

Inhibition of the integrase of human immunodeficiency virus (HIV) type 1 by anti-HIV plant proteins MAP30 and GAP31

(AIDS/antiviral target/DNA integration/antiviral agents)

SYLVIA LEE-HUANG*†, PAUL L. HUANG‡§, PHILIP L. HUANG‡§, ALDAR S. BOURINBAIAR*, HAO-CHIA CHEN¶, AND HSIANG-FU KUNG||

*Department of Biochemistry, New York University School of Medicine, New York, NY 10016; †American BioSciences, New York, NY 10021; ‡National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; §Laboratory of Biochemical Physiology, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21701

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ABSTRACT MAP30 (*Momordica* anti-HIV protein of 30 kDa) and GAP31 (*Gelonium* anti-HIV protein of 31 kDa) are anti-HIV plant proteins that we have identified, purified, and cloned from the medicinal plants *Momordica charantia* and *Gelonium multiflorum*. These antiviral agents are capable of inhibiting infection of HIV type 1 (HIV-1) in T lymphocytes and monocytes as well as replication of the virus in already-infected cells. They are not toxic to normal uninfected cells because they are unable to enter healthy cells. MAP30 and GAP31 also possess an N-glycosidase activity on 28S ribosomal RNA and a topological activity on plasmid and viral DNAs including HIV-1 long terminal repeats (LTRs). LTRs are essential sites for integration of viral DNA into the host genome by viral integrase. We therefore investigated the effect of MAP30 and GAP31 on HIV-1 integrase. We report that both of these antiviral agents exhibit dose-dependent inhibition of HIV-1 integrase. Inhibition was observed in all of the three specific reactions catalyzed by the integrase, namely, 3' processing (specific cleavage of the dinucleotide GT from the viral substrate), strand transfer (integration), and "disintegration" (the reversal of strand transfer). Inhibition was studied by using oligonucleotide substrates with sequences corresponding to the U3 and U5 regions of HIV LTR. In the presence of 20 ng of viral substrate, 50 ng of target substrate, and 4 μ M integrase, total inhibition was achieved at equimolar concentrations of the integrase and the antiviral proteins, with EC₅₀ values of about 1 μ M. Integration of viral DNA into the host chromosome is a vital step in the replicative cycle of retroviruses, including the AIDS virus. The inhibition of HIV-1 integrase by MAP30 and GAP31 suggests that impediment of viral DNA integration may play a key role in the anti-HIV activity of these plant proteins.

The integrase of human immunodeficiency virus (HIV) type 1 (HIV-1) is a critical target for selective antiviral therapy because it is responsible for the integration of viral DNA into the host genome (1–3). The integrated DNA, the provirus, serves as the template for the transcription of viral genes. Integration is thus essential for viral gene expression and the production of progeny viruses (4–8). The integration process requires two viral components: the integrase, a product of the *pol* gene, and specific cis-acting DNA sequences at the ends of the viral long terminal repeats (LTRs) (9–11). These sequences are highly conserved in all HIV genomes. The inaugural event leading to viral DNA integration is the binding of the integrase to the HIV LTR end sequences. This is followed by three sequential reactions. First, the 3' ends of the viral LTR DNA are processed by the removal of two nucleotides, GT, so that the 3' ends of both viral strands terminate with the

dinucleotide CA for precise integration. Next, there is strand transfer, a concerted cleavage–ligation reaction, in which the integrase makes a staggered cut in the target DNA and ligates the recessed 3' ends of the viral DNA to the 5' ends of the target DNA at the site of cleavage, resulting in a gapped intermediate. Finally, there is gap repair, the removal of the two unpaired nucleotides at the 5' end of the viral DNA and repair of the gap between the viral and the target DNA (12–16). In the presence of a DNA substrate that mimics the product of viral integration, integrase can catalyze the reversal of strand transfer, known as "disintegration" (17). In this reaction, the viral DNA is released and the target DNA is sealed. We have previously demonstrated that plant proteins MAP30 (*Momordica* anti-HIV protein of 30 kDa) and GAP31 (*Gelonium* anti-HIV protein of 31 kDa) are capable of topological inactivation of HIV LTR DNA (18–20). To delineate the antiviral mechanism of these compounds, we examined the effect of the proteins MAP30 and GAP31 on each of the reactions catalyzed by HIV-1 integrase. We report here our results of these investigations.

