

## Enhancing the Proteolytic Maturation of Human Immunodeficiency Virus Type 1 Envelope Glycoproteins

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Received 31 August 2001/Accepted 6 December 2001

**In virus-infected cells, the envelope glycoprotein (Env) precursor, gp160, of human immunodeficiency virus type 1 is cleaved by cellular proteases into a fusion-competent gp120–gp41 heterodimer in which the two subunits are noncovalently associated. However, cleavage can be inefficient when recombinant Env is expressed at high levels, either as a full-length gp160 or as a soluble gp140 truncated immediately N-terminal to the transmembrane domain. We have explored several methods for obtaining fully cleaved Env for use as a vaccine antigen. We tested whether purified Env could be enzymatically digested with purified protease in vitro. Plasmin efficiently cleaved the Env precursor but also cut at a second site in gp120, most probably the V3 loop. In contrast, a soluble form of furin was specific for the gp120–gp41 cleavage site but cleaved inefficiently. Coexpression of Env with the full-length or soluble form of furin enhanced Env cleavage but also reduced Env expression. When the Env cleavage site (REKR) was mutated in order to see if its use by cellular proteases could be enhanced, several mutants were found to be processed more efficiently than the wild-type protein. The optimal cleavage site sequences were RRRRRR, RRRRKR, and RRRKKR. These mutations did not significantly alter the capacity of the Env protein to mediate fusion, so they have not radically perturbed Env structure. Furthermore, unlike that of wild-type Env, expression of the cleavage site mutants was not significantly reduced by furin coexpression. Coexpression of Env cleavage site mutants and furin is therefore a useful method for obtaining high-level expression of processed Env.**

The Env glycoprotein complex mediates receptor binding and membrane fusion during human immunodeficiency virus type 1 (HIV-1) infection of susceptible cells (66). It is synthesized as a polypeptide precursor (gp160) that oligomerizes to form a heavily glycosylated trimer (20, 24). At a late stage of synthesis, most probably in the trans-Golgi network (TGN), gp160 is cleaved by furin (17, 18, 55–58) or other, related subtilisin-like proteases (17, 18, 28, 38, 58, 90) into the surface (SU; gp120) and transmembrane (TM; gp41) subunits (34, 43, 55–58, 82). Cleavage occurs at a motif at the gp120–gp41 junction that contains a basic amino acid tetrad, R-X-(R/K)-R (where X is any amino acid). The gp120 and gp41 proteins then remain noncovalently associated, forming the functional, native gp120<sub>3</sub>–gp41<sub>3</sub> complex (20, 24, 66).

During fusion, the gp120 protein interacts with the virus receptor and coreceptor on target cells. This triggers conformational changes that lead to the insertion of a hydrophobic fusion peptide, located at the N terminus of gp41, into the target cell membrane (66). Cleavage of gp160 is essential for

fusion, since uncleaved gp160 is fusion incompetent (9, 33, 39, 48). Generally, only cleaved Env is incorporated into virions (22), although uncleaved Env can be virion associated (39, 48). By analogy with other enveloped viruses such as influenza A virus (5, 32, 36, 40–42, 60), Semliki Forest virus (27, 71), and Newcastle disease virus (76), gp160 cleavage may induce a shift from a low-energy state to a metastable Env configuration that is capable of fusion. The common requirement for cleavage of an Env precursor in many families of enveloped viruses is an indication of the general importance of this event in virus assembly (8, 27, 42, 70, 79, 83, 89, 91).

HIV-1 Env is the focus of vaccine design strategies intended to elicit virus-neutralizing antibodies. To neutralize HIV-1, an antibody must be able to bind to the native, trimeric virus-associated Env complex (11, 12, 63, 64). Most Env-based vaccine candidates tested to date have been either monomeric gp120 subunits or various forms of the uncleaved gp160 or gp140 (gp120 plus gp41 ectodomain) precursor protein (4, 25, 26, 37, 47, 68, 80, 96–98). The use of uncleaved gp140 or gp160 protein has been considered necessary because the labile, non-covalent gp120–gp41 association in cleaved Env leads to the dissociation of gp120 from gp41 (30, 49, 54, 74). However, gp120 and uncleaved gp140 and gp160 proteins do not fully mimic the structure of the native trimeric Env complex. As a result, antibodies elicited to gp120 and uncleaved Env proteins can sometimes neutralize the homologous HIV-1 isolate but generally do not cross-neutralize heterologous primary isolates (4, 25, 68, 98).

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It is not yet clear what effect cleavage has on the overall structure of HIV-1 Env, especially from the perspective of antibody recognition. Cleavage of the influenza A virus hemagglutinin precursor (HA<sub>0</sub>) causes only localized refolding with little impact on its overall structure (15). However, it is not known how precise a model influenza A virus HA<sub>0</sub> is for HIV-1 gp160: The two viruses are distant relatives, and their fusion potentials are triggered by quite different mechanisms, so it may not be appropriate to extrapolate what has been learned from HA<sub>0</sub> to predict all aspects of gp160 structure and function. Indeed, uncleaved HIV-1 gp140 proteins are antigenically and, by implication, structurally different from cleaved proteins (6). Moreover, the Env proteins of several other viruses exhibit dramatic refolding upon cleavage (23, 27, 35, 62, 71, 73, 76, 79). Thus, the projecting domains of the trimeric spike precursor of Semliki Forest virus coalesce to form a compact, mature spike (27, 71). The structures of the mature forms of the tick-borne encephalitis virus (TBEV) E protein and the simian virus 5 (SV5) paramyxovirus F protein, as probed by antibodies, appear to be significantly different from those of the immature forms (23, 35, 73, 79). Of note, antibodies against the heptad repeat regions of the transmembrane domain of the SV5 F protein recognized only the uncleaved form (23). Overall, whether the above examples represent better paradigms than HA<sub>0</sub> for the structural impact of cleavage on HIV-1 Env is not known, but clearly they support further analysis of cleaved forms of HIV-1 Env.

Mimicking the native structure of Env may be a useful HIV-1 vaccine design strategy. Production of a native Env complex as a recombinant protein has, however, been hampered by the limited efficiency of Env cleavage (6, 38, 55, 57, 58, 90, 95) and by the instability of the complex after cleavage has occurred (30, 49, 54, 74). The SU-TM association in cleaved forms of Env can be stabilized by the introduction of appropriately positioned cysteine residues that form an intermolecular disulfide bond between gp120 and gp41 (6). However, to achieve full cleavage of the gp140 precursor in Env-transfected cells, it was necessary to coexpress furin (6). A disadvantage of this approach is that furin coexpression significantly reduced Env expression (6, 55, 57, 58, 95). Moreover, cleavage of some Env proteins was still not complete even with furin coexpression (18, 55, 58, 95). Changes in the gp120 variable loops (72, 85), elsewhere in Env (19, 51, 84, 88, 94), and at residues proximal to the cleavage site (2, 29, 33, 84) can all affect Env cleavage efficiency, usually unpredictably. Overall, cleavage efficiency is a function of the folding, oligomerization, and glycosylation of gp160, factors that influence the access of furin to its binding site at the gp120–gp41 juncture.

