Sequence specificity of Cr(III)–DNA adduct formation in the p53 gene: NGG sequences are preferential adduct-forming sites

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Hexavalent chromium [Cr(VI)] is a known etiological factor in human lung cancer. Cr(VI) exposure-related lung cancer morbidity rate for ex-chromate workers with ≥9 years of exposure is >20 times higher than that of non-smokers (15). The underlying mechanisms of how chromate exposure enhances lung carcinogenesis remain unclear. One possible mechanism is that mutations induced by Cr(III)–DNA adducts initiate lung carcinogenesis.

More than 50% of lung cancers have been found to contain a mutation in the p53 gene, and the remaining lung cancers frequently have dysfunctional genes either upstream or downstream of the p53 gene (16,17). These findings strongly suggest that the p53 gene plays a crucial role in lung carcinogenesis. Moreover, the p53 mutational spectrum in lung cancer has been found to closely mirror the binding spectrum of lung cancer etiological agents (18,19). It has been reported that mutations in the p53 gene in lung cancers from workers with Cr(VI) exposure are concentrated in the coding strand of exons 7 and 8 (20,21). Thus in order to elucidate the role of Cr(III)–DNA adduct formation in lung carcinogenesis it is crucial to map Cr(III)–DNA adduct distribution at the nucleotide level in the p53 gene.

It has been well established that nucleotide excision repair (NER) is the major mechanism for the repair of bulky DNA damage in both mammalian and Escherichia coli cells (22,23). In E. coli cells the repair of bulky DNA damage is carried out by the concerted work of three gene products—UvrA, UvrB and UvrC—which recognize and subsequently incise the damage (22,23). We have found that in vitro these three proteins together (termed as UvrABC nuclease) can incise bulky DNA damage specifically and quantitatively, and we have successfully used the UvrABC nuclease incision method to identify the DNA-binding sequence specificity of bulky carcinogens such as benzo[a]pyrene diol epoxides (BPDE), 2-acetylaminofluorene, 4-hydroxy-aminobiphenols, trans-4-hydroxy-2-nonenes and mitomycin C (24–28). In this study we have used the UvrABC nuclease incision method to determine both the DNA-binding specificity of Cr(III) and histidine(His)-conjugated Cr(III) in the PCR-amplified p53 gene.

Introduction

Environmental chromium (Cr) contamination is ubiquitous. Cr is widely used in the chemical industry, artistic paints, anticorrosion paints, electroplating and stainless steel welding (1,2). Cigarette smoke is also rich in Cr content (2,3). Although Cr (III) is unable to penetrate human cells and thus Cr(III) exposure does not pose a health danger, Cr(VI) exposure, in contrast, causes cell transformation, chromosomal aberration and sister-chromatid exchange (3,4–12). It has been proposed that upon uptake by cells Cr(VI) is sequentially reduced to Cr(V), Cr(IV) and finally Cr(III) by various cellular reducing agents (13). Intracellular Cr(III) then forms covalent bonds with various amino acids, ascorbic acids and glutathiones (9,14). Intracellular Cr(III) and ligand-conjugated Cr(III) can form covalent binary and ternary DNA adducts in genomic DNA; these binary and ternary Cr(III)–DNA adducts have been shown to have different mutagenic potencies, with ternary Cr(III)-ligand–DNA adducts being much more potent than binary DNA adducts (10). Cr(III) is also able to bind to phosphate groups in the DNA backbone through electrostatic interactions, although the frequency and the effects of this binding remain unclear.

Cr(VI)-containing compounds are well-known carcinogens in both humans and animal models (3). It has been shown that the lower respiratory tract is the target organ of Cr(VI) compound exposure (3,4–7), and it has been reported that the lung cancer morbidity rate for ex-chromate workers with ≥9 years of exposure is >20 times higher than that of non-smokers (15). The underlying mechanisms of how chromate exposure enhances lung carcinogenesis remain unclear. One possible mechanism is that mutations induced by Cr(III)–DNA adducts initiate lung carcinogenesis.

