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Characterization of Human Immunodeficiency Virus Type 1 Mutants with Decreased Sensitivity to Proteinase Inhibitor Ro 31-8959

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A human immunodeficiency virus type 1 (HIV-1) variant with highly reduced susceptibility to Ro 31-8959, an inhibitor of the viral proteinase, has been selected by repeated passage of wild-type virus in CEM cells in the presence of increasing concentrations of the inhibitor. Peptide sequences of the proteinase of selected virus were obtained from proviral DNA. Sequence comparison to wild-type (wt) proteinase demonstrated two amino acid substitutions in the resistant virus, a Gly to Val exchange at position 48 and a Leu to Met exchange at position 90. Furthermore, sequences of intermediate passage virus suggest contributions from positions 12, 36, 57, and 63 in early steps of resistance development. The selected virus showed a ca. 40-fold increase in 50% inhibitory concentration of Ro 31-8959. Growth kinetics of resistant virus were comparable to wild-type virus and the resistant genotype proved to be stable in the absence of inhibitor. Directed mutagenesis of the HIV-1 HXB2 proteinase at positions 48 and 90 suggested that each mutation alone led to a moderate decrease in sensitivity of the recombinant virus to proteinase inhibitor. However, a recombinant virus carrying both mutations in the proteinase gene showed a significant reduction in its sensitivity to Ro 31-8959 thus proving the importance of these exchanges for the resistance phenotype.

INTRODUCTION

Retroviral genomes encode an aspartyl proteinase whose activity is essential for processing of the *gag* and *gag–pol* polyprotein precursors (Dickson *et al.*, 1984; Kräusslich and Wimmer, 1988). The proteinase of human immunodeficiency virus (HIV) is part of the *gag–pol* ORF (open reading frame) and forms active homodimers in assembling viral particles. Inactivation of the proteinase, e.g., by substitution of alanine for the catalytic aspartic acid, yields nonprocessed precursor proteins and immature noninfectious progeny virus, as does inhibition of proteinase by specific inhibitors (Kohl *et al.*, 1988; Le Grice *et al.*, 1988; Mous *et al.*, 1988; Seelmeier *et al.*, 1988). HIV proteinase has thus been recognized as an attractive target for directed and specific antiviral therapy. Ro 31-8959 is such a specific inhibitor of HIV-1, HIV-2, and SIV proteinases (Roberts *et al.*, 1990). It is a transition state mimetic based on a natural cleavage site with the Phe–Pro scissile bond being replaced by a hydroxyethylamine moiety. Its potent and selective antiviral activity in chronic and acute HIV infections *in vitro* has been reported and the compound is currently undergoing clinical trials (Craig *et al.*, 1991).

The rapid development of resistance to nucleoside or nonnucleoside inhibitors of HIV reverse transcriptase has become a major obstacle in their clinical use as antiviral drugs (Larder *et al.*, 1989a, 1991; Rooke *et al.*, 1989, 1991; Land *et al.*, 1990; Japour *et al.*, 1991). A number of mutations in the reverse transcriptase gene have been described which confer decreased sensitivity to different inhibitors or may even lead to multidrug resistance (Larder and Kemp, 1989b; Nunberg *et al.*, 1991; Richman *et al.*, 1991; Richman, 1992). Rapid identification of specific mutations underlying the development of resistance will be increasingly important to monitor ongoing therapy. It is thus of significance for new drugs to identify such mutations and prove their role in drug susceptibility. We have previously selected in cell culture HIV-1 variants with reduced sensitivity to Ro 31-8959 and report here on the molecular basis of the resistant phenotype (Craig *et al.*, 1993b).

MATERIAL AND METHODS

Cells, virus, and selection

The details of cell lines, virus, and selection conditions have been described (Craig *et al.*, 1993). HIV-1 strain GB8 was passaged on CEM cells in the presence of increasing concentrations of the specific proteinase inhibitor Ro 31-8959 (*N-tert*-butyl-decahydro-2-[2(*R*)-hydroxy - 4 - phenyl - 3(*S*) - [[*N* - (2 - quinolylcarbonyl) - L -

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asparaginyl]amino]butyl] - (4a*S*, 8a*S*) - isoquinoline - 3(*S*) - carboxamine methanesulfonate) referred to as compound XVII in Roberts *et al.* (1990). Virus was characterized for sensitivity to each inhibitor by a syncytium reduction assay and p24 ELISA.

Cos-1 cells were grown in Dulbecco's modified Eagle medium and MT-2 or CEM cells in RPMI 1640 medium (Gibco BRL), each supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (2 m*M*).

