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Biochemistry

Active human immunodeficiency virus protease is required for viral infectivity

(aspartyl protease/active-site mutation/gag p55/polyprotein processing/*in vitro* assay)

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ABSTRACT **Retroviral proteins are synthesized as polyprotein precursors that undergo proteolytic cleavages to yield the mature viral proteins. The role of the human immunodeficiency virus (HIV) protease in the viral replication cycle was examined by use of a site-directed mutation in the protease gene. The HIV protease gene product was expressed in *Escherichia coli* and observed to cleave HIV gag p55 to gag p24 and gag p17 *in vitro*. Substitution of aspartic acid residue 25 (Asp-25) of this protein with an asparagine residue did not affect the expression of the protein, but it eliminated detectable *in vitro* proteolytic activity against HIV gag p55. A mutant HIV provirus was constructed that contained the Asn-25 mutation within the protease gene. SW480 human colon carcinoma cells transfected with the Asn-25 mutant proviral DNA produced virions that contained gag p55 but not gag p24, whereas virions from cells transfected with the wild-type DNA contained both gag p55 and gag p24. The mutant virions were not able to infect MT-4 lymphoid cells. In contrast, these cells were highly sensitive to infection by the wild-type virions. These results demonstrate that the HIV protease is an essential viral enzyme and, consequently, an attractive target for anti-HIV drugs.**

The mature retroviral proteins encoded by the *gag*, *pol*, and *env* genes are derived from polyprotein precursors by post-translational cleavage (1). In human immunodeficiency virus (HIV), the gag, pol, and env polyproteins are processed to yield, respectively, the viral structural proteins p17, p24, and p15—reverse transcriptase and integrase—and the two envelope proteins gp41 and gp120. Viral-encoded proteases have been biochemically characterized for several retroviruses, including the avian myeloblastosis virus (AMV; ref. 2) and the murine (MLV; ref. 3), bovine (BLV; ref. 4), and feline (FeLV; ref. 5) leukemia viruses. *In vitro* experiments with purified proteases from these viruses have confirmed that these enzymes are capable of cleaving the gag precursor polypeptides (3–6). Genes that would encode analogous proteases have been identified for all of the retroviruses whose genomes have been sequenced, including HIV-1 (7–9). All of these retroviral proteases share limited amino acid homology with the active site residues of the family of aspartyl proteases represented by renin and pepsin (10). Among these proteins, the sequence Asp-Thr-Gly is highly conserved, with the invariant aspartic acid being the active site aspartyl residue in the aspartyl proteases.

The necessity of these proteolytic processing steps in the life cycle of the retroviruses remains to be determined. Deletion of a region of the Moloney murine leukemia virus (MoMLV) genome that encodes the protease resulted in the production of noninfectious virions that contained unprocessed gag and pol precursor polypeptides (11, 12). Although other processes could have been impaired by this large deletion in the *gag–pol* gene, this observation suggests that the MoMLV protease plays an essential role in maintaining the infectious nature of the virus. The *gag–pol* structure of HIV differs from that of MoMLV. Whereas the gag proteins of MoMLV are translated sequentially in the order p15, p12, p30, and p10 (13–15), the HIV gag products are translated sequentially as p17, p24, and p15 (7–9), where HIV gag p24 is analogous to MoMLV gag p30. In addition, the HIV pol polypeptide is translated after a frameshift from the *gag* reading frame (16) and the MoMLV pol polypeptide is translated in the *gag* reading frame after suppression of a nonsense codon (3). These differences preclude a direct inference from the experiments with MoMLV on the role of the HIV protease.

In the case of HIV-1, the protease would be translated as the N-terminal region of the *pol* reading frame. Proteolytic activity has been observed to occur during expression in microbial cells of polypeptides that contain the 10-kDa polypeptide whose N-terminal sequence would initiate at pol residue 69 (ref. 17; numbering is according to ref. 8). We have recently observed that a polypeptide having the sequence pol 69–167, produced either by chemical synthesis or by expression in *Escherichia coli* or yeast cells, has the ability to cleave HIV gag p55 to give gag p24 and gag p17 (unpublished observations). The conserved Asp-Thr-Gly sequence occurs at positions 25–27 within this polypeptide. By analogy with the aspartyl proteases, Asp-25 might be critical for the activity of the HIV protease. In this report, we have examined the effect of replacing the Asp-25 residue with an asparagine residue on the ability of the HIV protease to cleave gag p55 and on the ability of the corresponding mutant HIV to replicate in cells.

