Volume 300, number 1, 97–100 FEBS 10857 March 1992

RNase H activity of HIV reverse transcriptases is confined exclusively to the dimeric forms

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Received 30 January 1992

A method for the rapid preparation of a defined substrate to monitor RNase H activity has been developed. Using this substrate, we have investigated the RNase H activities of the different forms of recombinant HIV-1 and HIV-2 reverse transcriptase (RT) in detail. As we report here, RNase H activity is associated only with the dimeric forms (p51/p66 or p66/p66) of the enzymes

HIV; Reverse transcriptase; RNase H; Dimerization

1. INTRODUCTION

In addition to their DNA polymerase activity, retroviral reverse transcriptases possess an RNase H activity which degrades the RNA moiety of an RNA/DNA hybrid. The RNase H activity is essential for the process of conversion of genetic information from the single stranded RNA form in the virion into the double stranded DNA form in infected cells. Formally, during synthesis of the minus strand of DNA complementary to the viral RNA genome, the RNAse H activity is required to degrade the RNA strand in the DNA/RNA hybrid to allow plus strand DNA synthesis. The actual mechanism appears to involve the simultaneous action of both the polymerase and RNase H activities, so that as the DNA strand grows, the RNA template is degraded [1].

The reverse transcriptases of HIV-1 and HIV-2 are heterodimeric proteins consisting of one subunit of molecular weight of approximately 66 kDa and another of molecular weight of 51 kDa, which represents a carboxyterminally truncated form of the larger subunit [2–7]. The difference polypeptide between these two subunits contains the RNAse H activity of the reverse transcriptase [8,9]. There is still controversy regarding the biological significance of the dimeric organization, which is characteristic of the known lentiviral RTs. In particular there is no agreement about the significance of the monomeric and homodimeric species which have been isolated from recombinant expression systems.

Using recombinant enzyme preparations we have investigated the biochemical properties of monomeric and dimeric forms of HIV-1 and HIV-2 RT. We were particularly interested to determine whether the RNAse H activity is influenced by subunit interaction. We describe here experiments which clarify the dependence of the RNAse H activity on the state of dimerization of the reverse transcriptase molecule.

Abbreviations: BSA, bovine serum albumine; *E. coli*, *Escherichia coli*; FPLC, fast protein liquid chromatography; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; RT, reverse transcriptase.

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2. MATERIALS AND METHODS

2.1 *Proteins*

Recombinant RTs from HIV-1 and HIV-2 were expressed in *E. coli* and purified as described previously [7,10].

The coding sequence of the RT RNase H domain of HIV-1_{BRU} starting with Tyr^{427} was amplified by PCR. By using appropriate oligonucleotides for PCR amplification we added four codons (Met-Ala-Asn-Glu) to the N terminus and a TAA stop codon to the 3′ terminus of the coding sequence. Flanking *NcoI* restriction sites were also introduced. The fragment was digested with *NcoI* and then ligated with the prokaryotic expression vector pKK233-2 [11] which had been linearized with *NcoI*. *E. coli* CGSC 6662 cells which contained the compatible repressor plasmid pDMI.1 [12] were transformed with the expression construct. The bacteria were grown in LB medium to an OD_{578} of ca. 0.5 and induced with 20 mg/ml isopropyl-1-thio-β-D-galactopyranoside for 5 h.

