

An *Escherichia coli* Expression Assay and Screen for Human Immunodeficiency Virus Protease Variants with Decreased Susceptibility to Indinavir

LAURENCE MELNICK,* SHIOW-SHONG YANG, RICK ROSSI,
CHARLIE ZEPP, AND DONALD HEEFNER
Sepracor Inc., Marlborough, Massachusetts 01752

Received 17 April 1998/Returned for modification 30 June 1998/Accepted 12 September 1998

We have developed a recombinant *Escherichia coli* screening system for the rapid detection and identification of amino acid substitutions in the human immunodeficiency virus (HIV) protease associated with decreased susceptibility to the protease inhibitor indinavir (MK-639; Merck & Co.). The assay depends upon the correct processing of a segment of the HIV-1 HXB2 gag-pol polyprotein followed by detection of HIV reverse transcriptase activity by a highly sensitive, colorimetric enzyme-linked immunosorbent assay. The highly sensitive system detects the contributions of single substitutions such as I84V, L90M, and L63P. The combination of single substitutions further decreases the sensitivity to indinavir. We constructed a library of HIV protease variant genes containing dispersed mutations and, using the *E. coli* recombinant system, screened for mutants with decreased indinavir sensitivity. The discovered HIV protease variants contain amino acid substitutions commonly associated with indinavir resistance in clinical isolates, including the substitutions L90M, L63P, I64V, V82A, L24I, and I54T. One substitution, W6R, is also frequently found by the screen and has not been reported elsewhere. Of a total of 12,000 isolates that were screened, 12 protease variants with decreased sensitivity to indinavir were found. The L63P substitution, which is also associated with indinavir resistance, increases the stability of the isolated protease relative to that of the native HXB2 protease. The rapidity, sensitivity, and accuracy of this screen also make it useful for screening for novel inhibitors. We have found the approach described here to be useful for the detection of amino acid substitutions in HIV protease that have been associated with drug resistance as well as for the screening of novel compounds for inhibitory activity.

High-level resistance to human immunodeficiency virus (HIV) protease inhibitors is accompanied by multiple mutations in the HIV protease (4, 5, 13, 14, 23). Even in selective cell culture systems, significant resistance appears to require multiple substitutions (5, 25). Resistant viruses with a single substitution generally are not found in clinical isolates, and the level of resistance increases with the acquisition of additional substitutions. Certain specific substitutions occur at high frequency in response to selective pressure from a number of different protease inhibitors. For example, statistical analysis shows that 11 different substitutions are associated with indinavir (MK-639) resistance in clinical isolates (4). In cell culture studies at least three of these substitutions are required to achieve a detectable level of resistance, and subsequent additions of other members of the observed 11 substitutions lead to even greater levels of resistance. In the case of resistance to Abbott's protease inhibitor, ritonavir (ABT-538), nine different codons are selected in response to monotherapy (23). Seven of these nine substitutions are identical to the substitutions observed to develop in response to monotherapy with indinavir.

The observations that high-level resistance to various protease inhibitors requires multiple amino acid substitutions and that common substitutions occur for protease inhibitors for which clinical data are available suggest common pathways of protease evolutionary escape from drug inhibition.

The independent recurrence of a limited number of resistance-incurring mutations as an evolutionary response by a

pathogen to chemotherapy is reminiscent of a number of studies focused on bacterial evolution of drug resistance. For example, in the work of Hedge and Spratt (10, 11) on PBP 3 and mutations that accompany resistance to the β -lactam antibiotics, they concluded that some alterations created a binding protein with lowered affinity for the inhibitor, while other substitutions were "compensatory" and increased the catalytic efficiency of the enzyme. Taken together these sequential substitutions defined an evolutionary escape pathway. The same appears to apply for substitutions in the HIV protease; some substitutions, such as the V82A and I84V substitutions in the cases of resistance to ritonavir and indinavir, decrease the affinity of the enzyme for the inhibitor. Other substitutions such as M46I, which by itself appears to have little influence on the enzyme-inhibitor interaction (5, 9), are frequently found in association with other substitutions which do lower the affinity of the protease for the inhibitor.

The complexity of the evolution of resistance to protease inhibitors and the limitations inherent in cell culture studies, which only partly reflect in vivo results (28), prompted us to develop a simplified assay system that would not require intact virus but that would accurately indicate the effects of protease genotype on drug susceptibility. The ultimate goal of this work was to develop a simplified screening system that will allow the accurate, prospective determination of resistance-conferring substitutions which occur in response to a particular inhibitor. This system will also allow estimates of the frequency at which these substitutions arise. Such a system not only would allow the rapid identification of single amino acid substitutions that decreased the affinity of the protease for the inhibitor but would also provide information on the total number of such substitutions that decreases the sensitivity of the protease for

* Corresponding author. Mailing address: Sepracor Inc., 111 Locke Dr., Marlborough, MA 01752. Phone: (508) 357-7416. Fax: (508) 357-7467. E-mail: lmelnick@sepracor.com.

the inhibitor while retaining activity on a truncated version of the natural substrate. Furthermore, this information would be obtained without the need for the handling of pathogenic virus. Such a simple assay could also be used for the rapid identification of alternative inhibitors that could effectively inhibit proteases containing substitutions that conferred resistance to another compound. The system does not depend upon cell culture or intact pathogenic virus but still allows the direct identification of single protease mutations that may be associated with drug resistance and the subsequent rapid identification of inhibitors that block the resistant variants. Below we describe an *Escherichia coli*-based recombinant system that appears to meet many of these requirements.

MATERIALS AND METHODS

Plasmid construction. The *E. coli* plasmid pL124.23 provides isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible expression of a portion of the HIV type 1 (HIV-1) Pol polyprotein including the complete protease and reverse transcriptase as well as 52 amino acids at the N terminus of HIV protease and 141 amino acids of the integrase gene extending from the C terminus of HIV reverse transcriptase. HIV-1 HXB2 DNA in pL124.23 was derived from plasmid pART-2, which was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, Rockville, Md., and which was contributed by Ronald Swanstrom (20, 21). Plasmid pART-2 was cut with the restriction enzymes *Bgl*II and *Eco*RI to derive a 2.6-kb DNA segment that was ligated into the similarly digested vector pTrcHisC (Invitrogen) to derive plasmid pL124.23. The ligation places the HIV coding sequences in frame with an encoded peptide including a polyhistidine sequence of the pTrcHisC vector. This construct was used to transform *E. coli* Top10 cells (Invitrogen) and the expression of HIV polyprotein segment induced by the addition of 50 mM IPTG. Expression of HIV polyprotein was analyzed as described below by Western blot analysis and enzyme-linked immunosorbent assay (ELISA)-based detection of reverse transcriptase activity.

Plasmids pL129.33 and pL141.2, which contain the HIV protease-reverse transcriptase polyprotein gene segment, were derived from pL124.23 and contain the *f1* region for phage particle production. These vectors were used for site-directed mutagenesis experiments. The restriction enzymes *Nco*I and *Hind*III were used to excise the 2.6-kb HIV polyprotein gene segment from pL124.23. This segment was ligated to a similarly cut pALTER-Ex1 vector (Promega) to derive plasmid pL129.33. Plasmid pL141.2 contains the same HIV protease insert as pL129.33 but in the reverse orientation. pET21c (Novagen)-derived HIV protease expression vectors were used for production of purified HIV protease protein. pL319.1 expresses native HIV protease under the tightly regulated T7 *lac* promoter in BL21(DE3)pLysS cells (33). To construct pL319.1, the HIV protease gene was amplified by PCR from the template DNA of plasmid pL124.23 by using primers for attachment of a *Hind*III restriction site at the 3' end of the amplified *pol* gene segment. The amplified DNA was digested with *Bam*HI and *Hind*III to derive a 0.75-kb segment containing the entire HIV protease gene and the 169 bp upstream of the HIV protease gene as well as 284 bp of the 3' adjacent reverse transcriptase gene. The vector pET21c was similarly cut, and the HIV DNA was ligated to encode an in-frame fusion to the T7 tag peptide and place expression under regulation of the T7 promoter. Plasmid pL343.1 contains the HIV protease mutations K55N and L90M, and pL345.1 contains the HIV protease polymorphism L63P. These plasmids are otherwise identical to pL319.1. Replacement of the native HIV protease gene in pL319.1 was achieved by replacement of a 0.58-kb *Bam*HI-*Bsr*GI DNA segment with similar DNA sequences from a plasmid used for site-directed mutagenesis of the HIV protease gene.

