Demethylation of DNA by purified chick embryo 5-methylcytosine-DNA glycosylase requires both protein and RNA

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Received March 7, 1997; Revised and Accepted April 22, 1997

ABSTRACT

We have previously purified and characterized a 5-methylcytosine (5-MeC)-DNA glycosylase from 12 day old chick embryos [Jost, J.P. et al. (1995) J. Biol. Chem. 270, 9734–9739]. The activity of the purified enzyme is abolished upon treatment with proteinase K and ribonuclease A. RNA copurifies with 5-MeC-DNA glycosylase activity throughout all chromatographic steps and preparative gel electrophoresis. RNA with a length of ∼300–500 nucleotides was isolated from the gel purified enzyme. Upon extensive treatment with proteinase K, the gel eluted and labeled RNA did not show any significant change in molecular mass. The purified RNA incubated alone or in the presence of Mg²⁺ and deoxyribonucleotide phosphates had no 5-MeC-DNA glycosylase or demethylating activities. However, activity of 5-MeC-DNA glycosylase could be restored when the purified RNA was incubated with the inactive protein, free of RNA.

INTRODUCTION

There is strong evidence to suggest that the pattern of DNA methylation is vital for the normal development of vertebrates (1–5). It is believed that at least three different components are needed for the establishment of a specific methylation pattern: DNA methyltransferases, the demethylation system of DNA (passive and/or active) and the determination factors (cis and trans). DNA methyltransferases and the demethylation system are CpG or CpXpG specific but not strictly sequence specific whereas the determination factors should be sequence and developmental stage specific. Active DNA demethylation could be carried out by a 5-methylcytosine (5-MeC)-DNA glycosylase (6) or by nucleotide excision (7). In our present case we show evidence that RNA copurifies with 5-MeC-DNA glycosylase and that both RNA and protein are necessary for the activity of the enzyme.

MATERIALS AND METHODS

Purification of chick embryo 5-MeC-DNA glycosylase

Purification of crude nuclear extracts from 12 day old chick embryos, chromatography on heparin–sepharose, DEAE–sepharose, carboxymethyl (CM)–sepharose and affinity chromatography on DNA-Dynabeads were carried out as previously described (6). Following chromatography on CM–sepharose we included an additional purification step on butyl–sepharose. The ammonium sulfate sediment from the active fraction eluted from CM–sepharose was slowly dissolved in 1.2 M (NH₄)₂SO₄ containing 20 mM HEPES pH 7.5, 10 mM EDTA, 100 mM KCl, 2 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Non-solubilized protein was sedimented by centrifugation and discarded. The supernatant fraction, containing 10–15 mg protein was loaded onto a 10 ml column of butyl–sepharose 4B equilibrated with 1.7 M (NH₄)₂SO₄, 20 mM HEPES pH 7.5 and 1 mM DTT. The column was step-eluted with buffers containing 1.7, 1.5 and 1.3 M down to zero M (NH₄)₂SO₄. The main peak of activity eluting with 0.85 M (NH₄)₂SO₄, was precipitated with 0.47 g/ml of solid (NH₄)₂SO₄. The supernatant fraction, containing 10–15 mg protein was loaded onto a 10 ml column of butyl–sepharose 4B equilibrated with 1.7 M (NH₄)₂SO₄, 20 mM HEPES pH 7.5 and 1 mM DTT. The column was step-eluted with buffers containing 1.7, 1.5 and 1.3 M down to zero M (NH₄)₂SO₄. The main peak of activity eluting with 0.85 M (NH₄)₂SO₄, was precipitated with 0.47 g/ml of solid (NH₄)₂SO₄. The butyl–sepharose chromatography was followed by affinity chromatography on DNA-Dynabeads and preparative SDS–polyacrylamide gel electrophoresis exactly as described previously (6). The enzyme purified from the affinity column was precipitated with acetone. The 1 M NaCl eluate containing the active enzyme was diluted with water to 0.15 M NaCl and 4 vol acetone was added. Precipitation was carried out in silicone treated Eppendorf tubes and the precipitate was kept at −80°C until further use. Similarly the enzyme eluted from preparative gels with 0.15 M NaCl, 10 mM HEPES pH 7.5, 1 mM EDTA, 0.5% SDS, 1 mM DTT and 50 µg bovine serum albumin (BSA) (RNase and DNase free) was filtered.

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through a 0.45 µm Millipore Ultrafree filter and precipitated as indicated above, where indicated BSA was replaced by 100 µg dextran-T 70 as carrier. The enzyme was recovered from the acetone precipitate by centrifugation for 20 min at 12 000 r.p.m. in an HB-4 Sorval rotor at 2°C. The sediments were washed three times with cold acetone (acetone:water, 9:1 v/v) by vigorous vortexing and centrifugation at 17 000 r.p.m. for 5 min in an SS-34 Sorval rotor.