The cells were harvested by centrifugation and resuspended in buffer A (50 mM Tris-HCl pH 8.0, 5 mM DTT, 1 mM EDTA, 0.5% NP-40, 0.4 mM PMSF, 1 mM Americain, 0.1 mM benzamidine). Following cell lysis by sonification, insoluble material was removed by centrifugation (30 min, 80,000 × g). Nucleic acids were precipitated from the cell extract with 40 mg/ml streptomycin sulfate and removed by centrifugation (20 min, 48,000 × g). The supernatant was dialyzed overnight against buffer B (50 mM Tris-HCl pH 7.6, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF). All following purification steps were performed using a FPLC system (Waters). The crude extract was applied to a DEAE fractogel column (Superformance, 26 × 300 mm, Fractogel EM[] DEAE 650, Merck) in buffer B. Bound protein was

eluted with a step gradient (50 mM, 130 mM and 200 mM NaCl in buffer B). Fractions containing RNase H were collected and the protein was precipitated with 80% ammonium sulfate. Following centrifugation (20 min, 48,000 × g) the pellet was resuspended in buffer C (25 mM Tris-HCl pH 8.2, 1 mM DTT, 1 mM EDTA) containing 75 mM NaCl and applied to a gel filtration column (HiLoad, 26 × 600 mm, Superdex 200; Pharmacia) in buffer C + 75 mM NaCl. The recombinant protein was finally purified by anion- exchange chromatography on a Q-Sepharose column (HiLoad, 16 × 100 mm; Pharmacia). The fractions from the gel filtration column containing RNase H were diluted by the addition of 2 vols. of buffer C and applied to the column in buffer C. The bound protein was eluted by a step gradient (50 mM, 100 mM and 200 mM NaCl in buffer C). The purified protein was then dialyzed against 20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 15% glycerol and stored at −80°C.

2.2. *Preparation of the RNase H substrate*

The plasmid pBS-Endo (pBluescript KS containing the coding sequence for the endonuclease from HIV-1_{BRU}) was linearized with *Xho*I. Transcription of DNA into RNA was carried out in a total vol. of 50 μl containing 40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 100 μg/ml BSA, 60 U RNasin (Promega), 0.5 mM rNTP (GTP, CTP and UTP), 0.05 mM ATP [α-^{32}P]ATP, 400 Ci/mmol or [^{3}H]ATP, 44 Ci/mmol; Amersham), 2–5 μg linearized vector DNA and 100 U T7 RNA polymerase (New England Biolabs). After 2 h at 37°C the reaction was stopped by the addition of 20 mM EDTA and purified using Quiagen columns (according to the instructions of the manufacturer: Diagen; Düsseldorf, Germany). The purified RNA was dissolved in hybridization buffer (80% formamide, 400 mM NaCl, 1 mM EDTA, 50 mM HEPES pH 6.4) and the concentration was calculated from the amount of incorporated radioactivity. The corresponding DNA fragment was amplified by PCR from the plasmid pBS- Endo using PCR primers hybridizing to the T3 and T7 promotor sequences respectively. PCR conditions were: 67 mM Tris-HCl pH 8.8, 10% DMSO, 0.16 mg/ml BSA, 2 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 7 mM β-mercaptoethanol, 200 μM dNTP each, 0.4 μM primer, 15 pM vector and 2 U *Taq* DNA polymerase (Beckman) in a 50 μl reaction mixture. 40 cycles (1 min at 90°C, 1 min at 50°C and 10 min at 70°C) were performed. The product was purified by HPLC gel filtration in 20 mM Tris-HCl pH 7.5, 100 mM NaCl (columns see 2.5). After ethanol precipitation, the fragment was dissolved in TE buffer and the concentration was determined by measuring the absorbance at 260 nm. Subsequently the DNA was lyophilized and redissolved in 50 μl hybridization buffer containing the labelled RNA. The molar ratio of RNA to the corresponding DNA strand was 1:3. The mixture was incubated for 15 min at 85°C followed by 3 h at 55°C. To remove the formamide, the hybridization mixture was applied to a sepharose (CL- 6B, Pharmacia) column and eluted with TE (10 mM Tris-HCl pH 7.8, 1 mM DTT) buffer. Digestion of the final preparation with S1 nuclease led to degradation of ca. 20% of the radioactively labelled RNA to acid soluble material, suggesting a contamination with single stranded RNA. The remaining 80% of the RNA could be completely degraded by reverse transcriptase.