Site-directed mutagenesis. Mutagenesis was carried out by mismatch primer extension with the Promega pALTER system. To compare the activities of the proteases from the mutants obtained by site-directed mutagenesis to the activity of HIV protease expressed from pL124.23 or from library plasmids (see below), we transferred the mutagenized DNA from pALTER mutagenesis vectors pL129.33 or pL141.2 to the pTrcHisC vector. These vectors contain precisely the same configuration of HIV Pol protein gene segment as pL124.23 or the library plasmid vectors.

Construction of the L191 library containing dispersed mutations within the HIV protease gene. The HIV protease gene including bases 2090 to 2576 (GBVRL:HIVHXB2CG numbering [GenBank]) was amplified by error-prone PCR (1, 2) to distribute an estimated average of two to three mutations within each HIV protease gene variant. The reverse transcriptase-integrase region of HIV protease comprising bases 2516 to 4644 (GBVRL:HIVHXB2CG numbering) was also amplified, but by using conditions favoring high-fidelity PCR. Ligation of the mutagenized protease and nonmutagenized reverse transcriptase genes was achieved by PCR. The ligated DNA was isolated from agarose gels (30), and restriction digestion was performed with the HIV *pol* gene native sites *Bgl*II and *Eco*RI to produce a 2.6-kb DNA segment containing the *pol* gene

sequence from bases 2096 to 4644 (GBVRL:HIVHXB2CG numbering). This segment was subcloned into the *Bgl*II- and *Eco*RI-cut expression vector pTrcHisC. In this vector, library expression is under IPTG-inducible regulation.

The configuration of HIV *pol* genes in the L191 plasmid library is identical to the configuration of genes for the pL124.23 plasmid except for the mutations distributed within the protease gene of the L191 library plasmids.

Western blotting. The protein from crude cell extracts was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12% homogeneous gels (Novex, San Diego, Calif.) with Tris glycine buffer (pH 8.3, containing 0.1% sodium dodecyl sulfate) at 125 V. Protein bands were then electrophoretically transferred to a nitrocellulose membrane with a Novex Xcell II Blot module according to the manufacturer's protocol. After blocking with 5% nonfat milk in phosphate-buffered saline, the blot was incubated overnight at 4°C with rabbit serum to HIV-1 reverse transcriptase (catalog no. 634; AIDS Research and Reference Reagent Program) (27) at a 1:2,000 dilution. The membrane was then treated with protein A-peroxidase conjugate (Sigma, St. Louis, Mo.) at a 1:800 dilution at room temperature for 1 h. Reactive protein bands were then visualized by staining the membrane with 3,3'-diaminobenzidine-cobalt chloride solution.

Microbe-based assay system. Colonies of clones of *E. coli* Top10 (Invitrogen) containing either plasmid pL124.23 or a plasmid from the L191 HIV protease gene variant library were picked from solid Luria-Bertani (LB) medium supplemented with ampicillin (200 μ g/ml) and inoculated into 20 μ l of LB medium containing ampicillin and indinavir (49.31 μ g/ml), and the culture was incubated at 30°C overnight with shaking.

The next day 1 μ l of each overnight culture was inoculated into 20 μ l of LB medium supplemented with ampicillin and indinavir (at the indicated concentration), and the culture was incubated for 4 h with shaking at 30°C. The cultures were then induced by the addition of 1 μ l of 1 M IPTG and incubation was continued for 3 h. The cultures were then frozen at -80°C for 20 min and thawed at 30°C for 10 min. This freeze-thaw procedure was repeated three additional times. Following the final thaw the cultures were centrifuged at 10,000 \times g for 7 min. The 20 μ l of supernatant was then assayed for reverse transcriptase activity with the Boehringer Mannheim reverse transcriptase assay nonradioactive kit (Boehringer Mannheim GmbH, Mannheim, Germany). The incubation period for this ELISA was 3 h. The V_{max} (in milli-optical density units per minute), a measure of HIV reverse transcriptase activity in the cell extracts, was measured with a Thermo max microplate reader (Molecular Devices, Sunnyvale, Calif.) at 30°C.

Isolation and assay of HIV protease. *E. coli* BL21(DE3)pLysS (34) was the host for the pET21c plasmid which contains an insert (GBVRL:HIVHXB2CG numbering; bases 2096 to 2833) coding for the native HXB2 protease or mutant proteases. A single colony of the clone of interest was inoculated into 10 ml of LB medium supplemented with ampicillin (200 μ g/ml), and the culture was incubated overnight at 30°C. The next day the culture was diluted 50-fold into fresh medium and was incubated with shaking at 37°C to an A_{600} of 0.4 to 0.6. Expression of the protease was then induced by the addition of IPTG to a final concentration of 1 mM, and incubation was continued for 3 h. The cells were then harvested, and the paste was frozen at -80°C until processing. All steps of protease purification were carried out at 4°C. The protease was isolated from inclusion bodies essentially by the procedure described previously (19), and the protease activity was measured as described previously (24). The enzyme sample was incubated at 37°C in morpholinoethanesulfonic acid (MES) buffer (50 mM; pH 6; containing 1 mM EDTA, 1 mM dithiothreitol, 200 mM NaCl, and 0.1% Triton X-100) with the substrate AcSQNPYV-NH₂ at 1.18 mM. The product, AcSQNY, was separated on a reverse-phase high-pressure liquid chromatography column (250 by 4.6 mm; Alltech, Deerfield, Ill.), and quantitation was by determination of the area under the curve of the identified product peak.

For determination of 50% inhibitory concentrations (IC_{50} s), inhibitor stock solutions were prepared in 50% ethanol. The enzyme sample and inhibitor were incubated at ambient temperature in MES buffer for 10 min prior to the addition of substrate, and the complete reaction mixture was incubated at 37°C (the final concentration of ethanol in the reaction mixture was 2.5%). Samples were taken at the indicated times, and the quantity of product formed was determined by high-pressure liquid chromatography analysis. The equation $IC_{50} = (V \times 1) / (V_0 - V)$, where I is the inhibitor concentration, V is the initial hydrolysis rate in the presence of inhibitor, and V_0 is the initial hydrolysis rate in the absence of inhibitor, was used to calculate the IC_{50} (18).

Infected PBMC culture studies. pLN4-3-infected peripheral blood mononuclear cell (PBMC) culture studies were done in the laboratory of Douglas Richman (28).

RESULTS

Western blots and immunodetection of processed reverse transcriptase. Plasmid pL124.23 expresses an HIV gene segment that includes the entire protease and reverse transcriptase genes and that also includes DNA encoding the 52 amino acids of the Gag polyprotein preceding the N terminus of the protease and 141 amino acids of the integrase following the C