MATERIALS AND METHODS

Antiviral Proteins. Homogeneous MAP30 and GAP31 were used in these studies. These anti-HIV agents were prepared as described previously (20–22).

HIV-1 Integrase. Homogeneous recombinant HIV-1 integrase expressed in *Escherichia coli* was kindly supplied by Steve Hughes of the National Cancer Institute and Anna M. Skalka of Fox Chase Cancer Center.

Oligonucleotide Substrates. Oligonucleotide substrates were synthesized on an Applied Biosystems model 380 B DNA synthesizer and purified by gel electrophoresis. The sequences of the oligonucleotides correspond to the U3 and U5 ends of HIV LTR. Three types of substrates were synthesized, including (i) the 21-nucleotide minus strand of HIV-1 U3 LTR, 5'-GAGTGAATTAGCCCTTCCAGT-3', and the U5 LTR, 5'-GTGTGGAAAATCTCTAGCAGT-3', as well as their complementary strands, for assaying the 3'-processing reaction; (ii) the 19-mer U3-GT, 5'-GAGTGAATTAGCCCTTCCA-3', and U5-GT, 5'-GTGTGGAAAATCTCTAGCA-3' (i.e., U3 and U5 minus the 3'-end dinucleotide GT) for assaying heterologous integration; and (iii) a 38-mer, 5'-TGCTAGTTCTAGCAGGCCCTTGGGCCGCGCTTGCCG-3', known as the dumbbell substrate, for assaying the disintegration reaction (23).

Abbreviations: HIV, human immunodeficiency virus; HIV-1, HIV type 1; GAP31, *Gelonium* anti-HIV protein of 31 kDa; LTR, long terminal repeat; MAP30, *Momordica* anti-HIV protein of 30 kDa.

†To whom reprint requests should be addressed.
§Present address: Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.

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Radiolabeling and Preparation of the Substrates. The U3 LTR, U5 LTR, U3-GT, U5-GT, and the dumbbell substrate, 1 μ g each, were 5'-end labeled with 100 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) by 20 units of polynucleotide T4 kinase in a final volume of 40 μ l of kinase buffer (Boehringer Mannheim) at 37°C for 60 min. The reaction was stopped by the addition of EDTA to 25 mM and the kinase was inactivated by heating to 65°C for 10 min. The unincorporated label was removed by two passages through a Sephadex G-25 spin column (Boehringer Mannheim). The purified labeled oligonucleotide was then annealed with an equimolar amount of its unlabeled complementary strand in 10 mM Tris-HCl, pH 7.5/1 mM EDTA/100 mM NaCl at 95°C for 5 min followed by slow cooling. The dumbbell substrate was annealed by itself under the same conditions.

3'-Processing Reaction. The 3'-processing reaction was carried out in integrase buffer (buffer IN: 20 mM Hepes, pH 7.5/10 mM MnCl₂/1 mM dithiothreitol/0.5% Nonidet P-40) containing 20 ng of the annealed double-stranded substrate U3 or U5 LTR and 40 pmol of HIV-1 integrase in the presence or absence of MAP30 or GAP31 in a final volume of 10 μ l. Incubation was for 60 min at 37°C. The reaction was stopped by the addition of 10 μ l of stop buffer [90% (vol/vol) formamide/0.025% bromophenol blue/0.025% xylene cyanol/89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.0]. The samples were heated at 95°C for 3 min and electrophoresed on a 20% polyacrylamide denaturing (7.5 M urea) gel in TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.0). The results were visualized by autoradiography of wet gels.

