical coiled-coil motif has been widely described (1, 2). Heptad amino acid repeats (a-b-c-d-e-f-g) are the hallmark of this structure which is driven by apolar residues buried in a interface formed by two (or more) α-helical chains in the coiled-coil structure (3, 4). Positions a and d of the heptad form the characteristic 3–4 hydrophobic repeat, which has been identified in the primary sequence of more than 200 proteins (5).

Coiled-coils are also found as components of eukaryotic transcription factors (6). In the eukaryotic bZIP family of proteins, a coiled-coil motif of 3–4 heptads in length enables dimerization and positioning of the two polypeptide chains into a DNA binding unit (7, 8). The involvement of the leucine zipper coiled-coil is also a centerpiece of the basic region helix-loop-helix motif (16–19). Most of these bacterial examples however, lack biophysical evidence supporting the presence of a coiled-coil. Nevertheless, one cannot exclude the possibility that the leucine repeats, in these so-called zipper regions of the bacterial regulators, could mediate dimerization, if not by coiled-coil-like interaction, by way of another novel conformation. Indeed, the X-ray crystal structures of two other bacterial gene regulators, catabolite gene activator protein and the lac repressor, show that they contain short coiled-coil motifs enabling subunit interaction (20, 21).

The TlpA protein encoded by the Salmonella typhimurium virulence plasmid forms an elongated homodimer coiled-coil (15, 22). Here we show that TlpA has an ability to autoregulate its own gene by sequence-specific binding to its promoter DNA, an intriguing finding when one considers the sparse occurrence of extensive coiled-coils in bacterial proteins. As a first step toward dissecting the role of the coiled-coil domain in TlpA, we constructed a panel of mutant proteins lacking various portions of the reading frame. Purified mutant proteins were subjected to DNA binding and transcription assays. Based on these results we could localize the DNA-binding region, at the N terminus adjacent to the predicted coiled-coil. Evidence for in vivo chain exchange also points to TlpA’s flexibility as a gene regulator.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Methods for DNA manipulation and transformation have been previously described (23). All enzymes were used as suggested by the manufacturers (Boehringer Mannheim; New England Biolabs).

Plasmids pMR11, p3062, and p3062d1, inclusive of tlpa or deleted fragments thereof, were available from previous work (15, 22). In the p3062 series, tlpa is under the control of the tac promoter of pKK223-2 (Pharmacia Biotech, Inc.), whereas in the pMR series tlpa is contained in pUC19 (New England Biolabs) and expressed from its native promoter.

pMR12 and p3062d5 were manufactured by replacing in tlpa the region of codons 31 to 371 with a PCR-generated fragment encoding residues 43–371. The oligonucleotides used for PCR were AGATATGGAAGCTACCAGC and ACCGATATCACTCGGCTTACTCA, p3062d3 was produced by deleting the Sall-Xhol fragment in tlpa of p3062. p3062d2 and p3062d4 were produced by deleting, respectively, the 465- and 234-bp PvuII fragments of tlpa in p3062.

The pOP tlpa-lacZ transcription fusion constructs were based on the pACYC184 vector (New England Biolabs) containing a lacZ cartridge in the BamHI–SalI sites (pKTH2090) (24). To insert intact tlpa before lacZ, tlpa was transferred as a Smal fragment into Bluescript SK+ (Stratagene), and subsequently as a HindIII–BamHI fragment into...
pKTH3090 to generate pOF14. The truncated tlpA region that regulates lacZ expression in pOF6 was generated by PCR using S. typhimurium pEX102 virulence plasmid DNA as a template. Oligonucleotide CCTGGCAAGGAGAGGCGGCTCAT was used for pOF6, and as a second primer, CAGCTGCGTGGTCTGCGC. Next, the resulting PCR fragment was cloned into Bluescript SK+ from the resultant plasmid. The inserts were cut out as BamHI-HindIII fragments before ligation into the corresponding cloning sites of pKTH3090. Finally, all lacZ fusion constructs were supplemented with chloramphenicol acetyltransferase gene block (Pharmacia) inserted into the HindIII in front of the tlpA promoter in an opposite orientation to prevent readthrough from the plasmid.