**Purification of other proteins**

The translation initiation factor eIF2γ was purified from 12 day old chick embryos ribosome as described by Stringer et al. (12). The crude 0.4 M KCl ribosome extract was fractionated by (NH₄)₂SO₄, dialyzed and chromatographed on a DEAE–sepharose column (12). Mismatch-specific thymine-DNA glycosylase was purified from HeLa cells as described by Neddermann and Jiricny (13).

**Enzyme assay**

The assays for 5-MeC-DNA glycosylase and G/T mismatch-DNA glycosylase were carried out as described previously (6). The hemimethylated, end-labeled oligonucleotide (only the upper strand is shown) 5'-GGATTTCCTGTCAGCGTGACm-CGGAGCTGAAAACACATTGACCCCGTG-3' was used as the standard substrate throughout all experiments. The reaction product was heat or alkaline denatured and analyzed on a 20% polyacrylamide–urea sequencing gel.

**Peptide sequencing**

The preparative SDS/10% PAGE was followed by electroblotting of the protein onto poly (vinylidene difluoride) membranes (Immobilon from Millipore). Tryptic digestion of the bands containing 5-MeC-DNA glycosylase activity was carried out according to Bauw et al. (14), essentially as described by Mayer et al. (15). Additional washing of the membranes with 0.8% Triton was included prior to blocking with poly(vinylpyrrolidone). The peptides were purified by reverse phase HPLC and sequenced as described (16).

**RNA purification and radioactive labeling**

The purified enzyme fraction was dialyzed against 0.15 M NaCl, 20 mM HEPES pH 7.5, 5 mM EDTA. SDS was added to a final concentration of 0.5% and the protein was digested for 1 h in the presence of 200 µg/ml proteinase K. Samples were extracted four times with phenol and chloroform and the RNA was ethanol precipitated. Upon sedimentation the RNA was dissolved in 0.2 M NaCl and precipitated twice with ethanol. RNA dissolved in sterile water was stored for short periods of time at –80°C or for longer times in liquid nitrogen. RNA was labeled in a total volume of 10 µl containing 30 µCi [γ-32P]dATP, 5 µl polynucleotide kinase, 5 mM DTT and 50 U ribonuclease inhibitor in 1× polynucleotide kinase buffer. Upon 30 min incubation at 37°C the reaction mixture was mixed with an equal volume of 2× loading buffer and fractionated on a 10% SDS–polyacrylamide gel.

**Chemicals and enzymes**

Benzamidine was purchased from Fluka AG (Buchs/SG, Switzerland), PMSF, Pefabloc, proteinase K, ribonuclease A (DNase free) and the inhibitor of ribonuclease were obtained from Boehringer Mannheim. Polynucleotide kinase and restriction enzymes were obtained from Biofinex (Praraun, CH-1724, Switzerland). Heparin–sepharose CL-6B, DEAE–sepharose, CM–sepharose fast flow and butyl–sepharose were obtained from Pharmacia Biotech Inc. Dynabeads–Spleptavidin were purchased from Milan Analytica AG (CH-1634, LaRoche, Switzerland). Collodion dialysis bags were from Sartorius AG (D-3400, Göttingen, Germany). [α-32P]dATP and [γ-32P]ATP triethylammonium (3000 Ci/mmol) were purchased from Amersham.

**RESULTS**

**Purified 5-MeC-DNA glycosylase is sensitive to proteinase K and ribonuclease A**

Recently it has been shown that the demethylation of DNA is dependent only on RNA and that an extensive proteinase K treatment of crude cell lysates from mouse myoblasts did not alter the enzymatic removal of 5-methylcytidine from a methylated DNA substrate (7). In sharp contrast the results presented in Figure 1 show that a treatment of the purified 5-MeC-DNA glycosylase (fraction eluted from heparin–sepharose and from CM–sepharose) with 100 µg/ml proteinase K abolishes the activity of the enzyme completely whereas the non-specific cleavage sites on the DNA substrate (7). In sharp contrast the results presented in Figure 1 show that a treatment of the purified 5-MeC-DNA glycosylase (fraction eluted from heparin–sepharose and from CM–sepharose) with 100 µg/ml proteinase K abolishes the activity of the enzyme completely whereas the non-specific cleavage sites on the DNA substrate (7). In sharp contrast the results presented in Figure 1 show that a treatment of the purified 5-MeC-DNA glycosylase (fraction eluted from heparin–sepharose and from CM–sepharose) with 100 µg/ml proteinase K abolishes the activity of the enzyme completely whereas the non-specific cleavage sites on the DNA substrate (7).
fluoride (Pefabloc) to all incubation mixtures of Figure 2 did not change the results. This strongly indicates that the decrease in 5-MeC-DNA glycosylase activity observed upon ribonuclease treatment may be due to the destruction of an enzymatically active RNA present in the 5-MeC-DNA glycosylase preparation.