Strand-Transfer (Integration) Reaction. The strand-transfer reaction was assayed by the integration of viral DNA into heterologous plasmid DNA. U3-GT or U5-GT was used as the viral substrate and pUC18 plasmid DNA was used as the target. The reaction mixture contained 20 ng of the viral substrate, 50 ng of target substrate, and 40 pmol of HIV-1 integrase in 10 μ l of buffer IN in the presence or absence of MAP30 or GAP31. The reaction was carried out at 37°C for 60 min and stopped by adding SDS to 0.1%. Products of integration were separated on a 1% agarose gel in Tris-phosphate buffer (36 mM Tris/33 mM NaH₂PO₄/1 mM EDTA, pH 8.0). The results were visualized by autoradiography of dried gels.

Disintegration Reaction. The disintegration activity of HIV-1 integrase was assayed by the disintegration of the annealed dumbbell substrate (20 ng) in buffer IN. The reaction conditions were identical to those described for 3' processing. The results were analyzed by electrophoresis on an 18% polyacrylamide denaturing gel and visualized by autoradiography.

DNA Topological Activity. The topological activity of HIV-1 integrase, MAP30, and GAP31 was monitored by the conversion of supercoiled plasmid DNA to relaxed (nicked) and linear forms. The reaction was carried out in a final volume of 10 μ l containing 180 ng of supercoiled pUC18 substrate, in buffers with various divalent cations and concentrations. Buffer I (10 mM Tris-HCl, pH 7.5/50 mM NaCl/5 mM MgCl₂/1 mM dithiothreitol) contains 5 mM Mg²⁺ as the sole divalent ion. Buffer II (20 mM Hepes, pH 7.5/5 mM MnCl₂/1 mM dithiothreitol) contains 5 mM Mn²⁺ and no Mg²⁺. Buffer III (20 mM Hepes, pH 7.5/10 mM MnCl₂/1 mM dithiothreitol) contains 10 mM Mn²⁺. The assays were conducted at 37°C for 60 min. The samples were analyzed by electrophoresis in 1% agarose in Tris-phosphate buffer. Gels were stained with ethidium bromide (0.5 μ g/ml) for 30 min and destained in distilled water prior to photodocumentation using a short-wavelength UV lamp.

RESULTS

Inhibition of 3'-Processing Activity of HIV-1 Integrase by MAP30 and GAP31. The 3'-processing activity of HIV-1

integrase was monitored by a dinucleotide cleavage reaction using a 21-bp double-stranded oligonucleotide that mimics the U3 or U5 end of HIV-1 LTR. The reaction scheme is depicted in Fig. 1A. The specific cleavage of the dinucleotide GT from the 3' end of the labeled minus strand of the substrate results in the formation of a 3' recessed product which is 19 nucleotides in length. The appearance of the shorter product was monitored by polyacrylamide gel electrophoresis as seen in Fig. 1B. In the presence of MAP30 or GAP31, a dose-dependent inhibition of the cleavage of the 3' dinucleotide GT was detected with both U3 and U5 substrates. The degree of inhibition depends on the concentrations of both HIV-1 integrase and the antiviral proteins. At equimolar concentrations, 100% inhibition was observed.

Inhibition of Strand-Transfer Activity of HIV-1 Integrase by MAP30 and GAP31. The effect of MAP30 and GAP31 on the integration (strand-transfer) activity of HIV-1 integrase was measured by a quantitative heterologous integration assay. To focus the assay on strand transfer, supercoiled pUC18 plasmid DNA was used as a heterologous target, and 3'-recessed U3 and

