Estradiol receptor potentiates, in vitro, the activity of 5-methylcytosine DNA glycosylase

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Abstract **At a concentration of 5×10^{-9} M of hemi-methylated DNA (one order of magnitude below the K_m), MCF-7 (a human breast carcinoma cell line) nuclear extracts potentiate the activity of 5-methylcytosine DNA glycosylase (5-MCDG, alias G/T mismatch DNA glycosylase). Depending on the ratio between MCF-7 nuclear extracts and 5-MCDG, there is an up to 10-fold increase in 5-MCDG activity. The potentiation of 5-MCDG by MCF-7 nuclear extracts requires an estradiol response element adjacent to the hemi-methylated site. Depletion of the estradiol receptor from MCF-7 nuclear extracts with specific antibodies abolishes the potentiation of 5-MCDG activity. The estradiol receptor present in MCF-7 nuclear extracts can be precipitated with antibodies directed against 5-MCDG. Reciprocally, antibodies directed against the estradiol receptor precipitate 5-MCDG. The results indicate the formation of a complex between the estradiol receptor and 5-MCDG.**

Key words: DNA demethylation; Targeting demethylation; Protein–protein interaction; Hemi-methylated DNA; Estradiol response element

1. Introduction

It has been shown that throughout vertebrate development there are dynamic changes in DNA methylation responsible for the establishment of specific methylation patterns [1,2]. In terminally differentiated cells, hormones can trigger changes in chromatin structure and DNA methylation of specific genes. For example, a single injection of estradiol given to immature chickens leads to the demethylation of the promoter region and the activation of the hepatic vitellogenin gene [3,4]. Similarly, in hepatoma cells, glucocorticoids induce DNA demethylation of the promoter region and the activation of the tyrosine aminotransferase gene [5,6]. In both cases, the local demethylation of the target gene promoters creates a priming and a developmental gene memory for subsequent hormone responses [3–6]. While studying the mechanisms of hormone-dependent DNA demethylation we observed that overexpression of 5-methylcytosine DNA glycosylase (5-MCDG) in human kidney cells resulted in the demethylation of an enhancer region composed of tandem repeats of retinoid receptor binding sites [7]. In these cells the retinoid receptor was physically associated with 5-MCDG (G/T mismatch DNA glycosylase). Such an association has already been described by others [8]. Similarly we show here, in vitro, that the estradiol receptor associates with 5-MCDG and potentiates the activity of the glycosylase only when the DNA substrate concentration is below the K_m.

2. Materials and methods

2.1. Assay of 5-MCDG

5-MCDG was tested as previously described [9]. MCF-7 nuclear extract and recombinant human 5-MCDG (alias G/T mismatch DNA glycosylase, EMBL, accession number U51166) were pre-incubated for 30 min on ice before the addition of the labeled DNA substrate. The concentration of the hemi-methylated substrate was 5×10^{-9} M (below the K_m of 5-MCDG which is 8×10^{-8} M). After 30 min incubation at 37°C, samples were diluted to 150 μl with water and treated with 25 μg proteinase K for 15 min. After phenol and chloroform extractions, samples were precipitated with ethanol. The sediments were dissolved in 10 μl 0.1 M NaOH, and hydrolyzed for 3 min at 95°C. After addition of 10 μl formamide loading dye, samples were heated for 1 min at 95°C and loaded onto a 20% polyacrylamide DNA sequencing gel. Table 1 shows the synthetic oligonucleotides used in the experiments.

2.2. Preparation of nuclear extracts from MCF-7 and HeLa cells

MCF-7 cells are derived from human breast carcinoma and contain high levels of the estradiol receptor whereas HeLa cells, a human uterine cervical carcinoma cell line, has no traces of the estradiol receptor. Nuclear extracts were prepared from semi-confluent cells using nuclear and cytoplasmic extraction reagents from Pierce. Alternatively, cells were fractionated as previously described [10].

The estradiol binding assay was carried out essentially as described by Best-Belpomme et al. [11].

