AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 19, Number 9, 2003, pp. 779–784

Short Communication

Polymorphisms in p1-p6/p6* of HIV Type 1 Can Delay Protease Autoprocessing and Increase Drug Susceptibility

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ABSTRACT

Maturation of infectious human immunodeficiency virus type 1 (HIV-1) particles requires proteolytic cleavage of structural polyproteins by viral protease. Inhibition of protease is a powerful tool for the treatment of HIV infection. Using a well-established phenotypic drug susceptibility assay, we found that sequences outside of the protease gene can modulate the susceptibility to protease inhibitors (PIs). Chimeric viruses carrying p1-p6/p6* sequences from patient isolates in the context of an NL4-3 molecular clone exhibited increased PI susceptibility. Furthermore, this phenotype was associated with a delay in protease autoprocessing in virions and a reduction in replication capacity. We propose that the interplay between protease and the C terminus of Gag is critical for proper protease activity and mismatches between these regions can reduce viral replication and increase drug susceptibility.

In HIV-1, STRUCTURAL AND NONSTRUCTURAL PROTEINS are synthesized from a single RNA template to yield two main polyproteins, Gag and Gag–Pol. The 55-kDa Gag precursor contains the matrix (MA), capsid (CA), p2, nucleocapsid (NC), p1, and p6 proteins. The Gag–Pol polyprotein is generated by a −1 ribosomal frameshift at the NC–p1 junction, occurring with a frequency of about 5%. The p160-kDa Gag–Pol precursor consists of the Gag products followed by a transframe protein referred to as p6*, the viral protease (PR), reverse transcriptase (RT), and integrase (IN).[1]

The viral protease is a member of the aspartic acid protease family, active only as a homodimer. PR is responsible for its own release from Gag–Pol and the processing of Gag and Gag–Pol precursors to yield structural and functional viral proteins. The temporal regulation of PR activity in the viral replication cycle is critical for proper virion assembly and maturation. Premature activation of the PR or partial inhibition of its activity leads to defects in viral assembly and consequently to the formation of noninfectious particles. Sequential processing of Gag and Gag–Pol polyproteins results in discrete intermediates appearing transiently before the final products (for review, see Pettit *et al.*[2]). Such intermediates may be important for viral morphogenesis but are incompatible with virus infectivity.

The mature 11-kDa PR is liberated from the Gag–Pol polyprotein by a two-step mechanism. The initial step involves the hydrolysis of the peptide bond at the p6*–PR junction via an intramolecular mechanism.[3] This cleavage at the N terminus of PR is accompanied by a large increase in PR activity. The flanking C-terminal sequences are cleaved via an intermolecular process.[4] The molecular mechanisms leading to PR activation are currently unknown. The p6* sequence upstream of the PR region has not been ascribed a specific function and is in a position corresponding to the prosegment observed in other aspartic PRs.[5] On the basis of this analogy, it has been suggested that the p6* region may serve to regulate PR activity and that autocatalytic release of PR from p6* may be a key event in HIV polyprotein processing. In this study, using recombinant viruses containing p1-p6/p6* and PR sequences derived from patient viruses, we characterize the interplay between p6/p6* sequences and PR activity, autoprocessing, and susceptibility to protease inhibitors (PIs).

Drug susceptibility was determined with PhenoSense HIV.[6] In this assay, resistance test vectors are constructed by inserting amplified patient-derived HIV-1 sequences into a modified retrovirus vector derived from the pNL4-3 molecular clone (Fig. 1A). Viral stocks were prepared by cotransfecting 293 cells with

