Volume 283, number 2, 298–302 FEBS 09784 June 1991

ADONIS 001457939100494L

Rapid purification and characterisation of HIV-1 reverse transcriptase and RNaseH engineered to incorporate a C-terminal tripeptide α-tubulin epitope

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Received 6 March 1991

The C-termini of p66 and p51 forms of HIV-1 reverse transcriptase have been engineered to contain a Glu-Glu-Phe sequence recognized by a monoclonal antibody to α-tubulin, YL1/2. Mutated RTs were purified in a single step using peptide elution from columns of immobilized YL1/2. The known sequence requirements of the YL1/2 epitope are consistent with protein eluting from the column with an intact C-terminus. Kinetic parameters of these mutated RTs are essentially unchanged from wild-type enzyme. The p15 RNaseH domain has been purified using this method and shown to have low enzyme activity compared to the parental p66 subunit.

HIV reverse transcriptase; RNaseH; Protein engineering; α-Tubulin epitope; Immunoaffinity purification

1. INTRODUCTION

Reverse transcriptase is an enzyme of central importance in the replication of HIV, the causative agent of AIDS [1]. Part of the HIV-1 *pol* gene codes for a p66RT subunit that is cleaved at the C-terminal region by the HIV protease to give a p66/p51 heterodimer, the form of RT observed in virions [2]. An RNaseH domain has been identified within the last 120 amino acids of the C-terminal region of the p66 subunit by sequence analysis [3] and functional studies [4].

We have previously described immunoaffinity chromatography of RT using immobilized monoclonal antibodies raised to HIV-1 RT [5]. Elution of RT from these columns requires vigorous conditions which may have deleterious effects on enzyme structure. This together with the C-terminal heterogeneity observed in these preparations [6], may contribute to the disorder observed in three different crystal forms of RT when examined in an X-ray beam [7]. We thus wished to devise a mild isolation procedure which could produce protein with an intact C-terminus.

The work of Wehland et al. [8] on the detailed mapping of the amino acid sequence requirement for the binding of α-tubulin to the monoclonal antibody YL1/2 [9] showed that an essentially linear epitope was located at the C-terminus of the polypeptide chain. There was a requirement for a C-terminal aromatic residue (either tyrosine or phenylalanine) containing a free carboxyl group, preceded by an acidic residue (glutamic or aspartic acid). A further adjacent acidic residue greatly increased binding. It was also demonstrated that the loss of the C-terminal aromatic residue abolished the binding of α-tubulin as well as related peptides to the YL1/2 antibody [8]. We therefore reasoned that incorporation of this epitope into HIV RT and RNaseH could afford a method of purifying these proteins with a homogeneous C-terminus using a simple peptide elution procedure from columns of immobilized YL1/2 antibody. Incorporation of this α-tubulin epitope into p66RT and RNaseH can be achieved by mutating the C-terminus from Lys-Val-Leu to Glu-Glu-Phe. The site of HIV protease cleavage of the p66RT subunit has been shown to be between a Phe and Tyr giving a p51 subunit with a C-terminal sequence of Glu-Thr-Phe [10]. Therefore only a single change of Thr to Glu is required to incorporate the α-tubulin epitope at the C-terminus of the p51 polypeptide. In changing the natural amino acid sequence of a protein there is the possibility of changing the biological characteristics of that protein. It has, however, previously been shown that the C-terminus of HIV-1RT

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Abbreviations: AIDS, acquired immune deficiency syndrome; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; p66RT(KVL), p66RT(EEF), p51RT(ETF), p51RT(EEF), RTs with apparent molecular wts of 66 kDa and 51 kDa with the C-terminal amino acid sequence according to the single letter code; p15RNaseH(EEF), ribonucleaseH with apparent molecular wt of 15 kDa with the C-terminal amino acid sequence according to the single letter code; PFA, phosphonoformate; SDS-PAGE, sodium dodecylsulphate/polyacrylamide gel electrophoresis; RTMab3, RTMab8, monoclonal antibodies raised against reverse transcriptase; IPTG, isopropyl-β-D-thiogalactopyranoside

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is not essential for activity as several amino acids can be deleted without affecting DNA polymerase or RNaseH enzymic activities in vitro [5,11].

