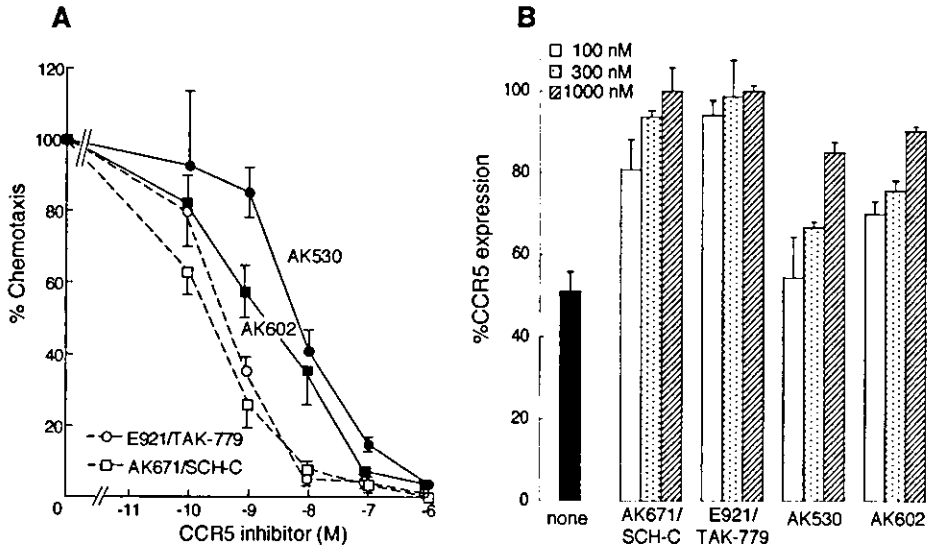

FIG. 7. AK602 allows RANTES-induced chemotaxis and CCR5 internalization. (A) $CCR5^+$ MOLT4 cells were exposed to various concentrations of AK530, AK602, E921/TAK-779, or AK671/SCH-C, thoroughly washed, plated onto the upper chamber of the ChemTx System, exposed to 0.5 nM RANTES contained in the lower chamber, and incubated for 4 h; the number of the cells which migrated to the lower chamber was determined, and chemotaxis was calculated. (B) $CCR5^+$ CHO cells were exposed to 10 nM RANTES in the presence or absence of various concentrations of each CCR5 inhibitor and washed with acidic solution for removal of the cell-bound RANTES (21). The amount of cell surface CCR5 was subsequently determined with monoclonal antibody 3A9 (BD PharMingen), which recognizes the N terminus of CCR5 and competes with none of the CCR5 inhibitors tested. In panel A, the level of chemotaxis suppression by TAK-779 and SCH C was greater than that by AK530 and AK602 at four concentrations examined, although complete suppression was seen only at the highest concentration of the AK compounds, 1 μM. However, in panel B, the level of CCR5 internalization suppression by TAK-779 and SCH-C was greater than that of the AK compounds at all three concentrations examined.

derivatives stems from their inhibition of gp120 binding to CCR5, as reported for other CCR5 inhibitors such as TAK-779 (3), although the binding pocket (or subsite) of CCR5 for certain SDP derivatives (such as AK530) apparently does not quite overlap the rgp120/sCD4 complex binding site of CCR5 (Fig. 3B). It is also possible that the conformational changes ensuing upon AK602's binding to CCR5 could differ from that ensuing upon AK530's binding to CCR5, thereby producing differences in gp120/sCD4 binding and anti-HIV activity.

It is generally noted that although the determination of any binding sites with antibodies provides "indirect" evidence, in many cases it gives good insights (14). Indeed, SCH-C has been reported to induce conformational changes in CCR5 and bind to its transmembrane (TM) domain, thereby blocking HIV-gp120 binding to CCR5. In our data, SCH-C completely blocked the binding of the "multidomain"-reactive monoclonal antibody 45523, which reportedly causes conformational changes in CCR5, while it only moderately blocked the binding of the ECL2B-specific monoclonal antibody 45531 (Fig. 4). In contrast, AK602 completely blocked the binding of both 45523 and 45531. Considering that monoclonal antibody 45531's CCR5 binding is closely linked to amino acids 184 to 189 of ECL2B, as shown by Lee and colleagues (14), it was thought that the binding site of AK602 includes ECL2B or is vicinal to it. Indeed, our recent analysis with the alanine-scanning algorithm showed that AK602 totally failed to bind to a CCR5 mutant when a K191A substitution was introduced (Maeda et al., unpublished data), corroborating and extending the idea that AK602's binding site involves the ECL2B domain.

It is noted that the IC_{50} of AK602 against HIV-1 as determined in peripheral blood mononuclear cells (0.4, 0.1, and 0.2 nM against $HIV\text{-}1_{Ba\text{-}L}$, $HIV\text{-}1_{JR\text{-}FL}$, and $HIV\text{-}1_{MOKW}$, respectively: Table 1) are substantially lower than the K_d of AK602 (2.9 nM) and the IC_{50} of AK602 for its inhibition of rgp120/sCD4 complex binding to CCR5 (2.7 nM). The anti-HIV-1 IC_{50}s of AK602 are also lower than the IC_{50}s of AK602 for its inhibition of MIP-1α-induced Ca^{2+} influx (39.8 nM; unpublished data) and that for its inhibition of CCR5 internalization (≈300 nM; unpublished data).

One possible explanation for these inconsistencies is the different cell lines employed for each assay. However, it is of note that when we determined the IC_{50} values against several R5 HIV strains and K_d values of AK602 in MAGI/CCR5 cells (18), AK602's IC_{50}s (≈0.2 nM) were reproducibly lower than AK602's K_d (3.8 nM) (data not shown). Thus, one can postulate that for the inhibition of HIV-1 infection by CCR5 inhibitors, not all CCR5 molecules might have to be occupied. In this regard, our studies with ^3H-labeled AK602 and $CD4^+$ target cells expressing CCR5, MAGI/CCR5 (18) and U373-MAGI (34), have shown that less than 30% of HIV-1 infection occurred when approximately 50% of CCR5 molecules were bound by AK602, and at its anti-HIV-1 IC_{50} concentration, AK602 was found to bind to 5 to 20% of CCR5 molecules on the target cells (Maeda et al., unpublished data). These data suggest that when one of the multimerized CCR5 molecules is bound or occupied by AK602, inhibition of the cell is likely to be blocked, although further stoichiometric analyses need to be conducted.

It has been thought that individuals carrying a gene encoding a mutant form of CCR5 called Delta32 are resistant to HIV-1

infection and apparently do not have significant health problems (2, 15, 23, 25). One can assume that individuals with homozygous CCR5-Delta32 might inherently have certain defenses which could compensate for the deficiency of CCR5. In this regard, there has been a report that individuals carrying homozygous CCR5-Delta32 have longer survival of renal transplants than those with other genotypes, suggesting that such individuals might have compromised graft rejection immunity (7). Moreover, Woitas et al. have reported that individuals with homozygous CCR5-Delta32 have significantly higher levels of hepatitis C virus in blood than their counterparts who have wild-type CCR5, suggesting that the CCR5-Delta32 mutation may be an adverse host factor in hepatitis C virus infection (35), although others have recently argued against a role of CCR5 in susceptibility to hepatitis C virus infection or response to antiviral therapy (9). Thus, sustained, long-term suppression of the effect of CC-chemokines/CCR5 interactions, in particular in those who carry wild-type CCR5 and might not have a possible compensatory mechanism for the absence of CCR5, might produce adverse effects, and caution should be used in the development of chemokine receptor antagonists as potential therapeutics for HIV-1 infection.

In this respect, SDP derivatives such as AK602 can preserve CC-chemokine/CCR5 interactions at their anti-HIV activity-exerting concentrations; they allow RANTES and MIP-1β binding to $CCR5^+$ cells and their functions at anti-HIV-1 concentrations. In contrast, two previously published CCR5 inhibitors, TAK-779 and SCH-C, fully blocked CC-chemokine/CCR5 interactions (Fig. 5 and 7). It is of note that AK602's complete inhibition of the binding of MIP-1α was not surprising because in the initial search of lead compounds, we sought compounds that blocked the binding of ^{125}I-labeled MIP-1α to $CCR5^+$ CHO cells and MIP-1α-elicited cellular Ca^{2+} mobilization, as described previously (17).

In support of the above observation, the results of competitive biding assays with [^{3}H]AK602 and [^{125}I]RANTES and their corresponding unlabeled agents clearly indicated that AK602 and RANTES bind simultaneously to CCR5 (Fig. 6). Moreover, AK602 allowed $CCR5^+$ MOLT4 cells to undergo RANTES-elicited chemotaxis (Fig. 7A) and $CCR5^+$ CHO cells to internalize CCR5 in response to RANTES (Fig. 7B) at concentrations much greater than AK602's anti-HIV-1 activity-exerting concentration in peripheral blood mononuclear cells. However, it is worth noting that although AK602 blocked the binding of [^{125}I]RANTES to $CCR5^+$ CHO cells only by ≈40% at micromolar concentrations (Fig. 5A), it virtually completely blocked the RANTES-induced chemotaxis at micromolar concentrations, as examined in $CCR5^+$ MOLT4 cells (Fig. 7A). This apparent inconsistency could be explained by the different cell lines employed for each assay and the fact that the number of CCR5 molecules in $CCR5^+$ CHO cells (≈5×10^5/cell) is substantially different from that of $CCR5^+$ MOLT4 cells (≈1×10^5/cell), and thus, AK602 could more efficiently block the chemotaxis of MOLT4 cells. It is also possible that AK602 may more effectively block CCR5 multimerization, which is reportedly important for the functionality of the G protein-coupled receptor (29), rather than the RANTES binding block to CCR5 per se. However, it is not clear yet whether AK602's unique profile that AK602 partially allows RANTES and MIP-1β to bind to CCR5 despite AK602's tight binding to CCR5 brings about a clinical advantage. This can be examined only in the setting of clinical trials and careful clinical investigation in long-term treatment with such an agent.

Several HIV-1 variants which acquired resistance to CC-chemokines, including MIP-1α and CCR5 inhibitors, have been reported. Trkola et al. described that when HIV-1 was passaged in the presence of increasing concentrations of a CCR5-specific, structurally SCH-C-related CCR5 inhibitor, AD101, an escape mutant which contained 22 amino acid substitutions in the gp120 subunits emerged as early as after 19 passages (31). This escape mutant showed a >20,000-fold resistance to AD101 and was similarly resistant to SCH-C compared with wild-type HIV-1, suggesting that HIV-1 can acquire the capability of using CCR5 bound to certain classes of CCR5 inhibitors for its entry into the target cell (31). Maeda et al. reported that HIV-1_{JR-FL}, following in vitro selection against MIP-1α over 3 months, acquired amino acid substitutions in the V2 and V3 regions of HIV-1 gp120 and became four- to sixfold more resistant to MIP-1α, MIP-1β, and RANTES (18). In this regard, as of this writing, we have passaged HIV-1_{Ba-L} in $CD4^+$ $CCR5^+$ PM1 cells (16) in the presence of moderately increasing concentrations of AK602 in one selection experiment and aggressively increasing concentrations of AK602 in another selection experiment over 22 months (45 passages); however, the virus has acquired no detectable resistance to AK602 and no significant amino acid substitutions (Nakata et al., unpublished data).