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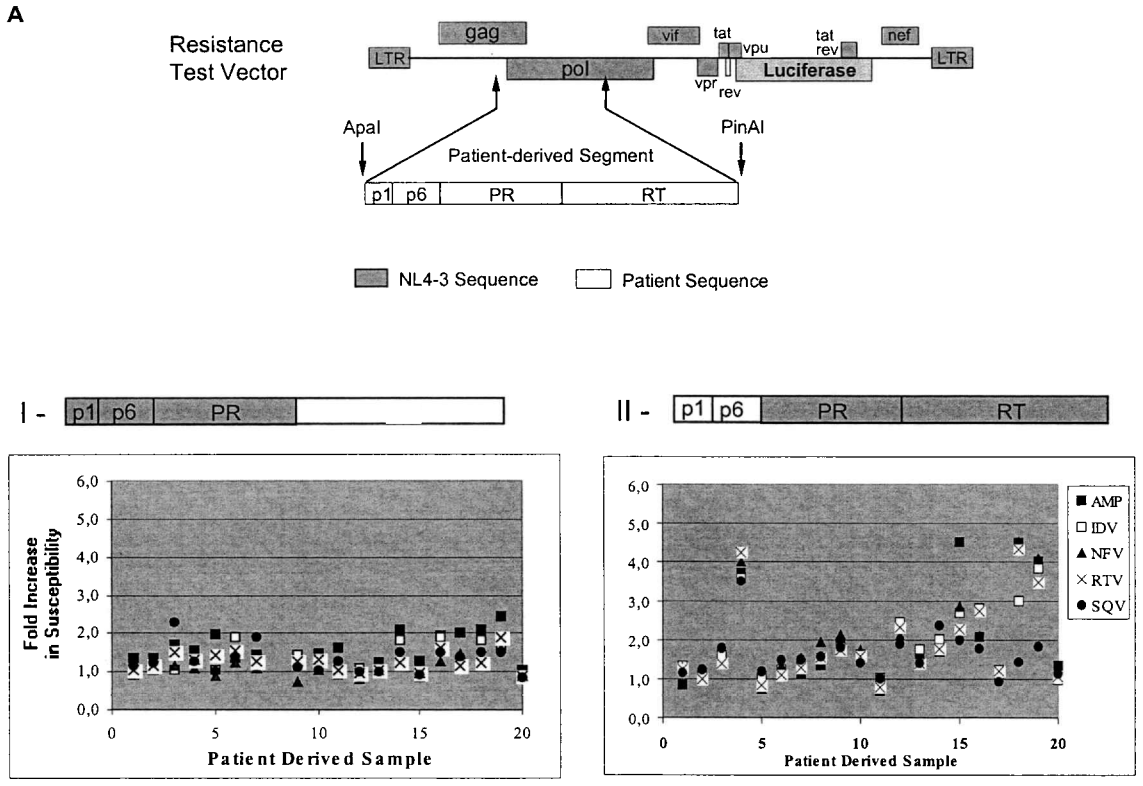

FIG. 1. Effect of patient isolate Gag and RT sequences on HIV-1 PI susceptibility. (**A**) Schematic representation of the resistance test vector. Recombinant viruses were constructed by exchanging Gag or RT sequences of the NL4-3 reference virus (shaded) with the respective sequences from patient isolates (open) displayed in Table 2. (**B**) Fold increase in susceptibility to PIs using PhenoSense HIV. Recombinant viruses carrying RT (panel I) or p1-p6 sequences (panel II) from patient isolates are indicated.

the resistance test vector DNA and an expression vector that produces envelope proteins from Abelson murine leukemia virus (A-MLV). Pseudotyped virus particles were harvested from the transfected cells and were used to infect fresh 293 cells. Resistance test vector DNAs also contain a luciferase indicator gene cassette within the envelope region and the production of luciferase in infected cells is dependent on the completion of one round of viral replication. Luciferase activity is used to compare the ability of the virus to replicate in the presence and absence of antiretroviral drugs. Analysis of the distribution of phenotypic susceptibility to antiretroviral drugs in 9913 viruses identified a group of viruses with increased susceptibility to one or more PI compared with the NL4-3 reference virus (Table 1). We defined the hypersusceptible(HS) phenotype as a 50% infective concentration (IC_{50}) that is more than 2.5-fold lower than the IC_{50} of the reference virus, corresponding to a fold change of 0.4 or less. This represents approximately the 10th percentile of the distribution of fold change values among wild-type viruses, and is well outside assay reproducibility limits.[6a] The HS phenotype was more frequently observed for amprenavir (APV) and saquinivir (SQV) (13.6 and 7.9%) than for indinavir (IDV), ritonavir (RTV), or nelfinavir (NFV) (6.6, 6.2, and 2.9%, respectively) (Table 1). To understand the cause of the PI HS phenotype, we evaluated 20 randomly selected patient samples that displayed increased susceptibility to at least one PI and no resistance (fold change, >2.5) to any other PI (Table 2). In this set of viruses 3- to 6-fold increases in PI susceptibility were often observed. The pro-

