2-Chloro-2'-deoxyadenosine monophosphate residues in DNA enhance susceptibility to 3'→5' exonucleases

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2-Chloro-2'-deoxyadenosine triphosphate, a purine nucleotide analogue and potent antileukaemic agent, was incorporated into double-stranded 36-mers in place of dATP to investigate the effects of 2-chloroadenine (ClAd) on DNA polymerase-associated 3'→5' exonuclease activity. ClAd residues within one strand of duplex DNA did not inhibit exonuclease activity; on the contrary, ClAd-containing minus strands were digested to a greater extent than was control DNA in the absence of deoxyribonucleoside triphosphates by *Escherichia coli* Klenow fragment, yeast DNA polymerase II and T4 DNA polymerase. After a 30 min incubation with 5 units of Klenow fragment, ~65% of control DNA remained in DNA fragments of 26 bases or larger

compared with only ~25% of ClAd-substituted substrates. Unsubstituted plus strands opposite a ClAd-containing strand were likewise digested more quickly by 3'→5' exonuclease, but only in the vicinity of the ClAd sites. Approx. 63% of the plus strands from ClAd-containing oligomers were less than 24 bases in length after a 25 min digestion period with Klenow fragment compared with only ~32% of control DNA. Such results indicate that, unlike other base modifications such as pyrimidine dimers, methoxy psoralen adducts and certain nucleoside analogues, all of which inhibit or decrease the rate of strand degradation by 3'→5' exonucleases, incorporated ClAd enhances strand degradation of duplex DNA.

INTRODUCTION

2-Chloro-2'-deoxyadenosine (CldAdo, cladribine), a chlorine-substituted deoxyadenosine analogue, has proven to be very effective in producing complete remission with relatively few relapses in patients with hairy cell leukaemia (Piro et al., 1990; Tallman et al., 1992; Estey et al., 1992). Other recent clinical studies have indicated the therapeutic value of CldAdo in the treatment of a variety of lymphoid and myeloid malignancies including paediatric leukaemia (Santana et al., 1991, 1992), adult chronic lymphocytic leukaemia (Piro et al., 1988), low-grade lymphoma (Kay et al., 1992) and cutaneous T-cell lymphoma (Saven et al., 1992). Toxic side effects from a single course of CldAdo treatment (0.1 mg/kg per day, continuous infusion for 7 days) are extremely limited (primarily bone marrow suppression) with no significant nausea, vomiting, skin rash or hair loss (Beutler, 1992; Tallman et al., 1992).

The exact biochemical mechanism of action of this compound has been investigated but remains somewhat unclear. CldAdo is effectively taken up by the cell and is phosphorylated (Carson et al., 1980; Blakley et al., 1986; Griffing et al., 1989). The presence of the halogen atom on the purine ring renders the nucleoside resistant to cellular adenosine deaminase and subsequent inactivation (Montgomery, 1982). In addition, CldAdo inhibits cellular ribonucleotide reductase, decreases DNA synthesis and is incorporated into cellular DNA (Carson et al., 1980, 1983; Blakley et al., 1986; Parker et al., 1988; Griffing et al., 1989; Hirota et al., 1989). As a consequence of these activities, CldAdo is highly toxic to human T- and B-lymphoblasts in culture (Huang et al., 1981, 1986; Carson et al., 1980, 1983; Blakley et al., 1986; Avery et al., 1989).

At the molecular level we have demonstrated that CldATP, the triphosphate derivative of CldAdo, was utilized as a substrate during *in vitro* DNA synthesis by human DNA polymerases α

and β (Hentosh et al., 1990), human immunodeficiency virus (HIV) reverse transcriptase (Hentosh et al., 1991) and several bacterial and phage DNA polymerases (Hentosh et al., 1991). However, the overall rate and extent of strand elongation was diminished compared with control reactions (Hentosh et al., 1990, 1991). Chunduru et al. (1993) further reported that, for both human polymerase α and β , the CldATP concentration required for the half-maximum rate of *in vitro* extension was only twice the value of dATP. This suggests that there are only minor differences in the relative K_m values and that the analogue is incorporated efficiently. However, the kinetics of subsequent extension of 3' CldAMP termini differed for the two polymerases. Elongation by polymerase α was highly dependent on DNA sequences downstream of the CldAMP incorporation site, ranging from near-normal rates to virtually no further extension after incorporation of four consecutive CldAMP residues. In contrast, rate constants for incorporation of additional nucleotides by polymerase β at all sites examined were drastically reduced relative to control reactions, and CldAMP acted as a virtual chain terminator. Thus decreased *in vitro* elongation in the presence of CldATP may be attributed not to reduced incorporation of the analogue but rather to slow or inefficient extension of CldAMP termini at specific sites.