2.3. *Determination of the RNase H activity*

The reaction solution contained: 50 mM Tris-HCl pH 8.0, 80 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, 5 pmol RNA/DNA hybrid and enzyme. Reactions were incubated for 10 min at 37°C and stopped by the addition of 2 ml ice-cold 5% trichloroacetic acid. Acid-insoluble material was collected on nitrocellulose filters (Schleicher and Schuell, BA 85) and radioactivity retained on the filters was measured by scintillation counting. 1 U of RNase H activity catalyzes the release of 1 nmol rNMP from a RNA/DNA hybrid in 10 min at 37°C.

2.4. *Preparation and characterization of anti HIV-1 RT monoclonal antibodies*

The preparation and characterization of 23 monoclonal antibodies against HIV-1 RT will be described in detail elsewhere (Resch et al., submitted). Briefly, mice were immunized with purified recombinant HIV-1 RT and production of hybridomas was performed as described by Pawlita et al. [13]. Monoclonal antibodies were purified from hybridoma cell supernatants as well as from ascites fluid using a commercially available kit (MAPS II kit, Bio-Rad) following the instructions of the manufacturer. All preparations were proven to be free of protease and nuclease activities. Epitope mapping was performed using a set of 11 C-terminal deletion mutants of HIV-1 RT. The interaction of the antibodies with the truncated proteins was investigated by immunoblotting [14].

2.5. *HPLC size exclusion chromatography*

Chromatography was performed using two HPLC columns in series (Bio-Rad TSK-250 followed by Bio-Rad TSK-125; both 7.5 × 300 mm). The columns were eluted with 200 mM potassium phosphate pH 6.5 at 1 ml/min.

3. RESULTS AND DISCUSSION

For determination of the RNase H activity of HIV RT we developed a simple method for the rapid preparation of large amounts of a defined RNA/DNA hybrid as outlined in Fig. 1. The method is based on the production of a 'runoff' RNA transcript of defined length by T7 polymerase, followed by hybridization to the complementary DNA strand. The DNA fragment was amplified by PCR and hybridization was carried out under conditions favouring formation of RNA/DNA hybrids. The resulting hybrid, which was labelled uniformly with [^{3}H]- or [^{32}P]ATP in the RNA strand, consisted of a 1043 base DNA strand derived from the HIV-1 endonuclease sequence and a complementary RNA strand of 966 bases. The degradation of the RNA

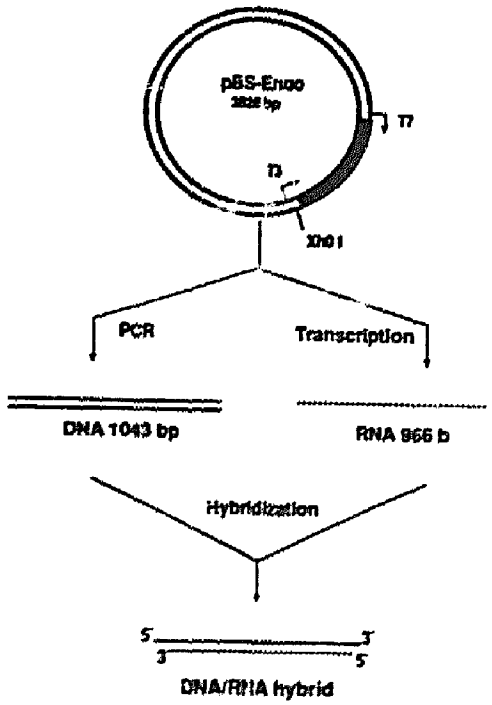

Fig. 1. Preparation of the RNA/DNA hybrid. For details see materials and methods. The resulting hybrid has 5'- and 3'-DNA overhanging ends of 53 and 24 bases respectively.