Here, we have investigated several ways to produce proteolytically cleaved HIV-1 Env proteins: the use of purified proteases to cleave purified Env, coexpression with Env of full-length and soluble forms of furin, and mutation of the cleavage site to enhance its processing by cellular proteases. We report that coexpression of Env cleavage site mutants with furin is a useful method for obtaining significant amounts of processed Env. The generation of stable, cleaved, oligomeric Env complexes for immunogenicity and structural studies is a complex, multistage process. Here, we describe a possible solution to one of the obstacles: the inefficiency of cellular proteases at cleaving Env proteins when these are expressed at high levels.

## MATERIALS AND METHODS

**Plasmids and mutagenesis.** The pPPI4 plasmid, which expresses soluble gp140 lacking the transmembrane and intracytoplasmic domains of gp41, has been described elsewhere (6, 72, 86). Unless specified otherwise, the Env glycoproteins expressed in this study were derived from the HIV-1 JR-FL molecular clone, a subtype B R5 primary isolate. However, we also expressed gp140 proteins from the subtype B molecular clones HXB2, 89.6, 89.6<sub>KB9</sub>, DH123, and Gun-1<sub>WT</sub>, as previously described (6), and from a subtype C South African isolate, DU151, using a pT7blue-based source plasmid provided by Lynn Morris and Maria Papathanopoulos (National Institute of Virology, Johannesburg, South Africa) and Carolyn Williamson (University of Cape Town, Cape Town, South Africa). The gp140 proteins from SIVmac and SIVmne were expressed in a manner similar to that for HIV-1 JR-FL. Some of the above gp140 proteins were also made as mutants that contained cysteine substitutions designed to introduce an intermolecular disulfide bond between gp120 and gp41; the positioning of this disulfide bond corresponds to that of the one introduced into JR-FL gp140, to make the protein designated SOS gp140 (gp140<sub>SOS</sub>), as described elsewhere (6). Wild-type gp140 proteins that lack the SOS mutations but retain the native SU/TM cleavage site are designated gp140<sub>WT</sub>. Other gp140 proteins were mutated to replace the wild-type SU/TM cleavage site REKR (see below) and are designated as follows: gp140<sub>RRRRKR</sub>, gp140<sub>RRRRKR</sub>, gp140<sub>RRRRKR</sub>, gp140<sub>KKRRKR</sub>, and gp140<sub>RRRRKR</sub>. All amino acid substitutions were performed by using the Quikchange site-directed mutagenesis kit (Stratagene Inc.) with appropriate primers. Plasmid pSV7D was used to express full-length JR-FL gp160 for infectivity and fusion assays (21). Mutants of this protein were constructed and named analogously to the pPPI4 gp140 mutants. Vesicular stomatitis virus (VSV) G protein was also expressed by the pSV7D plasmid (21). Furin was expressed from plasmid pcDNA3.1-Furin as previously described (6). A stop codon was introduced within the furin gene in place of the codon for residue E-684, to make plasmid pcDNA3.1-furin<sup>Δ</sup>TC. This mutation truncates furin close to the C-terminal end of its ectodomain, leading to the expression of a secreted, active form of furin (65). A pGEM furin source plasmid was obtained from Gary Thomas and Sean Molloy (Vollum Institute, Portland, Oreg.) (52, 53).

**Anti-HIV-1 and anti-SIV antibodies and sera.** Monoclonal antibody (MAb) B12 recognizes an epitope in the C2 domain of gp120 that is preferentially exposed on denatured forms of the molecule (1). This was provided by George Lewis (Institute of Human Virology, Baltimore, Md.). MAb 2F5 recognizes a neutralizing epitope in the C-terminal region of the gp41 ectodomain (59) and was provided by Hermann Katinger (Polymun Scientific Inc., Vienna, Austria). Simian immunodeficiency virus (SIV) immune globulin (SIVIG) was purified from the sera of SIVmac251-infected rhesus macaques as previously described (7). Purified human immune globulin from HIV-1-infected people (HIVIG) was obtained from John Mascola (Vaccine Research Center, National Institutes of Health [NIH], Washington, D.C.).

**Transfection, immunoprecipitation, and Western blotting.** Transfection and metabolic labeling of 293T cells and immunoprecipitations were performed as described previously (6, 72) by using HIVIG or SIVIG to precipitate the labeled HIV-1 or SIV proteins, respectively. Ten micrograms of each plasmid was used for transfections in duplicate 10-cm-diameter cell culture plates, unless otherwise stated. When two plasmids were cotransfected, we used 10 μg of each plasmid to transfect a 10-cm-diameter plate of 293T cells. In other experiments, purified gp140 proteins were analyzed by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using either MAb 2F5 or MAb B12 as a probe (6, 72).

**Measurement of Env expression and cleavage efficiency.** Densitometry measurements were performed using ImageQuant and NIH Image software. Env cleavage efficiency was calculated by the formula (density of gp120 band)/(combined density of gp120 + gp140 bands or gp120 + gp160 bands) after the background density was subtracted in each case. The values obtained were reproducible for each protein within a 6% deviation from the value presented in each case. Env expression was calculated by combining the densities either of the gp120 and gp140 bands or of the gp120 and gp160 bands and subtracting the background density. In each gel, expression is recorded as a ratio relative to the standard used for normalization in that particular experiment.

**Vaccinia viruses.** Vaccinia virus v-VSE5 (expressing full-length SIVmne Env under the control of the 7.5K promoter) has been described previously (67). Vaccinia virus v-VS4 expresses SIVmne Gag-Pol under the control of the 11K promoter (S.Hu, unpublished data). Vaccinia virus vv:hfur, expressing full-length human furin, was obtained from Gary Thomas and Sean Molloy (52). For protein production, BSC40 cells were infected at a multiplicity of infection (MOI) of 5. Supernatant proteins metabolically labeled with [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methi-

online were collected 2 days later. Samples were processed in a manner similar to that for the transfected cell supernatants above.

**Purified Env proteins, enzymes, and in vitro enzymatic digestion.** Purified human furin was purchased from Affinity Bioreagents Inc., Golden, Colo. This is a soluble form of furin with the transmembrane domain and cytoplasmic tail removed. The specific activity of 1 U of furin is the amount required to release 1 pmol of fluorogenic substrate peptide in 1 min. Purified human plasmin was purchased from Sigma Chemical Co. For determination of optimal digestion conditions, a highly purified gp140<sub>SOS</sub> protein was used (prepared by Progenics Pharmaceuticals Inc.). This particular early production batch of protein was approximately 50% cleaved. Purified gp140<sub>SOS</sub> was found to be monomeric, in contrast to an earlier report on unpurified gp140<sub>SOS</sub> (6), probably because of instability between the gp41 subunits (N. Schuelke et al., unpublished data). As a control, JR-FL gp120 produced and purified in the same manner was used (86). For plasmin digestions, 8  $\mu$ g (approximately 60 pmol) of gp140<sub>SOS</sub> or gp120 was incubated at 37°C with 200 pmol (approximately 0.2 U) of plasmin in 0.1 M Tris-HCl (pH 7.0) in a total volume of 80  $\mu$ l. For furin digestions, 8  $\mu$ g (approximately 60 pmol) of gp140<sub>SOS</sub> was incubated at 37°C with 20 U of furin in 100 mM HEPES–1 mM CaCl<sub>2</sub> (pH 7.5) in a total volume of 80  $\mu$ l. The digests were then analyzed by SDS-PAGE and Western blotting. In experiments in which furin digestions were performed at various pHs, we used the same concentration of furin in a buffer consisting of 50 mM Tris maleate and 10 mM CaCl<sub>2</sub>, essentially as described by Stadler et al. (79).