It has been previously reported (6) that the gel purified 5-MeC-DNA glycosylase also had a G/T mismatch-DNA glycosylase activity (see also Fig. 4). As shown in Figure 3, the activity of the G/T mismatch-DNA glycosylase present in the preparation of 5-MeC-DNA glycosylase (fraction post CM–sepharose) is also sensitive to proteinase K (Fig. 3A) and to ribonuclease A (Fig. 3B).

**RNA co-purifies and co-migrates with highly purified 5-MeC-DNA glycosylase activity**

Since there was evidence that RNA might be essential for the activity of 5-MeC-DNA glycosylase it was reasonable to investigate whether the gel purified enzyme also contained RNA. Figure 4 shows that the two protein bands b and c eluted from the preparative gel (panel A) when denatured and slowly reanimated regained activity for both 5-MeC-DNA glycosylase and G/T mismatch DNA glycosylase (panels B and C). All other fractions eluted from the gel were inactive. The active enzyme eluted from bands b and c was pooled and acetone precipitated. RNA present in the precipitate was labeled as described in Materials and Methods. Labeled RNA was then separated on a 10% polyacrylamide–SDS gel typically used for the proteins. Lane 1 of Figure 5A shows that the labeled material migrated exactly at the position of the active enzyme (mw between 50 and 53 kDa) and an additional smaller band is visible at 44 kDa. Evidence that it is RNA is shown in lane 2 of Figure 5A where the labeled preparation was hydrolyzed for 30 min at 56°C in 0.1 M NaOH. Lane 3 of the same figure shows that following an extensive proteinase K treatment there was no significant shift in the migration of the labeled RNA. A control incubated with [γ-32P]ATP but in the absence of polynucleotide kinase gave no trace of labeling (Fig. 5A, lane 4) indicating that the enzyme eluted from the gel had no endogenous kinase activity. When the labeled RNA, migrating at the position of the 5-MeC-DNA glycosylase, was extracted, denatured and rerun on a denaturating polyacrylamide gel, an approximate length of 300–500 nt was obtained (Fig. 5B, lane 1). Some product of degradation of the labeled RNA was also seen below the main bands. Further tests carried out with the nucleic acids extracted from the 5-MeC-DNA...
In the experiment presented in Figures 1 and 2 it is evident that preparation alone has no catalytic activity. Purified RNA from 5-MeC-DNA glycosylase deoxyribonuclease I (Fig. 6, lanes 5 and 6) was sensitive to ribonucleases (Fig. 6, lanes 3 and 4) but not to glycosylase eluted from CM–sepharose indicated that it is different system such as ribonuclease P also requiring protein and the full activity of the 5-MeC-DNA glycosylase. However, in the presence of both protein and RNA are absolutely required for the full activity of 5-MeC-DNA glycosylase. Therefore, a combination of the purified RNA with the active enzyme did not result in any modification of its activity. From the above experiments it is obvious that both protein and RNA are required for the full activity of 5-MeC-DNA glycosylase.

RNA it was shown that RNA alone could regain full catalytic activity under certain conditions of high Mg$^{2+}$ concentrations (17). In Figure 7A, addition of more purified RNA to the already active enzyme did not result in any modification of its activity. When under the same incubation conditions, the same increasing concentrations of RNA alone were incubated in the absence of protein (panel B), no trace of 5-MeC-DNA glycosylase could be observed. A treatment of the reaction products with 0.1 M NaOH for 30 min at 90°C did not result in any specific cleavage of the labeled DNA template indicating that no removal of 5-MeC in the absence of phosphodiester bond cleavage had occurred (results not shown). Inclusion in the reaction of 10, 30 and 60 mM MgSO$_4$ (panel C) did not result in DNA cleavage in the presence of RNA alone and further incubation in the presence of all four deoxyribonucleotide phosphates had no effect on DNA demethylation (data not shown).