MATERIALS AND METHODS

***In Vitro* HIV Protease Assay.** The HIV protease bacterial expression vector pPRT was derived from pKK233-2 (Pharmacia). The DNA sequence between the *Eco*RI and *Nco* I sites of the plasmid pKK233-2 was replaced with a synthetic *trp* promoter and a prokaryotic ribosome binding site. A *Nco* I/*Hin*dIII gene fragment derived by modification of the 5′ portion of the *pol* gene of the HIV NY5 isolate (18) was inserted downstream of the *trp* promoter between the *Nco* I and *Hin*dIII sites. This fragment encodes a polypeptide that initiates with Met-Ala followed by residues corresponding to pol 70–167 (protease 2–99) and terminates with a nonsense codon. For construction of pPRT(Asn-25), a bacteriophage M13 subclone was made containing the *Nco* I to *Hin*dIII protease fragment and oligonucleotide-directed mutagenesis was performed to change the codon for Asp-25 (GAT) to that for Asn (AAT) (19). The *Nco* I/*Hin*dIII protease fragment was then subcloned into *Nco* I/*Hin*dIII-digested pPRT.

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Abbreviations: HIV, human immunodeficiency virus; MoMLV, Moloney murine leukemia virus.

During the construction of the pPRT plasmid, a plasmid was isolated that lacked the codon for the N-terminal methionine residue (pPRT-ATG). Details of these plasmid constructions will be published elsewhere. The HIV gag p55 substrate was expressed in yeast cells and isolated as a crude cytosolic preparation (to be described elsewhere). Rabbit anti-HIV protease antisera were raised against a peptide having the sequence NH_2-Glu-Glu-Met-Ser-Leu-Pro-Gly-Arg-Trp-Pro-Lys-Met-COOH, which corresponds to residues 34–44 of the protease (unpublished data).

For expression of bacterially produced HIV protease, *E. coli* strain HB101 carrying the protease expression plasmids were grown to stationary phase in L broth (20) containing ampicillin (100 mg/liter) (Sigma). Cells were collected by centrifugation and resuspended in an equal volume of minimal medium (M9CA; ref. 20) supplemented with indolacrylic acid (20 μg/ml) (Sigma) and grown at 37°C for 2.5 hr. Cells from a 5-ml culture were lysed in 0.25 ml of sample buffer A [50 mM Tris·HCl, pH 6.8/20% (vol/vol) glycerol/1% $NaDodSO_4$/240 mM 2-mercaptoethanol], and the proteins were separated by electrophoresis on 16% $NaDodSO_4$/polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were incubated with rabbit anti-HIV protease antiserum (1:1000 dilution) in hybridization buffer (25 mM Tris·HCl, pH 7.5/150 mM NaCl/1% powdered milk/0.5% bovine serum albumin (Sigma)/1 mM $CaCl_2$/20 μM EDTA) at 22°C for 1–3 hr and washed in 25 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Tween 20 (Bio-Rad). Immunoreactive proteins were detected by autoradiography after incubation for 1–3 hr at 22°C with ^{125}I-labeled protein A (2 μCi; 1 Ci = 37 GBq; Amersham) in hybridization buffer.

To examine the activity of the bacterially expressed HIV protease, cells from a 5-ml culture of *E. coli* HB101 expressing the wild-type or mutant HIV protease were pelleted and resuspended in 0.25 ml of 50 mM Tris·HCl, pH 7.8/100 mM NaCl/5 mM EDTA/1 mM dithiothreitol/lysozyme (10 mg/ml)/1 mM phenylmethylsulfonyl fluoride (Sigma)/aprotinin (10 ng/ml) (Sigma)/10 μM *trans*-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64, Sigma). Cells were kept on ice for 30 min and then disrupted by sonication for 4 min at 4°C. An aliquot (10 μl) of the soluble fraction was incubated with 4 μl of a crude preparation of yeast-produced HIV gag p55 (1 mg/ml) in a final volume of 20 μl and a final concentration of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.5). Reaction mixtures were incubated at 37°C for 1 hr and quenched by the addition of 10 μl of sample buffer A. Products were analyzed by electrophoresis on 16% $NaDodSO_4$/polyacrylamide gels, electroblotted onto nitrocellulose membranes, and incubated first with murine monoclonal antibodies (DuPont) against HIV gag p24 and gag p17 (1:1000 dilution), and then with ^{125}I-labeled goat anti-mouse antisera (2 μCi; Amersham) as described above.

