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Mutating P_2 and P_1 residues at cleavage junctions in the HIV-1 pol polyprotein

Effects on hydrolysis by HIV-1 proteinase

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Mutations were introduced into the P_2 and P_1 positions of the junctions, (a) linking reverse transcriptase (RT) and integrase (IN) (-Leu*Phe-) and (b) between the p51 and RNase H domain (-Phe*Tyr-) within p66 of RT in the HIV-1 pol polyprotein. Processing by HIV proteinase (PR) *in cis* was monitored upon expression of these constructs in *E. coli*. Whereas the presence of Leu or Phe in P_1 permitted rapid cleavage at either junction, substitution of a β-branched (Ile) hydrophobic residue essentially abolished hydrolysis. By contrast, placement of a β-branched (Val) residue in the P_2 position flanking such -Hydrophobic*Hydrophobic- junctions resulted in effective cleavage of the scissile peptide bond. Gly in P_2, however, abrogated cleavage. The significance of these findings in terms of PR specificity, polyprotein processing and the generation of homodimeric (p51/p51) RT for crystallisation purposes is discussed.

HIV-1 proteinase; Specificity; Polyprotein processing; Cleavage site mutagenesis

1. INTRODUCTION

The gag and gag-pol polyproteins of the human immunodeficiency virus (HIV) are processed into individual components by the virally encoded proteinase (PR). Proviral clones incorporating a mutationally inactivated PR are unable to generate infectious viral particles [1]. Thus, there has been considerable interest in PR as a target for antiviral chemotherapy [2]. To facilitate the development of specific inhibitors, many previous reports have utilised synthetic peptide substrates, sometimes containing chromophoric residues [e.g. 3–5], to help to elucidate the molecular topography of the active site of this important enzyme [6]. Since the natural substrates for PR are large multidomain polyproteins, the susceptibility to hydrolysis of the peptide bond at each of the cleavage junctions might be influenced significantly by conformational constraints placed upon it because of its position in a polypeptide strand located between two domains. The presence of either an -Aromatic*Pro- or a -Hydrophobic*Hydrophobic- scissile peptide bond at the cleavage junctions in the HIV-1 polyprotein reflects the somewhat unusual ability of PR to attack these two different types of site (for a review, see [7]). In the former, the nature of the residue present in P_1 position has been shown to be important [8] and in one recent report, the contribution of residues flanking the -Aromatic*Pro- bond has been investigated by mutagenesis [9]. Little is known about the requirements for effective cleavage at -Hydrophobic*Hydrophobic- junctions; it was considered of importance therefore to examine whether the preferences established in peptide substrates for the P_2 and P_1 positions were reflected in the cleavage of polyproteins altered by mutagenesis at such junctions.

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Abbreviations: PR, proteinase; RT, reverse transcriptase; IN, integrase. The nomenclature system of Schechter and Berger [19] i.e. P_2-P_1*P_1'-P_2' is used to depict amino acids adjacent to the residues in the P_1 and P_1' positions which contribute the scissile peptide bond (indicated by an asterisk*).

2. MATERIALS AND METHODS

A *Bgl*II–*Nde* I fragment from the HTLV-IIIB provirus [10] was used as the source of the HIV-1 pol open reading frame. Construction of the plasmid (pPolCG) in which this fragment was placed under the control of an IPTG-inducible promoter has been described previously [11]. To facilitate the substitution of residues contributing to the reverse transcriptase (RT)-integrase (IN) cleavage junction in the pol polyprotein, silent mutations were introduced by means of an oligonucleotide-directed mutagenesis kit (Amersham, UK). Appropriate oligonucleotides were used to introduce unique *Hin*dIII (AAATTA→AAGCTT) and *Cla*I (ATAGAT→ATCGAT) restriction endonuclease sites on the RT and IN sides of the junction respec-

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tively. By this means, synthetic oligonucleotides encoding the desired substitutions in the amino acid sequence (See legend to Figs. 1 and 2) were then ligated into this plasmid following digestion with these restriction endonucleases. To facilitate the removal of the sequence encoding IN, a silent *Bgl*II (AAATAT→AGATCT) site was introduced at the RT-IN junction in one of these constructs (pPolHC P_1F).