2. MATERIALS AND METHODS

2.1. *Preparation of RT and RNaseH constructs containing the C-terminal Glu-Glu-Phe motif*

Mutations were introduced into the HIV-1 RT gene by site-directed mutagenesis and cloned into the high level expression plasmid pKK233-2 as described previously [12]. The coding sequence for RNaseH was produced from the p66RT(EEF) construct by PCR using primers incorporating *Nde*I and *Hin*dIII restriction sites at the 5' and 3' ends, respectively. The 380 bp fragment obtained was cloned into pT7-7 containing the T7 RNA polymerase promoter and a 420 bp *Xba*I/*Hin*dIII fragment sub-cloned into M13mp19 for sequencing.

2.2. *Expression of polypeptides containing the Glu-Glu-Phe motif*

High level expression of the recombinant p66RT(EEF) and p51RT(EEF) and RT extraction were carried out as described previously [12]. The extract was dialysed into 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, before loading on the YL1/2-Sepharose column.

For expression of the recombinant p15RNaseH(EEF), the pT7-7 plasmid was used to transform *E. coli* strain BL21 DE3 lacIq [18]. Bacteria were grown in 2XTY medium with ampicillin and tetracycline selection until the OD_{590} equalled 0.5 and then induction was carried out with IPTG (1 mM). Cells were harvested after 4 h by pelleting (10000 rpm, 5 min, 4°C) and resuspended in 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, and disrupted by sonication. Cell debris was removed by centrifugation at 40000 rpm for 10 min before application to the YL1/2-Sepharose columns.

2.3. *Preparation of YL1/2 Sepharose antibody column*

YL1/2 rat ascites fluid was obtained from Serotec (Oxford, UK). IgGs were purified from this by ammonium sulfate fractionation and ion exchange on DEAE-cellulose (DE52, Whatman) and coupled to CNBr-activated Sepharose (1 g/10 mg of IgG) following the manufacturers' instructions (Pharmacia). Columns of YL1/2-Sepharose were equilibrated in 50 mM NaCl, 50 mM Tris-HCl, pH 7.5. Elution conditions are described in section 3. Asp-Phe, Glu-Glu and Asp-Phe (OMe) were obtained from Sigma.

2.4. *Purification of p66RT(KVL) and p51RT(ETF)*

These were purified on RTMab8- and RTMab3-Sepharose immunoaffinity columns as described previously [5] and protein concentration of purified protein determined based on OD_{280} of 1 = 0.5 mg/ml.

2.5. *Assay for reverse transcriptase*

Assays were carried out using poly(rA)-$(dT)_{18}$ (Pharmacia) as template/primer with the incorporation of [^{3}H]dTTP (Amersham) as described previously [12]. K_m measurements for dTTP were determined using saturating levels of rA-dT (50 μg/ml) and varying dTTP. IC_{50} determinations for phosphonoformate (PFA) were measured at a dTTP concentration of 5 μM. Kinetic data were analysed using non-linear least-squares [13].

2.6. *Assay of RNaseH*

RNaseH assays were carried out at 37°C for 15 min in 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT plus [^{35}S]RNA/DNA hybrid containing 2×10^5 cpm (M13 synthesised using RNA polymerase and [^{35}S]UTP). Samples were precipitated on glass fibre filters (Whatman GF/C) using 5% TCA and washed in the same reagent. Thermal inactivation studies at 45°C were carried out as described previously [14].

2.7. *SDS-PAGE and Western blotting*

These were performed using a Tris-glycine buffer system [15]. Western blots were probed with either YL1/2 monoclonal antibody [9] or a mixture of two monoclonals to RT, RTMab3 and RTMab8 [5].

