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# Ubiquitination of the Human Immunodeficiency Virus Type 1 Env Glycoprotein

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**Expression of the human immunodeficiency virus type 1 (HIV-1) Env glycoprotein is stringently regulated in infected cells. The majority of the glycoprotein does not reach the cell surface but rather is retained in the endoplasmic reticulum or a *cis*-Golgi compartment and subsequently degraded. We here report that Env of various HIV-1 isolates is ubiquitinated at the extracellular domain of gp41 and that Env expression could be increased by lactacystin, a specific proteasome inhibitor, suggesting that the ubiquitin/proteasome system is involved in control of expression and degradation.**

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp160 is produced as a precursor polyprotein, which is cleaved in a Golgi or post-Golgi compartment by a cellular furin-type protease into gp120 and gp41 subunits (13, 19, 22, 25, 26). Although Env cleavage efficacy during its transport through the secretory pathway depends on both cell type and virus isolate, it is very inefficient; the majority of the Env glycoproteins remain uncleaved and retained in the endoplasmic reticulum (ER) or a *cis*-Golgi compartment (8). Accordingly, the majority of the gp160 glycoproteins remain endoglycosidase H sensitive, which is indicative of proteins that do not reach the medial Golgi (14, 27). Retained and probably misfolded Env is subsequently degraded, which was reported to occur in either lysosomes (31) or a Golgi-associated compartment (14). However, the current view of protein degradation holds that lysosomes are reached through either endosomes or late Golgi compartments.

The ER is the entry site of proteins into the secretory pathway. It is responsible for proper folding of proteins before transport to the *cis*-Golgi compartment and acts to ensure that misfolded and nonassembled proteins are eliminated (reviewed in references 10 and 15). Recently, the ubiquitin/proteasome system located in the cytoplasm was identified as the major site of degradation for ER-resident proteins as well as proteolytic substrates of the secretory pathway destined for degradation (3, 28). Proteolytic degradation of these proteins appears to be preceded by a retrograde transport to the cytosol (17, 30). Recently, it was reported that the chaperone GRB78 BiP is linked to this retrograde protein translocation for ER degradation (12, 18, 23). In HIV-infected cells, GRB78 BiP was shown to bind to Env (9) together with other ER-based chaperones including calnexin (16, 21) and calreticulin (21). As the majority of Env is retained in the ER and subsequently degraded without reaching late Golgi compartments, we asked whether the ubiquitin/proteasome system is involved in HIV-1 Env degradation and whether ubiquitinated Env can be detected in infected cells.

First, we tested whether Env glycoprotein of the MN isolate can be reprecipitated using antiubiquitin and anti-Env antibodies (Fig. 1a). Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Env glycoprotein was detected