Elimination of the IN domain generated pPRRT1. A similar strategy was employed with this plasmid to introduce amino acid substitutions (See legend to Figs. 3 and 5) at the p51-RNase H junction within RT. Briefly, silent mutations were introduced on the p51 side of the junction site to introduce a *Nar*I (GGAGCA→GGCGCC) site. Appropriate oligonucleotides encoding the desired amino acid substitutions were then readily ligated into this plasmid following digestion with *Nar*I and *Nhe*I (since an *Nhe*I recognition sequence was already present on the RNase H side of the junction). Following all mutagenesis experiments and the insertion of reconstruction oligonucleotides, each nucleotide sequence was confirmed by dideoxysequencing [12].

In some plasmids, the PR encoding region was also deleted to permit the expression of RT alone. This was accomplished by utilising the plasmid pRTIN [11] from which a fragment encoding the authentic N-terminus of RT was taken.

E. coli strain M15 [pDM1.1] was used throughout for expression studies [13]. Following induction with IPTG (400 μg/ml), culture aliquots (1 ml) were removed at the time intervals indicated in the figure legends and collected by centrifugation. Pelleted cells were resuspended in 200 μl of SDS-PAGE sample buffer [14] and lysed by heating to 95°C. Following electrophoresis [14], the fractionated proteins were transferred to nitrocellulose membranes [15]. Immunodetection was carried out using a monoclonal antibody against PR (generously provided by Dr. Mary Graves, Roche Inst., Nutley, NJ, USA) or antisera to RT or IN which were raised in rabbits against purified p66 RT and p32 IN respectively [11]. Oligonucleotides were synthesized on a Gene Assembler (Pharmacia Biotechnology, Milton Keynes, UK).

3. RESULTS AND DISCUSSION

Maturation of the HIV-1 pol polyprotein requires cleavage at 4 sites, all by the viral proteinase (Fig. 1). Two sites are at the N- and C-termini of PR itself (both involving -Phe*Pro- bonds); the p51-RNase H junction (-Phe*Tyr-) exists within the p66 subunit of RT and the C-terminal residue of RT is linked directly to the N-terminal residue of IN, thus forming a -Leu*Phe- junction. Upon expression of *E. coli* harbouring the plasmid pPolCG, cleavage by PR *in cis* of all of these bonds in the wild-type PR-RT-IN polyprotein was examined. Using a monoclonal antibody (anti-PR), the release of mature PR with the anticipated M_r of 11 000 was established (Fig. 1, panel a1). Similarity, using two polyclonal antisera (anti-IN and anti-RT, respectively) authentic IN (Fig. 1, panel a2) with the expected M_r of 32 000 and the two subunits of RT (M_r 66 000 and 51 000) (Fig. 2, lane 1) were also shown to have been released by rapid cleavage of the 110 kDa polyprotein precursor.

When the -Leu*Phe- bond constituting the RT-IN junction was mutated to -Ile*Phe-, the release of mature PR (Fig. 1, panel b1), perhaps not unexpectedly, was unaffected. By contrast, immunostaining for IN (Fig. 1, panel b2) revealed that very little authentic IN had been released Instead, a protein with a higher M_r of approx. 47 000 was evident so that cleavage at or