**Viral infectivity and cell-cell fusion assays.** Pseudotyped luciferase reporter viruses were produced by calcium phosphate transfection. Thus, 293T cells were cotransfected with 5  $\mu$ g of the Env-deficient NL4-3 HIV-1 construct pNL-luc and with 15  $\mu$ g of a pSV7D vector expressing either the full-length JR-FL Env glycoproteins or the positive control VSV-G protein (21). The pNL-luc virus carries the luciferase reporter gene. The pSV7D plasmids expressed either wild-type gp160 (gp160<sub>WT</sub>) or a mutant with a cleavage site modified from REKR to RRRRRR, designated JR-FL gp160<sub>RRRRRR</sub>. Supernatants containing pseudotyped viruses from transfected cells were harvested after 48 h and filtered through a 0.45- $\mu$ m-pore-size filter. Viral stocks were then standardized for p24 protein content by enzyme-linked immunosorbent assay (21), and infections were performed using HeLa-CD4-CCR5 cells. Infectivity was expressed as light units per nanogram of p24 protein in the viral inoculum (21).

Cell-cell fusion activity was measured by a fluorescent cytoplasmic dye transfer assay, as described elsewhere (50). Briefly, 293T cells on a 6-cm-diameter dish were transfected with 10  $\mu$ g of the pSV7D vector expressing full-length JR-FL Env and were then labeled with 1.5  $\mu$ M acetoxymethyl calcein (Molecular Probes, Inc., Eugene, Oreg.) in 2 ml of phosphate-buffered saline, according to the manufacturer's instructions. Cells were detached from the dish by incubation in phosphate-buffered saline supplemented with 0.5 mM EDTA and 0.5 mM EGTA and were then transferred into a centrifuge tube. Approximately  $5 \times 10^6$  CEM.NKR.CCR5 cells (87) were suspended in 2 ml of Opti-MEM (Gibco) containing 100  $\mu$ M 7-amino-4-chloro methylcoumarin (CMAC; Molecular Probes) and incubated for 30 min at 37°C. After extensive washing to remove the remaining free dye, the effector and target cells were mixed, transferred into polylysine-coated, 8-well chambered slides, and incubated for 2 h at 37°C. The extent of fusion was determined by fluorescence video microscopy by normalizing the number of fusion products (stained with both cytoplasmic markers) against the number of target cells that were in contact with the effector cells.

## RESULTS

**Enzymatic processing of purified, uncleaved gp140<sub>SOS</sub>.** In principle, one way to achieve Env cleavage is to treat purified Env proteins in vitro with proteases capable of recognizing the gp120–gp41 cleavage site. The highly active subtilisin family protease plasmin was previously reported to cleave recombinant gp160 into gp120–gp41, whereas other trypsin-like proteases lacked this ability (61). Plasmin is also capable of processing influenza virus HA<sub>0</sub> at the cell surface (32). We therefore evaluated the effect of plasmin on a preparation of purified, soluble gp140<sub>SOS</sub> that was 50% cleaved. The partially cleaved gp140<sub>SOS</sub> preparation was incubated with an excess of plasmin for 2 or 16 h at 37°C, and the proteins were analyzed by SDS-PAGE and Western blotting using the anti-gp41 MAb 2F5 (Fig. 1). After 2 h of plasmin treatment, there was a

reduction in the intensity of the uncleaved gp140 band, but the longer reaction time (16 h) was required for processing to be complete (Fig. 1A). This is consistent with the previous report on gp160 cleavage by plasmin (61). However, when a Western blot of the 16-h plasmin digest was probed with the gp120-specific MAb, B12, it was clear that plasmin also digests gp120 into fragments, one of which is about 70 kDa (Fig. 1B). MAb B12 recognizes an epitope in the second conserved domain of gp120, N-terminal to the V3 loop (1). Thus, plasmin cleaves gp120 internally, most likely at the site in the V3 loop that is a substrate for other tryptic proteases and that typically yields 50- and 70-kDa fragments (16, 49, 75). Although plasmin does process the gp120–gp41 cleavage site, the use of this enzyme to enhance Env cleavage is not, therefore, a practical technique.

We next investigated whether soluble furin would cleave gp140 efficiently but with greater specificity. Cleavage of gp140 was virtually undetectable after a 2-h digestion (data not shown). However, when the digestion period was increased to 16 h, soluble furin significantly, albeit incompletely, cleaved gp140<sub>SOS</sub> into gp120 without causing additional gp120 degradation (Fig. 1C; compare the first and third lanes). The efficiency of cleavage of gp140 by soluble furin was low, as shown by the following calculation: 1 U of soluble furin can process 1 pmol of fluorogenic peptide substrate in 1 min (3). If gp140 were an equally efficient substrate, the 8  $\mu$ g of gp140, containing approximately 4  $\mu$ g (30 pmol) of uncleaved gp140, would be digested by 20 U of furin within 2 min. However, only 50% of the gp140 was actually processed after 16 h. If we assume that the rate of processing was uniform over this period, gp140 was cleaved at 0.7 fmol/min; thus, gp140 is  $\sim$ 1,000-fold less efficiently cleaved by furin than are model peptides.

The pH of the furin digest may affect its efficiency. For example, the mildly acidic pH of the exocytic pathway alters the structure of the TBEV Env precursor to permit an increase in cleavage efficiency (79). Hence, furin is able to cleave the TBEV Env precursor in vitro at pH 6.2 but not at pH 7.5 (79). Furthermore, NH<sub>4</sub>Cl treatment of cells, which raises the pH of the secretory pathway, can interfere with HIV-1 Env processing (93). We therefore investigated whether a mildly acidic pH might allow more-efficient cleavage of gp140 by soluble furin during a 16-h incubation. The optimal pH for Env cleavage was found to be 5.8 (Fig. 1D), in contrast to a report that furin was most active (>80%) at a pH in the range of 6.5 to 8 (17, 18). However, even at pH 5.8, gp140 was only about 60% cleaved (Fig. 1D), so optimizing the reaction pH was insufficient to achieve complete cleavage. Overall, we conclude that because a large excess of furin is required to achieve only a modest degree of gp140 cleavage, this also is not a practical technique for routine use.