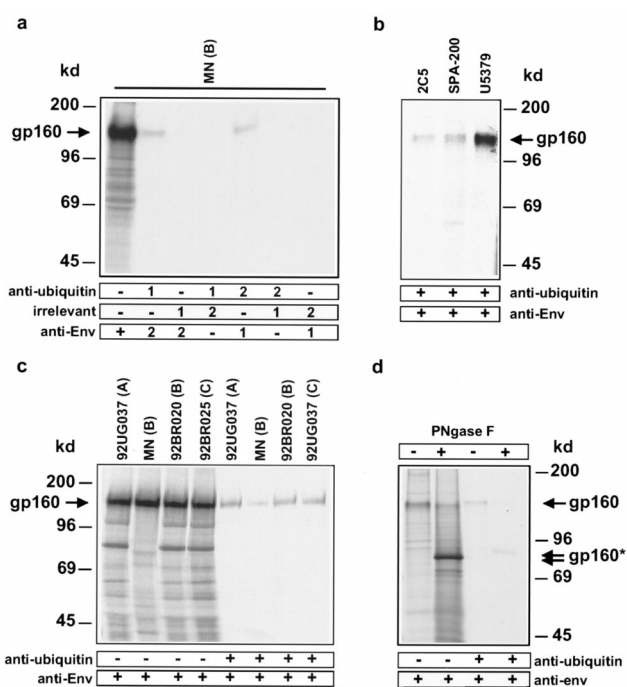


FIG. 1. Env from various HIV-1 subtypes is ubiquitinated. (a) 293T cells transiently transfected with Env MN were pulsed for 1 h with [<sup>35</sup>S]Cys-Met, lysed, and precipitated with either polyclonal antiserum U5379 against ubiquitin (Sigma, St. Louis, Mo.), a human antiserum detecting HIV-1 Env (95-2), or an irrelevant antiserum. Subsequently, lysates were reprecipitated with either of the three antibodies and analysed by SDS-PAGE (8% gel) and autoradiography. The numbers 1 and 2 refer to the sequential order of precipitation. (b) Ubiquitinated Env of cells metabolically labeled for 1 h with [<sup>35</sup>S]Cys-Met can be detected by precipitation using monoclonal antibody 2C5 (Calbiochem, La Jolla, Calif.) or polyclonal antisera SPA-200 (Stressgene, Victoria, British Columbia, Canada) and U5379 (Sigma) against ubiquitin and reprecipitation with anti-Env. (c) 293T cells were either infected with recombinant vaccinia virus (NIBSC/MRC AIDS Reagent Project) expressing Env of the 92UG037 (subtype A), 92BR020 (subtype B), or 92BR025 (subtype C) isolates or transiently transfected with a plasmid expressing Env of the MN (subtype B) isolate (1). Cell lysates of cells metabolically labeled for 1 h with [<sup>35</sup>S]Cys-Met were either precipitated with human antiserum 95-2 against HIV-1 or first precipitated with polyclonal serum U5379 against ubiquitin and reprecipitated with antiserum 95-2 (derived from an HIV-1-infected individual). Immunoprecipitates were subjected to SDS-PAGE on an 8% gel. (d) Cells transiently transfected with Env MN were labeled for 1 h with [<sup>35</sup>S]Cys-Met, lysed, and precipitated with either antiserum 95-2 or polyclonal antiserum U5379 and subsequently reprecipitated with 95-2. The immunoprecipitates were either digested with PNGase F (Roche, Mannheim, Germany) (gp160\*) overnight or left untreated (gp160) and subjected to electrophoresis.

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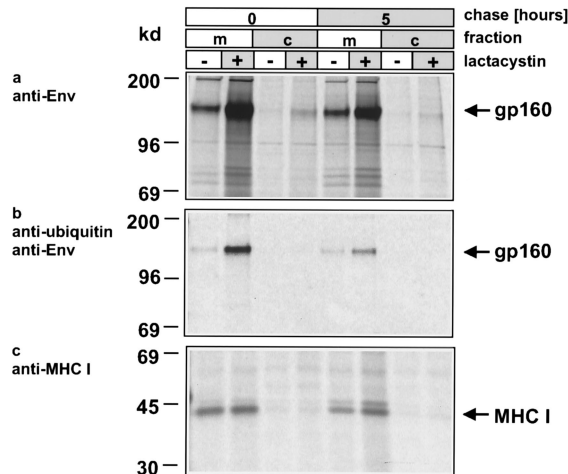


FIG. 2. Ubiquitinated Env is membrane associated, and its expression is increased by proteasome inhibitors. Transiently transfected 293T cells expressing Env MN were pulsed for 1 h with [ $^{35}$ S]Cys-Met and subsequently chased for 0 and 5 h. Cells were pretreated with 5  $\mu$ M lactacystin (+) or culture medium alone (-) for 1 h. Lactacystin was also present during the pulse and chase periods. Cells were detached with 1 mM EDTA-phosphate-buffered saline (PBS) and resuspended in hypotonic buffer (10 mM HEPES, 10 mM KCl, 10 mM MgCl<sub>2</sub> [pH 7.6]). Subsequently, cells were disrupted using a 25-gauge syringe, nuclei were removed by centrifugation at 1,000  $\times$  g for 10 min, and membrane (m) and cytosolic (c) fractions were isolated by centrifugation at 20,000  $\times$  g for 15 min. The pellet was resuspended and washed once in hypotonic buffer. The lysates were split and either precipitated with human antiserum 95-2 against HIV-1 (a), first precipitated with a polyclonal serum against ubiquitin (U5379) and reprecipitated with antiserum 95-2 (b), or precipitated with a monoclonal antibody against MHC class I (W6/32) (c). Immunoprecipitates were subjected to electrophoresis on either an 8% (gp160) or 12% (MHC class I) gel.

