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Characterization of a protein recognizing minor groove binders-damaged DNA

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ABSTRACT

By using electromobility shift assay (EMSA), we have identified a protein able to recognize the DNA only if it was previously reacted with minor groove binders. This protein binds with very high affinity AT containing DNA treated with minor groove binders such as distamycin A, Hoechst 33258 and 33342, CC-1065 and ethidium bromide minor groove intercalator, but not with major groove binders such as quinacrine mustard, cisplatin or melphalan, or with topoisomerase I inhibitor camptothecin or topoisomerase II inhibitor doxorubicin. This protein was found to be present in different extracts of human, murine and hamster cells, with the human protein which appears to have a molecular weight slightly lower than that of the other species. This protein was found to be expressed both in cancer and normal tissues. By using molecular ultrafiltration techniques as well as southwestern analysis it was estimated that the apparent molecular weight is close to 100 kDa. We can exclude an identity between this protein and other proteins, with a similar molecular weight previously reported to be involved in DNA damage recognition/repair, such as topoisomerase I, mismatch repair activities such as the prokaryotic MutS protein and its human homologue hMSH2 or proteins of the nucleotide excision repair system such as ERCC1, -2, -3 and -4.

INTRODUCTION

Anticancer agents which bind in the minor groove of DNA represent a new class of antineoplastic drugs under clinical investigation. CC-1065 and tallimustine belong to this class and both compounds have shown high antitumor activity *in vitro* and *in vivo* in murine tumors and in human tumors transplanted in nude mice (1–4). Their interaction with DNA has been characterized in detail and both revealed a high sequence-specific DNA interaction (5–7). For tallimustine in particular, alkylation of N3-adenine in the sequence 5′-TTTTGA has been reported, representing the highest sequence-specific interaction ever observed for a small molecule interacting with DNA (5). The mechanism by which the alkylation induced by tallimustine and CC-1065 lead to the cytotoxicity is still unclear. It has been postulated (8,9) that the very low number of alkylations produced by tallimustine and CC-1065 could hit crucial genomic sequences and, for example, block the DNA binding of transcription factors recognizing at least part of the sequence alkylated by the drug. This possibility is supported by the finding that *in vitro* tallimustine has been reported to be able to block the binding of transcription factors to their consensus sequence in DNA (8,9).

Another important factor determining the cytotoxicity of anticancer alkylating agents is the mechanism by which the DNA lesions are repaired. For drugs which alkylate DNA in the major groove, different mechanisms of repair have been characterized, some of which are specific for the drug–DNA adduct formed (10–13).

Relatively little is known about the repair mechanisms for the minor groove binders. Methyl- and ethyl-adducts on N3-adenine can be repaired by the enzyme 3-methyladenine glycosylase (3-MAG) which recently has also been shown to be able to repair adducts formed by melphalan (14). This enzyme, however, does not seem to play a role in the repair of adenine adducts formed by tallimustine and CC-1065, as the introduction of the bacterial gene encoding 3-MAG in different human cells did not change the sensitivity of these cells to the drugs (15).

Here we report the characterization and initial purification of a protein of ~100 kDa which recognizes DNA treated *in vitro* with minor groove binders. This protein does not recognize major groove binders/DNA complexes and thus appears to be specific for drugs which bind AT-rich regions in the minor groove.

MATERIALS AND METHODS

Drugs

All drugs were prepared as 20 mM stock solutions: distamycin A, tallimustine and CC-1065 were dissolved in dimethylsulfoxide, while cisplatin (DDP), melphalan (L-PAM), doxorubicin, camptothecin and ethidium bromide were dissolved in water. For further dilutions bidistilled-sterile water was used.

Cell lines

L1210 mouse leukemia and M5076 mouse reticulum cell sarcoma were transplanted intraperitoneally in CDF1 and C57Bl/6J mice respectively. Procedures involving animals and

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their care are conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 February 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

At day 5 (L1210) or 11 (M5076) after transplant, mice were sacrificed and cells recovered from peritoneum.

