DEGRADATION OF HIV-1 INTEGRASE BY THE N-END RULE PATHWAY

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SUMMARY

Human immunodeficiency virus type-1 (HIV-1) integrase catalyzes the irreversible insertion of the viral genome into host chromosomal DNA. We have developed a mammalian expression system for the synthesis of authentic HIV-1 integrase in the absence of other viral proteins. Integrase, which bears a N-terminal phenylalanine, was found to be a short-lived protein in human embryo kidney 293T cells. The degradation of integrase could be suppressed by proteasome inhibitors. N-terminal phenylalanine is recognized as a degradation signal by a ubiquitin-proteasome proteolytic system known as the N-end rule pathway. The replacement of N-terminal phenylalanine with either methionine, valine or glycine, which are stabilizing residues in the N-end rule, resulted in metabolically stabilized integrase proteins (half-life of N-terminal Met-integrase was at least 3hr.). Conversely, the substitution of N-terminal phenylalanine with other destabilizing residues retained the metabolic instability of integrase. These findings indicate that the HIV-1 integrase is a physiological substrate of the N-end rule. We discuss a possible functional similarity to the better understood turnover of the bacteriophage Mu transposase, and functions of integrase instability to the maintenance and integrity of the host cell genome.
INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) integrase (IN) belongs to an ancient group of enzymes, the polynucleotidyl transferases. These proteins related to HIV-1 integrase play a number of major roles in all organisms, from the transposases of prokaryotic insertion elements to mammalian enzymes that are required for the formation of the immune system, such as the RAG1/RAG2 recombinases (1,2). The retroviral integrase mediates an obligatory step in the viral life cycle by permanently joining viral genomic DNA to the chromosomal DNA of the infected host (3). This step is typical of the retroviruses, and is required for efficient expression and stable maintenance of their genomes. Biochemical reconstitution of the reactions catalyzed by recombinant HIV integrase in vitro

1 Abbreviations Used: HIV-1, human immunodeficiency virus type 1; IN, integrase; PIC, preintegration complex; Ub, Ubiquitin; EGFP, enhanced green fluorescent protein; Asp, aspartic acid, Glu, glutamic acid; Phe, phenylalanine; Gly, glycine; His, histidine; Leu, leucine; Met, methionine; Asn, asparagine; Gln, glutamine; Arg, arginine; Val, valine; Trp, tryptophan.
has revealed the mechanistic aspects of integration, which can be mediated in the absence of any other viral or host protein, reviewed in (4). Current understanding of the reactions catalyzed by integrase has recently led to the development of a potent inhibitory molecule that specifically blocks HIV-1 replication at integration (5).

Viral integrase is synthesized in infected cells as a component of a large precursor protein (Gag-Pol, 160 kilodaltons). The Gag-Pol polyprotein is packaged into the virion in a step that precedes endoproteolytic processing by the viral protease. This step occurs soon after viral budding to generate the mature 32 kilodalton integrase protein. Upon infection, integrase is released into the cytoplasm of the target cell as part of the viral preintegration complex (PIC). The PIC is a large (28nm) (6), subviral complex that provides a sequestered environment within the host cell where reverse transcription of viral RNA into linear DNA takes place; part of this complex is then transported to the nucleus (7). As would be expected, integrase contains a potent nuclear localization signal (NLS) that is apparently required for nuclear transport of the PIC (8-10) (D. Kaufman and M. Muesing, unpublished observations), a step that is critical for infection of non-dividing cells.

