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Role of Conserved gp41 Cysteine Residues in the Processing of Human Immunodeficiency Virus Envelope Precursor and Viral Infectivity

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All animal retroviruses whose nucleotide sequences have been determined contain two or three closely spaced cysteine residues in the extracellular domain of the *env*-encoded transmembrane protein. Using human immunodeficiency virus type 1 gp41 as a working model, the functional significance of these highly conserved cysteines was investigated. We report here that substituting the two conserved cysteine residues in this domain of gp41 with glycine residues resulted in the loss of viral infectivity, which could be attributed to severe impairment in the processing of gp160 precursor to gp120.

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that displays extensive genetic variability among different isolates (1, 9, 10, 20, 28, 29, 31, 34). This genomic diversity is most pronounced within the *env* gene, which encodes an outer coat protein, gp120, and a transmembrane protein, gp41 (2, 31). Despite the envelope sequence variability in gp120, there are conserved sequences interspersed among the variable regions, several of which have been shown to have critical functions (11, 12, 14, 21, 34).

Within the sequence of gp41, there are three cysteine residues, designated Cys-598, Cys-604, and Cys-764 in Fig. 1, which are highly conserved by different HIV-1 isolates (20). Cys-764 is located downstream from the hydrophobic membrane anchor region of gp41 and presumed to be in the intracellular domain of gp41. In contrast, both Cys-598 and Cys-604 are located upstream from the membrane anchor region and are believed to be in the extracellular domain of gp41.

The presence of two closely spaced cysteine residues, Cys-598 and Cys-604, in the extracellular domain of the *env*-encoded transmembrane protein is not unique to HIV-1. In fact, such cysteine residues have been found in the transmembrane proteins of all animal retroviruses with known nucleotide sequences (32). These conserved cysteine residues are located downstream from a region which has a high probability of forming an α-helix structure (6) and are in most cases followed by consensus N-linked glycosylation sequences (6, 32). The number of conserved cysteine residues in this region varies from two to three, with the latter number being found most often in those retroviruses whose extracellular envelope proteins are linked to their respective transmembrane proteins by disulfide bonds (15, 17, 19, 23, 24). In HIV-1, the presence of only two conserved cysteine residues in this region of gp41 is consistent with previous observations that gp120 was not linked to gp41 by disulfide bonds (3, 12).

Despite the highly conserved nature of these closely spaced cysteine residues, little is known about whether they form disulfide bonds and whether they have any functional significance. Recently, it was reported that sera from HIV-1-infected people reacted better to synthetic gp41 peptides containing both cysteine residues than to peptides containing only one of the two cysteine residues (7). This finding supports the notion that a cystine loop is formed by the two cysteine residues in the extracellular domain of gp41. In this study, we replaced the two closely spaced cysteine residues in the extracellular domain of gp41 and a third cysteine residue in the intracellular domain of gp41 with glycine residues to explore functional significance of these conserved cysteine residues.

Three gp41 mutant viruses, 598, 604, and 764, were derived from an infectious molecular clone, HXB2 (4), by oligonucleotide-directed mutagenesis (Fig. 1). A 2.7-kb *Sal*I-*Bam*HI fragment of HXB2 was introduced into M13mp18. All mutations were generated by the uracil-containing single-strand template method (13). Three mutagenic oligonucleotides, 5′ATTTGGGGTgGCTCTGGAAAA3′, 5′GGAAAAC TCATTgGCACCACTG3′, and 5′CGGAGCCTGgGCCTCT TCAG3′ (the lowercase letter indicates the position of the mutation) were synthesized for the generation of cysteine-to-glycine mutations at positions Cys-598, Cys-604, and Cys-764, respectively. Cloning was done by standard molecular biology procedures, and DNA sequencing was performed to verify the mutated sequences (27).