TABLE 1. DISTRIBUTION OF PROTEASE INHIBITOR SUSCEPTIBILITY, USING A DATABASE OF 9913 PATIENT-DERIVED VIRUSES

	Protease inhibitor				
	APV	*IDV*	*NFV*	*RTV*	*SQV*
Resistant (%)[a]	2920 (29.5)	3713 (37.5)	4792 (48.3)	4052 (40.9)	3280 (33.1)
Susceptible (%)	5646 (57.0)	5542 (55.9)	4831 (47.7)	5251 (53.0)	5854 (59.1)
Hypersusceptible (%)[b]	1347 (13.6)	658 (6.6)	290 (2.9)	610 (6.2)	779 (7.9)

Abbreviations: APV, amprenavir; IDV, indinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir.
[a]IC_{50} 2.5-fold higher than that of the reference virus.
[b]IC_{50} 2.5-fold lower than that of the reference virus.

TABLE 2. PATIENT-DERIVED VIRUSES DISPLAYING INCREASED SUSCEPTIBILITY TO PROTEASE INHIBITORS[a]

Sample	PR genotype	Fold increase in susceptibility				
		APV	IDV	NFV	RTV	SQV
1	E35D, N37D, L63P, V77I, I93L	2.7	1.7	0.9	2.7	3.1
2		3.0	2.9	2.5	2.6	3.1
3	T12A, N37C, R41N, I62V, L63P, V77I	4.9	2.6	1.6	2.7	4.0
4	M36I, I62V, I93L	4.2	4.7	4.0	5.2	3.6
5	M36I, R41K, R57K, I64V	5.2	2.5	1.7	1.9	2.9
6	L63A	2.0	2.0	1.7	2.4	2.7
7		3.8	2.2	1.7	3.6	2.8
8	T12I, I62V, L63N	2.7	2.5	1.9	1.8	2.8
9	L63Q, I64V	3.1	2.5	2.0	3.1	2.0
10	K14R	2.8	2.5	2.2	2.4	2.4
11		2.6	2.5	2.3	2.0	2.8
12	L63A, E65D	2.9	1.9	2.4	2.2	2.6
13	N37C, L63A, V77I	3.4	3.0	2.6	3.3	3.1
14	E35D, N37D, R57K, L63A, V77I	4.8	2.9	2.9	4.6	5.6
15	I15V, G16E, N37T, P39S, D60E, Q61E, L63P, V77I	6.1	2.9	3.7	2.8	1.8
16	N37K, R41K	3.2	3.6	2.2	3.2	2.3
17	G16E, N37S, I62M, L63P, V77I	5.3	4.7	4.4	4.3	4.5
18	N37S, R41K	4.3	4.5	4.9	5.2	3.8
19	N37S, R41K	3.3	4.2	5.4	6.2	4.0
20	N37T, R41K, I62V	2.9	3.5	5.9	3.9	3.1

Abbreviations: PR, protease; for PIs, see Table 1.
[a]Values indicate the ratio between the IC_{50} of the reference virus and the IC_{50} of the patient sample (IC_{50} Ref % IC_{50} Pat.).

tease genotypes of several of these viruses were either identical to NL4-3 or the differences were limited to common polymorphisms (Table 2). This observation led us to consider that mutations outside of protease could alter PI susceptibility. To investigate this possibility, we analyzed the impact of RT and p1-p6/p6* sequences derived from PI HS viruses on the PI susceptibility of viruses containing wild-type PR. To this end, we generated chimeras, using the NL4-3-based resistance test vector carrying either amino acids 20 to 305 of RT, or the 3′ terminus of Gag encoding p1-p6/p6* derived from PI HS patient isolates (Fig. 1).