PCR, cloning, and DNA sequencing

DNA was extracted from infected cells (passage 7, 8, 11, and 14, selection at 40, 80, 320, and 640 n*M* Ro 31-8959, respectively) by proteinase K digestion in the presence of SDS/EDTA and subsequent phenol/chloroform extraction. DNA was precipitated in isopropanol, washed in 70% ethanol, and dissolved in TE buffer (pH 8.0, Sambrook *et al.,* 1989). The viral proteinase gene was amplified by polymerase chain reaction (PCR, Horn *et al.,* 1985; Saiki *et al.,* 1988) in 50-μl final volume in 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% gelatine, 2 μM of each oligonucleotide primer, 250 μM of each dNTP, and 5 units of Thermus aquaticus DNA polymerase (Ampli Taq, Perkin–Elmer–Cetus). Cycling conditions were as follows: 4 min/94° denaturation followed by the addition of DNA polymerase and 25 cycles of 40 sec/58° annealing, 60 sec/72° elongation, and 40 sec/94° melting. The reaction was finished by 4 min/72° elongation. The oligonucleotide primers were as follows: Sense primer — 5′GTCGACTAGTCAGAGCCAACAGCCCCACCAGAAGAGC — corresponding to position 1730–1757 of HIV-1 Bru with an *Spe*I site added at its 5′ end, and antisense primer — 5′CAGCTGCGGCCGCTCTTCTGTCAATGGCCATTGTTTAAC — corresponding to position 2192–2217 of HIV-1 Bru with a *Not*I site added to its 5′ end (Wain-Hobson *et al.,* 1985). PCR products were separated on agarose gel and purified on glasmilk (GeneClean, Bio 101; La Jolla, CA). Purified fragments were digested with *Spe*1/*Not*1 and ligated into a correspondingly digested and dephosphorylated pBluescript II SK (–) vector (Stratagene). Plasmid DNA was prepared by a modified SDS/alkaline lysis method (Magic miniprep, Promega) and the sequence of the insert was determined by the dideoxy chain termination method (Sequenase sequencing kit, USB) and labeling with [^{35}S]dATPαS (Amersham, >37 TPq/mmol). Sequences were analyzed and compared using the GCG sequence analysis software (Genetic Computer Group, University of Wisconsin). The statistical significance of amino acid changes between the wild-type and passage 11 virus was assessed by means of 2 × 2 contingency tables (Bailey, 1973).

Mutagenesis of HIV-1 BRU proteinase

The following mutations were introduced into the HIV-1 BRU proteinase by PCR using the appropriate mismatched pairs of primers (No *et al.,* 1989): Gly48 → Val, Leu90 → Met, and the double mutant containing both exchanges. The mutation oligonucleotides for the Gly48 → Val exchange were BRU nucleotide position 1968–1986 5′AAAATGATAGTGGGAATTGG (sense) and 5′CCAATTCCCACTATCATTTT (antisense) and for the Leu90 → Met exchange, Bru nucleotide position 2090–2111 5′GGAAGAAATCTGATGACTCAG (sense) and 5′CTGAGTCATCAGATTTCTTCC (antisense). The mutated positions are underlined. The flanking oligonucleotide primers were 5′TTTAGGGAAGATCTGGCCTTCCTAC (sense, BRU nucleotide position 1635–1652), encompassing the *Bgl*II site at position 1641, and 5′AAGCACATTGTACTGATATCTAATCCC (antisense, BRU nucleotide position 2553–2577), encompassing the *Eco*RV site at position 2578. The *Bgl*II/*Eco*RV fragment was inserted into a *Pst*I/*Sal*I fragment (nucleotide 1418–5785) of the HIV-1 HXB2 genome which was finally introduced into a complete HXB2 genome (Wain-Hobson *et al.,* 1985; Wong-Staal *et al.,* 1985). The details of the experimental strategy for the construction of an HXB2 clone with the Gly48 → Val/Leu90 → Met double mutation will be described in detail elsewhere (Dean L. Winslow *et al.,* submitted for publication). In brief, a full length infectious molecular clone of HIV-1 HXB2 was digested at the unique *Nco*I site and the two halves were subcloned into separate plasmid vectors. The 5′ genomic fragment was further modified by adding a *Esp*3I site near the 3′ end of the proteinase gene to make a cassette shuttle vector in which the wild-type proteinase coding region can be conveniently replaced by, e.g., mutant proteinase genes. Plasmids containing the 5′ and 3′ halves of the virus genome are linearized at their *Nco*I site, ligated, and transfected into MT-2 cells to recover infectious mutant virus.