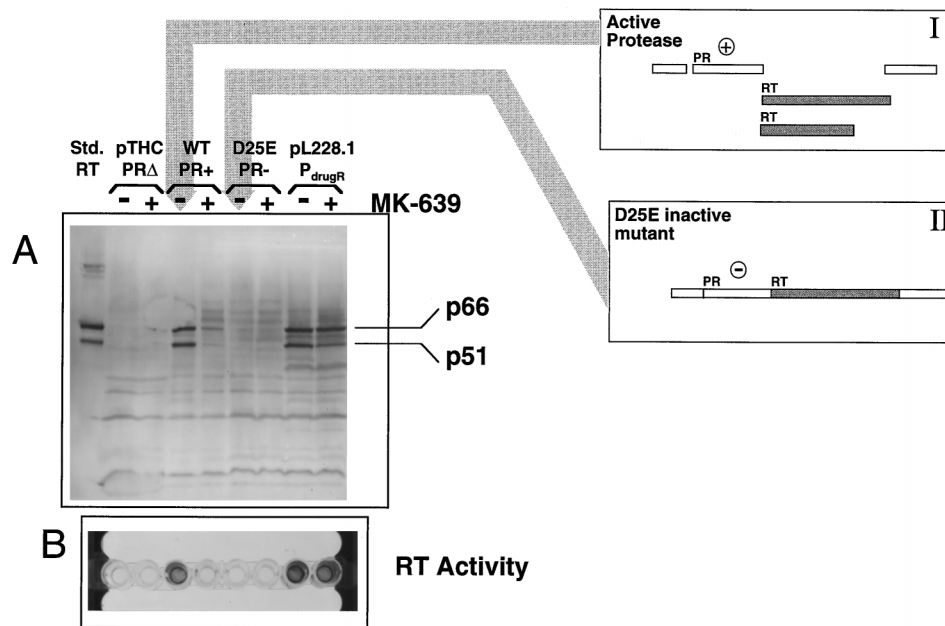


FIG. 1. HIV protease (PR) processing of the *E. coli*-expressed polyprotein segment, reverse transcriptase (RT) activity, and inhibition by indinavir (MK-639). The basis for the *E. coli* expression assay for HIV protease activity is that, to achieve a high level of reverse transcriptase activity, HIV protease must be active to excise reverse transcriptase from the *E. coli*-expressed polyprotein segment. Extracts from *E. coli*-expressed protease-reverse transcriptase polyprotein segments were examined by Western blot analysis, and reverse transcriptase activity was examined by ELISA. The lanes of the Western immunoblot are labeled as follows: pTHC, pTrcHisC (Invitrogen), the expression plasmid without HIV DNA sequences; WT, plasmid pL124.23, which is pTrcHisC containing native HXB2 HIV protease-reverse transcriptase sequences; D25E, plasmid pLD25E, which contains the HIV protease portion of the protease-reverse transcriptase polyprotein that expresses a D25E-substituted, inactive HIV protease; pL228.1, which expresses a protease region with four substitutions encoding M46I, L63P, V82T, and I84V. Plasmids pLD25E and pL228.1 are identical to pL124.23 except for mutations within the protease-encoding DNA. Western blot analysis and the ELISA for reverse transcriptase activity are described in the Materials and Methods section of the text. (A) Western blot analysis. Polyclonal anti-HIV reverse transcriptase antibody was used to detect *E. coli*-expressed antigen. The reverse transcriptase standard displays the expected two antibody-reacting bands, p51 and p66, in approximately equimolar amounts. Samples to which indinavir was not added display these bands for *E. coli* extracts from cells expressing native protease (wild type) and drug-resistant (pL228.1) protease. The bands are not detected for the polyprotein segment containing the D25E inactive protease. For native (wild-type) protease (WT lane), the addition of indinavir prevents the appearance of the p51 and p66 bands. In contrast, for the drug-resistant genotype sample, pL228.1, the p51 and p66 bands appear, despite incubation of expressing cells in the presence of MK-639. (B) Reverse transcriptase ELISA. Cells containing the described plasmids were grown in 20- μ l aliquots of medium with or without indinavir and were induced with IPTG. Following permeabilization of the cells by freeze-thaw cycles and centrifugation, the 20- μ l supernatants were assayed for RT activity. Reverse transcriptase activity is indicated by dark coloration in the photograph. Clones containing the pTrcHisC construct lacking the HIV sequences (plasmid without insert) did not exhibit reverse transcriptase activity. A solution of buffer (data not shown) also gave a negative result. Assay of the supernatant from induced cells containing pL124.23 with the described HIV sequences and grown in medium without indinavir revealed high levels of reverse transcriptase activity. Supernatants from these cells grown in the presence of indinavir contained little detectable reverse transcriptase activity. However, indinavir does not prevent the indication of reverse transcriptase activity from pL228 expressing a drug-resistant protease. For the inactive protease mutant D25E, no reverse transcriptase activity is detected. High levels of RT activity correspond with the appearance in the Western immunoblot (A) of the p51 and p66 RT heterodimer bands.

terminus of the p66 reverse transcriptase. Others had previously established that truncated versions of the Gag-Pol polyprotein, although in different expression vectors, were expressed and processed in *E. coli* (3, 21). The Western blot in Fig. 1A indicates that this is also true with the construct described here. Extracts of induced cells expressing the pL124.23 polyprotein segment display antigens that comigrate with the correctly processed HIV-1 reverse transcriptase antigens p66 and p51. Plasmid pLD25E expresses the same polyprotein segment as described for pL124.23, except that for pLD25E, site-directed mutagenesis was used to replace the active-site aspartate at position 25 with glutamate. *E. coli* extracts expressing the pLD25E variant do not show the p66 and p51 antigens of the reverse transcriptase. Rather, higher-molecular-weight protein bands become apparent. When the host cells expressing plasmid pL124.23 (native HIV-1 protease) are grown and induced in the presence of indinavir, the reverse transcriptase antigens p66 and p51 are not observed in the Western blot, consistent with effective inhibition of processing of polyprotein by this protease inhibitor.

Bacterium-expressed HIV protease can indicate evidence of drug resistance. Plasmid pL228.1 expresses a highly drug resistant protease variant within the same polyprotein segment

as described for pL124.23. For pL228.1, site-directed mutagenesis was used to insert DNA substitutions encoding the four protease amino acid substitutions, M46I, L63P, V82T, and I84V, which are associated with high-level resistance to indinavir and other protease inhibitors (4, 5). The Western blot analysis results presented in Fig. 1A indicate that extracts from cells induced for expression from pL228.1 contain the correctly processed p51 and p66 reverse transcriptase antigen bands for cells grown without indinavir and also for cells grown in the presence of indinavir. For expression from pL124.23 (native protease), indinavir prevents processing of detectable reverse transcriptase antigen bands, but for pL228.1, which expresses drug-resistant protease, indinavir fails to inhibit polyprotein processing, and correctly processed reverse transcriptase antigen bands are readily detectable.

Although Western blotting can be used to detect protease activity, an ELISA is more adaptable to high-throughput screening. Therefore, we examined the usefulness of a commercially available HIV reverse transcriptase assay system (Boehringer Mannheim GmbH) for detection of HIV reverse transcriptase activity as an indication of proper processing of the polyprotein.

