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Mutational Analysis of the Human Immunodeficiency Virus Type 1 *env* Gene Product Proteolytic Cleavage Site

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The structural requirements for proteolytic cleavage of the human immunodeficiency virus type 1 *env* gene product, gp160, to gp120 and gp41 have been assessed by specific mutagenesis of the sequence Lys Ala Lys Arg Arg Val Val Glu Arg Glu Lys Arg located between amino acids 500 and 511, i.e., at the putative C terminus of gp120. The basic amino acids underlined have been mutated, individually and in combination, to neutral amino acids, and the cleavability of the mutated *env* gene products was examined after expression in CV-1 cells. The results show that the replacement of Arg-511 (cleavage presumably occurs C terminal to this amino acid) with Ser completely abolishes recognition and cleavage by the cellular protease(s), i.e., the remaining basic amino acids in the vicinity do not serve as alternative substrates. However, Arg-508 and Lys-510 are important features of the recognition site since, when they are individually changed to neutral amino acids, cleavage is severely impaired. The basic amino acids 500, 502, and 504 are, individually, not important for cleavage, since their individual replacement by neutral amino acids does not impair cleavage. However, when all four basic amino acids 500, 502, 503, and 504 are changed to neutral amino acids, cleavage is almost completely abolished. This shows that the sequence Arg Glu Lys Arg at the cleavage site is alone not sufficient for cleavage but that a contribution of other amino acids is required, whether the other amino acids provide a basic character or a certain structure in the vicinity of the cleavage site. When noncleavable or poorly cleavable mutant *env* genes are expressed from the infectious plasmid pNL4-3 in CD4$^+$ human lymphoblastoid cells, noninfectious virus, incapable of spread throughout the culture, is produced.

Human immunodeficiency virus type 1 (HIV-1), like other retroviruses, recognizes its target cell as a result of a specific interaction between the viral surface glycoproteins and the cellular receptor for the virus. In the case of HIV-1, the cellular receptor is the CD4 molecule present on the surfaces of helper T lymphocytes and some other cell types (6, 16, 20). After interaction of the viral glycoprotein with the receptor, virus uptake by the host cell is initiated in a process involving fusion between viral and cellular membranes. The HIV-1 surface glycoproteins are encoded by the *env* gene whose primary gene product is a glycosylated precursor, gp160, which undergoes several processing steps, including oligosaccharide modification and proteolytic cleavage, to yield the mature glycoprotein complex which contains probably four subunits (27, 31). Each subunit consists of the transmembranous glycoprotein, gp41, noncovalently associated with gp120. These HIV-1 glycoprotein molecules have been quite extensively studied with regard to the binding sites of neutralizing antibodies; in addition, attempts have been made to locate functional domains (see reference 18). Membrane fusion induction, which is CD4 dependent and encoded entirely within the *env* gene (19), involves an amino acid sequence located near the N terminus of gp41 (the so-called fusion peptide) which shows a certain sequence homology to fusion peptides of other viral glycoproteins inducing fusion (8). Mutagenesis of amino acids in this region exquisitely influences the ability to induce cell fusion (syncytium formation) (4, 18). In order for the fusion peptide to be functional, proteolytic cleavage of the precursor gp160 has to have taken place (15, 21). In fact, in the case of influenza virus, an exact N-terminal location of the fusion peptide in the transmembranous hemagglutinin subunit 2 is essential for viral infectivity (9). Proteolytic cleavage of gp160 to gp120 and gp41 occurs intracellularly, possibly in the Golgi complex (36), and is essential for HIV infectivity (21). Expression of the HIV *env* gene product in a number of different cell types leads to proteolytic cleavage to gp41 and gp120. An exception may be insect cells (30). This shows that the (cellular) enzyme(s) capable of cleavage is virtually ubiquitous and can recognize the HIV *env* gene product cleavage site. Like the cleavable glycoprotein precursors from orthomyxoviruses (2), paramyxoviruses (10, 34), and other retroviruses (see reference 26), the HIV *env* gene product contains several basic amino acids N terminal to the glycoprotein precursor cleavage site. The new N terminus of gp41 begins at amino acid position 512 (35) (BH10 numbering; 28). It has been demonstrated for influenza A virus hemagglutinin (13) and paramyxovirus F_0 protein (25) that relatively minor changes which, however, partially reduce the basic character of the cleavage site prevent cleavage from occurring. For HIV-1, it has been shown that gross alterations of the sequences amino terminal to the cleavage site also abolish cleavage (15, 21). In this study, a detailed analysis of the cleavage site has been performed by mutating, individually and in combination, the basic amino acids in this region.

