The DNA-binding domain of human c-Abl tyrosine kinase promotes the interaction of a HMG chromosomal protein with DNA

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ABSTRACT

The biological activity of the c-Abl protein is linked to its tyrosine kinase and DNA-binding activities. The protein, which plays a major role in the cell cycle response to DNA damage, interacts preferentially with sequences containing an AAC motif and exhibits a higher affinity for bent or bendable DNA, as is the case with high mobility group (HMG) proteins. We have compared the DNA-binding characteristics of the DNA-binding domain of human c-Abl and the HMG-D protein from Drosophila melanogaster. c-Abl binds tightly to circular DNA molecules and potentiates the interaction of DNA with HMG-D. In addition, we used a series of DNA molecules containing modified bases to determine how the exocyclic groups of DNA influence the binding of the two proteins. Interfering with the 2-amino group of purines affects the binding of the two proteins similarly. Adding a 2-amino group to adenines restricts the access of the proteins to the minor groove, whereas deleting this bulky substituent from guanines facilitates the protein–DNA interaction. In contrast, c-Abl and HMG-D respond very differently to deletion or addition of the 5-methyl group of pyrimidine bases in the major groove. Adding a methyl group to cytosines favours the binding of c-Abl to DNA but inhibits the binding of HMG-D. Conversely, deleting the methyl group from thymines promotes the interaction of the DNA with HMG-D but diminishes its interaction with c-Abl. The enhanced binding of c-Abl to DNA containing 5-methylcytosine residues may result from an increased propensity of the double helix to denature locally coupled with a protein-induced reduction in the base stacking interaction. The results show that c-Abl has unique DNA-binding properties, quite different from those of HMG-D, and suggest an additional role for the protein kinase.

INTRODUCTION

The c-Abl proto-oncogene on human chromosome 9 encodes a non-receptor tyrosine kinase (1). The protein is primarily localised in the cell nucleus (2) and plays an important role in cell regulation. Overexpression of c-Abl causes cell growth arrest in G1 phase (3,4). Genotoxic drugs such as the antimetabolite ara-C and the DNA alkylating agent methylmethane sulphonate (MMS) activate c-Abl kinase activity leading to a complete G1 arrest in MCF-7 carcinoma cells (5). There is now strong evidence that c-Abl plays a role in the cell cycle response to DNA damage (6,7).

Substrates of c-Abl include the c-CRK adaptor protein (8), the protein tyrosine phosphatase SHPTP1 (9) and a group of proteins named Abi, for Abl interactor proteins (10,11). The growth suppression activity of c-Abl requires its tyrosine kinase activity and is highly dependent upon p53 (12). The retinoblastoma (Rb) protein (13) and Cdc2 kinase (6) may also be required.

Unlike other tyrosine kinases, c-Abl binds to DNA. This unique function of c-Abl is regulated during cell cycle progression. As cells enter mitosis, hyperphosphorylation of Ser/Thr residues inactivates DNA binding (14). The DNA binding properties of c-Abl are not well understood. Initially, it was shown that the protein binds to a palindromic element, EP, of the hepatitis B virus enhancer (15). A more recent study proposed that c-Abl binds essentially to A/T-rich DNA sequences via the cooperative action of three high mobility group 1-like boxes (HLB). The authors also suggested that c-Abl exhibits a higher affinity for bent or bendable DNA rather than A/T sequences per se (16). Recently, we showed that the DNA-binding domain of human c-Abl interacts preferentially with sequences containing an AAC motif. The c-Abl–DNA interaction mainly involves contacts within the minor groove of the double helix (17). The authors also suggested that c-Abl exhibits a higher affinity for bent or bendable DNA rather than A/T sequences per se (16). Recently, we showed that the DNA-binding domain of human c-Abl interacts preferentially with sequences containing an AAC motif. The c-Abl–DNA interaction mainly involves contacts within the minor groove of the double helix (17). The potential analogy between c-Abl and high mobility group (HMG)-1 proteins proposed by Miao and Wang (16) prompted
us to examine in more detail the DNA-binding characteristics of the DNA-binding domain of human c-Abl and a typical HMG protein. For this study, we selected the HMG-D protein from Drosophila melanogaster which binds preferentially to sites containing TTG/CAA steps and to distorted DNA structures (18), as is the case with c-Abl. The results reported here establish unequivocally that c-Abl has unique DNA-binding properties, quite different from those of HMG-D. Moreover, the investigation led to the unexpected finding that c-Abl enhances the binding of HMG-D to DNA, suggesting an additional role for the protein kinase.

