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Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160

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ABSTRACT **The synthesis and processing of the human immunodeficiency virus 1 (HIV-1) envelope precursor glycoprotein gp160 was studied in an infected CD4$^+$ lymphocytic cell line. Surprisingly, only a small percentage (5–15%) of gp160 is cleaved to produce the mature gp120 component. Intracellular sorting results in the transfer of most uncleaved gp160 to lysosomes, where it is degraded, while gp120 is transported to the cell surface and subsequently secreted. Cleavage of gp160 to generate gp120 occurs intracellularly and can be inhibited by NH_4Cl. Taken together, these results indicate that intracellular cleavage of gp160 determines the intracellular transport and survival of the envelope glycoproteins necessary to produce infectious virus.**

Human immunodeficiency virus 1 (HIV-1) is a human retrovirus and the primary etiological agent associated with the acquired immunodeficiency syndrome (AIDS). Like other retroviruses, the HIV-1 genome encodes envelope glycoproteins, which project from the membrane surface of mature particles. The external glycoproteins of several viruses are synthesized as precursor molecules, which are ultimately processed to mature proteins that mediate functions required for infection (1). These include the gp70/p15E envelope gene products of the murine retroviruses, the HA1/HA2 hemagglutinin proteins of the influenza virus, and the F1/F2 fusion proteins of Sendai virus (1, 2). The same is true for HIV-1, in which the gp160 glycoprotein precursor is cleaved to form the external gp120 and gp41 membrane-associated envelope components (3, 4). The cleavage of gp160 is essential for HIV-1 infectivity (5). Functional studies indicate that gp120 is responsible for the adsorption of virions to the CD4 receptor (6, 7), while gp41 mediates the fusion of viral and cellular membranes (8, 9). Expression of HIV-1 envelope proteins on the surface of infected cells can also lead to cell-to-cell fusion, resulting in the formation of syncytia (10).

While information continues to accumulate pertaining to the functional characterization of the envelope glycoproteins, the intracellular synthesis and processing of these proteins is not well understood. In this study we have quantitatively analyzed the fate of newly synthesized gp160 during productive HIV-1 infection of CD4$^+$ cells. Pulse–chase analyses indicate that only a small fraction of the precursor gp160 is processed into the mature gp120 component. The uncleaved gp160 is efficiently transported to and degraded within lysosomes, while a greater proportion of the gp120 remains stable and is eventually secreted from the cell.

MATERIALS AND METHODS

Cell Cultures. A3.01, a human lymphocytic leukemia cell line (11), was maintained in RPMI 1640 medium (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (RPMI/FCS). Peripheral blood lymphocytes were grown in RPMI/FCS medium containing 0.25 μg of phytohemagglutinin per ml for 3 days prior to infection. The cells were then transferred to and maintained in RPMI/FCS medium containing human interleukin 2 (Electro-Nucleonics) during infections.

Virus Stock and Infections. The HIV-1 virus used in this study was generated from an infectious molecular clone of HIV-1, pNL4-3 (12). A3.01 cells (1×10^7) were infected in 1 ml of medium at a multiplicity of 1×10^{-3} for 2 hr at 37°C. Virus/cell suspensions were then transferred to 75-cm^2 culture flasks and maintained in 25 ml of medium.

Metabolic Labeling and Pulse–Chase Protocols. Five to 7 days after infection, cells were washed and incubated in methionine-free medium containing 5% fetal calf serum for 10 min at 37°C and then pulse-labeled with [^{35}S]methionine (200 μCi/ml; 1 Ci = 37 GBq) for 30 min at 37°C. Cells were then resuspended in complete medium and chased for various time periods at 37°C. At the end of the pulse and chase periods, cell aliquots were removed and washed with ice-cold phosphate-buffered saline (pH 7.4; PBS) prior to cell lysis.