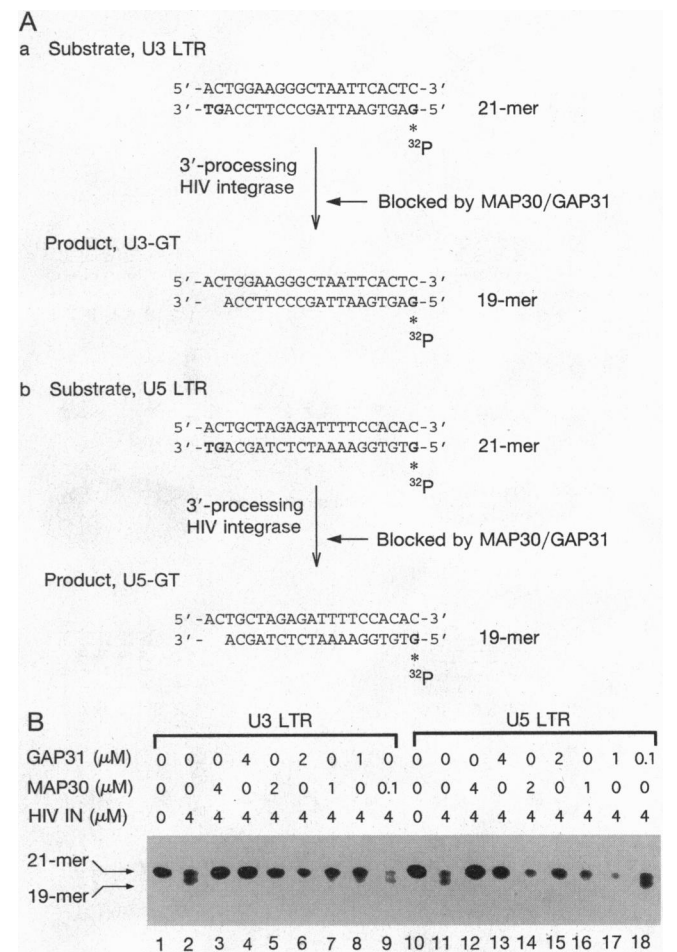


FIG. 1. (A) Schematic representation of the 3'-processing reaction of HIV integrase. (a) The 3' processing of double-stranded U3 LTR by HIV integrase: specific cleavage of the dinucleotide GT from the 3' end and formation of the 3'-recessed U3-GT. (b) The 3' processing of double-stranded U5 LTR by HIV integrase: specific cleavage of the dinucleotide GT from the 3' end and formation of the 3'-recessed U5-GT. (B) Inhibition of 3'-processing activity of HIV integrase by MAP30 and GAP31. The 3'-processing activity of HIV integrase (IN) was assayed by specific cleavage of 5'- 32 P-labeled 21-mer U3 and U5 LTR substrates. The addition of MAP30 and GAP31 caused dose-dependent inhibition in HIV-integrase-mediated cleavage of the substrates. The effect was monitored by the inhibition in the formation of labeled 19-mer products.

U5 oligonucleotides, which already lack the 3' GT dinucleotides, designated as U3-GT and U5-GT, were used as viral substrates. Under these conditions any inhibition detected must be specific for strand transfer and not for 3' processing. The general scheme of the reaction is shown in Fig. 2A. Incubation of 5'-labeled U3-GT or U5-GT with unlabeled pUC18 in the presence of integrase results in the integration of the labeled oligonucleotide