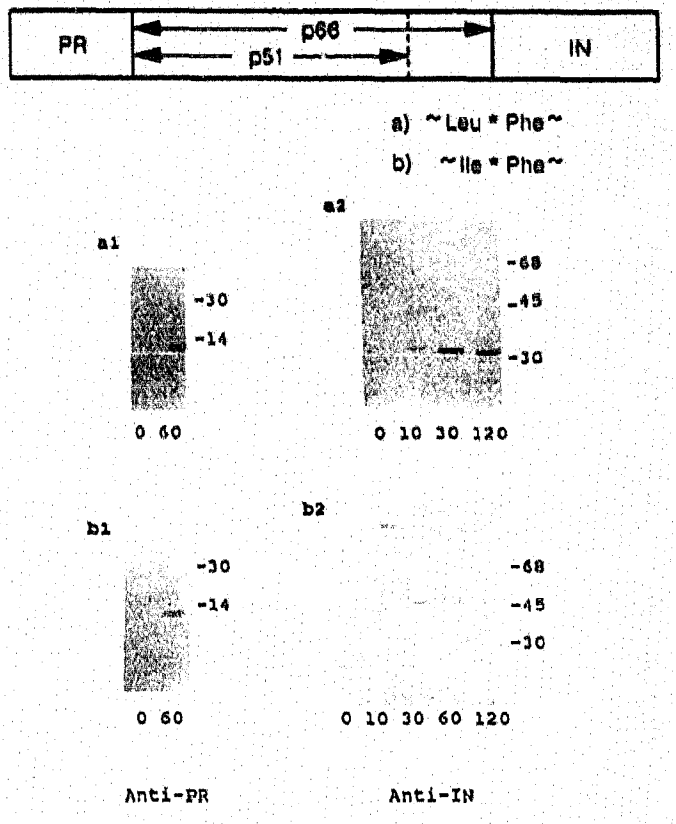

Fig. 1. Processing of full-length pol polyprotein with the junction between the RT and IN components (indicated by *) as -Leu*Phe- (panel a) or -Ile*Phe- (panel b) bonds respectively. (Mature RT is a heterodimer of 66 kDa and 51 kDa subunits; the latter is generated by proteolytic removal of the RNase H domain from the C-terminus of p66 subunit forms). *E. coli* harbouring the plasmids pPolCG (panel a) and pPolHC P_1I (panel b) were induced with IPTG, after which aliquots were removed and processed (see section 2) at the indicated times (between 0 and 120 min). Immunodetection was carried out using a monoclonal antibody to PR (panels a1 and b1) and polyclonal antiserum to IN (panels a2 and b2). Markers of M_r 68 000, 45 000, 30 000 and 14 000 migrated as indicated.

near the RT-IN junction would not appear to have taken place. Parallel immunostaining of the *E. coli* extracts for the appearance of RT revealed a band at 51 kDa (Fig. 2, lane 2). By contrast, in the wild-type construct, two bands of apparent M_r 51 000 and 66 000 were generated (Fig. 2, lane 1), confirming that cleavage within one subunit had generated the well-documented heterodimeric form of RT [16]. In the Ile mutant, since authentic p66 was not produced yet p51 was evident (Fig. 2, lane 2) and since no mature IN was generated (Fig. 1, panel b2), the C-terminal 15 kDa RNase H domain of RT appears to be still attached to the N-terminus of IN. Thus, replacement of the wild-type Leu with Ile would appear to abolish cleavage at the RT-IN junction. Under these circumstances, the internal site within RT was cleaved in both subunits, thus generating homodimeric (p51/p51) RT. Synthetic peptides containing a β-branched (Ile or Val) residue in the

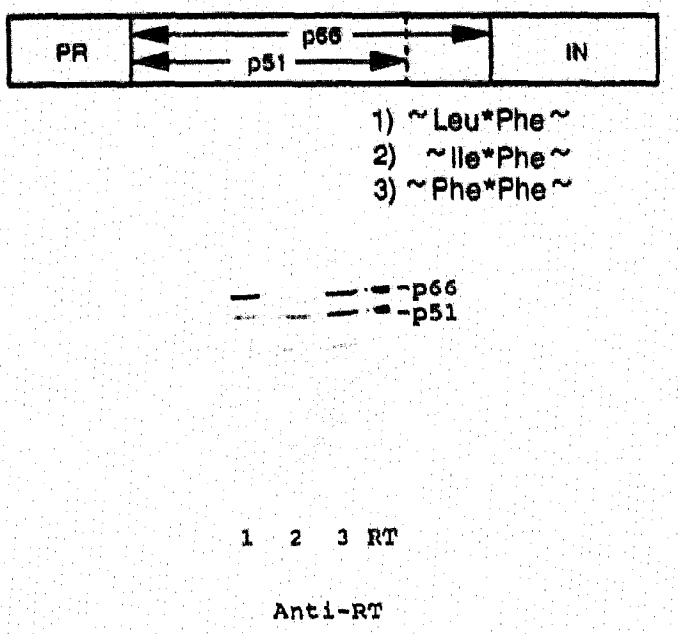

Fig. 2. Processing of full-length pol polyprotein with the RT-IN junction (*) as -Leu*Phe- (lane 1), -Ile*Phe- (lane 2) or -Phe*Phe- (lane 3) bonds respectively. *E. coli* harbouring the plasmids pPolCG, pPolHC P_1I and pPolHC P_1F (lanes 1, 2 and 3, respectively) were induced with IPTG for 60 min. Immunodetection was with a polyclonal antiserum to RT. The lane identified as RT contained purified authentic RT.

P_1 position have also been shown to be resistant to hydrolysis by PR [3]. In this earlier work, a Phe residue was however accommodated readily in the P_1 position. In keeping with these observations, the introduction of a Phe residue into the P_1 position at the RT-IN junction (thus generating a -Phe*Phe- site) allowed normal processing of RT by PR (Fig. 2, lane 3) and IN was also released in authentic (32 kDa) form (not shown).