Sequence Analysis—COILS2 program based on a previous colis program (5, 11) was used with a 28 residue window, MDITK sequence profile, and a weighting of a and d with a factor of 2.5.

Proteins—Plasmids producing the recombinant proteins TlpA (p3062) and dTlpI-5 (p3062d1-d5) were harbored in Escherichia coli TG1 (Amersham Corp.). Overnight cell cultures were harvested for the purification and processed as described previously (22). All mutant TlpAs were purified according to the procedure previously described (22), except for TlpA, dTlpI, and dTlp5, which were further purified on high performance liquid chromatography anion exchange chromatography followed by size exclusion chromatography with resulting species being more than 95% pure. As a control for the other protein isolates, a TG1 strain transformed with the vector pKK223 (Pharmacia) was induced with subject to parallel to our previous purification procedure (22). The resultant fraction exhibited no DNA binding activity even when tested at high concentration in gel mobility shift assay (data not shown).

Gel Mobility Shift Assays and DNAase I Footprinting—DNA fragments for footprinting or the mobility shift assays were end-labeled following calf intestinal phosphatase (Promega) dephosphorylation (when necessary) with [γ-32P]ATP (Amersham) using polynucleotide kinase (Boehringer Mannheim) following published protocols (23). A 223-bp fragment containing the target sequence was PCR-amplified with oligonucleotides CTCGGGACATGCGTGGCAGCAGTTCGTCTACAGCGAGGGCGAGGCAG (a 249-bp control fragment was analyzed using the same primer pair). The amplified DNA was subsequently subjected in parallel to our previous purification procedure (22). The resultant fraction exhibited no DNA binding activity even when tested at high concentration in gel mobility shift assay (data not shown).

Gel Mobility Shift Assays—Three 32P-labeled DNA fragments were separated by gel electrophoresis (1%). The resulting gels were either autoradiographed or analyzed by PhosphorImager (Molecular Dynamics Inc.) for DNA binding activity.

RESULTS

TlpA Protein Interacts with the 5’ End of lacZ Gene—Deletions in tlpA lead to increased levels of expression (22), which suggests an autoregulatory control of transcription. Seeking to establish a functional role for TlpA it was therefore obvious to test whether this protein would have DNA-binding capabilities.

Electron Microscopy—TlpA, dTlpI, and dTlp5 in phosphate buffer were diluted in distilled water to a protein concentration of about 0.03 mg/ml. 5-μl droplets were applied to carbon films which were glow-discharged in air and were stained with 0.5% uranyl acetate (pH 5.0) for 1 min. The specimens were examined in a J eol 1010 microscope operated at 100 kV.

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Region Outside the Coiled-coil Abolishes DNA Binding—Encouraged by the finding that purified TlpA bound DNA (Fig. 2), we constructed and purified various TlpA mutant proteins (Fig. 3A) for more detailed study of the DNA-protein interaction. Mutant proteins were designed such that the deleted residues spanned overlapping segments covering most of the protein sequence and the predicted coiled-coil sequence (Fig. 3B). Using a prediction algorithm COILS2 (11) we can distinguish areas of high coiled-coil forming probability which is shown to cover about two thirds of the protein (Fig. 3B). Since the binding was directed to the 5’ end of the SmaI DNA fragment (Fig. 1A), we thus selected a 223-bp fragment spanning this region for PCR amplification and used it in screening the mutant protein panel. In addition, a 249-bp control fragment comprising...
the binding buffer. TlpA addition leads to a disappearance of the 5' end fragment in TlpA. Abbreviations: X, no protein added; T, TlpA; Xh, XhoI; Sna, SnaBI.

Fig. 3. A, schematic representation of deleted regions in TlpA amino acid sequence. Internal (dTlp1-dTlp5) deletion derivatives of TlpA are shown underneath with the line symbol indicating residues present in each protein. All deletions are in frame, i.e., the proteins are translated in their entirety but lacking the residues indicated. B, plot of the probability of coiled-coil formation. Probability P(S) is shown as a function of amino acid residue number in TlpA protein. Figure was calculated and produced with the COILS2 program (5, 11).

