Point mutations in conserved amino acid residues within the C-terminal domain of HIV-1 reverse transcriptase specifically repress RNase H function

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Two single site substitutions ($E^{478} \to Q$ and $H^{539} \to F$) were introduced into the C-terminal RNase H domain of HIV-1 reverse transcriptase. These mutant proteins were expressed in *Escherichia coli* and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography. Both enzymes are clearly defective in RNase H function, but exhibit wild type reverse transcriptase activity.

RNase H; HIV-1 reverse transcriptase; Site-directed mutagenesis

1. INTRODUCTION

Retroviruses contain an RNase H activity which cleaves the RNA within a RNA-DNA hybrid [1–3]. This activity is an integral part of the reverse transcriptase (RT) enzyme [4,5]. The RNase H domain of retroviral RTs resides in the C-terminal part of the protein which displays partial homology with *Escherichia coli* RNase H. This functional assignment [6] was verified by proteolytic fragmentation [7], deletion studies [8,9], linker insertion [10] and site-directed mutagenesis [11].

In the course of retroviral replication, the RT-associated RNase H activity probably serves a variety of functions. Synthesis of minus strand cDNA (the first intermediate in retrotranscription of viral genomic RNAs) initiates from a tRNA primer hybridized to a complementary sequence downstream of the 5' LTR denoted the primer binding site (pbs). The template RNA which has been copied into cDNA is then very likely cleaved by RNase H to allow base pairing of the second template RNA and elongation of the minus strand cDNA [12]. RNase H digestion of the resulting DNA-RNA hybrid generates polypurine RNA fragments with free 3' hydroxyl ends which can serve as primers for the synthesis of plus strand cDNA [13,14]. Finally, the tRNA primer has to be removed by RNase H in order to allow completion of plus strand cDNA synthesis [15]. At least in the case of Moloney Murine Leukemia Virus (MMuLV), this RT-associated RNase H activity is indispensable for viral replication and loss of function cannot be complemented by a cellular

counterpart [11]. RNase H might therefore be a promising target for a new class of antiviral drugs.

Such an approach requires a more profound insight into the structure and function of HIV-1 RT-RNase H. To this end, we have constructed mutant enzymes carrying amino acid exchanges at conserved positions within the RNase H domain. With the help of these subtly altered proteins, we have been able to pinpoint residues important for RT-RNase H function.

2. MATERIALS AND METHODS

2.1. Cloning and mutagenesis of RT-RNase H

The complete HIV-1 RT-fragment (1680 bp) was excised from vector p6HRT [16] with *Bam*HI and *Hind*III and ligated into the polylinker of M13mp18. Mutagenesis was performed using the Amersham M13 mutagenesis kit [17]. The mutated DNAs were sequenced, cloned back into p6HRT and transformed into *E. coli* strain M15, harboring *lac* repressor plasmid pDMI.1 [18]. For heterodimeric enzyme production, these constructs were cut with *Eco*RV and *Sal*I, and the resulting 1.3 kb fragment was ligated into vector p6HRT-PROT [16], followed by transformation into *E. coli* strain M15:pDMI.1.

2.2. Expression and purification of p66 and p66/51 RT

Two liter L-broth cultures of recombinant strains were grown to $OD_{600} = 0.6$ and RT expression was induced by addition of IPTG to a final concentration of 400 mg/l. After 4 h, cells were harvested and lysed at 4°C in 50 mM NaH_2PO_4-Na_2HPO_4 (pH 7.8), 1 mM PMSF, 1 mg/ml lysozyme (20 min with stirring). NaCl was added to an end concentration of 300 mM and cells were sonicated for 3×30 s at 300 W, using a Labsonic sonicator with a 4 mm microtip (B. Braun). After centrifugation (30 min at $100\,000 \times g$, 4°C), the supernatant was loaded onto a 2 ml Ni^{2+} NTA column [19] equilibrated with at least 10 column-vols lysis buffer + 300 mM NaCl. The column was washed with 200 mM NH_4Ac (pH 6.5), 300 mM NaCl, and 10% glycerol until a stable 280 nm baseline was obtained. The hexahistidine tagged enzymes were eluted from the column with a pH gradient (pH 6.5–4.0) in the same buffer. Fractions (1 ml) were im-