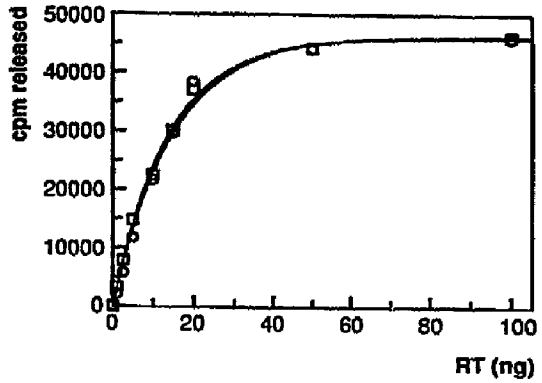

Fig. 2. Comparison of the RNase H activity of HIV-1 (circles) and HIV-2 (squares) reverse transcriptase heterodimers as a function of the enzyme concentration.

moiety of the hybrid was followed by the decrease of acid-precipitable radioactivity upon incubation with RT.

The different forms of HIV-1 and HIV-2 RT were incubated with this substrate to determine their specific RNase H activities. Preparations of heterodimeric reverse transcriptase from HIV-1 had specific activities of ca. 400 U/mg. The same activity was found for the dimeric form of the 66 kD; subunit of reverse transcriptase. It is of interest to note that recombinant reverse transcriptase from HIV-2 [7] exhibits a comparable RNase H activity (Fig. 2), in contrast with earlier findings which suggested that the activity was 10 times lower than in the case of HIV-1 [15]. This activity arose exclusively from the recombinant protein, and not from contaminating *E. coli* RNase H, as proven by the result that it could be completely inhibited by a monoclonal antibody (designated MAb 2; Fig. 3). This antibody belongs to a set of 23 monoclonal antibodies

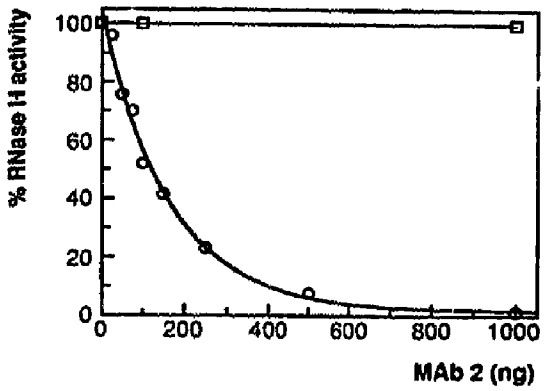

Fig. 3. Inhibition of HIV-1 reverse transcriptase RNase H activity by a purified monoclonal antibody. See text for details. A preincubation of enzyme and antibody was performed for ca. 10 minutes on ice before starting the reaction by adding the substrate. The amount of heterodimeric RT was held constant at 20 ng. The 100% value corresponds to a specific activity of 400 units/mg. Circles, HIV-1; squares, HIV-2.

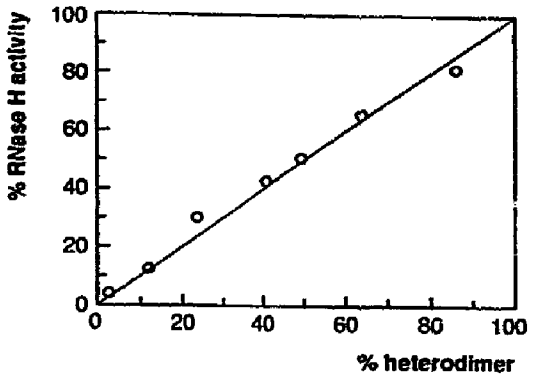

Fig. 4. Specific RNase H activity of HIV-1 reverse transcriptase (p51/p66) as a function of the degree of dimerization. The heterodimer was dissociated by treatment with 15% acetonitrile and re-association was initiated by lowering the acetonitrile concentration to 3%. For details see text and refs. [5,7]. RNase H activity was measured throughout the course of redimerization and the dimer content was determined in parallel by HPLC gel filtration. The 100% value corresponds to a specific activity of 400 U/mg.