In contrast to recombinant HIV integrase, which has been engineered with a N-terminal methionine (Met), viral integrase is excised from the Gag-Pol polyprotein and bears N-terminal phenylalanine (Phe) (11). In previously studied model proteins, N-terminal phenylalanine is part of a degradation signal termed N-degron. This signal is recognized by the ubiquitin (Ub)/proteasome-dependent N-end rule pathway (12). The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal residue. The recognition of N-degrons by the Ub ligase (E2-E3) of the N-end rule leads to the conjugation of (Ub), in the form of a multi-Ub chain, to one or more of the internal lysine (Lys) residues. The resulting Ub-containing protein is processively degraded by the 26S proteasome.
To study the authentic HIV-1 integrase protein in the absence of viral infection in human cells, and to test whether integrase is a physiological substrate for the N-end rule pathway, we have adapted the ubiquitin fusion technique first described by Varshavsky and co-workers (13). This method involves the fusion of an ubiquitin monomeric moiety immediately adjacent to the N-terminal amino acid of the protein of interest, which in this case results in an ubiquitin-HIV-1 integrase fusion protein (Ub-Phe-IN). Fusions to Ub are known to be co-translationally cleaved at the Ub-protein junction. This approach offers the advantage of an often large increase in yield of the studied protein, as well as the ability to produce any desired residue at the protein’s N-terminus (14,15).

Our results identify HIV-1 integrase as a short lived protein degraded by the N-end rule pathway. Integrase is the second viral protein that is known to be a substrate for this pathway. This observation may have implications with respect to HIV-1 replication, host cell viability and the immunological surveillance of cells infected with the pathogen. We discuss these results in light of the experimental observation that inhibition of proteasome function greatly increases the intrinsic infectivity of HIV-1 (16), along with the implications of proteasome-mediated metabolism of viral protein products in the context of HIV-1 antigen presentation (17-19). Finally, we compare the results obtained here with that obtained for the bacteriophage Mu transposase; a phage recombinase subject to the activity of the molecular chaperone (ClpX) and its associated proteolytic form, ClpXP (20-23).
EXPERIMENTAL PROCEDURES

**Plasmids.** pEGFP*IRES-Ub-X-IN CTE (where X stands for the different IN N-terminal residues) and pEGFP*IRES ATG were constructed using pEGFP-C1 (Clontech) as backbone, and by applying standard molecular biology techniques. PCR fragments used in cloning were sequenced either with the T7 Sequenase v2.0 7-deaza-dGTP sequencing kit (Amersham Pharmacia Biotech or USB Corporation) or by automated sequencing (ABI Prism 377XL DNA Sequencers) performed by The Rockefeller University Protein and DNA Technology Center. All clones were correct by DNA sequence; one clone pEGFP*IRES-Ub-L-IN CTE contains a conservative point mutation at E48 of integrase that does not alter the amino acid at this position.

**Chemicals.** Proteasome inhibitors clasto-lactacystin β-lactone and MG132 were purchased from Calbiochem. Cycloheximide was purchased from Aldrich. Stock solutions were prepared in dimethyl sulfoxide (DMSO).

**Tissue Culture and Transfections.** Human embryo kidney (HEK) 293T cells were cultured in Dulbecco modified minimal Eagle’s medium (DMEM) 10% fetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin. Transfections were performed using calcium phosphate precipitation in either 6 well plates or 60mm dishes with 1µg or 4µg of DNA respectively. Cells were harvested typically 40-44 hours post-transfection. Since integrase partitions preferentially to the nuclear compartment (8,10), (D. Kaufman and M. Muesing, unpublished results), nuclear extracts were prepared utilizing a hypotonic lysis step followed by high salt extraction of the nuclei, as described (24).
**Western Immunoblot.** Equal volumes of nuclear extracts were loaded onto 10% acrylamide NuPage pre-cast mini-gels (Novex) and electrophoresed under reducing conditions. After transfer to Hybond-P filters (Amersham), blots were blocked with phosphate-buffered saline (PBS) containing 0.2% Tween (PBS-T) plus 5% nonfat dry milk (Carnation). Integrase was detected by incubating the filters overnight at 4°C with anti-IN antibody EL 896 or EL 892 (see below), followed by incubation with anti-rabbit horseradish peroxidase (HRP) -conjugated antibody (Amersham) and development with ECL-Plus (Amersham). For GFP detection, blots were stripped with 0.2N NaOH and washed extensively with water and PBS-T; filters were then blocked, incubated overnight at 4°C with anti-GFP monoclonal antibody (cat# 8362-1, Clontech), probed with anti-mouse HRP-conjugated antibody (Amersham), and developed as above.