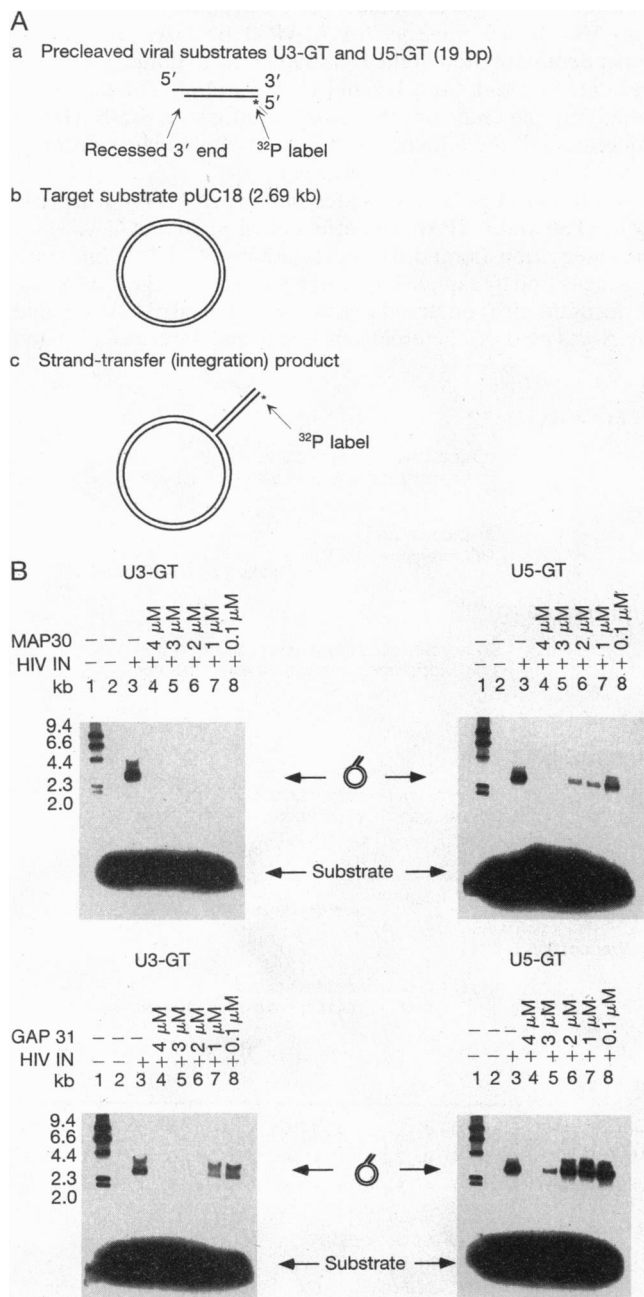


FIG. 2. (A) Schematic representation of strand transfer (integration) of precleaved viral substrate, U3-GT and U5-GT, into heterologous target substrate pUC18 DNA. (B) Inhibition of strand-transfer (integration) activity of HIV-1 integrase by MAP30 and GAP31. Integration was monitored by the conversion of the plasmid into labeled DNA. HIV integrase (IN) was 4 μ M in all assays. Lanes 1, 5'- 32 P-labeled size marker, *Hind*III fragments of λ phage DNA. Lanes 2, target substrate, pUC18; because it was unlabeled, it was not seen in the autoradiogram. Lanes 3, the product of integration by HIV integrase. The integration of the 5'- 32 P-labeled U3-GT or U5-GT into pUC18 resulted in the appearance of a labeled band at 2.69 kb, corresponding to the size of pUC18. Lanes 4-8, the addition of MAP30 or GAP31 to final concentrations of 4, 3, 2, 1, and 0.1 μ M, respectively.

into the target plasmid. Integration was monitored by the conversion of unlabeled plasmid to labeled DNA. The results are shown in Fig. 2B. Both MAP30 and GAP31 exhibited dose-dependent inhibition on the strand-transfer activity of HIV-1 integrase, and total inhibition was observed at stoichiometric concentrations of the HIV inhibitor and HIV integrase. The reaction proceeded more efficiently with U5-GT than with U3-GT. These results are in agreement with those reported by others (24). It is noteworthy that the U3 LTR contains a specific integrase-binding site, the homopurine motif 5'-GGAAGG-3'. The U5 LTR does not possess this target motif, but it was more readily integrated. In terms of inhibition by the anti-HIV agents, U3 was also distinct from U5: its integration was inhibited to a much greater extent than that of U5. It has been reported that the sequences of both the viral and target substrates affect HIV-1 integrase-mediated integration *in vitro* (9, 15, 24, 25). The relative efficiency of U3 and U5 integration is not equivalent, and it was suggested that integration of U3 LTR is the rate-limiting step in the overall integration process. The less insertion and the greater inhibition of U3 LTR demonstrated its uniqueness and its specificity for the anti-HIV agents.