Molecular proviral DNAs containing the designed mutations were transfected into COS-1 cells by the DEAE-dextran method (35). Forty-eight hours posttransfection, reverse transcriptase (RT) activities were detected in supernatants of wild-type and all three gp41 mutant virus-transfected COS-1 cultures. To determine the infectivity of gp41 mutant viruses, we used cell-free supernatants from each of the COS-1 transfectants to infect $CD4^+$ Sup-T1 cells. The infected Sup-T1 cultures were monitored for RT activity and syncytium formation to determine the infectivity of the gp41 mutants. As with the culture infected by the wild-type virus, both RT activity and syncytia were detected in cultures

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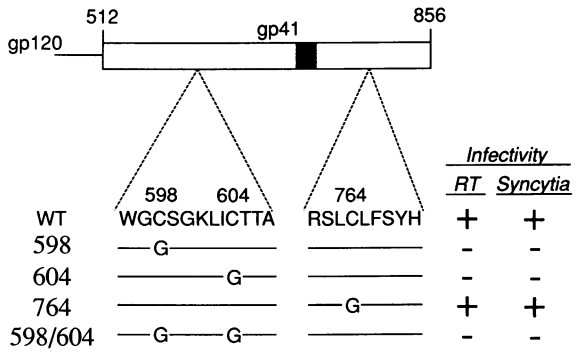

FIG. 1. Phenotypes of HIV-1 gp41 mutants. Substitutions of cysteine residues with glycine residues were introduced at positions 598, 604, and 764. Amino acid residues identical to those of the wild-type (WT) virus are indicated by straight lines.

infected by mutant 764 (Fig. 1). In contrast, mutants 598 and 604 showed no signs of syncytium formation, and the RT activity detected in cultures infected by these mutants did not rise above background during the 4-week follow-up period. Because RT activity in the inoculum for mutants 598 or 604 was equal to or higher than that of the wild-type virus, the loss of infectivity by these mutants was probably due to defects caused by substituting the highly conserved cysteine residues with glycine residues.

The HIV-1 envelope glycoproteins, gp120 and gp41, like those of other retroviruses, are synthesized in the endoplasmic reticulum as a polyprotein precursor, gp160, which is cleaved during transport to the cell membrane (2, 22, 30, 33). The lack of endoproteolytic cleavage of gp160 has been shown to result in the loss of viral infectivity (5, 8, 18). To explore the possibility that the two closely spaced cysteines of gp41 might be critical for efficient cleavage of gp160 and thus result in the loss of infectivity by mutants 598 and 604, we further examined the expression of gp160 and gp120 by gp41 cysteine mutants. For this purpose, four envelope expression vectors designated pBWT, pB598, pB604, and pB764, respectively, were constructed for the wild-type and mutant viruses by cloning a 3.1-kb *Sal*I-*Xho*I fragment of the wild type or mutant viruses 598, 604, and 764 into the *Xho*I site of pBaby (26). The cloning strategy for the construction of these expression vectors was similar to that described previously for another HIV envelope expression vector, pSVX (26).

Forty-eight hours posttransfection, cell lysates prepared from COS-1 transfectants were analyzed for the expression of gp160 and gp120 by Western blot (immunoblot), using a representative HIV-1-positive human serum and a goat anti-gp120 serum designated G-85, which was raised against a recombinant gp120 protein, gp120(343C) (16). This recombinant gp120 protein contains amino acid residues 343 to 512 of the BH10 clone, according to the numbering system of Ratner et al. (25). As shown in Fig. 2, both gp160 and gp120 were detected in COS-1 cells transfected with pBWT or pB764. In contrast, only gp160 was detected in COS-1 cells transfected with plasmid pB598 or pB604 using the same human serum (Fig. 2A). A small amount of gp120 was detectable in these cells using the G-85 serum (Fig. 2B). These findings suggest that either one of the two closely spaced cysteine residues in the extracellular domain of gp41 was critical for efficient cleavage of gp160 precursor. Western blot analysis was also done with lysates prepared from COS-1 cells transfected with the wild-type or mutant virus constructs. The results obtained from this analysis also showed inefficient processing of gp160 by mutants 598, 604, and 598/604 (data not shown).