**Degradation Rate Quantitation** HEK 293T cells were transfected in 60 mm dish format with 4µg plasmid DNA encoding Ub-Met-IN and Ub-Gln-IN. After 16-20 hours of transfection, the cells were split 1:4 in 6-well dishes and the day after treated with 100µg/ml cycloheximide for 0, 20, 50 and 110 minutes for the Ub-Gln-IN transfections, and 0, 50, 110 and 180 minutes for the Ub-Met-IN transfections. For each sample, 7.15µg of protein nuclear extract were immunoblotted and visualized using anti-integrase antibody EL 892 . Quantitation of band density was performed with NIH Image 1.62 software. Relative values were referred to standard curves obtained from dilutions of time point 0 min. samples of each experiment. Figure 4B represents the mean relative values and standard deviations of two independent experiments.

**Preparation of Polyclonal Antibodies to Integrase.** The antisera, EL896 and EL892 directed to the carboxyl terminus and amino terminus of HIV-1 integrase respectively, were raised by immunizing...
rabbits against peptides prepared as follows: peptides corresponding to the C-terminal residues (amino acids 273-288; Ac-KQMAGDDCVASRQDED-OH) and N-terminal residues (amino acids 1-16 Ac-FLDGIDKAQDEHEKYH-OH) of HIV-1 integrase were synthesized, purified to 90%, and covalently coupled at a 1:50 w/w ratio to the keyhole limpet hemocyanin (KLH) as carrier protein, (Chiron Mimotopes, Victoria, Australia).
RESULTS

Expression Vector for the Synthesis of Authentic HIV-1 Integrase in Mammalian Cells

The first amino acid of viral integrase is phenylalanine and is invariant among all genetic subtypes of HIV-1 (25). However, the conservation of this residue is not dictated by HIV protease specificity constraints, since its target cleavage sites have a wide degree of degeneration (26). In order to express authentic integrase, we employed a technique previously designed to express proteins which have an N-terminal amino acid that differs from methionine (13). This takes advantage of potent cellular de-ubiquitylating enzymes that cleave rapidly at the junction between the carboxyl terminus of ubiquitin and the amino terminus of a fused protein domain. Therefore, the coding sequence for human ubiquitin was fused with that of authentic HIV-1 integrase to generate Ub-Phe-IN, specifying phenylalanine as the N-terminal amino acid (Figure 1). The vector was designed to contain the coding sequence of the enhanced green fluorescent protein (EGFP) under the control of the CMV promoter, along with an internal ribosome entry site (IRES) upstream of the Ub-Phe-IN gene segment. This arrangement creates a bicistronic transcriptional unit (EGFP-IRES-Ub-Phe-IN) in which EGFP expression can be used to monitor the efficiency of DNA transfection and serves as an internal control for the synthesis of the EGFP-IRES-Ub-Phe-IN bicistronic transcript (Figure 1).

Expression of Authentic HIV-1 Integrase is not Detected in the Absence of Proteasome Inhibitors.

The HIV-1 integrase protein is readily detected in lysates prepared either from purified virions (27,28) or from cultures of infected T-cells (data not shown). However, in the absence of other viral gene products, the expression of authentic integrase (Phe-IN) could only be detected when specific
inhibitors of the proteasome function were added to the transfected cell culture (Figure 2). The failure to detect Phe-IN was not due to lack of Ub-Phe-IN mRNA expression, since approximately equal amounts of the EGFP control protein were present in all samples (Figure 2). These results strongly suggest that integrase is a short-lived protein degraded by a proteasome-dependent pathway.