Inhibition of Disintegration Activity of HIV-1 Integrase by MAP30 and GAP31. Disintegration, the reverse of integration, is a concerted strand-cleavage and ligation reaction (24). This reaction is depicted in Fig. 3A. Strand cleavage takes place precisely at the junction between the viral and the target sequences. The dumbbell substrate is a 38-mer oligonucleotide that mimics the recombination intermediate of retrovirus integration. It contains virus-specific, U5 LTR, and arbitrary target DNA sequences (23, 24). The folded structure of the annealed substrate shown in Fig. 3A is based on reports obtained from hairpin formation by similar sequences (23, 24, 26, 27). The two unpaired 5' nucleotides of the viral DNA, which are not required for disintegration, are not included in the substrate. The viral sequence is in the stem of the hairpin loop and the target sequence is in the base of the dumbbell. Disintegration of the dumbbell by HIV-1 integrase is expected to give two products, a 14-mer hairpin loop viral sequence and a 24-mer closed circular target DNA. These results are shown in Fig. 3B. When the 5'-labeled dumbbell was used as the substrate, HIV-1 integrase catalyzed the formation of a 14-mer labeled product and an unlabeled 24-nucleotide target DNA. The labeled hairpin loop was detected by autoradiography of the gel, whereas the 24-mer product, the unlabeled target DNA, was not seen in the autoradiography. This product was seen by UV shadow of the gel or by the use of 3'-labeled substrate. These results indicate that the antiviral proteins are able to inhibit the cleavage and/or release of the viral DNA from the integrated complex. The disintegration assay using the dumbbell substrate (23) was designed to determine the kinetics and the turnover characteristics of the integrase (23, 24). Correct strand cleavage and ligation of the substrate promote catalytic turnover of the integrase (23). Thus inhibition of disintegration by MAP30 and GAP31 may also affect catalytic recycling of the HIV-1 integrase.

Comparison of the DNA Topological Activity of HIV-1 Integrase, MAP30, and GAP31. HIV-1 integrase, MAP30, and GAP31 all exhibit topological activity on supercoiled plasmid and viral DNAs. It is important to define the similarities and differences between these proteins in their DNA topological activity. Several interesting points found are shown in Fig. 4: (i) HIV-1 integrase demonstrated an absolute requirement of Mn^{2+} for activity, whereas MAP30 and GAP31 were active with both Mn^{2+} and Mg^{2+} . In buffer I, in the presence of 5 mM Mg^{2+} and no Mn^{2+} , HIV-1 integrase was inactive, whereas MAP30 and GAP31 were active. They totally converted the supercoiled substrate to relaxed (95%) and linear (5%) forms. In buffer II, in 5 mM Mn^{2+} and no Mg^{2+} , all three proteins showed identical activity and catalyzed complete conversion of