Jurkat, K562, U937, Molt-4 and Cem cells were grown in RPMI medium. CHO and LoVo in D-MEM medium. All the media were supplemented with 10% foetal calf serum

Protein extracts

Nuclear extracts were obtained from mammalian cells and tissues as described by Dignam (16) and protein concentration was measured by using the BioRad Protein Assay (BioRad).

Oligonucleotides

The synthetic oligonucloetides (Duotech), containing the tallimustine target sequence (underlined) were: upper strand: 5′-GATATTTTGATGTGGGCTGCA-3′; lower strand: 3′-ACGTCTATAAAACTACACCCG-5′. Lower and upper strands were annealed to give a 17 bp double-stranded DNA with a four bases overhang at each end which was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and purified through 12% non denaturing polyacrylamide gel.

For DNA affinity purification step, the upper strand oligonucleotide was synthesized (Duotech) with 3′-biotinylated adenine.

Electromobility shift assay (EMSA)

Aliquots of 0.5–1 ng of ^{32}P-labeled double-stranded oligonucleotide were incubated at room temperature with different concentrations of drugs in the presence of 3 µg poly(dI–dC) in 1× binding buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.01 M NaCl, 0.2 mM DTT, 1% glycerol); after 30 min nuclear extracts were added and incubation prolonged for further 30 min. The mixture was run in 5% polyacrylamide gel in 0.25× TBE for 1.5 h at 150 V. The gel was dried and autoradiographed.

Molecular ultrafiltration

Microcon (Amicon) at 30, 50 and 100 kDa membrane cut-off were used. L1210 nuclear extracts (20–40 µg) were added to the sample reservoir and centrifuged at 14 000 *g* to obtain desired final volume, then the retained fraction was recovered according to the manufacturer's instructions and assayed by EMSA.

Southwestern assay

Nuclear extracts were separated on 8% SDS–PAGE. After separation, proteins were electroblotted onto nitrocellulose membrane in transfer buffer (50 mM Tris, 100 mM glycine, 0.01% SDS, 20% methanol) for 2 h at 50 V. The nitrocellulose-blotted proteins, after Ponceau red staining, were *in situ* renaturated by 24 h incubation in renaturation buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 5% non-fat dried milk; 5 ml/cm^2) at 4°C with gentle shaking. The membrane was incubated in 0.5 ml/cm^2 of hybridization buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 0.25% non-fat dried milk) containing 10–20 ng ^{32}P-labeled DNA (preincubated with 250 µM distamycin A for 30 min at room temperature), 10 µg/ml poli(dI–dC) and 250 µM distamycin A for 4–6 h at room temperature with gentle rocking, washed three times in washing buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 0.25% non-fat dried milk and 250 µM distamycin A) for 20 min at room temperature and then autoradiographed.

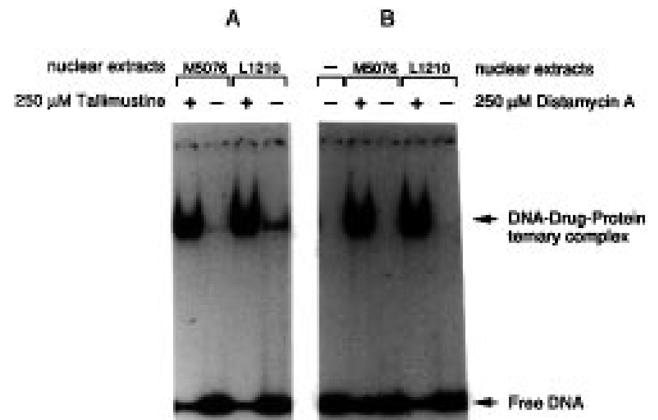

Figure 1. EMSA showing the formation of a retarded band upon incubation of nuclear extracts from M5076 and L1210 cells with labeled oligonucleotide pretreated with tallimustine (**A**) or distamycin A (**B**).

Immunoprecipitation

Jurkat nuclear extracts were incubated 1 h at 4°C with monoclonal antibody anti-human topoisomerase I (kindly obtained from Dr I. Scovassi, Pavia, Italy), then Protein A–Sepharose, prewashed three times with PBS, was added and the incubation was continued overnight at 4°C.