**HIV-I Integrase is Degraded by the N-end Rule Pathway.**

The N-terminal residue of mature HIV-1 integrase protein is Phe, a strongly destabilizing residue in both mammalian and yeast N-end rule pathways (12). To determine whether integrase is indeed a substrate for the N-end rule pathway, we substituted the N-terminal Phe in Phe-IN (produced from Ub-Phe-IN) with 10 other residues. The corresponding plasmids, EGFP-IRES-Ub-X-IN, are isogenic with EGFP-IRES-Ub-Phe-IN except for the identity of the codon specifying the residue at the Ub-IN junction. The N-terminal residues used encompass the four classes of amino acids in the N-end rule: primary (Leu, Arg, His), secondary (Asp, Glu), tertiary (Gln, Asn) and stabilizing (Met, Val, Gly) residues, primary residues being the least stable (12). Figure 3 shows that the steady-state levels of wild-type Phe-IN molecule and of the Arg-IN and the His-IN mutant proteins were detectable in this assay only when the transfected cell culture was pretreated overnight with a proteasome inhibitor prior to lysis. Leu-IN was barely detectable in the absence of inhibitor, whereas the levels of integrase derivatives bearing secondary (Asp and Glu) and tertiary (Asn and Gln) destabilizing N-terminal residues were, as expected, increasingly higher. Taken together, these results indicate that HIV-1 integrase is a physiological substrate of the Ub/proteasome-dependent N-end rule pathway.

**Kinetics of the in vivo Degradation of Integrase.**

To monitor the *in vivo* decay of Phe-IN (wild-type integrase) and some of its N-terminal
derivatives, we carried out a cycloheximide chase analysis. In this assay, the second day after DNA transfection, the translational inhibitor cycloheximide was added to cell cultures at sequential time points, nuclear extracts were then made simultaneously of samples with different periods of treatment. This analysis is particularly relevant because it does not examine newly synthesized molecules but monitors the turn-over of existing proteins, a scenario that more closely reflects the viral replication cycle in which native integrase is not produced in the infected cell but is delivered in the cytoplasm from the moment of entry of the virus. Figure 4A shows the qualitative results of such a kinetic analysis for native integrase and three of its N-terminal substitution mutants (one mutant was chosen from each N-degron family; see above). We were unable to detect wild-type integrase at any experimental time point using this assay. However, it was possible to compare and quantitate by densitometry (see Material and Methods) the decay of a tertiary and a stabilizing N-degron mutants, Ub-Gln-IN and Ub-Met-IN respectively (Figure 4B). Figure 4B shows a clear difference in the rate of degradation between the two IN mutants. A conservative estimate of the half-life of Met-IN is ≥3 hours whereas the half life of Gln-IN, as estimated by curve interpolation, is 47 min.
DISCUSSION

Metabolic instability, like that observed for HIV-1 integrase, is a common property of several rate-limiting enzymes, transcription factors and critical regulatory proteins (12). It is unclear why HIV-1 integrase has evolved to include this mechanism of post-translational regulation. To our knowledge the only other viral protein that has been shown to be regulated by the N-end rule is the RNA polymerase (nsP4) of Sindbis alphavirus (29). Integrase is one of only two processed HIV-1 proteins (p6 being the other) (16), with an N-terminal amino acid residue belonging to the primary N-end degron class (p6 has not been investigated with respect to the N-end rule).

While expression of authentic integrase in the absence of other viral protein has revealed that HIV-1 integrase is subject to rapid degradation, it is clear that integrase is protected during a large portion of the viral life cycle. Integrase stability is not affected by N-end rule degradation during its synthesis in the producer cell as an unprocessed domain of the Gag-Pol polyprotein, nor during its subsequent packaging into the virion particle. At what stage of the HIV-1 life cycle does integrase become exposed to the host proteasome? Although the maturation of integrase from the Gag-Pol precursor is complete after viral budding from the infected cell, the potential for contact with the host proteolytic machinery can occur only after viral membrane fusion and entry of the pre-integration complex into the cytoplasm of the infected target cell. During the initial steps of infection, the HIV-1 genome and its associated nucleoproteins (including integrase) are released into the cytoplasm as a high molecular weight subviral organelle that has been operationally defined as the viral pre-integration complex (PIC) (3). The PIC provides an isolated environment within the host cell for the reverse transcription of viral RNA into blunt-ended, linear DNA, as well as providing the signals necessary for
targeting and entry of the large macromolecular complex into the cell nucleus. Although the exact composition of the complex before and after nuclear translocation remains unclear (3), integrase and the viral genomic DNA necessarily must be transported to the nucleus in order to transfer the viral genome into the host chromosomal DNA.