independently of the sequential order of precipitation if anti-ubiquitin and anti-Env antibodies were used, but not if either of the two antibodies was replaced by an irrelevant antibody. To exclude that cross-reactivity of the antiubiquitin antibody caused this result, we tested three different commercially available mono- and polyclonal antibodies (Fig. 1b). As shown with Env 92BR020, all three antibodies were able to reprecipitate Env, indicating specific recognition of the antigen. Subsequently, we tested glycoproteins of different HIV-1 isolates expressed by either recombinant vaccinia virus or transient transfection with Env expression plasmids for ubiquitination (Fig. 1c). Env glycoproteins from all HIV-1 isolates tested, including MN (subtype B) and the primary isolates 92UG037 (subtype A), 92BR020 (subtype B), and 92BR025 (subtype C), were found to be ubiquitinated. Moreover, ubiquitinated Env was precipitated from 293T kidney carcinoma cells (Fig. 1), as well as C8166 and Jurkat T lymphocytes, indicating that ubiquitination of Env is not cell type specific (data not shown). Although the molecular weight was difficult to evaluate due to high glycosylation, we could detect no major differences in size between total Env and ubiquitinated Env, suggesting that only a single ubiquitin molecule (8.4 kDa) had been added. To evaluate the number of ubiquitin residues linked to the glycoprotein, we treated the immunoprecipitates with peptide N-glycosidase (PNGase F), which completely removes carbohydrate moieties (Fig. 1d). A size difference of approximately 8 kDa corresponding to one ubiquitin molecule could be detected between deglycosylated total and ubiquitinated Env. No ubiquitinated Env could be found within total deglycosylated Env, probably reflecting the low quantity of the ubiquitinated form in relation to total Env.

Misfolded soluble and membrane proteins in the ER are

dislocated to the cytosolic proteolytic system by retrograde transport, in which at least for some proteins components of the Sec61 translocon and the proteasome appear to be involved (17, 30). We thus tested by particulate fraction experiments whether ubiquitinated Env is membrane associated or can be found in the cytosol (Fig. 2). Transfected 293T cells were metabolically labeled for 1 h and chased for 0 and 5 h. Cells were disrupted and separated into membrane and cytosolic fractions by centrifugation. After both 0 and 5 h of chase, ubiquitinated Env was found only in the membrane (pellet), not in the cytosolic fraction (supernatant). Due to the low Env cleavage efficiency in 293T cells, almost no gp120 could be detected even after 5 h of chase. To control for proper fractionation, major histocompatibility complex (MHC) class I molecules were precipitated from both fractions. As previously reported, MHC class I molecules were precipitated from the membrane but not in the cytosolic fraction under the conditions used (30). Therefore, we have no evidence for a contamination of the two fractions and conclude that the main portion of ubiquitinated Env is still membrane bound and not located within the cytosol in soluble form. Addition of ubiquitin, Env and ubiquitinated Env after 0 and 5 h of chase (Fig. 2). Thus, these data suggest that ubiquitinated Env is at least partly degraded by proteasomes and that Env is ubiquitinated prior to translocation into the cytosol.