Cells, Transfections, and Infections. SW480 human colon carcinoma cells (21) were grown in Dulbecco's modified Eagle's medium (Cellgro, Mediatech, Washington, DC) supplemented with 10% fetal calf serum. MT-4 human lymphoid cells (22) were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum. SW480 cells were transfected with 10 μg of DNA per 10^6 cells in a 10-cm dish by the calcium phosphate method (23). For infection of MT-4 cells, 1.0×10^6 cells were pelleted and resuspended in 1 ml of medium taken from the transfected SW480 cultures 48 hr posttransfection. Adsorption was allowed to proceed for 1 hr at 37°C with constant agitation, followed by the addition of 2 ml of fresh medium. Cells then were placed in individual wells of 48-well cell culture plates (Costar, Cambridge, MA) and incubated at 37°C.

Construction of HIV Protease Mutant Provirus. The HIV protease mutant provirus PM-3 was constructed by subcloning the 2.1-kilobase (kb) *Hin*dIII/*Kpn* I fragment of the HIV provirus pNL4-3 (24) containing the protease gene into bacteriophage M13 and performing oligonucleotide-directed mutagenesis as described for pPRT(Asn-25). The fragment was ligated with *Nde* I/*Kpn* I-digested pUC-18 together with the 3.4-kb *Nde* I/*Hin*dIII fragment of pNL4-3 containing the 5′ long terminal repeat and *gag* sequences. The resulting plasmid first was digested with *Eco*RI, the end was made flush by Klenow polymerase I and then partially digested with *Bcl* I, and the 6.6-kb fragment was isolated. This fragment was ligated with the *Bcl* I/*Eco*RI *pol* and *Eco*RI/*Nru* I *env* fragments of pNL4-3 to give PM-3. The wild-type control WT-6 was made by replacing the 6.9-kb *Sma* I/*Sal* I fragment containing the mutation with the identical fragment from pNL4-3.

Analysis of Viral Proteins. For analysis of HIV proteins in transfected SW480 cells, one 10-cm dish of cells was starved for 4 hr, beginning 24 hr posttransfection, in medium lacking methionine. Cells were then labeled for 18 hr with 1 mCi of [^{35}S]methionine (Amersham) in 10 ml of serum-free medium and lysed in 0.4 ml of RIPA buffer (1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$/150 mM NaCl/10 mM Tris·HCl, pH 7.2). The resultant extract was incubated with 50 μl of fixed washed *Staphylococcus aureus* (Pansorbin, Calbiochem) for 10 min at 0°C, clarified by centrifugation, and incubated with 5 μl of anti-HIV antibody-positive antiserum from a seropositive healthy individual for 1 hr at 0°C. This antiserum is highly reactive with the HIV gag p24 protein and its precursor gag p55. Pansorbin (50 μl) was then added and incubation was continued for 10 min at 0°C. The mixture was centrifuged for 15 min at 2000 × *g* and the resulting pellet was washed three times with 0.5 ml of RIPA buffer, once with 0.5 ml of 150 mM NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA, and resuspended in 55 μl of sample buffer B (62.5 mM Tris·HCl, pH 6.8/10% glycerol/2% $NaDodSO_4$/5% 2-mercaptoethanol). Proteins were eluted from the bacteria by boiling and analyzed by electrophoresis on 10% $NaDodSO_4$/polyacrylamide gels followed by autoradiography.

For analysis of viral proteins in the supernatants of transfected SW480 cells, supernatant from a 10-cm dish of [^{35}S]methionine-labeled cells was clarified by centrifugation and concentrated 500-fold by using the Centriprep 10 System (Amicon). The concentrated supernatants were diluted to 0.4 ml with RIPA buffer, immunoprecipitated with human anti-HIV antibody-positive antiserum, and analyzed as described above.