MATERIALS AND METHODS

Construction of the expression plasmid pLExHIVenv. A fragment consisting of simian virus 40 (SV40) sequences 4710 to 4100/2770 to 2533 (nucleotide numbering; 5) containing the SV40 small t intron (4638/4571) and the T-gene polyadenylation signal (23) was inserted into a Bluescript vector (Stratagene, San Diego, Calif.) at the *Asp*718 site. The resulting plasmid was linearized with *Eco*RI and *Xho*I and ligated with two other fragments. One fragment consisted of

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SV40 nucleotides 2533 to 5243/1 to 294, contained the SV40 origin of DNA replication and the T-antigen gene, and had been excised from an intermediate plasmid with *Eco*RI and *Sal*I. The second fragment was from HIV-1 strain BH10 (12), was excised with *Sal*I and *Xho*I (nucleotides 5818 to 8926; 28), and contained both exons of *tat* and *rev* as well as the *U* and *env* genes. The resulting expression plasmid, pLExHIVenv/I, was similar to an expression plasmid described previously (29) and was capable of replication in monkey CV-1 cells. A related vector, pLExHIVenv/II, was identical to that described above, except that the polyadenylation signal was derived from the herpes simplex virus thymidine kinase gene. In this case, the SV40 splice and polyadenylation signals have been replaced by herpes simplex virus nucleotides 46697 to 46067 (22).

Oligonucleotide-directed mutagenesis of pLExHIVenv and transfer of mutated *env* genes into the infectious proviral clone pNL4-3. Single-stranded DNA was prepared from the Bluescript-based vector, pLExHIVenv, essentially as described by the manufacturers of Bluescript. The mutagenesis protocol was based on that described by Zoller and Smith (37). In addition to the mutagenizing oligonucleotide (see below), the T7 primer (localized on Bluescript, nucleotides 622 to 638) and sometimes a wild-type HIV *env* oligonucleotide spanning nucleotides 7620 to 7639 (BH10 isolate; 28) were employed to prime the fill-in reaction with Klenow enzyme. Bacterial colonies obtained after transformation were analyzed by two consecutive rounds of colony hybridization by using the ^{32}P-labeled mutagenizing oligonucleotide as a probe, followed by washing steps at increasing temperatures. Mutant plasmid DNA was subjected to restriction analysis to exclude recombinants with gross rearrangements which, for unknown reasons, were found in approximately one-third of the plasmids. The mutations were confirmed by nucleotide sequence analysis. The following oligonucleotides were employed to generate the mutants. For each mutant, the name (based on the position of the mutated amino acid) is given, followed by the parent plasmid, the position, and the sequence of the oligonucleotide, with the mismatched nucleotide underlined.

MUT 511, pLExHIVenv/I, 7784 to 7805, AGCGCAGTGGGAATAGGAGCTT

MUT 510, pLExHIVenv/I, 7771 to 7792, GCAGAGAGAAAACAGAGCAGTG

MUT 508, pLExHIVenv/I, 7761 to 7782, GAAGAGTGGTGCAGAGCGAAAA

MUT 510+511, pMUT510, 7784 to 7805, AGCGCAGTGGGAATAGGAGCTT

MUT 508+510, pMUT510, 7761 to 7782, GAAGAGTGGTGCAGAGCGAAAA

MUT 504, pLExHIVenv/II, 7760 to 7781, AGAAGCGTGGTGCAGAGAGAAA

MUT 502, pLExHIVenv/I, 7743 to 7764, CACCCACCAAGGCAAACAGAAG

MUT 500, pLExHIVenv/I, 7737 to 7758, GAGTAGCACCCACCAACGCAAA

MUT 500+502+503+504/P, pLExHIVenv/II, 7735 to 7774, AGGAGTAGCA CCCACCAACGCAAACAGCAGCGTGGTGCAG (precursor for MUT 500+502+503+504)

MUT 500+502+503+504, pMUT500+502+503+504/P, 7747 to 7768, CACC AACGCAATCAGCAGCGTG

The mutations which had been generated in pLExHIVenv were transferred to the infectious plasmid pNL4-3 (1) by replacing a *Sal*I-*Bam*HI fragment spanning nearly the whole length of the *env* gene (pNL4-3 [1]; nucleotides 5785 to 8465) with the same fragment from pLExHIVenv (BH10 nucleotides 5818 to 8504).

Electrotransfection of CV-1 and Jurkat cells. Electroporation was carried out essentially as described previously (7). Approximately 5×10^6 subconfluent monkey CV-1 cells, maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, were trypsinized and electrotransfected with pLExHIVenv and mutants thereof (10 to 20 μg of DNA) at 200 V. The other parameters were as described previously (7). After electrotransfection, the CV-1 cells were plated onto three 6-cm dishes. Jurkat human T-lymphoma cells (33) (2×10^7 cells), maintained in RPMI 1640 medium containing 10% fetal calf serum, were electrotransfected with 10 μg of pNL4-3 DNA (1) or derivatives thereof at 250 V.