2.8. *Protease digestion of p66RT(EEF)*

Digestion of p66RT(EEF) (100 μg) with HIV protease (6 μg) was carried out in 50 mM Tris-HCl, pH 7.6, 0.6 M KCl for 60 min at 37°C. Digestion with immobilised carboxypeptidase A (Sigma) was carried out at 22°C in 50 mM NaCl, 10 mM HEPES, pH 8.0.

3. RESULTS

3.1. *Purification of p66RT(EEF) and p51RT(EEF)*

p66RT(EEF) in a bacterial extract was removed by passage through a YL1/2-Sepharose under low salt conditions. After washing in running buffer, the column was eluted with 5 mM Asp-Phe (Fig. 1). By contrast, no protein was eluted with either Glu-Glu or Asp-Phe(OMe). Analysis of the eluted fractions on SDS-PAGE gels showed the peak eluting with Asp-Phe was largely p66RT' (Fig. 2). The small amount of p51RT present is due to proteolysis as has been observed previously [6]. RT with the normal C-terminus, p66RT(KVL), did not bind to the YL1/2-Sepharose column, neither did p66RT(EEF) that had been digested with carboxypeptidase A. p51RT(EEF) was purified as described for p66RT(EEF). A comparison of specific activities, K_ms for dTTP, IC_{50}s for PFA for normal and mutated p66 or p51 forms of RT are shown in Table I. The RNaseH activity of p66RT(EEF) and p66RT(KVL) are shown in Fig. 3. An alternative elution procedure was to wash the YL1/2-Sepharose column with 0.2 M

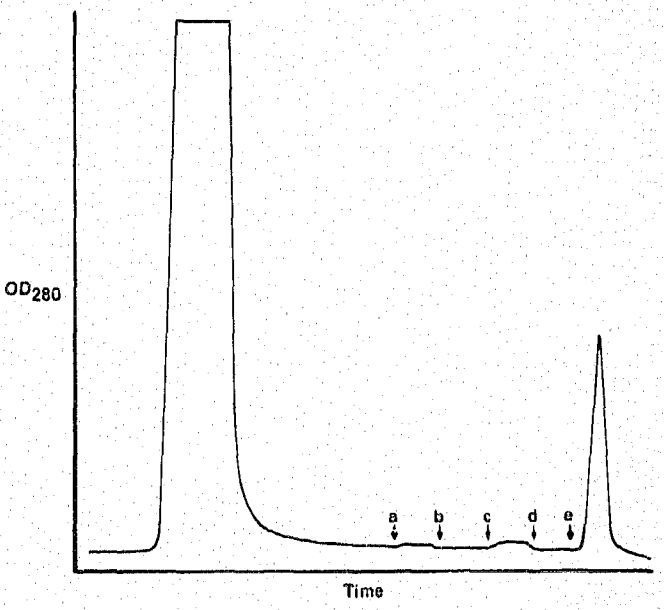


Fig. 1. Elution profile of p66RT(EEF) from YL1/2-Sepharose. The column was run as described in section 2 and eluted as follows: (a) 5 mM Glu-Glu; (b) running buffer; (c) 5 mM Asp-Phe(OMe); (d) running buffer; (e) 5 mM Asp-Phe.

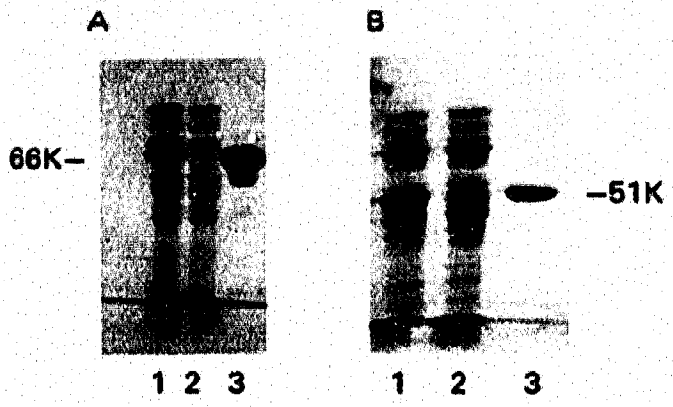


Fig. 2. SDS-PAGE of RT samples run on YL1/2-Sepharose. (A) 1 = column load of bacterial extract containing p66RT(EEF); 2 = run through; 3 = elution with 5 mM Asp-Phe. (B) 1 = column load of bacterial extract containing p51RT(EEF); 2 = run through; 3 = elution with 5 mM Asp-Phe.

