Excision of Nucleoside Analogs from DNA by p53 Protein, a Potential Cellular Mechanism of Resistance to Inhibitors of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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Received 15 August 2004/Returned for modification 12 October 2004/Accepted 28 December 2004

We investigated the ability of p53 in cytoplasm to excise nucleoside analogs (NAs). A decrease in incorporation of NAs by human immunodeficiency virus type 1 reverse transcriptase and their excision from DNA by p53, provided by the cytoplasmic fraction of LCC2 cells, suggest that p53 in cytoplasm may act as an external proofreader for NA incorporation.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) was previously found to be a successful target for the treatment of AIDS (10). The enzyme catalyzes the transcription of viral genomic single-stranded RNA into double-stranded DNA in the cytoplasm (9). HIV-1 RT lacks 3′→5′ exonuclease activity and exhibits low fidelity of DNA synthesis during both RNA→DNA and DNA→DNA replication steps (5, 6, 13, 16, 19, 21). HIV RT readily utilizes many nucleoside analogs (NAs), and the incorporation of nucleoside RT inhibitors (NRTIs) leads to DNA chain termination (25). Although NRTIs reduce the viral load in HIV-1-infected individuals, mutations in HIV-1 RT give rise to resistance (26). The resistance mutations either decrease the incorporation efficiency of the NRTIs or increase their removal from the extended primer (2, 8, 17, 25).

Removal of drugs by 3′→5′ exonuclease activity intrinsic to DNA polymerase or by external proofreading activity associated with some polymerases or proteins may be viewed as a potential cellular mechanism of resistance to drugs (23, 27). The tumor suppressor protein p53 displays intrinsic 3′→5′ exonuclease activity and may provide a proofreading function for exonuclease-deficient DNA polymerases (3, 4, 12, 15, 18, 22, 24). p53 in cytoplasm preferentially removes 3′-terminal mispaired nucleotides from RNA/DNA and DNA/DNA template-primers incorporated by HIV-1 RT (7). Furthermore, the protein may recognize and remove incorporated NAs from DNA both in vitro and in whole cells (11). Hence, it was of interest to test the possibility that p53 in cytoplasm may play a role in the removal of incorporated NA from the 3′ end of DNA. We utilized cytoplasmic fractions of LCC2 cells, expressing high levels of wild-type p53 (wt p53) with intrinsic 3′→5′ exonuclease activity (14), as an experimental model system. Two experiments were done to evaluate the involvement of p53 in cytoplasm in (i) incorporation of NA by HIV-1 RT and (ii) excision of incorporated NA.

NA incorporation by HIV-1 RT in the presence of the cytoplasmic fraction (4 μg) of LCC2 cells was studied with both DNA/DNA and RNA/DNA substrates (experiment i). The DNA primers were end labeled at the 5′ end and annealed to the template RNA or DNA as described previously (5, 6). The sequences of the template-primers are given in the figures. The incubation mixture (10 μl) contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mg of bovine serum albumin (BSA)/ml, 5′-end-labeled substrates, and NA. The reaction products of NA incorporation or excision were analyzed by electrophoresis through 16% polyacrylamide gel electrophoresis (PAGE) and were detected by autoradiography (7, 14). The results of the primer extension assays show the incorporation of ddCTP by HIV-1 RT opposite template G at site 10, 6, or 5 of template DNA (Fig. 1A, lane 1) or at site 2009 of template RNA (Fig. 1B, lane 1) in the presence of ddATP and ddCTP with running-start substrates, following the incorporation of two running-start A’s. HIV-1 RT displays NA incorporation capacity using DNA/DNA and RNA/DNA standing-start template-primers (wherein the target template residue immediately follows the 3′-terminal end of the primer) as well. The 17mer product is accumulated following the incorporation of ddATP (Fig. 1A, lane 4, B, lane 4) or ddCTP (Fig. 1A lane 7, and B, lane 7). Interestingly, the incorporation of either ddCTP, ddATP, or ddTTP was reduced in the presence of cytoplasmic extract of LCC2 cells with both DNA/DNA (Fig. 1A, lanes 2, 5, and 8) and RNA/DNA (Fig. 1B, lanes 2, 5, and 8) template-primers. Indeed, the decrease in the amount of ≥19mer and 17mer products was observed, and products longer than 16mer were formed. The decrease in NA incorporation (Fig. 1A and B, lanes 3, 6, and 9) was also detected in the presence of purified p53 (100 ng). Thus, p53, purified or in cytoplasmic fraction, substantially reduced the number of NAs incorporated into DNA. In control experiments, no reduction in incorporation of either ddCTP or ddATP was observed in the presence of cytoplasmic fractions of H1299 (p53-null) cells (4 μg) with DNA/DNA substrate (Fig. 1C, lanes 2 and 4, respectively).

