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Engineering human immunodeficiency virus 1 protease heterodimers as macromolecular inhibitors of viral maturation

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ABSTRACT **Dimerization of human immunodeficiency virus type 1 protease (HIV-1 PR) monomers is an essential prerequisite for viral proteolytic activity and the subsequent generation of infectious virus particles. Disruption of the dimer interface inhibits this activity as does formation of heterodimers between wild-type and defective monomers. A structure-based approach was used to identify amino acid substitutions at the dimer interface of HIV-1 PR that facilitate preferential association of heterodimers and inhibit self-association of the defective monomers. Expression of the designed PR monomers inhibits activity of wild-type HIV-1 PR and viral infectivity when assayed in an *ex vivo* model system. These results show that it is possible to design PR monomers as macromolecular inhibitors that may provide an alternative to small molecule inhibitors for the treatment of HIV infection.**

Crystallographic structures of human immunodeficiency virus 1 protease (HIV-1 PR) complexed to peptide mimetics and nonpeptide inhibitors have played a major role in obtaining highly potent and specific small molecule inhibitors against this PR. Although a select group of these inhibitors have demonstrated efficacy in cell culture and clinical studies, the emergence of variants of HIV-1 PR resistant to the various structurally diverse PR inhibitors is now well documented (ref. 1 and references therein). However, the initial antiviral efficacy of these inhibitors still validates the PR as a therapeutic target. While strategies involving multiple PR inhibitors may eventually block the emergence of resistant variants, an alternative approach that is less sensitive to point mutations may be preferable.

Defective gene products that can associate with wild-type (WT) gene products and lead to inactive heteromeric complexes has been proposed for a number of HIV proteins (2). This type of dominant negative inhibition has been described for mutants of gag (3) and rev (4), and formation of inactive protein complexes has actually been demonstrated for rev variants (5). Furthermore, these defective gene products have shown antiviral effects when expressed in HIV-1-infected T cells (6–8). This macromolecular inhibition of viral gene products involves protein–protein interactions between WT and trans-dominant protein monomers. By judiciously targeting unique interfacial regions in viral gene products that are less sensitive to resistance mutations, defective gene products can then be designed as effective antivirals.

The dimeric HIV-1 PR plays a crucial role in the maturation of viral structural (gag) and enzymatic (pol) proteins. HIV-1 particle maturation and assembly must occur in a coordinated fashion to yield infectious virus (9). The gag and pol proteins are synthesized as polyproteins of either 55 kDa (gag) or 160 kDa (gag–pol). The monomers of the PR are expressed as part of the gag–pol fusion protein, and PR activation is thought to take place in the plasma membrane of a cell during viral budding. When two PR monomers assemble, each contributes an aspartic acid residue at the active site of the enzyme, and the combination of the two aspartic acids provides the machinery required for peptide bond hydrolysis. Catalytically compromised HIV-1 PR variants inhibit the processing of polyprotein precursors and the subsequent generation of mature virions. This occurs with a direct correlation between the levels of PR activity and virus infectivity, revealing a threshold of proteolytic processing required for the production of infectious virus (10). In addition, PR monomers in which the active site Asp-25 is replaced with asparagine appear to act in a trans-dominant inhibitory fashion and interfere with the activity of WT dimers (11). These results suggested that defective PR monomers could be designed and effectively used to interfere with HIV-1 infection. We have modeled a number of HIV-1 PR structure complexes to aid in the design of mutations in the PR dimer interface to promote heterodimer formation and decrease the stability of the mutant PR homodimer interaction. These studies have led to the construction of proviral plasmids carrying the designed defective PRs. Transient transfection of 293T cells with equimolar amounts of the WT plasmid (HIV-gpt) and the plasmids carrying the designed PRs showed decreased particle maturation and infectivity, with some of the defective PRs exhibiting greater inhibitory effects than the others. Furthermore, these designed PR subunits appear to be trans-dominant inhibitors since the activity of WT dimers can be titrated out in a dose-dependent fashion.