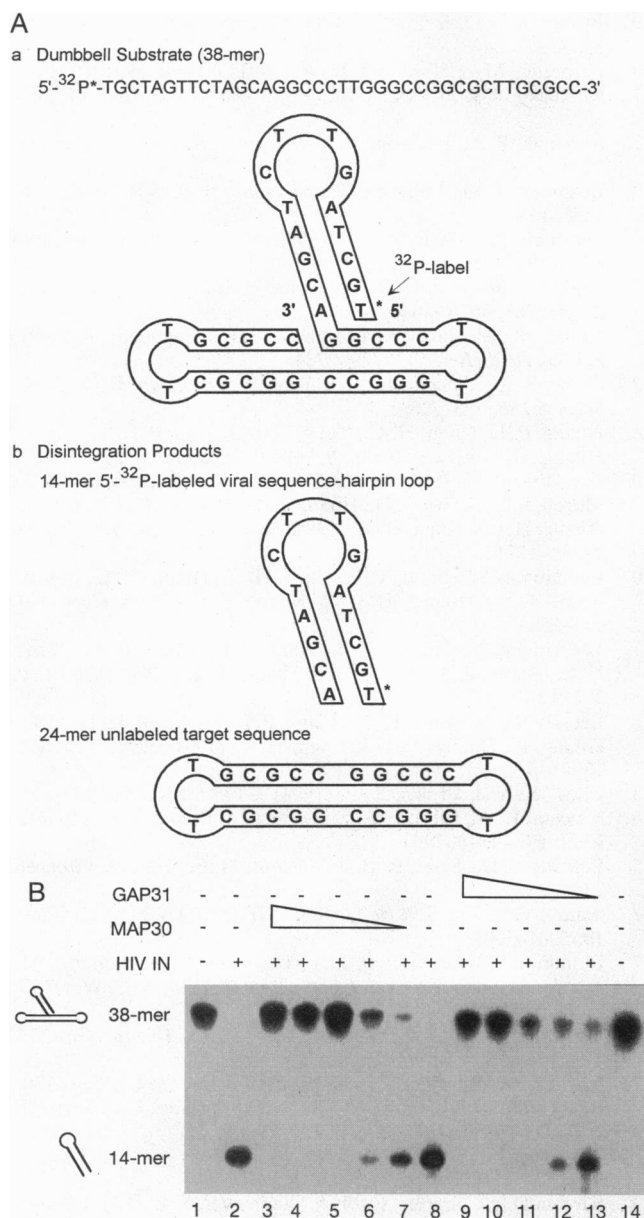


FIG. 3. (A) Schematic representation of the disintegration activity of HIV-1 integrase. (a) The 5'-labeled 38-mer dumbbell substrate with the predicted secondary structure. (b) Disintegration yields a ³²P-labeled 14-mer consisting of the viral sequences in the hairpin stem and a 24-mer unlabeled target sequence that has been repaired. (B) Inhibition of disintegration activity of HIV-1 integrase by MAP30 and GAP31. Lanes 1 and 14, the 38-mer dumbbell substrate. Lanes 2 and 8, the 5'-³²P-labeled 14-mer disintegration product [HIV integrase (IN) at 4 μM]. Lanes 3, 4, 5, 6, and 7, HIV integrase reaction mixtures contained 4, 3, 2, 1, and 0.1 μM MAP30. Lanes 9, 10, 11, 12, and 13 contained 4, 3, 2, 1, and 0.1 μM GAP31.

supercoiled substrate to equivalent amounts of relaxed and linear forms. In buffer III, in 10 mM Mn²⁺, the concentration used in integrase assays, all three proteins displayed analogous activity and generated similar products with an increased formation of linear DNA. (ii) The relaxed product of HIV-1 integrase was topologically active and could be supercoiled by DNA gyrase, whereas the products of MAP30 and GAP31 were inactive substrates for the gyrase.

DISCUSSION

Studies of compounds derived from medicinal plants have yielded basic knowledge that is critical to modern medicine.

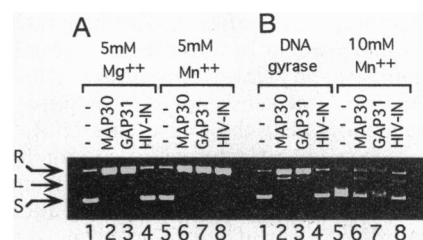


FIG. 4. Comparison of the topological activities of HIV-1 integrase, MAP30, and GAP31 with supercoiled plasmid DNA, pUC18. (A) Lanes 1–4, buffer I (5 mM MgCl₂); lanes 5–8, buffer II (5 mM MnCl₂). Lanes 1 and 5, no addition; lanes 2 and 6, 3 and 7, and 4 and 8, addition of MAP30, GAP31, or HIV integrase (IN), respectively, each at 4 μM. S, supercoiled; R, relaxed circle; and L, linear form of the DNA. (B) Lane 1, supercoiled DNA control. Lanes 2–4, samples from lanes 6–8 in A, purified and treated with DNA gyrase. Lanes 5–8, same as A, except in buffer III (10 mM MnCl₂).

MAP30 is isolated from *Momordica charantia*, commonly known as bitter melon. This plant is indigenous to China. Its extracts have been used for centuries as antiinfection and antitumor agents. Recently there has been intense community interest in the use of bitter melon as a therapy for AIDS. Encouraging results have been reported (28). GAP31 comes from *Gelonium multiflorum*, an ancient medicinal plant from the Himalayan mountains. This plant has also been widely used against common infections. The discovery that MAP30 and GAP31 are potent inhibitors of HIV-1 integrase suggests that these antiviral agents act on a critical target indispensable to the life cycle of the AIDS virus.