**Incomplete processing of recombinant Env proteins by cellular proteases and the effect of coexpressing recombinant furin and Env.** We examined the extent of endogenous gp120–gp41 cleavage of seven HIV-1 and four SIV gp140 proteins by immunoprecipitation using HIVIG or SIVIG, as appropriate. Although the cleavage site (REKR) was conserved among all seven HIV-1 isolates (JR-FL, HXB2, 89.6, 89.6 KB9, DH123, Gun1 WT, and DU151), the gp140<sub>SOS</sub> cleavage efficiency (defined in Materials and Methods) varied from 38 to 58%, and in no case was cleavage complete (Fig. 2A). Similar results were obtained with the corresponding seven HIV-1 gp140<sub>WT</sub> pro-



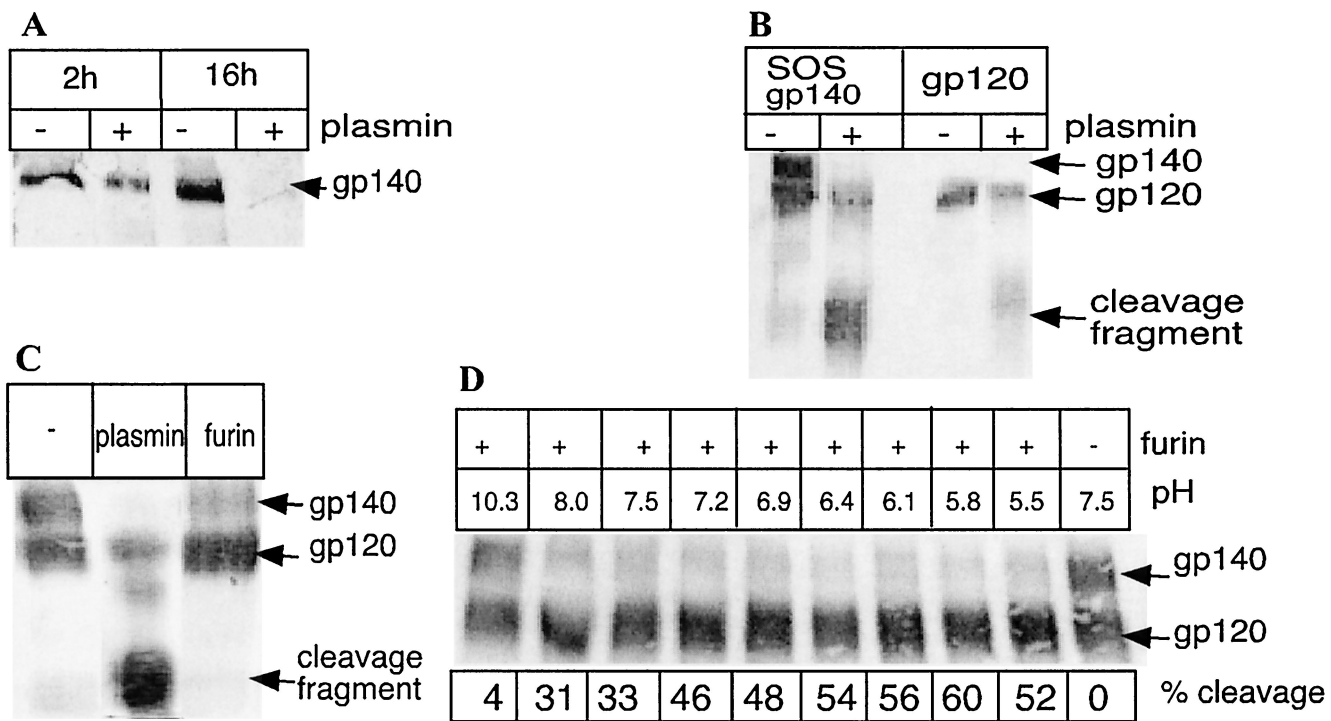


FIG. 1. In vitro enzymatic cleavage of Env with plasmin or soluble furin. Eight micrograms of partially (~50%) cleaved, purified JR-FL gp140<sub>SOS</sub> or gp120 was incubated with a protease for various times at 37°C. The Env proteins were then analyzed by reducing SDS-PAGE and Western blotting. (A) gp140<sub>SOS</sub> proteins from a 2- or 16-h plasmin (20 μg) digest. The Western blot was probed with the anti-gp41 MAb 2F5. (B) gp140<sub>SOS</sub> and gp120 samples from a 16-h plasmin (20 μg) digest. The Western blot was probed with the anti-gp120 MAb B12. (C) gp140<sub>SOS</sub> was incubated at pH 7.5 with or without plasmin or soluble furin for 16 h at 37°C. The Western blot was probed with MAb B12. (D) JR-FL gp140<sub>SOS</sub> was incubated with or without furin for 16 h at the pH indicated. The Western blot was probed with MAb B12. The percent cleavage achieved by soluble furin was calculated as described in Materials and Methods and was expressed with reference to the control in the last lane (no furin; 0% cleavage).

teins that lack the SOS cysteine substitutions (data not shown). These proteins are secreted as mixtures of gp120 and un-cleaved gp140 despite retaining the REKR cleavage site, because proteolysis is inefficient in the absence of cotransfected furin (6). The cleavage efficiency was generally slightly higher for each gp140<sub>WT</sub> protein than for the corresponding gp140<sub>SOS</sub> mutant (data not shown). The cleavage efficiencies of different Env proteins are unpredictable and are likely to be affected by folding differences related to variation in the primary amino acid sequence (2, 19, 29, 33, 51, 84, 85, 88, 94). This is demonstrated by the virtually identical cleavage efficiencies of the JR-FL and DU151 envelope proteins, despite the fact that the DU151 envelope protein is expressed at much lower levels than the JR-FL protein.

Incomplete cleavage was also observed with the SIVmac251 and SIVmne gp140<sub>SOS</sub> proteins and the SIVmne gp140<sub>WT</sub> protein, each of which has an RNKR cleavage site motif. In contrast, cleavage of the SIVmac251 gp140<sub>WT</sub> protein was almost complete (Fig. 2A). Since the cleavage site motifs in the mutant and wild-type proteins are identical, indirect factors such as differences in folding must influence cleavage efficiency.

**Coexpression of furin reduces the expression of Env proteins.** We next examined the effects of coexpressing furin with JR-FL gp140<sub>SOS</sub> and gp140<sub>WT</sub> proteins, since this has previously been shown to increase cleavage efficiency (6, 55, 95).

Varying amounts of the full-length, furin-expressing plasmid pcDNA3.1-furin, were cotransfected with a constant amount of an Env-expressing plasmid (Fig. 2B). Expression of sufficient furin resulted in almost complete (>90%) cleavage of both forms of gp140 protein, but it also caused a significant reduction in overall Env expression, as measured by a decrease in the combined intensity of the gp140 and gp120 bands. We considered whether the use of too much transfected DNA might explain the poor secretion of envelope glycoproteins seen when furin is coexpressed. However, when we used three-fold less of each plasmid in a repeat experiment, the results were essentially identical to those shown in Fig. 2B (data not shown). This, together with our observations that furin cotransfection has no overtly cytotoxic effects, implies that the reduction in envelope glycoprotein secretion is not merely related to an “overburdening” of the transfected cells with plasmid DNA.