FIG. 2. Gel mobility shift assay with the tlpA gene fragments. Fragments derived from differentially digested tlpA SmaI block, with either XhoI or SnaBI, were radiolabeled and mixed with TlpA or only the binding buffer. TlpA addition leads to a disappearance of the 5' end fragment in TlpA. Abbreviations: X, no protein added; T, TlpA; Xh, XhoI; Sna, SnaBI.

Fig. 4. Gel mobility shift assays. A, specificity controls for TlpA binding. TlpA was mixed with either the putative target or noncognate DNA and subjected to a mobility shift assay. Same DNA fragments were used at more than 600-fold excess to compete the binding. B, binding of TlpA mutant proteins to target DNA competed with noncognate DNA. Abbreviations: T, TlpA; T1, dTlp1; T2, dTlp2; T3, dTlp3; T4, dTlp4; C, control DNA fragment; F, DNA fragment which includes the target.

start point delineated previously (22) and a 35 element (TTTATT) where only three out six consensus residues are present (Fig. 5B). Also a striking GT-rich stretch is evident in the footprint (Fig. 5B).

Mutant Protein Lacking 13 Amino Acids Shows Loss of Function—Satisfied with the fact that dTlp1 showed no intent to interact with DNA in gel mobility shift assays or footprinting, we created a new mutant, dTlp5. dTlp5, analogous to dTlp1 but with a smaller deleted region, was constructed in order to identify the residues responsible for the protein-DNA interaction more closely. The new mutant protein, dTlp5, lacking 13 amino acids (31–43; Fig. 3A) showed no evidence of binding compared to wild-type TlpA in mobility shift assay (Fig. 6, lanes 2 and 4). Lanes containing no protein or dTlp1 or dTlp5 looked indistinguishable (Fig. 6, lanes 1, 3, and 4).

Nonbinding Mutants Are as Helical as the Wild Type—The structural integrity of the mutant proteins, as compared to TlpA, was monitored using circular dichroism spectroscopy, to rule out massive perturbation of the folding as a cause for loss of biological activity. The CD spectra of dTlp1 (82% α-helical) and dTlp5 (82% α-helical) mutants are nearly indistinguishable from the wild-type TlpA (85% α-helical; Fig. 7), indicating that their α-helicity and most probably their coiled-coil forming potential remains intact despite the deleted segments. dTlp1 and dTlp5 Form Oligomers in Electron Microscopy—To assess whether the binding differences should depend on oligomer state and to see any differences in the morphology of higher order structure, the solutions of mutant proteins were examined for their capacity to form higher protein-protein organization in electron microscopy. TlpA as shown before (15) produced all levels of assembly where stacks of small fibers (data not shown) could be seen culminating in the filament network (Fig. 8A). dTlp1 also showed a similar propensity (Fig. 8B). Surprisingly we could not find filament networks of same morphology in the dTlp5 preparation, although it also was able to assemble into smaller filament stacks (Fig. 8C).

Transcription Assays Show Repressor Mechanism of TlpA Action—In order to ascertain the activity of TlpA on its promoter in vivo, transcriptional lacZ (β-galactosidase) fusions to the tlpA gene were constructed (Fig. 1, B and C). Since footprinting had shown that TlpA binds to promoter elements, we expected this to have an effect on transcription as well. Basal transcription levels of the fusions were measured in strains co-transformed with an isogenic plasmid construct containing no tlpA (Table I). The fusion construct pOF6 (Fig. 1B) contained only a portion of the tlpA reading frame, so that promoter activity could be measured without interference from TlpA. Construct pOF6 had a basal activity of 1170 Miller units (Table I), indicating that the fragment
preceding lacZ, indeed contains an active promoter. Fusion pOF14 capable of expressing TlpA, had a 6-fold reduced basal activity, of 155 Miller units (Table I) as compared to pOF6, indicating that full-length TlpA exerted a significant repressor activity on the tlpA promoter. As a further confirmation, TlpA effects were measured in trans with strains carrying a transcription fusion co-transformed with a plasmid expressing TlpA from its own promoter or a synthetic promoter (Table I). Fusion pOF6 showed a reduced activity when TlpA was present in trans of 245 units and at higher level TlpA production upon induction of the synthetic promoter of 50 units (Table I). dTlp5 on the other hand was not able to repress pOF6, supporting our in vitro data which showed a lack of interaction region in that protein (Fig. 6). The relative amounts of TlpA produced from a wild-type promoter or a synthetic tac promoter is roughly illustrated by whole cell lysates of E. coli harboring appropriate constructs. TlpA produced from tlpA promoter cannot be distinguished in Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis gels, whereas tac-directed TlpA expression shows massive production as indicated by the appearance of an intense TlpA band in the gel (15). The reason for using both sources of TlpA production was to exclude the possibility of interference of normal cellular function upon massive overproduction of TlpA which previously has been shown to literally pack the whole cell with this protein (15).