Abbreviations: APRT, adenine phosphoribosyltransferase; Cr, chromium; NER, nucleotide excision repair; PAH, polynuclear aromatic hydrocarbon.
Materials and methods

Materials

The Cr(III) source was CrCl3·6H2O (Sigma, St Louis, MO) and the CrCl3 solutions were freshly prepared before each experiment. L-His was obtained from Sigma and L-[14]H]His (50 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). [γ-32P]ATP (3000 Ci/mmol) was purchased from NEN (Boston, MA). T4 polynucleotide kinase and Taq DNA polymerase were purchased from Promega (Madison, WI). Primers were synthesized by the Midland Certified Reagent Co. (Midland, TX). The plasmid DNA was used as double-stranded pGEM-zf11(+)-APRT (adenine phosphoribosyltransferase). [pGEM-APRT] was constructed by inserting a 3.9 Kb Bam HI fragment into the template for DNA amplification. The 5'-end-32P-labeled amplified DNA fragments were purified by electrophoresis on an 8% polyacrylamide gel.

DNA fragment isolation and 5'-end-32P-labeling

Methods for preparing single 5'-end-32P-labeled exons 5, 7 and 8 of the p53 gene DNA fragments were the same as previously described (25). The oligonucleotide primers used for amplifications are: 5'-CAACTCTGTCTCTCTCATCTAC3'- (primer 1) and 5'-TCTCTCAGCCGCCCCGCTCTCCTAC3'- (primer 2) for exon 5; 5'-GCACGTGGCCTCATTCTGGGCGTCG3'- (primer 3) and 5'-CACGACGACCAGTGGCTGACG3'- (primer 4) for exon 7; and 5'-ACTGCTCTCTGTCTTCTTTTCTAC3'- (primers 5) and 5'-CTTCTCCTCCCTACGGCGTCCTT3'- (primer 6) for exon 7. Primers 1, 3 and 5 were 5'-end-32P-labeled with [γ-32P]ATP (30). Plasmid DNA containing the human p53 gene (pAT153 p53); obtained from L. Crawford and S.P. Tuck, Imperial Cancer Research Laboratories, UK, London) was used as the template for DNA amplification. The 5'-end-32P-labeled amplified DNA fragments were purified by electrophoresis on an 8% polyacrylamide gel.

Cr(III) modification of the plasmid and the DNA fragments

Plasmid DNA (pGEM-APRT) or 5'-end-32P-labeled exons 5, 7 and 8 were modified with Cr(III) by mixing pGEM-APRT plasmid (1 μg) or 5'-end-32P-labeled exons 5, 7 and 8 (2 × 106 c.p.m., ~20 ng) with different concentrations of CrCl3 (0.5–5 μM) in MES buffer [final concentration 10 mM (pH 6.3), and total volume of 100 μl]. The mixtures were incubated for 30 min at 37°C. The unreacted Cr(III) was removed by centrifugation in a filter device (YM-30 Amicon), and the filter was washed twice with 500 μl H2O. The modified DNAs were recovered by washing the filter with 10 μl H2O.

Modifications of DNA with Cr(III)-His complex

To achieve Cr(III)-His-DNA modifications, the His-Cr(III) complex was prepared in the same manner except that only His solution was used. The amount [3H] incorporated from the control plasmid.

DNA purifications

DNA fragments were dissolved in a denaturing dye mixture containing 95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol and heated at 90°C for 5 min. The resulting DNA was separated by electrophoresis at 40 V/cm in an 8% (w/v) polyacrylamide sequencing gel containing 45% (w/v) urea in TBE buffer in parallel with Maxam and Gilbert sequencing reaction products (34). After electrophoresis, the gel was dried using a Bio-Rad gel dryer and exposed to Kodak X-omat RP film at −70°C. Band intensity in the autoradiograph was quantified by Chemi Imager. RI (relative intensity) of the bands was calculated by RI = I/Imax, where I is the intensity of each bands and Imax is the highest intensity of the bands in the autoradiograph.