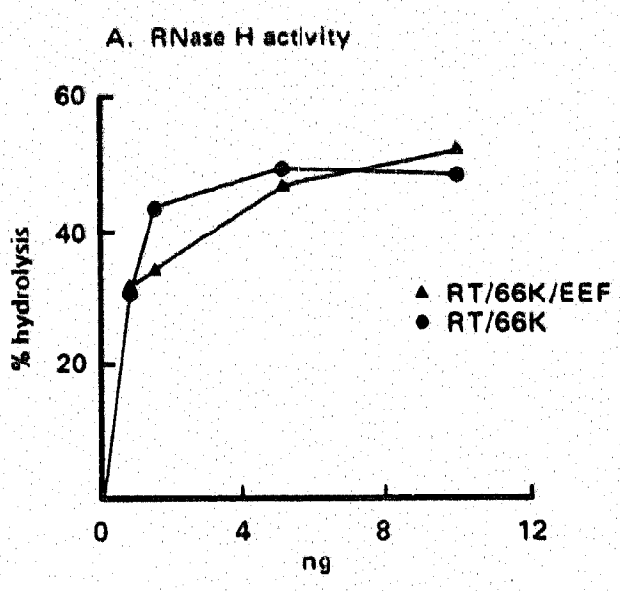
KCl to remove non-specifically bound protein and to elute the RT with 1 M KCl.

3.2. *Digestion of p66RT(EEF) with HIV protease and the identification of p15RNaseH(EEF)*

p66RT(EEF) purified on YL1/2-Sepharose as described above was subjected to proteolysis with homogeneous HIV protease. Analysis of this on SDS-PAGE gels followed by Western blots probed with either YL1/2 or RTMab3 and RTMab8 showed that a p15 polypeptide can be identified using the YL1/2 α-tubulin antibody but not with RTMab3 or RTMab8 (Fig. 4). This result indicated that it might also be possible to purify the 15K RNaseH domain using the approach described above.

3.3. *Purification of p15RNaseH(EEF) on YL1/2-Sepharose*

p15RNaseH(EEF) was purified from a crude *E. coli* extract to >95% purity in a single step on YL1/2-Sepharose using the procedure described above. Only low levels of RNaseH activity were detected for the p15 polypeptide which was at best 1000-fold lower than for the corresponding p66(EEF)RT protein.



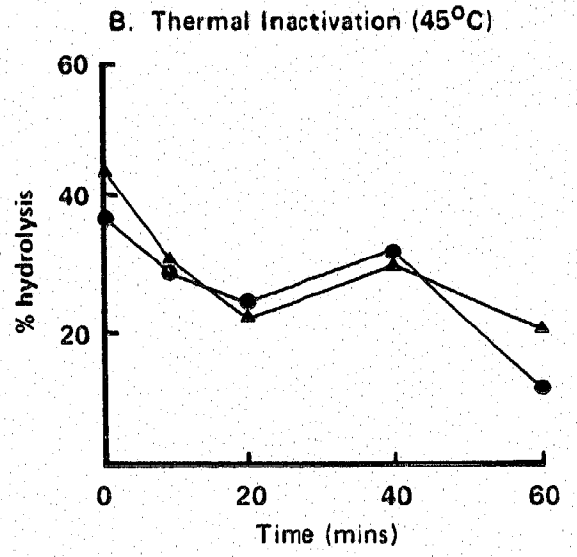


Fig. 3. Comparison of RNaseH activity of p66RT(EEF) and p66RT(KVL). (A) As a function of enzyme concentration. (B) Thermal inactivation as a function of time.

