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Legitimate and Illegitimate Cleavage of Human Immunodeficiency Virus Glycoproteins by Furin

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Coexpression of human immunodeficiency virus type 1 glycoproteins with the subtilisin-like protease furin leads to processing of gp160 and gp140, a truncated form of gp160, to gp120. In addition, we show that gp120 itself is further cleaved by furin at sites near the primary cleavage site and within the V3 loop.

The precursor envelope glycoprotein of human immunodeficiency virus (HIV) (gp160) is normally processed in mammalian cells to give the mature glycoproteins gp120 and gp41. Such processing is essential for infectivity (15), and cleavage occurs at one or two closely linked sites with the amino acid consensus sequence basic-X-basic-basic found at the gp120-gp41 junction (5). The cleavage recognition sequence of HIV type 1 (HIV-1) gp160 is shared with HIV-2, simian immunodeficiency virus, and also with a number of other viral glycoproteins and is the reported preferred substrate for the cellular enzyme furin, a subtilisin-like protease originally isolated as a member of a class of endopeptidases involved in prohormone processing (1, 10, 14). Recent evidence from studies of mammalian cells suggests that furin is indeed capable of cleavage of the influenza virus hemagglutinin precursor (26), the Newcastle disease virus glycoprotein (6), and the envelope of HIV-1 (8). However, the role of other factors in determining the specificity of glycoprotein cleavage remains open. For example, when the cytoplasmic domain of gp160 is deleted, a region that is some distance away from the cleavage junction, processing of the precursor molecule is reduced (7). Similarly, treatment of the precursor with drugs that inhibit glucosidase activity and lead to hyperglycosylation of the precursor also leads to inefficient processing (23, 24).

When expressed in insect cells with recombinant baculoviruses, HIV gp160 is largely unprocessed (16, 27), suggesting that insect cells are naturally deficient in furin activity or that the gp160 conformation is not suitable for cleavage. Here, we make use of this property to examine the cleavage of gp160 by furin provided in *trans*. We confirm that expression of furin alone leads to gp160 cleavage, and we show that both of the possible cleavage sites at the gp120-gp41 boundary are substrates for the reaction. However, we also show evidence for additional illegitimate cleavage of gp120 within a region of the molecule (the V3 loop) that does not contain sequences that match the preferred substrate specificity of furin.

In order to examine the cleavage of gp160 by furin, we constructed a furin recombinant baculovirus with a cDNA isolated from a mouse cDNA library (21) and carried out coinfection studies with recombinant baculoviruses producing (i) HIV-1 gp160 (16); (ii) gp140, a soluble gp160 derivative that has been truncated from amino acid 678 to remove the transmembrane domain; and (iii) HIV-1 gp120 (20). All of these recombinants contain sequences similar to the furin consensus cleavage site (Fig. 1); gp160 and gp140 contain both possible cleavage sites located at the gp120-gp41 boundary, and gp120 contains a single site, the more amino terminal of the two situated 12 amino acids upstream of the engineered carboxy terminus of the protein (20). Coinfection experiments were done under conditions previously shown to result in expression of both proteins within the same cell (18, 22), and the products of expression were analyzed by electrophoresis of supernatants on reducing or nonreducing gels and Western blotting (immunoblotting) as described previously (3) with site-specific or polyvalent antisera (Fig. 1). On nonreducing gels, coexpression of furin with both gp140 and gp160 led to the production of a band in the supernatants of coinfected cultures that comigrated with gp120 (Fig. 2). In gp140 coinfections, furin cleavage gave rise to a new gp120 band (Fig. 2; compare lane 4 with lane 3), whereas in the gp160 coinfection (which undergoes some endogenous cleavage [27]), the gp160 band was wholly converted to gp120 and the level of gp120 antigen increased accordingly (lanes 5 and 6). Coinfection of furin with gp120 did not change the pattern of antigen expression compared with gp120 infection alone (Fig. 2, lanes 1 and 2). Thus, the addition of furin alone is sufficient for cleavage of the glycoprotein in *trans*. Moreover, as the conformation of the substrate is clearly conducive to cleavage, we conclude that the low level of cleavage in the single infection with gp160 is due solely to the lack of endogenous furin activity in insect cells.