Expression and transfection studies

To assess the activities of the mutagenized proteinases, the processing of the *pol* ORF in *Escherichia coli* was studied (Mous *et al.,* 1988; Debouck *et al.,* 1987). The *pol* ORF was isolated from the proviral clones containing the mutated proteinases by PCR (sense primer: 5′TGACGGATCCCCTACAAGGGAAGGCCAGGGAATTTTCTTCAGAG, corresponding to nucleotides 2108–2141 of the HIV-1 HXB2 genome with a 5′ *Bam*HI site added; antisense primer: 5′TGACAAGCTTGGCACTACTTTTATGTCACTATTATCTTGT, corresponding to nucleotides 4981–5011 with an 5′ *Hin*dIII site). The purified and restricted PCR fragment was inserted into a pDS56/RBSII vector (pQE4, Diagen; Stüber *et al.,* 1990). After transformation into *E. coli* strain M4022, expression was induced with 2 m*M* IPTG. Cells were harvested and lysed in sample buffer, and proteins were separated by SDS–PAGE (Laemmli, 1970). Processing of *pol* was analyzed by Immuno-blot with a rabbit serum against HIV-1 reverse transcriptase (Towbin *et al.,* 1979).

Construction of HIV-1 HXB2 virus containing mutant proteinase

Proviral genome containing plasmid was transfected into Cos cells by the Lipofectin method (10 μg DNA/10^6 cells) following the manufacturer's instructions (Gibco BRL). Supernatants of transfected Cos were used to infect MT-2 cells. The resulting virus-containing supernatants were passaged again on MT-2 and subsequently used to assay for sensitivity to Ro 31-8959. MT-2 cells were infected for 1 hr with virus-containing supernatant, washed once, and aliquoted on microtiter plates in medium with inhibitor. After about 4 days when control cultures showed widespread syncytia formation, the supernatants were harvested and analyzed for virus growth by p24 ELISA (DuPont). Sensitivity of the HXB2 virus with the double mutation (Val48/Leu90) to proteinase inhibitor was assayed on PBMC following the protocol of the ACTG/DoD PBMC consensus assay (Japour *et al.*, 1993).

RESULTS

Selection of HIV-1 GB8 resistant to proteinase inhibitor

HIV-1 GB8 was passaged on CEM cells, a human T-lymphoblastoid cell line, in the continuous presence of the proteinase inhibitor Ro 31-8959 as described (Craig *et al.*, 1993b). Selection was started at 10 n*M* which corresponds approximately to 1.5 × 50% inhibitory concentration (IC_{50}) in this particular cell-virus system. When significant virus growth had occurred as judged by microscopic examination of the infected culture, i.e., observation of extensive cell clumping and syncitia formation, cell-free supernatant was harvested and used for *de novo* infection of CEM cells. Selection was continued in parallel cultures with either the previous inhibitor concentration maintained or at twice that concentration. Inhibitor concentrations were raised whenever growth at the previously highest concentration had occurred, otherwise selection was continued at the same concentration.