Detection of HIV antigens by indirect immunofluorescence and ELISA. For indirect immunofluorescence of CV-1 cells, electrotransfected cells were plated onto sterile glass cover slips in a plastic petri dish. At 48 h posttransfection (p.t.), the cover slips were washed once in 1 mM $MgCl_2$ in phosphate-buffered saline and fixed with acetone. Electrotransfected Jurkat cells were collected by low-speed centrifugation, suspended in 1/20 to 1/50 of the original volume, smeared onto glass slides, and, after drying, fixed with acetone. For double immunofluorescence of electrotransfected CV-1 cells, hamster anti-SV40 T-antigen serum at a dilution of 1/50 in phosphate-buffered saline (a kind gift of G. Sauer, Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany) and human anti-HIV-1 serum at a dilution of 1/200 in phosphate-buffered saline were employed together. The second antisera were fluorescein isothiocyanate-labeled goat anti-hamster immunoglobulin G and Texas red-labeled goat anti-human immunoglobulin G (both from Flow Laboratories, Inc., McLean, Va.). Transfected Jurkat cells were treated with the same dilutions of human anti-HIV serum and Texas red-labeled goat anti-human immunoglobulin G as described above. Immunofluorescence was carried out by using standard procedures. Enzyme-linked immunosorbent assays (ELISA) to detect HIV-1 antigen in the supernatants of Jurkat cells transfected with wild-type and mutant pNL4-3 plasmids were performed by using a commercial kit (Organon Teknika, Eppelheim, Federal Republic of Germany).

Labeling of transfected CV-1 cells with [^{3}H]glucosamine and immunoprecipitation of labeled HIV *env* gene products. CV-1 cells in a 6-cm dish, transfected with pLExHIVenv or mutants thereof, were labeled with 100 μCi of [^{3}H]glucosamine per ml (specific activity, 20 to 40 Ci/mmol) (Amersham Buchler, Braunschweig, Federal Republic of Germany) from 32 to 48 h p.t., and cell lysates and media were immunoprecipitated with 10 μl of a 1:1 mixture of rabbit anti-gp160 serum and rabbit anti-gp120 serum (kind gifts of M. Schwaller, National Institute of Medical Research, Mill Hill, London, England), plus 25 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) essentially as described previously (3). After electrophoresis in an 8% polyacrylamide gel and autoradiography, the relative intensities of the immunoprecipitated bands were determined by densitometric scanning of the autoradiogram.

RESULTS

Expression of pLExHIVenv in CV-1 cells. The HIV *env* gene expression plasmid, pLExHIVenv (Fig. 1), is a SV40 late expression vector similar to that described by Rekosh et al. (29) but differs in being based on the bacterial plasmid Bluescript which allows single-stranded DNA, suitable for site-directed mutagenesis, to be made directly from it, thus avoiding multiple recloning steps. In addition, it contains the splice-polyadenylation signals from SV40 (pLExHIVenv/I) or from the herpes simplex virus thymidine kinase gene (pLExHIVenv/II). At 48 h after transfection of CV-1 cells