It is worth noting that the anti-HIV-1 activity of AK602 is virtually unaffected by the presence of human serum proteins. For instance, the IC_{50} of AK602 against HIV-1_{Ba-L} in the presence of 10% fetal calf serum in culture medium was 0.4 ± 0.3 nM, while those of AK602 with 10 μM α1-acid glycoprotein and 45% human serum added to the culture medium were 0.8 ± 0.3 and 0.7 ± 0.7 nM, respectively. AK602 failed to induce Ca^{2+} flux, chemotaxis, or CCR5 internalization in $CCR5^+$ cells (Maeda et al., unpublished data). As far as the sensitivities of our methods used in the present work, AK602 is to be categorized as a nonagonist or antagonist. The phase 1 clinical trial of AK602 in HIV-1-seronegative individuals has recently been concluded, and no significant adverse effects have been documented. Considering that AK602 potently inhibited the replication of HIV-1 in vitro and in a nonobese diabetic-SCID mouse model (Nakata et al., unpublished data) and that AK602 has a favorable oral bioavailability in rodents, averaging 20 to 30% (unpublished data), the present data strongly suggest that AK602 is a promising CCR5 inhibitor as a potential therapeutic for HIV-1 infection.

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ELSEVIER

The current status of, and challenges in, the development of CCR5 inhibitors as therapeutics for HIV-1 infection

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The discovery of CCR5 as a HIV-1 co-receptor unfolded the cryptic and complicated process of HIV-1 cellular entry and has provided more than a few entry steps as possible modalities for effective viral intervention. The proof-of-principle has already been established for the use of entry inhibitors against HIV-1 and there is a cautious optimism that several CCR5 inhibitors might soon be added to our armamentarium for therapy of HIV-1 infection.

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Introduction

Highly active antiretroviral therapy has had a major impact on the AIDS epidemic in industrially advanced countries [1,2]. However, the limitation of antiviral therapy of AIDS is exacerbated by its complicated regimens, the development of drug resistant HIV-1 variants [3,4] and several inherent adverse effects [5,6]. Nearly a score of different drugs are now on the market targeting two viral enzymes: reverse transcriptase and protease. However, the present highly active antiretroviral therapy regimens are not well tolerated, and there are increasing concerns about the long-term metabolic side effects of protease inhibitors, notably poorly understood problems with fat metabolism [5,6]. Furthermore, the alarming rates of emergence and transmission of drug-resistant HIV variants has posed another formidable problem [7,8]. Thus, the identification of a new class of antiretroviral agents that has a unique mechanism of action and produces no, or minimal, side effects remains a crucial and imperative therapeutic objective [2]. In this regard, agents that block viral entry could be of particular therapeutic value. A proof of concept for this approach has been established with the use of T20, a gp41-based entry inhibitor that blocks the fusion process [9], as well as certain CCR5 receptor inhibitors.

CCR5 as a target for developing therapeutic agents

After the identification of CD4 as a primary receptor for HIV entry into the cells of the immune system, it soon became evident that CD4 alone was not sufficient to establish productive infection. It took another 10 years until 1996 for the G-protein-coupled seven-transmembrane chemokine receptors CXCR4 and CCR5 to be identified as coreceptors for HIV-1 entry [10–18]. HIV-1 infection is initiated by the attachment of the virus envelope glycoprotein, gp120, to CD4 on the target cell. Binding to CD4 triggers a conformational change in gp120, which exposes a binding site for a chemokine receptor that acts as a coreceptor [11,13,19–21]. Interaction with the coreceptor triggers a rearrangement of the transmembrane subunit of the envelope glycoprotein, gp41, which leads to fusion between the virus and cell membrane [9,22–24]. The predominant chemokine receptors employed as coreceptors for entry by primary isolates of HIV-1 are CCR5 and CXCR4, although other chemokine receptors including CCR2 and CCR3 can also be used by some virus isolates with much lower efficiency [25–29]. CCR5 is the most important coreceptor for the macrophage-tropic (now also designated as R5) strains that are commonly transmitted between individuals, whereas CXCR4 is the most relevant coreceptor for the T-cell-tropic (now also referred to as X4) isolates that emerge after several years of HIV-1 infection [29–31]. Furthermore, blocking the function of CCR5 might not significantly impact human health, as approximately 1% of Caucasians naturally lack CCR5 because of a protein-disrupting mutation with no apparent detectable consequences [32–35]. Thus, the HIV-1 coreceptor CCR5 represents a new therapeutic target for the development of novel antiretroviral agents [9,36–38], and extensive screening programs were launched to identify small-molecule CCR5 inhibitors. Over the past few years, we have already seen several unique small-molecule CCR5 inhibitors in the pipeline.

Small-molecule CCR5 inhibitors and their antiviral activity

Certain protein-based CCR5-targeting ligands, including aminooxypentane (AOP)-RANTES (regulated on

activation normal T cell expressed and secreted) [36,39], PRO140 [40,41] and LD78β derivatives [42], were reported in the late 1990s. Although many of these protein-based CCR5 inhibitors were valuable as research tools, their poor pharmacokinetics and only moderately potent anti-HIV activity hindered their potential as candidates for therapeutics. It is only recently that several small-molecule CCR5 inhibitors have been described in the literature, and their use is swiftly evolving and expanding.

TAK-779 and TAK-220

TAK-779 represents the first small-molecule CCR5 inhibitor published (Takeda Chemical Industries Ltd) in the scientific literature (Figure 1) that exerts potent activity against R5 HIV-1 *in vitro* [43]. However, TAK-779 was not orally bioavailable and the development of TAK-779 was discontinued because of irritation at injection sites. Subsequently, Takeda disclosed an orally bioavailable CCR5-specific inhibitor, TAK-220 (whose structure has not been disclosed as of this writing), which inhibits the replication of R5 HIV-1 isolates with EC_{50} and EC_{90} values of 1.1 nM and 13 nM, respectively. TAK-220 inhibits the binding of RANTES and macrophage inflammatory protein-1α to CCR5-expressing cells, but does not inhibit the binding of macrophage inflammatory protein-1β. When TAK-220 was administered orally to fasted rats and monkeys at a dose of 5 mg/kg, its bioavailability was reportedly 9.5% and 28.9%, respectively [44,45]. It is hoped that TAK-220 will enter Phase II clinical trials soon.

SCH-C and SCH-D

SCH-351125 (SCH-C) and SCH-D (Figure 1), reported by Schering-Plough Co., are orally bioavailable CCR5 inhibitors with potent *in vitro* antiviral activity [46,47]. SCH-C was administered to HIV-1-infected subjects in the setting of monotherapy over 10 days. Mean viral load reductions when administered 25–100 mg twice daily were increased by -1.50 $\log_{10}$. However, the compound was associated with cardiac adverse effects (e.g. QTc elongation) and was subsequently dropped from the pipeline. Another CCR5 inhibitor, SCH-D, has greater *in vitro* potency, and its *in vivo* efficacy has been evaluated in Phase I clinical trial as monotherapy with escalating doses of 10–50 mg twice daily over 14 days. No major adverse effects were seen, and a dose-related viremia decrease has been reported with mean reductions of -1.62 $\log_{10}$ [48••].

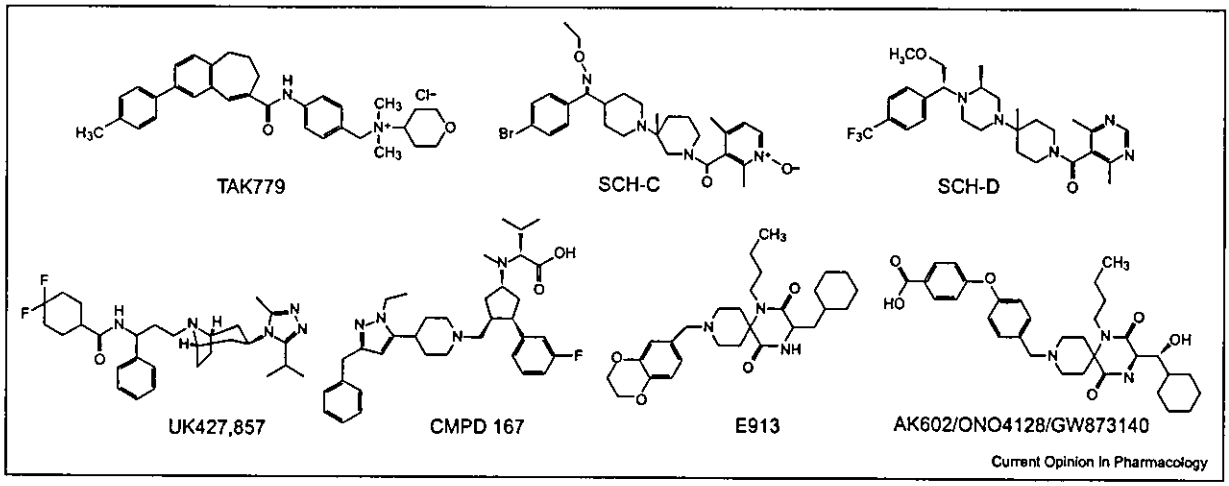
UK-427,857

Piperidine-based CCR5 inhibitors were reported by researchers at Pfizer Inc, including UK-427,857 (whose structure has not been disclosed in the scientific literature as of this writing), and have been examined in a short-term monotherapy involving R5-HIV-1-harboring patients with regimens of 25 mg four times daily, 100 mg twice daily, or placebo over 10 days. Patients receiving 100 mg twice daily had a mean decrease in viral load of 1.42 $\log_{10}$ from baseline. Mean CCR5 receptor saturation with 100 mg twice daily was in excess of 90% over the 10 day period. UK-427,857 was well tolerated, with no severe or serious adverse events [49••]. UK427,857 is currently undergoing Phase II clinical trials.

CMPD 167

Merck has reported a series of small-molecule CCR5 inhibitors [50–53]. One such compound, CMPD 167 (Figure 1), is a cyclopentane-based compound that produced a rapid and substantial (4- to 200-fold) decrease in plasma viremia in rhesus macaques chronically infected

Figure 1



Structures of CCR5 inhibitors.

with simian immunodeficiency virus. Merck also evaluated whether vaginal administration of gel-formulated CMPD 167 could prevent vaginal transmission of R5 virus. Complete protection occurred in two out of 11 animals, and early viral replication was less in the 11 macaques receiving CMPD 167 than in control animals, suggesting the possible practicability of small-molecule CCR5 inhibitors as a component of topical microbicide preparations to prevent HIV-1 sexual transmission [54•]. CMPD 167 is, however, no longer being developed as a therapeutic agent for HIV-1 infection.

E913 and AK602/ONO4128/GW873140

E913, a prototypic spirodiketopiperazine-based CCR5 inhibitor (Figure 1), was reported in 2001, and inhibited the replication of R5-HIV-1 and showed synergism when used with a CXCR4 inhibitor AMD-3100 in cellular assays [55]. AK602/ONO4128/GW873140 (Figure 1) has recently been developed following experiences with E913, which has a high affinity for CCR5 (K_D values of ~3 nM), potently blocks HIV-1-gp120/CCR5 binding, and exerts potent activity against a wide spectrum of R5-HIV-1 isolates, including multi-drug resistant HIV-1 strains (IC_{50} values of 0.1–0.6 nM) *in vitro* [56•]. In human peripheral blood mononuclear cell-transplanted R5 HIV-$1_{JR\text{-}FL}$-infected, non-obese diabetic-SCID interleukin-2 receptor γ-chain-knock out (NOG) mice, in which massive and systemic HIV infection occurred, AK602 produced ~2 $\log_{10}$ reduction in viremia in these mice (Nakata *et al.*, unpublished).