Table I

Comparison of the kinetic properties of normal and mutated RT

Mol. wt.	66K		51K	
C-terminus:	Lys-Val-Leu	Glu-Glu-Phe	Glu-Thr-Phr	Glu-Glu-Phe
Specific activity (nmol/min/mg of protein)	424 ± 16	496 ± 29	23 ± 4	113 ± 35
K_m dTTP (μM)	5.6 ± 0.4	8.2 ± 0.3	9.5 ± 2.3	11.7 ± 2.5
IC_{50} PFA (μM)	0.44 ± 0.03	0.35 ± 0.05	0.3 ± 0.08	0.55 ± 0.1

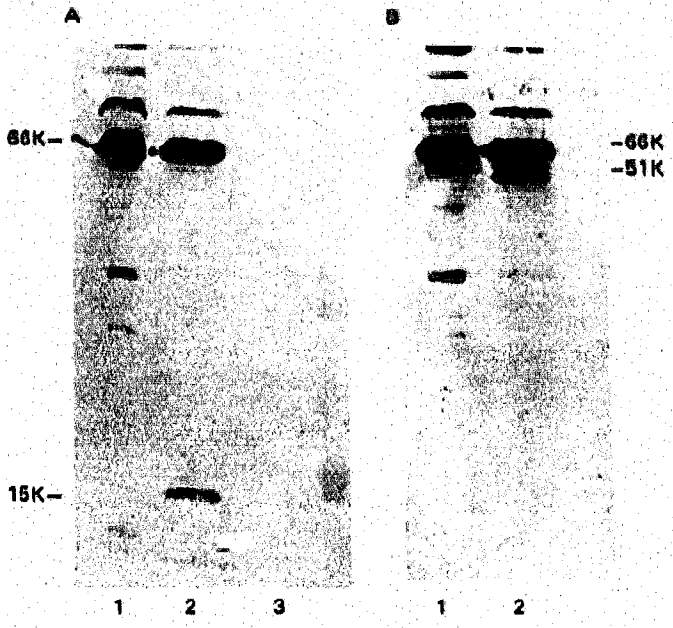


Fig. 4. Western blots of p66RT(EEF) digested with HIV protease. Samples were analysed on SDS-PAGE. (A) Probed with YL1/2 antibody. 1 = p66RT(EEF); 2 = p66RT(EEF) after digestion with HIV protease; 3 = HIV protease. (B) Probed with RTMab3 and RTMab8. 1 = p66RT(EEF); 2 = p66RT(EEF) after digestion with HIV protease.