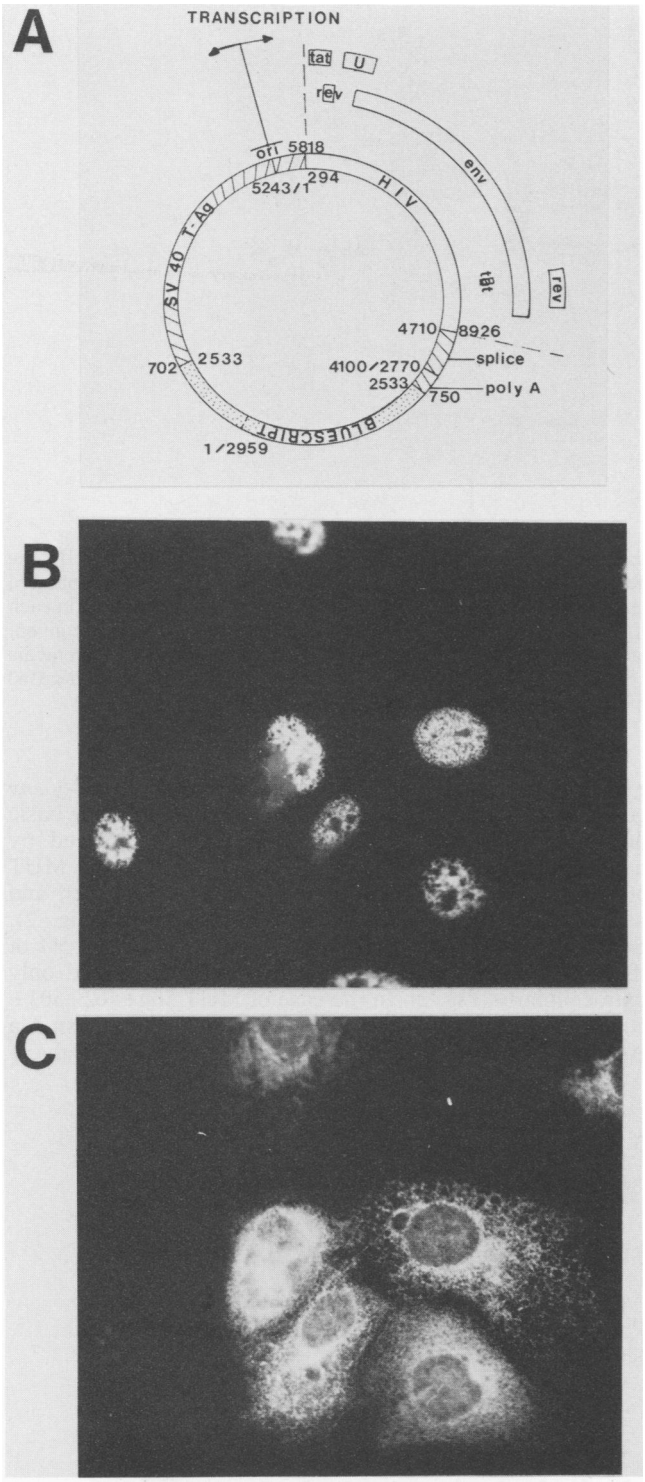

FIG. 1. Expression of pLExHIVenv in CV-1 cells. (A) pLEx HIVenv/I is depicted schematically. The vector consists of Bluescript sequences from nucleotides 750 to 2959/1 to 702 ( ), SV40 sequences from nucleotides 2533 to 5243/1 to 294 ( ), HIV-1 (strain BH10) sequences from nucleotides 5818 to 8926 ( ), and other SV40 sequences from nucleotides 4710 to 4100/2770 to 2533 ( ). The position of the SV40 origin of DNA replication (ori) and the positions of the HIV-1 *tat* exons (tat), the *rev* exons (rev), and the *U* and *env* genes are indicated. (B and C) Double immunofluorescence staining for SV40 T antigen (B) and HIV *env* gene products (C) in CV-1 cells 48 h after transfection with pLExHIVenv/I.

with pLExHIVenv, SV40 T antigen and HIV *env* protein could be detected simultaneously by using double indirect immunofluorescence. Antiserum to SV40 T antigen stained 20 to 30% of the transfected cells with a strong nuclear fluorescence consistent with the predominant nuclear localization of this antigen (Fig. 1B). Figure 1C shows staining of the same cells with anti-HIV serum, where reactivity was localized predominantly to a cytoplasmatic network interpreted as representing the endoplasmic reticulum and Golgi complex. This was consistent with the presence of the HIV *env* gene product in these organelles. Immunofluorescence using rabbit anti-gp120 or anti-gp160 serum showed an identical staining pattern (data not shown) confirming the expression of the HIV *env* gene. Only approximately 50% of the SV40 T antigen-expressing cells also expressed HIV glycoprotein (an example of such a T-antigen-positive, HIV *env*-negative cell can be seen in Fig. 1B and C; cell on the left). The reason for this behavior has not been examined. The level of HIV *env* expression as measured by immunofluorescence or by immunoprecipitation of HIV glycoproteins from transfected cells (see below) was comparable with either pLExHIVenv/I or pLExHIVenv/II.

Proteolytic cleavage of wild-type and mutant HIV *env* gene products in CV-1 cells. Mutants, carrying changes in the vicinity of the HIV *env* gene cleavage site, were prepared by using the procedure of Zoller and Smith (37). The amino acid changes which have been generated are shown in Fig. 3. The yield of colonies containing mutant plasmid was approximately 0.5 to 3% in the first screening round and 50 to 100% in the second screening round.

CV-1 cells, transfected with wild-type pLExHIVenv or mutants thereof, were labeled with [^{3}H]glucosamine at 32 to 48 h p.t., and cell lysates and media were immunoprecipitated with a 1:1 mixture of anti-gp120 and anti-gp160 sera. The intracellular HIV *env* gene products, gp160, gp120, and, on longer exposure, a weak band of gp41, generated from wild-type pLExHIVenv could clearly be seen (Fig. 2). Immunoprecipitation of HIV-containing lysates with normal rabbit serum and of labeled lysates from untransfected cells with anti-gp120 and anti-gp160 sera, which served as negative controls, did not precipitate these glycoproteins (data not shown). This demonstrates that proteolytic cleavage of the viral glycoprotein precursor had occurred in CV-1 cells, as has also been shown by others (29). The fact that relatively little gp41 could be detected was probably because it was being more poorly labeled (fewer glycosylation sites than gp120) and because it was less well recognized by the antisera employed. Immunoprecipitation from the media of cells transfected with wild-type pLExHIVenv revealed a small amount of gp120 (~10 to 20% of the gp120 found intracellularly) (Fig. 2). The relative amounts of radioactivity in the gp160 and gp120 bands were quantitated by scanning the autoradiograms. The amount of gp120 relative to the amount of gp160 plus gp120 was in the range of 25 to 50% with wild-type pLExHIVenv in different experiments. This value (the amount of proteolytic cleavage of wild-type *env* gene product under our assay conditions) was taken as 100%, and the values obtained with the mutants tested in the same experiment were calculated relative to this.