MATERIALS AND METHODS

Design of Heterodimers. The SYBYL modeling package (12) was used to investigate the impact of possible amino acid mutations of the 5HVP structure (13), the current highest resolution of all HIV-1 PR Protein Data Base structures. The PR inhibitor complexes 1HIV (14), 1HOS (15), 4HVP (16), 5HVP (13), 7HVP (17), 8HVP (18), and 9HVP (19) were overlayed on the 5HVP template with the SYBYL match algorithm using the following amino acids and their dimeric equivalents: {Asp-25} + {Gly-27} + {Ala-28} + {Asp-29} + {Asp-30} + {Val-32} + {Val-82} + {Ile-84}. Atoms of the bound inhibitors were extracted from the models, and the hydrophobic regions, defined simply as carbon atoms were used as templates to delineate hydrophobic pockets within the active site. Substitutions of interest were located by studying only those amino acid side chains of one monomer positioned within 5 Å of residues in the opposing subunit. While this included the amino- and carboxyl-terminal regions of the PR, generally defined as the dimer interface, no suitable mutation sites were found in this section of the molecule. Side-chain alterations were then sought for one monomer that might increase the strength of interaction with a WT monomer. Energy minimization was carried out using the standard SYBYL

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Abbreviations: PR, protease; WT, wild type.

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force field for each mutation to ensure that the new side chains could interact without major steric clashes. All residues within a 5-Å region around any substitutions were allowed to rearrange, while the remainder of the PR structure was held rigid. These studies resulted in the proposal of a number of amino acid replacements including Asp-25 → Lys, Asp-25 → Arg, Gly-49 → Trp, and Ile-50 → Trp.

These substitutions are likely to simultaneously enhance heterodimer stability through salt bridge formation and hydrophobic interactions in the WT P1 and P2′ pockets, while decreasing homodimer stability through electrostatic and steric repulsions.

Viral Constructs and Site-Directed Mutagenesis. The HIV-gpt proviral plasmid consists of the HIV-1 HXB2 sequences (the *env* gene, nt 6402–7620 replaced with the drug-selectable *Escherichia coli gpt* gene) cloned into the pBS plasmid (pBluescript, Stratagene). The HXB2-env plasmid encodes the gp160 envelope sequences from the HXB2 strain (nt 5999–8896) cloned into a simian virus 40 expression vector (20).

Mutagenesis was performed using standard methods (21). For introduction of mutants into the provirus, site-directed mutagenesis was carried out with the Bluescript-based phagemid pSpol, which contains a 4314-bp *Spe*I–*Sal*I (nt 1051–5366) fragment of the HIV-gpt plasmid encompassing the PR gene. Mutagenesis was carried out using four oligonucleotides singly or in combination. To introduce a lysine at position 25 of the PR, the oligonucleotide 5′-GAAGCTCTTTTAAAAACAG-GAGCAGAT-3′ was used and identified by restriction endonuclease screening with *Dra*I (underlined nucleotides). Arginine was introduced at position 25 using the oligonucleotide 5′-GAAGCTCTCTTAAGGACAGGAGCAGAT-3′ and identified by restriction endonuclease screening with *Afl*II (underlined nucleotides). Tryptophan was introduced at position 49 using the oligonucleotide 5′-AAAATGATAGGGT-**GG**ATTGGAGGTTTT-3′ and at positions 49 and 50 using the oligonucleotide 5′-AAAATGATAGGG**TGGTGG**G-GAGGTTTTATC-3′. Mutations are indicated in boldface type. The *Spe*I–*Sal*I fragment was then introduced into the HIV-gpt plasmid to generate the complete viral plasmid. All constructs were sequenced through the PR gene to confirm the mutations.

Cells. COS, 293, 293-T, and HeLaT4 cells were maintained in Dulbecco's modified Eagle's medium H21 (DMEM) supplemented with 10% fetal calf serum (GIBCO/BRL), penicillin (100 units/ml), and streptomycin (100 μg/ml). COS-7 cells were obtained through the American Type Culture Collection. HeLaT4 cells were obtained from R. Axel through the AIDS Research and Reference Program, National Institutes of Health. HeLaT4 cells were grown in the presence of G418 (400 μg/ml) to maintain CD4 expression.

Transfection, Infection, Isolation of Viral Particles, and Quantitation of p24. Transfection, infection, isolation of viral particles, and quantitation of p24 were carried out as described (11, 20, 22). Transfections utilizing 60 μg of HXB2-env, 10 μg of HIV-gpt, 10 μg of substituted HIV-gpt, and 10 μg of pHIVluc DNA (a construct encoding the luciferase reporter gene provided by M. Peterlin, University of California, San Francisco) were carried out to determine whether factors required for proper expression of HIV proteins were not limiting in the cells and that the level of expression of the engineered proteins was equivalent. Factors were not limiting as similar levels of luciferase expression resulted (data not shown).