Cell Lysis and Immunoprecipitation. Cellular lysates were made and immunoprecipitations were conducted as described (13). HIV-1 antibodies reactive with the envelope glycoproteins present in the serum from an AIDS patient were adsorbed to protein A-agarose beads (Bethesda Research Laboratories) and used for all immunoprecipitations. Immunoprecipitations with the patient serum were shown to be quantitative by sequential precipitations. HIV-1 envelope glycoproteins were also immunoprecipitated from the culture medium at the various pulse–chase time intervals after the addition of the cellular lysis buffer constituents. Precipitates were resolved by reducing sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) on 10% acrylamide/AcrylAide gels, and autoradiography was performed as described (13). Radioactivity present in protein bands was quantified by scanning densitometry with a Hoefer densitometer and was computer-analyzed by using the Appligration II data analysis program (Dynamic Solutions, Pasadena, CA).

Inhibition of Lysosomal Proteolysis. HIV-1-infected A3.01 cells (5–7 days after infection) were incubated in RPMI/FCS without additions (control) or in the presence of 20–40 mM NH_4Cl for 1 hr or 20 mM methionine methyl ester for 3 hr. Control and NH_4Cl-treated cells were then pulse-labeled with [^{35}S]methionine (200 μCi/ml) for 30 min at 37°C. Another sample of control cells and the methionine methyl ester-treated cells were pulse-labeled with [^{35}S]cysteine (200 μCi/ml). Samples were chased for various periods of time in complete medium. NH_4Cl and methionine methyl ester at the concentrations mentioned above were maintained through-

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Abbreviations: HIV-1, human immunodeficiency virus 1; AIDS, acquired immunodeficiency syndrome.

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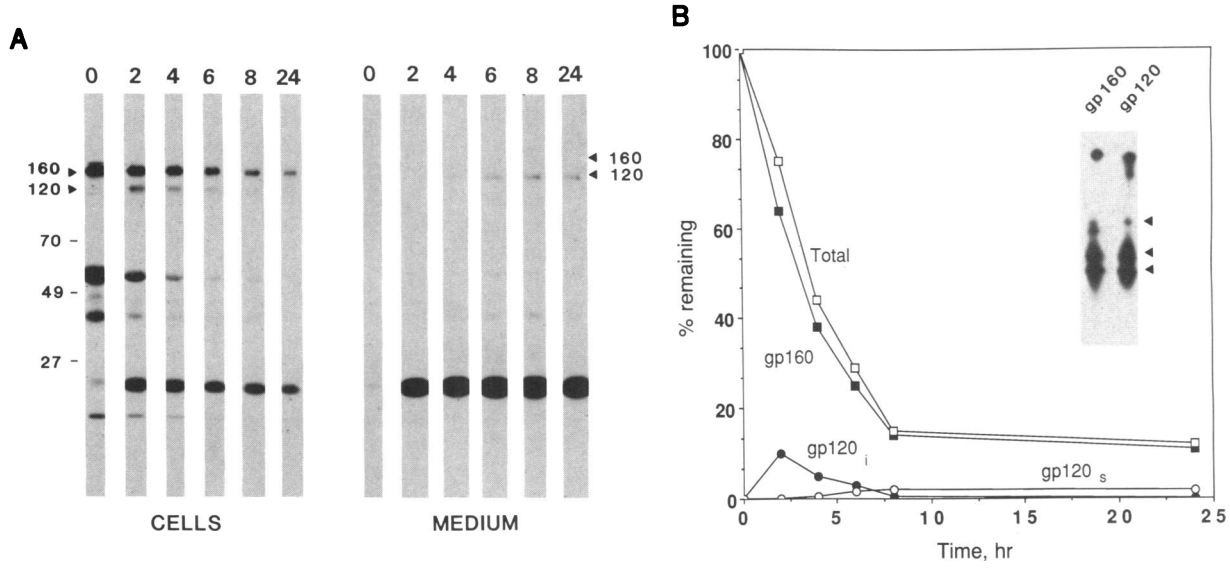