Figure 5. Labeling of the RNA from the pooled fractions b and c eluted from the SDS–PAGE shown in Figure 3. (A) The labeled RNA was separated on a 10% polyacrylamide–SDS gel as for the separation of the proteins. KDa are the protein size standards. Lane 1 shows the product of the polynucleotide kinase reaction. Lane 2 is the same as lane 1, except that the product of reaction was hydrolyzed with 0.1 M NaOH for 30 min at 56°C. Lanes 3 and 4 is the labeled product subjected to proteinase K treatment (2 mg/ml) for 30 min at 37°C. Lane 4 is the labeling reaction carried out in the absence of polynucleotide kinase. (B) The analysis of the RNA extracted from the boxed area of (A) on a 6% polyacrylamide–urea denaturing gel (lane 1). Lane 2 shows the polynucleotide size standards in bp.

Figure 6. Sensitivity of the labeled RNA to ribonucleases and deoxyribonuclease I. RNA present in the 5-MeC-DNA glycosylase eluted from the CM–sepharose column was labeled as outlined in Materials and Methods. Labeled RNA was separated on a 10% SDS–polyacrylamide gel as in Figure 5. Lanes 1, 3 and 5, RNA not denatured; lanes 2, 4 and 6, RNA denatured for 2 min at 95°C. Lanes 1 and 2 are the positive controls not treated with enzymes. Lanes 3 and 4, RNA treated for 1 h at 37°C with a mixture of ribonuclease A (10 µg/ml) and ribonuclease I (200 U/ml); lanes 5 and 6, RNA treated for 1 h at 37°C with deoxyribonuclease I (200 U/ml).

Glycosylase eluted from CM–sepharose indicated that it is sensitive to ribonucleases (Fig. 6, lanes 3 and 4) but not to deoxyribonuclease I (Fig. 6, lanes 5 and 6).

Purified RNA from 5-MeC-DNA glycosylase preparation alone has no catalytic activity

In the experiment presented in Figures 1 and 2 it is evident that the presence of both protein and RNA are absolutely required for the full activity of the 5-MeC-DNA glycosylase. However, in a different system such as ribonuclease P also requiring protein and glycosylase eluted from CM–sepharose column was labeled as outlined in Materials and Methods. Labeled RNA was separated on a 10% SDS–polyacrylamide gel as in Figure 5. Lanes 1, 3 and 5, RNA not denatured; lanes 2, 4 and 6, RNA denatured for 2 min at 95°C. Lanes 1 and 2 are the positive controls not treated with enzymes. Lanes 3 and 4, RNA treated for 1 h at 37°C with a mixture of ribonuclease A (10 µg/ml) and ribonuclease I (200 U/ml); lanes 5 and 6, RNA treated for 1 h at 37°C with deoxyribonuclease I (200 U/ml).

Reconstitution experiments with the protein free of RNA and the RNA free of protein could restore the 5-MeC-DNA glycosylase activity

From the above experiments it is obvious that both protein and RNA are required for the full activity of 5-MeC-DNA glycosylase. Therefore, a combination of the purified RNA with the inactive enzyme free of RNA should restore 5-MeC-DNA glycosylase activity. Figure 8, lane 1 shows a positive control of the active enzyme eluted from a CM–sepharose column. A ribonuclease A pretreatment of the same enzyme preparation results in the loss of 5-MeC-DNA glycosylase activity (lane 2). For details of the experiment see Figure 8 legend. The enzyme pretreated with ribonuclease A could not be restored by the addition of tRNA (Fig. 8, lane 3). The same results were obtained by the addition of the same amount of crude cellular RNA consisting mostly of rRNA (data not shown). The activity of the enzyme pretreated with ribonuclease A could be restored by the addition of the purified RNA (RNA purified from an active enzyme preparation) (Fig. 8, lanes 4 and 5). Lane 6 is a negative control (Fig. 8, lane 6).
control of the substrate incubated with BSA. Further reconstitution tests carried out with the same preparation of RNA, but with proteins smaller than 52 kDa (10–40 kDa) eluted from the 10% SDS-PAGE, did not yield any trace of 5-MeC-DNA glycosylase activity (results not shown).

DISCUSSION

The enzymatic activity of RNA either alone or in combination with proteins has been well documented. For example, the group I ribozymes and ribonuclease P are able to cleave single stranded DNA in vitro (19–21) and the group II ribozymes can also ligate DNA to RNA (22). Group II intron RNA can associate with a DNA endonuclease and cleave one strand of the DNA duplex while the protein associated with the DNA cleaves the other DNA strand in a site-specific manner (23). In the case of ribonuclease P the RNA alone catalyzes the reaction only in the presence of very high concentrations of Mg2+ (17) while in the presence of low concentrations of Mg2+ it requires the presence of a protein. Other enzymes such as mitochondrial DNA primase (24) and telomerase transferase (25) are also associated with RNA.