RESULTS

Designing Macromolecular Inhibitors. Computer modeling was used to design a monomer of the HIV-1 PR into a macromolecular inhibitor of the enzyme. The engineered substitutions were chosen to promote the formation of heterodimers between WT and substituted monomers while decreasing the stability of the mutant PR homodimer interaction. If equimolar concentrations of WT and substituted PR monomers associate in a random fashion, the probability of forming an active PR homodimer is 25% (Fig. 1). This value assumes similar dissociation constants for the WT and defective PR homodimers and heterodimers. If the dissociation constant for the heterodimer is lower than that of the WT homodimer, the percentage of active PR formed could be less than 25%. Similarly, if the self association of defective monomers is inhibited by design, an even lower percentage of active PR could result at equimolar concentrations of WT and defective monomers.

Favorable interactions were predicted with the active-site aspartate of the opposing monomer if Asp-25 was modified to either of the positive residues lysine or arginine. In principle, arginine or lysine side chains could be accommodated into the heterodimer without difficulty due to the solvent-accessible cavity surrounding the catalytic aspartates. However, a modified homodimer containing two arginines at positions 25 and 25′ was predicted to be more unstable due to consequent steric and electrostatic constraints. Replacement of Gly-49 or Ile-50 with a tryptophan would allow favorable hydrophobic interactions with either the S1 or S2′ site of an associated WT monomer. A double substitution converting both Gly-49 and Ile-50 to tryptophans could also be adapted in the model of the heterodimer interaction (Fig. 2). The introduced tryptophan side chains mimic the role of P1 and P2′ substrate side chains in forming the heterodimer with the WT monomer. A model of the heterodimer between WT monomer and the Trp-49/Trp-50 variant with arginine at position 25 suggested unfavorable electrostatic and steric interactions between the guanidinium and indole moieties. However, a variant containing all three changes Lys-25, Trp-49, and Trp-50 could be easily accomodated in the heterodimer interaction.

Expression of Designed PR Monomers Interferes with HIV-1 PR Activity. The efficacy of the designed macromolecular inhibitors was determined using a cell-based assay (11, 22). This assay monitors viral polyprotein processing and the formation of viral particles and is a direct and sensitive measure of HIV PR activity. The amino acid replacements were introduced into the HIV-1 PR gene expressed from an HIV-gpt plasmid (20) encoding the selectable marker guanine phosphoribosyltransferase (gpt) in place of the gp160 envelope gene. The consequences of coexpressing WT and engineered PR monomers on particle maturation were examined by transfection of HIV-gpt plasmids encoding WT and substituted PRs into human kidney 293 cells. At 1:1 ratios of variant to WT PR, the inhibitory effect of the variant PR on polyprotein processing varied depending on the designed amino acid substitution (Fig. 3). All engineered PRs were more effective at inhibiting proteolytic activity of WT PR than the original Asn-25 variant, as seen by the appearance of unprocessed p55 and partially processed p39 and p25 species (Fig. 3*a*). By comparing the ratio of fully processed CA (p24) to unproc-

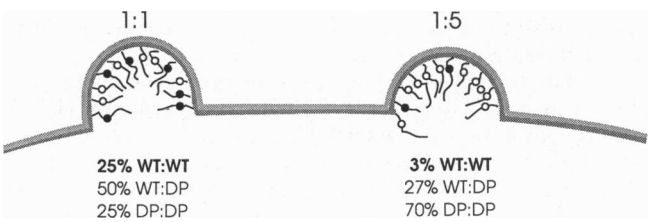

FIG. 1. Proposed mechanism of dominant negative inhibition of HIV PR activity. At the membrane, budding virions incorporate WT (●) and defective (DP, ○) PR polyproteins at the ratio set by the transfection. A cotransfection range from 1:1 to 1:9 (WT/DP) represents nearly two orders of magnitude difference between WT (1%) and defective proteins (99%) assuming similar dissociation constants for the dimeric proteins.