Sequence analysis of the proteinase of resistant virus

We initially sequenced 10 individual clones of the passage 11 (P11) viral proteinase following its PCR amplification from total DNA of infected CEM cells and cloning into pBluescript vector. Since this particular HIV-1 strain had not been molecularly characterized before, we sequenced for comparison 11 wild-type proteinase clones which had been isolated by the same procedure from HIV-1 GB8-infected cells without previous passages in the presence of Ro 31-8959. The deduced protein sequences were aligned and compared for amino acid exchanges (Fig. 1). Two striking differences between wt and P11 virus proteinases were noted, i.e., a glycine to valine exchange at peptide position 48 and a leucine to

```
     1                                                  50
Ro31 ---------- --t------- ---------- -----i---- ----------
Ro32 ---------- ---------- ---------- -----i---- -------v--
Ro33 ---------- ---------- ---------- -----i---- -------v--
Ro34 ---------- ---------- ---------- -----i---- -------v--
Ro35 ---------- ---------- ---------- -----i---- -------v--
Ro36 .......... ...------- ---------- -----i---- -------v--
Ro37 ---------- ---------- ---------- -----i---- -------v--
Ro38 ---------- ---------- ---------- -----i---- -------v--
Ro39 ---------- ---------- ---------- -----i---- -------v--
Ro41 ---------- ---------- ---------- -----i---- -------v--
Wt17 ---------- ---------- ---------- ---------- ----------
Wt16 ---------- ---------- ---------- -----i---- ----------
Wt15 ---------- -i-------- ---------- ---------- ----------
Wt14 ---------- -i-------- ---------- -----i---- ----------
Wt13 ---------- ---------- ---------- ---------- ----------
Wt12 ---------- ---------- ---------- ------s--- ----------
Wt11 ---------- ---------- ---------- -----i---- ----------
Wt10 ---------- ---------- ---------- -----i---- ----------
Wt09 ---------- ---------- ---------- -----i---- ----------
Wt08 ---------l ---------- ---------- ------s--- ----------
Wt07 ---------- -i-------- ---------- ---------- ----------
Wt-C PQITLWQRPV VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI

Ro-C PQITLVQRPV VTIKIGGQLK EALLDTGADD TVLEEINLPG RWKPKMIVGI
                 •                    •          •

     51                                                 99
Ro31 ------rr--- -vl------- ---------- ---------- --i-----
Ro32 ------r--- --v------- ---------- ---------m ---------
Ro33 ------rh-- --v------- ---------- ---------m -.........
Ro34 ------r--- --v------- ---------- ---------m -.........
Ro35 ------r--- --v------- ---------- ---------m ---------
Ro36 ------r--- --v------- ---------- ---------m ---......
Ro37 ------r--- --v------- ---------- ---------m ---------
Ro38 ------r--- --v------- ---------- ---------m ---------
Ro39 ------r--- --v------- ---------- ---------m ---------
Ro41 --i---r--- --v------- ---------- ---------m ---------
Wt17 ---------- ---------- ---------- ---------- ----------
Wt16 ------r--- --v------- ---------- ---------- ----------
Wt15 ------r--- --v------- ---------- ---------- -hv-------
Wt14 ---------- ---------- ---------- ---------- ----------
Wt13 ---------- ---------- ---------- ---------- ----------
Wt12 ------r--- ---------- ---------- ---------- ----------
Wt11 ---------- ---------- ---------- ---m------ ----------
Wt10 ---------- ---------- ---------- ---------- ----------
Wt09 ---------- ---------- ---------- ---------- ----------
Wt08 ------r--- ---------- ---------- ---------- ----------
Wt07 ---------- ---------- ---------- ---------- ----------
Wt-C GGFIKVKQYD QILVEICGHK AIGTVLVGPT PVNIIGRNLL TQLGCTLNF

Ro-C GGFIKVRQYD QIVVEICGHK AIGTVLVGPT PVNIIGRNLM TQLGCTLNF
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FIG. 1. Comparison of proteinases from HIV-1 GB8 wild-type virus and virus selected for resistance to Ro 31-8959 (passage 11). Proteinase gene was amplified by PCR from proviral DNA and inserted into pBluescript, and the sequences from individual clones were obtained. An alignment of sequences from 11 wild-type (wt07-17) and 10 passage 11 genes (Ro31-39, 41) is shown. – indicates that all sequences share the same residue at the given position; deviations from the consensus are indicated by lower case letters. The one-letter code for amino acids is used. Wt-C and Ro-C are the consensus sequences of either all wt clones or all P11 clones. Positions which are considered to be relevant for resistance are underlined by a dot, positions 48 and 90 are additionally highlighted by bold lettering.

methione exchange at position 90. The two amino acid exchanges are both caused by base transversions. The Gly48 → Val exchange is consequent to a G → T mutation whereas a T → A mutation underlies the Leu90 → Met exchange. In addition we observed sequence heterogeneities at several other positions (i.e., positions 12, 36, 37, 57, 63) in the wt sequences where the selected virus proteinase displayed a more homogenous pattern.