A multiple dose escalation study of AK602 was conducted in healthy subjects with doses of 200–800 mg twice daily (once daily on the first day) over seven days by GlaxoSmithKline (USA). AK602 was well tolerated with no serious adverse events, and >97% CCR5 occupancy was recorded at 2 h and 12 h after multiple dosing. The prolonged CCR5 occupancy *in vivo* suggests a long half-life for AK602 binding to the receptor [57]. A Phase II clinical trial of AK602 in HIV-1-infected subjects is now underway.

Challenges and unknowns of CCR5 inhibitors

Substantial progress has been made in the development of CCR5 inhibitors for treatment of subjects harboring both wild-type and drug-resistant HIV-1. Indeed, more than a handful of CCR5 inhibitors are currently in various stages of clinical and preclinical development. We are at another new forefront in antiviral research that could lead to new modality of viral intervention. It is true that clinical proof-of-principle has now been obtained for the use of entry inhibitors as therapy for AIDS. However, we can only be cautiously optimistic for the future of CCR5 inhibitors: there are still various challenges and unknowns associated with these inhibitors. They include the elucidation and understanding of the mechanism of their anti-HIV-1 activity, viral tropism, drug resistance issues, and possible long-term adverse effects.

Mechanisms of anti-HIV-1 activity of CCR5 inhibitors

Development of a reliable CCR5 structural model and the delineation of its interaction with CCR5 inhibitors should provide reasonable insights into the structural determinants responsible for ligand–receptor affinity. Such modeling efforts would also help to molecularly modify and optimize existing CCR5 inhibitors, which could lead to the identification of more potent drug candidates [53]. Dragic *et al.* [58] reported that the binding site for TAK-779 on CCR5 is located near the extracellular surface of the receptor, within a cavity formed between transmembrane helices. Tsamis *et al.* [59] have also examined the CCR5 binding profile of SCH-C and shown that SCH-C binds within a putative ligand-binding cavity formed by certain transmembrane helices, suggesting that the binding of small molecules to the transmembrane domain of CCR5 might disrupt the conformation of its extracellular domain, thereby inhibiting ligand binding to CCR5.

We have characterized the CCR5 binding profile of AK602 and its interactions with HIV-1-gp120, CC-chemokines and CCR5. AK602, despite its potent anti-HIV activity, preserves both RANTES binding to CCR5 and RANTES functions, including CC-chemokine-induced chemotaxis and CCR5 internalization [56]. Detailed studies with a panel of mutated CCR5 species have shown that AK602 directly interacts with the extracellular loop-2, and that unlike SCH-C and TAK-779, both of which have binding sites reportedly located in the transmembrane domains, the binding sites of AK602 are clustered around the interface of extracellular loop-2 (Maeda *et al.*, unpublished).

Viral resistance to CCR5 inhibitors

When Trkola *et al.* [60] passaged a R5 primary HIV-1 isolate in culture with a SCH-C-related compound, AD101, an escape mutant emerged with a >20 000-fold resistance to AD101 by 19 passages. Their data suggested that selection pressure appeared to act on an Env subunit, but no acquisition of CXCR4 use occurred. It is as yet unclear whether HIV-1 can develop such a significant resistance against other CCR5 inhibitors, but their data suggest that HIV-1 acquires the ability to use CCR5 despite the inhibitor, first by requiring lower levels of CCR5 for cellular entry and then by using the drug-bound form of the receptor.

Viral tropism changes in the presence of CCR5 inhibitors

One theoretical concern for the long-term protection against R5-HIV-1 infection is the possible change of viral tropism that enables the virus to use the CXCR4 receptor, which occurs naturally by evolution of the virus during the disease process. In the aforementioned studies by

Trkola *et al.* [60], AD101 failed to generate X4 virus. One challenging issue is to determine at which stages of HIV-1 infection CCR5 inhibitors can clinically be used most effectively. At an early stage of infection when R5-HIV-1 phenotype is predominant, it might be desirable to prevent CCR5 usage as completely as possible. At a later stage when viruses have developed the ability to use CXCR4 for cellular entry, blocking CCR5 may inhibit the predominant R5-HIV-1 population, but might also provide a selective advantage to HIV-1 that has evolved to use CXCR4. The possible tropism changes with the use of CCR5 inhibitors have been controversial. It is hoped that new data from clinical trials with CCR5 inhibitors will enable us to use these agents most effectively.

Combination use of CCR5 inhibitors

The orderly and cooperative nature of the HIV-1 entry process suggests the possibility for synergism among inhibitors targeting different steps in the viral entry. As with other classes of antiretroviral drugs, entry inhibitors are unlikely to be used as a single agent because of insufficient potency and the risk of emergence of resistant variants [7]. Moreover, there are concerns about selecting viruses with CCR5 inhibitors because of the infection with R5 and X4 viruses [61]. Tremblay *et al.* [62,63] studied anti-HIV-1 interactions of SCH-C with other antiretroviral agents *in vitro*. Synergism was seen with nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors and protease inhibitors at all inhibitory concentrations evaluated. Synergistic interactions have also been seen with combinations of SCH-C and T-20 [62], and with TAK-220 plus various reverse transcriptase, protease and fusion inhibitors *in vitro* [63]. We also determined whether AK602 provided synergisms with other HIV-1 inhibitors. It was noted that the most potent synergism was seen when AK602 was combined with AMD-3100 against the mixture of R5 and X4 HIV-1 (Nakata *et al.*, unpublished). No antagonistic anti-HIV effects or synergistic toxicity was seen in any combinations.

These data taken together suggest that CCR5 inhibitors may be combined with existing anti-HIV-1 drugs, although it remains vital that combinations of CCR5 inhibitors with other anti-HIV agents be validated by controlled clinical trials. This point is well supported by the limited success recently observed in clinical trials with once-daily dosing of triple-nucleoside combinations [64].

Adverse effects of CCR5 inhibitors

In the clinical trails of SCH-C, unexpected cardiac adverse effects, in particular QTc elongation, emerged and there has been a concern that such cardiac effects could be inherent to other CCR5 inhibitors. However, SCH-D, UK-427,857 and AK602 do not produce such effects in humans, and the observed cardiac adverse effects in relation to SCH-C are thought to have been be associated with SCH-C administration.

It was initially thought that individuals carrying a gene encoding a mutant form of CCR5 called Δ32 did not have significant health problems. However, it has recently been reported that individuals carrying homozygous CCR5-Δ32 have longer survival of renal transplants than those with other genotypes, suggesting that such individuals might have compromised graft-rejection immunity [65]. Moreover, Woitas *et al.* [66] have reported that individuals with homozygous CCR5-Δ32 have significantly higher viremia levels of hepatitis C virus than their counterparts with wild-type CCR5, suggesting that the homozygous CCR5-Δ32 mutation may be an adverse host factor in HCV infection. Thus, the sustained, long-term suppression of the effect of CC-chemokines/CCR5 interactions, particularly in those who carry wild-type CCR5 and might not have a possible compensatory mechanism(s) for the absence of CCR5, might produce adverse effects, and caution should be used in the development of chemokine receptor antagonists as potential therapeutics for HIV-1 infection. It should be noted, however, that only clinical data for the compounds herein described will determine their safety.

Conclusions

Since CCR5 was first identified as an HIV co-receptor in 1996, the cryptic and complicated process of HIV-1 entry has continued to unfold and, indeed, more than a few steps (i.e. attachment, co-receptor interactions and fusion) have now been targeted for therapeutic intervention. Several potent CCR5 inhibitors have undergone preclinical and clinical development. Clinical proof-of-principle has already been established for the use of entry inhibitors, and combinations of entry inhibitors with other existing anti-HIV-1 agents could emerge as a new paradigm for therapy of HIV-1 infection. Successful drug development in this area should further validate the small-molecule approach for drug discovery efforts against diseases associated with protein–protein interactions.

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Amino Acid Insertions near Gag Cleavage Sites Restore the Otherwise Compromised Replication of Human Immunodeficiency Virus Type 1 Variants Resistant to Protease Inhibitors

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A variety of amino acid substitutions in the protease and Gag proteins have been reported to contribute to the development of human immunodeficiency virus type 1 (HIV-1) resistance to protease inhibitors. In the present study, full-length molecular infectious HIV-1 clones were generated by using HIV-1 variants isolated from heavily drug-experienced and therapy-failed AIDS patients. Of six full-length infectious clones generated, four were found to have unique insertions (TGNS, SQVN, AQQA, SRPE, APP, and/or PTAPPA) near the p17/p24 and p1/p6 Gag cleavage sites, in addition to the known resistance-related multiple amino acid substitutions within the protease. The addition of such Gag inserts mostly compromised the replication of wild-type HIV-1, whereas the primary multidrug-resistant HIV infectious clones containing inserts replicated significantly better than those modified to lack the inserts. Western blot analyses revealed that the processing of Gag proteins by wild-type protease was impaired by the presence of the inserts, whereas that by mutant protease was substantially improved. The present study represents the first report clearly demonstrating that the inserts seen in the proximity of the Gag cleavage sites in highly multi-PI resistant HIV-1 variants restore the otherwise compromised enzymatic activity of mutant protease, enabling the multi-PI-resistant HIV-1 variants to remain replication competent.

Currently available combination chemotherapy with reverse transcriptase inhibitors (RTIs) and protease inhibitors (PIs) for human immunodeficiency virus type 1 (HIV-1) infection and AIDS have been shown to suppress the replication of HIV-1 and extend the life expectancy of HIV-1-infected individuals (8, 41). In the course of treatment, however, drug-resistant HIV-1 variants often emerge, which has been a major factor contributing to treatment failure (8, 16, 26, 27).

HIV-1 also develops high levels of resistance against multiple antiviral drugs by accumulating a variety of amino acid substitutions near (and beyond) the active sites of target viral enzymes (5, 20, 35–37), whereas such multiple mutations can often compromise the enzymatic functions of the viral protease and reverse transcriptase (RT) (7, 10, 17, 24, 34, 39). In the case of HIV-1 resistance to an RTI, amino acid changes in the polymerase are virtually fully responsible for the viral acquisition of resistance to RTIs. Indeed, the introduction of such amino acid changes into the polymerase can generally convert a wild-type HIV-1 to a nucleoside reverse transcriptase inhibitor (NRTI)-resistant HIV-1 variant (21). However, in the case of HIV-1 resistance to PIs, the mere introduction of amino acid substitutions seen within the viral protease of PI-resistant variants to wild-type HIV-1 in many cases results in impaired replication competence of the virus (4, 7, 21, 33). Indeed, when HIV-1 develops resistance to PIs, the virus is known to add further amino acid substitutions often located outside the protease that do not confer resistance on HIV-1 per se but improve the otherwise compromised catalytic functions of protease (3, 18). For example, several amino acid substitutions have been seen in the cleavage sites of the Gag proteins in HIV-1 resistant to PIs (6, 9, 25, 43). These substitutions have been shown to compensate for the reduced catalytic activity of mutant proteases. Moreover, certain amino acid substitutions in noncleavage sites have been shown to contribute to the development of high levels of viral resistance to multiple PIs (15).