FIG. 1. Synthesis, processing, and degradation of the HIV-1 envelope glycoproteins gp160 and gp120. (*A*) Pulse–chase-labeled HIV-1 proteins recovered from infected A3.01 cells and the culture medium. Hours of chase are identified above each lane. Arrowheads identify the HIV-1 envelope precursor gp160 and the mature gp120 cleavage product. Additional numbers refer to protein molecular weight standards. (*B*) Quantitation of radioactivity present in protein bands from *A*. $gp120_i$, intracellular gp120; $gp120_s$, secreted gp120; Total, the addition of gp160 radioactivity in all its forms ($gp160_i$, $gp120_i$, and $gp120_s$ as well as envelope $gp41_i$ and $gp41_s$ calculated from the amount of gp160 cleaved). The additional prominent protein bands observed in *A* at $M_r \times 10^{-3}$ of 55, 41, 24, and 17 are all *gag* gene products. (*B inset*) Peptide maps of gel-purified bands corresponding to gp160 and gp120. Peptide mapping was performed by the method of Cleveland *et al* (14) with *Staphylococcus aureus* V8 protease at 20 μg/ml.

out the experiment. HIV-1 envelope proteins were isolated from cellular lysates by immunoprecipitation and resolved by SDS/PAGE; the amount of gp160 remaining at each time point was quantitated as described above.

Proteinase K Treatment of Infected Cells. HIV-1-infected A3.01 cells (5 days after infection) were pulse-labeled for 30 min at 37°C with [^{35}S]methionine and chased for 2 and 4 hr. At each time point, cells were washed once and resuspended in ice-cold PBS. Aliquots (1 ml) containing 5×10^6 intact cells were incubated on ice for 30 min in the absence or presence of 12.5 μg of proteinase K per ml. An additional treatment included the solubilization of cells in PBS containing 0.5% Triton X-100 and incubation in the presence of proteinase K as described above. Phenylmethylsulfonyl fluoride as added to a final concentration of 1 mM to inhibit proteinase K, and the intact cells were washed twice with ice-cold PBS. HIV-1 envelope proteins were immunoprecipitated from lysates of intact and solubilized cell treatments and analyzed by SDS/PAGE as described above.

Immunofluorescence Staining. HIV-1-infected A3.01 cells 5 days after infection were either untreated or treated with 6 μg of proteinase K in 1 ml of ice-cold PBS as described above. After the addition of phenylmethylsulfonyl fluoride, cells were washed twice with ice-cold PBS and preparations were made from cytocentrifuged cells (5×10^5 cells per slide) for fluorescence microscopy. Cytopreparations were fixed for 5 min at 4°C in either 3.7% paraformaldehyde for surface immunolabeling or with acetone for intracellular detection of HIV-1 envelope proteins. The slides were overlayed with a mouse monoclonal antibody to HIV-1 gp160/120 (clone 2E12.1; Epitope, Beaverton, OR) and rhodamine-conjugated goat anti-mouse IgG. The slides were mounted in 0.05 M Tris, pH 8.4/0.02 M dithiothreitol/50% glycerol to enhance the signal and viewed with a 100-W mercury lamp.

RESULTS AND DISCUSSION

The kinetics of HIV-1 envelope synthesis and processing are shown in Fig. 1*A*. During the pulse period, the only HIV-1