Substituting one of the two closely spaced cysteine residues of gp41 with a glycine residue may leave the second cysteine unpaired. This may cause the formation of intermolecular disulfide bonds and thus hamper endoproteolytic cleavage of the gp160 precursor. To provide direct proof that both cysteines in the extracellular domain of gp41 were critical for the cleavage of gp160 precursor, we constructed a mutant virus designated 598/604, which had both cysteine residues replaced by glycine residues, using the same strategy as described above. When expressed, this mutant gp160 is expected to be free of unpaired cysteines and does not have internal or intermolecular disulfide bonds linking residues 598 or 604. As shown in Fig. 1, the Sup-T1 culture infected by mutant virus 598/604 did not form syncytia and had no detectable RT activity. Western blot analysis of COS-1 cells transfected with the envelope expression vector pB598/604, which had a 3.1-kb *Sal*I-*Xho*I fragment of mutant virus 598/604 cloned into the *Xho*I site of pBaby (26), also showed very poor processing of gp160 (Fig. 2). By radioimmunoprecipitation analysis, there was no evidence indicating that the lack of detection of gp120 in the cell lysates was

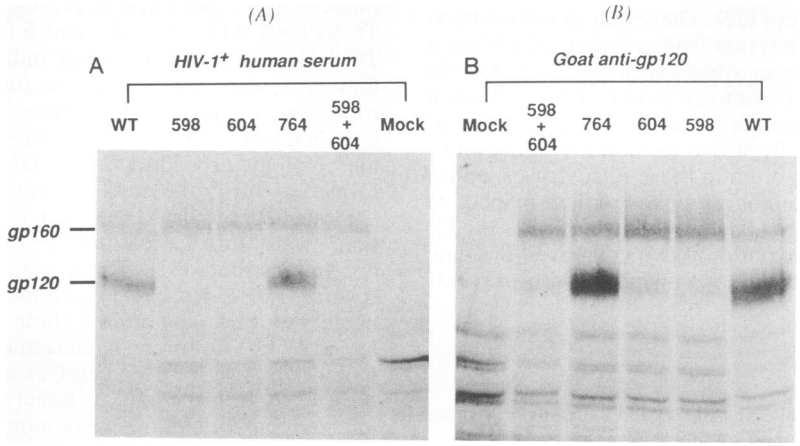

FIG. 2. Western blot analysis of the expression of gp160 and gp120 in COS-1 transfectants. The positions of gp160 and gp120 are indicated. Cell lysates from COS-1 cells transfected with plasmid pBWT, pB598, pB604, pB598/604, or pB764 or buffer only (mock transfected) were reacted with an HIV-1 positive human serum sample (A) and a goat anti-gp120 serum, G-85 (B). WT, wild type.

due to excessive secretion of gp120 (data not shown) (11). These findings strongly suggest that a configuration determined by Cys-598 and Cys-604 is critical for the recognition of the envelope precursor protein by as yet undefined proteases.

Thus far, the only conserved motif known to be critical for the cleavage of envelope precursors of a diverse family of animal retroviruses is the amino acid sequence K/R-X-K-K/R, which is located immediately adjacent to the site of cleavage of envelope precursors. In addition, in the case of the HIV-1, only the last Arg in the C terminus of this sequence motif was shown to be critical for the cleavage of envelope precursor protein, since nonconservative substitutions at other sites had no effect on the cleavage of the envelope precursor (5). Our finding that only the two closely spaced cysteine residues in the extracellular domain of gp41, but not the conserved cysteine residue in the intracellular domain, were critical for the cleavage of HIV-1 envelope precursor indirectly supports the presence of a cystine loop formed by the two highly conserved cysteine residues in the extracellular domain of gp41 (7). We demonstrated in this study that disrupting the formation of this loop reduced the efficiency of cleavage of envelope precursor. Taken together, our observations suggest strongly that the cleavage of the HIV-1 envelope precursor by the intracellular protease requires, in addition to the previously identified linear sequence motif, a higher order of substrate structure involving a highly conserved cystine motif in the extracellular domain of gp41. Because of the highly conserved nature of this cystine motif, an attractive hypothesis is that this motif will also be found critical for the efficient cleavage of envelope precursors of other animal retroviruses.

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