The addition of certain amino acids can also contribute to the development of viral resistance. Winters et al. identified a 6-bp insert between codons 69 and 70 of the RT gene in HIV-1 isolated from NRTI-treated patients and conducted elegant site-directed mutagenesis studies showing that the insert alone confers on HIV-1 reduced susceptibility to multiple NRTIs (40). Peters et al. have also recently identified the duplication of a proline-rich motif, Ala-Ala-Pro (APP), in the PTAP motif of the Gag protein in HIV-1 variants isolated from patients with AIDS receiving NRTIs, including didanosine (ddI), stavudine (d4T), zidovudine (AZT), and lamivudine (3TC), and have shown that this addition could improve assembly and packaging at membrane locations, resulting in increased infectivity and viral resistance to NRTIs (28).

In the present study, we identified unique insertions (TGNS, SQVN, AQQA, SRPE, APP, and/or PTAPPA) near the p17/p24 and p1/p6 Gag cleavage sites, in addition to the known resistance-related multiple amino acid substitutions within the pro-

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TABLE 1. Patient profiles

Patient	No. of CD4 cells/mm^3	No. of HIV RNA copies/ml	Duration (mo) of antiviral therapy	Anti-HIV-1 agents received[a]
B	361	247,000	64	AZT, ddI, ddC, d4T, 3TC, ABC, IDV, RTV, SQV, APV, DLV
C	3	554,000	46	AZT, ddI, ddC, d4T, 3TC, ABC, IDV, RTV, SQV, APV
G	568	60,000	81	AZT, ddI, ddC, d4T, 3TC, ABC, IDV, SQV, APV, DLV
EV	185	68,000	24	AZT, 3TC, ABC, RTV, SQV, APV, EFV
ES	8	42,000	34	AZT, ddI, ddC, d4T, 3TC, ABC, IDV, RTV, APV, EFV, HU
EY	140	10,000	36	AZT, ddI, d4T, 3TC, ABC, IDV, RTV, APV, EFV, HU

[a] ddC, zalcitabine; EFV, efavirenz; HU, hydroxyurea. Levels of HIV RNA in serum were determined by using a branched DNA assay.

tease in full-length molecular infectious multidrug-resistant HIV-1 (HIV_{MDR}) clones generated from HIV-1 variants isolated from patients with AIDS who had received 7 to 11 anti-HIV-1 drugs over 24 to 81 months and had lost response to any existing antiviral drugs (except for tenofovir and enfuvirtide at the time). Virologic and biochemical studies demonstrated that whereas these inserts mostly compromise the enzymatic functions of the wild-type protease, they restore the Gag processing by the mutant protease and enable PI-resistant HIV variants to remain replication competent.

MATERIALS AND METHODS

Patients. Patients with AIDS were enrolled into a randomized clinical study of amprenavir (APV) and abacavir (ABC) (11, 42). The clinical characteristics of the patients are presented in Table 1. Other medications, including antiretroviral therapies, were permitted with the exception of other PIs (indinavir [IDV], ritonavir [RTV], and saquinavir [SQV]), non-NRTIs (nevirapine [NPV] and delavirdine), and agents available on expanded access, which were prohibited for the first 12 weeks of therapy with APV. These patients were put on the study under an investigational new drug application by Critical Care Medicine Department, Clinical Center, National Institutes of Health. For the present study, 20 patients were randomly chosen from the enrollees who had failed the APV-plus-ABC therapy.

Cells and viruses. MT-4 and PM1 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (HyClone, Logan, Utah), 50 U of penicillin/ml, and 50 μg of streptomycin/ml. Peripheral blood mononuclear cells (PBMC) obtained from healthy donors were stimulated by phytohemagglutinin (PHA) in RPMI 1640-based medium containing interleukin-2 (5 ng/ml; R&D Systems, Minneapolis, Minn.) for 2 days (PHA-PBMC) before HIV-1 exposure. Clinical HIV-1 strains were isolated as previously described (37) by culturing PBMC obtained from patients with AIDS. Thus obtained HIV-1 isolates were passaged once or twice in PHA-PBMC.

Generation of full-length molecular HIV_{MDR} clones. To generate full-length molecular infectious HIV_{MDR} clones from multidrug-resistant clinical HIV-1 isolates, the PCR-mediated recombination (PMR) method was used (12). First, we amplified an upstream proviral DNA fragment (5′ DNA fragment, 5,337 bp) and a downstream proviral DNA fragment (3′ DNA fragment, 5,042 bp), both of which shared an overlapping sequence (730 bp), by using the primer pair 5LTR (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CT TGG AAG GGC TAA TTT GGT CCC AAA AAA GAC-3′) plus pol-2 (5′-GTC TAC TTG TGT GCT ATA TCT CTT TTT CCT CC-3′) and the primer pair pol-1 (5′-GCA TTC CCT ACA ATC CCC AAA G-3′) plus 3LTR (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT GCT AGA GAT TTT CCA CAC TGA CTA AAA GG-3′), respectively. The DNA recombination sequence, *att*B, was tagged at the 5′ ends of 5LTR and 3LTR for subsequent cloning. Thus obtained 5′ and 3′ DNA fragments were joined by using PMR. The PMR reaction was performed using the standard condition for ExTaq polymerase (Takara, Kyoto, Japan) with 40 pmol of *att*B1 (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CT-3′) and *att*B2 (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT-3′) adapter primers and the 5′ and 3′ DNA fragments (100 ng each) in a 50-μl reaction solution. Thermal cycling was carried out as follows: 95°C for 2 min, followed by 15 cycles of 95°C for 10 s, 55°C for 30 s, and 68°C for 8 min, and followed finally by 68°C for 10 min. Thus amplified *att*B-flanked full-length HIV-1 was cloned into pcDNA3.1 according to the manufacturer's instructions (Gateway Cloning System; Invitrogen, Carlsbad, Calif.).

Generation of molecular HIV_{NL4-3} clones containing a Gag insert. To generate molecular infectious HIV-1 clones carrying the wild-type protease plus an insert, site-directed mutagenesis by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) was performed as described previously (15). In brief, each desired insert (9 to 18 nucleotides) was introduced into the BssHII-SmaI fragment of the wild-type HIV_{NL4-3} (nucleotides 712 to 2591), which encodes Gag and protease, by site-directed mutagenesis. The fragment was subsequently introduced into $pHIV_{NLSma}$, which had been created to have a

TABLE 2. Sensitivity of HIV-1 isolated from heavily drug-experienced individuals against NRTIs and PIs

Virus	IC_{50}[a] (μM) (fold change)								
	AZT	ddI	3TC	ABC	IDV	RTV	SQV	NFV	APV
$HIV\text{-}1_{wt}$[b]	0.0060 (1)	0.20 (1)	0.22 (1)	0.13 (1)	0.017 (1)	0.055 (1)	0.0090 (1)	0.030 (1)	0.015 (1)
$HIV\text{-}1_{B}$	0.42 (70)	2.8 (14)	>100 (>455)	5.0 (22)	>1 (>77)	>1 (18)	0.25 (28)	>1 (>33)	0.25 (17)
$HIV\text{-}1_{C}$	0.42 (70)	1.6 (8)	>100 (>455)	4.4 (34)	0.39 (23)	>1 (18)	0.039 (4)	0.40 (13)	0.31 (21)
$HIV\text{-}1_{G}$	0.54 (90)	1.2 (6)	>100 (>455)	2.2 (17)	0.17 (10)	>1 (18)	0.033 (4)	0.095 (3)	0.22 (15)
$HIV\text{-}1_{ES}$	0.75 (125)	4.3 (>22)	0.46 (2)	4.3 (33)	>1 (>77)	>1 (18)	0.040 (4)	0.24 (8)	0.73 (49)
$HIV\text{-}1_{EV}$	0.53 (88)	4.9 (>25)	>100 (>455)	5.0 (22)	0.86 (51)	>1 (18)	0.50 (56)	0.60 (20)	0.55 (37)
$HIV\text{-}1_{EY}$	0.72 (120)	1.5 (>8)	0.97 (4)	1.6 (12)	0.33 (19)	0.62 (11)	0.18 (20)	0.18 (6)	0.61 (40)

[a] The IC_{50} values were determined by using PHA-PBMC exposed to HIV-1s (50 $TCID_{50}$ dose/10^5 PBMC) and the inhibition of p24 Gag protein production as an endpoint. All drug sensitivities were determined in triplicate. The values shown are representative of two or three separate experiments. The numbers in parentheses represent the fold changes of the IC_{50} values against each isolate compared to the IC_{50} values against $HIV\text{-}1_{wt}$. See Table 1, footnote *a*, and Materials and Methods for other abbreviations. NFV, nelfinavir.

[b] $HIV\text{-}1_{wt}$ was a pretherapy clinical HIV-1 strain, $HIV\text{-}1_{RSHIPRO}$ (36).

TABLE 3. Amino acid substitutions in protease and RT

Patient	Amino acid substitutions[a]	
	PR	RT
B	L10I, K14R, L33I, M36I, M46I, I54V, K55R, I62V, L63P, A71V, G73S, V82A, L90M, I93L	V35I, M41L, K43E, S48T, $D67_{DELETED}$, T69G, K70R, L74I, A98G, L100S, Q102K, K103N, M135I, V179I, M184V, G196E, T215F, K219Q, L227H
G	L10I, V11I, T12E, I15V, L19I, R40K, M46L, L63P, A71T, V82A, L90M	D67G, S68G, T69D, K70R, V118I, E122K, I135T, M184V, Q197K, T215F, D218E, K219Q
EV	L10V, T12E, G16A, L19I, K20R, L33F, E35D, M36I, M46I, I49V, F53L, I54V, K55R, G57K, D60E, I62V, L63P, A71V, V82A, L90M	M41L, K43E, E44A, T58A, D67E, S68G, L74V, V75M, L100I, Q102N, K104T, V118I, I135T, I142V, T165I, Q174N, Y181I, M184V, L210W, R211K, T215Y, H221Y, V245E
ES	L10I, M46L, K55R, I62V, L63P, I72L, G73C, V77I, I85V, L90M	M41L, K43E, E44D, D67N, T69D, L74V, K101E, V118I, E122K, D123E, Y181C, G190S, G196E, E203V, Q207E, L210W, R211K, L214F, T215Y, V245K

[a] Amino acids different from those of the consensus HIV_{NL4-3}.

SmaI site by changing two nucleotides (2590 and 2593) of $pHIV_{NL4-3}$. The generated molecular HIV-1 clones containing Ala-Gln-Gln-Ala (AQQA), Pro-Thr-Ala-Pro-Pro-Ala (PTAPPA), Thr-Gly-Asn-Ser (TGNS), Ala-Pro-Pro (APP), Ser-Arg-Pro-Glu (SRPE), and Ser-Gln-Val-Asn (SQVN) were designated HIV_{AQQA}, HIV_{PTAPPA}, HIV_{TGNS}, HIV_{APP}, HIV_{SRPE}, and HIV_{SQVN}, respectively.