Supernatant was recovered and assayed by EMSA and western blot. Immunoprecipitated complexes were washed three times with PBS and resuspended in loading buffer (1× = 50 mM Tris pH 6.8, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol, 100 mM DTT).

Both supernatant and immunoprecipitated complex were fractionated onto 8% SDS–PAGE, electroblotted to nitrocellulose membrane, and topoisomerase I was detected by using specific antibodies and the ECL method (Amersham).

Ammonium sulfate precipitation

Cold saturated ammonium sulfate solution was added to the extracts on ice to obtain the desired final saturation percentage; the solution was chilled on ice for 30–60 min then centrifuged at 14 000 *g* for 30 min at 4°C. The pellet was resuspended in buffer D (20 mM HEPES pH 7.4, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA pH 8, 0.5 mM PMSF, 0.5 mM DTT, 0.5 µg/ml Aprotinin) and dialyzed against the same buffer. The amount of precipitated protein was determined by using the BioRad Protein Assay (BioRad) while the activity recovered was estimated by EMSA.

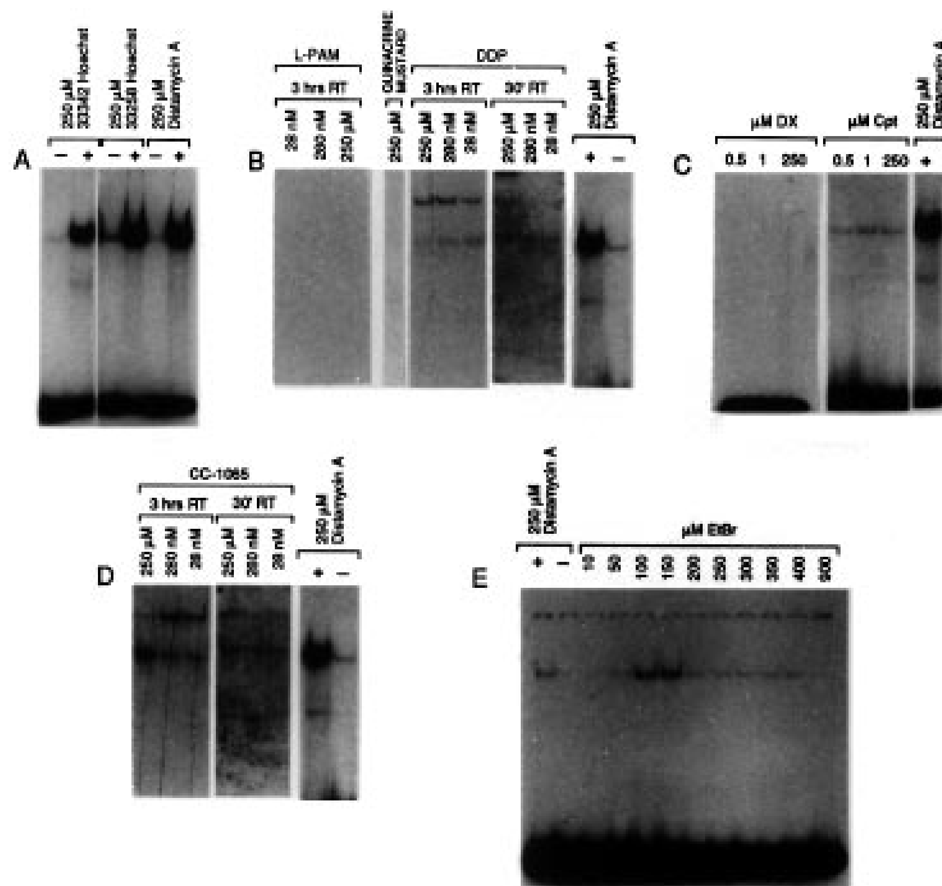

Figure 2. EMSA of labeled oligonucleotide incubated with 5 µg of Jurkat nuclear extracts in the absence (–) or presence (+) of different concentrations and times of incubations, as indicated, of Hoechst 33342, 33258 (**A**), L-PAM and DDP (**B**) DX and camptothecin (**C**), CC-1065 (**D**) and ethidium bromide (**E**). A lane containing DNA incubated with 250 µM distamycin A is present in each panel for comparison.