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mediately neutralized with 0.5 vols of 1 M Tris-HCl (pH 8.2), 300 mM NaCl, and 10% glycerol. The protein concentration of pooled fractions was determined using the Biorad protein assay and the purity of the enzymes was checked on a 10% SDS-polyacrylamide gel (see fig.2). Pooled samples were adjusted to 50% glycerol and stored at −20°C. The overall yield of RT varied between 3 and 10 mg per liter of culture.

2.3. RT- and RNase H filter assay

Reverse transcription reactions were performed as described previously [16] using poly(rC):oligo(dG) as substrate.

For the RNase H-activity assay, the DNA-RNA hybrid was prepared by unspecific transcription of M13mp18 ssDNA with E. coli RNA polymerase in 40 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 250 μM ATP, CTP, GTP, 100 μM UTP, and 50 μM [^{35}S]UTP (800 Ci/mmol) at 37°C for 60 min. Unincorporated nucleotides were removed by Sephadex G-25 gel filtration. RNase H reactions were performed at 37°C in 50 mM Tris-HCl (pH 8), 5 mM DTT, 50 mM KCl and 8 mM MgCl₂, in the presence of 50000 cpm of DNA-RNA hybrid. The different enzyme preparations were added at a concentration of 2.5 μg/ml. At times indicated in fig.3B, 10 μl aliquots were spotted on DEAE filter papers (Whatman DE 81) and dried. The filters were washed 3 × 10 min in 1 × SSC (0.15 M NaCl, 0.015 M Na₃-citrate, pH 7.6), 2 × 10 min in 96% EtOH, and dried. The amount of RNA remaining on the filters was determined by scintillation counting.

2.4. In situ RNase H-activity assay

In situ gel assays were performed essentially as described earlier [5]. For the in situ gel assay [α-^{32}P]UTP-labelled DNA-RNA hybrid was used. Samples containing 2 μg of monomeric or 4 μg of heterodimeric RT were loaded on a 10% SDS-polyacrylamide gel containing 6 × 10⁶ cpm of DNA-RNA hybrid. After electrophoresis, the enzymes were renatured by washing 3 × 20 min in 50 mM Tris-HCl (pH 7.8), and 3 mM β-mercaptoethanol at 37°C. The enzyme reaction was performed in 50 mM Tris-HCl (pH 8), 2 mM MnCl₂, 2 mM DTT, and 50 mM NaCl at 37°C for 2 h, with three changes of buffer. The gel was stained in Coomassie blue (0.2%), 10% acetic acid, and 50% methanol for 30 min, destained in 5% methanol, and 4.2% acetic acid, dried and exposed on Kodak X-AR 5 film.

3. RESULTS AND DISCUSSION

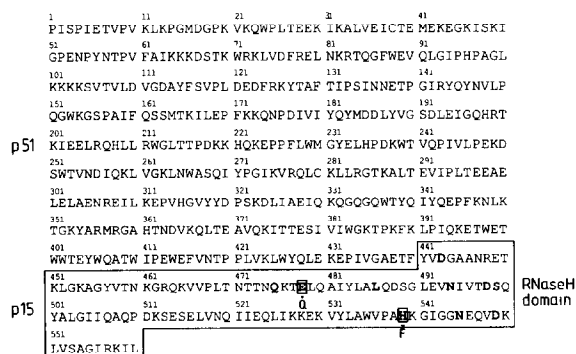Fig.1 shows the positions of the mutations introduced into the RNase H domain of HIV-1 RT. Amino acids

