The Unmethylated State of CpG Islands in Mouse Fibroblasts Depends on the Poly(ADP-riboyl)ation Process

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In vivo and in vitro experiments carried out on L929 mouse fibroblasts suggested that the poly(ADP-riboyl)ation process acts somehow as a protecting agent against full methylation of CpG dinucleotides in genomic DNA. Since CpG islands, which are found almost exclusively at the 5’-end of housekeeping genes, are rich in CpG dinucleotides, which are the target of mammalian DNA methyltransferase, we examined the possibility that the poly(ADP-riboyl)ation reaction is involved in maintaining the unmethylated state of these DNA sequences. Experiments were conducted by two different strategies, using either methylation-dependent restriction enzymes on purified genomic DNA or a sequence-dependent restriction enzyme on an aliquot of the same DNA, previously modified by a bisulfite reaction. With the methylation-dependent restriction enzymes, it was observed that the HpaII tiny fragments greatly decreased when the cells were preincubated with 3-aminobenzamide, a well known inhibitor of poly(ADP-riboyl) polymerase. The other experimental approach allowed us to prove that, as a consequence of the inhibition of the poly(ADP-riboyl)ation process, an anomalous methylation pattern could be evidenced in the CpG island of the promoter fragment of the Hif9 gene, amplified from DNA obtained from fibroblasts preincubated with 3-aminobenzamide. These data confirm the hypothesis that, at least for the Hif9 promoter region, an active poly(ADP-riboyl)ation protects the unmethylated state of the CpG island.

During pre-implantation development, most DNA sequences undergo extensive demethylation. This unmethylated state is maintained through the blastula stage to the time of embryo implantation, when a burst of de novo methylation generates a bimodal pattern, characterized by unmethylated CpG islands versus the bulk of genomic DNA, which is highly methylated (1). A problem yet to be solved is the identification of different cis-acting signals and trans-acting protein factors that may play a key role in defining the bimodal pattern of methylation involved in cell differentiation and gene expression.

Our previous in vitro experiments, carried out with the aim of individuating chromatin proteins involved in determining and/or maintaining in some way the DNA methylation pattern, have shown that histone H1 (2, 3), through its variant H1e (4, 5), is a chromatin protein that is able to greatly inhibit (>90%) methylation of double-stranded DNA. Moreover, gel retardation experiments have emphasized that H1e is the only variant able to bind CpG-rich sequences, both unmethylated CpG-rich double-stranded oligonucleotides and double-stranded DNA purified from chromatin fractions enriched in CpG islands. Further experiments have evidenced that the inhibition of in vitro enzymatic DNA methylation by histone H1 was essentially due to the poly(ADP-riboyl)ated isoform of this protein and/or to the long and branched protein-free ADP-ribose polymers (6).

In vivo experiments carried out on L929 mouse fibroblasts preincubated for 24 h with or without 8 mM 3-ABA,1 a well known inhibitor of poly(ADP-riboyl) polymerase, confirmed the existence of a negative correlation between poly(ADP-riboyl)ation and DNA methylation processes. A block of the poly(ADP-riboyl)ation process allowed, in the isolated nuclei, a considerable increase in the susceptibility of DNA to methylation by endogenous DNA methyltransferase. Subsequent methylation by exogenous enzymes was, as a consequence, severely reduced.

The aim of this paper was to verify if, in cells preincubated with 3-aminobenzamide, the DNA methyltransferase becomes able to modify the unmethylated state of CpG islands, which normally remain untouched by the action of DNA methyltransferase even though they are located in the promoter region of housekeeping genes (7–9), which are permanently accessible to the transcription factors. Our results, obtained using two different experimental approaches, allowed us to observe that the block of the poly(ADP-riboyl)ation process modifies the methylation pattern of CpG islands in general and in particular introduces an anomalous methylation pattern in the Hif9 promoter region (10) when it is purified from cells treated with 3-aminobenzamide. These data confirm the hypothesis that, at least for the Hif9 promoter region, an active poly(ADP-riboyl)ation protects its unmethylated state.

EXPERIMENTAL PROCEDURES

Chemicals and Materials—L929 mouse fibroblast cells were a kind gift of Roger L. P. Adams (Institute of Biomedical and Life Sciences, University of Glasgow). All materials for cell culture were obtained from Life Technologies, Inc. S-Adenosyl-l-[methyl-3H]methionine (S- [3H]AdoMet; specific activity of 70–80 Ci/mmol), [a-32P]dCTP (6000 mCi/mmol), [γ-32P]dATP (specific activity of 3000 Ci/mmol), and the Megaprime DNA labeling system were from Amersham International plc. Proteinase K (EC 3.4.21.14), trypsin (EC 3.4.21.4), T4 polynucleotide kinase (DNA nucleotidyltransferase (DNA-directed), EC 2.7.7.7),

1 The abbreviations used are: 3-ABA, 3-aminobenzamide; S-AdoMet, S-adenosyl-l-methionine; bp, base pair; PCR, polymerase chain reaction.
Klenow fragment of DNA polymerase I, and 50-pb ladder molecular size markers were from Boehringer Mannheim. The BstUI restriction enzyme and SsrI methylase were from New England Biolabs Inc. MspI and HpaII restriction enzymes, Taq DNA polymerase (EC 2.7.7.7), and the Wizard DNA Clean-Up system were from Promega. S-Aminobenzimidazole (10 mM) was included in the methionine-free Opti-MEM I Reduced Serum Medium Screen membrane was from NEN Life Science Products. The PCR purification kit, nucleotide removal kit, and QIAEX II gel extraction kit were from QIAGEN Inc. All other chemicals used were of the highest purity commercially available.