We have identified at least three activities that may be critical to the antiviral action of MAP30 and GAP31. The first is an *in vitro* RNA N-glycosidase activity that cleaves the link between a ribose and a purine in 28S rRNA, specifically at A-4324 or G-4323, and thus inhibits ribosomal function in cell-free assays (29, 30). The second is a DNA topological inactivation activity that renders viral and plasmid DNA topologically inactive (18–20). The third is the inhibition of HIV-1 integrase reported in this article. It is important to define the extent to which these activities, or whether all of them, contribute to anti-HIV activity. These studies should provide basic insights on how to enhance anti-HIV activity while eliminating other activities that do not contribute to anti-HIV function. We have cloned and expressed MAP30 (20) and GAP31. Identical to their natural counterparts, the recombinant products are also active in these reactions.

Viral DNA integration is a nonhomologous recombination event, and the role of integrase in this process has been extensively investigated. The 3'-processing and strand-transfer reactions are thought to take place via a single transesterification event (4–8). In 3' processing, the integrase activates the phosphodiester bond at the site of cleavage to nucleophilic attack by nucleophiles (8–15). In strand transfer, although it engages in a similar mechanism, the integrase is also required to position the 3'-OH end of the viral DNA for nucleophilic attack on a phosphodiester bond in the target DNA (8). In disintegration, the integrase is required to cleave the phosphodiester bond at the junction of the viral and target DNA, liberate the viral DNA, and seal the gap in the target DNA (17, 23). The inhibition of 3' processing by MAP30 and GAP31 indicates that these antiviral proteins are capable of blocking the specific actions of integrase in the U3 and U5 regions of HIV LTR. The inhibition of strand transfer with precleaved substrates U3-GT and U5-GT indicates that these antiviral proteins are effective in interrupting the positioning of the 3'-OH end of the viral substrate to the target DNA, independent of 3' processing. Finally, the inhibition of disintegration suggests that these antiviral proteins effect cleavage at the viral and host DNA junction, or block the release of the viral DNA

from the integration intermediate, and prohibit the turnover of the integrase, independent of the other two reactions.

Retroviral integration involves two substrates that engage in distinct roles. The viral substrate is recognized by specific nucleotide sequences near the end of the LTR. The target substrate is recognized at internal sites with little known sequence preferences. However, the site of integration is not totally random (31). The basis for specific locations for integration remains to be elucidated. It has been reported that chromatin structure, genomic DNA sequence, and other host cell factors can affect target site selection (32). *In vivo* studies also suggest that retroviral integration takes place preferentially at regions of transcriptionally active and DNase I-hypersensitive sites (33). HIV-1 integrase, MAP30, and GAP31 are all capable of binding to viral and target substrates. They are equally effective in relaxing supercoiled target DNA and making specific double-strand nicks in the target DNA. Integrase prepares the substrates for integration, whereas MAP30 and GAP31 block viral integration. Factors that promote viral integration have been reported, including integrase interacting protein, a human homolog of yeast transcription factor SNF5 (34). To our knowledge, MAP30 and GAP31 are the first reported protein factors that block the sites of recombination and prevent viral integration.

In the design and development of specific antiviral agents, information on the fine structure of the viral target as well as its inhibitors is of prime importance. The three-dimensional structure of the catalytic domain of HIV-1 integrase has been reported (3). This central core domain is evolutionarily conserved in all retroviral integrases. In addition to catalytic activity it also contains the determinants for recognition of the CA/GT dinucleotides that are present at the LTR ends of HIV-1 and all retroviruses (35–37). We have crystallized MAP30 and GAP31 (38) and are analyzing the fine structure of these proteins. Knowledge of the interactions between the integrase and MAP30 or GAP31 in the presence of the viral and target substrates should be useful in designing selective HIV inhibitors.

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