Fig.1. Amino acid sequence of HIV-1 RT. Residues in the RNase H domain that are conserved between several retroviral RNase H domains and E. coli RNase H [6] are shown in bold face. Amino acids that were mutated are boxed.
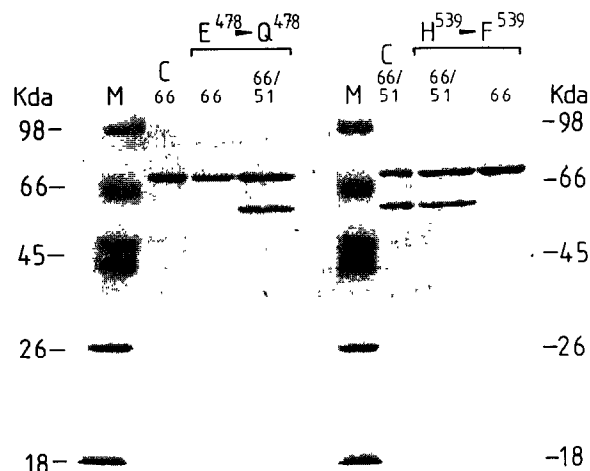
Fig.2. Mutant and wt RT enzymes after Ni²⁺ NTA chromatography of the crude lysate. Lanes M, mol. wt markers; lanes C 66 and C 66/51, wt homodimeric and heterodimeric RT, respectively. Lanes 66 and 66/51 marked E⁴⁷⁸ → Q⁴⁷⁸, homo- and heterodimeric E → Q RT mutant; lanes 66/51 and 66 marked H⁵³⁹ → F⁵³⁹, hetero- and homo-dimeric H → F mutant RT. 1 (2) μg of homodimeric (heterodimeric) RTs were loaded onto the gel.