MATERIALS AND METHODS

Proteins

HMG-D contains a single HMG domain followed by a basic region and an acidic tail (19,20). In the experiments presented here, we used the HMG-D100 construct which corresponds to the first 100 amino acids, i.e. the HMG domain plus the basic region but lacking the acidic tail. The HMG-D100 protein, overproduced in Escherichia coli, was purified according to a standard procedure (18) and stored at −20°C in 50 mM HEPES buffer pH 7.5, containing 1 mM EDTA and 50% glycerol. The procedure for the expression and purification of the DNA-binding domain of c-Abl has recently been reported (17,21). Briefly, the protein containing a GST portion and a (His)6 tail was expressed in E.coli strain JM109 by induction with IPTG and then purified using Ni columns.

Preparation of DNA fragments containing modified bases

Molecules containing normal or modified bases (having inosine, 2,6-diaminopurine, 5-methylcytidine or uracil in place of guanosine, adenine, cytidine or thymine, respectively) were synthesised by PCR using the primers 5'-AATTCGGTTACCTTTAA TC and 5'-TCGGGAACCCCCACCACGGG bearing a 5'-OH or 5'-NH2 terminal group so as to permit 5'-phosphorylation of one strand only. The template 160 bp tyrT(A93) fragment containing the E.coli tyrT promoter (22) was cut out of plasmid pKmp27 (23) by digestion with restriction enzymes EcoRI and AvaI. This template bore a 5'-phosphate due to the action of EcoRI and thus only the newly synthesised DNA (with normal or modified nucleotides) could be labelled by the kinase. Twenty amplification cycles were performed, each cycle consisting of the following segments: (i) for normal, M DNA, U DNA and DAP DNA, 94°C for 1 min, 37°C for 2 min and 72°C for 10 min; (ii) for I DNA and 1+DAP DNA, 84°C for 1 min, 30°C for 2 min and 62°C for 10 min. The purified PCR products were 5'-end-labelled with [γ-32P]ATP in the presence of T4 polynucleotide kinase and the labelled DNA was isolated by 6% polyacrylamide gel electrophoresis.

Gel retardation analysis

PCR-generated 5',32P-end-labelled tyrT(A93) DNA fragments (10 000 c.p.m., 1–5 ng) were incubated with the protein for 30 min at 4°C in 10 µl of the binding buffer [50 mM HEPES, pH 7.9, 100 mM KCl, 1 mM DTT, 5 mM MgCl2, 0.1 mM EDTA, 20% glycerol, 500–1000-fold poly(dI-dC)·(dI-dC), 1 mM PMSF] and then loaded directly onto a 6% polyacrylamide gel. After 120 min electrophoresis at 100 V at room temperature in 0.7x TAE buffer (4.7 mM Tris–HCl, 2.3 mM Na acetate, 0.7 mM EDTA, pH 7.9). Dried gels were scanned with a PhosphorImager.

Ligation experiments

Oligonucleotide 05, corresponding to the c-Abl specific binding site (17), or oligonucleotide 3A containing three repeats of six adenines separated by 5 bp were cloned between EcoRI and BamHI restriction sites of the pBS3SK vector. The XbaI–BamHI DNA fragment of plasmid pcDNA3 was then incorporated in the pBS3SK-05 or the pBS3SK-3A vector digested by the same restriction enzymes so as to obtain an EcoRI and an EcoRV restriction site on both parts of the cloned 05 or 3A sequences. The 58 and 74 bp DNA fragments used for the ligation experiments were obtained by EcoRI or EcoRV digestion, respectively, of the pBS3SK-05-modified plasmid, whereas the 68 and 84 bp DNA fragments were obtained by digestion with EcoRI or EcoRV restriction enzyme of the pBS3SK-3A-modified vector. The 121 bp length fragment was obtained by XhoI digestion of the pBS3SK-05 vector. In each case, the 5'-end-labelled DNA fragments were incubated in the presence of the test protein in 1x ligase buffer for 20 min at room temperature prior to adding 1 U of T4 DNA ligase (Life Science) for 15 min at room temperature and then denatured by heating for 15 min at 65°C. Samples were incubated with 30 U of exonuclease III (Boehringer) for 1 h at 37°C. Ligation products were resolved by electrophoresis on a 5% polyacrylamide gel.