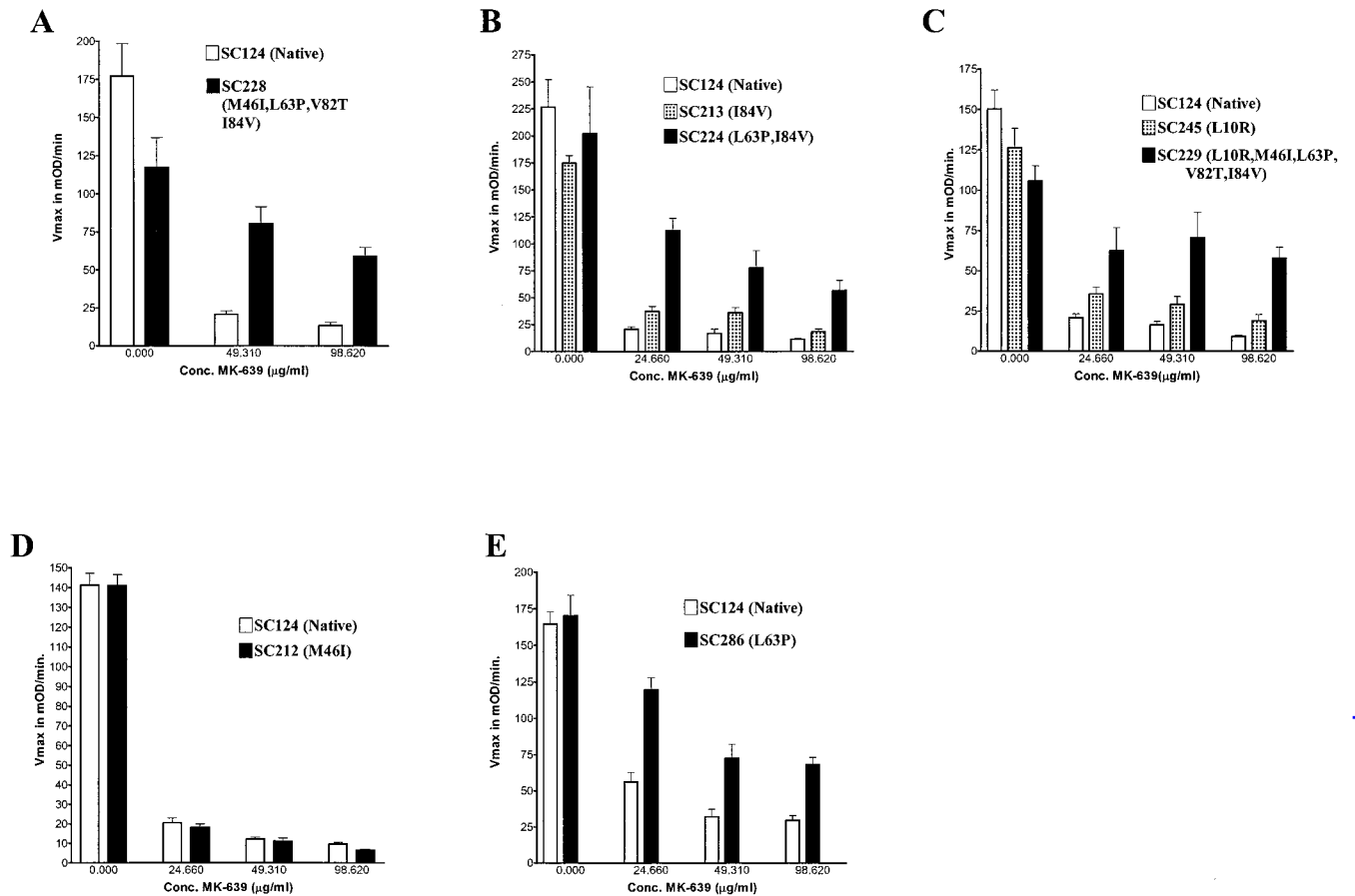


FIG. 2. *E. coli* expression assay: sensitivities of HIV protease variants to indinavir (MK-639). HIV protease variants obtained by site-directed mutagenesis and library-discovered HIV protease variants were assayed for the resistance contribution of single and combined mutations. Mutant HIV protease genes are configured to be a portion of a polyprotein gene segment as described for plasmid pL124.23. A reverse transcriptase ELISA was used to determine reverse transcriptase activity as an indirect indication of HIV protease processing efficiency, as described in the Materials and Methods section. HIV protease-expressing *E. coli* cells were grown in different concentrations of indinavir as indicated on the x axes. Reverse transcriptase activity is represented as V_{max} . Six separate determinations for each protease construct at each inhibitor concentration were averaged to derive the results described above. (A) Native HIV protease and HIV protease with the M46I, L63P, V82T, and I84V substitutions. (B) Native HIV protease I84V-substituted HIV protease and L63P-I84V-substituted HIV protease. (C) Native HIV protease, L10R-substituted HIV protease, and HIV protease with the L10R, M46I, L63P, V82T, and I84V substitutions. (D) Native HIV protease and M46I-substituted HIV protease. (E) Native HIV protease and L63P-substituted HIV protease.

ELISA for HIV reverse transcriptase activity indicates HIV protease activity. Extracts from cells expressing the HIV polyprotein segments from plasmids pL124.23 (native protease), pLD25E (inactive protease), and pL228.1 (drug-resistant protease) were assayed for reverse transcriptase activity as described in Materials and Methods. All of these plasmids contain DNA for expression of the same HIV polyprotein segment, but each has different mutations within the HIV protease gene. Extracts from control vector pTHC (pTrcHisC) containing no HIV DNA were also assayed. Reverse transcriptase activity is detected from extracts of cells induced for expression from plasmids pL124.23 (native protease) and pL228.1 (drug-resistant HIV protease). These reverse transcriptase activity levels are much higher than the levels detectable from cells induced for expression from pLD25E (inactive protease). Furthermore, growth of cells in the presence of indinavir significantly reduces the level of detectable reverse transcriptase activity for expression from pL124.23. In contrast, indinavir has a pronouncedly lower effect on the reverse transcriptase activities of extracts from cells expressing protein from pL228.1 (drug-resistant HIV protease). Protease expressed from pL228 contains the four resistance-associated mutations M46I, L63P, V82T, and I84V. The data suggest that

indinavir is less effective at inhibiting pL228 protease containing these four substitutions than it is at inhibiting the native HXB2 protease. Thus, protease amino acid substitutions that resulted in indinavir resistance in intact virus in both clinical and cell culture isolates also appear to lower the sensitivity of the protease to indinavir in our simplified assay system.

Western blot analysis of polyprotein processing and ELISA for reverse transcriptase activity indicate correspondence between reverse transcriptase maturational processing and detected reverse transcriptase activity for polyproteins containing different genotype proteases in the absence or presence of the protease inhibitor (Fig. 1B).

In the absence of indinavir the average reverse transcriptase activity observed with the native sequences is about 1.5 times that observed with the mutant protease sequences (Fig. 2A). This is in agreement with earlier observations (9) that proteases with these substitutions are catalytically less active than the nonmutated protease. In the presence of indinavir, on the other hand, the amount of recovered processed reverse transcriptase activity is pronouncedly greater for the multiply mutated protease than for the native protease. Under conditions of inhibition by indinavir, the *E. coli*-expressed drug-resistant protease variant shows a greater ability to process polyprotein

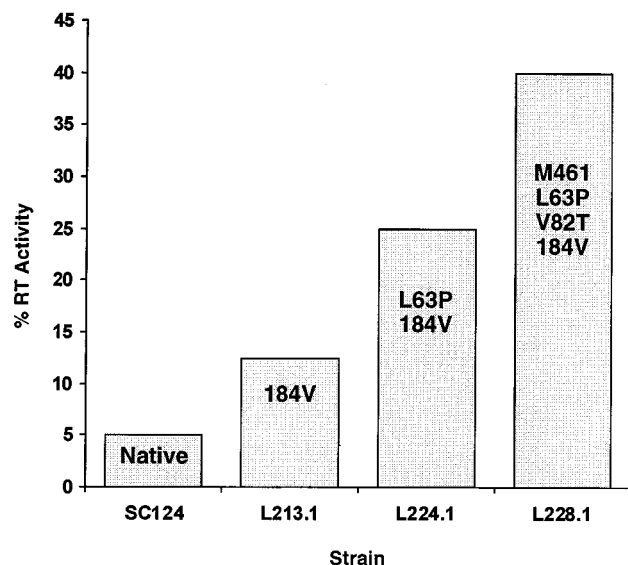


FIG. 3. *E. coli* expression assay: effect of additive substitutions on the susceptibility to indinavir of HIV protease variants. Site-directed mutagenesis was used to construct HIV HXB2 protease variant genes which were inserted into the *E. coli* expression vectors as a portion of a polyprotein gene segment as described in the text for the plasmid pL124.23. Cells were grown in the presence of 99 mg of indinavir per ml, as described in the text. The reverse transcriptase ELISA was used to determine activity as described in Materials and Methods. The reverse transcriptase (RT) activity from drug-treated cells was calculated as a percentage of the activity from cells grown without drug. Each assay was repeated at least six times, and averaged readings from a Molecular Devices plate reader are depicted. Incremental loss of sensitivity clearly coincides with additive effects for this set of mutations.

than the native variant, and this activity is reflected in the degree of activation of reverse transcriptase.

Protease genotype and reverse transcriptase activation. In order to determine the influence of single and specific combined HIV protease mutations on drug susceptibility, site-directed mutagenesis was used to construct a number of protease variants of known genotype. These mutant proteases were placed in expression vectors as portions of polyprotein gene segments identical to those in the pL124.23 expression construct. The reverse transcriptase ELISA was used to determine protease processing of reverse transcriptase at different concentrations of indinavir. Since the reverse transcriptase ELISA is catalytic, we expect a high degree of sensitivity to different protease activity levels. The results are summarized in Fig. 2 and 3.

The indinavir sensitivities of the HIV mutant proteases containing only the I84V substitution in the protease region or the combination of L63P and I84V were compared with that of the native protease (Fig. 2B). The mean activities of the constructs differ in the absence of inhibitor, with the native protease showing the highest level of activity in our assay system; in the presence of inhibitor the reduced sensitivities of proteases with the I84V substitution or the combination of the L63P and I84V substitutions are readily apparent. The reverse transcriptase ELISA shows decreased sensitivity to indinavir associated with the L63P substitution alone (Fig. 2E), and the possible effects of this polymorphism with respect to protein turnover are discussed below. It should be noted that the L63P variant by itself had higher than expected resistance in our assay. This may be related to the observation that the L63P variation, unlike the other resistance-conferring mutations that we have examined, does not decrease the activity of the uninhibited

protease. Both the I84V and L63P substitutions are commonly found, *inter alia*, in indinavir-resistant clinical isolates from patients who had undergone indinavir monotherapy. It must be noted that relatively high levels of indinavir are used in these experiments. This is required because of the poor permeation of this inhibitor into *E. coli* cells.

The L10R substitution is another substitution that is identified (4) as being strongly associated with indinavir resistance in clinical isolates, and as shown in Fig. 2C, this single substitution confers a marginal reduction in indinavir sensitivity in our assay system. When combined with the four substitutions M46I, L63P, V82T, and I84V, an even greater decrease in indinavir sensitivity was observed.