In their mature cleaved form, HIV envelope glycoproteins are not covalently linked and can be identified directly on nonreducing gels. The gp120 domain, however, has many disulfide bonds (13), and cleavage within the loop structures of the molecule is apparent only after resolution of the cleavage products on reducing gels (e.g., see reference 4). To control for the possibility that furin was also cleaving within the gp120 domain of the envelope but that the cleavage products remained covalently attached, we repeated the analysis of the supernatants from coinfected cultures but reduced the samples prior to electrophoresis. Under these conditions, each coexpression supernatant showed evidence of further cleavage within the gp120 domain to generate antigenic species of approximately 45 and 60 kDa (Fig. 3). The sizes of these two fragments are in keeping with a single additional cleavage of gp120 by furin in a region of the molecule that is bounded by cysteine residues linked by disulfide bonds within the folded molecule. The sizes of the secondary cleavage products (45 and 60 kDa) suggested that the additional furin site was situated somewhere in the middle section of the molecule, and to deter-

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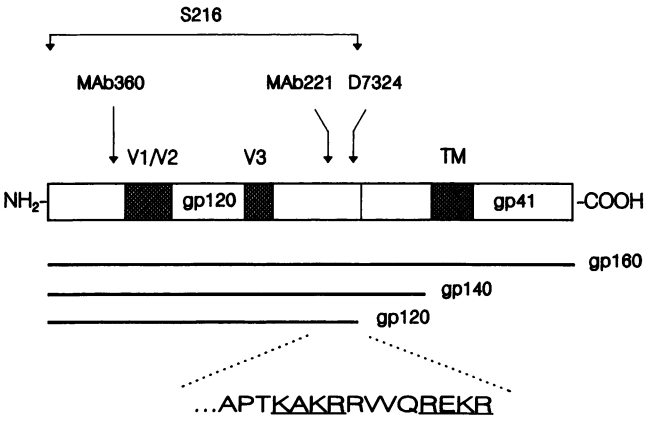

FIG. 1. The recombinant glycoproteins and antibodies used in this study. The full-length *env* gene product of HIV-1 is shown in schematic form with landmarks such as the variable (V) loops and transmembrane domain (TM) shown as shaded areas. Above gp160 are shown the epitopes for two different MAbs (MAb 360 and MAb 221), one site-specific peptide serum (D7324), and one polyvalent anti-gp120 serum (S216) used in this study. Below the diagram is a linear representation of the three different *env* recombinants used and the sequence detail of the carboxy terminus of gp120 showing the two possible cleavage sites (underlined).

mine further the position of the cleavage site, we carried out Western blotting of supernatants from a gp120-furin coinfection with monoclonal antibodies (MAbs) whose epitopes are known (Fig. 1). Western blotting with MAb 360, which recognizes the C1 domain of gp120, highlighted the 60-kDa species, while blotting with MAb 221, whose epitope lies within the terminal 44 amino acids of gp120 (19), highlighted only the 45-kDa species (Fig. 4). Recognition of either cleavage product was possible only when the samples had been reduced (Fig. 4). From the sizes and origins of the products and their disulfide linkage, these results show that the additional cleavage of HIV-1 envelope glycoproteins by