The cytoplasmic fraction of LCC2 cells was further assessed for NA excision from DNA/DNA and RNA/DNA template-primers containing ddATP (set I) or ddTTP (set II) at 3′ termini (substrates that were produced by HIV-1 RT) (exper-
FIG. 1. Incorporation of nucleoside analogs. (A) The DNA/DNA template-primer (set I) was incubated with HIV-1 RT (1.5 U), dATP, and ddCTP in the absence (lane 1) or presence of either the cytoplasmic fraction of LCC2 cells (cyt p53) (lane 2) or purified wt p53 (lane 3) (I). The DNA/DNA template-primer (set II) was incubated with HIV-1 RT (1.5 U), dATP, and ddATP in the absence (lane 4) or presence of either the cytoplasmic fraction of LCC2 cells (cyt p53) (lane 5) or purified wt p53 (100 ng) (lane 6) (II). The DNA/DNA template-primer (set II) was incubated with HIV-1 RT, dATP, and ddCTP in the absence (lane 7) or presence of either the cytoplasmic fraction of H1299 cells (4 ng) (lane 8) or purified wt p53 (100 ng) (lane 9) (III). (B) The experiments with RNA/DNA template-primers were conducted as described above for the DNA/DNA template-primer. (C) The DNA/DNA template-primer was incubated with HIV-1 RT, dATP, and ddCTP in the absence (lane 1) or presence of the cytoplasmic fraction of H1299 cells (4 ng) (lane 2) (I). The DNA/DNA template-primer was incubated with HIV-1 RT and ddATP in the absence (lane 3) or presence of the cytoplasmic fraction of H1299 cells (4 ng) (II). wt p53 was expressed by employing a recombinant baculovirus and was purified to apparent homogeneity (20). The position of the 16mer primer is indicated by an arrow.

Two DNA hybrids, one with all matched normal deoxynucleotides between the primer and template strands (16 out of 25) and one with NA at the 3' end of the primer and template strand (17 out of 25), were used as the substrates for excision reactions. The hydrolysis of 3'-terminal ddAMP or ddTMP was observed from DNA/DNA (Fig. 2A, lanes 2 and 3 and lanes 5 and 6) and RNA/DNA (Fig. 2B, lanes 2 and 3 and lanes 5 and 6) template-primers. Interestingly, the excision of mismatched deoxynucleotides by p53 was more effective than the removal of drug (11). Figure 2 shows that the removal of the NA (17mer) was slower than the excision of matched deoxynucleotides; longer incubation times were required for excision of the terminally incorporated analogs (70 min). The excision of NA is probably less efficient than the removal of mismatched or matched nucleotides. The fact that the exonuclease is able to remove NA from the 3' termini of both substrates demonstrates no difference in the nucleic acid nature (RNA versus DNA) of the template.

Following insertion of NA, DNA polymerase dissociates from the template-primer (e.g., DNA polymerase α in the nucleus or HIV-1 RT in the cytoplasm). The fact that p53 binds the 3' mismatch containing template-primer (R. Gedelovish, E. Novitsky, G. Rahav, and M. Bakhanashvili, unpublished data) and analog-containing DNA (11) suggests that the NA-DNA would be subjected to removal by the external proof-reading activity in the nuclear or cytoplasmic compartment of the cell. Although p53 protein rapidly accumulated in the nuclei of the drug-treated cells, the removal of the incorporated nucleotide (dFdCMP) from cellular DNA was slow in whole cells with wild-type (wt) p53 and was not detectable in cells harboring mutant p53. There is a possibility that the difference stems from the fact that p53 in nuclear extracts displays a relatively low level of 3'→5' exonuclease activity compared to that in cytoplasmic extracts. It was of interest to examine the removal of the 3'-terminal NA in the presence of the nuclear fraction of MCF-7 cells (4 μg) expressing high levels of p53. The excision of 3'-terminally incorporated NA (e.g., ddATP) from DNA with nuclear fractions of MCF-7 cells appeared to be less efficient (Fig. 2C, lanes 5 and 6). Recent studies showed that although p53 in complex with DNA-protein kinase exhibited 3'→5' exonuclease activity, it was unable to excise the incorporated gemcitabine from a DNA construct containing gemcitabine at the penultimate site and a matched pair at the 3' end (1). The behavior of p53 exonuclease probably depends on the nature of the substrate.