shown in bold face are conserved between most retroviral RNase H domains and E. coli RNase H [6]. The E⁴⁷⁸ → Q and H⁵³⁹ → F mutations were chosen because they: (i) affect highly conserved amino acid residues; (ii) are located at helix-sheet transitions that are spatially clustered as evident from a preliminary three-dimensional structure of E. coli RNase H (Morikawa,
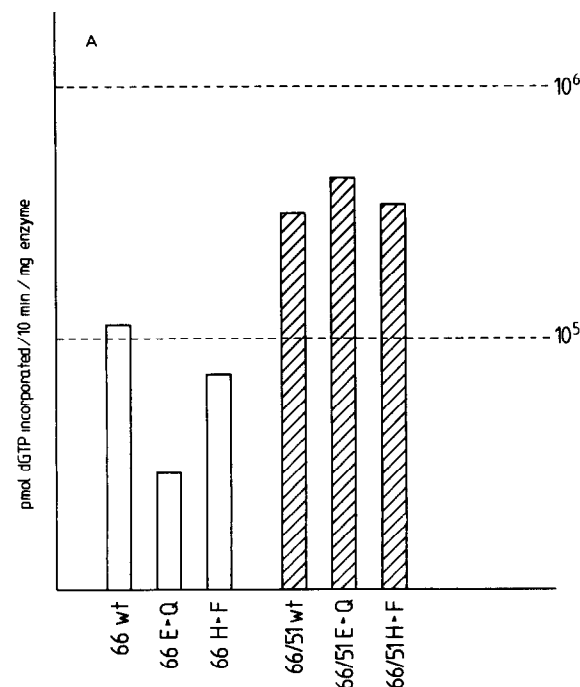
Fig.3A. RT activity filter assay of mutant and wt enzymes. Incorporation of [α-^{35}S]dGTP was determined in duplicate for five successive twofold dilutions of enzyme. Values within linearity were used for the computation of specific activities (displayed as bars on log scale).
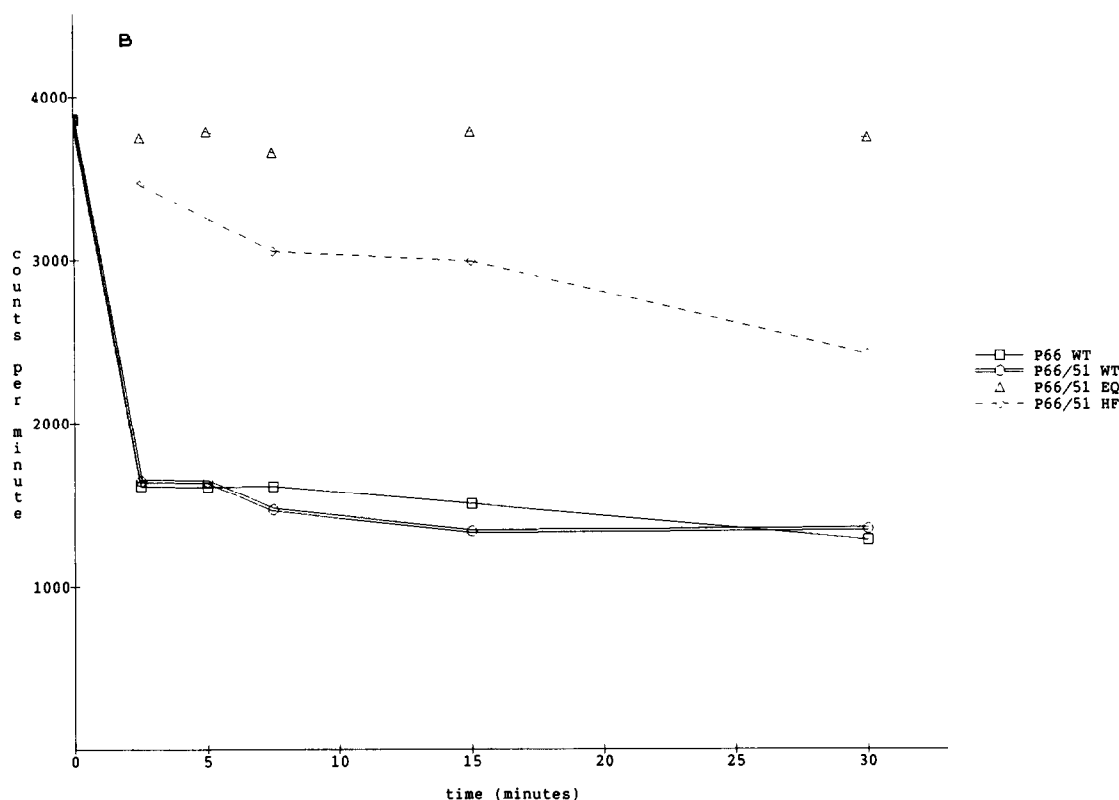
Fig.3B. RNase H activity filter assay of mutant and wt enzymes. The graphs show the loss of radioactivity from the DNA-RNA hybrid with time for mutant and wt RTs (as indexed in the figure). DNA-RNA hybrid was incubated at 37°C together with 125 ng (~ 1 pmol) of RT-RNase H. At the time points indicated, aliquots were withdrawn and spotted on a Whatman DE 81 filter. After several washings, the residual radioactivity on the filters was determined by scintillation counting.