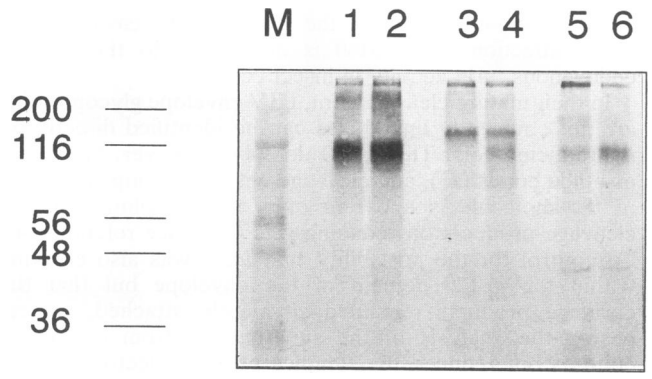

FIG. 2. Coexpression of gp160, gp140, and gp120 with furin. Coinfection experiments were done in 35-mm dishes with each virus added at a multiplicity of infection of 10. Two-day-postinfection protein in the supernatant was resolved on 8% nonreducing sodium dodecyl sulfate-acrylamide gels and Western blotted with S216, a polyvalent antiserum to gp120. Lanes 1 and 2, gp120; lanes 3 and 4, gp140; lanes 5 and 6, gp160. Lanes 1, 3, and 5 are infections with envelope-expressing viruses alone. Lanes 2, 4, and 6 are coinfections with the furin recombinant. Molecular sizes (M) are in kilodaltons and are shown on the left. The gp160 band is particularly faint as little antigen is released into the supernatant.

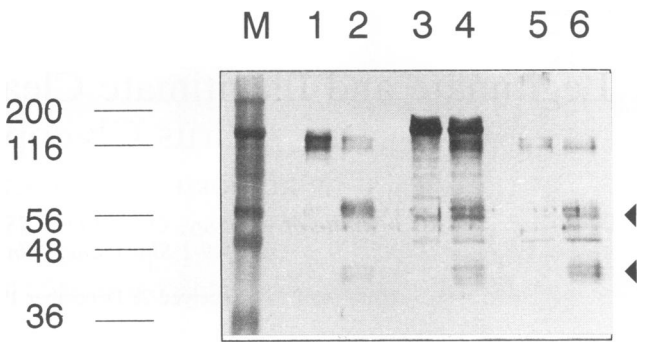

FIG. 3. Illegitimate cleavage of *env* glycoprotein revealed on reducing gels. The same samples used for Fig. 2 were resolved on 8% reducing gels (5% beta-mercaptoethanol in the sample buffer), loaded, and blotted as for Fig. 2. The appearance of new bands at approximately 45 and 60 kDa is indicated. M, molecular size markers (in kilodaltons).

furin occurs within the immunodominant V3 loop. To further map the cleavage site within the loop, Western blotting was done with two additional V3 loop-specific MAbs, 9305 and 9284 (DuPont). The epitopes recognized by these MAbs are overlapping (Fig. 5, upper section), and yet their blotting profiles are different. MAb 9305 gave the same pattern as MAb 221 and highlighted the carboxy-terminal 45-kDa band in furin-plus-gp120 coexpressions analyzed under reducing conditions (Fig. 5, lane 2). MAb 9284, however, failed to detect any cleavage product, although it reacted well with gp120 produced by the single infection (lane 1) and with the small amounts of uncleaved gp120 remaining in the gp120-plus-furin coexpression supernatant (lane 2). Thus, while the binding site for MAb 9305 is preserved following furin cleavage, the site recognized by MAb 9284 is destroyed. These data are consistent with cleavage by furin within the V3 loop sequence NTRKSIRIQRG.

In our original coexpression experiments, we noted no

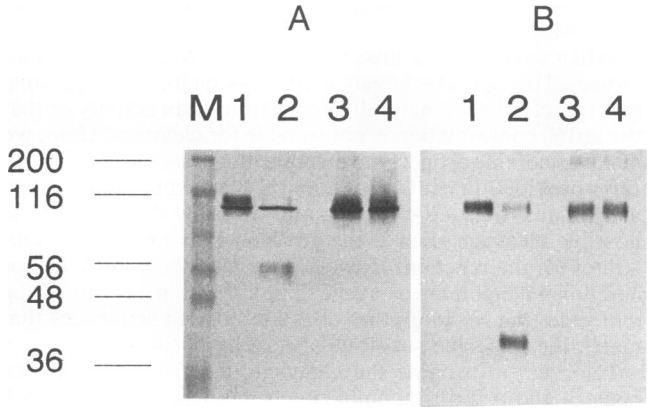

FIG. 4. Mapping the illegitimate cleavage products of gp120 with MAbs. gp120 expressed alone or with furin was resolved with or without reduction on polyacrylamide gels and blotted with MAb 360 (A) or MAb 221 (B) (Fig. 1). Lanes: 1, gp120 reduced; 2, gp120 plus furin reduced; 3, gp120 nonreduced; 4, gp120 plus furin nonreduced. The epitope for MAb 360 lies in the 60-kDa fragment, while that for MAb 221 lies in the 45-kDa fragment. The cleavage products are normally linked by a disulfide bond as they are observed only in the reduced samples (lanes 1 and 2). Molecular size markers (M) are shown in panel A and are indicated in kilodaltons.