Recently, five domains in the HIV-1 proteinase have been defined which are highly conserved among all known virus isolates (Fontenot *et al.*, 1992). Gly48 borders the conserved domain III and is part of the GGIGGFI motif which is found in HIV-1, HIV-2, and SIV, whereas

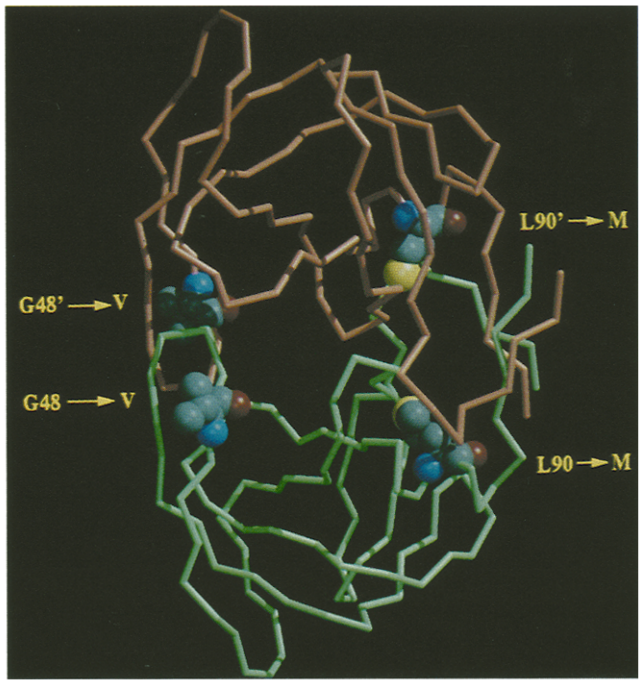

FIG. 2. Structural model of HIV-1 proteinase with inhibitor Ro 31-8959 showing mutations of G48V and L90M as solid spheres.

Leu90 borders the conserved domain IV and is also conserved among the three primate immunodeficiency viruses as well as in other retroviral proteinases. A structural model of the HIV-1 proteinase based on X-ray analysis places the G48 into the flexible "flap" covering the substrate binding site with the bulky aliphatic side chain of the mutant valine protruding into the interior of the binding site (Fig. 2). The Leu90 is localized to the core of the proteinase with no obvious contact to enzyme-bound substrate or inhibitor (Wlodawer and Erickson, 1993).

We also analyzed the sequence of virus proteinase at a later point of selection (passage 14, growth in 640 nM Ro 31-8959). However, no additional exchanges in the peptide sequence were observed (data not shown). Of 10 sequences of the P11-selected virus 1 had maintained the genotype of the wt proteinase sequence (Fig. 1, Ro31). This would indicate that there is still a heterogenous virus population with a minority of wt virus although we cannot rigorously exclude an accidental PCR contaminant.

It was of interest to determine the order of appearance of the mutations that led to the resistant phenotype. We thus obtained the proteinase sequence at the earlier passages 7 and 8 from virus grown at 40 and 80 n*M* Ro 31-8959, respectively (Fig. 3). P7 virus still showed the wt sequence at position Gly48 and Leu90. However, earlier heterogeneities in the wt sequences at positions 12(Iso/Thr), 36(Met/Ile), 37(Ser/Asn), 57(Lys/Arg), and 63(Leu/Val) had resolved into a homogeneous pattern in P7 virus, i.e., Thr12, Ile36, Asn37, Arg57, and Val63, respectively. The changed patterns at positions 36, 57, and 63 were statistically significant ($P = 0.009$, 0.003, and 0.002, respectively), while those at positions 12 and 37 were not ($P = 0.276$ and 0.262, respectively). Moreover, no single wt sequence had all residues at positions 36, 57, and 63 as found in the consensus P7 sequence. This suggests that during passages 1 to 7 a rare wt sequence had become the predominant genotype of the P7 viral population or that selection of spontaneously arising mutants at these positions had occurred. Further selection at a higher concentration of Ro 31-8959 led to the appearance of the Val48 mutant in P8 virus on the genotypic background of the P7 virus and subsequently at a later time point to the appearance of the Val48/Met90 double mutant as found in the passage 11 virus.

Comparison of cleavage site sequences

Mutations in the proteinase substrate-binding site could conceivably require compensating amino acid exchanges at or surrounding the substrate cleavage sites. Thus, we determined the peptide sequences within the p55 *gag* precursor and at the N- and C-termini of the proteinase itself. These cleavage sites include the Tyr–Pro (p17/p24), Leu–Ala (p24/X), Met–Met (X/p9), and Phe–Pro (p15/proteinase and proteinase/RT) scissile bonds. No consistent differences between wt virus and virus selected for resistance to Ro 31-8959 were observed (data not shown). We conclude that no such com-

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     1                                                            50
P7/9  ---------- ---r------ ---------- ---------- ----------
P7/8  ---------- ---------- ---------- ---------- ----------
P7/7  ---------l ---------- ---------- ---------- ----------
P7/6  ---------l ---------- ---------- ---------- ----------
P7/2  ---------- ---------- ---------- ---------- ----------
P7/11 ---------- ---------- ---------- ---------- ----------
P7-C  PQITLWQRPV VTIKIGGQLK EALLDTGADD TVLEEINLPG RWKPKMIGGI
                   •                    •
P8/1  ---------- ---------- ---------- ---------- -------v--
P8/2  --t------- ---------- ---------- ---------- -------v--
P8/3  ---------- ---------- ---------- ---------- -------v--
P8/4  ---------- ---------- ---------- ---------- -------v--
                                                          •

     51                                                           99
P7/9  ---------- ---------- ----l----- ---------- ----------
P7/8  ---------- ---------- ---------- ---------- ----------
P7/7  ---------- ---------- ---------- ---------- ----------
P7/6  ---------- ---------- ---------- ---------- -----a----
P7/2  ---------- ---------- ---------- ---------- ----------
P7/11 ---------- ---------- ---------- ---------- ----------
P7-C  GGFIKVRQYD QIVVEICGHK AIGTVLVGPT PVNIIGRNLL TQLGCTLNF
            •        •                                  •
P8/1  ---------- ---------- ---------- ---------- ----------
P8/2  ---------- ---------- --p------- ---------- ----------
P8/3  ---------- ---------- ---------- ---------- ----------
P8/4  ---------- ---------- ---------- ---------- ----------
```