Nuclear translocation of the PIC may well provide a step in the viral life cycle during which integrase is accessible to ubiquitylation and proteasomal degradation. Perhaps it is at this point that inhibition of proteasome function has the greatest effect with regard to the efficiency of HIV-1 infection (16). Conversely, a plausible role for the ubiquitin-proteasome pathway may involve the degradation of integrase after the enzymatic insertion of the provirus has been completed. It is known that integrase binds to the host protein BAF47/INI1 (30). This protein is a component of the hSWI/SNF chromosomal remodeling complex, interacts with c-myc (31), and has recently been identified as a new tumor-suppressor gene (32). It is possible to imagine that a prolonged interaction between IN and BAF47/INI1 results in death of the host cell, as described in yeast, where the expression of methionine-initiated (and thereby stable) HIV-1 integrase results in a lethal phenotype (33,34). Indeed this phenotype is completely reversed by inactivation of the yeast INI1 homolog SNF5 (35).

Another indication that the retroviral integrase might be toxic for the host cell comes from a report in which cells deficient for the DNA repair enzyme, DNA-dependent protein kinase (DNA-PK), or components of the DNA-PK pathway, Ku86 or XRCC4, die through apoptosis upon infection with retroviral vectors (36). Control vectors that were integration-defective did not have this effect. In this report, it was concluded that the integration process requires the DNA-PK pathway for repair (36). Such a repair pathway could be overridden in the cause of DNA damage, for instance damage mediated by the catalytic properties of integrase or by the persistence of a stable integrase protein that is inherently long-lived. In any case, the end point of of retroviral infection might be prematurely
terminated with the apoptosis of the host cell. In this scenario, cell viability, required for the production of progeny virus, is dependent on rapid proteasome-mediated degradation of integrase, a protein no longer required by the virus nor tolerated by the cell.

Regardless of the reason for the existence of the N-terminal degron of integrase, it is clear from a phylogenetic alignment of all known lentiviral integrase proteins that a primary destabilizing N-degron residue (either Phe or Trp) is found at the junction between reverse transcriptase and integrase. The conservation of a primary destabilizing N-degron residue in the lentiviral family is particularly striking for two reasons. First, the viral protease is not limited to viral substrates in which the amino terminus of the cleaved protein is from the set of amino acids specifying a primary N-degron (26). Second, non-lentiviral retroviruses do not exhibit strict conservation of a primary N-degron amino acid at the N-termini of their respective integrases (3,11,37,38).

Integrase, like Mu transposase (MuA), is part of a broad family of enzymes that catalyzes the cleavage and joining reactions of DNA elements. Indeed the crystal structures of the two molecules are superimposable in many parts of the catalytic domain. MuA is essential for the transposition of *Escherichia coli* phage Mu. After recombination is complete the protein-DNA complex is remodeled by a host chaperone protein, ClpX, (passage from strand transfer complex STC1 to STC2) MuA is disassembled by yet unidentified factors (passage from STC2 to STC3), and at least *in vitro*, degraded by the protease complex formed by ClpX-ClpP (ClpXP) (21,23,39). This rather complicated succession of reactions permits the initiation of the Mu DNA replication. MuA is protected throughout the recombination process by the transposition activator, MuB, which recognizes a region of the transposase that overlaps with the ClpX binding region (22). Analogies can be found between Mu transposition and the HIV-1 integration process. It could be that integrase degradation mirrors to a certain extent that of MuA processing. Protection from degradation might be assured in the cytoplasm.
by the complex of the PIC proteins and in the nucleus by the hSWI/SNF nucleosome structure remodeling complex. After recombination it could be envisioned that integrase is recognized by the mammalian Ubr1 homolog, E3alpha ubiquitin ligase complex (40) and degraded by the proteasome; a pattern that resembles the ClpX-ClpXP sequence of events. Experiments are currently in progress to address these hypotheses.

Proteins degraded by the ubiquitin-proteasome system are efficiently presented by MHC class I molecules, and as a result help to enhance cytotoxic T lymphocyte (CTL) recognition (41,42). Siliciano and co-workers have recently provided evidence that CD8+ cells have much stronger de novo cytolytic response to the HIV-1 nef gene when the Nef protein is engineered to express a primary N-degron arginine at its N-terminus (Ub-Arg-Nef; native Nef is initiated by methionine) (17). This effect can also be demonstrated in vivo after immunization of mice with the modified viral antigen (17).