Strong association with drug resistance does not indicate that a particular substitution alone confers decreased affinity to the target protein. As shown in Fig. 2D, variant HIV protease with the M46I substitution, another substitution strongly associated with resistance to indinavir (5), ABT-538 (23), and VX-478 (25), did not exhibit decreased sensitivity in our assay system. Others (9, 32) have also noted in cell culture studies with intact virus as well as studies with the isolated enzyme that this single substitution did not significantly alter sensitivity to indinavir. The agreement between the findings of Gulnik et al. (9) and Schock et al. (32) serves to validate the relevance of the *E. coli* expression-based assay described here.

Although certain single substitutions in the HIV protease confer decreased sensitivity to indinavir, it is the accumulation of substitutions that confers higher levels of resistance. If the differences in the activities of the various constructs in the absence of inhibitor are taken into account, then the buildup of resistance due to combination of substitutions is observed (Fig. 3). The combination of the four substitutions M46I, L63P, V82T, and I84V in the protease is sufficient to confer detectable indinavir resistance upon intact virus in cell culture studies, whereas the single or double substitutions do not (5). In this sense, the *E. coli* expression-based assay system, which uses only a portion of the Gag-Pol polyprotein, is more sensitive to single amino acid substitutions than intact viral assay systems. At the same time, the additive effects of certain mutations by the *E. coli*-based assay reflect properties of cell culture-based systems for the evaluation of resistance.

Screening a library of protease variant genes. HXB2 protease sequences were mutagenized by error-prone PCR, reintroduced into expression vectors, and introduced into *E. coli* Top10 cells (Invitrogen) as described in Materials and Methods (Fig. 4). Between 25 and 50% of the mutagenized proteases remained active following mutagenesis, as judged by the recovery of active reverse transcriptase activity following induction of expression of the truncated polyprotein. Individual clones containing mutagenized protease variants were grown in the presence of indinavir, induced for HIV polyprotein segment expression, and assayed for reverse transcriptase activity. Because indinavir inhibits the HIV protease, most extracts contain little detectable reverse transcriptase activity. On the other hand, if the protease variant were resistant to indinavir we would expect polyprotein processing to occur, despite the presence of the protease inhibitor. In this case, reverse transcriptase is expected to display activity.

Figure 5 shows a photograph of the ELISA plate in which the first HIV protease mutant with decreased sensitivity to indinavir was found. Sequencing of the protease region revealed that this mutant protease, DLH310, contained two substitutions, K55N and L90M. The L90M substitution in the protease region has been reported to be present in virus mutants resistant to a number of protease inhibitors including indinavir (5, 5a, 23). What is more, studies with the isolated

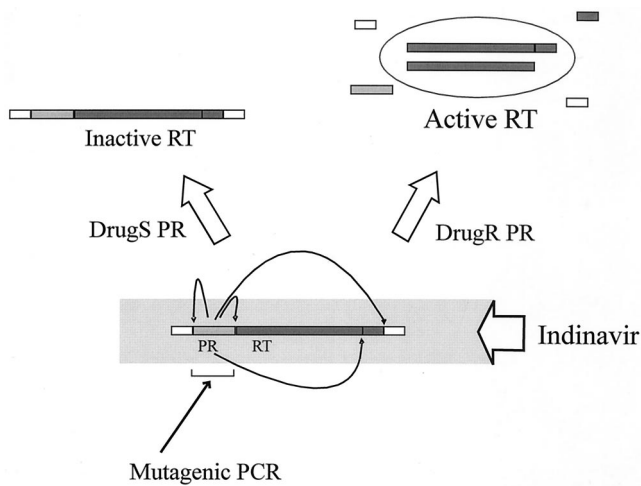


FIG. 4. Construction and screening of a library of HIV protease (PR) variants. Error-prone PCR was used to introduce dispersed mutations within the HIV protease gene. This mutagenized population of DNAs was ligated into the *E. coli* expression vector pTrcHisC (Invitrogen) along with unmutagenized DNA encoding the entire HIV reverse transcriptase (RT) gene. This plasmid library was used to transform *E. coli* Top10 cells (Invitrogen), and single colonies were distributed into individual microplate wells containing growth media and the protease inhibitor indinavir. IPTG was added to induce expression, cells were made permeable by freezing and thawing, and reverse transcriptase activity was assayed as described in Materials and Methods. DrugS, drug susceptible; DrugR, drug resistant.

protease containing the L90M substitution (22) show that this protease variant has a decreased affinity for indinavir, and that result agrees with those of our own studies with the isolated protease (see below). The protease variant containing the

K55N and L90M substitutions was purified and compared with the isolated, native HXB2 protease for sensitivity to indinavir. The IC₅₀ for the protease with the substitutions was determined to be 7.76 nM when assayed at pH 6.0 with AcSQNYP VV-HH₂ as the substrate, while an IC₅₀ of 2.27 nM was obtained for the native protease. Thus, as indicated by the microbial screen, the affinity of this protease variant for indinavir is decreased relative to that of the parent HXB2 protease.

In a screening of 12,000 isolates we discovered 12 variants with decreased affinity for indinavir (Tables 1 and 2). Six of the substitutions, at positions 24, 54, 63, 64, and 90, have been reported to be statistically associated with indinavir resistance in clinical isolates (4).

The substitution W6R is, in our reverse transcriptase ELISA, clearly associated with decreased indinavir sensitivity (this substitution was found in three of the isolates) and may represent an artifact of this system since it has not been reported by others to play a role in resistance to protease inhibitors. A possible role of this substitution and others in protein stabilization is considered in the Discussion section.

Stability of proteases with the L63P or the K55N and L90M substitutions. The L63P substitution is strongly associated with clinical resistance to indinavir, and this same substitution is associated with a lower level of sensitivity to indinavir in our assay system (Fig. 2E). It has been reported that the L63-I64 peptide bond is a junction of autolysis for purified HIV protease (29). Amino acid substitutions at this junction could possibly reduce autolysis and in this way increase enzyme stability. Accordingly, we compared the stabilities at 37°C of protease variants containing the L63P substitution or the K55N and L90M substitutions with that of the native protease (Fig. 6). Indeed, the L63P substitution appears to dramatically sta-

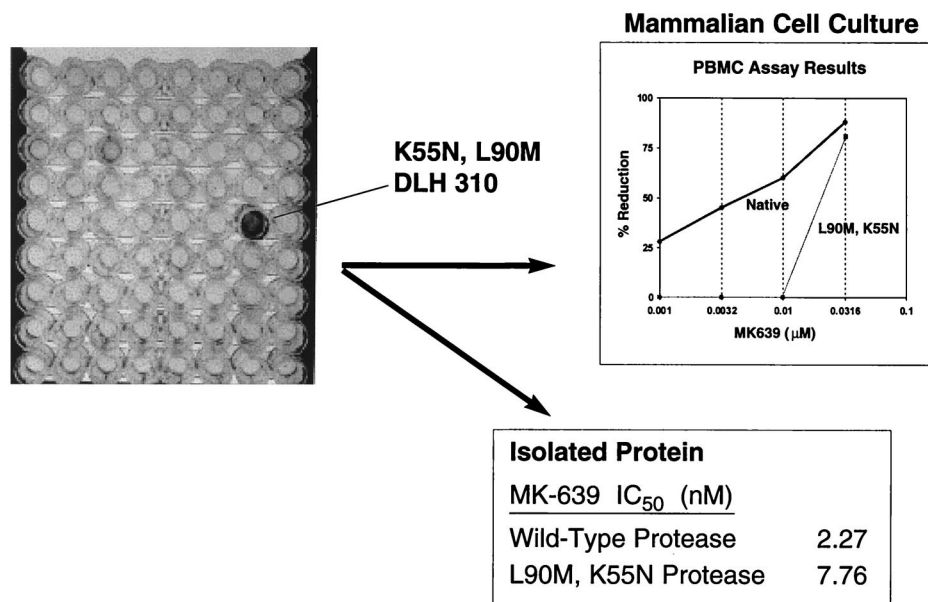


FIG. 5. Identification of the HIV protease variant with K55N and L90M substitutions from a library of protease variant genes by the reverse transcriptase ELISA-based screen. The reverse transcriptase ELISA was carried out in a 96-well microplate format. Each well contains material expressed by an individual *E. coli* colony that expresses a plasmid from a library of HIV protease variant genes. Individual colonies from the library were grown in microplate wells in the presence of indinavir (see Materials and Methods). For the *E. coli*-expressed protease-reverse transcriptase polypeptide segments, efficient activation of reverse transcriptase requires the activity of the HIV protease (Fig. 1). One microplate well shown above indicates a high level of reverse transcriptase activity. Since indinavir is expected to inhibit HIV protease activation of reverse transcriptase, only a protease variant with lowered susceptibility to inhibitor is expected to efficiently activate reverse transcriptase in the presence of inhibitor. The identified HIV protease variant was determined to contain the substitutions K55N and L90M. The selected protease gene containing the K55N- and L90M-encoding mutations was transferred to the vector pET21c for expression of protein for isolated protease experiments and to the vector pNL4-3 for virus-infected cell culture studies. The IC₅₀ for the protease isolated from the variant with the K55N and L90M substitution was heightened, and by using pNL4-3 infected PBMCs, the variant showed decreased susceptibility to indinavir (MK-639) (PBMC culture studies were done in the laboratory of Douglas Richman).