For analysis of HIV proteins in infected MT-4 cells, half of the cells in a single well of a 48-well cell culture plate were lysed in 55 μl of sample buffer B, and the proteins were separated by electrophoresis in 10% $NaDodSO_4$/polyacrylamide gels and electroblotted onto Immobilon membranes (Millipore). Membranes were incubated with human anti-HIV antibody-positive antisera (1:500 dilution) in 20 mM Tris·HCl, pH 7.5/500 mM NaCl/0.1% Tween 20/5% powdered milk/5% immunoglobulin-free fetal calf serum (GIBCO) at 22°C for 1 hr and then washed with 20 mM Tris·HCl, pH 7.5/500 mM NaCl/0.1% Tween 20. Immunoreactive proteins were detected by autoradiography after incubation with ^{125}I-labeled protein A (5 μCi; Amersham) in the same buffer.

Immunofluorescence Analysis. SW480 or MT-4 cells were harvested, washed in phosphate-buffered saline (PBS), resuspended in 100 μl of PBS/H_2O (1:1), spotted onto slides, dried, and fixed in methanol/acetone (1:1) at −20°C for 10 min. The fixed cells were incubated with human anti-HIV antibody-positive antiserum (1:100 dilution) in PBS for 1 hr at 37°C, washed with PBS containing 0.02% Tween 20, and then incubated with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (Kirkegaard and Perry Laboratories, Gaithersburg, MD; 1:30 dilution) in PBS for 1 hr at

37°C. The resulting fluorescence was visualized by epifluorescence microscopy with a Zeiss Axioplan microscope.

RESULTS

Bacterial Expression of Mutant HIV Protease. A bacterial expression vector for the HIV protease was constructed that produces enzymatically active HIV protease (unpublished data). This plasmid contains a gene fragment from the HIV NY5 isolate (18) spanning the protease coding region under the control of the tightly regulated bacterial *trp* promoter. By using oligonucleotide-directed mutagenesis, a single base pair substitution, an A for a G, was made in codon 25, changing the codon for an aspartic acid residue to one for an asparagine. Cells containing either the wild-type (pPRT), the wild-type lacking the initiator ATG (pPRT-ATG), or the Asn-25 mutant [pPRT(Asn-25)] protease constructs were induced by growth in minimal medium containing indolacrylic acid, and the intracellular proteins were analyzed by NaDodSO$_4$/PAGE followed by immunoblot analysis with a polyclonal anti-protease antiserum. Whereas no immunoreactive protein was detected in cells containing pPRT-ATG, a single immunoreactive protein migrating with an apparent molecular mass of 10 kDa was observed in extracts from cells containing either pPRT or pPRT(Asn-25) (Fig. 1*A*). The level of protein produced by the pPRT(Asn-25)-containing cells was similar to that of cells containing the wild-type plasmid.

To determine the effect of the mutation on the activity of the enzyme, the ability of the protease produced by pPRT-(Asn-25)-containing cells to cleave the HIV gag p55 polyprotein precursor was examined. Extracts from cells expressing either the wild-type or mutant protease were incubated with yeast-produced HIV gag p55 *in vitro* and the products were analyzed by NaDodSO$_4$/PAGE followed by immunoblotting with a mixture of murine monoclonal antibodies against gag p24 and p17. As shown in Fig. 1*B*, the protease produced by pPRT cleaved p55 to give 24- and 17-kDa polypeptides that were immunologically indistinguishable from the viral p24 and p17 proteins. In contrast, no cleavage products were detected in reaction mixtures containing protease produced by pPRT(Asn-25) (Fig. 1*B*), indicating that the substitution of Asp-25 with an asparagine residue completely inhibited the ability of the enzyme to cleave p55.