The intracellular HIV *env* gene products generated after transfection of the various mutant pLExHIVenv derivatives are also shown in Fig. 2, and the data are quantitated in Fig. 3. In all cases in which proteolytic cleavage occurred, i.e., gp120 was generated, a small amount of gp120 (~10 to 20%) was detected in the supernatant (data not shown). Two clusters of basic amino acids, referred to as site 1 and site 2

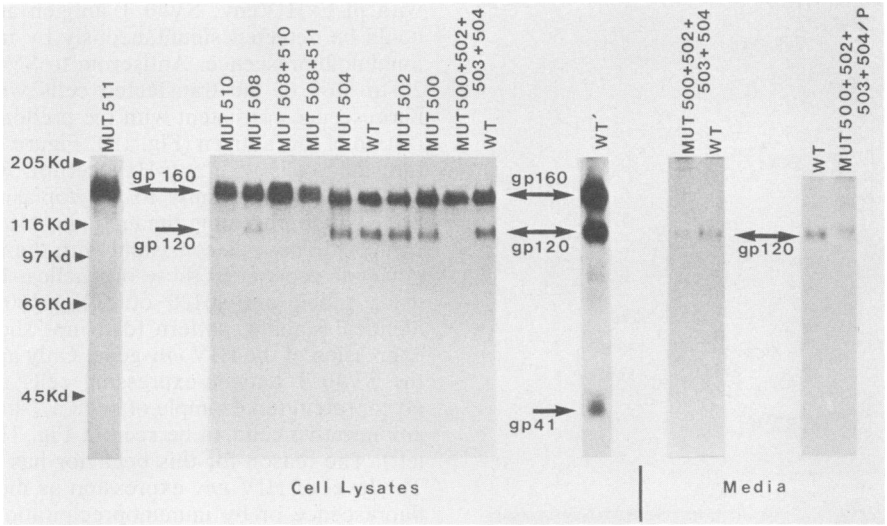

FIG. 2. Immunoprecipitation of HIV *env* gene products from CV-1 cells transfected with wild-type (WT) and mutated pLExHIVenv vectors. Immunoprecipitates from lysates or media from CV-1 cells transfected with pLExHIVenv and mutants thereof have been separated on an 8% polyacrylamide gel as described in Materials and Methods. The numbers above each lane refer to the amino acid(s) mutated in each individual mutant used for transfection (see also Fig. 3). The positions of molecular size markers and of gp160, gp120, and gp41 are given. WT' shows a longer exposure of the intracellular wild-type immunoprecipitate in order to visualize gp41. The immunoprecipitates from media shown here have been exposed longer than the immunoprecipitates from cell lysates. The relative amounts of intra- and extracellular gp160 and gp120 have been assessed by scanning the autoradiograms (same exposure times) (see Fig. 3). Kd, Kilodaltons.

(15, 21), can be observed in the vicinity of the HIV-1 *env* gene product cleavage site. Mutation of any of the three basic amino acids directly N terminal to the cleavage site (35) (i.e., site 1) severely impaired (MUT 508 and MUT 510) or completely prevented (MUT 511) proteolytic cleavage. The double mutation in MUT 508+510 completely abolished cleavage, as was, of course, also the case with the double mutation in MUT 508+511 (Fig. 2). However, single amino acid substitutions in three of the four basic amino acids further N terminal to the cleavage site (i.e., in site 2) (MUT 500, MUT 502, and MUT 504) had no significant effect on the extent of proteolytic cleavage of the respective HIV-1 *env* gene products in CV-1 cells. However, when all four basic amino acids in site 2 were simultaneously replaced by neutral amino acids (MUT 500+502+503+504/P and MUT 500+502+503+504), cleavage was virtually abolished and no intracellular gp120 could be detected at all (Fig. 2). Surprisingly, a small amount of cleaved product (1 to 5% of the amount of intracellular gp160) could be detected only extracellularly (Fig. 2). In the case of MUT 500+502+503+504/P, the amino acid sequence generated at site 2, Asn Ala Asn Ser Ser, represents a consensus sequence for N glyco-

	{------SITE 2-----}									{----SITE 1---}				NH_2-gp41				
	499	500	501	502	503	504	504	506	507	508	509	510	511	512	513	514	515	CLEAVAGE
WT	THR	LYS	ALA	LYS	ARG	ARG	VAL	VAL	GLU	ARG	GLU	LYS	ARG	ALA	VAL	GLY	ILE	+++
MUT 511	-	-	-	-	-	-	-	-	-	-	-	-	SER	-	-	-	-	-
MUT 510	-	-	-	-	-	-	-	-	-	-	-	ASN	-	-	-	-	-	+
MUT 508	-	-	-	-	-	-	-	-	-	SER	-	-	-	-	-	-	-	+
MUT 508+510	-	-	-	-	-	-	-	-	-	SER	-	ASN	-	-	-	-	-	-
MUT 508+511	-	-	-	-	-	-	-	-	-	SER	-	-	SER	-	-	-	-	-
MUT 504	-	-	-	-	-	SER	-	-	-	-	-	-	-	-	-	-	-	+++
MUT 502	-	-	-	ASN	-	-	-	-	-	-	-	-	-	-	-	-	-	+++
MUT 500	-	ASN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++
MUT 500+502+503+504/P	-	ASN	-	ASN*	SER	SER	-	-	-	-	-	-	-	-	-	-	-	±
MUT 500+502+503+504	-	ASN	-	ILE	SER	SER	-	-	-	-	-	-	-	-	-	-	-	±