FIG. 3. Comparison of proteinases from HIV-1 GB8 passage 7 (P7) and passage 8 (P8). Sequences were obtained as described in the legend to Fig. 1. – indicates that all sequences share the same residue at the given position; deviations from the consensus are indicated by lowercase letters. The one-letter code for amino acids is used. P7-C is the consensus sequence of the passage 7 sequences. Positions which are considered to be relevant for resistance are underlined by a dot; positions 48 and 90 are additionally highlighted by bold lettering.

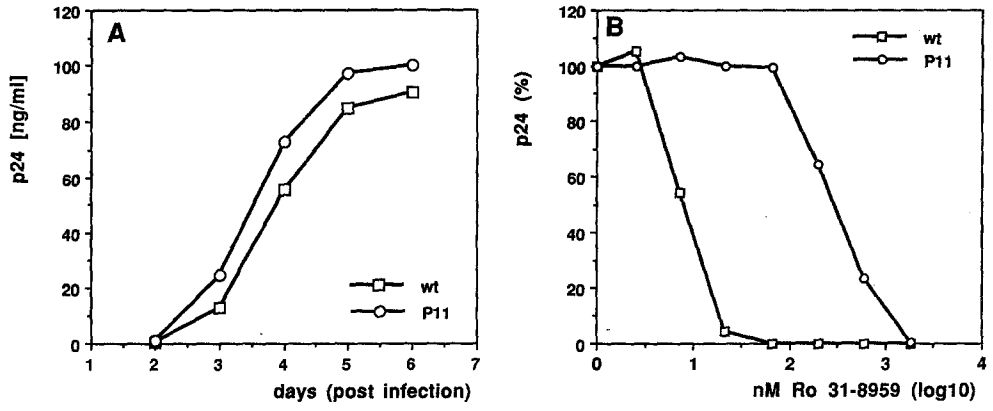

FIG. 4. (A) Growth kinetics of HIV-1 GB8 wild-type and P11 virus. CEM cells were infected with virus-containing culture supernatant, washed, and resuspended at 2.5 × 10^5/ml in fresh medium. Aliquots of the supernatant were removed at Days 2 to 6 postinfection and p24 antigen concentration was determined in an ELISA assay. (B) Sensitivity of HIV-1 GB8 wild-type and P11 to Ro 31-8959. Cells and infection were as described in (A). Inhibitor was added to 1800, 600, 200, 67, 22, 7.5, and 2.5 n*M* final concentration. When extensive syncitia formation in the control culture had developed, culture supernatants were harvested and p24 antigen was determined. Content of p24 in the supernatant is expressed as percentage of a control infected culture without inhibitor.

pensating exchanges are required for the mutant proteinase to process the p55 *gag* and p160 *gag–pol* precursor.

Characterization of the P11 virus

We compared wt and P11 virus with respect to growth in CEM cells and sensitivity to proteinase inhibitor. In acute infection, growth of resistant virus was as rapid as wt virus reaching the same level of p24 antigen in the culture supernatant within 4 to 5 days (Fig. 4A). Inhibition of virus growth by Ro 31-8959 was likewise assayed in acute infections of CEM cells. For wt virus an IC_{50} of 7 n*M* and an IC_{90} of 16 n*M* was determined. The corresponding values for P11 virus were 250 n*M* and 1500 n*M*, respectively (Fig. 4B). Thus, drug selection yielded a virus with a 30- to 100-fold decrease in sensitivity to proteinase inhibitor without any gross defects in viability. To assess the stability of the genotype of resistant virus we grew P11 virus for 20 passages in the absence of Ro 31-8959. Sequencing of the proteinase gene of such virus revealed no difference to P11 virus demonstrating the stability of the mutant genotype without selection pressure (data not shown).