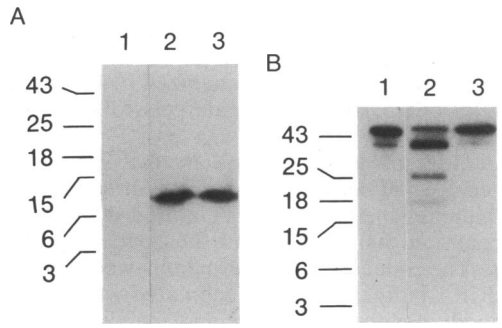

FIG. 1. Characterization of bacterially expressed HIV protease. (*A*) Immunoblot analysis of HIV protease expression. *E. coli* HB101 cells transformed with the HIV protease constructs were induced for expression and whole cell extracts were prepared. Proteins were separated by NaDodSO$_4$/PAGE in 16% gels, electroblotted onto nitrocellulose membranes, and incubated with rabbit anti-protease antiserum raised against HIV protease residues 34–44. Immunoreactive proteins were detected with ^{125}I-labeled protein A (Amersham), followed by autoradiography. Each lane contains extract from cells isolated from 0.25 ml of culture. Lanes: 1, pPRT-ATG; 2, pPRT; 3, pPRT(Asn-25). (*B*) HIV gag p55 cleavage assay. Cells isolated from 5 ml of induced cultures of *E. coli* HB101 containing the HIV protease constructs were disrupted by sonication in 0.25 ml of lysozyme-containing buffer. A 10-μl aliquot of the cell-free extract was added to 4 μl of yeast-expressed HIV gag p55 (1 mg/ml), the reaction mixture was made 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.5), and the volume was brought to 20 μl. After a 1-hr incubation at 37°C, the reaction was quenched by the addition of 10 μl of sample buffer B. Products (15 μl) were analyzed by NaDodSO$_4$/PAGE on 16% gels, electroblotted onto nitrocellulose membranes, and incubated with murine monoclonal antibodies (DuPont) against HIV gag p24 and p17. Immunoreactive proteins were detected with ^{125}I-labeled goat anti-mouse antiserum (Amersham) followed by autoradiography. Lane 1, HIV gag p55 alone. Extracts incubated with HIV gag p55 were from cells transformed with pPRT (lane 2) and pPRT(Asn-25) (lane 3). Molecular mass values of prestained markers (Bethesda Research Laboratories) are indicated in kDa.

HIV Protease Mutant Provirus. To examine the infectivity of HIV virions mutated at Asp-25 of protease, the identical A to G substitution was made in a gene fragment spanning the protease coding region of pNL4-3, an infectious HIV proviral DNA containing *gag* and *pol* sequences from the HIV NY5 isolate and *env* sequences from lymphadenopathy-associated virus (24). The virus, with some 5′ and 3′ flanking cellular sequences, was then reconstructed around this fragment (PM-3; Fig. 2). Detailed restriction mapping of the virus and sequencing of the region containing the mutation revealed no additional mutations (data not shown). As a control, a virus lacking the mutation, WT-6, was constructed by replacing the *Sma* I/*Sal* I fragment of PM-3 containing the mutation with the corresponding fragment from pNL4-3.

Transient Expression of Mutant Virus in SW480 Cells. Equivalent amounts of pNL4-3, WT-6, or PM-3 DNA were separately transfected into SW480 cells. SW480 cells are sensitive to DNA transfection and have been shown to transiently produce high levels of HIV (24). Immunofluorescence staining with human anti-HIV antibody-positive antiserum was used to determine whether the transfected proviral DNAs were capable of directing the synthesis of viral proteins. As determined by immunoprecipitation and immunoblot analysis of HIV lysates, this antiserum contains antibodies to all of the major viral proteins and is highly reactive with the HIV gag p24 protein and its precursor p55. Viral antigens were detected in ≈5% of the cells transfected with pNL4-3, WT-6, or PM-3. No antigens were observed in mock-transfected SW480 cells (data not shown). Thus, HIV antigens were produced in a similar number of SW480 cells by the mutant and wild-type proviruses, indicating that the transfection efficiency of each of the plasmids was similar.

To examine production and processing of the gag p55 precursor polyprotein in SW480 cells transfected with the mutant and wild-type proviral DNAs, the cells were labeled with [^{35}S]methionine and the intracellular viral proteins were immunoprecipitated with human anti-HIV antibody-positive antiserum and fractionated by NaDodSO$_4$/PAGE. As shown in Fig. 3*A*, p55 was detected in cells transfected with both the wild-type and mutant DNAs. However, whereas cells transfected with pNL4-3 and WT-6 contained p24, cells transfected with PM-3 did not. A higher level of the p55 precursor was present in cells transfected with the mutant virus (Fig. 3*A*). This observation is consistent with the absence of the p24 product in these cells and indicates that the mutant protease was completely blocked in its ability to process p55 *in vivo*.

The presence of viral proteins in the supernatants of the transfected SW480 cultures also was examined by radioactive labeling and immunoprecipitation with human anti-HIV antibody-positive antiserum. Viral proteins were detected in the supernatants from all of the cultures (Fig. 3*B*). However, whereas wild-type virions contained a relatively low level of p55 and abundant p24, mutant PM-3 virions contained a relatively high level of p55 and no p24. Thus, failure to cleave p55 did not prevent virion assembly and release.