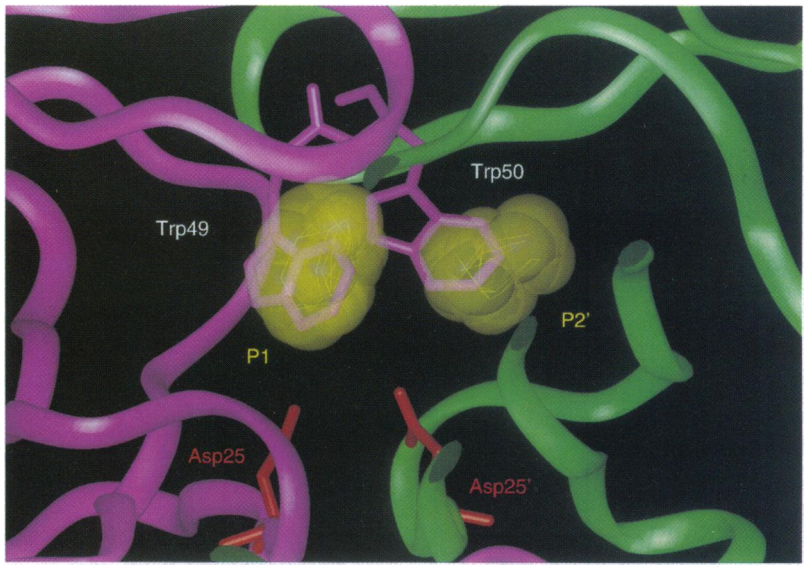

FIG. 2. Model of the predicted interactions of an HIV-1 PR heterodimer. Energy-minimized model of the Trp-49 and Trp-50 substitutions. A cross-section of the model is displayed to clarify the view of the active site. The tryptophan side chains at positions 49 and 50 of the defective PR monomer (magenta) are found to occupy the P1 and P2′ hydrophobic sites, respectively, of the opposing WT monomer (green). These sites are highlighted in yellow by atoms taken from numerous substrate analogues (14–19). The atoms were extracted from crystal structures overlaid with the model. The overlap between the substrate and the tryptophan side chain atoms illustrate the additional hydrophobic contacts possible between variant and the WT monomer. These additional interactions suggest that the Gly-49 → Trp and Ile-50 → Trp substitutions could enhance binding affinity on dimerization with the WT PR monomer and, thus, enhance the dominant negative effect.

essed p55 among the different variants, the following rank order of the effectiveness of the monomer to inhibit polyprotein processing by WT PR was established: Lys-25/Trp-49/Trp-50 > Arg-25 ≥ Lys-25 = Arg-25/Trp-49/Trp-50 ≥ Trp-49/Trp-50 > Trp-49 > Asn-25 (Fig. 3*b*). Notably, the inhibitory effect of the Trp-49 and Trp-49/Trp-50 variants is observed even when the catalytic aspartates are present at position 25 suggesting the tryptophans hinder the accessibility of the PR's natural substrates to the active site. It should be also noted that the effects of the designed variants on polyprotein processing have been underestimated since the p24 assay does not discriminate between p24 and partially processed p25.

Designed PR Monomers Reduce Viral Infectivity. The potency of the engineered PR inhibitor at reducing viral particle infectivity was determined by transfecting the human kidney cell line 293-T with a 1:1 ratio of WT and engineered PR, and HXB2-env (20) to provide gp160 env. A sample of the virions produced (Fig. 3*a*) was analyzed for quantitation of the ratio of p24 to total gag protein (Fig. 3*b*), while the remainder was used to screen for the presence of infectious particles by culturing HeLaT4 cells with transfected cell supernatants. Selection for the gpt marker gene permitted quantitation of the number of cells infected by recombinant viruses. The rank order of inhibitory effectiveness of the cotransfected engineered monomers on viral infectivity paralleled the polyprotein processing data (Fig. 3*c*). All designed PRs inhibited infectivity to a greater extent than the Asn-25 variant. Interestingly, reductions in the ratio of p24 to total and partially cleaved gag protein appeared to have a considerable inhibitory effect on viral infectivity. The ratio of p24 to total gag protein for the Lys-25/Trp-49/Trp-50 variant was approximately half that observed for the asparagine variant. However, viral infectivity was approximately 26-fold lower in the presence of the Lys-25/Trp-49/Trp-50 variant compared with the Asn-25 variant.