Ubiquitin is usually attached to lysine residues in long polymeric chains (7, 11) but can also be detected in monoubiquitinated (20, 29) and in lysineless proteins (5, 6). To determine the site of ubiquitination, C-terminal Env deletion mutants were tested by reprecipitation with antiubiquitin and anti-Env antibodies (Fig. 3a and b). Ubiquitinated Env MN was detected in transfected 293T cells after a 1-h pulse if gp160, gp140 $\Delta$ cyt, or gp130 $\Delta$ tm was expressed, but not for gp120, suggesting that ubiquitin is bound to the extracellular part of gp41. Further evidence for this binding site was received by proteinase K digest of isolated microsomes from 293T cells expressing Env (Fig. 3c). Ubiquitinated Env was detected by reprecipitation in undigested as well as in proteinase K-digested microsomes, indicating that proteolytic deletion of the cytoplasmic Env domain does not disrupt ubiquitination. Since we were not able to detect the proteinase K-digested Env protein by Western blot analysis with monoclonal antibody 1577 directed against residues 735 to 752 of gp41, the cytoplasmic gp41 domain had been completely degraded (Fig. 3d). Sequence comparison between the four ubiquitinated Env glycoproteins shown in Fig. 1 revealed three conserved lysine residues as potential sites of ubiquitination. Our results indicating that ubiquitinated Env is still membrane associated and that ubiquitin is bound to the extracellular, luminal part of gp41 suggest that ubiquitination, translocation, and ER degradation of Env constitute an integrated process, as recently discussed for other luminal and integral membrane proteins (4, 24). To test whether ubiquitinated Env can also be detected in HIV-1-infected cells, we metabolically labeled C8166 cells infected with either HIV-1 MN or HIV-1 MvP 899 and reprecipitated cell lysates with antiubiquitin and anti-Env antisera (Fig. 4). In accordance with the experiments shown in Fig. 3, mapping the ubiquitination site, we detected ubiquitinated gp160 but not gp120 in cells infected with both HIV-1 isolates.

In this study, we showed that the extracellular domain of HIV-1 gp41 is ubiquitinated. Since the proteasome inhibitor lactacystin increased the amount of Env, we assume that ubiquitination occurs prior to degradation in the proteasome. This is consistent with and might explain observations by us and other groups that the majority of Env molecules are retained in