2.3. Immunoprecipitations and Western blots

Specific antibody (1 μl) was added to a total reaction volume of 100 μl containing either 20 μg MCF-7 nuclear extract or 20 μg MCF-7 extracts pre-incubated on ice for 30 min with 0.25–2 μg of recombinant 5-MCDG. For the estradiol receptor we used a mixture of the three monoclonal antibodies AER311 and AER314 from Serotec, and NCL-ER-6711 from Novocastra Laboratories. The monoclonal antibody specific for G/T mismatch DNA glycosylase (5-MCDG) was YOHGL 2/99 from Serotec. The parallel controls received the same amounts of non-immune IgG. After 1–2 h incubation at 4°C, 1 μl of the second antibody (anti-mouse IgG) was added and the incubation continued on ice for 1 h. The immunoprecipitates were collected by centrifugation through a cushion of 400 μl 1 M sucrose prepared in phosphate-buffered saline containing 1% Triton. The centrifugation was for 20 min at $15\,000\times g$. The tubes were frozen in liquid nitrogen and the tips cut off and collected. The immunoprecipitates were either counted for radioactivity or further processed for immunoblots. For the immunoblots, the monoclonal antibodies were diluted according to the recommendations of the manufacturer. In specific cases the supernatant fractions were further incubated with the labeled DNA substrate and tested for 5-MCDG activity.

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2.4. Chemicals

[γ-^{32}P]ATP 3000 Ci/mmol and [2,4,6,7-^{3}H]17β-estradiol 120 Ci/mmol were obtained from Amersham. All oligonucleotides (methylated and non-methylated) were synthesized by Microsynth (Balgach, Switzerland).

3. Results

3.1. Nuclear extracts from MCF-7 cells potentiate the activity of 5-MCDG

At a concentration of hemi-methylated nucleotide below the K_m, MCF-7 nuclear extracts potentiated the activity of 5-MCDG. The potentiation was only observed at a specific ratio of MCF-7 nuclear extracts to 5-MCDG. Fig. 1 shows a titration curve of a constant amount of MCF-7 nuclear extract (20 μg) with increasing concentrations of 5-MCDG. The maximum stimulation is observed with 1 μg 5-MCDG (Figs. 1 and 2A, lanes 1 and 2). One of the possible reasons why is there a drop in 5-MCDG activity at higher concentrations of recombinant 5-MCDG may be due to the presence of some inhibitory bacterial proteins present in the preparation. As evidence for it, we tested different preparations of the recombinant 5-MCDG containing less bacterial protein (purified twice by affinity chromatography). These preparations gave a less prominent drop in activity when incubated at higher concentrations (results not shown). Similar results were obtained for G/T mismatch glycosylase while using oligonucleotide D from Table 1 (results not shown). MCF-7 extracts incubated alone with the hemi-methylated oligonucleotides showed only non-specific degradation of the substrate but no traces of the glycosylase activity (lane 3), thus excluding an additive effect between MCF-7 and 5-MCDG. The potentiation by MCF-7 nuclear extracts requires an estradiol re-

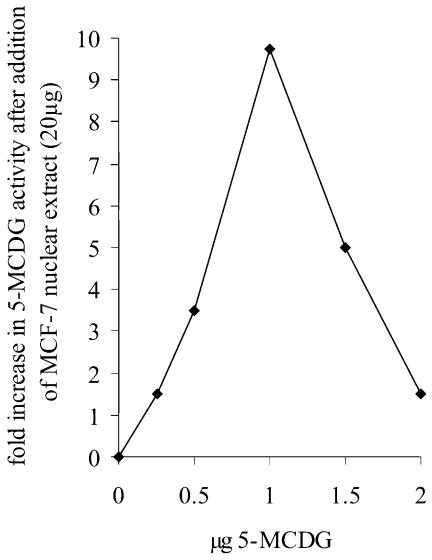

Fig. 1. Potentiation of 5-MCDG activity by MCF-7 nuclear extracts. A constant amount of MCF-7 nuclear extract (20 μg) was titrated with increasing concentrations of recombinant 5-MCDG produced in bacteria. The S.E.M. for each concentration point is about ±3% (three or four measurements per point).