Dose-Dependent Inhibition of Proteolytic Activity and Viral Infectivity in the Presence of the Designed Lys-25/Trp-49/Trp-50 PR Monomer. Titration of the effects of the Lys-25/Trp-49/Trp-50 on polyprotein processing and viral infectivity is displayed in Fig. 4. A dose-dependent inhibitory response on WT PR polyprotein processing is observed with increasing concentrations of the variant PR (Fig. 4*a*). The immunoblot analysis shows that, as the amount of DNA encoding the engineered PR increased at a fixed concentration of DNA encoding the WT PR, the ratio of p24 to total gag protein decreased and the presence of intermediate species p25 and p39/p41 was more apparent. Quantitation of p24 to total gag protein revealed a typical dose-dependent inhibitory response (Fig. 4*b*). As more HIV-gpt DNA encoding the substituted PR was added, inhibitory effects on the generation of infectious particles in relation to polyprotein processing dramatically augmented (Fig. 4*c*). At a 1:1 ratio of WT to the Lys-25/Trp-49/Trp-50 variant, a 2-fold reduction in p24 production resulted in approximately 50-fold reduction in the generation of infectious particles. This suggests that the build up of partially processed and unprocessed polyprotein precursors due to the formation of inactive heterodimeric PRs exerts an amplified inhibitory effect on viral assembly and hence infectivity. This might be caused by a second level of trans-dominant interference with virus assembly due to partially matured viral proteins.

DISCUSSION

We have used molecular modeling to design defective HIV-1 PR monomers and demonstrated that this strategy is a valid approach for designing macromolecular inhibitors. The designed defective PR monomers exert a trans-dominant inhibitory effect leading to the formation of catalytically compromised PR heterodimers *ex vivo*, ultimately yielding noninfectious viral particles. Depending on the amino acid substitution at the dimer interface, the extent of the inhibitory effect of the designed PR monomer on both polyprotein processing and subsequent infectivity varies significantly. All designed PR monomers were more effective inhibitors than the inactive PR variant that has an asparagine at position 25. The inhibitory effects of these mutations appear to be partially additive as observed by the Lys-25/Trp-49/Trp-50 variant, which is a more potent inhibitor than either the Lys-25 or Trp-49/Trp-50 variants. The titration curve of the Lys-25/Trp-49/Trp-50 variant parallels that observed for the Asn-25 variant (11) except for a shift in the inhibitory response. The inhibitory response on polyprotein processing and infectivity at a WT PR to Asn-25 variant ratio of 1:2 is similar to that observed at a