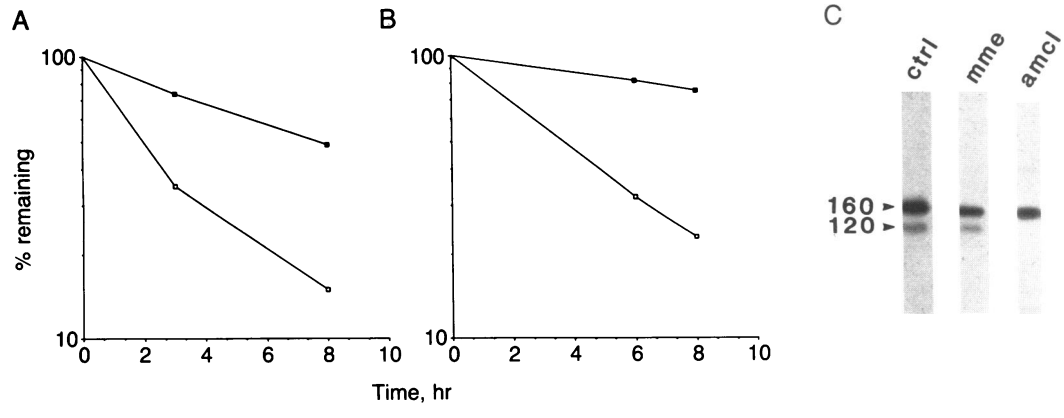

FIG. 2. Effect of lysosomotropic agents upon the degradation of gp160 and the production of gp120. The gp160 envelope glycoprotein was isolated by immunoprecipitation from pulse–chase-labeled HIV-1-infected A3.01 cells treated with 20 mM NH_4Cl and 20 mM methionine methyl ester, and the amount remaining at each time point was quantitated. (*A*) Degradation of gp160 in the absence (□) or presence (■) of NH_4Cl. (*B*) Degradation of gp160 in the absence (□) or presence (■) of methionine methyl ester. (*C*) Production of gp120 at the 2-hr chase point in the absence (ctrl) or the presence of 20 mM methionine methyl ester (mme) or 40 mM NH_4Cl (amcl).

envelope glycoprotein that incorporated radioactivity was gp160 (Fig. 1*A*). This pulse-labeled gp160 could be digested to the unglycosylated core polypeptide of 88 kDa (3) with endoglycosaminidase H (data not shown), consistent with localization of the newly synthesized gp160 to a premedial Golgi compartment (15). The gp120 envelope glycoprotein product was first observed after 2 hr of chase (Fig. 1*A*). Peptide mapping of the gp120 shown in Fig. 1 indicated that it was derived from gp160 (Fig. 1*B Inset*). Densitometer tracings of gp160 present during the chase periods showed that only 10–20% of the initial labeled gp160 remained after 8 hr (Fig. 1*B*). Possible explanations for this loss include: (*i*) processing to gp120 and gp41; (*ii*) secretion of gp160 or its processed forms or both; and (*iii*) degradation. Quantitation of the gp120 produced during the same chase-time periods revealed that relatively little gp160 is cleaved to gp120 (Fig. 1*B*). Secretion of the gp160 and gp120 glycoproteins was also monitored by quantitative immunoprecipitation from the medium (Fig. 1*B*). When one adds the amount of gp120 secreted over 24 hr with the amount remaining in the cells at the 24-hr chase time and corrects for the number of methionine residues in gp160 and gp120, the results of several experiments indicate that <15% of the gp160 is converted to gp120. In these studies, we were unable to quantitate the gp41 envelope cleavage product because of its comigration with a 41-kDa *gag*-encoded protein intermediate (16). A significant fraction of the cell-associated gp120 was slowly secreted into the medium (30–50% over 24 hr), while very little gp160 was released from the cells (<10% of "secreted" gp120). The envelope products found in the medium are referred to as secreted because we did not ascertain if they were particle-associated. The data from these pulse–chase experiments indicate that the vast majority of newly synthesized gp160 was neither cleaved nor secreted and most likely was degraded within the cell. In contrast, 30–50% of the gp120 produced was still detectable as a secreted or intracellular protein at the 24-hr time point. Although little cell lysis was observed over the course of these experiments, we cannot rule out that some of the glycoproteins observed in the medium, especially at late time points, was due to cell lysis.

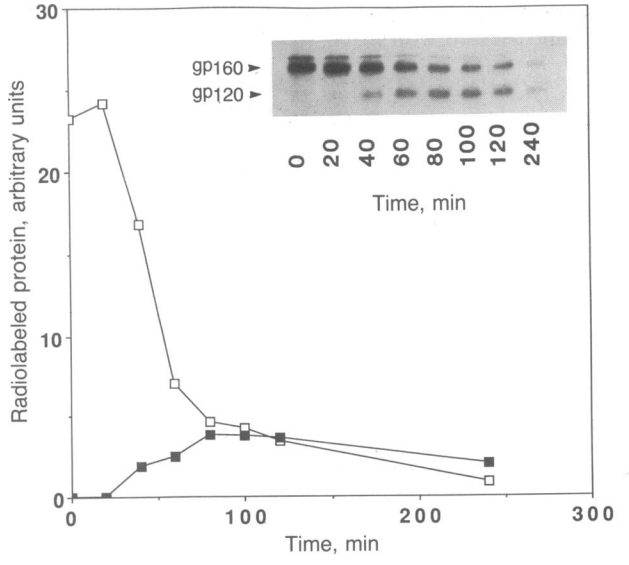

FIG. 3. Pulse–chase analysis of gp160 and gp120 envelope glycoprotein production during HIV-1 infection of peripheral blood lymphocytes. The plot shows the amount of labeled gp160 (□) and gp120 (■) present at the indicated time points during the pulse–chase. (*Inset*) gp160 and gp120 protein bands corresponding to the pulse–chase time points depicted in the plot.