Generation of molecular HIV_{MDR} clones lacking a Gag insert. Primers that lack the insert sequence were designed to generate molecular HIV_{MDR} clones lacking a Gag insert. Primers del17-1 (5′-GCA GCT GAC ACA GGA AAC AAC AGC CAG GTC AGC CAA AAT TAC-3′), del17-2 (5′-GTA ATT TTG GCT GAC CTG GCT GTT GTT TCC TGT GTC AGC TGC-3′), del17-3 (5′-GAG CAA AAC AAA AGT AAG AAA AAG GCA CAG CAA GCA GCA GCT GAC-3′), and del17-4 (5′-GTC AGC TGC TGC TTG CTG TGC CTT TTT CTT ACT TTT GTT TTG CTC-3′) were used to delete the inserts near the p17/p24 cleavage site, whereas del6-1 (5′-CTT CAG AGC AGA CCA GAG CCA ACA GCC CCA CCA GAA GAG AGC-3′), del6-2 (5′-GCT CTC TTC TGG TGG GGC TGT TGG CTC TGG TCT GCT CTG AAG-3′), del6-3 (5′-CAG AGC AGA CTA GAG CCA ACA GCC CCA CCA GCA GAG AGC TTC AGC-3′), and del6-4 (5′-CCT GAA GCT CTC TGC TGG TGG GGC TGT TGG CTC TAG TCT GCT CTG-3′) were used to delete the inserts near the p1/p6 cleavage site. Thus obtained 5′ and 3′ DNA fragments were joined by using PMR as described above.

Determination of the nucleotide sequences of the plasmids containing full-size molecular HIV-1 clones confirmed that each molecular clone generated had the desired mutations but no unintended mutations. Each recombinant plasmid was transfected into COS-7 cells with Lipofectamine 2000 reagent (Invitrogen), and the infectious virions thus obtained were harvested 48 h after transfection and stored at −80°C until use. In order to determine virus titers, PHA-PBMC (15,000 cells/well) in 96-well flat-bottom microtiter culture plates (Costar, Cambridge, Mass.) were exposed to each virus preparation that had been serially diluted. Culture supernatants were examined for the amounts of p24 Gag on day 7 of culture by using a commercially available radioimmunoassay kit (Dupont/NEN Research Products, Boston, Mass.). When the amounts of p24 Gag were <0.6 ng/ml, cultures were defined to be negative for the virus, and the 50% tissue culture infective dose ($TCID_{50}$) was determined by the method of Reed and Muench (32). All titration assays were performed in six replicates.

Generation of HIV_{NL4-3} carrying mutated protease with or without Gag inserts. To conduct experiments to examine the possible effects of the inserts identified in Gag on the proteolytic activity of mutated protease and the viral fitness, two infectious clones (HIV_B and HIV_{ES}) that have two inserts near the p17/p24 and p1/p6 cleavage sites were chosen. First, an EagI site was introduced into $pHIV_{NLSma}$ by changing two nucleotides (2215 and 2216) as described above, generating $pHIV_{NLEag/Sma}$. Using a pair of primers containing the EagI and SmaI sites, PCR products were generated with HIV_B and HIV_{ES} as templates, followed by digestion by both EagI and SmaI, thus generating the EagI-SmaI fragments for both HIV_B and HIV_{ES}. Each of the HIV_B and HIV_{ES} EagI-SmaI fragments was introduced into $pHIV_{NLEag/Sma}$, generating $HIV_{NL/B\text{-}Pr}$ and $HIV_{NL/ES\text{-}Pr}$, respectively. Subsequently, each pair of insertions (TGNS plus APP or AQQA plus PTAPPA) was introduced into $HIV_{NL/B\text{-}Pr}$ and $HIV_{NL/ES\text{-}Pr}$, thus generating $HIV_{NL/B\text{-}Pr/TGNS\text{-}APP}$ and $HIV_{NL/ES\text{-}Pr/AQQA\text{-}PTAPPA}$.

Replication kinetic assay. MT-4 cells (5×10^5) or PHA-PBMC (1.5×10^6) were exposed to each infectious virus preparation (30 $TCID_{50}$ in 1 ml of culture medium) for 2 h, washed twice with phosphate-buffered saline (PBS), and cultured in 1.5 ml of complete medium. Culture supernatants were harvested every 3 days, and the amounts p24 Gag were determined

CHRA. The competitive HIV replication assay (CHRA) was performed as previously reported (15, 22) with minor modifications. In brief, two titrated infectious clones to be examined in the assay were combined and added to freshly prepared MT-4 cells (3×10^5) or PM1 cells (3×10^5). To ensure that the two infectious clones to be compared were of an approximately equal infectivity, a fixed amount (30 $TCID_{50}$) of one infectious clone was combined with three different amounts (15, 30, and 60 $TCID_{50}$) of the other infectious clone. On day 1, one-third of infected cells were harvested and washed twice with PBS, and cellular DNA was purified. The purified DNA was subjected to nested PCR and sequencing as described below. The HIV-1 coculture that best approximated a 50:50 mixture on day 1 was further propagated; the remaining cultures were discarded. Every 4 to 10 days, the cell supernatant of the virus coculture (1 ml) was transferred to fresh uninfected cells (1.5×10^5 MT-4 cells or 1.5×10^5 PM1 cells in 1 ml), 8 ml of fresh culture medium was added on the following day, and a half of the medium was replenished with an equal volume of fresh culture medium every 3 to 4 days. The cells harvested at the conclusion of each passage were subjected to DNA extraction and then to direct DNA sequencing of the proviral DNA, and a viral population change was determined as previously reported (15, 22).

Western blot analysis. To analyze whether HIV-1 polyproteins in molecular HIV-1 clones were cleaved by the viral protease, Western blot analysis with the lysates of HIV-1-producing cells and cell-free virions was conducted. Briefly, at 48 or 72 h after transfection with plasmid preparations, COS-7 cells were washed with PBS and lysed in M-per solution (Pierce, Rockford, Ill.), and the cell lysates were subjected to Western blotting. Culture supernatants containing virions were harvested 48 h after transfection, filtered through 0.22-μm-pore-size Millex-GV membranes (Millipore, Bedford, Mass.), and centrifuged at 20,000 × *g* for 4 h to pellet virions, which were then lysed in M-per solution.

In the assay, samples were normalized based on the amounts of p24 Gag and subjected to electrophoresis on sodium dodecyl sulfate–15% polyacrylamide gel (Bio-Rad, Hercules, Calif.), followed by electroblotting onto nitrocellulose membranes. The HIV-1 Gag proteins were visualized with SuperSignal WestPico (Pierce) by using anti-p24 Gag antiserum or anti-p6 monoclonal antibody (Advanced Biotechnologies, Inc., Columbia, Md.). The anti-p6 monoclonal antibody in general does not recognize the p6 protein of HIV_{NL4-3} (9). Thus molecular HIV-1 clones with or without each insert containing the BssHII-SmaI fragment that encodes the entire *gag-pol* region of HIV_{HXB}, the p6 protein of which can be recognized by the antibody, were generated for Western blot analysis of the wild-type HIV_{NL4-3}-based clones.

The percent signal density of Gag products was analyzed on a Macintosh computer by using the NIH Image Program (developed at the U.S. National Institutes of Health <http://rsb.info.nih.gov/nih-image/>) and the percent density of p24 Gag (% $density_{p24}$) was determined by use of the following formula: % $density_{p24}$ = 100 × (the density of the p24 Gag signal)/(the cumulated density of all Gag signals).

Determination of nucleotide sequences. Determination of nucleotide sequences of HIV-1 was performed as described previously (15). In brief, high-molecular-weight DNA was extracted from HIV-1-infected cells by using a QIAamp DNA minikit (Qiagen, Valencia, Calif.), and the Gag and protease-

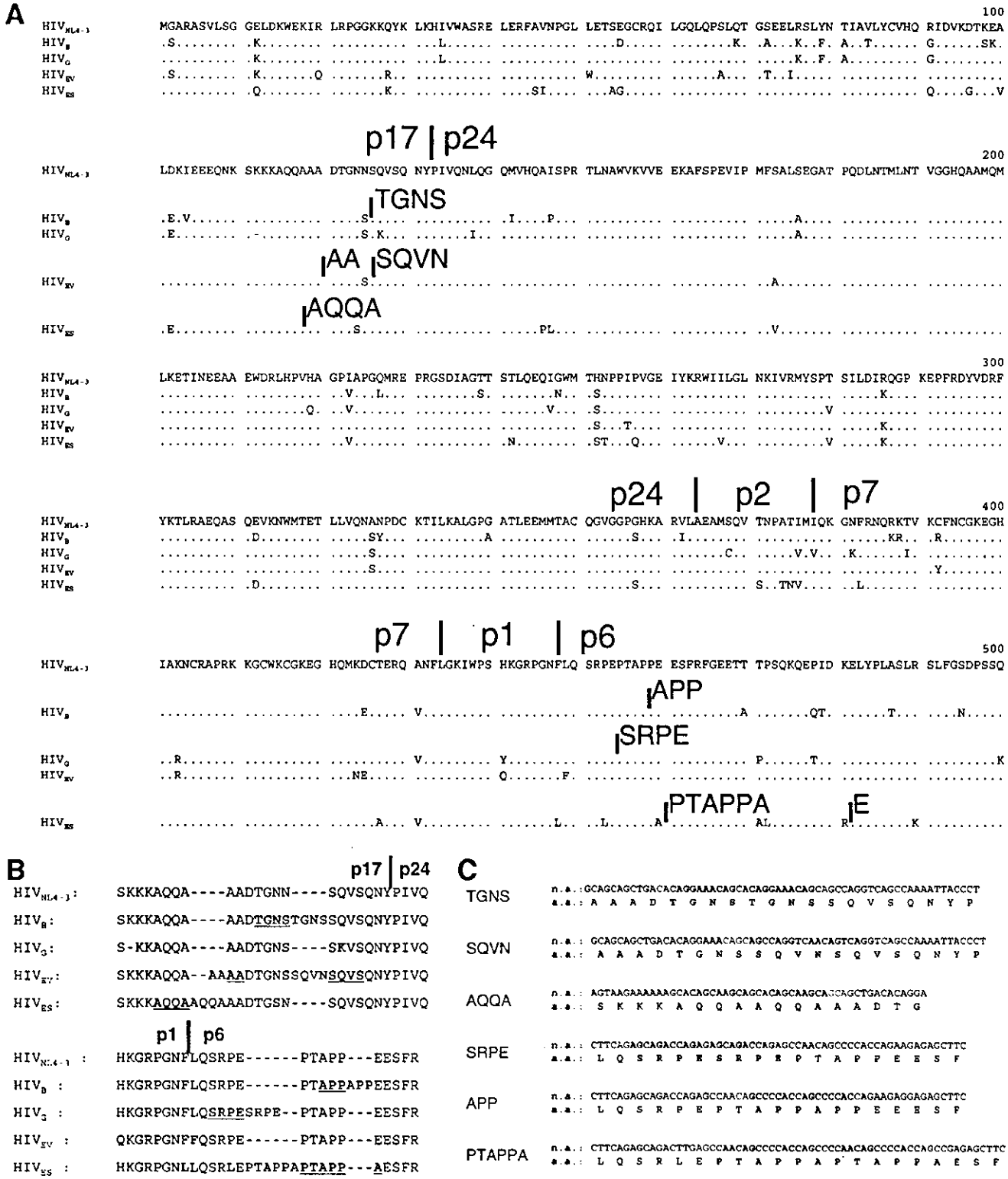


FIG. 1. Amino acid and nucleic acid sequences of molecularly cloned HIV_{MDR}. (A) Amino acid (AA) substitutions in the Gag protein and amino acid insertions clustered near the p17/p24 and p1/p6 cleavage sites in molecular infectious HIV_{MDR} clones. (B) Deduced amino acids sequences near the Gag p17/p24 and p1/p6 cleavage sites. The consensus sequence of HIV_{NL4-3} is shown in brown. Amino acid insertions are shown in red, whereas amino acids different from those of the consensus HIV_{NL4-3} are shown in blue. The amino acid stretches corresponding to the inserts are shown aligned and underlined. (C) Nucleic acid sequences for the insertions. The stretches associated with the duplication are shown in boldface. Note the presence of three sets of two to four nucleotides located in the 5′ end, center, and 3′ end of the associated stretches (shown in red).