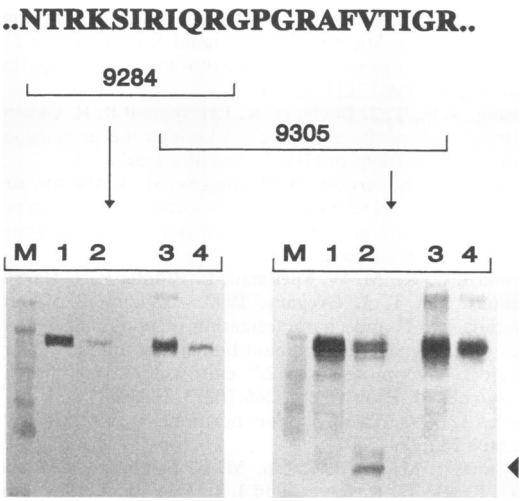

FIG. 5. Mapping the V3 loop cleavage by furin. The upper section of the figure shows a portion of the V3 loop of HIV-1_{LAI} with the epitopes recognized by two commercial V3 loop MAbs indicated. The antibodies were used as shown to Western blot the same samples as for Fig. 4. Lanes: M, size markers in kilodaltons as for Fig. 2; 1 and 3, gp120-only infection supernatant; 2 and 4, gp120-plus-furin supernatant. Lanes 1 and 2 are reduced, while lanes 3 and 4 are not. The position of the 45-kDa cleavage product highlighted by MAb 9305 is indicated.

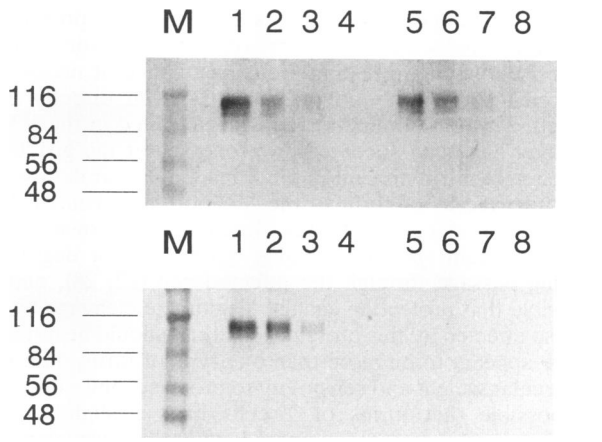

FIG. 6. Trimming of the gp120 carboxy terminus by coexpressed furin. gp120 was expressed alone or coexpressed with furin, and the antigen in the supernatant was detected by nonreducing gels and Western blotting with MAb 221 or D7324. Lanes: 1 to 4, twofold dilutions of gp120 only; 5 to 8, twofold dilutions of gp120 plus furin. The upper panel was blotted with MAb 221, and the lower panel was blotted with D7324. Molecular size markers on the left are in kilodaltons.