To determine if the gp160 loss could be due to lysosomal degradation, similar pulse–chase experiments were conducted in the presence of NH_4Cl or methionine methyl ester, both of which are known to inhibit lysosomal proteolysis (17, 18). Both agents significantly but incompletely inhibited the degradation of gp160 (Fig. 2 *A* and *B*), suggesting that the major cause of gp160 loss was due to lysosomal degradation. The majority of gp160 acquires partial resistance to endoglycosaminidase H, demonstrating that much of it leaves the endoplasmic reticulum and transits through the Golgi before

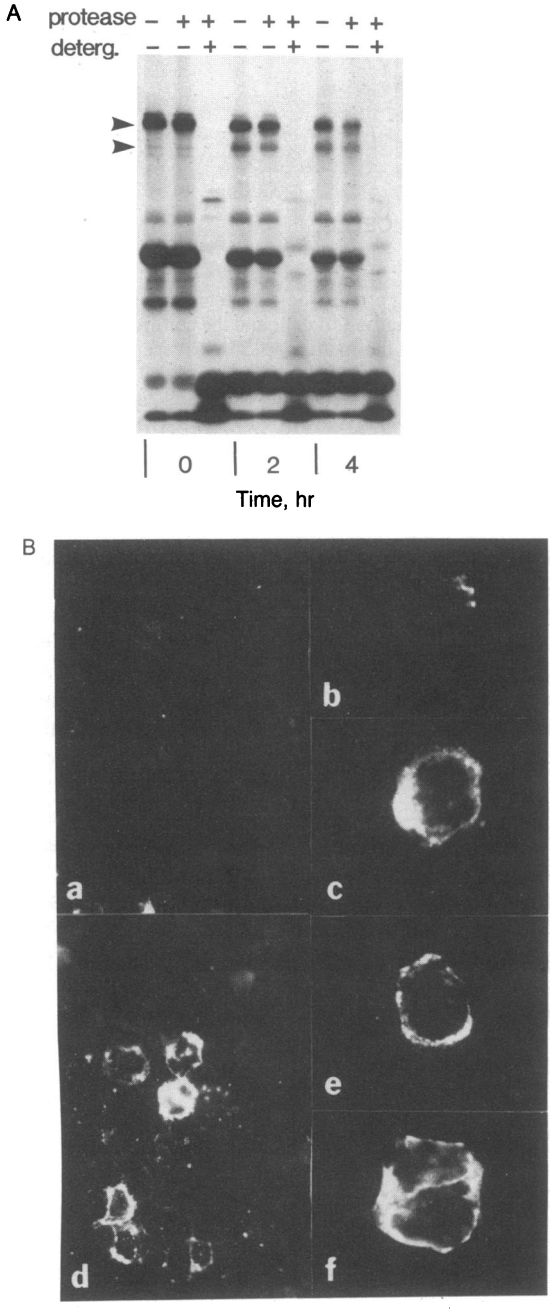

FIG. 4. Cleavage of gp160 to gp120 occurs intracellularly. (*A*) HIV-1-specific proteins immunoprecipitated from intact or solubilized pulse–chase-labeled infected A3.01 cells incubated in the absence (−) or the presence (+) of proteinase K and Triton X-100 (deterg.). Arrowheads indicate the positions of the gp160 and gp120 envelope glycoproteins. (*B*) Immunofluorescence staining of HIV-1-infected A3.01 cells with gp160/120 envelope-specific antibodies after treatment of intact cells with (*a*, *b*, and *c*) and without (*d*, *e*, and *f*) proteinase K. (*a*, *b*, *d*, and *e*) Presence or absence of surface-associated gp160/120. (*c* and *f*) Presence of intracellular gp160/120. (*a* and *d*, ×270; *b*, *c*, *e*, and *f*, ×690.)

being degraded (data not shown). However, the persistence of some fully endoglycosaminidase H-sensitive gp160 during the entire chase time period indicates that a fraction of gp160 does not leave the endoplasmic reticulum. Therefore, the NH_4Cl- and methionine methyl ester-insensitive degradations could be due to mechanisms not affected by these agents such as degradation from the endoplasmic reticulum (19).