Infectivity of Mutant Virus. To measure the infectivity of the mutant virions, supernatants from the transfected SW480 cells were applied to MT-4 lymphoid cells. MT-4 cells are

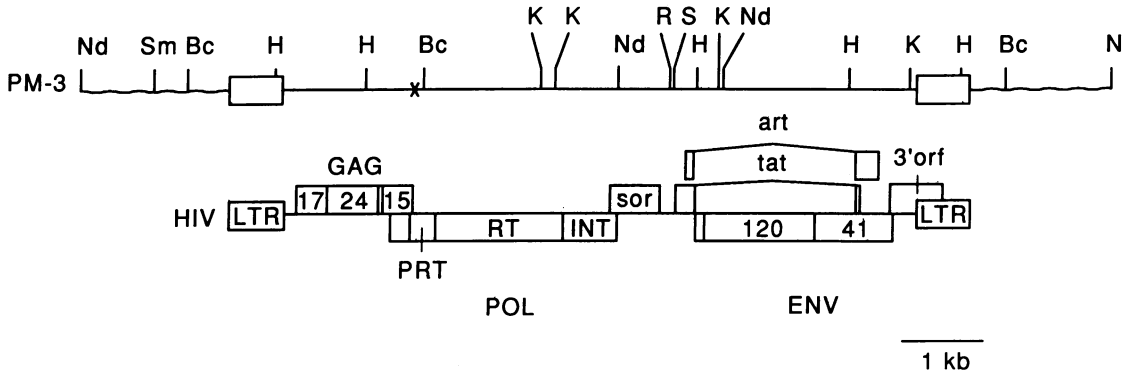

FIG. 2. Restriction map of HIV protease mutant provirus PM-3. Thin lines, viral sequences; wavy lines, flanking cellular sequences; ×, site of the A to G mutation; LTR, long terminal repeat; Bc, *Bcl* I; H, *Hin*dIII; K, *Kpn* I; N, *Nru* I; Nd, *Nde* I; R, *Eco*RI; S, *Sal* I; Sm, *Sma* I.

sensitive to infection by HIV and display virus-induced cytopathic effects (22). Infection was monitored by immunofluorescence and immunoblotting. The presence of viral antigens in MT-4 cells was detected in 100% of the cells infected with the wild-type viruses, pNL4-3 and WT-6, by immunofluorescence staining with human anti-HIV antibody-positive antiserum by 4 days postinfection. These cultures exhibited a total cytopathic effect by 6 days. Immunoblot analysis of the intracellular viral proteins produced in MT-4 cells infected with these wild-type viruses indicated that, as expected, p55 was processed to yield p24 (Fig. 4). In contrast, no viral antigens were detected by immunofluorescence or immunoblot analysis in MT-4 cells infected with the PM-3 mutant (Fig. 4). These cultures remained healthy and no viral antigens were noted up to 15 days postinfection. This result indicates that virions produced by HIV containing a mutation at Asp-25 of the protease are not infectious.