To confirm that the detected NA excision activity in the cytoplasmic fraction of LCC2 cells is attributed to p53, we immunodepleted p53 from the cytoplasmic extracts by using various preparations of antibodies to analyze whether it would eliminate the exonuclease function of the protein. The depletion of p53 protein by Do-1 (Fig. 3, lane 3), PAb-6 (lane 5), or PAb-11 (lane 6) anti-p53 antibodies leads to a significant decrease in NA excision activity. Conversely, nonspecific immunoprecipitation by anti-horse immunoglobulin G (heavy plus light chains) did not affect exonuclease activity (lane 4). Thus, the inhibition of excision reaction was most probably caused by eliminating the p53 exonuclease activity.

The possibilities that p53 exonuclease may increase the fidelity of HIV-1 RT in the cytoplasm (7) and excise the incor-
Incorporated NAs suggest that p53 could confer a cellular resistance mechanism to the antiviral compounds.

We are indebted to A. Hizi (Tel-Aviv University, Tel-Aviv, Israel) for supplying us with purified HIV RT and M. Oren (Weizmann Institute, Rehovot, Israel).

This research was supported by a grant from the Israel Cancer Research Fund (ICRF) and by grant no. 5891 from the Chief Scientist Office, Ministry of Health.

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12. Huang, P. 1998. Excision of mismatched nucleotides from DNA: a potential FIG. 2. Excision of nucleoside analogs by 3′→5′ exonuclease activity of p53. (A) Excision reactions were carried out with DNA/DNA template-primers containing 3′-terminal ddAMP (lanes 1 to 3) or ddTMP (lanes 4 to 6) in the absence (lanes 1 and 4) or presence of the cytoplasmic fraction of LCC2 cells (4 µg) (lanes 2, 3, 5, and 6). The substrates were incubated for 40 min (lanes 2 and 5) or 70 min (lanes 3 and 6). (B) Excision reactions were carried out with RNA/DNA template-primers containing 3′-terminal ddAMP (lanes 1 to 3) or ddTMP (lanes 4 to 6) in the absence (lanes 1 and 4) or presence of the cytoplasmic fraction of LCC2 cells (4 µg) (lanes 2, 3, 5, and 6). (C) Excision reactions were carried out with DNA/DNA substrates containing 3′-terminal ddAMP in the presence of cytoplasmic fractions of LCC2 cells (4 µg) (lanes 3 and 4) or nuclear fractions of MCF-7 cells (4 µg) (lanes 5 and 6). The substrates were incubated for 40 min (lanes 3 and 5) or 70 min (lanes 4 and 6). The positions of the 16mer (correct) and 17mer (with 3′-terminal NA) primers are indicated by arrows.

FIG. 3. 3′-Terminal nucleoside analog excision with p53-depleted cytoplasmic fractions of LCC2 cells. Excision reactions were carried out with DNA/DNA template-primer containing 3′-terminal ddAMP in the absence (lanes 1) or presence of the cytoplasmic fraction of LCC2 cells (4 µg) (lanes 2), the cytoplasmic fraction of LCC2 cells immunodepleted by the Do-1 anti-p53 monoclonal antibody (lane 3), the cytoplasmic fraction of LCC2 cells immunodepleted by anti-horse IgG (heavy plus light chains) (lane 4), the cytoplasmic fraction of LCC2 cells immunodepleted by PAb-6 anti-p53 monoclonal antibody (lane 5), or the cytoplasmic fraction of LCC2 cells immunodepleted by PAb-11 anti-p53 monoclonal antibody (lane 6). The positions of the 16mer (correct) and 17mer (with 3′-terminal NA) primers are indicated by arrows.
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