Because CldATP is not an absolute chain terminator for polymerase α , it is likely that its incorporation into cellular DNA will have significant effects on additional rounds of DNA synthesis and enzymic processes. Indeed, we recently demonstrated that template ClAd residues were also *in vitro* blocks to DNA synthesis by human polymerases α and β (Hentosh and Grippo, 1994). These findings together with the above results may explain, in part, the reduced cellular DNA synthesis and may contribute to the cytotoxic action of CldAdo. However, the response of 3'→5' exonuclease activities associated with many DNA polymerases to ClAd residues in DNA has not been addressed. Exo-

Abbreviations used: DDT, dithiothreitol; ClAd, 2-chloroadenine; CldAdo, CldAMP and CldATP, 2-chloro-2'-deoxyadenosine and its 5'-mono- and tri-phosphate forms; HIV, human immunodeficiency virus.

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nucleases play an important role not only in proofreading and error-correcting functions during DNA synthesis, but also in DNA-repair processes. Here we have examined whether CIAd residues within DNA impair DNA polymerase proofreading exonucleases in the same manner as other nucleoside modifications, such as pyrimidine dimers (Doetsch et al., 1985), methoxy psoralen adducts (Sage and Moustacchi, 1987) and specific nucleotide analogues (Huang et al., 1991, 1992).

EXPERIMENTAL

Materials

Sequence II was from United States Biochemical Corporation; exonuclease VII, T4 DNA polymerase, phenol and ultrapure dNTPs were from Bethesda Research Laboratories; *Escherichia coli* polymerase I (Klenow fragment) and T4 polynucleotide kinase were from Promega; [γ - 32 P]ATP was from Dupont–New England Nuclear; Sephadex G-50 was from Pharmacia; CldATP and a 36-base plus-strand oligonucleotide (5'-GAGTCGAC-CTGCAGGCATGCAAGCTTGGCACTGGCC-3') with complementary minus-strand primer 2B (5'-GGCCAGTGCCAAG) were kindly provided by Dr. R. L. Blakley, St. Jude Children's Research Hospital, Memphis, TN, U.S.A. Yeast polymerase II was the generous gift of Dr. Akio Sugino, NIEHS, Research Triangle Park, NC, U.S.A.

Radioactive labelling of oligonucleotides and synthesis of duplex 36-mer

Oligonucleotide primers were 5'-end-labelled with T4 polynucleotide kinase and [γ - 32 P]ATP by the forward kinase reaction as described previously (Hentosh et al., 1990). A 36-base-long oligonucleotide, representing a portion of the plus-strand sequence of M13mp18 DNA, was used as a template to synthesize enzymically complementary minus strands containing either four normal bases or CIAd in place of Ade. The location of the three CIAd sites and the synthesis of duplex 36-mer were as reported previously (Hentosh and McCastlain, 1991) (see also Figure 1). Briefly, 5'- 32 P-end-labelled primer 2B (≈ 4.8 pmol) was annealed to 4 pmol of plus-strand 36-mer and extended enzymically at 37 °C overnight with Sequenase II (13 units) in the presence of four normal dNTPs at 30 μ M each or in the presence of CldATP (30 μ M) in place of dATP in a final volume of 50 μ l. In other experiments, non-radioactive primer 2B was annealed to 32 P-labelled plus-strand 36-mer and enzymically extended in the same manner. DNA was then extracted by phenol and centrifuged through a G-50 Sephadex spin column to separate double-strand DNA from unincorporated nucleotides. After ethanol precipitation, DNA was dried and resuspended in water.

Digestion of DNA with DNA polymerase (3' $\rightarrow$ 5') exonuclease

Exonuclease reaction mixtures (final volume 50 μ l) contained approximately 1–2 pmol of double-stranded 36-mer, with or without CIAd present in the minus strand. Reaction buffers for each enzyme also contained the following: for Klenow fragment, 150 mM KCl, 300 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mg/ml BSA and 2 μ l of enzyme (0.1–5 units/ μ l); for phage T4 DNA polymerase, 33 mM Tris/acetate, pH 7.8, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM DTT, 0.1 mg/ml BSA and 0.005–6.0 units of T4 polymerase; for yeast DNA polymerase II, 50 mM Tris/HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, 2 mM DTT and ~ 40 ng (0.3 pmol) of enzyme. All reaction mixtures were incubated at

37 °C; at various times, samples (6–7 μ l) were removed and inactivated with an equivalent volume of stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue and 0.05% xylene cyanol FF). After heat denaturation at 90 °C for 4 min, reaction products were analysed by denaturing 15% polyacrylamide/7 M urea gel electrophoresis. Autoradiograms were obtained by exposing XAR-5 film overnight at –70 °C and scanned with an LKB laser densitometer.