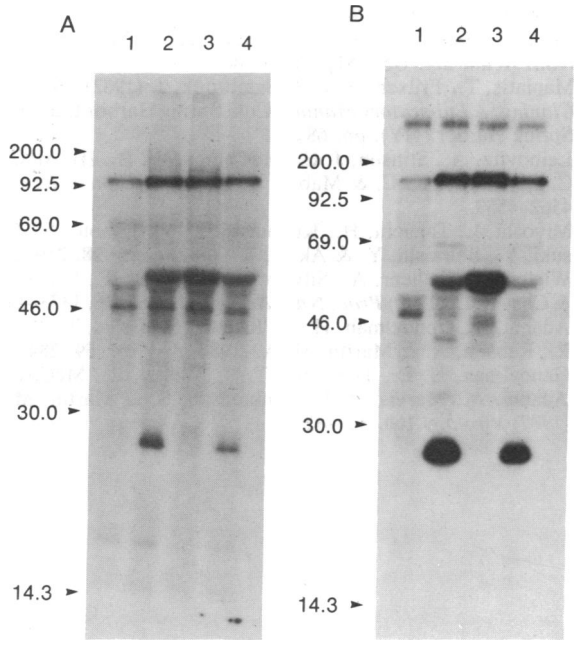

FIG. 3. Immunoprecipitation of HIV proteins from transfected SW480 cells. SW480 cells transfected with the Asn-25 protease mutant (PM-3) and wild-type (pNL4-3 and WT-6) HIV proviral DNAs were labeled with [^{35}S]methionine for 18 hr beginning 28 hr posttransfection. Intracellular HIV proteins from a 10-cm dish of cells (*A*) and viral proteins in 8.5 ml of the cell culture supernatants (*B*) were immunoprecipitated with human anti-HIV antibody-positive antiserum and analyzed by NaDodSO$_4$/PAGE in 10% gels, followed by autoradiography. Cells were mock-transfected (lane 1) or transfected with pNL4-3 (lane 2), PM-3 (lane 3), and WT-6 (lane 4). PM-3 and WT-6 lack flanking cellular sequences present in pNL4-3. Molecular mass values of ^{14}C-methylated markers (Amersham) are indicated in kDa.

DISCUSSION

Substitution of Asp-25 with an asparagine residue eliminated the ability of the HIV protease expressed in *E. coli* to cleave HIV gag p55. This observation demonstrates that Asp-25 is essential for activity of the enzyme, and strengthens the proposed association of the retroviral proteases with the family of aspartyl proteases that includes renin and pepsin (10). Analogy with pepsin and renin, in which the conserved aspartyl residue is at the active site, suggests that Asp-25 would participate in the catalytic mechanism of the enzyme. Therefore, its substitution might result in loss of enzymatic activity with no effect on conformation. Thus, this single nucleotide substitution afforded an ideal probe for the importance of the protease in the replication cycle of HIV.

The effect of this mutation in the HIV protease gene on viral replication and infectivity was examined by first trans-

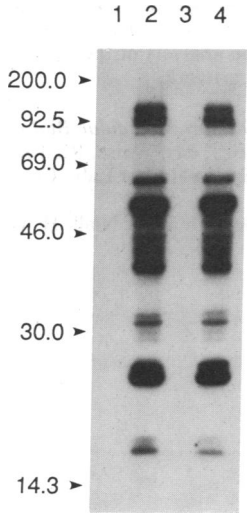

FIG. 4. Immunoblot of HIV proteins in infected MT-4 cells. MT-4 cells (1×10^6) were infected with 1 ml of supernatant from SW480 cultures that had been mock-transfected (lane 1) or transfected with pNL4-3 (lane 2), PM-3 (lane 3), or WT-6 (lane 4). Four days later, half of the cells were lysed and proteins from all of the extracts were separated by NaDodSO$_4$/PAGE in 10% gels, transferred to Immobilon membranes, and incubated with human anti-HIV antibody-positive antiserum. Immunoreactive proteins were visualized by incubation with ^{125}I-labeled protein A followed by autoradiography. Molecular mass values of ^{14}C-methylated markers (Amersham) are indicated in kDa.

fecting nonlymphoid SW480 cells with mutant proviral DNA. Viral particles produced as a result of this transfection were examined for the presence of viral proteins and analyzed for the ability to infect $CD4^+$ lymphoid cells. By this procedure, the individual steps of viral protein synthesis, release of virions and infectivity could be examined. Initial transfection of nonlymphoid cells with the mutant proviral DNA precluded the selection of revertant, infectious virions arising from second site mutations.

Incorporation of the protease Asn-25 mutation in the HIV provirus did not noticeably affect the expression of the gag polyprotein within transfected SW480 cells or the assembly and release of virions into the medium. Significantly, the virions produced by the Asn-25 mutant provirus contained gag p55 but were devoid of gag p24, reflecting a defect in *in vivo* proteolytic processing. Whereas both the mutant and wild-type proviruses directed the synthesis of virions, only the wild-type virions were infectious when assayed on the highly sensitive MT-4 lymphoid cells. Thus, inability of the mutant virus to infect MT-4 cells correlates with the loss of gag p55 cleavage. Studies of other retroviruses suggest that the viral protease might be responsible for cleavage of the *pol* gene product as well (11). Insufficient levels of viral protein obtained from the transfected cell cultures prevented the analysis of the processing of the *pol* and *env* gene products during viral maturation. The current experiment could not distinguish which of the proteolytic events is critical.

The HIV genome is known to encode three enzymes—reverse transcriptase, integrase, and protease. A previous study with a mutant that expressed a truncated *pol* gene product suggested that either reverse transcriptase or integrase or both were essential for viral infectivity (25). Here a single mutation that eliminated the activity of the HIV protease blocked the infectivity of the virus. These experiments, therefore, predict that inactivation of the HIV protease by a specific chemical compound should render the virus noninfectious.

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