DNA affinity purification

The 17 bp biotinylated oligonucleotide was bound to M-280 Streptavidin Dynabeads (Dynal) according to the manufacturer's instructions.

The purification step was performed as follows: into 1.5 ml eppendorf, ~76 pmol of oligonucleotide-beads were treated with distamycin A (same molar ratio distamycin A–DNA used in EMSA) in 1× binding buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.01 M NaCl, 0.2 mM DTT, 1% glycerol) for 30 min at room temperature, then ~300–400 µg of nuclear extracts were added in the presence of poly(dI–dC) and the incubation was continued for 30 min at room temperature. After supernatant removal, the DNA–beads pellet was washed three times with 1× binding buffer–distamycin A and bound proteins eluted in three elution steps with 100 µl of 1× binding buffer containing 1 M NaCl.

The eluate obtained was dialyzed against buffer D and the amount of proteins recovered and their activity were determined as described above.

RESULTS

We selected an oligonucleotide containing the preferred alkylation site for tallimustine (TTTTGA) but no sequences known to be recognized by transcription factors. Using this oligonucleotide in EMSA, we observed a retarded band only when the oligonucleotide was incubated with tallimustine and nuclear extracts. Figure 1A shows the results of an experiment in which murine nuclear extracts (from L1210 and M5076 cells) were used with a concentration of tallimustine of 250 µM. Similar results were obtained when other oligonucleotides containing the same 'core' sequence (TTTTGA) but with either blunt ends or a longer sequence were used (data not shown). *In vitro* treatment of oligonucleotide with the non alkylating distamycin A (Fig. 1B) gave a similar retarded band. The formation of the complex (presumably between DNA, drug and protein/s) was dose-dependent and we found 250 µM to be the optimal concentration for both tallimustine and distamycin A. By increasing the concentration of both distamycin A and tallimustine >500 µM, we could not detect an increase in the formation of the retarded band but rather a decrease mostly due to an increase of DNA remaining in the well.

To investigate whether the formation of the retarded band was restricted to this class of compounds, we analyzed by EMSA the ability of different compounds interacting with DNA to induce the formation of the complex (Fig. 2A–E). Hoechst 33258 and 33342, which interact with DNA in the minor groove, behaved in the same way as distamycin A and at 250 µM induced the formation of a similar retarded band (Fig. 2A).

Conventional major groove alkylating agents such as L-PAM, DDP and quinacrine mustard (Fig. 2B) did not induce the

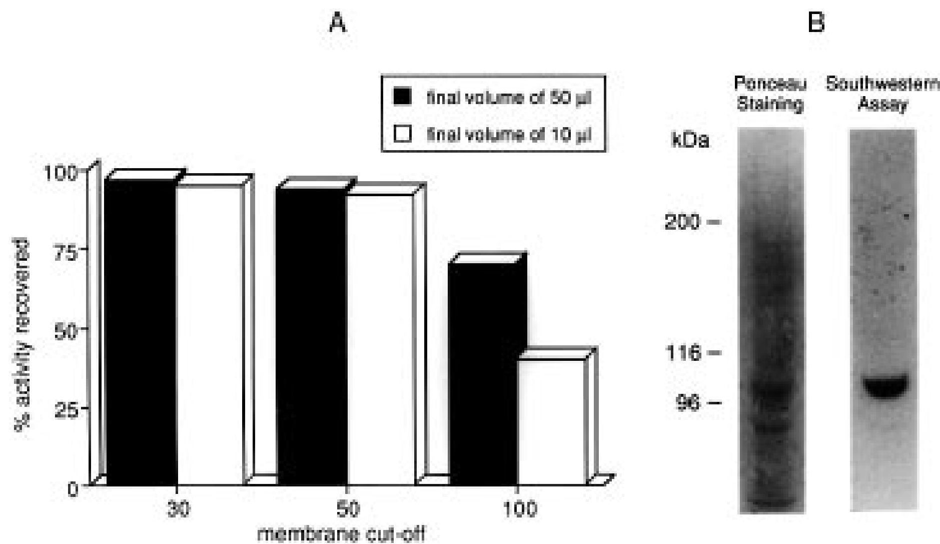

Figure 3. Estimation of apparent molecular weight of the protein. (**A**) Recovery of the activity of the protein after filtration through membranes at different cut-off. (**B**) Southwestern analysis of partially purified extracts. The lane stained with Ponceau before hybridization with oligonucleotide modified with 250μM distamycin A is also shown.