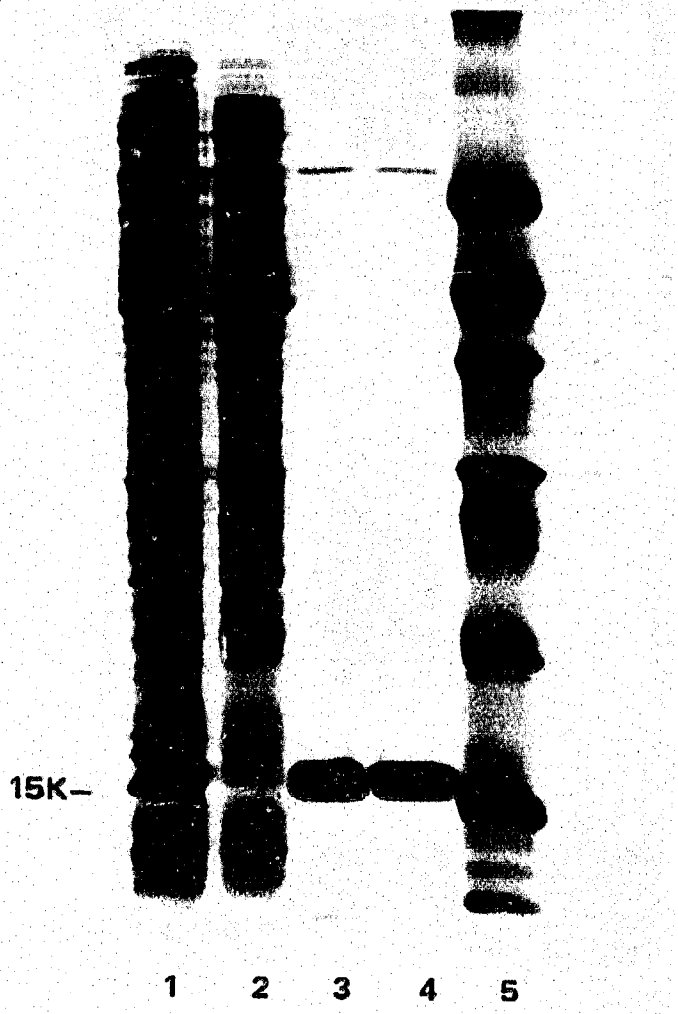


Fig. 5. SDS-PAGE of RNaseH run on YL1/2-Sepharose. 1 = column load of bacterial extract containing p15RNaseH(EEF); 2 = run through; 3,4 = elution with Asp-Phe; 5 = mol. wt. markers.

4. DISCUSSION

The work described here shows that engineering HIV-1 RT and RNaseH by site-directed mutagenesis to incorporate the C-terminal sequence Glu-Glu-Phe means that these proteins can be recognized by a monoclonal antibody, YL1/2, originally raised to a different protein, α-tubulin. Using YL1/2 immobilized on Sepharose provides a method for the rapid single step purification of p66 and p51RT, and p15RNaseH containing the Glu-Glu-Phe epitope. Using Asp-Phe to elute the columns gives very mild conditions compared to those normally required to elute antibody columns [5].

From the epitope mapping of YL1/2 it would be predicted that the proteins bound to the YL1/2 column would contain an homogenous C-terminus since a C-terminal aromatic residue is required [8]. The failure of proteins to be eluted by Glu-Glu or by Asp-Phe(OMe) is consistent with this. In addition we have shown that when p66RT(EEF) is subjected to carboxypeptidase A digestion, binding to the YL1/2-Sepharose column is eliminated. The specificity for carboxypeptidase A is such that aromatic residues are readily removed but acidic residues are only slowly released; it has been shown that carboxypeptidase A digestion of α-tubulin only releases the C-terminal tyrosine [16]. We are currently studying the crystallization properties of the RTs purified on YL1/2-Sepharose to ascertain whether crystal order is improved.

Our determination of various activity measurements for wild-type and mutated RTs indicates that specific activities, K_ms for dTTP and IC_{50}s for PFA are essentially unchanged as was the RNaseH activity of p66RT(EEF) compared to p66RT(KVL). Thus introducing these mutations does not affect these properties as would be predicted from earlier work on amino acid deletions from the C-terminus which had no effect on enzyme activity [5,11].

Our previous attempts to identify the p15RNaseH domain released after proteolysis of the p66RT subunit have proved unsuccessful [7]. In this study the digestion of p66RT(EEF) with HIV protease gave a p15 polypeptide that could be identified on Western blots probed with Yl1/2 anti α-tubulin antibody but not by RTMab8, an antibody previously shown to recognize an epitope in the RNaseH region [5]. This could in-

dicate that a conformational change occurs in the p15 polypeptide when it is released from the p66 subunit. High level expression of the p15RNaseH domain was achieved using the T7 system. Activity measurement indicates that the p15RNaseH(EEF) has only 1/1000th of the RNaseH activity of p66RT(EEF). This agrees with Becerra et al. (1990) [17] who reported that the bacterially expressed p15RNaseH domain had a very low level RNaseH activity. This could indicate that the isolated p15 polypeptide does not have significance as an RNaseH in the virus life cycle.

The use of YL1/2-Sepharose to purify proteins with the C-terminus mutated to incorporate the Glu-Glu-Phe or related motif thus could have wide application for single step purification of proteins in which the C-terminus is not critical for activity. In addition, incorporation of this epitope provides an immunological marker for assessing the expression of recombinant proteins.

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