Thus, whilst HIV PR can readily cleave -Leu*Aromatic- and -Phe*Aromatic- bonds, a subtle rearrangement of 4 carbon atoms in one side chain (Leu→Ile) is sufficient to alter the processing of a polypeptide of over 100 kDa in size. In order to investigate this remarkable effect further, the processing of a PR-RT diprotein was examined (i.e. with IN deleted). On expression of *E. coli* harbouring this plasmid (pPRRT1), mature PR was generated readily (not shown), reflecting rapid cleavage of the two -Phe*Pro- bonds at the extremities of PR. Immunostaining for RT indicated that authentic p66/p51 RT was generated also (Fig. 3, panel c). As mentioned earlier, the cleavaged junction within p66 (a -Phe*Tyr- bond) links p51 to the RNase H domain [8]. Substitution of the Phe residue in the P_1 position with Leu (Fig. 3, panel a) had no effect on the processing of RT by PR. By contrast, when an Ile residue was present in the P_1 position of this junction,

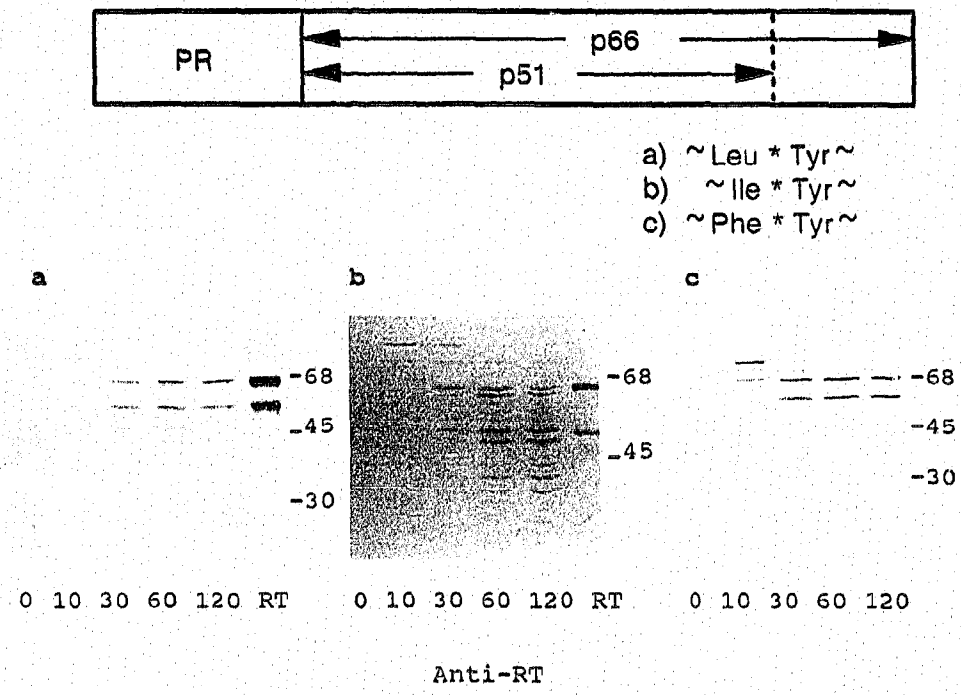

Fig. 3. Processing of PR-RT diproteins with the p51-RNase H junction (*) as -Leu*Tyr- (panel a), -Ile*Tyr- (panel b) or -Phe*Tyr- (panel c) bonds respectively. *E. coli* harbouring the plasmids pPRRT3, pPRRT2 and pPRRT1 (panels a, b and c, respectively) were induced with IPTG, aliquots were removed and processed (See Materials and Methods) at the indicated times (between 0 and 120 min). The samples in panel b were electrophoresed for longer than those in panels a and c for improved resolution. Immunodetection was with polyclonal antiserum to RT. Markers of M_r 68 000, 45 000 and 30 000 migrated as indicated. The lanes identified as RT contain purified authentic RT.

an altered processing pattern was observed (Fig. 3, panel b). Whilst it is evident that there is a band comigrating with the p66 marker, a further band which increased in intensity between the 30 and 60 min samples migrated just ahead of the p66 band. No authentic p51 appeared to be generated in this mutant (the gel in panel b is run further than those in panels a and c of Fig. 3 to substantiate this). Instead, 2 bands were observed which migrated slightly in front of and behind the p51 marker respectively. Thus, if PR is deterred from hydrolysing the p51-RNase H junction by the presence of for example an Ile residue in the P_1 position, it is possible that the attention of PR is deflected to alternative sites so that their (normally trivial) rates of cleavage become significant. Indeed, one such alternative site has been identified adjacent to the normal p51-RNase H junction [17]. Additional cleavages to generate a number of other degradation fragments are also evident in Fig. 3, panel b.