formation of the complex at the same concentrations used for distamycin A. Similarly neither topoisomerase I inhibitor (camptothecin) nor topoisomerase II inhibitor (doxorubicin) treatment of the oligunucleotide produced a retarded band (Fig. 2C). The N3-adenine alkylating agent CC-1065 was able to induce the formation of the complex at high concentrations (compared with those normally utilized to produce alkylations to N3-adenine in DNA fragments; Fig. 2D). Finally, another minor groove intercalator such as ethidium bromide was also able to induce the formation of a complex (Fig. 2E). These data altogether indicate that this is a protein recognizing DNA modified by minor groove binders. Although we did not carefully evaluate the affinity of the protein for the DNA modified by the different minor groove binders used, we estimated by densitometric scanning of the gels the percentage of DNA with a retarded migration induced after incubation with 250 μM of the different compounds. By analysing gels in which all the compounds were tested, the percentage of retarded DNA was very similar for all the examined compounds but ethidium bromide which was able to induce a greater formation of the retarded band (~2-fold greater than the other minor groove binders).

Two different approaches were used to determine the apparent molecular weight of the protein. Figure 3A reports the results obtained with the ultrafiltration with different membranes cut-off. As can be seen, only with the 100 kDa cut-off membrane was there a partial partition between the filtrate and the retained fraction.

To confirm these data we used southwestern analysis. Nuclear extracts were separated on SDS–PAGE, transferred to nylon filters and hybridized with the same oligonucleotide used for EMSA in the presence of distamycin A. A single signal corresponding to a band of ~100 kDa was found only in the lane hybridized with the oligo in the presence of 250 μM distamycin A (Fig. 3B).

The presence of this protein/factor recognizing DNA modified by minor groove binders was also demonstrated in different human cancer cells (Fig. 4). In all the nuclear extracts obtained from the human cell lines tested (Molt-4, K562, U937, Cem, Jurkat) we could observe the retarded band which, however, seemed to have a faster migration compared with that obtained from murine cells (this was particularly evident when the gels were run longer). In addition we observed the formation of the complex in normal murine tissues such as brain, spleen, kidney, thymus and lung, but not in liver (Fig. 5).

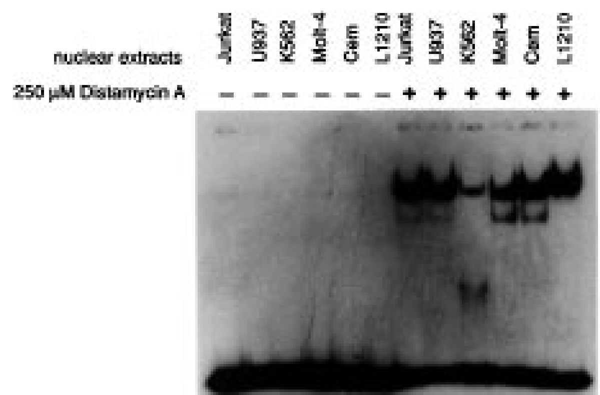

Figure 4. Presence of protein recognizing DNA modified by distamycin A in nuclear extracts from different cell lines. EMSA in the absence (–) or in the presence (+) of 250 μM distamycin A. Aliquots of 5 μg of nuclear extracts for each cell line were used.