Absorbance measurements

Absorbance measurements were performed with a Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. The quartz cuvette (10 mm path length) was placed in a thermostatically controlled cell holder. The measurements were performed at 260 nm in BPE buffer, pH 7.1 (6 mM Na2HPO4, 2 mM NaH2PO4, 1 mM EDTA) supplemented with 100 mM NaCl. Incubations with the test protein and the 56mer duplex were carried out in 1 ml solution directly in the quartz cell. Titrations were performed by adding aliquots of a concentrated protein solution to the DNA solution at a constant concentration (10 µM).

RESULTS

Circularisation experiments

Initial experiments were performed with the HMG protein alone to establish optimal conditions for the formation of DNA circles and to verify that the bound protein does effectively induce bending of the bound DNA molecules. A series of four radiolabelled DNA duplexes of 84, 74, 68 and 58 bp were prepared. Each fragment was incubated with DNA ligase for 30 min at room temperature with 50 nM HMG-D and the ligation products were separated by electrophoresis (Fig. 1A). In the absence of the protein, the ligation transformed the linear DNA molecules into linear dimers. Trimmers, tetramers and pentamers were also formed (not shown). In contrast, the same reaction carried out in the presence of HMG-D yielded circular DNA molecules which migrate more slowly than the linear DNA in the polyacrylamide gel. The reaction products were identified as circles by their complete resistance to digestion by exonuclease III. The formation of circles can be easily detected even with a substrate as short as 58 bp. This first set of experiments establishes unequivocally that HMG-D favours the formation of circular DNA and therefore attests that the HMG-D protein induces significant bending of the DNA, as anticipated. The effect of HMG-D is comparable, and perhaps even superior, to that of...
Figure 1. Circularisation experiments. (A) The 5’-end-labelled DNA substrate, 58, 68, 74 or 84 bp in length, was incubated for 30 min at room temperature without (−) or with (+) 50 nM HMG-D prior to adding 1 U of T4 DNA ligase. The band corresponding to circular DNA was identified by its resistance to exonuclease III. (B) The 121 bp fragment was incubated with increasing concentrations of HMG-D (µM as indicated). The lane marked ExoIII corresponds to the 121mer incubated with the HMG protein and the ligase and then treated with exonuclease III. This lane serves to identify the circular DNA product resistant to digestion by the exonuclease.

HMG-1, which permits the formation of circular products of 66 bp with duplex DNA fragments (24).

We repeated the experiment using a longer DNA fragment of 121 bp obtained by digestion of the pBS2SK-05 vector with XhoI. This fragment contains the c-Abl binding sequence. With this substrate, a tiny amount of circular product can be formed in the absence of HMG-D but the effect is extremely weak compared to what can be achieved in the presence of 25 or 50 nM HMG-D (Fig. 1B).

With the shortest fragment of 58 bp, the circular and linear DNA molecules are not well separated on native acrylamide gels. For this reason we selected the 68 bp duplex for subsequent experiments. The duplex fragment was incubated with 25 nM HMG-D in the presence and absence of the c-Abl protein at concentrations ranging from 10 to 300 nM. As in the earlier experiments, circular products resistant to exonuclease III were formed in the presence of HMG-D. The gel in Figure 2 shows that the yield of circle decreases in the presence of increasing concentrations of c-Abl. A concentration of ∼20 nM c-Abl is sufficient to reduce the formation of circular DNA species by 50% and at concentrations equal to 200 nM no more circles can be detected even when using 50 nM HMG-D. The majority of the radioactive material is recovered in the wells of the gel, reflecting the formation of higher order complexes (most likely higher molecular weight ligated DNA). These experiments suggest that c-Abl might bind particularly avidly to the small circles which are torsionally strained and thus have a propensity to form higher order complexes.