Fluorescence-activated Cell Sorter Analysis—Fluorescence-activated cell sorter analysis of L929 mouse fibroblasts, performed according to the previously described method (6), indicated that the cell cycle of synchronized fibroblasts was not influenced by the presence of 3-ABA. Evaluation of the “Residual” DNA Methyl-Accepting Ability—In nuclei, obtained from 6.5 × 10^6 L929 mouse fibroblasts (11) and preincubated for 24 h with or without 8 mM 3-ABA, the endogenous methyl-accepting ability was saturated by adding 16 μM unlabeled S-AdoMet for 1 h at 37 °C. The DNAs purified from these nuclei (2 μg) were used as substrates to evaluate their residual methyl-accepting ability in a final volume of 50 μl in the presence of 2.5 units of bacterial SsrI methylase using, as methyl donor, 80 μM S-AdoMet plus 100 μCi/ml S-[3H]AdoMet. The enzymatic reaction was carried out for 2 h at 37 °C and then was stopped by addition of 1% (w/v) SDS and 250 μg/ml proteinase K at 37 °C overnight. Purified DNAs were analyzed for their methyl-accepting ability (2), and incorporation of labeled methyl group was evaluated in a Beckman LS-6800 liquid scintillation spectrometer.

Bisulfite Reaction—The bisulfite reaction was carried out according to Frommer et al. (12) on a genomic DNA purified from nuclei (obtained from cells treated with or without 8 mM 3-ABA) in which the DNA methyl-accepting ability was saturated in the presence of 16 μM S-AdoMet, exploiting endogenous DNA methyltransferase activity. Before the reaction, the DNAs (2.5 μg) were digested for 4 h with 5 units of EcoRI restriction enzyme (that did not cut within the DNA regions that would subsequently be examined) and denatured by adding NaOH to a final concentration of 0.3 M for 15 min at 37 °C. The DNAs were incubated in the dark for 16 h at 55 °C in the presence of 0.5 mM tetramethylammonium chloride. The reaction (50 μl) was carried out under the following conditions: denaturation at 96 °C for 5 min, 95 °C for 1 min, 63 °C for 2 min, and 72 °C for 2 min for 37 cycles and a final cycle of 95 °C for 1 min, 63 °C for 2 min, and 72 °C for 6 min. Primers were removed from the PCR mixture using the QIAGEN PCR purification kit.

Methylation-dependent Restriction Analysis—Methylation-dependent restriction analysis was performed on 70–100 ng of DNA (20 μl) purified from nuclei (obtained from cells treated with or without 8 mM 3-ABA) in which the DNA methyl-accepting ability was saturated in the presence of 16 μM S-AdoMet, exploiting endogenous DNA methyltransferase activity. Samples were digested with MspI and HpaII restriction enzymes (30 units added three times) for 36 h at 37 °C. DNA fragments were resolved by electrophoresis on a 6% polyacrylamide I Klenow fragment (2 units) for 30 min at 37 °C, and the reaction was stopped by addition of NaEDTA to a final concentration of 25 mM. DNAs were precipitated in the presence of 3 μg of tRNA and stored at −80 °C. Samples were digested with 5 units of DpnI and amplified using random oligonucleotide primers. A mixture of amplified undigested DNA fragments was used as a labeled probe. A 50-pb ladder end-labeled with 10 μCi of [γ-32P]dATP in the presence of T4 polynucleotide kinase was used as marker after removing unincorporated [γ-32P]dATP with the QIAGEN nucleotide removal kit. Bands were evidenced by autoradiography on Kodak x-ray film.

RESULTS

Experiments were done to verify the possible direct role of poly(ADP-ribosyl)ation in maintaining the characteristic unmethylated state of CpG islands. The DNA used in the experiments was purified from nuclei obtained from cells in which the block of poly(ADP-ribosyl)ation allowed the introduction of new methyl groups. The DNAs used in the experiments were different in their residual methyl-accepting ability as this was reduced, according to previous results (6), to −30 ± 10% in the three different cell preparations examined (in comparison with the control taken as 100%) when DNA was purified from cells preincubated with 3-aminobenzamide.