Results

UvrABC nuclease is able to incise Cr(III)- and histidine-conjugated Cr(III)-modified plasmid DNA

It is well established that the NER enzyme UvrABC nuclease is able to recognize and incise a variety of bulky chemical carcinogen-induced DNA damage, including various forms of cis-platinum–DNA adducts, with a dual incision mode 7 nt 5' to and 4 nt 3' to the DNA adduct (35). These findings raise the possibility that the UvrABC nuclease is able to incise Cr(III)-DNA adducts, and that the incision can be used to identify the Cr(III)-DNA binding position. To test this possibility we modified supercoiled pGEM-APRT plasmid DNA with different concentrations of CrCl3 or histidine-conjugated Cr(III) and used these modified DNA as substrates for UvrABC nuclease incision. Results in Figure 1 (Panel A) show that the UvrABC nuclease is indeed able to cut Cr(III)- and (Panel B) Cr(III)-His–modified plasmid DNA, and the extent of incision is proportional to concentrations up to 3 μM for Cr(III) and up to 0.5 μM Cr(III)-His. UvrABC incisions plateau for Cr(III)- (Figure 2A) and Cr(III)-His–modified plasmids (Figure 2B) at an average of 1.4 and 1.2 incisions per plasmid, respectively.

Sequence specificity of Cr(III)-DNA adduct formation in the p53 gene

Since the UvrABC nuclease is able to cut Cr(III)-modified DNA and the extent of cutting is proportional to the concentration of Cr(III) used, we utilized the UvrABC incision

A)

![Fig. 1](http://carcin.oxfordjournals.org/)

UvrABC incision assay on Cr(III)- and Cr(III)-His–modified plasmids. pGEM-APRT plasmid DNA was modified with various concentrations of CrCl3 (0.5 μM) or histidine-Cr(III) (0–1.0 μM) and then reacted with UvrABC nuclease as described in Materials and methods. The resultant DNA was separated by electrophoresis in 1% agarose gels in TAE buffer. Typical ethidium bromide stained gels are presented.
method to identify the Cr(III)–DNA binding position in the human p53 gene. We found that bulky DNA adduct formation is mostly affected by primary DNA sequence and, if the binding is at a CpG site, by C5 cytosine methylation at CpG sequences (18,19,25,26). Therefore, we chose to use purified PCR-synthesized DNA fragments that would allow us to address these two possibilities. Single 5' end-32P-labeled exons 5, 7 and 8 of p53 gene fragments were prepared by PCR; these fragments were modified with different concentrations of CrCl3, and then reacted with UvrABC nuclease. The enzyme incision positions were identified by comparing the electrophoretic mobility of the incised fragments to Maxam-Gilbert sequencing reaction ladders. Results in Figure 3 A–C show that while the UvrABC nuclease does not produce specific incision bands in unmodified DNA fragments, it incises Cr(III)-modified fragments with specificity and the extent of incision is proportional to the concentrations of CrCl3 used for the modifications. It is well established that UvrABC nuclease consistently incises bulky DNA damage 6–8 nt 5' to and 3–4 nt 3' to a DNA adduct (22–24). If we assume that the mode of UvrABC nuclease incision on a Cr(III)-DNA adduct is consistent with its mode of incision for other bulky DNA adducts (i.e. 7 nt 5' to a DNA adduct), then the positions of Cr(III)-DNA adducts formed in exon 5 of the p53 gene fragment are: 1(TGG), 2(TGG), 3(GGG), 4(NGGT), 5(CGG), 6(NGG), 7(CGC), 8(TGG) and 9(NGG). Eight out of the nine Cr(III)-adducted guanine residues are located at the first or second guanine of a NGG triplet sequences, and one is located at a CGC sequence. Those guanine residues in the triplet sequences identified as 1, 2–3, 4, 5–6, 7, 8 and 9 are located in codons, 137, 146, 154, 158, 160 and 161, respectively (Figures 3A and 4A).