Transcription Assays Suggest in Vivo Chain Exchange—Transcription assays provided us with an opportunity to test whether TlpA would undergo chain exchange in vivo as has been demonstrated in vitro (15). As an indirect readout of heterodimer formation, we used the transcription activity of fusion pOF14 expressing full-length TlpA in trans supplemented with dTlp5. We reasoned that if brought in trans, a mutant TlpA containing a defective DNA-binding domain could activate the pOF14 transcription by forming heterodimers not
capable of repressing the tlpA promoter. Indeed, when brought in trans to pOF14, the nonbinding TlpA derivative, dTlp5, showed an 8-fold activation of that fusion from 155 to 1250 units (Table I). dTlp5 protein expressed from its native promoter on a multicopy plasmid can also be detected in Coomassie Blue-stained gels, probably due to the lack of the DNA-binding region and hence the inability to repress. This also indicates that there is a continuous supply of the dTlp5 protein available for heterodimer formation.

**DISCUSSION**

Previously we have described that *S. typhimurium* virulence plasmid codes for a coiled-coil protein TlpA which is a temperature-dependent chain-exchanging entity as shown in our in vitro system and is also able to form oligomeric structures resembling intermediate filaments in morphology (15, 22). The biological function (if any) of oligomerization remains at present unresolved, yet our new finding that TlpA is a gene regulator fits well with the flexibility offered by the monomer exchange phenomenon.

The sequence specificity of binding by TlpA is clearly demonstrated by footprinting and gel mobility shift assays. Binding was directed to specific target DNA, and could be competed only with a fragment containing this sequence (Fig. 4A). The inability to see a well resolved TlpA-DNA complex in gel mobility shift assays could indicate that protein-protein associations produce different sized oligomers (15) which dissipate the label throughout the running lane. The most apparent explanation for this is that a dimer binds DNA and undergoes higher oligomer interactions, producing complexes with different compositions. Then again, in vivo, the amount of TlpA may be so low that the issue of oligomerization may be redundant. Footprinting also showed a preferred region of interaction (Fig. 5) and identified the −10 and −35 elements in the broad protected region. Hydroxyl radical footprinting will hopefully shed more light on whether the large footprint is due to steric hindrance caused by TlpA or binding to several operator sites in the promoter region.