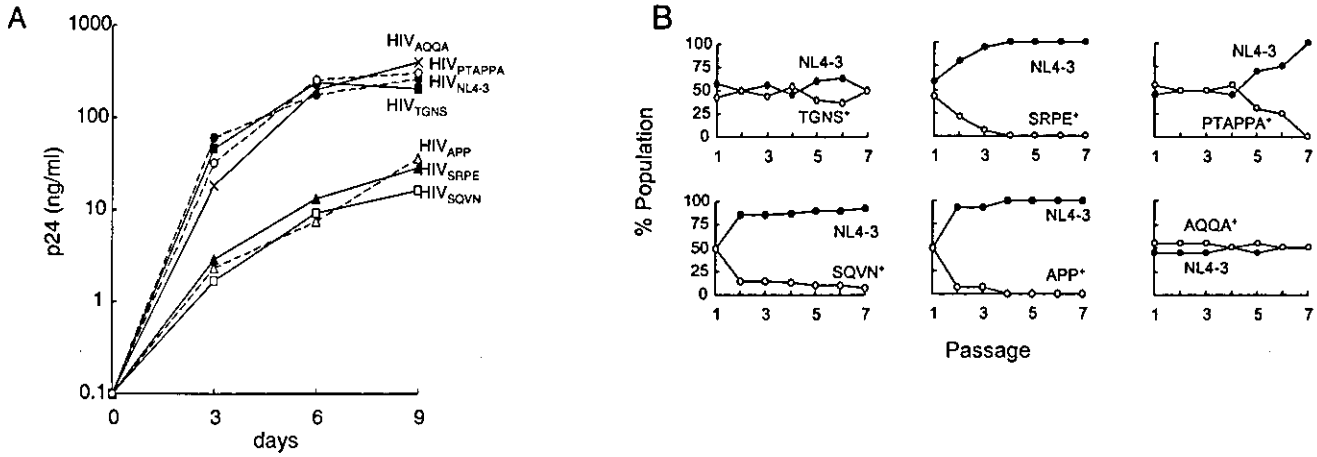


FIG. 2. Replication profiles of the wild-type HIV-1 with or without an insert. (A) Replication kinetic assay with MT-4 cells was conducted with HIV_{NL4-3} and HIV_{NL4-3} clones with various inserts. The production of p24 Gag protein by MT-4 cells into the culture supernatants was monitored over 9 days. (B) Competitive HIV replication assay. Six pairs of HIV_{NL4-3} and HIV_{NL4-3} clones with an insert were propagated in MT-4 cells, and the percent proportion of each virus was determined over seven passages. When the cells harvested at the conclusion of the last passage were subjected to DNA extraction and direct DNA sequencing, no deletions or additional amino acid changes in the p17/p24 and p1/p6 Gag cleavage sites were identified.

encoding regions of HIV-1 were amplified by using nested-PCR with Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, Calif.). The primers used were Seq1 (5′-GTA TGG GCA AGC AGG GAG CTA GAA CGA TTC-3′) and Seq2 (5′-GGG TAT TAC TTC TGG GCT GAA AGC CTT CTC) for the first PCR of the p17/p24 Gag cleavage site, Seq3 (5′-TGT AAA ACG GCC AGT TGT AGA CAA ATA CTG GGA CAG CTA CAA CCA-3′) and Seq4 (5′-CAG GAA ACA GCT ATG ACC CTT TTA CCC ATG CAT TTA AAG TTC TAG GTG-3′) for the second PCR of the p17/p24 Gag cleavage site, Seq5 (5′-AGG GCT GTT GGA AAT GTG GAA AGG AAG G-3′) and Seq6 (5′-TCT TCT GTC AAT GGC CAT TGT TTA AC) for the first PCR of the p1/p6 Gag cleavage site and protease-encoding region, and Seq7 (5′-TGT AAA ACG ACG GCC AGT TAG GGA AGA TCT GGC CTT CC-3′) and Seq8 (5′-CAG GAA ACA GCT ATG ACC TAC TGG TAC AGT CTC AAT AGG-3′) for the second PCR of the p1/p6 Gag cleavage site and PR encoding region. The products of the

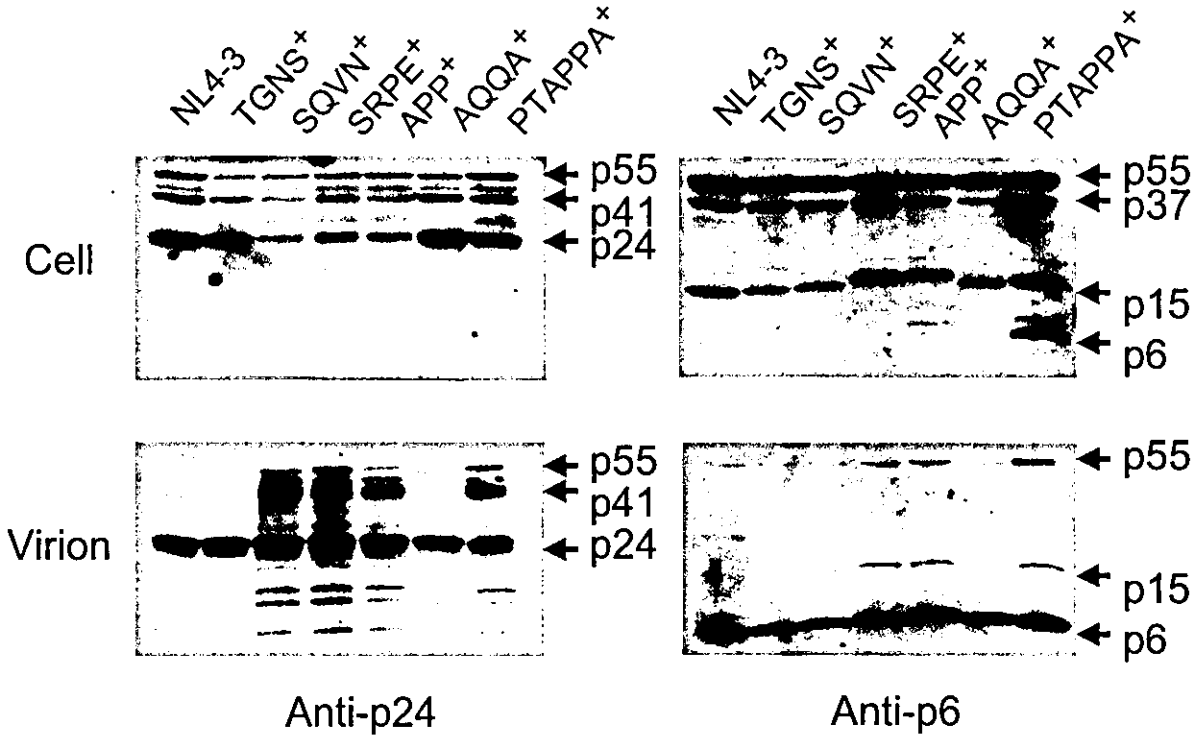


FIG. 3. Gag processing in HIV_{NL4-3} with or without an insert. The lysates of COS-7 cells producing HIV_{NL4-3} or HIV clones containing an insert and the virion lysates were examined for the Gag processing. The samples were prepared 48 h after transfection and subjected to Western blotting with anti-p24 antiserum and anti-p6 monoclonal antibody. The positions corresponding to the sizes of the fully cleaved mature proteins (p24 and p6) and the immature proteins (p55, p41, p37, and p15) are indicated by arrows.

TABLE 4. p24 signal density of each virus in a Western blot

Strain	Protease	Gag	Gag insertion at site:		Day posttransfection	% Density$_{p24}$[a]	Corresponding figure
			p17/p24	p1/p6			
NL4-3	WT[b]	WT			2	43.5	Fig. 3
	WT	WT	TGNS		2	46.3	Fig. 3
	WT	WT	SQVS		2	31.7	Fig. 3
	WT	WT		SRPE	2	29.6	Fig. 3
	WT	WT		APP	2	23.9	Fig. 3
	WT	WT	AQQA		2	49.9	Fig. 3
	WT	WT		PTAPPA	2	37.1	Fig. 3
	WT	WT			3	73.5	Fig. 5A
B	B	B	TGNS	APP	3	10.8	Fig. 5A
	B	B		APP	3	2.4	Fig. 5A
	B	B	TGNS		3	4.7	Fig. 5A
G	G	G		SRPE	3	45.4	Fig. 5A
	G	G			3	29.1	Fig. 5A
EV	EV	EV	SQVN		3	14.3	Fig. 5A
	EV	EV			3	7.3	Fig. 5A
ES	ES	ES	AQQA	PTAPPA	3	53.7	Fig. 5A
	ES	ES		PTAPPA	3	26.5	Fig. 5A
	ES	ES	AQQA		3	14.6	Fig. 5A
NL4-3	B	WT			2	10.5	Fig. 6
	B	WT	TGNS	APP	2	24.2	Fig. 6
	ES	WT			2	19.9	Fig. 6
	ES	WT	AQQA	PTAPPA	2	33.2	Fig. 6

[a] Calculated as 100 × (p24 signal density/total Gag product signal densities) in a Western blot of cell lysates with anti-p24 antisera. The values for % density are to be compared within the same figure.
[b] WT, wild type.

second PCR were directly sequenced by using M13 forward and reverse dye-labeled primers with an Applied Biosystems model 3100 automated DNA sequencer.

RESULTS

Examination of clinical profiles of patients with AIDS. We first determined the sequence of the protease-encoding region of 20 primary HIV-1 strains isolated from patients with AIDS who were enrolled in salvage therapy clinical trials in the Clinical Center of the National Cancer Institute (11, 42). We chose six primary strains that harbored the highest numbers of drug resistance-associated amino acid substitutions in the RT and protease and generated full-length molecular infectious clones. The clinical profiles of the six patients are given in Table 1. These individuals had received 7 to 11 anti-HIV-1 drugs over 24 to 81 months and no longer responded to any existing antiviral drugs (except for tenofovir and enfuvirtide at the time), showing low $CD4^+$ counts ranging from 3 to 568 cells/mm^3 and moderately to high levels of viremia (1×10^4 to 5.54×10^5 RNA copies/ml). As shown in Table 2, all of the HIV-1 strains isolated from these patients were found to be highly resistant to all anti-HIV-1 drugs used (four NRTIs and five PIs) in the present study.