TABLE 1. Amino acid substitutions in HIV-1 HXB2 protease with lowered susceptibility to indinavir discovered by screening of 12,000 HIV protease variants in *E. coli*^a

Variant	Substitution at the following position:														Reference(s)
	6 (W) ^b	14 (K)	24 (L)	43 (K)	53 (F)	54 (I)	55 (K)	63 (L)	64 (I)	70 (K)	82 (V)	85 (I)	90 (L)	91 (T)	
DLH310							N						M		5
10K283	R														29
DLH1863	R														29
DLH3218	R				Y										29
DLH3519				T	L										17, 35
DLH7956														A	16
DLH8860													M		5
DLH8968			I				P		R						5
DLH9423	E											T			5
10K201								V			A				5
10K707						T									5
10K711					I										

^a The screening in *E. coli* described in the legend to Fig. 1 was used to search a library of HIV protease variants. A total 12,000 colonies were screened for HIV protease-dependent reverse transcriptase activity in the presence of indinavir. Those colonies which expressed indinavir-resistant HIV protease variants were retested, and for those that again showed resistance, the DNA was sequenced and the inferred amino acid substitutions were entered into the table. Amino acid substitutions indicated in boldface type coincide with resistance-conferring genes found in either clinical isolates resistant to indinavir (5) or mammalian cell culture resistance to other protease inhibitors (16, 17, 34). Note that one or two amino acid substitutions are sufficient to show significant decreased sensitivity to indinavir (also see Fig. 2 and 3). This is in marked contrast to the results of mammalian cell culture studies, in which the appearance of indinavir resistance requires at least three amino acid substitutions (5).

^b Letters in parentheses indicate the native amino acids.

bilize the protease relative to the stability of the native protease (half-lives, 28 versus 7 h) and especially relative to the stability of the mutant protease containing the combination of substitutions K55N and L90M (half-life, 2.8 h). Thus, these data suggest that one of the important roles of the L63P substitution could be to stabilize mutant proteases. Some suggestion of this is given in Fig. 3, in which it is seen that protease containing the L63P and I84V substitutions in combination is more active, i.e., more effectively processes the polyprotein substrate, in the absence of inhibitor than the protease variant with only the I84V substitution.

DISCUSSION

The HIV protease has been a prime target of drugs that have been developed for the past decade; and a number of highly specific, high-affinity inhibitors have been developed, including saquinavir, ritonavir, indinavir, and nelfinavir (6), and have been approved for clinical use. These compounds, when used at a high dosage, strongly repress virus levels in plasma and stimulate a concurrent rise in CD4⁺ counts. Monotherapy with each of these inhibitors (indinavir [5], saquinavir [31], ritonavir [23], amprenavir [Vx-478] [7], and nelfinavir [26]) results in the appearance of resistant variants, leading to a rise in blood viral RNA levels and a decrease in CD4⁺ counts. It is this problem of resistance to the protease inhibitors that prompted us to develop the screening system that we describe here.

We felt that there was a clear need for a simple, sensitive, nonbiased screening or selection system that would allow the rapid detection of amino acid substitutions in the HIV protease that would give rise to resistance to a particular protease inhibitor.

We used a recombinant *E. coli* system in which a segment of the HIV Gag-Pol polyprotein gene was under control of a regulated promoter. Induction led to expression of a protein consisting of six histidine residues, 52 amino acids of the Gag protein, the entire protease and reverse transcriptase sequences, and 141 amino acids of the integrase. This truncated version of the Gag-Pol polyprotein was found to be properly processed in the host cell, resulting in easily detectable (by an ELISA) HIV reverse transcriptase activity. Protease inactivated by site-di-

rected mutagenesis (D25E) or through the inclusion of indinavir in the growth medium did not process the polyprotein, and reverse transcriptase activity was not found. This system provides for the rapid colorimetric assay of active HIV protease while using a set of substrates that is similar to the natural substrates for the enzyme.

Using site-directed mutagenesis we constructed a protease containing the multiple substitutions M46I, L63P, V82T, and

TABLE 2. *E. coli* expression screen and 11 clinically identified resistance-conferring mutations^a

Amino acid position contributing to clinical resistance to indinavir	Variation found in screen of <i>E. coli</i> expression library	Decreased susceptibility by site-directed mutagenesis
10		Yes
20		ND ^b
24	Yes	
46		No
54	Yes	
63	Yes	
64	Yes	
71		ND
82	Yes	
84		Yes
90	Yes	

^a The 11 positions at which amino acid substitutions were identified to contribute to clinical resistance to indinavir are indicated (5). All high-level-resistant protease variants contain substitutions at either of two positions, position 82 or 90 (5). The amino acid positions which were found to be altered by using the reverse transcriptase assay to screen 12,000 *E. coli* library colonies and which match the amino acid substitutions in the resistant clinical isolates are also indicated. The results of site-directed mutagenesis experiments to construct protease variant genes which contain specific amino acid substitutions are also presented. These altered HIV protease genes were inserted into the *E. coli* expression vector pL124.23 to enable us to report protease activity as a function of reverse transcriptase activity. Decreased susceptibility was detected by the reverse transcriptase ELISA. By the *E. coli* expression assay, the screen identified 6 of the 11 previously identified positions which are known to be associated with clinical resistance to indinavir. Substitutions at 2 more of the 11 positions, positions 84 and 10, also show lowered susceptibility using the *E. coli* based assay. A substitution at position 46 does not show altered susceptibility by the *E. coli*-based screen.

^b ND, not done.

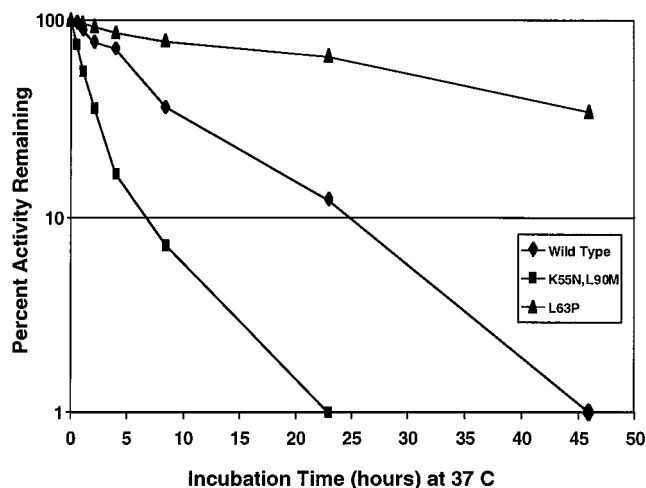


FIG. 6. Isolated HIV protease containing the L63P polymorphism displays increased thermostability. The L63P polymorphism contributes to high levels of resistance to indinavir in clinical and cell culture studies (4, 5). Using site-directed mutagenesis we constructed HIV protease containing the L63P substitution. The variant protease as well as the native protease and the L90M-K55N variants were expressed by using the pET21c vector, and isolated protein was examined for activity after incubation at 37°C. The graph indicates the greater stability of the L63P variant compared to the stabilities of both the wild-type and the L90M and K55N double mutant. It is interesting that an autolytic junction within the HIV protease is located between residues L63 and I64 (29). This suggests the possibility that the L63P polymorphism could contribute to drug resistance by extending the protein half-life through decreased autolysis at the amino acid junction at positions 63 and 64.

I84V. These substitutions have been found in viral clinical isolates which are resistant to indinavir and have been demonstrated to confer indinavir resistance on HIV in cell culture (5). In our assay system, constructs containing these substitutions in the protease portion of the truncated Gag-Pol polyprotein produce a protease, upon induction, with reduced sensitivity to indinavir (as indicated by elevated levels of reverse transcriptase activity). Thus, for the protease variant containing these four substitutions, the results obtained with the recombinant microbe assay reflect the results obtained with clinical isolates and in cell culture systems.