The viruses carrying only RT sequences from the patient isolates displayed normal levels of susceptibility to all the PIs (Fig. 1B, panel I). This result indicates that in the 20 samples analyzed, mutations in RT (many of them associated with resistance; data not shown), did not affect PI susceptibility. In contrast, 25% of the viruses (5 of 20) carrying only the 3′ end of the *gag* region from the patient isolates partially retained the increased PI susceptibility phenotype (Fig. 1B, panel II), indicating that mutations downstream of amino acid 418 of Gag or in p6* in the Pol frame can alter PI susceptibility of a wild-type protease. We chose these five chimeric viruses, which retained the HS phenotype observed in the patient isolate, as a tool to investigate the relationship between p1-p6/p6* sequences and PR activity and PI susceptibility.

Nucleic acid sequencing of the C-terminal patient-derived portion of Gag was performed. Despite the presence of two open reading frames within the same region this sequence demonstrated extensive variability, in agreement with previous studies.[7] Some of the mutations observed in the amino acid alignments of Gag and Gag–Pol appeared unusual on the basis of comparisons with samples from several large HIV-1 sequence databases (NCBI and Los Alamos), indicated in boldface in Fig. 2. The C-terminal Gag sequences of viruses 18 and 19 were isolated from viruses obtained from the same patient in 1999 and 2000, respectively. During that year, the patient virus developed resistance to delaviridine (DLV), efavirenz (EFV), and nevirapine (NVP), yet the genotype within p1-p6/p6* and the increased susceptibility to PIs were maintained.

Previous studies have shown that sequences within p6 are involved in the late stages of the viral life cycle, participating in Gag–Pol packaging, Vpr incorporation, and budding.[8,9] Mutations within a conserved P(T/S)AP motif (also known as the "late" or L domain) arrest viral release at a late stage.[10,11] It has been shown that in HIV-1 this motif specifically interacts with a cellular protein (Tsg101), which might facilitate the budding process.[12–14] Sequence analysis of the five viruses used in this study indicates that the P(T/S)AP motif is absolutely conserved (Fig. 2A). Nonetheless, to evaluate other possible defects during particle formation, we compared the amount of virus released from cells transfected with the resistance test vector DNA obtained from PI HS and sensitive viruses. The amount of viral particles produced was quantified by measuring virion-associated RNA, using real-time polymerase chain reaction (PCR) (TaqMan). Briefly, viral supernatants obtained from transfected 293 cells and a mock-transfected sample were lysed in a buffer containing 0.6% Nonidet-P40 (NP-40), 50 mM Tris-HCl (pH 8), 10 mM dithiothreitol (DTT), 10 mM $MgCl_2$, and 50 mM KCl and incubated for 30 min on ice. The lysate was diluted 10-fold with buffer lacking NP-40, and 5 μl was added to an RT-PCR reaction mix (0.3 mM dNTPs, 1 nM primers, 1 nM TaqMan probe, RNase inhibitor, murine leukemia virus [MuLV] RT, 5 mM $MgCl_2$, AmpliTaq Gold, and 1× TaqMan buffer). The samples together with the RNA copy number stan-