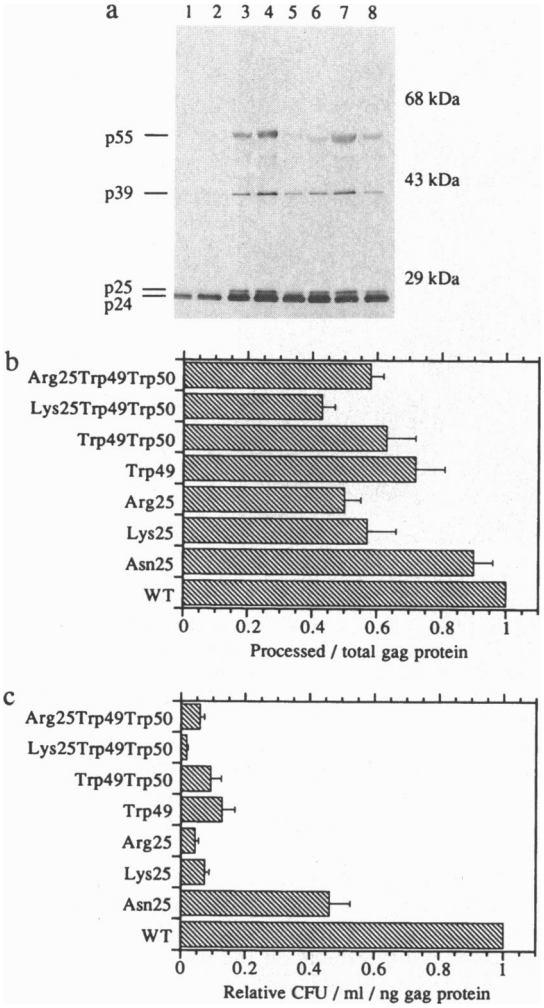

FIG. 3. Inhibition of polyprotein processing by cotransfection of WT and engineered proviral DNA. (*a*) Immunoblot analysis of the inhibition of HIV-1 PR polyprotein processing by cotransfection of plasmids encoding WT and engineered PR monomers at a 1:1 ratio. Lanes: 1, WT; 2, Asn-25; 3, Lys-25; 4, Arg-25; 5, Trp-49; 6, Trp-49/Trp-50; 7, Lys-25/Trp-49/Trp-50; 8, Arg-25/Trp-49/Trp-50. 293-T human kidney cells were cotransfected with 60 μg of HXB2-env (20) in addition to 10 μg of WT and 10 μg of substituted HIV-gpt constructs by a modified calcium phosphate procedure (22). At approximately 48 h after transfection, viral capsids were isolated from culture supernatants by sucrose sedimentation (22). Proteins were separated by SDS/PAGE on 10% gels and analyzed by immunoblot analysis using an antibody against p24 protein (American Biotechnologies, Cambridge, MA). (*b*) Quantitation of the effects of engineered monomers on HIV-1 polyprotein processing and infectivity. Purified viral capsids were quantitated by p24 ELISA before and after digestion with exogenous viral PR. The p24 value obtained for untreated samples indicated the amount of processed capsid protein (and partially processed p25). The p24 value obtained after *in vitro* processing indicated the amount of total capsid protein (unprocessed and processed). (*c*) Supernatants from the experiments discussed in *a* and *b* were used to infect HeLaT4 cells to determine the titer of infectious particles present. These results represent the mean of at least five experiments.

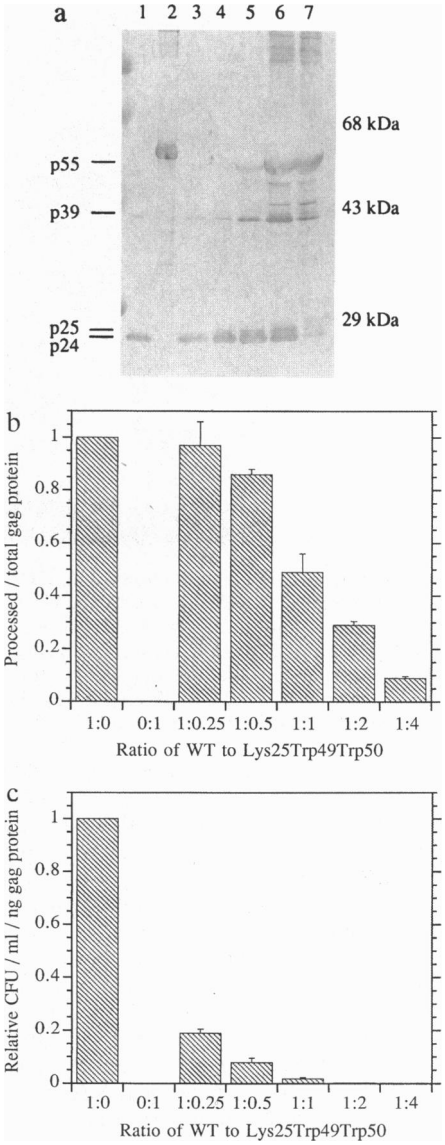

FIG. 4. Dose-dependent effects of the Lys-25/Trp-49/Trp-50 variant on polyprotein processing and generation of infectious particles. (*a*) Immunoblot analysis showing the dose-dependent effects of the Lys-25/Trp-49/Trp-50 variant on polyprotein processing by cotransfection of plasmids encoding WT PR and the Lys-25/Trp-49/Trp-50 variant. Lanes: 1, WT only; 2, Lys-25/Trp-49/Trp-50 only; 3–7, titration of WT with a 0.25, 0.5, 1, 2, or 4 molar equivalents of HIV-gpt–Lys-25/Trp-49/Trp-50 DNA, respectively. 293-T human kidney cells were cotransfected with 60 μg of HXB2-env (20) in addition to 10 μg of WT and 0, 0.25, 0.5, 1, 2, or 4 molar equivalents of HIV-gpt–Lys-25/Trp-49/Trp-50 DNA. Culture supernatants were collected at approximately 48 h after transfection, and the supernatants were analyzed as described in Fig. 3. Quantitation of the ratio of p24 (+p25) to total gag protein (*b*) and the number of infectious particles per ng of total gag protein (*c*) was as described in Fig. 3. These results represent the mean of two experiments.