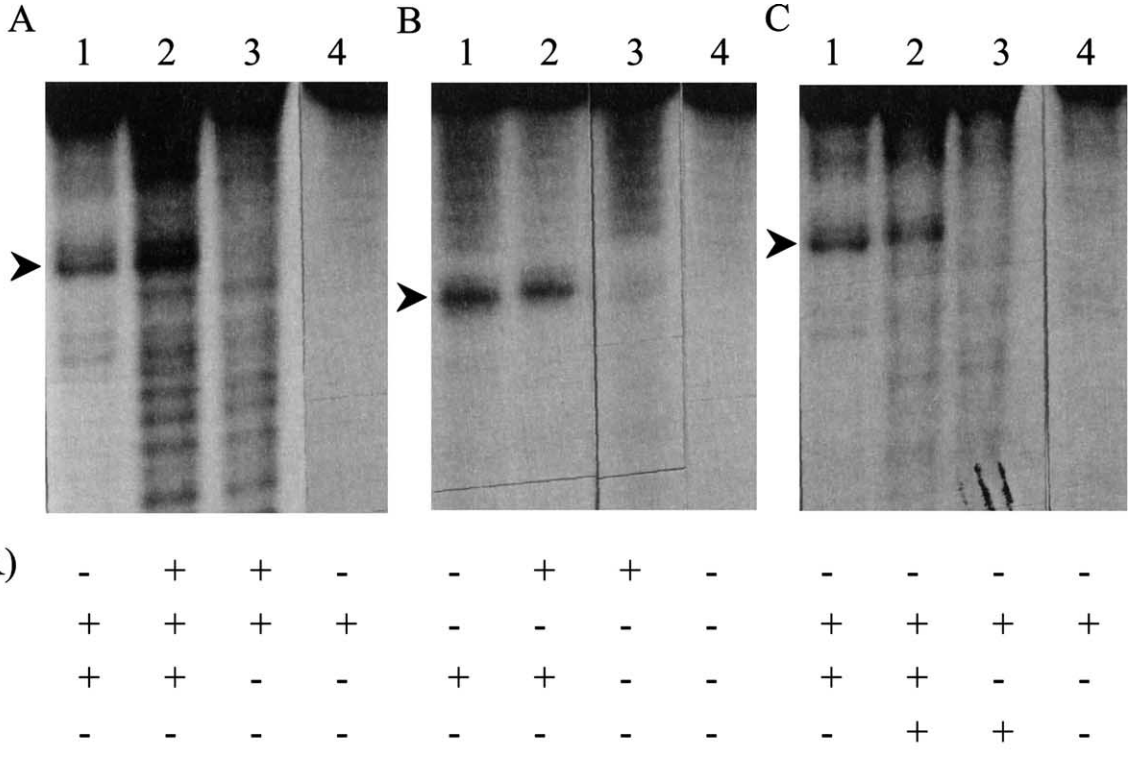

Fig. 2. Interaction between MCF-7 nuclear extracts and 5-MCDG. The reaction product was analyzed on a 20% polyacrylamide sequencing gel. The arrowhead shows the position of the reaction product of the glycosylase. In A the oligonucleotide substrate (oligonucleotide A) has an estradiol response element (ERE) and a methylated CpG. In B, the substrate has no ERE (oligonucleotide C) but one methylated CpG. In C, the substrate is oligonucleotide A but MCF-7 nuclear extracts have been replaced by HeLa extracts. Lanes 1 are the substrates incubated with 5-MCDG alone. Lanes 2 combine 5-MCDG with either MCF-7 nuclear extracts which contain estradiol receptors or HeLa nuclear extracts (which have no estradiol receptor). Lanes 3 are either MCF-7 or HeLa nuclear extracts incubated alone with the DNA substrate. Lanes 4 are the controls with DNA alone.

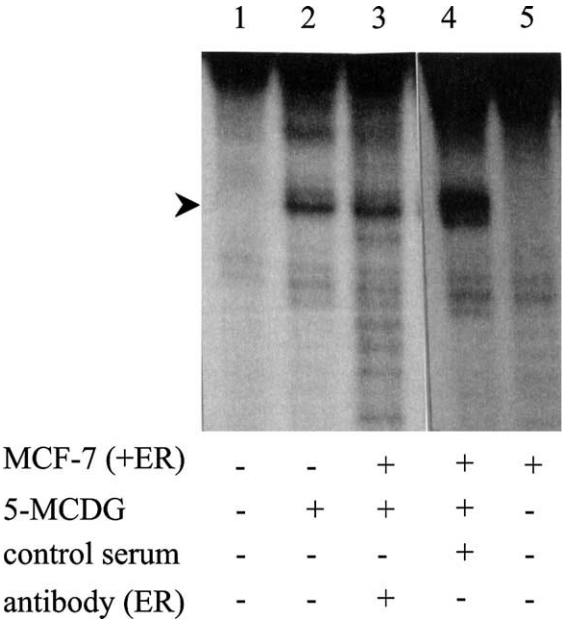

Fig. 3. Effect of a depletion of the estradiol receptor from MCF-7 nuclear extracts on the activity of 5-MCDG. The reaction product was analyzed on a 20% polyacrylamide sequencing gel. The arrowhead shows the position of the reaction product of the glycosylase. Lane 1 is the DNA substrate incubated alone. In lane 2, 5-MCDG is incubated alone with substrate A. In lane 3, MCF-7 nuclear extract has been treated with antibodies against the estradiol receptor and incubated with 5-MCDG and substrate A. Lane 4 is the same as lane 3 except that the MCF-7 nuclear extracts were treated with non-immune IgG. Lane 5 is a control of non-treated MCF-7 nuclear extracts incubated with DNA substrate A.