Knowing the apparent molecular weight we tested the possibility that the activity we found was ascribable to proteins previously reported to be involved in damaged-DNA recognition/repair. Figure 6A reports the experiments performed using anti-topoisomerase I antibodies. Immunodepletion of topoisomerase I from nuclear extracts of Jurkat cells did not modify the amount of retarded band while the addition of human purified topoisomerase I to DNA–distamycin A complex did not result in the formation of any retarded band thus excluding that our activity

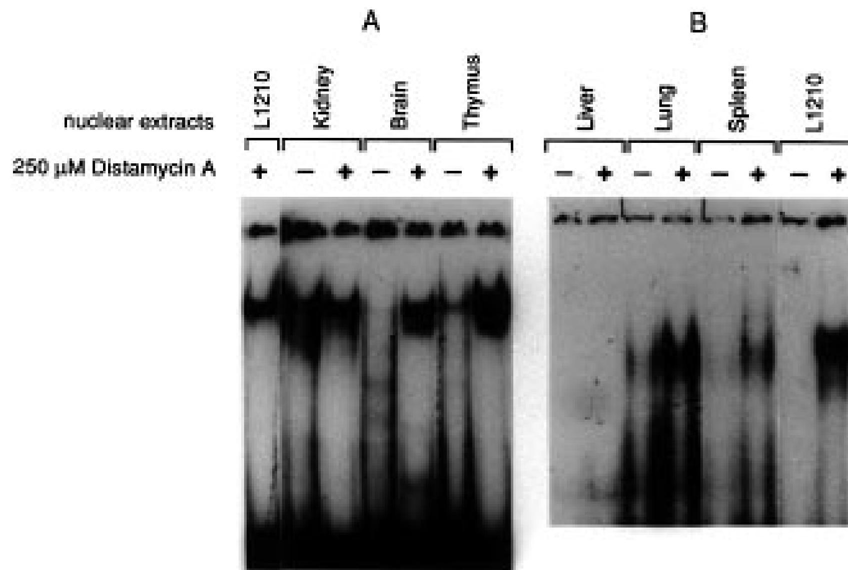

Figure 5. Determination of the activity of the protein in extracts from different murine tissue in the presence (+) or absence (–) of 250µM distamycin A.