prepared against recombinant HIV-1 RT [16; Restle et al., submitted] and has been shown to recognize an epitope located between amino acids 300 and 350 of the protein (for details see section 2). Since this antibody recognizes an epitope which is not part of the RNase H domain, it is evident that the inhibition cannot arise from cross reactivity with contaminating RNase H from other sources. HIV-2 RT, which does not cross react with this monoclonal antibody, shows no reduction of RNase H activity in a control experiment (Fig. 3). This demonstrates that the inhibition of the HIV-1 enzyme occurs in a specific manner.

We have previously shown that the RNA-dependent DNA-polymerase activity of reverse transcriptase from HIV-1 and HIV-2 is only present in dimeric forms of the enzyme (51 kDa homodimers, 66 kDa homodimers or 66/51 kDa heterodimers) and that the monomeric forms show no activity [5,7]. The results shown in Fig. 4 demonstrate that this is also true for the RNase H activity. As we have described earlier [5], dimeric HIV RT can be dissociated by the addition of acetonitrile. Upon reduction of the organic solvent concentration by dilution of the sample, slow ($t_{1/2}$ approximately 12 h at the concentration of 6 μM used here) quantitative re-association of the subunits to the native heterodimer occurs. This re-association process can be studied in a time-dependent manner by high resolution HPLC size exclusion chromatography. Using this technique we found that there is also a linear relationship between the degree of dimerization and the specific RNase H activity of the RT heterodimer (Fig. 4). Similar results were obtained for the 66 kDa preparations (data not shown), whereas neither the dimeric nor the monomeric form of the 51 kDa subunit showed detectable activity. Preparations of reverse transcriptase from HIV-2 showed similar behaviour (data not shown). We therefore conclude

that RNase H activity of reverse transcriptases from HIV-1 and HIV-2 is confined to the dimeric forms.

Reports on the RNase H activity of the isolated RNase H domain of HIV-1 reverse transcriptase have been inconsistent, with claims of active [1,9,17,18] and inactive [19] preparations. We have also investigated recombinant RNase H with respect to its enzymatic properties. Expression of the C-terminal domain in *E. coli*, starting with Phe^{440} of the RT coding sequence, was not successful. In contrast, chosing Tyr^{427} as the starting point [17] led to efficient expression in the range of 10–15% of soluble protein. The recombinant protein was purified as described in section 2. Upon purification by FPLC gel filtration, the recombinant protein was separated into two fractions which appeared identical on SDS-PAGE. These probably correspond to monomeric and dimeric forms of the protein with molecular weights of 16 and 30 kDa, respectively, as verified by high resolution HPLC size exclusion chromatography. However, it was not possible to induce dissociation of the putative dimeric form or association of the monomeric form by dilution or concentration of the protein solution respectively, so that we believe that they represent forms of the protein which are folded differently. Very low (ca. 50-fold lower than the reverse transcriptase heterodimer) RNase H activity was detected in the monomeric form of the protein. This activity could not be inhibited by another monoclonal antibody (designated MAb 1; [16]. Restle et al., submitted) which recognizes an epitope in the RNase H region of HIV-1 RT (epitope between amino acids 528 and 560) and was found to inhibit the RNase H activity of the 66/51 kDa heterodimer to the extent of 20%. Thus, we conclude that the enzymatic activity detected in the monomeric form of the recombinant RNase H domain probably arises from contaminating *E. coli* RNase H. No activity could be detected in the dimeric form, which is probably due to the fact that the FPLC gel filtration column is able to separate this form from the *E. coli* protein, which has a molecular weight of about 15 kDa.

Recent evidence suggests that the essential enzymatic functions of HIV-reverse transcriptase reside in the 66 kDa subunit in heterodimers [20,21]. In spite of this, as our results show, the dimeric organisation of the enzyme appears to be a prerequisite for the expression of these activities.