sponse element GGTCAxxxTGACC present in the vicinity of the hemi-methylated CpG site. Fig. 2B clearly shows that in the absence of the estradiol response element (oligonucleotides B or C from Table 1) there is no potentiation of 5-MCDG by MCF-7 nuclear extracts (Fig. 2B, compare lanes 1 and 2). These results indicate that the potentiation of 5-MCDG by MCF-7 nuclear extract may require the estradiol receptor. When 5-MCDG is incubated with an oligonucleotide duplex containing an estradiol response element but with a human cell extract devoid of the estradiol receptor (such as HeLa cell nuclear extracts), no potentiation takes place (Fig. 2C, compare lanes 1 and 2). Taken together, these results strongly indicate that the estradiol receptor may be responsible for the potentiation of 5-MCDG activity.

Table 1
Sequences of the oligonucleotides used as substrates (only the methylated strand is shown)

	ERE
A	5'CACGGGATCAATGTGTTCTTTCAGCTCMGGTCAGCGTGACCAGGAATACC3'
B	5'CACGGGATCAATGTGTTCTTTCAGCTCMAATATATATAAAAAGGAATACC3'
C	5'ATATATATATATATATATATATATATCMGGATATATATATATATATATAT3'
D	5'CACGGGATCAATGTGTTCTTTCAGTTCCGGTCACGCTGACCAGGAATACC3'
	ERE

Oligonucleotide A contains an estradiol response element (ERE) next to the methylated CpG. Oligonucleotides B and C have one methylated CpG but no ERE. Oligonucleotide D has one ERE and one G/T mismatch (underlined).

3.2. The estradiol receptor present in MCF-7 nuclear extracts is responsible for the in vitro stimulation of 5-MCDG

Preliminary experiments indicated that monoclonal antibodies against the human estradiol receptor could form a complex with the native receptor without inhibiting the potentiation of 5-MCDG activity. Consequently, it was necessary to first deplete the MCF-7 extracts of the estradiol receptor and then incubate them with 5-MCDG. This was achieved as outlined in Section 2. The results given in Fig. 3 (compare lanes 2 and 3) clearly show that after depletion of the estradiol receptor from MCF-7 nuclear extract, no potentiation of 5-MCDG took place. There is, however, a higher background for lanes 4 and 5 of Fig. 3 than for lane 3. This is not due to a longer exposure time of the autoradiograms. As seen in lane 5 an incubation of the labeled substrate with MCF-7 nuclear extracts gives a background of non-specific degradation. When the complex of MCF-7 and 5-MCDG is precipitated with antibody against the estradiol receptor and sedimented through sucrose there is less DNase present in the supernatant fraction. In contrast, in the absence of precipitation (non-immune serum) there is a higher level of non-specific degradation of the substrate. Further tests indicated that the stimulation of 5-MCDG by MCF-7 nuclear extracts did not require the presence of 17β-estradiol and that a recombinant estradiol receptor produced in bacteria was far less potent than the estradiol receptor present in nuclear extracts from MCF-7 cells (data not shown).

3.3. 5-MCDG is physically associated with the estradiol receptor

As a first test for a possible association of the estradiol receptor with 5-MCDG, the estradiol receptor present in MCF-7 nuclear extracts was labeled with 10^{-8} M [^{3}H]17β-estradiol and further incubated with increasing concentrations

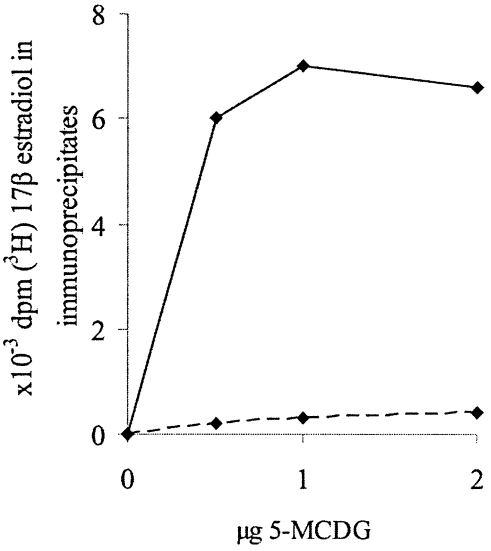

Fig. 4. Precipitation of [^{3}H]estradiol-labeled MCF-7 nuclear extracts with antibodies against 5-MCDG (G/T mismatch DNA glycosylase). The solid and dotted lines represent the precipitation of [^{3}H]estradiol-labeled MCF-7 and HeLa nuclear extracts respectively. Experiments were carried out as outlined in Section 3. A constant amount of labeled nuclear extract (20 μg) was titrated with increasing concentrations of recombinant human 5-MCDG. The average S.E.M. for each concentration point is about ±5% (average of three measurements).