personal communication); and (iii) should not impair correct folding of the protein as predicted by the Chou-Fasman [20] or Delphi [21] algorithms. In fact, the band pattern of peptides produced by partial hydrolysis with three different proteases of wild-type (wt) and mutant RTs on immunoblots stained with polyclonal α-RT antibodies is practically identical (T. Naas, personal communication). This may be regarded as indirect evidence against a major distortion of the protein's tertiary structure, a problem frequently encountered with linker insertion mutants. The substitutions constructed by oligonucleotide directed mutagenesis in M13 as described in section 2 were re-cloned either into the p6HRT expression vector to produce a p66 homodimer RT or into the p6HRT-PROT expression vector containing a functional HIV-1 protease cassette. In the latter case, proteolytic processing of the p66 homodimer generates a p66/51 heterodimer RT which is the form found in virions [22]. Bacteria transformed with these constructs were grown in parallel and the expression of RT was induced by addition of IPTG. Overexpressed wt and mutant RTs were purified on Ni^{2+} NTA columns under native conditions (see section 2). The material recovered is greater than 90% pure (as judged from the Coomassie-stained SDS gel shown in fig.2).
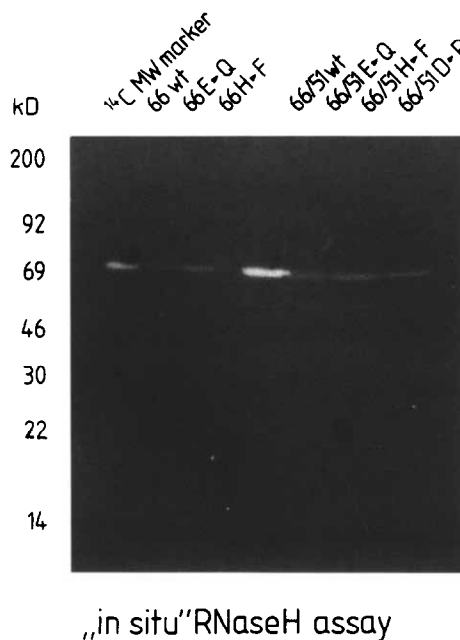

„in situ"RNaseH assay

Fig.4. In situ gel analysis of mutant and wt enzymes. Mutant and wt polypeptides were separated on a SDS-polyacrylamide gel containing ^{32}P-DNA-RNA hybrid. After electrophoresis the proteins were renatured by removing the SDS. RNase H cleavage is induced by addition of Mn^{2+}, after which the gel is fixed, stained and autoradiographed. The zones of RNase H activity appear as translucent bands on a dark background of undigested DNA-RNA hybrid.

In order to ascertain that the mutations chosen affected only RNase H activity, mutant and wt RTs were tested for their RT activity by measuring the amount of $[\alpha$-$^{35}S]dGTP$ incorporated into a poly(rC):oligo(dG) hybrid. In this assay, the mutant 66/51 RTs reproducibly perform as well or even better than the wt enzyme (fig.3A). On the other hand, the mutant p66 homodimers (66 H → F, 66 E → Q) containing the mutations on both polypeptide chains display a reduced RT activity. In numerous RT assays, we have observed that heterodimeric p66/51 RT is consistently 2–5 times more active than the p66 form. The p51 form of RT alone, however, is almost devoid of RT activity ([9] and our unpublished results). The proper conformation of the enzyme thus seems to be dependent on the presence of at least part of the C-terminal RNase H domain. Interestingly, this asymmetric configuration is also found in other retroviral polymerases [15].

Next, we compared the RNase H activity of our mutants and wt RT species. As can be seen in fig.3B, both wt forms of RT released about the same amount of radioactivity (~60–70% of the input RNA-DNA hybrid) with almost identical kinetics. On the other hand, the 66/51 H → F mutant shows markedly reduced activity, liberating only 35% of the input radioactivity with a much slower kinetic. In the 66/51 E → Q mutant, RNase H activity is almost totally abolished (less than 3% release of radioactivity). This would imply that Glu^{476} plays a very important role in RNase H function.

To ensure that the RNase H activities measured are indeed properties of the p66 (p66/51) RTs (and do not result from *E. coli* RNase H contamination), we have performed an RNase H in situ gel analysis (fig.4). RNase H activity can only be detected in the p66 species of RT, again demonstrating that it resides in the C-terminal part. The very light bands that can be seen around the position of p51 (in the lanes denoted 66/51) are most likely due to quenching and do not represent true RNase H activity, since a similar effect is also seen with BSA (not shown).

In conclusion, we have identified two amino acids in the C-terminal part of HIV-1 RT that are likely to be components of the active site of RNase H. Of course, definite proof of this concept requires further experiments. These mutants are now being used for mapping the binding region of the enzyme to a DNA-RNA substrate, which so far has been hampered by the RNase H cleavage of the RNA.

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