To verify the effect of Env and furin coexpression with a different form of Env protein and a different expression system, we expressed SIVmne E11S gp160 in BSC40 cells from a recombinant vaccinia virus, both alone and together with Gag and Pol. Coexpression of Env with Gag-Pol enabled us to examine the efficiency with which full-length, membrane-bound gp160, secreted as pseudovirions, was cleaved into gp120-gp41 complexes (Fig. 2C). The expression of SIVmne gp160 was approximately 10-fold higher after vaccinia virus

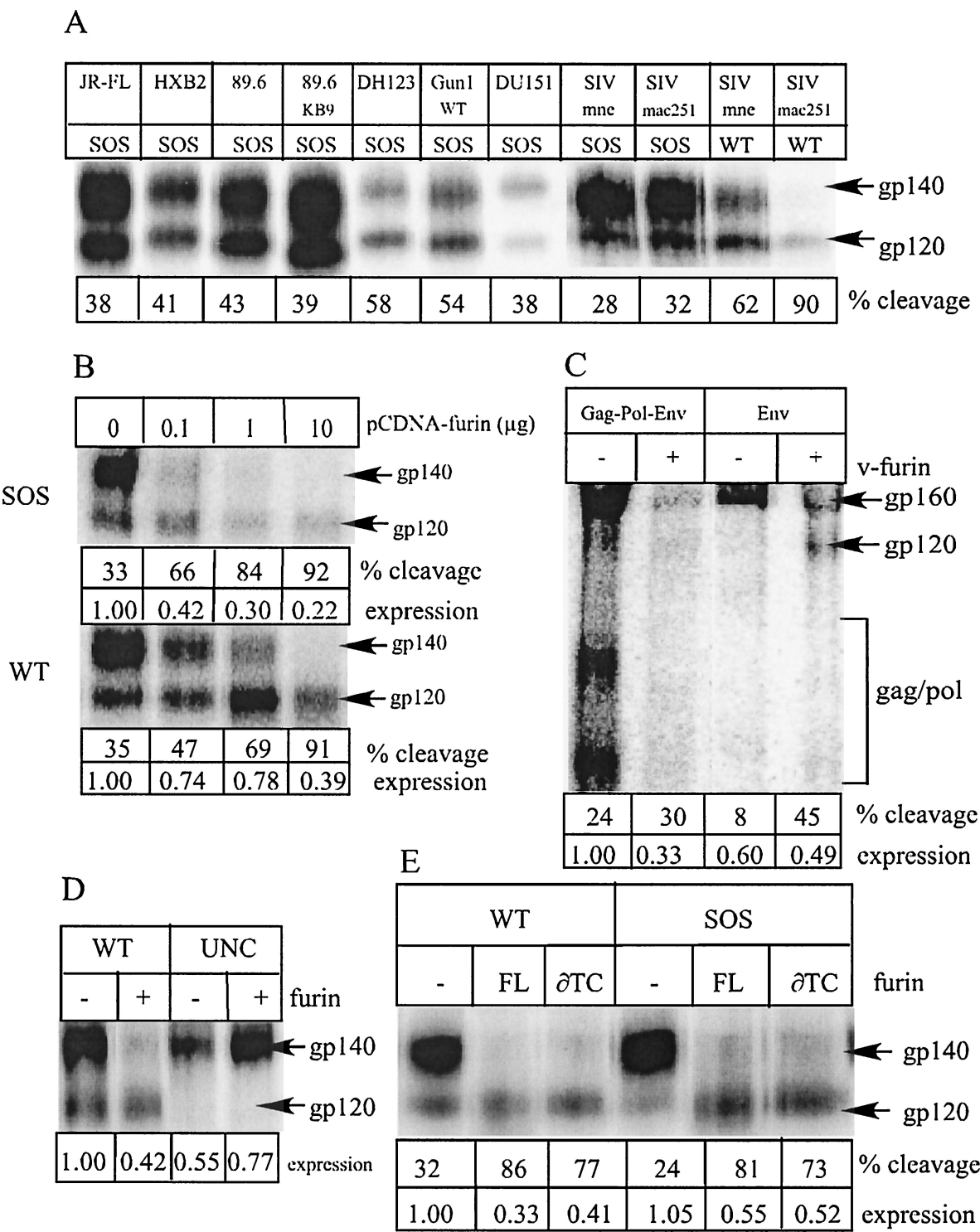


FIG. 2. Cleavage and expression of Env proteins with or without furin. Culture supernatants containing <sup>35</sup>S-labeled Env proteins were immunoprecipitated with either HIVIG or SIVIG as appropriate and then analyzed by reducing SDS-PAGE. Results shown are representative of three repeats. In each panel, the percent cleavage was calculated as described in Materials and Methods. Additionally, the relative expression of the gp120-plus-gp140 or gp120-plus-gp160 bands was calculated and expressed as a ratio relative to a standard (expression defined as 1.00) in each gel. (A) Soluble gp140 proteins were expressed in 293T cells transfected with pPPI4-based plasmids. (B) The JR-FL gp140<sub>SOS</sub> and gp140<sub>WT</sub> proteins were expressed as in panel A but in the presence of variable amounts of coexpressed, full-length furin. (C) BSC40 cells were infected with vaccinia viruses v-VS4 (expressing SIVmne Gag-Pol) and/or v-VSE5 (expressing SIVmne Env) at an MOI of 5, as indicated. Some of the cells were coinfectd, also at an MOI of 5, with the vaccinia virus vv:hfur expressing full-length furin (second and fourth lanes). (D) JR-FL gp140<sub>WT</sub> and gp140<sub>UNC</sub> proteins were expressed in 293T cells transfected with pPPI4-based plasmids, with or without cotransfection of full-length furin. (E) JR-FL gp140<sub>WT</sub> and gp140<sub>SOS</sub> proteins were expressed in 293T cells transfected with pPPI4-based plasmids, alone or with cotransfection of either full-length furin (FL) or truncated, soluble furin (δTC), as indicated.

v-VSE5 infection than after transfection of the pPPI4-based plasmid encoding the identical gp160 (data not shown). In the absence of furin, gp160 cleavage was very low (Fig. 2C, first and third lanes). The extent of cleavage was increased only modestly by furin coexpression, but there was a substantial reduction in the overall expression of Env (Fig. 2C, second and fourth lanes). Indeed, when Gag-Pol and Env were coexpressed along with furin, the Env proteins were barely detectable (Fig. 2C, second lane). Gag-Pol was also immunoprecipitated at diminished levels when furin was coexpressed (Fig. 2C, second lane). This may be because the precipitation of Gag-Pol from pseudovirions occurs indirectly via antibody reactivity with surface Env, and Env expression is reduced by coexpression of furin. Alternatively, the reduction in Gag-Pol immunoprecipitation could be explained by nonspecific competition for protein expression when furin is coexpressed. We believe, however, that the inhibitory effect of furin is probably specific to its substrate, Env in this case. Some full-length gp160 was present in the supernatant even in the absence of coexpressed Gag-Pol (Fig. 2C, third and fourth lanes). This full-length gp160 may be associated with cellular vesicles (31) or could have been released from dead cells.

Overall, it is clear that furin expression has qualitatively similar effects on both gp140 and gp160 proteins irrespective of the expression system. Moreover, the increase in Env expression in the vaccinia virus system is associated with a further reduction in the extent of Env cleavage.