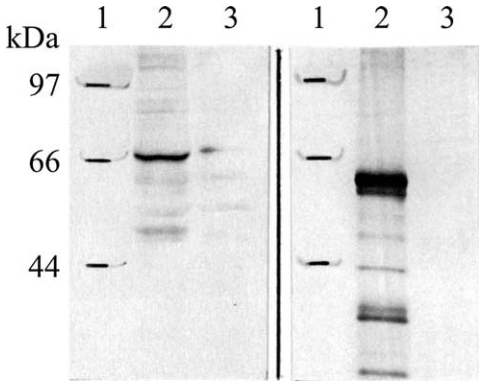

Fig. 5. Immunoprecipitation and Western blot of the estradiol receptor–5-MCDG complex. In the left hand panel the precipitation of the complex was carried out with anti-G/T mismatch DNA glycosylase and the Western blot was carried out with estradiol receptor antibody. In the right hand panel, the precipitation was done with an antibody against the estradiol receptor and the Western blot carried out with an antibody against G/T mismatch DNA glycosylase. Lanes 1 are the molecular weight size markers. The specific immunoprecipitations are shown in lanes 2. In lanes 3 the immunoprecipitation was carried out with non-immune reacting IgG.

of recombinant 5-MCDG. After 1 h incubation on ice to allow the formation of a complex, the reaction mixture was immunoprecipitated with antibodies against 5-MCDG as outlined in Section 2. Fig. 4 shows that an estradiol binding protein (presumably the estradiol receptor) co-precipitated with 5-MCDG. The same experiment repeated with HeLa nuclear extracts which have no estradiol receptor gave no precipitate (dotted line of Fig. 4).

The immunoprecipitates were further characterized by Western blots. Fig. 5 (left hand panel) shows that the protein that co-precipitates with 5-MCDG is indeed the estradiol receptor. The reciprocal immunoprecipitation was carried out with antibodies against the estradiol receptor. Fig. 5 (right hand panel) shows that the protein which co-precipitates with the estradiol receptor is 5-MCDG.

4. Discussion

We have previously shown that 5-MCDG and G/T mismatch DNA glycosylase activities share the same protein [12]. We also know that this protein can associate with the retinoic acid receptor [7,8]. Such an association has been shown, in kidney cells, to be instrumental for the selective demethylation of a methylated transgene containing repeats of retinoic acid receptor binding sites [7]. Our present results also indicate that the association of the estradiol receptor with 5-MCDG may explain the specific targeting of DNA demethylation by estradiol-controlled genes. As we have previously observed, in vivo demethylation of the estradiol-controlled vitellogenin gene starts in the vicinity of the estradiol response element [4] and an association of 5-MCDG with the estradiol receptor could possibly target this reaction more precisely. Concomitant with the demethylation reaction of the vitellogenin promoter, there are also changes in chromatin structure [13–15]. Since the same protein has so far two different catalytic functions (the removal of 5-methylcytosine and the correction of G/T mismatches) it is conceivable that the two reactions also share the same enzymes (AP lyase, DNA polymerase β, and DNA ligase) as the heterochromatin opens up and becomes associated with transcription factors. In addition, one cannot exclude the possibility that 5-MCDG (alias G/T mismatch DNA glycosylase) may also be part of the transcription machinery. For example, it has been shown recently that G/T mismatch DNA glycosylase associates with the transcription coactivators CMP and p300 acetylase [16]. In this system, G/T mismatch DNA glycosylase stimulates CBP transcriptional activity in transfected cells and serves as a substrate for CBP/p300 acetylation [16]. On the other hand, the estradiol receptor can be associated with other proteins, causing alterations of large-scale chromatin structures [17]. In this context it is not surprising that 5-MCDG (alias G/T mismatch DNA glycosylase) associates with the estradiol receptor forming a complex that could selectively target DNA repair and demethylation of genes in an open structure of chromatin.

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