To establish whether PR was indeed responsible for the anomalous cleavage patterns in Fig. 3, panel b, RT was expressed alone (i.e. in the absence of PR). Upon induction of *E. coli* harbouring the plasmid pRT1 (encoding wild-type RT), a single band comigrating with the p66 marker was observed with antiserum to RT (Fig. 4, panel b). Similarly, expression of a mutant RT (in which the internal p51-RNase H junction was -Ile-Tyr-) also produced only one band of 66 kDa (Fig. 4, panel a). The absence of any other significant bands would suggest that *E. coli* proteinases were unable to cleave p66 RT specifically into p66/p51 heterodimers or indeed into any other fragments. Thus, PR must be responsible for generating normal p66/p51 RT as well as the abnormally processed products seen in Fig. 3, panel b. By isolation of these recombinant protein substrates and incubation of the purified variants with homogeneous PR in vitro, it will be possible not only to establish the sites of cleavage but also to perform quantitative analyses to determine kinetic parameters for the rate of cleavage in vitro.

From these mutations at the internal RT and the RT-IN junctions, it is evident that as was found previously for synthetic peptides [3-5], the presence of a β-branched (Ile) residue in the P_1 position is unfavourable for hydrolysis of polyproteins by HIV PR whereas the isomeric (Leu) residue together with other hydrophobic residues (such as an aromatic Phe) permit rapid and specific cleavage. Indeed, the presence of a β-branched residue appears to be so unacceptable that the attentions of the enzyme are deflected to other peptide bonds which otherwise would not be attacked.

In an attempt to establish the activity requirements of PR further, the effect of substitution in the P_2 position (Thr→Val or Gly) at the p51-RNase H junction was examined. Processing of a PR-RT diprotein containing a -Val-Phe*Tyr- sequence (Fig. 5, panel a) resulted in

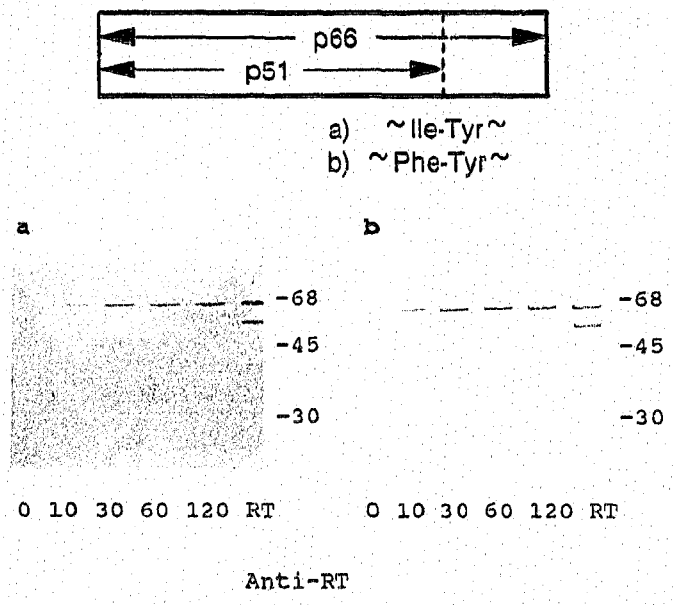

Fig. 4. Expression of *E. coli* harbouring the plasmids pRT2 and pRT1 which contain -Ile*Tyr- (panel a) or -Phe*Tyr- (panel b) bonds at their p51-RNase H junction. The cultures were induced with IPTG, aliquots were removed and processed (see section 2) at the indicated times (between 0 and 120 min). Immunodetection was carried out using polyclonal antibodies to RT. Markers of M_r 68000, 45000 and 30000 migrated as indicated. The lanes identified as RT contain purified authentic RT.