FIG. 3. Mutations generated in the HIV-1 *env* gene product cleavage site and their effects on proteolytic cleavage. The sequence between amino acids 499 and 515 in the vicinity of the cleavage site (arrow), the positions of the two clusters of basic amino acids conserved in all HIV-1 isolates (site 1 and site 2) of the wild-type *env* gene product, as well as the amino acid changes generated in the mutants are given. The basic amino acids in the wild-type sequence are underlined. Dashes indicate wild-type sequences in the mutants. The extent of proteolytic cleavage is expressed relative to that obtained with the wild-type *env* gene product (100%): −, no detectable cleavage; ±, 1 to 5% cleavage; +, 5 to 20% cleavage; ++, 20 to 50% cleavage; and +++, 50 to 100% cleavage. The asterisk on Asn-502 in MUT 500+502+503+504/P shows the new potential site for N glycosylation. WT, Wild type.

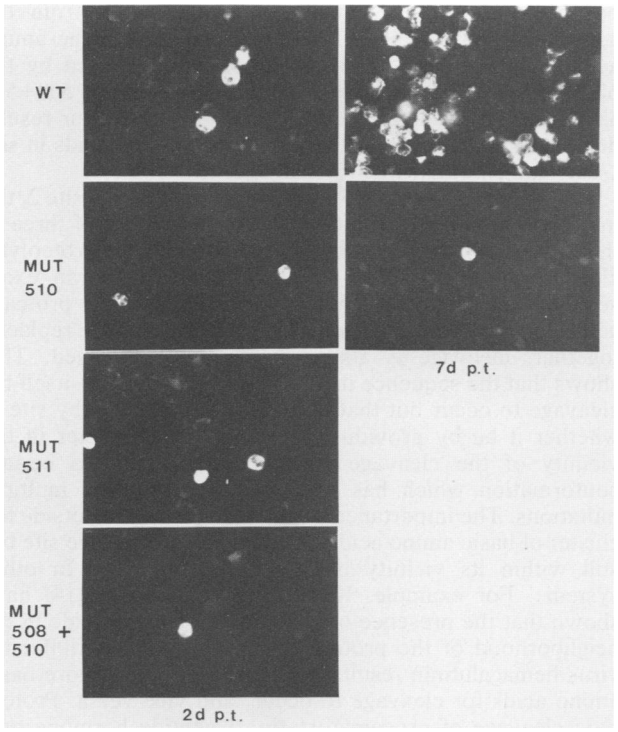

FIG. 4. HIV-1 immunofluorescence staining of permissive Jurkat T lymphocytes after transfection with pNL4-3 and *env* cleavage mutants thereof. The left panel shows Jurkat cells stained with a serum sample from a patient with acquired immunodeficiency syndrome 2 days (d) after transfection with wild-type (WT) pNL4-3, MUT 510, MUT 511, and MUT 508+510 (from top to bottom). The right panel shows immunofluorescence staining of the same transfected cultures 7 days after transfection with wild-type pNL4-3 (top) and MUT 510 (bottom).

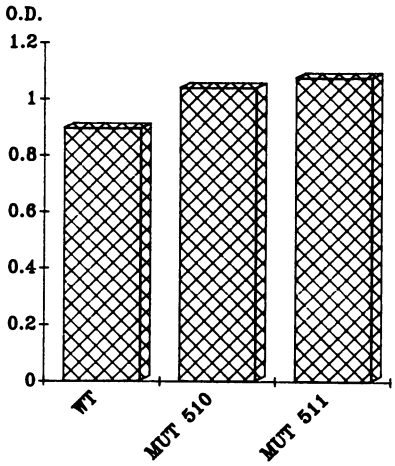

FIG. 5. HIV-1 antigen in the cell-free supernatants of Jurkat cells transfected with wild-type (WT) and mutant pNL4-3 vectors. The supernatants of transfected Jurkat cells were collected 2 days after transfection and clarified by low-speed centrifugation. Equivalent dilutions (in this experiment, 1:10) were reacted in a commercial ELISA test for HIV antigen. The absorbances of the color reactions obtained are shown for the wild-type and mutant MUT 510 and MUT 511. O.D., Optical density.

sylation. In fact, the gp120 product precipitated from the supernatant was slightly larger than the wild-type gp120, indicating that the new glycosylation site was probably being used (Fig. 2). Since, in this case, it was possible that cleavage was occurring to such a limited extent because of the presence of a putative oligosaccharide near the cleavage site, MUT 500+502+503+504 was generated by changing amino acid 502 in MUT 500+502+503+504/P from Asn to Ile (Fig. 3). However, again, cleavage was virtually abolished and only a very small amount of gp120, in this case having the same size as wild-type gp120, could be detected, again only in the medium (Fig. 2).