Mutagenesis of HIV-1 proteinase

Laboratory strains of HIV-1 grown in either T-cell lines or monocytic cells are highly susceptible to the inhibitory activity of Ro 31-8959. The IC_{50} in acute infection models is usually less than 5 n*M*. To obtain more definite evidence that the Gly48 → Val and Leu90 → Met exchanges lead to a resistant phenotype both mutations were introduced either alone or in combination into the HIV-1 HXB2 proteinase by PCR-directed mutagenesis. The mutated proteinase gene was inserted into the HXB2 *pol* gene and cloned into *E. coli* vector pQE4 which allows expression under the control of the *lac*I repressor (Stüber *et al.*, 1990). After induction of transformed bacteria, the activities of the mutated proteinases were monitored by immunoblot analysis for processing of the reverse transcriptase precursor to p66/p51 (Fig. 5). Cleavage of the *pol* gene product was observed for all *pol* constructs containing either wild-type or mutated proteinases proving that both Val48 and Met90 mutations are compatible with enzyme activity. However, examination of processing kinetics indicated consistent differences in the time course of p66/p51 appearance. Cleavage by the Val48 proteinase was reproducibly delayed when compared to the wt, Met90, or double mutant enzyme. Processing was incomplete at early times postinduction and reached wt levels only after 3 to 4 hr. Moreover, processing of p66 to p51 seemed to be less efficient in both single mutants. This would point to quantitative differ-

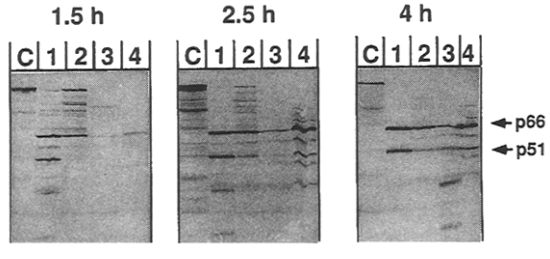

FIG. 5. Activity of mutant proteinase in *E. coli*. The *pol* open reading frame of HIV-1 HXB2 containing a wild-type proteinase (lanes 1), a G48V mutant (lanes 2), a L90M mutant (lanes 3), or a G46V/L90M mutant proteinase (lanes 4) was expressed in *E. coli* as described under Material and Methods. Cells were harvested at the indicated times after induction; lysed proteins were resolved by SDS–PAGE. Processing of the *pol* gene product to p66/p51 was analyzed by immunoblot with an antireverse transcriptase rabbit serum. As a control for processing by the viral proteinase a *pol* ORF with an inactivated proteinase (D25A) was run in parallel (lanes C).

TABLE 1

SENSITIVITY OF HIV-1 HXB2 WILD-TYPE VIRUS AND PROTEINASE MUTANTS TO RO 31-8959

Virus	IC_{50}[a]	IC_{90}[a]
HXB2 wt	1.7 n*M* MT-2	8.9 n*M* MT-2
	6.0 n*M* PBMC	n.d.
HXB2 G48-V	13.5 n*M* MT-2	20.0 n*M* MT-2
HXB2 L90-M	5.7 n*M* MT-2	15.1 n*M* MT-2
HXB2 G48V + L90M	120.0 n*M* PBMC	n.d.

Note. Virus was prepared by transfection of Cos cells with plasmids containing proviral genomes of HIV-1 HXB2 with either wild-type or mutant proteinase and cocultivation with MT-2 cells. Infection, inhibitor treatment, and p24 assay were as described in the legend to Fig. 4 except that MT-2 cells were used. Sensitivity of the double mutant virus was assayed on PBMC following the ACTG consensus protocol.

[a] $IC_{50/90}$, 50/90% inhibitory concentration of Ro 31-8959; n.d., not determined.

ences in proteinase activity between wt, single mutants, and double mutants. It should be noticed that these differences were uniform among a number of individual clones tested for each proteinase mutant.

Sensitivity of mutant HXB2 virus to Ro 31-8959

Infectious HIV-1 HXB2 proviral genomes containing either the single mutant proteinases Gly48 → Val and Leu90 → Met or the double mutant proteinase were constructed by directed mutagenesis and virus was recovered via transfection and passage on MT-2 cells. Sensitivity to Ro 31-8959 was assayed in acute infections of either MT-2 cells or stimulated PBMC by measuring the p24 level in the culture supernatants. Introduction of either mutation at position 48 or at position 90 led to a small but reproducible increase in $IC_{50/90}$, which supports their proposed significance in the evolution of resistance to Ro 31-8959. The values for IC_{50} and IC_{90} derived from these titrations are shown in Table 1. The single mutations raised the IC_{50} for Ro 31-8959 by 3.4- and 8-fold, respectively, with a concomitant increase in IC_{90}. Growth of the double mutant virus in the presence of inhibitor yielded an IC_{50} value of 120 n*M* which represents a ca. 20-fold increase over the corresponding value for the wild-type virus in the PBMC assay system. These results indicate that the presence of both mutations in the proteinase gene is necessary to give a Ro 31-8959 resistance phenotype.