**Influence of the furin substrate sequence on Env expression in the presence of coexpressed furin.** The coexpression of furin has previously been reported to reduce the expression of several furin substrates, perhaps due to the complexing and retention of the nascent proteins with furin in the TGN rather than to any overtly toxic effect of furin on the cells (52, 58, 81). To investigate this, we determined whether the reduction in Env expression caused by furin coexpression required that the Env protein exhibit a furin-recognition motif (Fig. 2D). We observed that furin coexpression had little effect on expression of the JR-FL gp140<sub>UNC</sub> protein (Fig. 2D, third and fourth lanes). In this protein, the KRRVVQREKRAV furin recognition sequence at the gp120–gp41 interface has been replaced by LRLRLRLRLRLR (6), so the protein is no longer a furin substrate. Although, in this experiment, gp140<sub>UNC</sub> expression was slightly increased in the presence of furin, the increase was not usually observed in repeat assays (data not shown). The lack of effect of furin on gp140<sub>UNC</sub> expression contrasts markedly with its substantial inhibition of the expression of the gp140<sub>WT</sub> and gp140<sub>SOS</sub> proteins, which have unmodified cleavage site sequences (Fig. 2D, first and second lanes, and Fig. 2B). These results are consistent with the hypothesis that furin-induced reduction in Env expression is attributable to the formation of Env-furin complexes that are retained within the cell.

**A soluble form of furin for Env cleavage.** In an attempt to overcome the apparent formation of furin-Env complexes in the TGN, we coexpressed Env with a soluble form of furin. The proteolytic activity of furin is contained entirely in its luminal domain, and soluble forms of the enzyme retain enzymatic activity (52, 53, 65). When we expressed JR-FL gp140<sub>WT</sub> and gp140<sub>SOS</sub> in the presence of full-length furin, we saw the expected reduction in Env expression (Fig. 2E, lanes FL). How-

ever, essentially the same result was observed when a soluble form of furin (furin $\delta$ TC) was used instead of the full-length, membrane-bound enzyme (Fig. 2E, lanes  $\delta$ TC). Thus, although the presence of the furin recognition sequence is important (Fig. 2D), the direct retention of Env in complex with furin in the TGN may not be the entire explanation for the reduction in Env expression upon furin coexpression (38, 52, 57, 93).

**Altering the cleavage sequence can increase Env processing by cellular proteases.** Our next approach toward increasing the efficiency of gp140 cleavage was to vary the furin recognition sequence. The rationale for this is partly derived from studies of other RNA viruses. For example, some influenza A virus variants have evolved proteolytic cleavage sites in the HA<sub>0</sub> precursor protein that contain basic residue insertions. This is associated with increased cleavage efficiency and a gain in viral virulence. Thus, whereas avirulent clones contain only a single arginine residue within the HA<sub>0</sub> cleavage site, the corresponding sites of virulent clones contain multiple basic residues, leading to motifs such as RRRKKR (5, 32, 36, 40, 41, 60). Biochemical evidence using peptide cleavage assays has confirmed that multiarginine stretches are highly efficient substrates for furin (13). The most efficiently recognized target sequences consist of hexa- or hepta-arginine repeats; for example, a peptide with the recognition sequence RRRRRR was cleaved approximately 50 times more efficiently than one with the RRRR motif (13).

In contrast to influenza A virus, HIV-1 and SIV strains contain only simple R-X-(R/K)-R furin recognition sequences. We therefore introduced basic amino acids into the cleavage sites of the JR-FL gp140<sub>SOS</sub> and gp140<sub>WT</sub> proteins. The mutated gp140 proteins were processed more efficiently than those containing the normal REKR motif, although none of the mutants was completely cleaved by endogenous cellular proteases (Fig. 3A; Table 1).

Two of the most efficiently cleaved mutants contained the RRRRRR or RRRKKR motif (Fig. 3A; compare first, third, and fifth lanes). When furin was coexpressed, these mutants were 100% cleaved, compared to only about 90% cleavage for wild-type Env (or less than 90% in other experiments [data not shown]). An unexpected finding was that furin did not reduce the overall expression of the cleavage site mutant gp140 proteins, whereas, as noted above, it significantly diminished the expression of wild-type gp140 (Fig. 3A; compare second, fourth, and sixth lanes). This was confirmed when the RRRRRR gp140 mutant was coexpressed with variable amounts of pcDNA3.1-furin (0.1, 1, or 10  $\mu$ g) (Fig. 3B). In this experiment, furin coexpression actually increased the overall amount of Env protein secreted, although an increase was not always seen with this or related mutants in other experiments.

The expression levels and cleavage efficiencies of a selection of gp140 mutants with basic insertions into the REKR cleavage site are summarized in Table 1. The closely related mutants RRRKKR, RRRRKR, and RRRRRR all had similar properties, in that cleavage was enhanced in the absence of cotransfected furin and was complete in the presence of furin, but without a significant decrease in the extent of Env expression. The mutants KKRKKR and RERRRKKR were also better cleaved than the wild-type protein, and their expression was unaffected by furin cotransfection. However, they were expressed at lower levels than the other mutants and less well



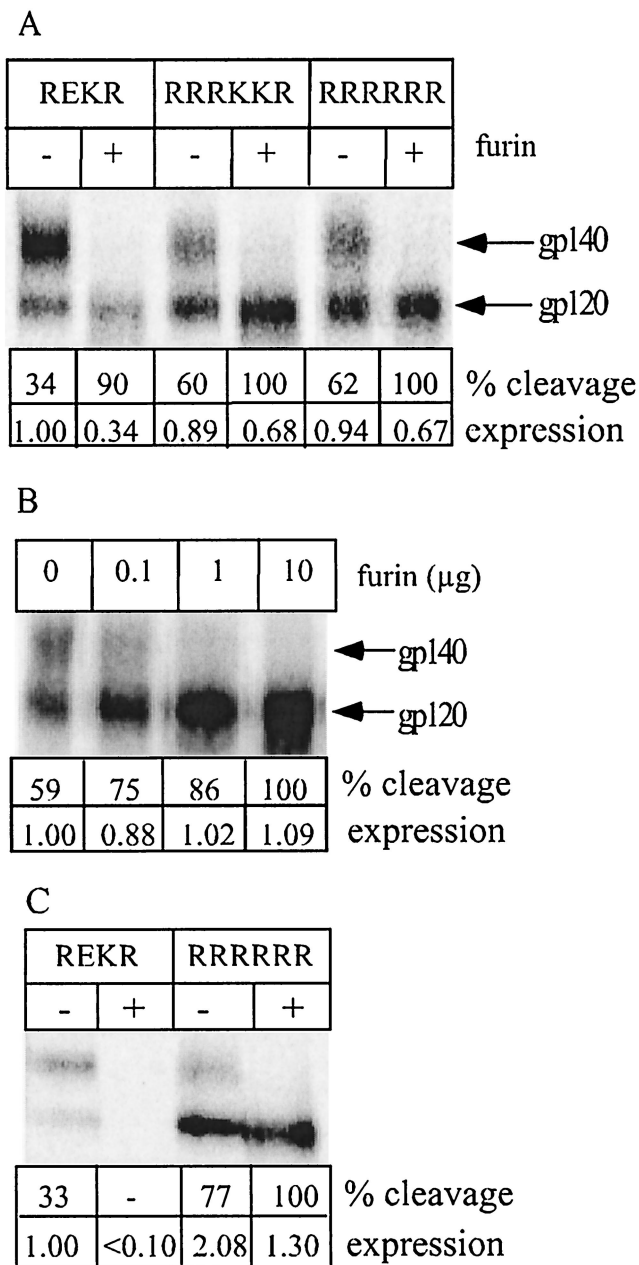


FIG. 3. Altering the cleavage sequence can increase Env processing by cellular proteases. (A) JR-FL gp140<sub>SOS</sub> with the wild-type REKR (first and second lanes) or the mutant RRRKRR (third and fourth lanes) or RRRRRR (fifth and sixth lanes) cleavage site sequence was expressed in the absence (first, third, and fifth lanes) or presence (second, fourth, and sixth lanes) of cotransfected full-length furin. (B) JR-FL gp140<sub>SOS</sub> RRRRRR was expressed with 0, 0.1, 1, or 10 μg of cotransfected full-length furin. (C) DU151 gp140<sub>SOS</sub> (first and second lanes) and its RRRRRR mutant (third and fourth lanes) were expressed in the absence (–) or presence (+) of cotransfected full-length furin. All samples were labeled and immunoprecipitated as described in the legend to Fig. 2. The percent cleavage and relative expression of Env were calculated as for Fig. 2.