Multiple amino acid substitutions are required for indinavir resistance to be apparent in cell culture studies or in clinical isolates (5). In the case of our microbial screen, however, single or double substitutions are sufficient for resistance to become evident. For example, the I84V substitution is one of the mutations associated with indinavir resistance in clinical isolates, but this single substitution is not sufficient to confer detectable resistance in cell culture studies (5). In this sensitive microbial screen, however, this single amino acid substitution clearly decreases the sensitivity of the HIV protease to indinavir (similar results are obtained with the single substitutions L10R and L63P). Using the isolated enzyme variant with the I84V substitution, we and others (9) have shown that this protease variant exhibits lowered sensitivity to indinavir (and other protease inhibitors), even though this decreased sensitivity is not apparent in cell culture studies (5). The combination of the substitutions L63P and I84V confers an even greater decrease in sensitivity to indinavir, although again, in cell culture studies this combination of substitutions does not confer detectable resistance on the intact virus. Thus, one of the advantages of this recombinant microbe-based system, in addition to authenticity, is sensitivity; single or double substitutions that do not confer sufficient levels of resistance to be detectable in cell culture are readily apparent by this simple recombinant mi-

crobe-based assay. What is more, the simple additive effect of substitutions can easily be demonstrated with this system.

The single substitution M46I does not in itself confer a detectable decrease in sensitivity to indinavir in our system. Furthermore, the indinavir IC_{50} for isolated protease containing this substitution is not elevated. Thus, the role of this substitution in conferring elevated resistance to indinavir must become apparent only in the presence of other substitutions. Indeed, others have shown that this substitution alone confers no detectable resistance on intact virus or reduced sensitivity on the isolated enzyme to a variety of protease inhibitors including indinavir (5, 23, 25, 26) but is strongly associated with resistance to a number of protease inhibitors.

In our screen for variants resistant to indinavir, we discovered isolates DLH310 (with the K55N and L90M substitutions) and DLH8860 (with the L90M substitution). Interestingly, the L90M substitution is one of two mutations either of which was always associated with resistance to indinavir in one study (5). This substitution alone decreases the sensitivity of the protease to indinavir but also could possibly destabilize the protease since the stability of the protease appears to be adversely affected by this substitution.

The limited screen (12,000 mutated variants, between 25 and 50% of the proteases of which retained activity) that we carried out to detect resistance-conferring substitutions resulted in the isolation of 12 protease variants that appeared to exhibit decreased sensitivity to indinavir. The amino acid substitutions of these protease variants selected for reduced susceptibility to indinavir are presented in Tables 1 and 2. Included among these isolates were the mutations L90M, I54T, V82A, I64V, L63P, and L24I. This represents 6 of the 11 substitutions reported as being strongly associated with resistance (4). Our site-directed mutagenesis studies demonstrated that the L10R and I84V substitutions conferred a detectable decrease in indinavir sensitivity, and thus, if these were present in our screen, they would have been detected (in subsequent screens for resistance to indinavir in combination with other inhibitors [not described in this paper], the I84V substitution did indeed appear). The M46I substitution was not detected in our screen, but as mentioned above, this substitution alone confers only a slight detectable alteration in the enzyme (9). We have not observed substitutions at codon 71 or 20, which have been reported to be correlated with indinavir resistance, and have not as yet examined the substitutions at these positions using site-directed mutagenesis experiments. Substitutions that are associated with indinavir resistance in the *E. coli*-based assay but that are not reported to be common in indinavir-resistant clinical isolates include F53Y, -L, or -I, K55N, and T91A. Substitutions at positions 53 and 91 have been reported to be associated with resistance to other, similar protease inhibitors (16, 17, 34).

The W6R substitution appears to confer lowered indinavir susceptibility in our screen. It is interesting that the junction at positions L5 and W6 appears to be a site for HIV autolysis (29). A possible mechanism by which the W6R substitution appears to result in drug resistance would be by decreasing autolytic protease turnover. The contribution of this type of mechanism to clinical resistance with regard to polymorphisms at position 63 is discussed below. Other substitutions that are common in our screen, at F53 (I, L, or Y), could also effect autolysis. Rose and coworkers (29) report that, for HIV-2 protease, the G52 and F53 amino acids comprise an autolysis junction. The amino acid sequences surrounding G52 and F53 are highly similar for HIV-1 and HIV-2, and the amino acid numbering corresponds for the two strains; i.e., residues 52 and 53 are G and F, respectively, for both HIV-1 and HIV-2.

Substitutions at amino acid 53 have not reported by others to be associated with indinavir resistance, although substitutions at this codon do seem to be involved in resistance to other protease inhibitors (17, 34).

The L63P substitution, alone or in combination with other mutations, decreases the sensitivity of the protease to indinavir. We have presented data demonstrating that this substitution decreases the IC_{50} of indinavir for the protease, and we have also demonstrated that this substitution increases the thermostability of the isolated protease at 37°C. This could contribute to drug resistance by decreasing protein turnover.

The *E. coli*-based system was designed to identify resistance-associated alterations which occur within the HIV protease. This cannot include all viral variations which contribute to drug resistance. For example, specific alterations within HIV protease substrates have been associated with increased levels of drug resistance (8, 35). These mutations appear to compensate for other resistance mutations within the protease that compromise catalytic activity. In general, critical resistance-conferring alterations of the HIV protease catalytic pocket result in a reduction in enzyme activity (9, 12, 15). This sets up a selection (or the requirement for a precondition) for mutations that partially restore the impaired activity of catalytically altered resistant mutants. Simplified systems for the identification of resistant variations, such as the one described in this paper, can be expected to identify critical pocket-altering mutations as well as the subset of compensatory mutations which occur within the protease.

In conclusion we have described a microbe-based HIV protease assay system that can be used to screen for mutant proteases that are resistant to a particular protease inhibitor. The system uses a truncated version of the Gag-Pol polypeptide, and many of the mutations that are associated with indinavir resistance in clinical isolates are also detected by this screening system. Cell culture systems used to select for indinavir-resistant HIV variants reveal only variants with multiple substitutions in the HIV protease. In the highly sensitive system described here, the effect of single substitutions can be ascertained. The mutations detected in this screening system have also been demonstrated to confer decreased sensitivity on the isolated HIV protease. Furthermore, single substitutions such as M46I, which do not confer lowered sensitivity in the microbial screen, also have no effect on the IC_{50} for the isolated enzyme. Finally, this simple recombinant assay system may be used to screen libraries of potential protease inhibitors for activity against native or mutant proteases.