Spread of HIV infection in CD4$^+$ Jurkat cells transfected with infectious plasmids containing wild-type or mutated HIV *env* cleavage sites. *env* gene fragments from wild-type BH10, MUT 510, MUT 511, and MUT 508+510 were transferred to plasmid pNL4-3 (1). The resulting wild-type and mutant derivatives of pNL4-3 were transfected into CD4$^+$ Jurkat T-lymphoid cells, and the cells were examined for the production of HIV antigen at different intervals p.t. Immunofluorescence of transfected cells (Fig. 4) showed that the initial transfection efficiency measured 2 days p.t. was approximately the same with wild-type or any of the three mutant derivatives tested (approximately 0.5 to 1% HIV antigen-positive cells). In another experiment, HIV antigen concentrations in the cell-free supernatants, collected 2 days p.t. from Jurkat cells transfected with wild-type MUT 510 or MUT 511 pNL4-3 plasmids, were measured by ELISA analyses (Fig. 5). The values obtained were similar to each other, again indicating that the transfection efficiencies were comparable and that, initially, the same amount of virus was being released from cells transfected with wild-type or mutant pNL4-3 plasmids. However, as can be seen by immunofluorescence at 7 days p.t., only the wild-type plasmid has led to the production of infectious viruses which resulted in the spread of the virus infection in the culture (Fig. 4). In the case of MUT 510, only very few infected cells could be observed 7 days p.t., and with MUT 511 and MUT 508+510, immunofluorescence revealed the virtual absence of antigen-positive cells (data not shown). The immunofluorescence assays in Fig. 4 were done by using a serum sample from a patient with acquired immunodeficiency syndrome, but the same results were obtained by using rabbit anti-gp120 serum (data not shown). The wild-type plasmid used in the experiment in Fig. 4 was the original pNL4-3 plasmid. The same result was obtained by using the recombinant derivative in which the wild-type *Sal*I-*Bam*HI fragment from BH10 replaced the pNL4-3 fragment. These results show that the cells transfected with pNL4-3 derivatives containing the noncleavable (MUT 511 and MUT 508+510) or poorly cleavable (MUT 510) *env* fragments fail to produce infectious virus.

DISCUSSION

Proteolytic cleavage of the HIV-1 *env* gene product is an essential step in the production of infectious HIV-1. The amino acid sequence at the cleavage site reveals two clusters of basic amino acids at positions 508 to 511 (Arg Glu Lys Arg; site 1) (cleavage presumably normally occurs C terminal to Arg-511, [35]) and at positions 500 to 504 (Lys Ala Lys Arg Arg; site 2) (BH10 numbering [28]; Fig. 3). These two basic sites are well conserved in all HIV-1 isolates sequenced so far, as well as in some animal lentiviruses, the human spumaretrovirus, and, although less well conserved, in HIV-2 and simian immunodeficiency virus (24). In analogy to the situations already described for the viral glycoprotein

precursors from orthomyxoviruses and paramyxoviruses and from other retroviruses (2, 10, 13, 25, 26, 34), it is very likely that these basic amino acids, at least in site 1, are necessary for recognition by, and enzymatic activity of, the (cellular) protease(s) performing cleavage. It has already been shown that complete replacement of site 1 with acidic and neutral amino acids abolishes cleavage of gp160 and infectivity of HIV-1 (21). The aim of this study has been to further analyze the cleavage site at the level of the individual amino acid and assess the importance of site 2 in protease activity by mutating the basic amino acids in site 1 and site 2, individually and in combination, and examining whether proteolytic cleavage of the resultant mutated *env* gene products could occur or not.

Mutations near the gp160 cleavage site were generated in the eucaryotic expression vector pLExHIVenv (Fig. 1), and their effects were analyzed after transfection into monkey CV-1 cells. First of all, the extent of cleavage and the localization of the wild-type HIV *env* gene products were established. The amount of immunoprecipitated gp120 relative to that of gp120 plus gp160 was about 30% in CV-1 cells metabolically labeled with [^{3}H]glucosamine from 30 to 48 h p.t. Recently, it has been shown that in T lymphocytes producing infectious HIV, only a minor portion of the gp160 synthesized is proteolytically processed and exported from the cell, while the rest is degraded intracellularly (36). Apparently, this also occurs in CV-1 cells and could explain why, even after long-term labeling, the amount of precursor, gp160, is in excess of the amount of products, gp120 and gp41. In addition to this intracellular localization, about 10 to 20% of the total gp120 could be found in the supernatant of transfected CV-1 cells, presumably because of a dissociation of the weakly associated gp120-gp41 complex (32) at the cell surface (Fig. 2).

Considering first of all the role of the basic amino acids in site 1 in substrate recognition, mutation of Arg-511 (immediately N terminal to gp41) to Ser (MUT 511) completely abolished cleavage, as did the double mutations in MUT 508+511 (Fig. 2 and 3). McCune et al. (21) have previously reported that the replacement of site 1 (Arg Glu Lys Arg) with the sequence Gly Glu Glu Phe completely blocked cleavage. This, however, was a major change and did not give information as to the role of individual amino acids in protease recognition. In a recent report, Kieny et al. (15) have mutated site 1 to Asn Glu His Gln, i.e., again a major change; however, they reported that partial cleavage still occurred. The reason for this discrepancy is not known but may be due to the cell type used for *env* expression or to the vaccinia virus vector employed by Kieny et al. (15), providing functions not present in an uninfected cell. The fact that the single amino acid change in MUT 511 completely prevented cleavage shows that the protease enzyme could not alternatively use any of the potential trypsinlike sites in the vicinity, including site 2, which, with the sequence Lys Ala Lys Arg Arg, could possibly have provided a recognition site for the processing protease.