The positions of Cr(III)-DNA adduct formation in exon 7 of the p53 gene fragment are: 1(TGG), 2(TGG), 3(GGG), 4(CGG), 5(NGG), 6(TGA), 7(CGG), 8(NGG), 9(AAG), 10(NGG), 11(NGG), 12(AAG), 13(NGG) and 14(AAG). Out of the 14 Cr(III)-adducted guanine residues 12 are located at the first or the second guanine of a NGG triplet sequence, while 1 is located at a TGA sequence and 1 at a AGA sequence. The guanine residues in the triplet sequences identified as 1, 2–3, 4–5, 6, 7–8, 9–10, 11, 12–13 and 14 are located in codons, 243, 244, 245, 246, 248, 249, 258, 258, 261 and 263, respectively (Figures 3B and 4B).

The positions of Cr(III)-DNA adduct formation in exon 8 of the p53 gene fragment are: 1(TGG), 2(GGG), 3(NGGA), 4(AGA), 5(AGA), 6(AGA), 7(AGG), 8(AGG), 9(GGG), 10(NGG), 11(AGC), 12(AGG), 13(GGG), 14(GGA). Out of the 14 guanine residues 10 are located at the first or the second of a triplet NGG sequence. The guanine residues in the triplet sequences identified as 1–3, 4, 5, 6, 7–9, 10–11, and 12–14 are located in codons, 279, 280, 281, 291, 293, 294 and 302, respectively (Figures 3C and 4C).

The extent of Cr(III)-guanine adduct formation at these sequences is presented in Figure 4A–C. Altogether 37 Cr(III) binding sites were identified in exons 5, 7 and 8 of the p53 gene; 30 of these binding sites were in NGG triplet sequences. These results suggest that guanines in NGG triplet sequences are preferable binding sites for Cr(III), with GGG sequences being the most preferable site for Cr(III) binding.

Sequence specificity of Cr(III)-His–DNA adduct formation in the p53 gene

It has been found that most of intracellular Cr(III) is conjugated with various amino acids, glutathiones, and ascorbic acids (9,14). The majority of the Cr(III)–DNA adducts found in genomic DNA are ternary ligand-Cr(III)–DNA adducts, and a minority are binary Cr(III)–DNA adducts (9,14). The ligand conjugated with Cr(III) may affect the Cr (III) binding sequence specificity. To test this possibility we modified p53 DNA fragments with histidine-conjugated Cr(III) and then identified the Cr(III)-His binding sequences by the UvrABC incision method. Results in Figure 5A–C show that the UvrABC nuclease incises Cr(III)-His modified DNA in the same manner as Cr(III)-modified DNA, indicating that ligand-conjugated Cr(III) has the same DNA-binding sequence specificity as Cr(III); i.e. NGG is the preferential site for Cr(III)-His-DNA adduct formation, with GGG sequences being the most preferable site for Cr(III) binding.
Discussion

It has long been recognized that the UvrABC nuclease is able to incise DNA damage induced by a variety of agents, ranging from DNA helix-stabilizing agents such as CC-1065 and anthramycin to helix-destabilizing agents such as UV, BPDE and cis-platinum (36–39). Although the precise mechanisms that enable this repair enzyme system to possess such versatility remain unclear, this system have been widely used for identifying the DNA-binding sequence specificity of many carcinogens and drugs (24,27). In this study we found that UvrABC is able to incise Cr(III)- and Cr(III)-His–modified plasmids, and the level of incision is proportional to the Cr(III) and Cr(III)-His concentrations used for modification. These results suggest that the amount of incision represents the amount of Cr(III)- and Cr(III)-His–DNA adducts formed in the plasmid enzyme system.