Figure 2. HMG-induced formation of circular DNA molecules is inhibited by c-Abl. The radiolabelled 68 bp fragment was incubated with 25 nM HMG-D prior to adding c-Abl at the indicated concentration (µM). The circular DNA products formed in the presence of the HMG protein are resistant to digestion by exonuclease III (lane marked ExoIII).

Competitive binding experiments

Next we investigated the effect of c-Abl on the binding of HMG-D to DNA and vice versa. A radiolabelled 56mer duplex containing the consensus binding sequence for c-Abl was used for these experiments. The substrate was incubated either with a fixed concentration of c-Abl in the presence of increasing concentrations of HMG-D or with a given concentration of HMG-D in the presence of increasing concentrations of HMG-D or with a given concentration of HMG-D in the presence of increasing concentrations of c-Abl. The results are presented in Figure 3. It can be seen that HMG-D has little, if any, effect on the binding of c-Abl, whereas under identical conditions, c-Abl significantly promotes the interaction of HMG-D with the DNA fragment. In the presence of 250 nM c-Abl, and also with higher concentrations, the yield of DNA–HMG protein complex is considerably increased. The results indicate that there is no direct competition between the two proteins for binding to DNA but, in contrast, c-Abl potentiates the interaction with HMG-D.

Protein binding to DNA substrates containing natural and modified nucleotide bases

The exocyclic groups of the DNA base pairs represent critical recognition elements for the binding of proteins and small molecules. In previous studies we have shown that the 2-amino group of guanine residues, which is the only hydrogen bond donor group exposed in the minor groove of DNA, governs the sequence-selective binding of a large variety of antibiotics and drugs (25). In addition we showed that this substituent can affect both the intrinsic curvature and the bendability of DNA as well as the recognition of DNA by a major groove-binding protein such as the E.coli factor for inversion stimulation (FIS) (26). In the major groove of the double helix, the 5-methyl group of thymines can also serve as a regulatory element for protein–DNA interaction. HMG-D is particularly sensitive to the presence or absence of the exocyclic methyl group of thymidine in the major groove since the removal of that group, achieved by T→U substitution, strongly promotes interaction of the chromosomal
protein with DNA (27). The study of DNA containing modified bases is a method of choice to compare the binding properties of the two proteins HMG-D and c-Abl.

A series of seven DNA fragments 160 bp in length containing either the canonical A-T and G-C base pairs or D-T, I-C, A-U or Mc-C-G base pairs were prepared by PCR using the methodology described previously (25,26). The various DNA fragments synthesised by this means differ only by the presence or absence of the purine 2-amino group in the minor groove or the pyrimidine 5-methyl group in the opposite major groove, the rest of the molecules being identical. The fragments, each uniquely labelled at the 5′-end, were incubated with c-Abl at concentrations ranging from 100 nM to 2 µM. The extent of protein binding varies significantly from one DNA species to another (Fig. 4). On the one hand, the two DNA species containing diaminopurine residues (DAP and I+DAP DNA) display very little interaction with c-Abl. Only a faint band corresponding to the protein–DNA complex was detected with 1 µM c-Abl. On the other hand, the two DNA fragments containing 5-methylcytosine residues seem to bind c-Abl much more strongly than does normal DNA. For example, with 100 nM c-Abl no protein–DNA complex was detected with the DNA containing canonical bases whereas a significant proportion of c-Abl–DNA complex is observed with the M and U+M DNA. Titration experiments revealed that the incorporation of 5-methylcytosine residues enhances the binding of c-Abl to DNA ∼3–5-fold. The substitution of inosine for guanosine residues also increases the interaction of c-Abl with the target sequence but the enhancement is less marked than that observed with the C→M replacement. Surprisingly, the incorporation of uridine seems to reduce slightly the interaction of c-Abl with DNA.