WT PR to Lys-25/Trp-49/Trp-50 variant ratio of 1:0.25. This indicates a lower dissociation constant for the WT monomer and triple variant heterodimer in comparison to the WT and Asn-25 variant heterodimer. These results suggest an approach that can be used in conjunction with or as an alternative to small molecule inhibitors for controlling the enzymatic activity of HIV-1 PR. Directly analogous to medicinal chemical approaches that are used to optimize a lead compound, protein engineering methods were used to optimize the initial macromolecular inhibitor HIV PR D25N. Presumably the more potent second-generation inhibitors have higher affinity for the WT monomer and lower affinity for self-interaction due to the engineered substitutions. Subsequent biochemical analysis of the various heteromeric complexes will be necessary to determine the basis of the increased potency of the designed macromolecular inhibitors on maturation and viral infectivity.

All possible substitutions that can be introduced into the dimer interface to promote heterodimerization have not been

exhausted. For example, one possible site for substitution exists at Leu-23. Inspection of the dimer reveals a small cavity in the region of Leu-23 on the opposing monomer formed by amino acids Thr-26, Gly-27, Ala-28, Asp-29, and Arg-87. This cavity is occupied with a water molecule in the crystallographic structures analyzed (13–19). Amino acid substitutions for Leu-23 would be designed to place the side chain of the resulting variant into the cavity to displace or to interact with the water molecule. Modeling Leu-23 as a tyrosine suggests that the tyrosine side chain is able to extend into the cavity, placing its hydroxyl close to the water position. Other large polar amino acids may also work in a similar manner. NMR evidence has recently suggested that the active-site aspartates of the HIV-1 PR can exist in a neutral state (23). If this is the case, replacement of the aspartate for a neutral residue may be preferable. Glutamine provides one possibility since its longer side chain could displace the catalytic water while forming neutral hydrogen bonds to the WT catalytic aspartate. Analysis of the effects of these substitutions on HIV PR heterodimerization must await further experimentation.

Unlike a small molecule inhibitor, a macromolecular inhibitor can have a greater interacting surface area with its target. This greater surface area can be an advantage in overcoming the effects of amino acid substitutions that result from the error prone replication cycle of HIV. The potent small molecule inhibitors of HIV PR analyzed in this study bind tightly to a defined area of the PR active site. Resistance mutations can have a concerted effect on the fit of the entire small molecule and reduce its binding efficiency. Our engineered alterations of HIV-1 PR have been targeted to the catalytically crucial (Asp-25) or the flexible flap regions (Gly-49–Ile-50) of the enzyme. Attempts of the virus to escape the catalytic site mutation in our engineered PR monomers would render the WT PR monomer inactive. Furthermore, alterations at the flap region should be able to adjust to mutations that can occur in the WT monomer. We make this assertion since, contrary to the case of a small molecule inhibitor, small adjustments of the flap regions are unlikely to cause concerted disruption at the remainder of the binding interface. Thus, our macromolecular inhibitors should be less prone to resistance than the small molecule inhibitors. To begin to address this issue, selected trans-dominant PR inhibitors described herein are currently being expressed intracellularly in human T-cell lines (24). These cell lines can then be challenged with both WT and HIV PR inhibitor resistant strains to determine the sensitivity of these cell lines to HIV viral replication.

To our knowledge, this is the first report of designed macromolecular inhibitors against HIV-1 viral maturation and should be applicable to other multimeric targets. Reverse transcriptase (RT) and integrase (IN) were expressed in our viral constructs. In a host cell, these proteins could interfere with normal cellular function. Rather than constructing pol truncations, RT and IN activity could be eliminated by judicious active-site mutations that maintain overall protein structure. Efficacy of such therapeutic constructs might be enhanced by converting RT and IN into trans-dominant inhibitors in tandem with the PR variants, thus interrupting more than one enzymatic activity. Other HIV-1 proteins have been shown to inhibit HIV infection in a trans-dominant fashion (6, 7). Therefore, obstacles could be set at different stages of the viral life cycle. Clearly, advances have to be made regarding the delivery of such macromolecular inhibitors to $CD4^+$ cells. In theory, stem cells could be designed to constitutively overexpress these trans-dominant inhibitors upon HIV infection by using a promoter that is activated when the cell becomes infected (25, 26). The trans-dominant inhibitor could then prevent the formation of new infectious particles. In summary, the use of defective PR monomers as trans-dominant PR inhibitors offers a unique possibility for a single or multifaceted gene therapy approach in the clinical treatment of HIV-1 infection.

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