We next asked whether the pattern of envelope processing observed in the A3.01 T-cell leukemia line is representative of other infected $CD4^+$ cells. Pulse–chase experiments of HIV-1-infected, phytohemagglutinin-stimulated peripheral blood lymphocytes revealed a similar pattern to that observed in the continuous T-cell line (Fig. 3). However, the degradation of gp160 was even more rapid than that observed in the A3.01 cells ($t_{1/2}$ after a 20-min lag, 20–40 min versus 2–3 hr). As in the cell line, conversion to gp120 in peripheral blood lymphocytes continued to be low (10–20% of gp160).

During the course of experiments conducted in the presence of NH_4Cl, we noticed a profound effect on gp120 production. Concentrations of 40 mM NH_4Cl completely inhibited gp120 formation (see Fig. 2*C*); virtually no gp120 was detected in the medium even 24 hr after the pulse (data not shown). The absence of proteolytic processing in the presence of NH_4Cl suggested that cleavage of gp160 takes place in an acidic compartment. Partial inhibition was also observed with the weak base chloroquine (data not shown), which also supports the conclusion that an acidic compartment is required for gp160 cleavage. Alternatively, NH_4Cl could interfere with the transport of gp160 to an intracellular site at which cleavage takes place. In contrast to NH_4Cl, methionine methyl ester had no effect on gp120 production (Fig. 2*C*). Thus, the failure of methionine methyl ester to affect the cleavage of gp160 despite its ability to inhibit lysosomal degradation suggests that the proteolytic processing of gp160 occurs at a nonlysosomal site.

To verify that the gp120 detected during the chase period was produced intracellularly, intact or solubilized infected cells were incubated with proteinase K at different points of the pulse–chase. Cell surface molecules should be digested by such treatment. After inhibition of the enzyme with phenylmethylsulfonyl fluoride, the proportion of gp160 and gp120 digested was assessed. Although virtually all of the gp160 and gp120 was digested in the solubilized cell preparations, <10–20% of the gp120 associated with intact cells was accessible to proteinase K (Fig. 4*A*). The small reduction in the amount of gp160 and gp120 observed after protease treatment of intact cells at the 4-hr time point could be due to partial cellular permeabilization by the enzyme and/or the presence of gp160 and gp120 on the surface of the infected cells. To confirm that the proteinase K was indeed digesting HIV-1 envelope proteins located on the cell surface, immunofluorescence analyses using gp160/120-specific antibodies were conducted. Surface-associated envelope proteins were efficiently digested, while the intracellular envelope proteins were unaffected (Fig. 4*B*). Taken together, these results support the conclusion that cleavage of gp160 to generate gp120 occurs within the virus-producing cell and that, at any given time, the majority of cell-associated gp160 and gp120 is present intracellularly.

Our results address several features of HIV-1 envelope glycoprotein synthesis and processing. Only a small minority of the precursor gp160 glycoprotein is cleaved into gp120. This cleavage occurs intracellularly and is dependent on the action of an NH_4Cl-sensitive, presumably acid-dependent, protease. At present, we have no explanation for the inefficient cleavage of the HIV-1 gp160 envelope protein. More complete proteolytic processing with little degradative loss of envelope glycoproteins has been reported for a number of RNA viruses (20). However, comparable studies carried out with the retrovirus rous sarcoma virus indicate that the precursor envelope glycoprotein Pr92 is both rapidly degraded and inefficiently cleaved to the mature products gp85 and gp37 (21–25). Thus, this may be a general feature of retroviral glycoprotein maturation.