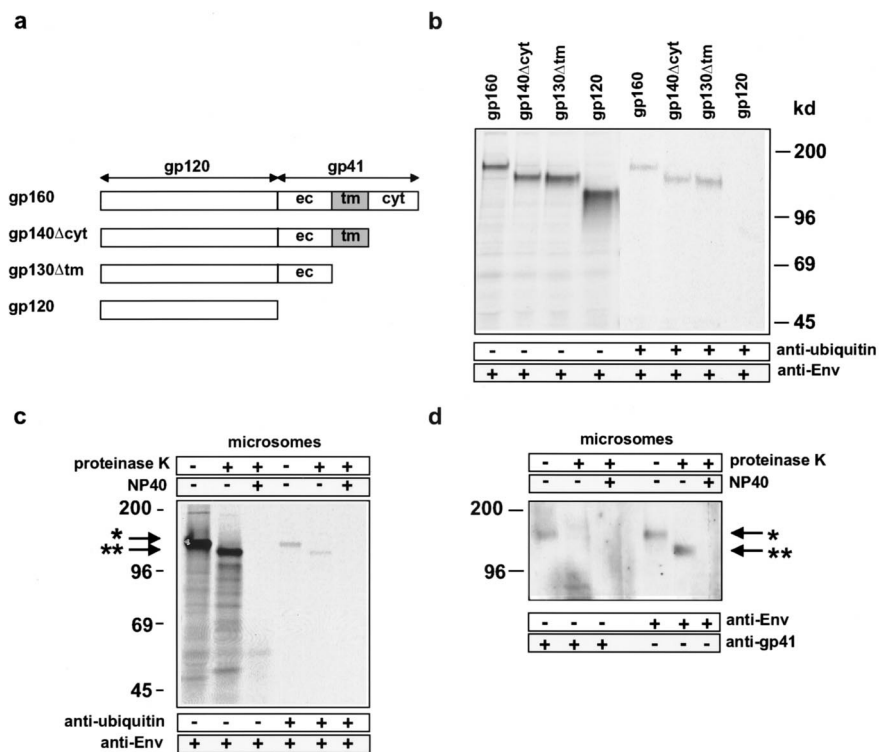


FIG. 3. The extracellular domain of gp41 is ubiquitinated. (a) Schematic diagram of the Env MN deletion mutants used. (b) Env MN deletion mutants were transiently transfected into 293T cells. Cell lysates of metabolically labeled cells were either precipitated with human antiserum 95-2 against Env or first precipitated with polyclonal serum U5379 (Sigma) against ubiquitin and reprecipitated with antiserum 95-2. Immunoprecipitates were subjected to SDS-PAGE on an 8% gel. (c) 293T cells transiently transfected with Env MN deletion mutants were metabolically labeled for 1 h. Subsequently, cells were detached with 1 mM EDTA-PBS, washed with PBS, resuspended in lysis buffer (140 mM NaCl, 5 mM MgCl<sub>2</sub>, 20 mM Tris-HCl [pH 7.6]) without NP-40, and homogenized in a cell homogenizer. Nondisrupted cells and nuclei were removed by low-speed centrifugation, and microsomes were isolated by centrifugation for 1 h at 100,000 × g. The microsome fraction was resolved and subjected to either proteinase K (0.4 mg/ml, final concentration) or proteinase K and NP-40 (1%) digestion for 30 min. Full-length Env (\*) or Env lacking the cytoplasmic domain (\*\*) was either precipitated with human antiserum 95-2 against HIV-1 or first precipitated with a monoclonal antibody against ubiquitin and reprecipitated with antiserum 95-2. Immunoprecipitates were subjected to SDS-PAGE on an 8% gel. (d) Untreated, proteinase K-treated, or proteinase K- and NP40-treated microsome fractions were analyzed by Western blotting using human antiserum 95-2 against Env or monoclonal antibody 1577 directed against the cytoplasmic domain of gp41.

the ER and stay endoglycosidase H sensitive (14, 27). Intriguingly, recent reports suggested alternative explanations for ubiquitinated viral proteins. It was shown that p6<sup>gag</sup> of HIV-1 and simian immunodeficiency virus, as well as p12<sup>gag</sup> of murine

leukemia virus and US9 of herpes simplex virus type 1 are ubiquitinated and can be found within virus particles, in addition to free ubiquitin (2, 5, 20). The significance of ubiquitinated viral proteins thus remains unclear. Ubiquitinated viral proteins might have escaped from degradation by the proteasome in cells with high levels of viral proteins. Alternatively, it has been suggested that ubiquitinated viral proteins have specific roles in viral pathogenesis using the host cell ubiquitin/proteasome machinery (5, 20).

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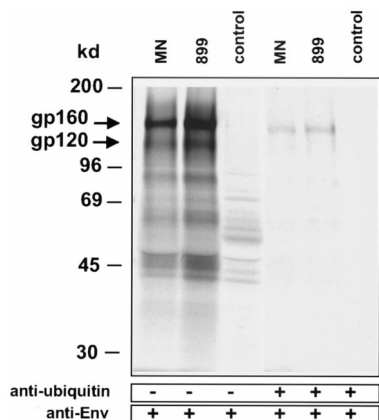


FIG. 4. gp160 is ubiquitinated in HIV-1-infected cells. C8166 cells infected with either HIV-1 MN or HIV-1 MvP 899 or mock-infected control cells were metabolically labeled with [<sup>35</sup>S]Cys-Met for 15 h and subsequently lysed without chase. Cell lysates were either precipitated with human antiserum 95-2 against Env or first precipitated with polyclonal serum U5379 (Sigma) against ubiquitin and reprecipitated with antiserum 95-2.

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