Acknowledgements: We thank Ursula Rühl and Sabine Zimmermann for excellent technical assistance. This work was supported by the Bundesministerium für Forschung und Technologie (BMFT), Grant FKS III/ 007/90.

REFERENCES

[1] Schatz, O., Mous, J. and LeGrice, S.F.J. (1990) EMBO J. 9, 1171–1176.
[2] DiMarzo Veronese, F., Copeland, T.D., DeVico, A.L., Rahman, R., Oroszlan, S., Gallo, R.C. and Sarngadharan, M.G. (1986) Science 231, 1289–1291.
[3] Lightfoote, M.M., Coligan, J.E., Folks, T.M., Fauci, A.S., Martin, M.A. and Venkatesan, S. (1986) J. Virol. 60, 771–775.
[4] Lowe, D.M., Aitken, A., Bradley, C., Darby, G.K., Larder, B.A., Powell, K.L., Purifoy, D.J., Tisdale, M. and Stammers, D.K. (1988) Biochemistry 27, 8884–8889.
[5] Restle, T., Müller, B. and Goody, R.S. (1990) J. Biol. Chem. 265, 8986–8988.
[6] DeVico, A.L., Copeland, T.D., Veronese, F.D., Oroszlan, S., Gallo, R.C. and Sarngadharan, M.G. (1989) AIDS Res. Hum. Retroviruses 5, 51–60.
[7] Müller, B., Restle, T., Kühnel, H. and Goody, R.S. (1991) J. Biol. Chem. 266, 14709–14713.
[8] Johnson, M.S., McClure, M.A., Feng, D.-F., Gray, J. and Doolittle, R.F. (1986) Proc. Natl. Acad. Sci. USA 83, 7648–7652.
[9] Hansen, J., Schulze, T., Mellert, W. and Moelling, K. (1988) EMBO J. 7, 239–243.
[10] Müller, B., Restle, T., Weiss, S., Gautel, M., Sczakiel, G. and Goody, R.S. (1989) J. Biol. Chem. 264, 13975–13978.
[11] Amann, E. and Brosius, J. (1985) Gene 40, 183–190.
[12] Certa, U., Bannwarth, W., Stüber, D., Gentz, R., Lanzer, M., LeGrice, S.F.J., Guillot, F., Wendler, I., Hunsmann, G., Bujard, H. and Mous, J. (1986) EMBO J. 5, 3051–3056.
[13] Pawlita, M., Mushinski, E., Feldmann, R.J. and Potter, M. (1981) J. Exp. Med. 154, 1946–1956.
[14] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[15] Hizi, A., Tal, R., Shaharabany, M. and Loya, S. (1991) J. Biol. Chem. 266, 6230–6239.
[16] Restle, T., Sczakiel, G., Müller, B., Pawlita, M. and Goody, R.S. (1991) VII International Conference on AIDS, Florence.
[17] Becerra, S.P., Clore, G.M., Gronenborn, A.M., Karlstrom, A.R., Stahl, S.J., Wilson, S.H. and Wingfield, P.T. (1990) FEBS Lett. 270, 76–80.
[18] Stammers, D.K., Tisdale, M., Court, S., Parmar, V., Bradley, C. and Ross, C.K. (1991) FEBS Lett. 283, 298–302.
[19] Hostomsky, Z., Hostomska, Z., Hudson, G.O., Moomaw, E.W. and Nodes, B.R. (1991) Proc. Natl. Acad. Sci. USA 88, 1148–1152.
[20] Cheng, N., Painter, G.R. and Furman, P.A. (1991) Biochem. Biophys. Res. Commun. 174, 785–789.
[21] Wu, J.C., Warren, T.C., Adams, J., Proudfoot, J., Skiles, J., Raghavan, P., Perry, C., Potocki, I., Farina, P.R. and Grob, P.M. (1991) Biochemistry 30, 2022–2026.