The Ub-Phe-IN integrase expression construct described here, and its mutant derivatives, will be instrumental for future dissection of the interaction of the host ubiquitin-proteasomal pathway with HIV-1 integrase and, in a larger sense, with viral life cycle, in addition to the role of the primary N-degron of integrase in the context of the host immunologic response to active HIV-1 replication.
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FIGURE LEGENDS

Figure 1  Description of the bicistronic HIV-1 integrase expression vector, pEGFP*IRES-Ub-X-IN CTE.  The following elements were used: CMV, human cytomegalovirus promoter/enhancer region; EGFP, enhanced green fluorescent protein; IRES, EMCV internal ribosome entry sequence; Ub, human ubiquitin monomeric moiety; X, different integrase N-terminal residues used in these experiments; IN, HIV-1 R7/3 integrase; CTE, Mason-Pfizer virus constitutive transport element.  Transcription of the bicistronic mRNA is initiated at the CMV promoter/enhancer.  The location of the translational start and stop for EGFP and the Ub-X-IN fusion protein are shown.  The downward arrow denotes the location of the post-translational cleavage between ubiquitin and X-integrase.

Figure 2.  Stability of integrase is regulated by the proteasome.  1µg of HIV-1 integrase expression vector, pEGFP*IRES-Ub-F-IN CTE (Fig. 1), EGFP expression vector pEGFP*IRES ATG (C-1), or empty vector pcDNA3.1(+) (Invitrogen) (C-2), were transfected into HEK 293T (250,000 cells/well, in 6-well dishes, plated the day before).  Cells were treated 36-40 hr after transfection with either proteasome inhibitors {MG132 (50µM), clasto-lactacystin β-lactone (10µM)}, drug-free diluent DMSO (1:100 final dilution), or medium alone (Medium). Three hours after treatment, nuclear extracts were prepared and analyzed with SDS-PAGE and immunoblotting.  The blots were probed with anti-integrase antibody, stripped, and reprobed with anti-GFP antibody.

Figure 3  The identity of the N-terminal residue of integrase determines its stability.  Plasmid DNA specifying the expression of wild-type integrase (Ub-F-IN), N-terminal integrase mutants (Ub-M-
IN, Ub-V-IN, Ub-G-IN, Ub-Q-IN, Ub-N-IN, Ub-E-IN, Ub-D-IN, Ub-L-IN, Ub-H-IN, Ub-R-IN), the EGFP expression vector pEGFP*IRES ATG (C-1) were transfected as above in HEK 293T cells. Eighteen hours after transfection the cells were treated either with \textit{clasto}-lactacystin \textit{β}-lactone (10µM) or drug-free diluent DMSO (1:100 final dilution). After an additional twenty hours of cell growth, the transfected cells were harvested, nuclear extracts prepared, the proteins separated by SDS-PAGE and analyzed by immunoblotting as described in Fig. 2.

\textbf{Figure 4. The degradation rate of integrase is influenced by its N-terminal residue.} A) Plasmid DNA specifying the expression of wild-type integrase protein (Ub-F-IN), a subset of the Met, Gln, Glu, N-terminal integrase mutants (Ub-M-IN, Ub-Q-IN, and Ub-E-IN), the EGFP expression vector pEGFP*IRES ATG (C-1), or an empty vector pcDNA3.1(+) (C-2), were transfected as above in HEK 293T cells. The second day after transfection cells were treated for 0, 30, 60 or 120 minutes with 100µg/ml cycloheximide. The zero time-points received drug-free diluent DMSO (final dilution 0.1%). Nuclear extracts were prepared and analyzed as in Fig. 2. B) Decay curves for Ub-M-IN and Ub-Q-IN. Equal amounts of protein nuclear extracts from each time point were analyzed by western blot and quantitated by densitometry.

Results were expressed relative to standard curves obtained by dilution of each time point 0 min..
Cycloheximide Chase

% of time points 0 min

Time (min)

Ub-M-IN

Ub-Q-IN
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