The first experimental approach was carried out according with the method used by Bird et al. (7–9) to evidence the clusters of unmethylated CpG dinucleotides in genomic DNA named CpG islands. The methylation state of CpG islands was investigated by digesting, with methylation-dependent restriction enzymes, the genomic DNAs purified from nuclei (obtained from cells treated with or without 3-aminobenzamide) in which the DNA methyl-accepting ability was saturated in the presence of 16 μM S-AdoMet, exploiting endogenous DNA methyltransferase activity. As shown by gel electrophoresis, those “HpaII tiny fragments,” which typically appear following digestion of genomic DNA with HpaII, were present when the DNA was purified from control cells, but were greatly decreased if the DNA was purified from cells preincubated with 3-ABA (Fig. 1).

In the second experimental approach, fragment 1482–1773 of the mouse CpG island HpaII promoter region was amplified by PCR after the bisulfite reaction, which converts cytosine to uracil, but 5-methylcytosine does not react (12). Since this reaction immortalizes the methylation state of CpG sites on DNA, the fragment, even after amplification, retains the memory of the original methylation pattern. Following amplification of the DNA fragment, Taq polymerase added thymine, whereas the 5-methylcytosine residues were amplified as cytosine, so that we replaced the use of methylation-dependent restriction enzymes with sequence-dependent ones.

As the sequence-dependent restriction enzyme, we chose the BstUI enzyme, which recognizes and cuts CGCG sequence. The use of this enzyme (14, 15) allowed us to observe alterations in the methylation pattern only if both cytosines were methylated in the sequence since the methylation of only one cytosine or the absence of 5-methylcytosine produces TGGG, CGT, or TGTG sequences that BstUI cannot recognize and cut.

Following digestion of PCR-amplified DNA fragments with the BstUI restriction enzyme, it was possible to observe an anomalous methylation pattern when the HpaII promoter region was purified from fibroblasts preincubated with 3-ABA. In fact, Southern blot analysis of digestion products showed the pres-
24 h, was saturated in the presence of 16 mM 3-ABA incubated without (control) or with (3ABA) 3-aminobenzamide for 24 h, was saturated in the presence of 16 mM S-AdoMet, exploiting endogenous DNA methyltransferase activity. Methylation-dependent restriction analysis was performed on DNAs (70–100 ng) purified from these nuclei by adding 30 units of MspI (M) and HpaII (H) (added three times) for 36 h at 37 °C. Digested fragments were labeled by the end-fill reaction in the presence of polymerase I Klenow fragment and [α-32P]dCTP and evidenced by autoradiography of 2% agarose gel. The lane labeled G at the top and UN at the bottom represents DNA purified from L929 mouse fibroblasts that was not subjected to 3-ABA treatment, to the methylation step, or to digestion, but was incubated in parallel with other samples. The lane labeled m represents the pL9.2 plasmid digested with MspI (marker). Results from three different cell preparations are reported.

Discussion

How the CpG islands maintain their unmethylated state despite being rich in CpG dinucleotides is still an intriguing unanswered question. The problem is made interesting by the fact that the pattern of CpG islands remains unmethylated despite the fact that they are correlated mainly with the housekeeping genes (8), which are located in the decondensed chromatin structure. In this chromatin region, to which the transcription factors and the enzymes involved in the transcription process have easy access, the DNA methyltransferase should have easy access, too (17). Taking into consideration the importance that this biological process assumes for the regulation of gene expression (16), many researchers are working with the aim to individuate some cis-acting and/or trans-acting factors that directly or indirectly play a role in regulating the methylation pattern of CpG islands, specially since in vitro experiments (18, 19) have shown that CpG islands are not by themselves unmethylatable.

“Centers of methylation” able to prevent the methylation pattern of flanking DNA sequences (20–28) as well as some sequence motifs that are intrinsically protected against de novo methylation (20, 29) have been identified. Parallel research carried out to individuate trans-acting factors capable of binding methylated DNA has met with great success, but although these proteins (30–44) are considered to be important in mediating the methylation-dependent repression of the genes, the simplest possibility that there are trans-acting factors directly associated with CpG islands, capable of preventing the access of DNA methyltransferase to those DNA regions, has been difficult to demonstrate up to now.

As this post-synthetic modification introduces several negative charges into the modified proteins (45), it must be clarified if the poly(ADP-ribosylation) is the only factor responsible for these changes or if additional factors are involved. As this post-synthetic modification introduces several negative charges into the modified proteins (45), it must be clarified if the poly(ADP-ribosylation) is the only factor responsible for these changes or if additional factors are involved.
Starting from the simplest hypothesis that there is a protein or another kind of molecule directly involved in protecting the unmethylated state of CpG islands, we propose histone H1 either covalently (46) modified by ADP-ribose polymers and/or noncovalently (47) linked to long and branched ADP-ribose polymers as the protein responsible for this protection. A possible mechanism is that histone H1 in its covalently modified isoform could position itself on the CpG islands, for which it shows a greater affinity (6), and, when there, attract the long and branched polymers that inhibit methylation of double-stranded DNA (6), thus preventing DNA methyltransferase from having access to these DNA regions. Histone H1 could participate in this role through its genetic variant H1e, which is (i) the only one involved in inhibiting the participation in this role through its genetic variant H1e, which

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