The purine 2-amino group apparently exerts a comparable effect on the binding of c-Abl and HMG-D. We have shown previously that deletion of the 2-amino group from guanosines significantly enhances the interaction of HMG-D with DNA (27). The effect was attributed to the increased flexibility of the DNA that results from G→I substitution. In agreement with this interpretation, we showed that addition of the 2-amino group to the adenine residues abolished the interaction of HMG-D with DNA, most likely as a result of a concomitant reduction in the flexibility of the DNA (27). The results obtained with c-Abl are comparable to those reported for HMG-D, but the situation is clearly different with regard to the major groove substitution.

Concerning the effects of U→C substitution, the results with c-Abl presented in Figure 4 differ profoundly from those obtained with HMG-D. Indeed, deletion of the 5-methyl group of thymidine (T→U substitution) significantly reinforces the interaction of HMG-D with DNA. In contrast, the addition of a 5-methyl group to the cytosine residues (C→M substitution) markedly decreases the capacity of HMG-D to interact with the DNA. A typical example of the gel shift obtained with the N, U, M and U+M DNA in the presence of HMG-D is presented in Figure 4. The results with the uridine-substituted DNA are in agreement with those previously published (27). It is striking to note that the binding of the protein to the methylated M DNA is significantly reduced whereas the interaction with the doubly substituted U+M fragment is strongly enhanced and even superior to that detected with the U DNA.

This set of experiments also establishes that c-Abl and HMG-D respond very differently to deletion or addition of the 5-methyl group of pyrimidine bases in the major groove. Addition of the methyl group favours the binding of c-Abl to DNA but inhibits the binding of HMG. In sharp contrast, deletion of the methyl group promotes the interaction of the DNA with HMG-D but diminishes its interaction with c-Abl. It is interesting how such a small group can exert quite opposite effects on protein–DNA recognition.
Figure 5. Gel retardation analysis of the interaction of HMG-D protein with normal and modified DNA containing uracil (U) and/or 5-methylcytosine (M) in place of thymidine and/or cytosine, respectively. The protein concentration (µM) is indicated at the top of each lane.

Competition experiments with modified DNA

As shown in Figure 5, HMG-D binds poorly to the DNA containing 5-methylcytosine residues. However, in the presence of c-Abl the interaction is considerably potentiated. With 0.1 µM c-Abl, a clear band corresponding to the HMG–M DNA complex can be detected whereas no retarded band was observed in the absence of c-Abl (Fig. 6). The reverse situation occurs with the DNA containing U residues. With the doubly substituted U+M DNA, an intense retarded band can be detected with HMG-D alone whereas the addition of c-Abl decreases HMG–DNA complex formation. At 0.5 µM c-Abl practically no complex between HMG-D and the U+M DNA is formed. With the uridine-containing DNA, the two proteins have antagonistic effects.

Spectrophotometric analysis

In parallel to the gel shift experiments, we measured the absorbance changes at 260 nm in the presence of HMG-D or c-Abl. The 56mer duplex fragment (as used in Fig. 3) was incubated with increasing concentrations of the two DNA-binding proteins. As shown in Figure 7, the absorbance of DNA at 260 nm remains practically unchanged in the presence of increasing concentrations of HMG-D or bovine serum albumin (BSA), used as a control. In contrast, the same experiments with c-Abl showed a marked increase in the absorbance of DNA bases. The hyperchromic effect is directly proportional to the protein concentration. The spectrophotometric measurements suggest that c-Abl disrupts the base stacking and perhaps induces a melting of the double helix, at least locally.

DISCUSSION

The aim of the present study was to compare the DNA binding properties of c-Abl and a typical HMG protein. Two years ago, Miao and Wang (16) proposed that mouse c-Abl contains three HMG-like domains responsible, at least in part, for the interaction of c-Abl with bent or easily bendable sequences. More recently, we showed that human c-Abl can bind not only to a variety of distorted DNA structures but also to standard duplex sequences containing an AAC triplet motif (17). The consensus binding sequence, $5'$-A¹C²AAC³AAC⁴C⁻, was deduced from CASTing experiments (21). Such a sequence also represents a preferred binding site for the HMG-D protein, which is an architecture-specific protein that preferentially binds to DNA containing the trinucleotide TTT/CAA (18). Thus there were good reasons to believe that c-Abl and HMG-D might share common DNA binding characteristics. In fact, the results reported here show that the two proteins have distinct DNA-binding properties. HMG-D induces DNA bending whereas c-Abl, which fails to bend DNA (17), binds tightly to HMG-induced bend structures such as circular DNA.