than wild-type gp140 proteins containing the standard REKR motif. The effects of the basic residue insertions were similar whether the test protein was gp140<sub>WT</sub> or gp140<sub>SOS</sub>, although some of the gp140<sub>WT</sub> proteins were expressed at slightly higher

levels than the corresponding gp140<sub>SOS</sub> proteins (Table 1). To examine whether enhancement of cleavage by furin also occurred in other cell lines, we performed the same experiments with BSC40 cells. As was seen in 293T cells, the mutant envelope proteins were more fully cleaved in BSC40 cells than was the wild-type, REKR-containing protein (data not shown). Hence, the effect of the cleavage-enhancing substitutions in Env is not unique to 293T cells but may be generally applicable to other cell lines that might be used to express recombinant envelope glycoproteins.

To address whether the insertion of basic amino acids into the proteolytic cleavage site had a general effect on cleavage efficiency (i.e., was not restricted to the JR-FL clone), we mutated the cleavage site of gp140<sub>SOS</sub> of the subtype C primary isolate DU151 from REKR to RRRRRR. In the absence of cotransfected furin, the unmodified DU151 gp140<sub>SOS</sub> protein was partially cleaved (Fig. 3C, first lane). When furin was coexpressed, Env expression was significantly reduced, in some experiments to the extent that the Env proteins were no longer visible (Fig. 3C, second lane; also data not shown). In contrast, the RRRRRR mutant was more efficiently cleaved in the absence of furin, and was fully cleaved in the presence of furin. Furthermore, the overall expression of Env was greater than that of the wild-type gp140 (Fig. 3C; compare second and fourth lanes).

**Effect of cleavage site mutations on HIV-1 infectivity.** We examined whether Env mutants containing basic cleavage site insertions were still functional for virus infection, using an Env-pseudotype assay (21). The JR-FL gp160<sub>RRRRRR</sub> mutant expressed by the pSV7D plasmid in 293T cells could successfully pseudotype pNL-luc, producing a virus capable of infecting HeLa-CD4-CCR5 cells. The infectivity of the JR-FL gp160<sub>RRRRRR</sub> Env pseudotype was about three- to fourfold lower than that of the JR-FL gp160<sub>WT</sub> pseudotype, but still in the range we find to be typical of pseudotyped virus stocks (Fig. 4A). In an independent test of the functional activity of the mutant Env, we examined the ability of the JR-FL gp160<sub>RRRRRR</sub> mutant to mediate cell-cell fusion, using a fluorescent cytoplasmic dye transfer assay (50). The modest, and statistically insignificant, increase in fusion with the RRRRRR mutant (Fig. 4B) may occur because it is expressed at fivefold-higher levels than the wild-type gp160 by Western blot analysis of cell lysates (data not shown). Overall, the REKR-to-RRRRRR substitution does not globally disrupt the Env conformation required for fusion and infection.

DISCUSSION

The Env proteins of most enveloped viruses, including HIV-1, are synthesized as inactive precursors that are proteolytically processed to attain full functional activity. In the case of HIV-1, the gp160 precursor is cleaved into a fusion-active gp120–gp41 complex. The structures of a monomeric gp120 core fragment (45) and a postfusion form of gp41 (14, 46, 92) have been determined. However, little is known about the structure of either uncleaved gp160 or the gp120–gp41 complex, although the latter is considered to be trimeric (14, 20, 46, 66, 92). The fusion-active complex is unstable, principally because the gp120–gp41 interaction is weak and gp120 is shed.

TABLE 1. Summary of expression and cleavage efficiencies<sup>a</sup> of gp140 proteins with mutant cleavage sites

Cleavage site	WT gp140		WT gp140 + furin		SOS gp140		SOS gp140 + furin	
	Cleavage (%)	Expression	Cleavage (%)	Expression	Cleavage (%)	Expression	Cleavage (%)	Expression
REKR	35	1.0	91	0.4	34	1.0	92	0.3
RRRKKR	66	0.9	100	0.7	60	0.9	100	0.8
RRRRKR	65	0.9	100	0.7	60	0.9	100	0.7
RRRRRR	71	1.0	100	0.9	62	0.9	100	0.9
KKRKKR	59	0.2	96	0.1	57	0.3	98	0.3
RERRKKR	58	0.4	97	0.3	55	0.4	100	0.4

<sup>a</sup> The cleavage efficiency of each gp140 cleavage site mutant is given as a percentage derived from densitometric analysis. Each percent cleavage value recorded is the mean from at least three individual experiments in which the values did not deviate by more than 6% from the mean. Combined expression of gp140 and gp120 is also given as a ratio relative to the level of expression of the parental gp140 plus gp120 observed in transfections with gp140<sub>WT</sub> or gp140<sub>SOS</sub>. Mean ratios from three repeats are given to the nearest decimal place and did not deviate more than 25% from this value. Data are shown for both gp140<sub>WT</sub> and gp140<sub>SOS</sub> proteins expressed both in the presence and in the absence of cotransfected furin.

We previously found that introducing a disulfide bond between gp120 and gp41 can prevent gp120–gp41 dissociation (6). Here we investigated methods to produce proteolytically processed proteins for future analytical and immunogenicity studies. The expression of gp140 or gp160 proteins at high levels usually leads to the production of a mixture of cleaved and uncleaved proteins, implying that processing of the cleavage site by host cell proteases is incomplete. Partial cleavage is a common phenomenon when Env is expressed in a variety of recombinant systems and cell lines (38, 55–58, 69, 78, 95). Differences in folding among natural and mutant Env proteins (19, 51, 84, 85, 88, 94) may affect the exposure of what is likely to be a loop structure containing the cleavage site (15). Another influence on Env cleavage is the direct or indirect masking of the furin recognition site by glycans (62). Overall, the accessibility of this site to the protease is a complex function of both Env folding and glycosylation (15, 58, 77). We first evaluated the possibility of cleaving unprocessed, purified Env proteins by adding a purified protease *in vitro*. Although plasmin could efficiently process the gp120–gp41 cleavage site, as has been previously reported (61), it also