DISCUSSION

In this study we present a phenotypic and genotypic description of an HIV-1 variant selected for resistance to proteinase inhibitor Ro 31-8959. Furthermore, we provide support for the proposed resistance genotype by directed mutagenesis of a wt HIV-1 HXB2 proteinase gene. Evolution of resistance in the case of the HIV-1 GB8 strain initially seemed to involve a selection of a rare, preexisting wt genotype. This led to a virus population with a homogeneous amino acid distribution at positions 12, 36, 57, and 63. A subpopulation with an *a priori* decreased sensitivity to Ro 31-8959 may thus exist in the previously nonselected HIV-1 GB8 wild-type virus population. However, titration of the P7 virus for its sensitivity to Ro 31-8959 gave variable values for the IC_{50}, which on an average were only slightly higher than for the wt virus (data not shown). The first *de novo* mutation at position 48 occurred within the background of this selected wt sequence followed subsequently by the second *de novo* mutation at position 90 to give the fully resistant virus. The importance of the positions 48 and 90 for the resistance phenotype could be confirmed by specific mutagenesis of the HIV-1 HXB2 proteinase. Either single mutation by itself resulted in a virus which showed only a modest reduction in its sensitivity to Ro 31-8959 when assayed in an acute infection experiment. However, combining the Val48 and Leu90 exchanges into one wt HXB2 proteinase gave rise to a virus which was markedly resistant to the inhibitor. The primary significance of these positions for development of resistance has been recently confirmed when proteinase sequences were obtained from proviral DNA of PBMC from patients after prolonged treatment with Ro 31-8959. In a number of integrated virus genomes proteinase genes with Val at position 48 and Leu at position 90 were observed (H. Jacobsen, unpublished results). The importance of positions 12, 36, 57, and 63 for the selection of the resistant HIV-1 GB8 remains unclear. Conceivably, residues at these positions could compensate for detrimental effects of the Val48/Leu90 mutations on proteinase activity and fitness of the virus. An ongoing biochemical analysis of single and double mutant proteinase in an *in vitro* assay will help to clarify the functional consequences of these mutations.

Molecular modeling based on structural data from the HIV-1 proteinase/Ro 31-8959 complex suggests that the Gly to Val exchange at position 48 may directly interfere with binding of the inhibitor to a substrate-binding site by steric interference of the Val side chain. However, the Leu90 → Met exchange alone also decreases sensitivity of the proteinase to the inhibitor as demonstrated by the mutagenesis of the HXB2 proteinase which led to a three- to fourfold increase in IC_{50}. Furthermore, a selection of HIV-2 Ben in the presence of Ro 31-8959 yielded a virus with a Leu90 → Met exchange as sole mutation which grew at 70 n*M* Ro 31-8959 (data not shown). Clearly, more structural data are necessary to define the precise contribution of Met90 to resistance. Recently a Val82 to Ala exchange has been described as being critical for resistance to C-2 symmetrical proteinase inhibitors without diminishing the activity of Ro 31-8959 (Otto *et al.*,

1993). We have previously prepared a Val82 → Phe mutant in HXB2 proteinase and found that the resulting virus grew only poorly with unaltered sensitivity to Ro 31-8959. Further selection experiments, possibly with other HIV-1 strains, may reveal additional mutations in the proteinase gene which reduce its sensitivity to Ro 31-8959.

Resistance to reverse transcriptase inhibitors has become a major problem in their clinical use. Careful selection of dosage, the alternating use of different inhibitors which are not subject to cross resistance, or combination therapy may be ways to prevent or delay onset of viral resistance. In the case of Ro 31-8959, clinical trials of phase II/III are underway and analysis of clinical isolates for changes in resistance to inhibitor will be of major importance. Combination of reverse transcriptase and proteinase inhibitors may turn out to be useful therapeutic regiments since cell culture studies indicate synergistic enhancement of antiviral activities (Craig *et al.*, 1993a). Furthermore, the existing data on resistance to proteinase inhibitors, albeit rather limited, suggest that combination of structurally different inhibitors may avoid cross resistance and thus increase their therapeutic potential.

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