The deletion, deletion or relocation of the 2-amino group of guanine residues (by virtue of combined A→D and G→I substitutions) produces more or less equivalent effects on bending of the two proteins. With both c-Abl and HMG-D, adding a 2-amino group to adenines restricts access of the protein to the minor groove whereas deleting this bulky substituent facilitates the protein–DNA interaction. These effects are reminiscent of those previously described with another DNA-binding protein, FIS, and may be correlated with modification of the bending and flexibility of the target sequences (26). As discussed in previous studies (26–29), replacement of adenine with diaminopurine reduces DNA bendability whereas comparable inosine-containing DNA molecules show increased anisotropic flexibility. It is not surprising that the two conformation-sensitive proteins c-Abl and HMG-D respond equally well to these modifications of DNA structure and dynamics.

Figure 6. Influence of c-Abl on the binding of HMG-D to normal and modified DNA. In each case, the tyrT fragment containing canonical bases or U and/or M residues was incubated with 0.1 µM HMG-D without or with c-Abl at 0.1, 0.25 or 0.5 µM. Note that c-Abl facilitates the binding of HMG protein to normal and M DNA but decreases binding to U and U+M DNA.
In contrast, c-Abl and HMG-D respond very differently to changes in the position of the pyrimidine 5-methyl group exposed in the major groove. We did not expect that the two proteins, which both interact primarily with the double helix via the minor groove, would be affected differently by addition or deletion of a pyrimidine methyl group. The enhanced binding of HMG-D to DNA containing uridine residues in place of thymidines has been attributed to an enhancement of DNA flexibility. The enhancement of HMG-D binding to the tyrT DNA was observed with c-Abl but not with FIS. T→U substitutions can result in a weakening of the stacking interactions between A-U pairs and their neighbours arising from the loss of van der Waals contacts between the 5-methyl group of thymine and the C5 of the adjacent thymine in a TT step (30).

The facilitated binding of c-Abl to DNA containing methylcytosine residues may also be due to an indirect influence of the methyl group on the deformability of the double helix consequent upon protein binding. In contrast to HMG proteins, c-Abl does not bend its target sequence but nevertheless is sensitive to the deformability of the DNA. At present it is difficult to explain why the binding of c-Abl to DNA is enhanced when C residues are replaced with M residues. One possible explanation, as yet purely conjectural, is that the C→M substitution exaggerates the propensity of the double helix to denature locally, thus favouring the interaction of c-Abl with M-containing DNA. The results in Figure 7 show that c-Abl decreases the hyperchromicity of the DNA bases and therefore suggest that upon binding to DNA the protein may reduce the base stacking interaction or perhaps even induce a local opening of the double helix. This hypothesis warrants further investigation but it would certainly satisfactorily account for the results of the competitive binding experiments. Binding of c-Abl to DNA might cause local, restricted opening of the double helix which would then facilitate the interaction of HMG-D with the deformed c-Abl-bound structure. The appearance of unpaired bases is expected to increase DNA flexibility (31) and thus in itself would facilitate HMG-D binding to adjacent sequences.

As well as complementing our knowledge of the unusual DNA-binding characteristics of c-Abl, this study also has certain functional implications. It is quite plausible that in the cell c-Abl potentiates the action of HMG proteins. By promoting the binding of architectural proteins, c-Abl could significantly modify the access of various enzymes to DNA. We might mention the possibility that the growth arrest of cells in G1 phase due to overexpression of c-Abl could be the consequence of recruitment by c-Abl of DNA-binding proteins such as HMGs capable of reducing the action of enzymes implicated in DNA replication or transcription. The c-Abl-mediated increased binding of architectural proteins to DNA might also reduce the activity of DNA repair enzymes, thereby contributing further to the growth-suppressant activity of c-Abl.

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