change of molecular weight for gp120 when coexpressed with furin and analyzed on nonreducing gels. The consensus cleavage site for furin has been reported to be Arg-X-Arg/Lys-Arg (10), corresponding to the most downstream site in gp160. Yet cleavage of gp160 at a second upstream site of similar characteristics (KAKR) has also been reported in 10 to 20% of processed molecules (5). This sequence lies within the gp120 coding sequence, 12 amino acids from the carboxy terminus, but the enzyme responsible for this cleavage has not been identified directly. To test whether furin was also responsible for this cleavage, we analyzed supernatants from a gp120-plus-furin coinfection with antisera capable of detecting the trimming of the carboxy terminus of gp120 that would be predicted to occur. Quantitation of the gp120 antigen in supernatants from a gp120-only culture compared with that from a gp120-plus-furin coinfection by blotting with MAb 221 (which recognizes an epitope within −44 to −17 amino acids from the carboxy terminus of gp120) showed that the quantity of antigen present in both cultures was the same (Fig. 6, upper panel). However, when the same titration of samples was blotted with D7324 (which recognizes the terminal 17 amino acids of gp120 and can bind only an untrimmed molecule [5]), the detection of gp120 in the coinfected culture supernatant was essentially abolished (Fig. 6, lower panel). These data indicate that coexpression of gp120 with furin leads to efficient trimming of the carboxy terminus of gp120 and indicate that the sequence KAKR is also a legitimate recognition sequence for the furin enzyme. The fact that only 10 to 20% of molecules cleave at this position during a productive HIV infection suggests that other factors, perhaps access to the site, moderate its cleavage. Interestingly, gp41 docking has been mapped to the terminus of gp120 (9), and it is possible that this occludes the upstream cleavage site in favor of the site downstream.

We have shown that coexpression of furin with a variety of HIV glycoprotein variants leads to cleavage of the molecule at a site consistent with the previously characterized gp120-gp41 junction. The fact that the truncated gp140 molecule as well as the full-length gp160 appears to be cleaved in *trans* suggests that loss of the cytoplasmic domain does not alter the accessibility of the furin cleavage site. Altered processing in mammalian cells, therefore, may be the consequence of a change in the intracellular routing of *env* following the deletion of the carboxy terminus (7) so that it fails to meet furin in the trans-Golgi compartment (2). We assume that this requirement may be bypassed in baculovirus-infected cells by the level of furin expression. In addition to gp160 and gp140, we also showed trimming of gp120 at a site near the carboxy terminus, consistent with recognition of the sequence KAKR by furin in addition to the previously characterized REKR and RERR sequences (10). As the characteristics of all these sites conform to the general pattern basic-X-basic-basic, we suggest that they can all be considered as legitimate recognition sites for furin, although from the qualitative nature of our data we cannot comment on the relative efficiency of cleavage at each site. However, we also observed cleavage within the gp120 molecule at a site shown to lie within the immunodominant V3 loop. There is no exact match for the furin consensus sequence within the loop, although substrates for a number of other proteases have been noted (4). The antibody binding data presented in Fig. 5 suggest cleavage by furin within the generally basic sequence NTRKSIRIQRG. Cleavage of dibasic residues has been reported for furin (2) but is more generally considered to be a property of the furin-related protease PC3, and in one reported experimental system, furin has been shown not to cleave at dibasic residues (10). Our results suggest that this is not wholly true and that the particular conformation of the substrate may be an important consideration.

Cleavage of the gp120 V3 loop has been suggested to be an essential step in the processes leading to virus cell fusion (17), and a number of nonfurin protease cleavage sites have been reported within the loop sequence (4). We suggest that an alternate explanation for these observations that would also allow for furin cleavage is that the V3 loop of HIV-1

gp120 is unusually cleavable as a result of its presentation within the molecule. If this were true, then caution should be exercised when suggesting the consequence or necessity of the cleavage event until its role has been directly established. As virion-bound gp120 is not cleaved in the V3 loop and yet has been successfully processed at the gp120-gp41 junction by furin present in the trans-Golgi, our data suggest that the access of furin to the V3 loop is restricted during normal virion egress. It has already been shown that the virion protein *vpu* can stabilize gp160 against degradation during passage through the infected cell (12, 28), and it is possible that protection against illegitimate cleavage by furin is also effected by this protein. Finally, it should be noted that there appears to be more than one type of furin, differing in molecular weight and sensitivity to protease inhibitors (26). It is possible that furins of T cells have a slightly altered substrate requirement compared with the furin expressed here. If premature cleavage of the V3 loop during virus maturation were a lethal event, then illegitimate cleavage by some furins might partly explain the observed link between the identity of the V3 loop and cellular tropism (11, 17, 25).

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