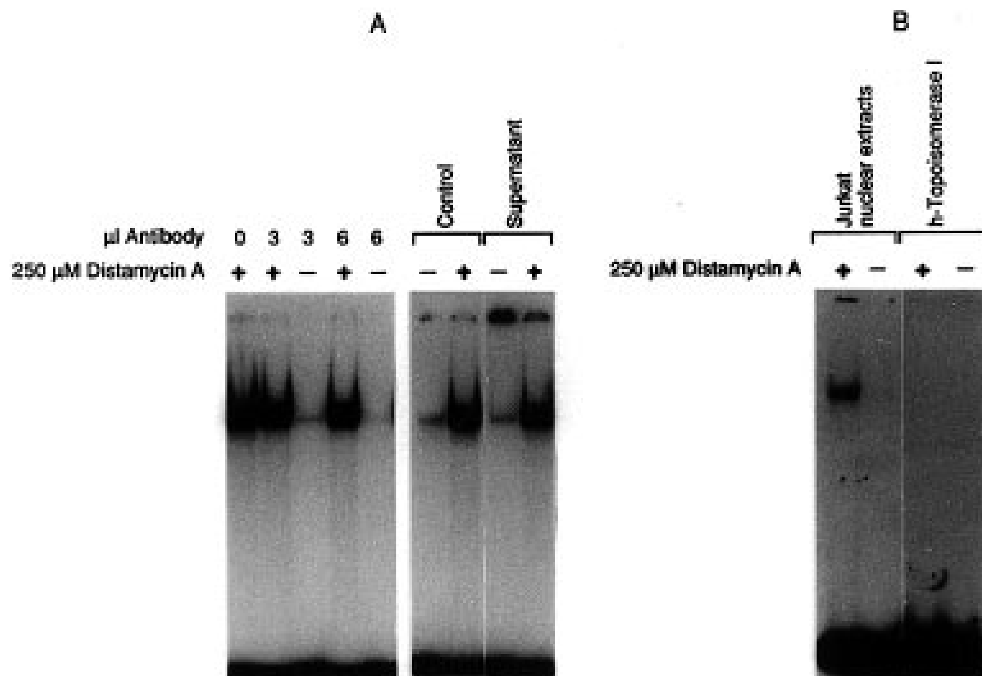

Figure 6. Jurkat nuclear extracts were incubated in the presence (+) or absence (–) of 250 µM distamycin A, with the addition of 3 or 6 µl of anti-topoisomerase I antibodies (**A**). In the same panel is reported the EMSA obtained from supernatant of Jurkat nuclear extracts obtained after immunoprecipitation with the same anti-topoisomerase I antibody. Control are extracts from Jurkat not immunoprecipitated. (**B**) EMSA with purified human topoisomerase I in the presence (+) or absence (–) of 250 µM distamycin A. The two lanes with total Jurkat nuclear extracts are reported for comparison.

can be attributable to topoisomerase I (Fig. 6B). We then used nuclear extracts from LoVo cells (which are deficient in the mismatch repair protein p100 hMSH2) and found that in this cell line we could detect a retarded band (Fig. 7A), again suggesting that our protein and hMSH2 are different. This was confirmed in experiments using the *Escherichia coli*-purified MutS protein (which is homologue to the human hMSH2) which was unable to recognize the DNA damaged by distamycin A (Fig. 7B).

We finally tested nuclear extracts obtained from different CHO-derived clones belonging to different excision repair complementing groups (ERCC1, -2, -3 and -4) involved in nucleotide excision repair. Figure 8 shows that in all the clones

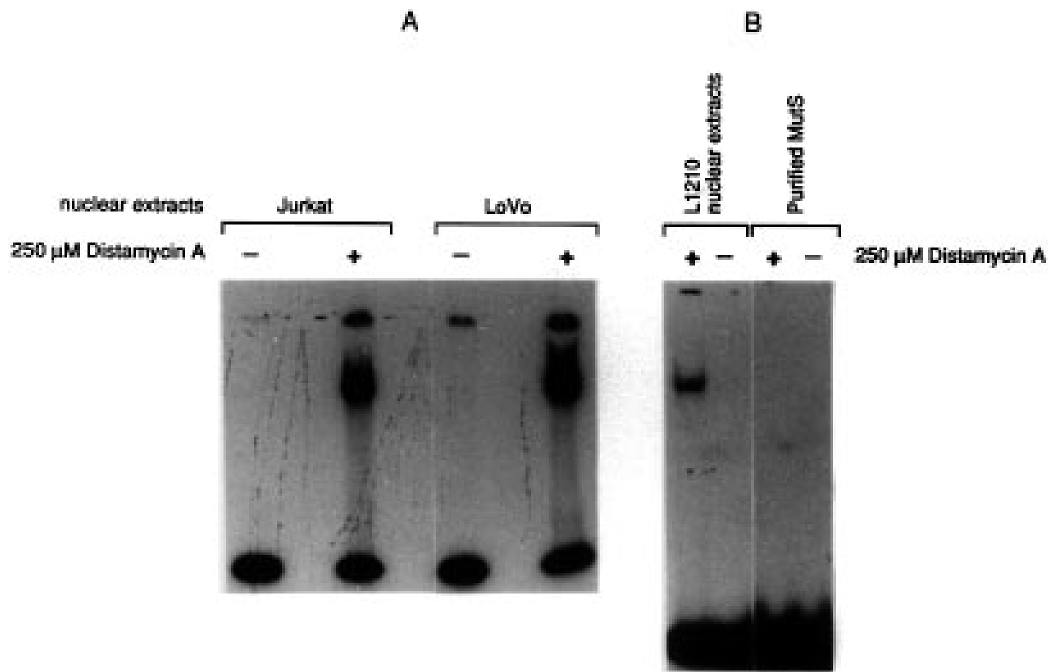

Figure 7. EMSA with nuclear extracts obtained from Jurkat or LoVo cell lines (**A**) or with purified *E.coli* MutS protein (**B**).