With our mutant-protein panel we have demonstrated the localization of the DNA-binding region with respect to the coiled-coil domain and also begun to probe the role of the latter in the binding. The only residues whose deletion leads to abolished DNA binding are those that map to the N-terminal portion of TlpA, adjacent to the predicted coiled-coil as delineated from lack of binding by dTlp1 and dTlp5 (Fig. 6). Deletions within the coiled-coil most adjacent to the DNA-binding region produced a different complex in the gel mobility shift assay as shown by dTlp2 and dTlp3 (Fig. 4). This can be interpreted as either a higher oligomer stabilization at the expense of any smaller complexes, or more likely as some loss of binding specificity, i.e. more protein is bound per DNA, suggesting coiled-coil serves a role in positioning the binding regions. Such an assumption is supported by the fact that dTlp4, with a considerable deletion in the coiled-coil but more distant to residues implicated in binding, showed a gel mobility shift pattern which was similar in appearance to that produced by the wild-type TlpA (Fig. 4). The capacity for oligomerization can be ruled out as a single key element of importance for binding since the DNA binding-deficient forms showed also a tendency for organized higher order protein-protein interactions. Based on the dominant negative phenotype of dTlp5 (and dTlp1, data not shown) we believe the binding structure to be minimally a dimer. From previous studies we can conclude that dTlp1 (referred to previously as the 41-kDa protein) (15) is able to form parallel unstaggered dimers with TlpA. dTlp5, which has a smaller deletion at the N terminus, shows a CD spectra nearly identical to TlpA and dTlp1, therefore we believe it also readily forms dimers (dTlp5 is rapidly oxidized into disulfide bridge dimers; data not shown). Also, the oligomer assembly of dTlp5 shows the capacity for formation of filament stacks in electron microscopy. It is unlikely, yet possible, that the inability to see a fully developed filament network in dTlp5 reflects an effect of the deleted residues, because dTlp1 with an overlapping but larger deleted segment shows all forms of oligomer arrangement. In any case, electron microscopy clearly shows that the fibrous appearance is intact, and this would not be expected in a randomly folded polypeptide preparation (nor would the highly helical CD spectra). Residues deleted in dTlp5 serve as a road map for identification of all of the critical residues which are needed for specific DNA interaction. We do not know to what extent the 13 amino acids are representative of the residues critical to function in the N-terminal DNA-binding region and whether the DNA-binding residues form an independent domain or are an extension of the coiled-coil. More detailed studies are underway to reveal the nature of the DNA-binding domain in TlpA, which could represent a novel combination of a DNA-binding structure coupled to a coiled-coil. At present we are unable to find any significant homology to known DNA-binding motifs in any part of tlpA sequence. Collectively our data indicates that TlpA consists of a functional outline where the N terminus is responsible for DNA binding and the adjacent long coiled-coil serves to dimerize the binding interfaces and position them for sequence specific contacts.

Transcription assays have shown that the region bound by TlpA contains an active promoter which can be repressed by TlpA in trans, but not by dTlp5 (Table I). These findings also set the stage for testing for in vivo chain exchange. Previously, we had shown that TlpA dimers at 37 °C are capable in monomer exchange with related partners, underscoring the dynamic nature of this protein (15). The transcription reporter construct pOF14 which is little active by itself, when propagated in a cell which also produces the DNA-binding mutant dTlp5, is activated to levels exhibited by the nonrepressed tlpA promoter. This can be easily explained if one considers that for every TlpA translated from the pOF14 transcript there is bound to be a dTlp5 partner protein for dimer formation or chain exchange between homodimers. Such heterodimers would be composed of one wild-type monomer with an intact recognition half-site and one monomer lacking this region. This
is analogous to eukaryotic CHOP or Id proteins which have a defective and a nonexistent DNA-binding domain, respectively, and form inactive heterodimers with other partners and thereby block transcription factors from binding to their targets (28, 29). The heterodimerization in the case of TlpA points also to the importance of the coiled-coil in organizing the structure into a binding proficient form.

A gene regulator such as TlpA would certainly have a role in the pathogenesis of virulent bacteria such as S. typhimurium, which is under constant pressure to sense its environment before entering the host, and while in the host as it progresses from one niche to another along its route of invasion experiencing changes in pH, temperature, and osmolarity (30). Coiled-coil structures which are known from many studies to respond to changes in the environment (31), could be ideal sensors to changes in the intracellular environment. An elevated temperature or osmolarity could affect the interactions within the coiled-coil domain and be sensed directly by the cytoplasmic TlpA. Temperature can of course influence TlpA activity, since it is analogous to eukaryotic CHOP or Id proteins which have a defective and a nonexistent DNA-binding domain, respectively, and form inactive heterodimers with other partners and thereby block transcription factors from binding to their targets (28, 29). The heterodimerization in the case of TlpA points also to the importance of the coiled-coil in organizing the structure into a binding proficient form.

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