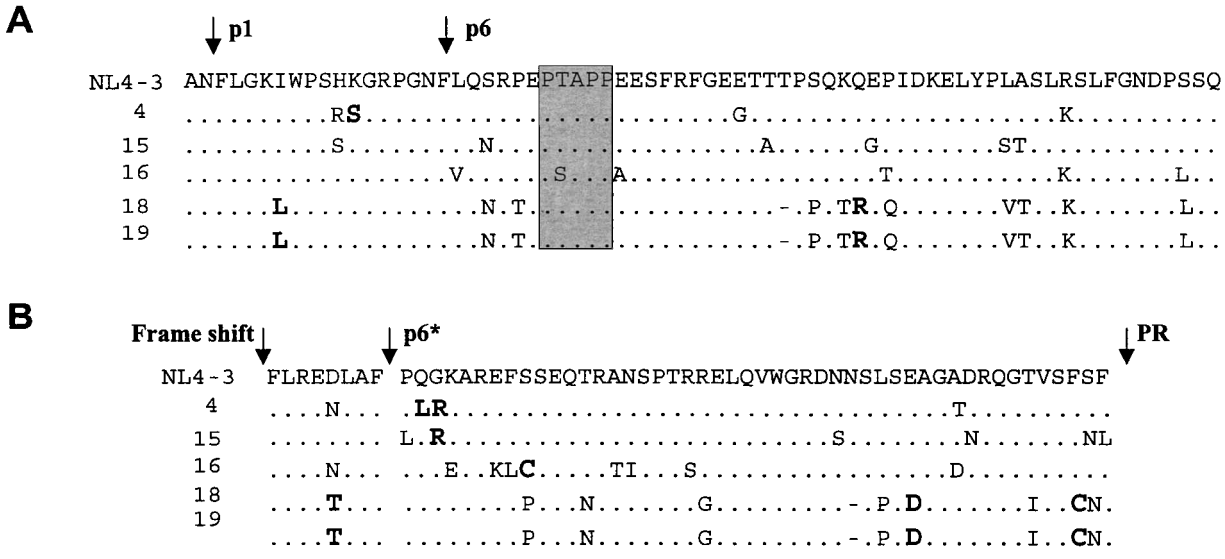

FIG. 2. Amino acid alignments of p1-p6/p6* sequences. (**A**) Amino acid sequences in the Gag open reading frame of patient isolates were aligned with the corresponding sequence of the NL4-3 reference virus. Amino acid residues that differ from the reference are indicated. The PR cleavage sites that yield p1 and p6 are indicated with arrows. The conserved sequence PTAPP is shown in gray. (**B**) Amino acid sequences in the Gag–Pol open reading frame of patient isolates were aligned with the corresponding sequence of the NL4-3 reference virus. The PR cleavage sites that yield p6* and PR and the frame shift site are indicated with arrows.

dard (obtained by *in vitro* transcription) were incubated for 2 hr at 37°C for the RT step and subjected to 40 cycles of PCR amplification (ABI 7700; Applied Biosystems, Foster City, CA). Transfection efficiency, determined by luciferase expression in the transfected cells (which is independent of viral replication), displayed less than 10% variability between samples transfected in the same experiment. No significant differences in viral RNA production were observed between the reference virus and samples 4, 15, 16, 18, and 19 (Fig. 3A). This was also confirmed by p24 quantification, using an enzyme-linked immunosorbent assay (ELISA) (data not shown). These results suggest that the patient p1-p6/p6* sequences did not alter viral particle production under our experimental conditions. To evaluate the ability of these pseudotyped viruses to enter and initiate a new round of viral replication, the viral particles obtained 48 hr after transfection were used to infect fresh 293 cells in the absence of drugs. After infection, the luciferase activity produced is absolutely dependent on functional PR and RT activities; therefore, we used luciferase expression to evaluate replication capacity (RC).[15–17] The RC of the HS viruses was surprisingly low and never exceeded 25% of that observed for NL4-3 reference virus (100%) (Fig. 3B). In addition, the replication of these HS viruses carrying only p1-p6/p6* from the patient isolates resembles the RC of the parental viruses carrying p1-p6/p6*, RT, and PR sequences from the patient samples (data not shown), indicating that the phenotype observed was not due to incompatibilities between Gag and PR sequences in the chimeric viruses. It is important to mention that not all HS viruses are necessary associated with low RC; analysis of other HS viruses not included in this study displayed a wide range of RCs.

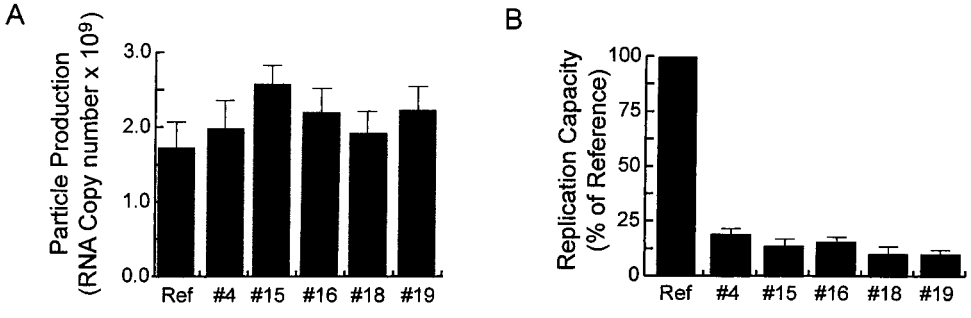

FIG. 3. Replication capacity of recombinant viruses containing p1-p6 sequences from patient isolates. (**A**) Amount of viruses produced by transfected cells as measured by virion-associated RNA. The mean values and standard deviation from two independent measurements are indicated. (**B**) Single-cycle replication capacity measured by luciferase activity in infected cells in the absence of drugs. The results are expressed as a percentage of the NL4-3 reference virus. The mean values and standard deviation from two independent experiments, each performed in duplicate, are indicated.