RESULTS

The 3' $\rightarrow$ 5' proofreading exonuclease associated with several DNA polymerases is inhibited by a number of DNA base modifications and nucleoside analogues. To determine the effects of CIAd residues on exonuclease activity, we incubated double-stranded 32 P-end-labelled 36-mers (Figure 1) with several concentrations of *E. coli* Klenow fragment in the absence of dNTPs (Figure 2). In substituted oligomers, CIAd residues occurred 4, 10 and 18 bases respectively from the 3' terminus of the labelled minus strand only. Ade sites in the sequence shown in Figure 2 represent corresponding sites of CIAd residues in substituted substrates. Digestion of both control and CIAd-substituted DNA with Klenow-fragment exonuclease resulted in small-sized hydrolysis products with increasing time of incubation; however, CIAd-containing DNA was digested to a greater extent at every time point than was control DNA, regardless of the concentration of Klenow fragment used. Densitometric analysis also clearly illustrated the more rapid hydrolysis of CIAd-containing oligomers (Figures 2b and 2c). For example, after a 30 min incubation with 5 units of Klenow fragment, approx. 65% of control DNA remained in DNA fragments equivalent to or longer than 26 bases (second cytosine from top) compared with $\sim 25\%$ remaining in this size range for CIAd-substituted substrates. Within that region, the exonuclease would encounter two CIAd residues. In addition, Klenow-fragment exonuclease stalled non-randomly on both substrates. The strongest sites of stalling, as determined by the density of individual bands, were primarily pyrimidine (cytosine and thymidine) positions throughout the DNA substrate; however, CIAd residues did not deter exonuclease activity, in contrast with the inhibitory effects of other base modifications.

Preferential cleavage of CIAd-containing DNA was not solely a characteristic of bacterial DNA polymerase exonuclease, but extended to yeast polymerase II, the properties of which are similar to those of mammalian DNA polymerase ϵ (Araki et al., 1992). Reaction mixtures were incubated with yeast polymerase II from 1 to 15 min; only two time points are depicted. As shown in the autoradiogram and accompanying densitometric scans (Figure 3), after either 2 or 10 min incubation, larger amounts of CIAd-substituted minus-strand DNA were hydrolysed than control DNA; control DNA was also digested by yeast polymerase II, but to a much lesser extent and with slower kinetics. By way of a quantitative comparison, there were no CIAd-containing oligomers equal to or larger than 33-mers remaining after

Figure 1 DNA sequence of double-stranded 36-base oligonucleotide used in exonuclease digestion reactions

Underlined area indicates primer 2B used in extension reactions with Sequenase to synthesize the minus strand. Positions of CIAd residues within the minus strand are designated (N).

Figure 2 Klenow-fragment 3' → 5' exonuclease digestion of control and CIAdA-substituted minus strands in duplex 36-mers

(a) The ³²P-primer 2B was annealed to a non-radioactive 36-mer, and synthesis of full-length minus strands containing Ade (A) or CIAdA (N) was conducted as described in the Experimental section. Duplex 36-mers were incubated in the absence of dNTPs with increasing amounts of Klenow fragment (units), and portions were removed at 10 and 30 min. Reaction products were analysed by PAGE (15% gels). PR indicates the original position of the 13-base primer. All bases marked A in the sequence shown are sites of CIAdA in the substituted substrate. (b, c) Densitometric scanning of hydrolysis products of (b) control 36-mers and (c) CIAdA-containing 36-mers resulting from 30 min incubations with 5 units of Klenow fragment. Numbered letters above peaks indicate nucleotide identity and position from the 5' end of the minus strand.

2 and 10 min incubation with yeast polymerase II. Instead, there was a preponderance of smaller DNA fragments in CIAdA samples; approx. 50 and 90 % respectively of the DNA fragments were 15 bases or less in length as determined from the densitometric scans (see Figures 3b and 3c for the 2 min data; the scan

Figure 3 Time course of yeast polymerase II 3' → 5' exonuclease digestion of control and CIAdA-substituted minus strands in duplex DNA

(a) Primer 2B was annealed to plus-strand 36-mer and extended as described in the Experimental section in the presence of dATP (A) or CIdATP (N). Duplex 36-mers were purified and incubated with yeast polymerase II, and samples were removed at the times shown. Other conditions were as specified for Figure 2. (b, c) Densitometric scanning of reaction mixtures from (a) incubated with yeast polymerase II for 2 min for (b) control 36-mers and (c) CIAdA-containing 36-mers.

for the 10 min time point is not shown). In contrast, after the 2 min incubation with yeast polymerase II, ~ 40 % of the control samples were 33 bases in length or longer; after 10 min, ~ 20 % were in this size range. This demonstrates the reduced or slower exonuclease activity on control DNA compared with the almost complete degradation of CIAdA-containing fragments. We have also observed similar trends with phage T4 DNA polymerase exonuclease on CIAdA-substituted oligomers (results not shown).