In addition to Arg-511, the other two basic amino acids in site 1 (Lys-510 and Arg-508) are important for recognition by, and enzymatic activity of, the protease(s) performing cleavage, since replacing these individually with neutral amino acids (MUT 510 and MUT 508) severely reduced, but did not completely abolish, cleavage. This is comparable to the situation with the Rous sarcoma virus glycoprotein precursor in which a single amino acid change comparable in its position to that in MUT 510 also resulted in cleavage being almost completely abolished (26). In this case, it could be shown that the residual cleavage resulted from the activity of another protease. The importance of the amino acids Arg-508 and Lys-510 is further strengthened by the observation that the double mutations in MUT 508+510 abolished cleavage completely. Taken together, our results demonstrate that each of the three basic amino acids in site 1 plays an essential role in cleavage.

If we analyze the role of the basic amino acids in site 2, the individual replacement with neutral amino acids of three of the four amino acids located there did not impair proteolytic cleavage, indicating that in contrast to site 1, an exact sequence in site 2 does not play a major role in protease activity. However, if all four basic amino acids are replaced together, cleavage is almost completely abolished. This shows that the sequence in site 1 is not sufficient in itself for cleavage to occur but that there is a contribution by site 2, whether it be by providing a more basic character in the vicinity of the cleavage site or by providing a certain conformation which has been destroyed by the multiple mutations. The importance of structural features outside the cluster of basic amino acids adjacent to the cleavage site but still within its vicinity has been demonstrated in other systems. For example, Kawaoka and Webster (14) have shown that the presence of a specific oligosaccharide in the neighborhood of the proteolytic cleavage site of influenza virus hemagglutinin results in a requirement for more basic amino acids for cleavage to occur, and vice versa. Proteolytic cleavage of prosomatostatin, a peptide hormone precursor, which is proteolytically processed at a cluster of basic amino acids probably by a protease similar to that used by viral glycoprotein precursors, is abrogated by mutations which destroy the predicted β-turn secondary structure in the vicinity of the cleavage site (11).

The lack of cleavage of the mutant HIV *env* gene products described here could, theoretically, be due to their inability to be transported to the site of proteolytic cleavage presumably in the Golgi complex. Kieny et al. (15) have, however, demonstrated surface expression, and thus presumably correct transport, of a noncleavable mutant in which all of the basic amino acids have been replaced with neutral and charged amino acids. Since the mutants described here contain far fewer changes than those of Kieny et al. (15), it is very likely that, in these cases also, normal transport to the cell surface occurs. Another related concern could be whether the mutant *env* gene products adopt a tertiary structure which prevents access of the cellular protease to the cleavage site. McCune et al. (21) have demonstrated that, in a mutant in which the entire site 1 at the cleavage site has been replaced by a chymotrypsin site, proteolytic cleavage by the cellular protease fails to occur, but the cleavage site is still accessible to external proteases and syncytium formation can be induced by the addition of chymotrypsin. Again, the mutants described here contain fewer amino acid changes than the mutant of McCune et al. (21), so it is likely that, in these cases also, the cleavage sites are accessible but no longer cleavable by the cellular protease.

Recombinant pNL4-3 vectors containing the mutations present in MUT 511, MUT 510, and MUT 508+510 were tested for their ability to produce infectious HIV-1 (Fig. 4 and 5). Although the initial transfection efficiency was comparable in each case (Fig. 4) and about the same amount of viral antigen was released from cells transfected with wild-type and mutant vectors 2 days p.t. (Fig. 5), only the wild-type vector led to a spreading infection. With MUT 510, in which gp160 is partially cleavable, a few antigen-positive cells were detected up to 7 days p.t. (Fig. 4). This may be

due to a low residual infectivity of this mutant. With MUT 511 and MUT 508+510, in which no cleavage of gp160 was observed, antigen-positive cells could be observed at day 2 p.t. but no longer at day 7 p.t., indicating that, as expected, only noninfectious virus was being produced.

The mutated *env* gene products still contain several basic amino acids at the cleavage site, so that it may be possible to specifically cleave the mutated gp160 by mild trypsin digestion and activate noninfectious mutant virus to infectivity, as has been described for several other viral glycoprotein precursors (17, 26). If this is the case, such mutant in vitro-activatable viruses should be useful tools to examine several aspects of the HIV-1 replication cycle. These aspects would include an analysis of the possible importance of an exact N terminus of gp41 in viral infectivity (e.g., MUT 511, on cleavage with trypsin, would lead to Ser-511 instead of Ala-512 being the N terminus of gp41). In addition, the possible requirement for a cleaved, fusion-competent viral glycoprotein complex in the cytopathogenicity of HIV-1 in cell culture and the role of a functionally competent glycoprotein in the phenomenon of *env* gene product-induced viral interference are questions which may be approached by using the mutant viruses described here.

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