Inserts identified in the Gag of HIV_{MDR} were duplicates. Determination of nucleotide sequences of these six molecular infectious HIV_{MDR} clones revealed that they contained 12 to 23 amino acid changes in the RT compared to the consensus nucleotide sequence (Table 3). Most of these amino acid substitutions were reportedly associated with resistance to NRTIs and/or non-NRTIs (38). These HIV_{MDR} clones also contained as many as 10 to 20 amino acid substitutions spread throughout the protease's amino acid sequence (Table 3). The nucleotide sequence determination of the entire *gag* region also revealed that each clone had a variety of amino acid substitutions spread throughout the Gag amino acid sequence, as shown in Fig. 1A. In addition to these amino acid substitutions, four of the six molecular clones were found to have amino acid insertions that were apparently clustered near the p17/p24 and p1/p6 cleavage sites (Fig. 1A). Interestingly, five of the six inserts had amino acid and nucleic acid sequences identical to those of the juxtapositioned stretches (Fig. 1B and C), strongly suggesting that the inserts represented duplicates, although the insert $SQVN_{AAC}$ differed from the adjacent stretch $SQVS_{AGC}$. For example, TGNS in HIV_B is a duplicate of the 5′-TGNS stretch, SRPE in HIV_G is a duplicate of the 5′-SRPE stretch, and PTAPPA in HIV_{ES} is a duplicate of the 3′-PTAPPA stretch (Fig. 1B and C). It is noteworthy that for five of six duplicates, three sets of two to four bases (shown in red in Fig. 1C) were recognized in the 5′ end, center, and 3′ end of each of the duplicate-associated stretches (shown in boldface in Fig. 1C), although for the SQVN insert the four bases seen in the center differed from the other two pairs of four bases.

Gag inserts compromise or do not affect the replication of HIV_{NL4-3}. In order to examine whether these unique inserts

affected the replication capability of HIV-1, we introduced each insert to a wild-type HIV-1 strain, HIV_{NL4-3}, and determined the replication profile of resulting infectious clones. As shown in Fig. 2A, all six newly generated molecular clones with inserts were clearly capable of replicating in culture. Three molecular clones—HIV_{AQQA}, HIV_{PTAPPA}, and HIV_{TGNS}—appeared to replicate comparably to the wild-type HIV_{NL4-3}. Three other molecular clones—HIV_{APP}, HIV_{SRPE}, and HIV_{SQVN}—appeared to be less replication competent than HIV_{NL4-3}. To confirm and corroborate these observations, we performed a CHRA in which two different clones were propagated in culture, and the percent population of each clone was determined, as previously reported (15, 22). Three molecular clones (HIV_{APP}, HIV_{SRPE}, and HIV_{SQVN}), which appeared to be less replication competent compared to HIV_{NL4-3}, were readily outgrown by HIV_{NL4-3} in CHRA. HIV_{PTAPPA} grew comparably to HIV_{NL4-3} by 4 weeks but then was outgrown by HIV_{NL4-3}. Two other molecular clones, HIV_{TGNS} and HIV_{AQQA} replicated comparably to HIV_{NL4-3}.

Gag processing of HIV_{NL4-3} carrying an insert. It was possible that the inserts introduced into the wild-type HIV_{NL4-3} impaired the activity of the wild-type protease of HIV_{NL4-3} to process the polyproteins, thereby largely compromising the replication of the molecular HIV_{NL4-3} clones containing an insert. Therefore, we performed a Western blot with anti-p24 antiserum and an anti-p6 monoclonal antibody by using the lysates of COS-7 cells producing insert-containing molecular HIV-1 clones and cell-free virions harvested on day 2 after transfection of COS-7 cells (Fig. 3). A dense p24 signal was detected in both the cell lysates and virion lysates of the wild-type HIV_{NL4-3} as examined with anti-p24 antiserum; however, all molecular clones carrying an insert produced substantially decreased amounts of p24 (Fig. 3) except for HIV_{TGNS} and HIV_{AQQA}. It is noteworthy that the two clones, HIV_{TGNS} and HIV_{AQQA}, which did not show a detectable difference in replication competence compared to HIV_{NL4-3} (Fig. 2A and B), had comparable amounts of p24 when both cell lysates and virions were examined (Fig. 3 and Table 4). HIV_{SQVN}, HIV_{SRPE}, and HIV_{APP}, which showed substantially decreased replication competence compared to HIV_{NL4-3} (Fig. 2A and B), had decreased p24 amounts compared to the wild type in the blotting of cell lysates. When analyzed by using densitometry, the decrease in the amounts of p24 in the case of APP added was by 45.1% (43.5 to 23.9% $density_{p24}$ [Table 4]). In the assay with HIV_{SQVN}, HIV_{SRPE}, and HIV_{APP} virions, significant amounts of noncleaved immature Gag protein precursors were also detected. HIV_{PTAPPA}, which comparably replicated initially but was ultimately overgrown by HIV_{NL4-3} (Fig. 2B), appeared to contain a slightly lower amount of p24 in the blotting of cell lysates, and more substantial amounts of immature Gag proteins were seen in virions.

When the cell lysates were examined with an anti-p6 monoclonal antibody, a significant amount of p6 was detected in HIV_{PTAPPA} and a small amount of p6 seen in HIV_{APP}. In contrast, when virions were examined, substantial amounts of the p6 proteins were seen in all clones, although small amounts of immature proteins were seen in HIV_{SRPE}, HIV_{APP}, and HIV_{PTAPPA}. It is of note that although the PTAP motif within the p6 Gag has recently been shown to recruit the human protein Tsg101 to facilitate HIV-1 budding (14, 30) significant

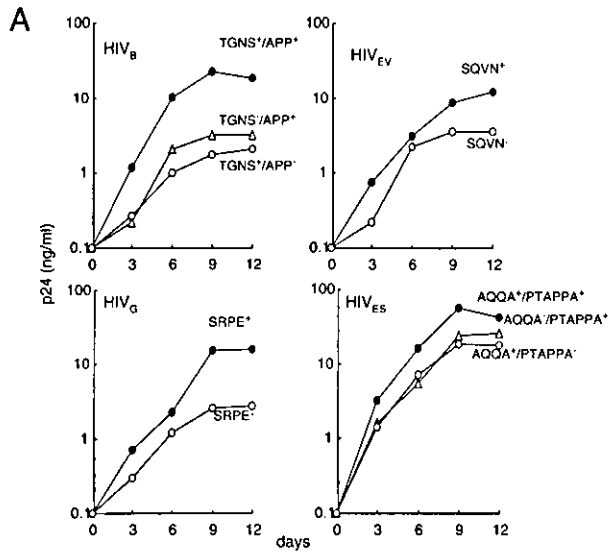


FIG. 4. Replication profiles of primary HIV_{MDR} and insert-lacking infectious clones. (A) Replication profiles of primary molecular HIV_{MDR} clones and insert-lacking infectious clones were determined in PHA-PBMC over 12 days. (B) CHRA. Six pairs of a primary HIV_{MDR} clone plus an insert-lacking HIV_{MDR} were examined (HIV_B, HIV_G, and HIV_{ES} in MT-4 cells and HIV_{EV} in PM1 cells), and the percent proportion of each virus was determined.

amounts of the mature p6 protein were seen in all of the virions, suggesting that none of the SRPE, APP, or PTAPPA inserts located near the PTAP motif blocked viral budding (Fig. 3).

We noted that the p15 (cell lysates) and p6 (virions) species in three clones—HIV_{SRPE}, HIV_{APP}, and HIV_{PTAPPA}—appeared to be of a slightly greater size(s) than those of other clones, presumably reflecting that these three clones contained the inserts within the p6 protein, thus being of a slightly larger size, although it is also possible that the p6 species in the three clones may represent undigested p1+p6 proteins.

Replication of HIV_{MDR} with or without Gag inserts. Next, we examined whether the Gag inserts affected the replication competence of the full-length primary HIV_{MDR} clones. We deleted each insert from molecularly cloned clinical isolates by using the PMR method and propagated each of them in MT-4 cells or PM1 cells. HIV_B, HIV_G, and HIV_{ES} replicated well and caused significant cytopathic effects in MT-4 cells (data not shown). In contrast, HIV_{EV} more efficiently propagated in PM1 cells than in MT-4 cells and did not induce cytopathic effects in MT-4 cells (data not shown). These data suggest that HIV_B, HIV_G, and HIV_{ES} are of the X4 lineage, whereas HIV_{EV} is of the R5 lineage. As shown in Fig. 4A, all four primary HIV_{MDR} clones appeared to propagate more efficiently than any Gag insert-deletant clones (i.e., clones with the Gag insert deleted) in culture over 12 days. To corroborate and extend this observation, we conducted the CHRA assay and compared the fitness of these molecular clones. The TGNS-deletant ($TGNS^-/APP^+$) and APP-deletant ($TGNS^+/APP^-$) clones were readily overgrown by the primary HIV_B clone ($TGNS^+/APP^+$) (Fig. 4B). The SQVN-deletant ($SQVN^-$) and SRPE-deletant ($SRPE^-$) clones were also readily overgrown by the primary HIV_{EV} ($SQVN^+$) and HIV_G ($SRPE^+$) clones,

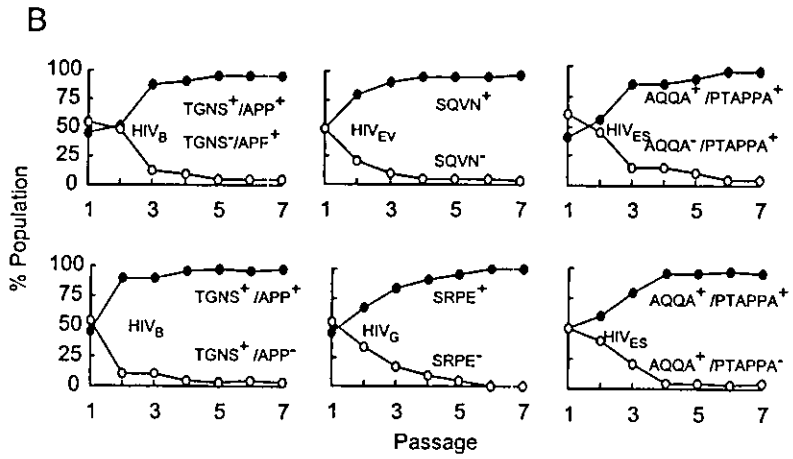


FIG. 4—*Continued.*

respectively. Moreover, the AQQA-deletant and PTAPPA-deletant clones were overgrown by the primary HIV_{ES} ($AQQA^+/PTAPPA^+$). These data strongly suggest that each insert conferred replication advantage on primary HIV_{MDR} clones.

Gag processing of HIV_{MDR} with or without Gag inserts. We sought to determine whether the inserts altered the Gag processing within the cells producing each HIV_{MDR} by using the Western blotting technique with anti-p24 antiserum and anti-p6 monoclonal antibody. As shown in Fig. 5A, a dense p24 signal and lower amounts of immature polyproteins were detected in the wild-type HIV_{NL4-3}. In contrast, all four HIV_{MDR} variants had substantially lower % $density_{p24}$ values than HIV_{NL4-3} (Table 4). We found that, in all HIV_{MDR} clones that were modified to lack an insert, even lower % $density_{p24}$ values than those of the original clones were identified (Fig. 5A and Table 4).