To better understand the cause of the low RC and the increased susceptibility to PIs of these viruses, we investigated the impact of p1-p6/p6* sequences from the patient isolates on protease autoprocessing in viral particles. Immunoblots employing rabbit polyclonal antibodies against PR protein were performed on NL4-3 reference and mutant particle preparations. Briefly, 24 hr after transfection the cells were refed with fresh medium and virus was harvested from the medium 4 hr later by centrifugation. Viral proteins in the pellets, containing equal amounts of viral particles (based on parallel p24 assays and RNA content), were analyzed by Western blots as previously described.[18] In the reference virus the vast majority of PR existed as the fully processed 11-kDa protein and little p6*-PR intermediate was detected (Fig. 4A, lane 1). In contrast, the four

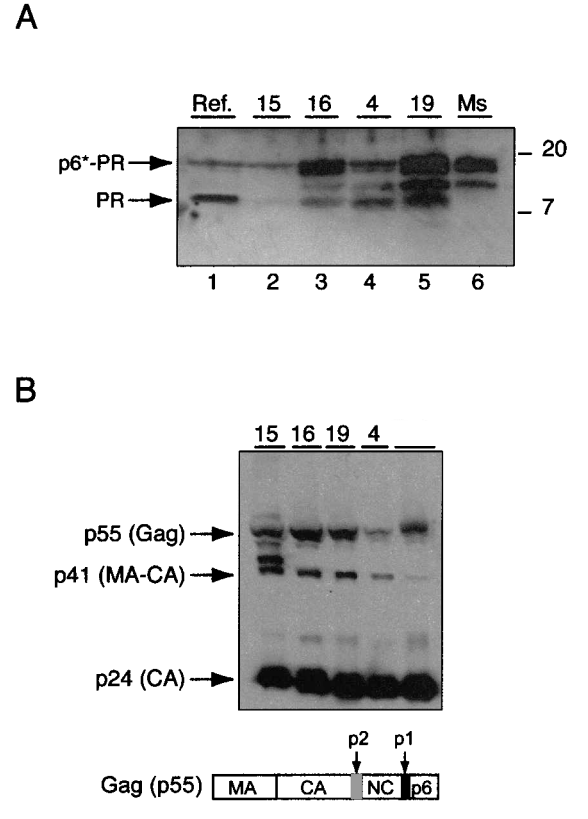

FIG. 4. Western blot analysis of particle-associated Gag and PR proteins. (**A**) Rabbit polyclonal antibodies against PR were used to detect precursors and mature 11-kDa PR. Viruses carrying p1-p6/p6* from patient isolates (15, 16, 4, and 19), NL4-3, and the site-directed mutant (Ms, in which the last three amino acids of p6* F-S-F were replaced by L-S-S) are indicated. Transfected cells were refed 24 hr after transfection, and the viral particles were harvested from cell culture medium 4 hr later. The amount of sample was normalized by quantification of viral particles. Molecular weight markers are indicated on the right and the mobility of PR protein and p6*-PR precursor is indicated on the left. (**B**) Western blot analysis with anti-p24 antibodies. Viruses carrying p1-p6/p6* from patient isolates (15, 16, 4, and 19) and NL4-3 reference are shown. The mobility of the viral protein $p55^{gag}$ (p55), the intermediate $p17^{MA}$-$p24^{CA}$ (p41), and the fully processed $p24^{CA}$ (p24) are indicated with arrows.