cleaved gp120 at a second site, most probably within the V3 loop. This renders its use impractical. Purified furin can also cleave secreted Env (17, 18, 38, 56), albeit at low efficiency (17). Our own findings were similar: Even when furin digestion of gp140<sub>SOS</sub> was performed at optimal pH with the enzyme in great excess, approximately 40% of the Env substrate remained uncleaved, suggesting that there may be a subpopulation of gp140 that is more resistant to cleavage. Furin is not an inherently inefficient enzyme—it is highly effective at cleaving synthetic peptides (13, 65)—but the conformation of its recognition site on gp160 limits its ability to cleave this particular substrate. That gp160 is an inherently poor substrate for furin is exemplified by a comparison of gp160 with anthrax toxin, which is cleaved by furin several orders of magnitude more efficiently than gp160 at pH 7.2 (53). One way to augment gp160 cleavage is to coexpress exogenous furin, but this can lead to a reduction in overall Env expression. The reduction of primary protein expression (including, but not limited to, HIV-1 Env) upon furin coexpression has been noted previously (6, 55, 57, 81, 90). One possible explanation is that furin may form stable complexes with Env proteins that it cleaves poorly, with these complexes being retained in the TGN or recycled to lysosomes rather than secreted (38, 52, 57, 81, 93). This idea is supported by our observation that furin coexpression with Env mutants containing either optimized or inactivated cleavage sites caused very little reduction in Env expression. Coexpression of Env with either the full-length (membrane-bound) or the soluble form of furin reduced gp140 expression, suggesting that this reduction can occur without a direct association of the furin–Env complex with a membrane. However, it has been shown that although truncated furin is shed into the culture medium, it can also still be isolated from membrane fractions, like its full-length counterpart (52). Although furin coexpression increases the cleavage of secreted gp140 proteins, it has been reported that this does not occur very efficiently with full-length gp160 molecules expressed on the cell surface (38, 99; P. Poignard, personal communication). In contrast, we and others (38, 57, 90, 95) have found that gp160 cleavage can be partially augmented by furin, at least under some experimental conditions. Clearly, then, there are poorly understood variables that affect different experimental systems differently, perhaps including the expression vectors, the particular Env gene, and the cell line used.

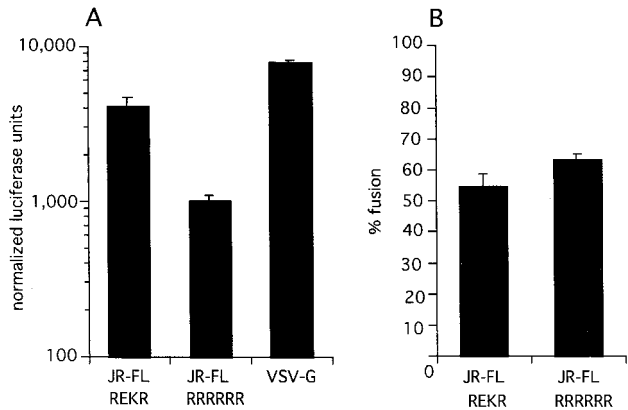


FIG. 4. Env cleavage site mutants are functional for infection and fusion. (A) The infectivities for HeLa-CD4-CCR5 cells of pNL-luc viruses pseudotyped with wild-type JR-FL gp160<sub>WT</sub>, the JR-FL gp160<sub>RRRRRR</sub> mutant, or VSV-G were measured by use of a single-round infection assay with a luciferase readout. Normalized luciferase values for negative-control viruses lacking envelope (derived from pNL-luc transfection supernatants) were <1 U. (B) Cell-cell fusion mediated by the gp160<sub>WT</sub> or gp160<sub>RRRRRR</sub> protein was analyzed in a dye transfer assay.



Our most successful strategy for improving Env cleavage efficiency involved mutating the furin recognition site. Studies of naturally occurring influenza A virus variants have revealed that insertion of basic amino acids in and near the cleavage site of the HA<sub>0</sub> protein is associated with enhanced proteolysis (5), and frequently also with increased host cell range and virulence (5, 32, 36, 40, 41, 60). Moreover, improved cleavage of the influenza B virus glycoprotein was previously achieved by Brassard and Lamb, who replaced the conserved monobasic cleavage site with the multibasic cleavage sites found in virulent influenza A virus clones (10). We therefore considered it possible that altering the conserved cleavage site (REKR) of HIV-1 Env might increase cleavage efficiency. We found that several variant furin recognition sequences, based on those found in HA<sub>0</sub> proteins from pathogenic influenza A virus strains, allowed enhanced cleavage of HIV-1 Env in the absence of coexpressed furin. The best of these variant sequences were RRRKKR, RRRRKR, and RRRRRR, which approximately doubled the extent of Env cleavage compared to that achieved with the wild-type sequence. Furthermore, coexpression of furin did not reduce the expression of Env proteins containing these mutated sequences but did allow the cleavage efficiency to now approach 100%. A consequence of the more-efficient cleavage of these improved furin substrates may be the more-rapid egress of Env from the secretory pathway, allowing a higher overall expression of fully processed Env. Furthermore, the REKR-to-RRRRRR mutation had little impact on the infectivity of Env-complemented reporter viruses or on Env-mediated membrane fusion. The cleavage site mutations do not, therefore, affect the overall folding of Env in any adverse manner, which is relevant to any consideration of the use of such Env mutants as vaccine antigens.

Although furin recognition of gp160 is rather inefficient, the strict conservation of the REKR sequence in HIV-1 (or of RNKR in SIV) suggests that this sequence confers a selective advantage on the virus. There are no examples of Env sequences with basic residue insertions adjacent to the consensus cleavage site motif (44), so a higher rate of Env cleavage may be disadvantageous. For example, a too-rapid destruction of the infected cell by fusion caused by high levels of processed, cell surface Env could reduce the yield of progeny virions from that cell. An immunological mechanism might be that uncleaved Env is actually beneficial to the virus by acting as a decoy that causes the induction of predominantly nonneutralizing antibodies (12, 63, 70).

Here we have demonstrated that we can produce HIV-1 Env mutants containing polybasic cleavage sites that are more-efficient substrates for furin than the consensus REKR sequence. Use of these Env mutants should simplify the production of significant amounts of cleaved Env, which may be of value for HIV-1 vaccine design and for structural studies. Whether monomeric or oligomeric forms of cleaved, stabilized Env proteins will turn out to be better immunogens than other forms of Env will be addressed in future studies. Although the purified form of SOS gp140 (JR-FL) is monomeric, we are now making progress at further stabilizing the Env complex to enable its production in an oligomeric, cleaved form (our unpublished data).

## ACKNOWLEDGMENTS

We are grateful to Sean Molloy and Gary Thomas for advice and for the vv:hfur virus. We thank Maceij Paluch and Daryl Schiller for technical assistance, Norbert Schülke for providing purified gp140<sub>SOS</sub>, and Gregory Melikyan and Levon Abrahamyan for assistance and advice with the fusion experiments. We thank Pascal Pognard for useful discussions.

This work was supported by NIH grants AI49566 (J.M.B.), AI49764 (Paul Maddon, Progenics Pharmaceuticals, Inc.), AI45463 (J.P.M.), and AI47735 (S.-L.H.). J.P.M. is an Elizabeth Glaser Scientist of the Pediatric AIDS Foundation and a Stavros S. Niarchos Scholar. The Department of Microbiology and Immunology at the Weill Medical College gratefully acknowledges the support of the William Randolph Hearst Foundation.

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