The anti-p6 monoclonal antibody did not recognize the p6 protein of HIV_G, HIV_{EV}, or HIV_{ES}; therefore, the profile of polyprotein processing was determined only for HIV_B. As shown in Fig. 5B, HIV contained a smaller amount of the p6 protein and larger amounts of precursor proteins than did HIV_{NL4-3}. It was found that when the TGNS or APP insert was deleted from HIV_B, even lower amounts of p6 protein and similar or much greater amounts of precursor proteins were detected compared to HIV_B.

Gag processing in HIV_{NL4-3} carrying a mutated protease with or without inserts. Finally, in order to examine more directly the possible effects of the inserts identified in Gag on the proteolytic activity of mutated proteases and the viral fitness, we generated four additional infectious clones, $HIV_{NL/B\text{-}Pr}$, $HIV_{NL/B\text{-}Pr/TGNS\text{-}APP}$, $HIV_{NL/ES\text{-}Pr}$, and $HIV_{NL/ES\text{-}Pr/AQQA\text{-}PTAPPA}$. When the lysates of COS-7 cells producing each of these clones were tested for the Gag processing profiles by using a Western blot, a relatively smaller amount of p24 Gag (% $density_{p24}$ = 10.5%) was detected in the lysates of COS-7 cells producing $HIV_{NL/B\text{-}Pr}$; however, the introduction of TGNS plus APP increased the p24 Gag amount (% $density_{p24}$ = 24.2%) (Fig. 6). The same was true for $HIV_{NL/ES\text{-}Pr}$ (% $density_{p24}$ = 19.9%) and HIVNL/ES-Pr/AQQA-PTAPPA (% $density_{p24}$ = 33.2%).

These data, taken together, strongly suggest that the inserts had rendered the polyproteins more excisable by the mutated and functionally compromised proteases and improved the otherwise reduced replicative competence of the HIV_{MDR} variants.

DISCUSSION

The prevalence of HIV-1 variants that contain the Gag inserts identified in the present study cannot be estimated from currently available data sets. In the present study, we examined HIV-1 variants isolated from 20 patients randomly chosen from those who had received a number of RTIs and PIs over long periods of time and were enrolled in the salvage therapy clinical trials (11, 42). Of these 20 HIV-1 isolates, 6 that harbored the greatest numbers of drug resistance-associated mutations were chosen, and a full-length molecular infectious clone was generated from each of the 6 isolates. Thus, it should not be surprising that Gag inserts were identified at such a high rate (four of the six clones) in the present study. It is noteworthy that the presence of Gag inserts is apparently not associated with drug resistance profiles of the clones (Table 2) or the numbers or positions of amino acid substitutions in the protease or RT (Table 3), although the number of the HIV_{MDR} clones examined in the present study is rather small and an analysis of greater numbers of HIV_{MDR} clones is warranted.

Although various inserts have been reported to occur around the p6 Gag's PTAP motif (13, 28), their exact amino acid sequences have not been well documented, and their frequency and role have been controversial. One study has shown that these insertions occurred with equal frequency in HIV-1 isolates from both drug-naive and drug-experienced patients (13), whereas other studies have shown that they are seen more frequently in HIV-1 from drug-experienced individuals (28). In the Los Alamos database (23), no insertions identical to those in the present study have been reported, although a number of other insertions have been seen. Of 114 wild-type HIV-1 isolates in the database, insertions of more than three amino acids occurred in four regions as follows: 5 (4.4%) near the p17/p24 cleavage site, 6 (5.3%) near the p2/p7 cleavage site, 6 (5.3%) near the p1/p6 cleavage site, and 15

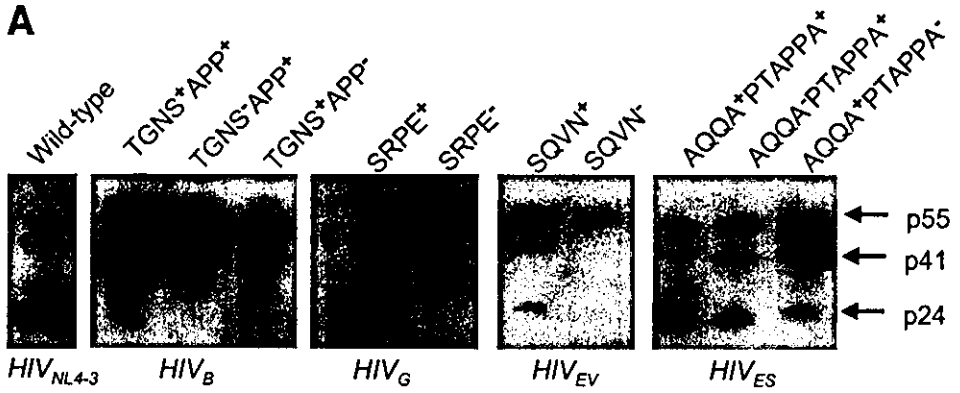
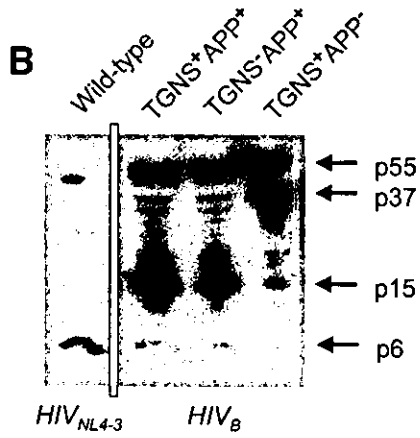


FIG. 5. Gag processing in molecular HIV_{MDR} clones and insert-lacking HIV_{MDR} clones. COS-7 cells producing primary molecular HIV_{MDR} clones and the corresponding insert-lacking HIV_{MDR} clones were harvested at 72 h after transfection, and their cell lysates were subjected to Western blot analysis with anti-p24 antiserum (A) and an anti-p6 monoclonal antibody (B). The positions corresponding to the sizes of the fully cleaved mature proteins (p24 and p6) and the immature proteins (p55, p41, p37, and p15) are indicated by arrows.



(13.2%) within p6. Considering that the incidence of insertions identified in the present study is very high (four of the six clones as described above), the present insertions are likely to be related to the long-term exposure of HIV-1 to antiviral drugs including multiple PIs. Indeed, a recently reported longitudinal study by Ibe et al. (19) demonstrated that certain insertions such as APP were identified in wild-type viruses at low percentages; however, with antiviral therapy started, more virions were found to harbor such insertions, suggesting that these insertions represent "polymorphisms" that are associated with drug resistance.

Pettit et al. have shown that the proteolytic processing of the Gag precursor by the viral protease occurs in a sequential manner and that the rates of cleavage at the five major Gag cleavage sites, including the p17/p24 and p1/p6 sites, differ by as much as 400-fold when full-length Gag protein is digested with wild-type HIV-1 protease in vitro (29). Although it is not clear whether the polyprotein is cleaved at similar rates by mutated proteases, it appears that the sequence of processing or the catalytic rates are not associated with the acquisition of the inserts seen in the present study.

The mechanism of the HIV-1 acquisition of the inserts observed in the present study is not known from the data presented. An extensive body of literature has demonstrated that HIV-1 RT is substantially error prone (1, 2, 31); however, mutations at Gag cleavage sites are rather limited, since not much flexibility is allowed near the scissile bond and the cleavage site must remain generally hydrophobic for the cleavage by the protease. One can presume that with highly mutated and enzymatically malfunctioning protease developed, the acquisition of inserts rather than developing cleavage site mutations should have been an efficient strategy for the virus to improve the otherwise deteriorated viral fitness by increasing the accessibility of the mutated protease to the cleavage sites and/or enhancing the cleavage sensitivity of the polyprotein to the mutated enzyme. It is also of note that the nucleic acid sequences of certain inserts has reportedly substantial variability in spite of the observation that the resulting amino acid sequence is relatively restricted (40). However, five of the six inserts studied here had a nucleic acid sequence identical to that of the juxtapositioned stretch, a finding that also corroborates that the inserts likely occurred through duplications. In addition, since three sets of two to four bases (Fig. 1C) were

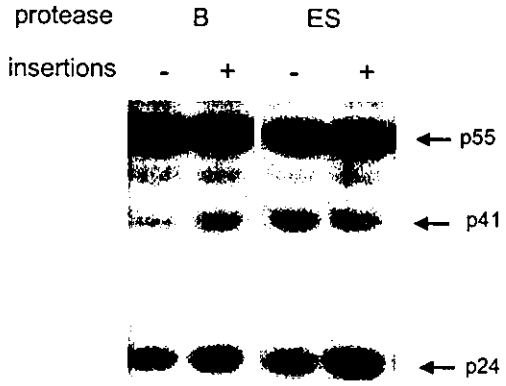


FIG. 6. Gag processing in HIV_{NL4-3} carrying a mutated protease with or without inserts. COS-7 cells producing four molecular clones (left pair, $HIV_{NL/B-Pr}$ and $HIV_{NL/B-Pr/TGNS-APP}$; right pair, $HIV_{NL/ES-Pr}$ and $HIV_{NL/ES-Pr/AQQA-PTAPPA}$) were harvested at 48 h after transfection, and their cell lysates were subjected to Western blot analysis with anti-p24 antiserum.

recognized in the 5′ end, center, and 3′ end of each of the duplicate-associated stretches, it is likely that a slippage or dislocation of the primer with respect to the template during DNA synthesis by RT occurred, although the possibility of involvement of polymerase errors, recombination, hypermutation, and instability cannot be fully ruled out.

It is noteworthy that the 449-Leu→Phe mutation at the p1/p6 site first reported by Doyon et al. (9) and Zhang et al. (43) is seen relatively often in HIV-1 variants resistant to PIs, suggesting that the mutated and enzymatically malfunctioning proteases excise the polyprotein at the p1/p6 site least efficiently, thus resulting in the elimination of HIV-1 with a "wild-type p1/p6 site" and the propagation of HIV-1 that acquired the 449-Leu→Phe mutation. It is plausible that with the inserts near the p1/p6 cleavage site obtained, the mutated proteases of HIV_B, HIV_G, and HIV_{ES} could excise the polyprotein efficiently. In this respect, it is possible that HIV_{EV} acquired the 449-Leu→Phe mutation to recover the catalytic activity and thus required no inserts close to the p1/p6 cleavage site. Similarly, the mutated proteases perhaps became less competent in excising the polyproteins at the p17/p24 cleavage site, and thus HIV-1 variants that acquired the insert(s) close to the site were presumably selected in the presence of the selection pressure imposed by PIs. It is also noteworthy that the introduction of the inserts to wild-type HIV-1 decreased the processing of polyproteins and viral fitness. We suggest that the presence of the inserts alters the conformation of the cleavage sites and limits the access of the wild-type protease to the cleavage sites and/or reduces the cleavage sensitivity of the polyprotein to the wild-type protease. This would explain why no insertions identical to those in the present study have been identified in wild-type HIV-1 isolates as described above.

Taken together, the results presented here establish that amino acid insertions in the proximity of Gag cleavage sites improve the otherwise compromised replication of HIV-1 variants that are highly resistant to multiple PIs. Further characterization of the factors related to the emergence of these insertions and biochemical studies of insertion-containing Gag polyproteins may open a new avenue to the intervention of HIV-1 highly resistant to multiple PIs.

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