Intriguingly, much higher concentrations of Cr(III) (3 μM) than Cr(III)-His (0.5 μM) are needed for plasmid modification to reach plateau levels of UvrABC incision (1.2–1.4 incision). It is reasonable to assume that Cr(III) has a higher binding affinity toward DNA than Cr(III)-His because water molecules in a Cr(III)- water complex are a better leaving group than His in a Cr(III)-His complex. It has been found that Cr(III) binds to both guanine residues and phosphate groups in DNA (40); if we assume that CrCl₃ generates the same amount of or more DNA adducts than the same concentration of Cr(III)-His, our results then raise the possibilities that: (i) the UvrABC nuclease incises only a particular class of DNA adducts, most likely the Cr(III)-guanine adduct, and (ii) that Cr(III)-phosphate backbone binding may interfere with UvrABC nuclese incision on Cr(III)-guanine adducts. While we are unclear on the exact structure of Cr(III)-guanine–DNA adducts that is recognized by UvrABC nuclease, we believe that because the histidine moiety of the Cr(III)-His-guanine adduct can partially insert into the DNA helix, causing base unstacking and a substantial degree of helix distortion, thereby allowing the UvrABC nuclease to efficiently recognize this type of adduct.

The reason for both Cr(III) and Cr(III)-His preferentially binding at –NGG- sequences is not clear. It has been found that GGG and GG sequences are much more nucleophilic than other sequences, and we found that the two strongest Cr(III)-binding sites are GGG sequences in exons 5 and 8 of the p53 gene. Therefore, it appears that the nucleophilicity of the sequences does indeed play an important role in Cr(III)–DNA binding (41,42). However, we also found Cr(III) binds much more strongly at an AGG sequence in codon 261 than at GGG sequences in codons 243 and 244 of exon 7. Furthermore, in exon 8, Cr(III) barely binds at a GGG sequence in exon 279, an AGG sequence in codons 285 and 286, and a CGG sequence in codon 282 of exon 8. Together these results suggest factor(s) other than nucleophilicity also play an important role in determining susceptibility to Cr(III) binding. Since Cr(III) has six binding sites that are able to interact with DNA, we suggest that Cr(III) and Cr(III)-His are able to form stable chelate adducts at NGG sequences. The possible structures of these adducts are presented in Figure 6.
It has been well established that individuals exposed to chromium have a high incidence of lung cancer, and most chromate factory workers who develop lung cancer were also cigarette smokers (3,6,7). Hence, it has been suggested that chromium exposure and cigarette smoke may have a synergistic or additive effect in inducing lung carcinogenesis (43). Consistent with this notion are our findings that Cr(III) and ligand-conjugated Cr(III) preferentially bind to NGG sequences, which include codons 245, 248 and 249 of the \( p53 \) gene, the mutational hotspots in cigarette smoke-related lung cancer. This DNA binding may contribute to chromium-related lung cancer. We previously found that polycyclic aromatic hydrocarbons (PAHs), the major carcinogen found in cigarette smoke, bind to all the \( p53 \) mutational hotspots that contain a –CG sequence in lung cancer; however, we also found that codon 249 (AGG) is not a preferential site for PAH binding, nor does the repair of PAH–DNA adducts formed at this codon differ from other codons (18,19,44). Cigarette smoke contains substantial amounts of chromium compounds, although the chemical forms of these chromium compounds are not known (45). Our finding that Cr(III) and Cr(III)-His bind strongly at codon 249 suggest that the etiological agent for lung cancer with codon 249 mutations is Cr(III). It should be noted that 55% of the codon 249 mutations found in cigarette smoke-related lung cancer are at the second base and 32% are at the third base of this codon; however, our results show that Cr(III) binds at the second base two times more frequently than at the third base of this codon (46). It is possible that the Cr(III)–DNA adducts formed at the second and third bases of this codon are repaired with different efficiencies and/or that they affect the fidelity of DNA replication differently.

We have recently found that the NER capacity is greatly reduced in lung cells exposed to Cr(VI); consequently,
exposure of these cells to DNA damaging agents such as BPDE results in a synergistic increase in mutations (47). These results, together with the results from the present study, suggest that chromium treatment induces two detrimental effects in cells: damaging DNA and reducing DNA repair capacity, and these two effects may contribute to chromium-related mutagenicity and carcinogenicity.

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