Figure 4 Klenow-fragment 3' → 5' exonuclease digestion of unsubstituted plus strands

(a) The ^{32}P -labelled plus-strand 36-mer was annealed to non-radioactive primer 2B, and synthesis of minus strands containing Ade (A) or CIAdA (N) was conducted as described in the Experimental section. Duplex 36-mers were incubated with 5 units of Klenow fragment/reaction, samples were removed at various time intervals, and reaction products were analysed as described for Figure 2. Asterisks indicate sites of CIAdA in the opposing minus strand. Bases are numbered from the 5' end of the 36-mer plus strand. (b, c) Densitometric analyses of plus-strand hydrolysis products of (b) control 36-mers and (c) CIAdA-containing 36-mers resulting from 25 min incubations with Klenow-fragment 3' → 5' exonuclease.

To determine whether preferential hydrolysis of analogue-containing DNA was due to regions of single-strandedness or to a reduced stability of duplex 36-mer, we incubated control and CIAdA-substituted DNA with *E. coli* exonuclease VII, which is specific for single-stranded DNA (Chase and Richardson, 1974). There was no apparent differential effect on the duplex DNA substrates (results not shown), suggesting the absence of single-

strand regions within CIAdA-substituted 36-mers. We then investigated the possibility of duplex instability by performing DNA polymerase 3' → 5' exonuclease digestions on double-stranded 36-mers that were 5'-end-labelled on the template plus-strand only, thus permitting us to assess hydrolysis of an unsubstituted DNA strand opposite a control or CIAdA-containing strand. Plus-strand 3' → 5' exonuclease on a CIAdA-containing duplex 36-mer must progress along a stretch of 18 bases before encountering the first dT:CIdA pair (Hentosh and McCastlain, 1991).

Figure 4 demonstrates that at early incubation times (2.5 and 7.5 min), Klenow-fragment digestion of plus-strands in duplex molecules proceeded at approximately the same rate regardless of the presence or absence of CIAdA residues in the opposing strand. The densitometric scans for either incubation period revealed almost identical fragment sizes and relative amounts of each DNA fragment for both control and CIAdA-substituted oligomers (not shown). The electrophoretic mobility and size range of the resultant digestion products established, moreover, that during either incubation time, the exonuclease had not reached the first dT:CIdA site. After 15 and 25 min, however, the digestion rate of CIAdA-containing substrate DNA increased, as shown by the appearance of a larger number of DNA fragments that were 23 bases or less. As determined by densitometric quantification of the relative amounts of each band, ~63% of the CIAdA-containing oligomers were less than 24 bases in length after a 25 min digestion period compared with only ~32% of control DNA (Figures 4b and 4c). The majority of the digested plus-strands from CIAdA substrates were quite small, only 3- and 4-mers. The site of increased activity corresponded roughly to the region of the first CIAdA residue in the opposing strand (see the asterisks in Figure 4a). Thus the greater exonucleolytic activity observed on both CIAdA-substituted and the opposing unsubstituted strands rules out specificity for CIAdA residues, but may represent the presence of localized regions of slight distortion, instability or bent DNA duplex surrounding the analogue that enhance accessibility to exonucleases.