REFERENCES

- Cadwell, R. C., and G. F. Joyce. 1994. Mutagenic PCR. *PCR Methods Appl.* 3:S136-S140.
- Cadwell, R. C., and G. F. Joyce. 1992. Randomization of genes by PCR mutagenesis. *PCR Methods Appl.* 2:28-33.
- Cheng, Y. S., M. H. McGowan, C. A. Kettner, J. V. Schloss, S. Erickson-Viitanen, and F. H. Yin. 1990. High-level synthesis of recombinant HIV-1 protease and the recovery of active enzyme from inclusion bodies. *Gene* 87:243-248.
- Condra, J. H., D. J. Holder, W. A. Schleif, O. M. Blahy, R. M. Danovich, L. J. Gabryelski, D. J. Graham, D. Laird, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, T. Yang, J. A. Chodakewitz, P. J. Deusch, R. Y. Leavitt, F. E. Massari, J. W. Mellors, K. E. Squires, R. T. Steigbigel, H. Teppler, and E. A. Emini. 1996. Genetic correlates of in vivo viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor. *J. Virol.* 70:8270-8276.
- Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Teppler, K. E. Squires, P. J. Deusch, and E. A. Emini. 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* 374:569-571.
- Craig, C., E. Race, J. Sheldon, L. Whittaker, S. Gilbert, A. Moffatt, J. Rose, S. Dissanayake, G. W. Chirn, I. B. Duncan, and N. Cammack. 1998. HIV protease genotype and viral sensitivity to HIV protease inhibitors following saquinavir therapy. *AIDS* 12:1611-1618.
- Deeks, S., M. Smith, M. Holodniy, and J. O. Kahn. 1997. HIV-1 protease inhibitors. A review for clinicians. *JAMA* 277:145-153.
- De Pasquale, M. P., R. Murphy, R. Gulick, L. Smeaton, J.-P. Sommadossi, V. Degruittola, A. Calendo, D. Kuritzkes, L. Sutton, A. Savara, and R. D'Aquila. 1998. Mutations selected in HIV plasma RNA during 141W94 therapy. *In Abstracts of the Fifth Conference on Retroviruses and Opportunistic Infections.*
- Doyon, L., G. Croteau, D. Thibeault, F. Poulin, L. Pilote, and D. Lamarre. 1996. Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. *J. Virol.* 70:3763-3769.
- Gulnik, S. V., L. I. Suvorov, B. Liu, B. Yu, B. Anderson, H. Mitsuya, and W. Erickson. 1995. Kinetic characterization and cross-resistance patterns of HIV-1 protease mutants selected under drug pressure. *Biochemistry* 34:9282-9287.
- Hedge, P. J., and B. G. Spratt. 1985. Amino acid substitutions that reduce the affinity of penicillin-binding protein 3 of *Escherichia coli* for cephalixin. *Eur. J. Biochem.* 151:111-121.
- Hedge, P. J., and B. G. Spratt. 1985. Resistance to beta-lactam antibiotics by remodelling the active site of an *E. coli* penicillin-binding protein. *Nature* 318:478-480.
- Ho, D. D., T. Toyoshima, H. Mo, D. J. Kempf, D. Norbeck, C.-M. Chen, N. E. Wideburg, S. K. Burt, J. W. Erickson, and M. K. Singh. 1994. Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. *J. Virol.* 68:2016-2020.
- Ives, K. J., H. Jacobsen, S. A. Galpin, M. M. Garaev, L. Dorrell, J. Mous, K. Bragman, and J. N. Weber. 1997. Emergence of resistant variants of HIV in vivo during monotherapy with the proteinase inhibitor saquinavir. *J. Antimicrob. Chemother.* 39:771-779.
- Jacobsen, H., K. Yasargil, D. L. Winslow, J. C. Craig, A. Krohn, I. B. Duncan, and J. Mous. 1995. Characterization of human immunodeficiency virus type 1 mutants with decreased sensitivity to proteinase inhibitor Ro 31-8959. *Virology* 206:527-534.
- Kaplan, A. H., S. F. Michael, R. S. Webbie, M. F. Knigge, D. A. Paul, L. Everitt, D. J. Kempf, D. W. Norbeck, J. W. Erickson, and R. Swanstrom. 1994. Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased sensitivity to an inhibitor of the viral protease. *Proc. Natl. Acad. Sci. USA* 91:5597-5601.
- Kozal, M. J., N. Shah, N. Shen, R. Yang, R. Fucini, T. C. Merigan, D. D. Richman, D. Morris, E. Hubbell, M. Chee, and T. R. Gingers. 1996. Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. *Nat. Med.* 7:753-759.
- Lee, T. G., Y. D. Kwon, H. I. Choi, H. J. Chang, S. Y. Hyang, S. C. Kim, and Y. J. Yoo. 1996. Analysis of HIV-1 variants resistant to irreversible protease inhibitors, LB71148 and LB71262. *In Abstracts of the XI International Conference on AIDS.*
- Lehr, P., A. Billich, B. Charpiot, P. Ettmayer, D. Scholz, B. Rosenwirth, and H. Gstach. 1996. Inhibitors of human immunodeficiency virus type 1 protease containing 2-aminobenzyl-substituted 4-amino-3-hydroxy-5-phenylpentanoic acid: synthesis, activity, and oral bioavailability. *J. Med. Chem.* 39:2060-2067.
- Lin, X. L., Y. Z. Lin, and J. Tang. 1994. Relationships of human immunodeficiency virus protease with eukaryotic aspartic proteases. *Methods Enzymol.* 241:195-224.
- Loeb, D. D., C. A. Hutchison, M. H. Edgell, W. G. Farmerie, and R. Swanstrom. 1989. Mutational analysis of human immunodeficiency virus type 1 protease suggests functional homology with aspartic proteinases. *J. Virol.* 63:111-121.
- Loeb, D. D., R. Swanstrom, L. Everitt, M. Manchester, S. E. Stamper, and C. A. Hutchison. 1989. Complete mutagenesis of the HIV-1 protease. *Nature* 340:397-400.
- Maschera, B., G. Darby, G. Palu, L. L. Wright, M. Tisdale, R. Myers, E. D. Blair, and E. S. Furfine. 1996. Human immunodeficiency virus. Mutations in the viral protease that confer resistance to saquinavir increase the dissociation rate constant of the protease-saquinavir complex. *J. Biol. Chem.* 271:33231-33235.
- Molla, A., M. Korneyeva, Q. Gao, S. Vasavanonda, P. J. Schipper, H. M. Mo, M. Markowitz, T. Chernyavskiy, P. Niu, N. Lyons, A. Hsu, G. R. Granneman, D. D. Ho, C. A. Boucher, J. M. Leonard, D. W. Norbeck, and D. J. Kempf. 1996. Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. *Nat. Med.* 2:760-766.
- Moore, M. L., W. M. Bryan, S. A. Fakhoury, V. W. Magaard, W. F. Huffman, B. D. Dayton, T. D. Meek, L. Hyland, G. B. Dreyer, B. W. Metcalf, J. E. Strickler, J. G. Gorniak, and C. Debouk. 1989. Peptide substrates and inhibitors of the HIV-1 protease. *Biochem. Biophys. Res. Commun.* 159:420-425.
- Partaledis, J. A., K. Yamaguchi, M. Tisdale, E. E. Blair, C. Falcione, B. Maschera, R. E. Myers, S. Pazhanisamy, O. Futera, A. B. Cullinan, C. M. Stuver, R. A. Byrn, and D. J. Livingston. 1995. In vitro selection and characterization of human immunodeficiency virus type 1 (HIV-1) isolates with reduced sensitivity to hydroxyethylamino sulfonamide inhibitors of HIV-1

- aspartyl protease. *J. Virol.* **69**:5228–5235.
26. **Patck, A. K., H. Mo, M. Markowitz, K. Appelt, B. Wu, L. Musick, V. Kalish, S. Kaldor, S. Reich, D. Ho, and S. Webber.** 1996. Antiviral and resistance studies of AG1343, an orally bioavailable inhibitor of human immunodeficiency virus protease. *Antimicrob. Agents Chemother.* **40**:292–297.
 27. **Porter, D. C., D. C. Ansardi, W. S. Choi, and C. D. Morrow.** 1993. Encapsulation of genetically engineered poliovirus minireplicons which express human immunodeficiency virus type 1 Gag and Pol proteins upon infection. *J. Virol.* **67**:3712–3719.
 28. **Richman, D., V. A. Johnson, D. L. Mayers, T. Shirasaka, M. C. O'Brien, and H. Mitsuya.** 1993. In vitro evaluation of experimental agents for anti-HIV activity, p. 12.9.1–12.9.21. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), *Current protocols in immunology*. J. Wiley, Brooklyn, N.Y.
 29. **Rose, J. R., R. Salto, and C. S. Craik.** 1993. Regulation of autoproteolysis of the HIV-1 and HIV-2 proteases with engineered amino acid substitutions. *J. Biol. Chem.* **268**:11939–11945.
 30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 31. **Schapiro, J. M., M. A. Winters, M. Vierra, S. Crawford, and T. C. Merigan.** 1998. Lymph node human immunodeficiency virus RNA levels and resistance mutations in patients receiving high-dose saquinavir. *J. Infect. Dis.* **2**: 477–480.
 32. **Schock, H. B., V. M. Garsky, and L. C. Kuo.** 1996. Mutational anatomy of an HIV-1 protease variant conferring cross-resistance to protease inhibitors in clinical trials. Compensatory modulations of binding and activity. *J. Biol. Chem.* **271**:31957–31963.
 33. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**:113–130.
 34. **Yamada, A., B. Anderson, S. Kageyama, J. Erickson, T. Ueno, H. Hayashi, and H. Mitsuya.** 1996. Detection of mutations conferring on HIV-1 drug resistance in patients receiving KNI-272. *In* Abstracts of the XI International Conference on AIDS.
 35. **Zhang, Y.-M., H. Imamichi, T. Imamichi, H. C. Lane, J. Falloon, M. B. Vasudevachari, and N. P. Salzman.** 1997. Drug resistance during indinavir therapy is caused by mutations in the protease gene and in its Gag substrate cleavage sites. *J. Virol.* **71**:6662–6670.