The 'demethylating' enzyme that we previously characterized as 5-MeC-DNA glycosylase (6) also requires RNA and protein for its catalytic activity. That RNA could be copurified with the enzyme activity through several chromatographic steps including gel electrophoresis (enrichment of enzyme activity is up to 50,000-fold) is an indication that the interaction between the RNA and the protein must be very strong. Even the presence of 1.7 M (NH4)2SO4 in the loading buffer of the butyl–sepharose column apparently did not dissociate the complex. However, the association of the RNA with the protein is most likely not covalent since an extensive proteolytic digestion of the complex of labeled RNA–protein with proteinase K did not result in a significant change in the electrophoretic mobility of the RNA (see Fig. 5). However, within the limits of error the detection of any smaller protein (mw 5 kDa) or peptide covalently linked to the RNA may not be possible. One could argue that the protein moiety of the enzyme is proteinase K resistant. This is unlikely to be the case since the enzyme activity is very sensitive to proteinase K. Since throughout the purification of 5-MeC-DNA glycosylase no special precaution was taken to protect RNA against nuclease degradation, it is quite possible that our highly purified enzyme is associated with only the 'core' RNA of a larger RNA molecule. Therefore, it is not yet possible to exclude the existence of a larger RNA having additional catalytic functions. Once the sequence of the 'core' RNA associated with the 5-MeC-DNA glycosylase is known, it will be of paramount importance to look in our original crude nuclear extracts for the presence of a larger RNA precursor. Such a putative larger RNA molecule should be tested then for other catalytic activities related to DNA demethylation. While using cell lysates, Weiss et al. (7) have identified the presence of RNA capable of replacing the methylated cytidine in the DNA by an unmethylated cytidine. However there are some major differences between their system and ours. For example they showed that the product of the reaction was insensitive to the cleavage with NaOH at the site of demethylation, suggesting the absence of abasic sugar which normally occurs during the glycosylase reaction. In this specific case it is possible that any abasic sugar is immediately cleaved by enzymes present in the crude cell lysate. Their results taken together also suggest that demethylation of DNA is taking place through a process of nucleotide replacement rather than the action of a 5-MeC-DNA glycosylase. Moreover another major difference between their results and ours is the relative insensitivity of their system to proteinase K. Any serious comparison of the two systems will have to await the cloning of the molecules responsible for the demethylation reaction.

The protein nature of 5-MeC-DNA glycosylase has remained very elusive so far. In spite of considerable efforts invested in the purification of the enzyme, no relevant sequence has yet been found. This is mainly due to the extremely low concentration of the enzyme present in the developing embryos and the chromatographic and electrophoretic behavior of the enzyme. For example, the activity eluted from preparative gels never gave a single sharp band and the fractions with the highest specific activity were always present on the gel as a diffuse band (Fig. 4A, band b). The sharp band c of Figure 4A has been further characterized by peptide sequencing. Nine oligopeptides had 100% identity with the translation initiation factor eIF2γ. We thought that this protein could either be part of the enzyme or it could be a major contaminant of 5-MeC-DNA glycosylase. The first possibility was tested by incubating increasing concentrations of the purified eIF2γ alone or with 5 µg of the purified RNA (same RNA preparation used for Fig. 7 experiments). The results clearly showed that eIF2γ is not the protein moiety of 5-MeC-DNA glycosylase (data not shown). Comparison of the results of Figures 1, 2 and 3 shows that the two enzymes activities of 5-MeC-DNA glycosylase and G/T mismatch-DNA glycosylase are copurifying and comigrating on the same position on SDS–polyacrylamide gels (see ref. 6 and Fig. 4). Both enzyme activities are sensitive to ribonuclease and proteinase K. Nedermaenn et al. (13) have isolated from HeLa cells a G/T mismatch-DNA glycosylase which has no demethylating activity. In our laboratory, all attempts to reconstitute 5-MeC-DNA glycosylase...
by combining the purified RNA from 5-MeC-DNA glycosylase and the G/T mismatch-DNA glycosylase purified from HeLa cells were negative, suggesting that the two enzymes isolated from chicken embryos and from HeLa cells are different (results not shown).

Obviously a different approach to purify the protein moiety of the enzyme is needed and the use of affinity resins containing the RNA may be one way to solve this problem.

ACKNOWLEDGEMENTS

We would like to thank Mrs Yan-Chim Jost for her technical assistance and for typing the manuscript. We are also grateful to Dr E. Oakeley, Dr J. Paszkowski and Dr W. Filipowicz for their critical reading of this manuscript.

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