DISCUSSION

DNA strands either containing or opposing CIAdA residues were more rapidly degraded than control DNA by 3' → 5' exonuclease activity associated with DNA polymerases. Other base modifications such as pyrimidine dimers (Doetsch et al., 1985), methoxy psoralen adducts (Sage and Moustacchi, 1987), certain nucleoside analogues [2',2'-difluorodeoxycytidine, 1- β -D-arabinofuranosylcytosine, 3'-azido-2',3'-dideoxythymidine and 2',3'-didehydro-2',3'-dideoxythymidine (Huang et al., 1991, 1992; Nickel et al., 1992)], major-groove C8-guanine *N*-acetyl-2-aminofluorene adducts (Bichara and Fuchs, 1985) and minor-groove N²-guanine 4-aminoquinoline 1-oxide adducts (Panigrahi Walker, 1990) have all been shown to inhibit or decrease the rate of strand degradation by 3' → 5' exonucleases. However, despite similarities in conformation and location, not all DNA adducts act in an analogous manner. Neither major-groove deacetylated C8-guanine 2-aminofluorene adducts (Bichara and Fuchs, 1985) nor 4-aminoquinoline 1-oxide-induced adducts at C8-guanine and N⁶-adenine sites (Panigrahi and Walker, 1990) block the action of exonucleases. Why some DNA modifications inhibit exonucleases and others do not is unclear; perhaps the relative degree of conformational distortion and position (major versus minor groove) of the adduct both play a role in inhibition of exonuclease activity. CIdAdo, unlike the above-mentioned nucleoside analogues, has a modified purine ring, whereas its sugar group is intact. While it is obvious from the above

examples that either minor- or major-groove adducts can inhibit exonucleases, the chlorine substitution within the minor groove may not be as bulky as 4-aminoquinoline 1-oxide adducts or pyrimidine dimers to cause the enzymes to fall off the DNA or impede their progress.

The cause of greater hydrolysis of ClAdc-containing DNA is also unclear. Some insight may be found in previous reports in which we demonstrated that double-stranded oligomers containing either Ade or ClAdc had similar mobility on non-denaturing gels, indicating that ClAdc-substituted duplex DNA was as stable as control DNA (Hentosh and McCastlain, 1991). However, single-stranded ClAdc-containing DNA migrated more slowly than normal DNA on denaturing gels (Hentosh and McCastlain, 1991). Such decreased gel mobility has often been associated with a bend or perturbation in a DNA strand (Marini et al., 1982; Gupta et al., 1988; Voight and Topal, 1990). In this study, the digestion rate of unsubstituted strands opposite a ClAdc-containing strand increased only near the site of the analogue. This result, together with the finding of decreased denaturing gel mobility, suggests that there may be localized instability or a bend in the helix in the region surrounding a CldA:dT base pair. Such a natural bend in DNA might enhance either binding of Klenow fragment to DNA or accessibility to and activity of its 3' → 5' exonuclease. This possibility is of interest in the light of recent crystallographic findings by Beese et al. (1993) that duplex DNA is severely bent when bound to Klenow fragment. The authors have suggested that duplex destabilization results from such bending and enhances the sensitivity of 3' termini to mismatched bases. They further speculate that polymerases lacking an editing function bind only straight duplex DNA, as does HIV reverse transcriptase, whereas those with an editing exonuclease, such as Klenow fragment, would bind bent DNA. Therefore it appears that a bend in the DNA duplex may be important for coupling polymerase/exonuclease activities. Furthermore, an oligomer with a pre-existing bend might enable exonucleases to function faster or more efficiently by positioning a CldA:dT mispair better within the exonuclease catalytic site.

The results from this study may explain in part previous observations that Klenow fragment and T4 DNA polymerase, both of which have 3' → 5' exonuclease activity, displayed greatly reduced *in vitro* strand extension in the presence of CldATP (Hentosh et al., 1991). As discussed earlier, K_m values for dATP and CldATP are fairly similar. However, a terminal CldA:dT pair represents a mismatched or incorrect base pair and will slow down Klenow or T4 polymerization of the next correct dNTP as it does for polymerases α and β . In an extensive kinetic study to examine Klenow-fragment synthesis on mismatched termini, Kuchta et al. (1988) reported that a conformational change slows dissociation of incorrect DNA products from the polymerase leading to an increased lifetime of the Klenow-DNA complex containing a misincorporated base. The increased complex lifetime is sufficient in turn to allow 3' → 5' editing activity to remove terminal mismatched bases. As shown in this study, ClAdc residues, unlike 2',2'-difluorodeoxycytidine or 2',3'-didehydro-2',3'-dideoxythymidine analogues, are excellent substrates for exonucleases. In addition, we previously observed in our synthetic assays using Klenow fragment and T4 DNA polymerase that many DNA fragments accumulated one nucleotide before a CldATP-incorporation site (Hentosh et al., 1991). The pause sites and limited strand elongation most likely reflect incorporation of the analogue followed by efficient exonucleolytic

removal of terminal ClAdc residues. Possible implications of enhanced exonuclease activity during DNA-repair processes await experimental data regarding the recognition of ClAdc by repair enzymes.

We thank Dr. D. Peffley for support and helpful discussions, Dr. A. Sugino for a gift of yeast polymerase II and Dr. R. L. Blakley for a generous supply of CldATP. This research was supported by grant CA55414 from the National Cancer Institute. The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

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