p6* mutants displayed different abnormalities in PR autoprocessing. Viruses 4, 16, and 19 contained mostly unprocessed p6*-PR intermediate, suggesting a defect in PR release from Gag–Pol. In addition, an extra protein of about 15 kDa was observed in these three viruses but was absent in the reference virus. Because virus 18 was identical in sequence to virus 19, it was excluded from this analysis. Virus 15 also displayed altered processing but the defect was qualitatively different compared with the other samples. In this virus, little mature PR was observed (Fig. 4A, lane 2). As a control, we also evaluated a virus with a mutation at the p6*-PR junction known to alter PR processing, in which the last three amino acids of p6* F-S-F were replaced by L-S-S (S mutant). The Western blot analysis of this mutant revealed accumulation of the p6*-PR precursor whereas the mature PR was completely absent (Fig. 4A, lane 6). Moreover, the S mutant was noninfectious in cell culture, confirming that processing between p6* and PR is required for viral viability.[18]

Western blots using anti-p24 antibodies were also performed to analyze processing of Gag polyprotein in the HS viruses. Wild-type and mutant viruses were harvested from the medium at different times after transfection. Viruses were collected from the medium as described above and 5×10^9 viral particles per sample were analyzed. Near-complete cleavage of the Gag polyprotein to mature $p24^{CA}$ was observed in wild-type particles (Fig. 4B, lane 15). Particles from viruses 16, 19, and 4 showed incomplete processing of p55 and accumulation of p41 (Fig. 4B). The particles from virus 15, which displays the least amount of mature PR (Fig. 4A, lane 2), shows the most pronounced accumulation of intermediates p41 and p45. These results indicate that the defects in PR autoprocessing and low RC of these HS viruses correlate with abnormal Gag processing, and that the activity of a wild-type PR and PI susceptibility can be modulated by sequences located upstream of the protease gene.

Taken together, our observations indicate that viruses with increased susceptibility to PIs can be associated with a delay in the release of mature PR, and that sequences within p1-p6/p6* are sufficient to confer this phenotype in the cases analyzed here. We hypothesize that decreased PR activity is responsible for both low RC and increased PI susceptibility in these viruses. Importantly, however, we have observed many viruses with PI HS that do not have reduced RC (data not shown), indicating that this is only one of several potential explanations for the HS phenotype. Viruses 15, 18, and 19 display amino acid substitutions near the p6*-PR cleavage site that may affect processing at this site.

The region that flanks the N terminus of PR has been postulated to have a regulatory role in PR activation. This hypothesis is supported by the observation that removal of the p6* region enhances Gag processing in an *in vitro* translation system, suggesting negative regulation of PR by p6*.[19] In addition, several peptides derived from p6* can inhibit purified HIV PR activity *in vitro*.[20,21] These previous *in vitro* studies together with our observations using viral particles argue strongly that important regulatory determinants of protease autoprocessing reside within p6* sequences. It remains unclear why these viruses should maintain polymorphisms in p6/p6* that impair viral replication. It is possible that mutations in other parts of the genome, beyond the PR region, compensate for these de-

fects in the intact patient virus. Alternatively, it is plausible that certain polymorphisms associated with decreased replication appeared in response to immune surveillance or other selective pressures present in the host. Interestingly, unusual polymorphisms in p6/p6* associated with slow growth phenotypes have been found in nonprogressive infections.[18] In addition, it has been reported that mutations within p6/p6* are associated with resistance to RT inhibitors. In this case, using an *in vitro* translation system, a delay in protease maturation was also observed.[22,23]

The fact that the viruses used in our study were originally selected on the basis of their increased susceptibility to PR inhibitors, and that they all share defects in PR maturation, suggests that drug susceptibility can be modulated by altering PR autoprocessing. The identification of novel targets against HIV-1 is clearly needed to expand and improve therapeutic strategies. A report demonstrated that antisense peptide nucleic acids (PNAs) directed against p6* of HIV-1 abolish virion production by up to 99% in cell culture.[24] Further elucidation of the molecular processes that lead to PR activation could help to improve existing therapies against HIV and to identify novel drug targets.

ACKNOWLEDGMENTS

We are grateful to the ViroLogic Clinical Laboratory for performing the PhenoSence assay and to H.G. Krausslich for PR antibodies and experimental suggestions. We also thank Jeannette Withcomb, Terri Wrin, and Amalio Telenti for useful discussions.

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