While the vast majority of gp160 is transported to and degraded in lysosomes, a greater proportion of the gp120 generated is stable and is secreted into the medium, presumably as a virion component. Thus, intracellular cleavage of gp160 is not only required for functional activation of the envelope glycoproteins (5) but is essential for the transport and survival of gp120. The degree of intracellular sorting of

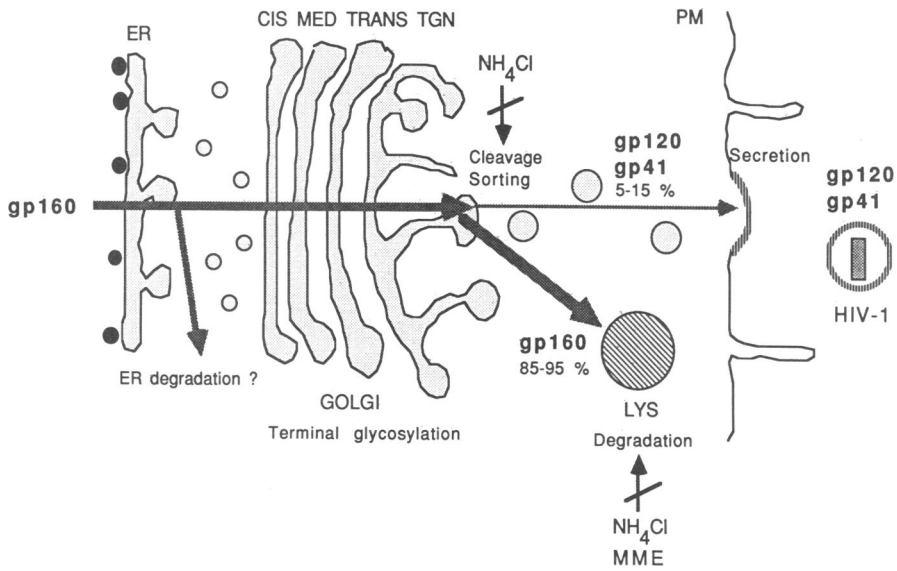

FIG. 5. A model for the sorting and degradation of newly synthesized HIV-1 envelope glycoproteins. HIV-1 envelope glycoproteins are synthesized as a precursor (gp160) in the endoplasmic reticulum (ER). The newly synthesized gp160 is transported through the Golgi system, where the carbohydrate chains are terminally modified. gp160 is then cleaved by a relatively inefficient process to the mature envelope glycoproteins gp120 and gp41. Cleavage occurs in an intracellular nonlysosomal acidic compartment and can be inhibited by weak bases. Some of the gp120 and gp41 are transported to the cell surface and secreted as viral particles, while the uncleaved gp160 is delivered to lysosomes for degradation. MED, medial Golgi cisternae; TGN, trans-Golgi network; PM, plasma membrane; Lys, lysosome; MME, methionine methyl ester. Crossed arrows indicate inhibition by pharmacological agents.

HIV-1 envelope proteins in infected cells is striking. This is summarized in Fig. 5. Why most gp160 is routed to lysosomes, while gp120 is transported to the plasma membrane and secreted, is unknown. We cannot rule out the possibility that gp160 goes to lysosomes via endocytosis from the plasma membrane. Perhaps the cellular mechanism(s) that directs the gp160 to lysosomes involves the intraction of gp160 with CD4 (26) or some other cellular component. Whatever the mechanism, the massive delivery of gp160 late in infection might alter lysosomal function or stability or both and thereby contribute to the cytopathic effects of the virus.

An unexpected result from our studies was the marked sensitivity of gp160 cleavage to NH_4Cl. A recent report has shown that NH_4Cl treatment of HIV-1-producing T cell and monocyte cell lines results in a slight reduction of particle-associated reverse transcriptase activity and a major loss (>95%) in titer of infectious virions (27). Coupled with our findings, these results suggest that, in the presence of NH_4Cl, noninfectious nucleocapsids that lack gp120 are released into the medium and point to the intracellular cleavage of gp160 as a requirement for infectivity. The NH_4Cl effect also suggests a vulnerable site in the life cycle of HIV-1 that may be amenable to antiviral therapy.

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