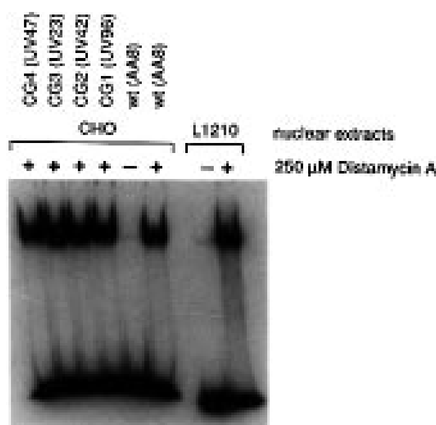

Figure 8. EMSA with nuclear extracts obtained from clones derived from CHO wild-type cells which are deficient in the expression of NER genes. For comparison the shift obtained with extracts from murine L1210 cells is reported.

(wild-type or mutant), we could detect a retarded band in EMSA, indicating that our protein does not belong to the four ERCCs and also that activity is detectable in hamster cells.

In order to sequence this activity for further molecular and functional characterization, we tried its purification from nuclear extracts obtained from murine L1210 or human Jurkat cells by using heparin–Sepharose chromatography, anion and cation exchange chromathography and hydrophobic interaction chromatography without obtaining a significant purification of the activity. By differential precipitation in ammonium sulfate, we obtained a 3-fold purification at 45% of ammonium sulfate. The 0–45% ammonium sulfate fraction has been used to purify the activity further using DNA-affinity. With this technique we lost most of the activity although some remained (as assessed also by southwestern analysis) and got a ~20-fold purification factor. With this purification we could not get any reliable sequence.

DISCUSSION

We have characterized a protein which is able to recognize DNA modified by compounds interacting with DNA in the minor groove. This protein migrates as a single retarded band in electromobility shift assays when nuclear extracts obtained from different cell types were incubated with DNA modified by minor groove but not major groove interacting agents. With the exception of ethidium bromide (which was slightly more efficient) all the different minor groove binders were able to induce roughly the same percentage of the retarded band at equal concentration.

We estimated, by southwestern analysis and ultrafiltration, the molecular weight ~100 kDa. Around this molecular weight, different proteins interacting with DNA and possessing repair activity have been characterized. Among these there is topoisomerase I which has been implicated in DNA transcription and repair (17,18). This protein was a strong candidate because of its affinity for AT-rich sequences in DNA, but we could exclude the identity between our protein and topoisomerase I. In addition, since in cells immunodepleted of topoisomerase I we could still detect a binding to damaged DNA, we even could exclude any possible interaction between topoisomerase I and this protein.

Other proteins, possible candidates for the activity we described, were excluded (for example proteins of the mismatch repair and of the excision repair). We have tested so far the strongest candidates but, of course, this does not exclude the possibility that other proteins known to participate in an indirect way to the repair of DNA lesions could have identity with our protein.

The protein was found to be expressed in almost all the cancer cells and normal tissues examined and we detected its activity in cells from humans, mice and hamster. The human protein bound to the modified oligo seemed to have a different migration on polyacrylamide gels which can be due either to a shorter amino acid sequence or to a different posttranslational modification.

It is interesting, although at present without interpretation, that the only tissue (among those examined) in which we could not detect the retarded band was the liver.

Since this protein specifically recognizes DNA damaged by minor groove but not major groove binders, it is attractive to speculate that it can be regarded as a totally new protein or eventually a known protein with a totally new function. In fact, to our knowledge no protein/s recognizing DNA damaged (not necessarily alkylated) by minor groove binders have been characterized.

Considering the emerging and promising activity of minor groove alkylating agents as anticancer agents (1–4,19), the knowledge of the possible repair/recognition mechanisms is important in order to improve their use. Purification and further characterization of this protein will therefore give us important clues for this relatively new class of compounds.

Its purification was very difficult because typical purificative steps did not give the expected results. For example, this activity cannot be considered a classic DNA-binding protein, since its DNA basal affinity is very low, and in fact, the commonly used transcription factors purification steps such as heparin–Sepharose chromatography, anion and cation exchange chromathography and hydrophobic interaction chromatography, were not able to produce a significative specific activity increment in this case.

We are at present trying some additional purificative steps to obtain sufficient purified material to be sequenced.

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