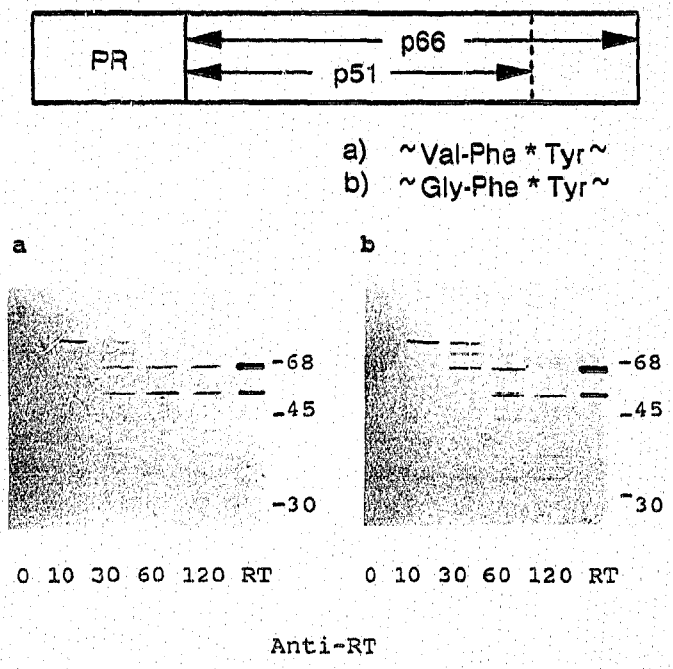

Fig. 5. Expression of *E. coli* harbouring the plasmids pPRRT4 and pPRRT5 which contain -Val-Phe*Tyr- (panel a) or -Gly-Phe*Tyr- (panel b) bonds at their p51-RNase H junction. The cultures were induced with IPTG, aliquots were removed and processed (see section 2) at the indicated times (between 0 and 120 min). Immunodetection was carried out using polyclonal antiserum to RT. Markers of M_r 68000, 45000 and 30000 migrated as indicated. The lanes identified as RT contain purified authentic RT.

processing of RT equivalent to that observed earlier for the wild-type -Thr-Phe*Tyr- bond (Fig. 3, panel e). With Gly in P_2 at this junction, a precursor of comparable size (M_r approx. 86 000) to the Val and Thr containing diproteins was observed in the 10 min time point (Fig. 5, panels a and b). However, a considerably different pattern of hydrolysis of this by PR was apparent at subsequent time points (Fig. 5, panel b). Only a single band of 66 kDa (with no band at 51 kDa) was observed at 30 min. This would suggest that hydrolysis of the -Gly-Phe*Tyr- site was impaired since a band corresponding to the p51 marker was observed at this time in both the wild type (Fig. 3, panel e) and Val containing mutant (Fig. 5, panel a). Sixty minutes after induction of the Gly containing mutant, in addition to the 66 kDa band, a band was observed co-migrating with the p51 marker. In the 120 min post-IPTG induced sample, however, the 66 kDa band was no longer present whereas the 51 kDa band persisted. This would appear to suggest that PR had quantitatively processed the RNase H domain from p66 RT in this mutant. Thus, the presence of a Gly residue in the P_2 position adjacent to the scissile peptide bond not only retards the initial rate of cleavage at the -Phe*Tyr- bond but ultimately influences the susceptibility to attack such that cleavage is achieved within both p66 subunits. Whether this takes place at the alternative site that is located 4 residues upstream [17] requires further investigation.

These substitutions were introduced into the P_2 position because previous work had shown that a β-branched residue in P_2 was optimal for cleavage of peptide substrates containing a -Hydrophobic*Hydrophobic- scissile bond [5] whereas Gly in this position effectively stopped hydrolysis [4,5,18]. The more complicated effects observed in the Gly containing mutant polyprotein may well be a reflection of the presence in such substrates of additional cleavage sites. The quantitative conversion of p66 to the 51 kDa form in this mutant (Fig. 5, panel b) permits the isolation of homodimeric (p51/p51) RT. However, p51 RT thus obtained would not be authentic since it contains one alteration (Thr→Gly) adjacent to the C-terminal residue. By contrast, from the construct described in Fig. 2, lane 2, i.e. by mutation of the junction between RT and IN, it was possible to alter processing such that an authentic homodimeric (p51/p51) RT was apparently generated. Isolation of these two variants of homodimeric RT would not only permit their characterisation free of the RNase H domain but also facilitate crystallisation (which has been difficult to attain for the heterodimeric enzyme) and the development of RT inhibitors as alternatives to AZT, DDC, etc. Thus, elucidation of the subtle requirements that must be met for effective cleavage of viral polyproteins by PR have considerable implications for the development of anti-viral therapeutic agents.

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