Uncleaved *env* gp160 of human immunodeficiency virus type 1 is degraded within the Golgi apparatus but not lysosomes in COS-1 cells

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Abstract The fate of newly synthesized human immunodeficiency virus type 1 *env* gp160 was examined in COS-1 cells. The results of morphological chase experiments involving cycloheximide demonstrated that gp160 was retained in the Golgi apparatus for longer than the half-life of the molecule. The degradation of gp160 was insensitive to both bafilomycin A_1 and leupeptin (< 0.2 mM), which block lysosomal proteolysis. However, degradation was effectively suppressed by leupeptin at higher concentrations, maximally at 1.7 mM. Furthermore, undegraded gp160 was accumulated in the Golgi apparatus, but was not detected in lysosomes. These results indicate that in COS-1 cells gp160 is not degraded in lysosomes, but rather that degradation takes place in the Golgi apparatus.

Key words: HIV-1; *env* gp160; Degradation; Golgi apparatus

1. Introduction

During the transport of human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins through the secretory pathway, the precursor polyprotein, *env* gp160, is cleaved into noncovalently associated external gp120 and membrane-spanning gp41 subunits. The cleavage is an essential step in the maturation of the glycoprotein into a functional complex [1,2], and takes place in the Golgi apparatus [3,4]. Although the cleavage efficiency depends on the host cells or strain of virus used [5], the processing of gp160 into the gp120-gp41 complex normally occurs in a very inefficient manner in both peripheral blood lymphocytes (PBLs) and T cell lines [5,6]. It has been reported that the excess, uncleaved gp160 is transported from the Golgi apparatus to lysosomes, where it is degraded [6].

Previously, however, we [3] and others [4,7] reported that most newly synthesized gp160 remained sensitive to endoglycosidase H (endo-H) digestion throughout the chase period. It follows that the great majority of gp160 retains the *N*-linked high mannose oligosaccharide structure, and that the glycoproteins have not yet been modified by *N*-acetylglucosaminyltransferase I and Golgi α-mannosidase II in the medial Golgi apparatus, i.e. they have not acquired hybrid or complex sugar side chains [8–10]. In contrast, gp120 is partially endo-H-resistant [3,4,7], suggesting that processing of gp160 to the gp120-gp41 complex takes place prior to the creation of hybrid or complex oligosaccharide units on gp120, and that gp120-gp41 but not gp160 is further translocated through the medial and *trans*-Golgi compartments to the plasma membrane [3]. gp160 was, however, found to be associated with the Golgi apparatus, which can be stained with wheat germ agglutinin (WGA) or antibodies to the c-*myc*-tagged human KDEL receptor [3].

To clarify the incompatibility between the oligosaccharide structures and subcellular localization of gp160, and the reported site of degradation [6], we examined the fate of excess, newly synthesized gp160 in COS-1 cells transiently expressing the HIV-1 *env*, *vpu* and *rev* genes [3]. Our results point to the presence of a new, distinct pathway for the degradation of *env* gp160. A morphological chase experiment showed that gp160 remained associated with the Golgi region throughout its lifetime (i.e. it is degraded there). The degradation of gp160 was insensitive to drugs that block lysosomal proteolysis. In this paper we describe these results and discuss possible roles of the disposal of gp160 in the life cycle of HIV-1.

2. Materials and methods

2.1. Plasmids

pTK19 [3], a HIV-1 *rev*, *vpu* and *env* expression plasmid, was used to study the intracellular degradation of *env* gp160. pHE22M, which expresses the *c-myc*-tagged human homologue of the yeast HDEL receptor (*ERD2*), was described previously [3]. The β-galactosidase expression vector, pCH110, was purchased from Pharmacia.

2.2. Cells and antibodies

COS-1 cells (ATCC CRL-1650) were maintained in Dulbecco's modified Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (D10). Pooled sera from asymptomatic HIV-1-positive donors, which reacted strongly with *env* gp160 and the gp120/gp41 complex, rabbit anti-c-Myc tag antiserum, and a mouse monoclonal antibody specific for *env* gp160 (ADP330) were as described [3]. Rabbit anti-gp120-gp160 antiserum was a gift from Rod Daniels (National Institute for Medical Research, London). A mouse monoclonal antibody (1B5) reacting with proteins found in prelysosomes-late endosomes was kindly provided by M. Marsh (MRC Laboratory for Molecular Cell Biology, University College, London). Cycloheximide (CHX) was purchased from Sigma, and HPLC-purified leupeptin and bafilomycin A_1 (more than 95% pure) were acquired from Sigma and Wako Pure Chemicals (Japan), respectively.

2.3. DNA transfection

3×10^5 COS-1 cells per T25 flask were transfected by the calcium phosphate method [11], using 5 μg of expression plasmid(s) and up to 8.4 μg of pUC12 as a carrier DNA. 1.6 μg of β-galactosidase expres-

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Abbreviations: HIV-1, human immunodeficiency virus type 1; PBLs, peripheral blood lymphocytes; endo-H, endoglycosidase H; ER, endoplasmic reticulum; D10, Dulbecco's modified Eagle's MEM supplemented with 10% heat-inactivated fetal calf serum; CHX, cycloheximide; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; hEGF, human epidermal growth factor; MEM/BSA, Eagle's MEM containing 0.1% BSA; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; WGA, wheat germ agglutinin; V-ATPase, vacuolar type H^+-ATPase; BMA, bafilomycin A_1; Leu, leupeptin

This study is dedicated to the memory of Akio Ohyama.

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sion vector (pCH110) was also included as an internal control to examine the transfection efficiencies of pTK19 and pHE22M in COS-1 cells. The efficiencies ranged from 10 to 20% in the series of experiments, but those in individual culture flasks within an experiment were comparable, as determined by a colorimetric assay (data not shown [12]).

2.4. Protein labelling of transfected cells

40–48 h after transfection, the cells were washed and incubated in methionine-free medium containing 10% FCS for 1 h at 37°C, and then pulse-labelled with [^{35}S]methionine (100 μCi/ml; 1000–1200 Ci/mmol) for 30 min at 37°C. The medium was then replaced with the complete medium, and the cells were chased for various times up to 21 h. At the end of the pulse and chase periods, aliquots of cells were removed and washed with ice-cold PBS prior to cell lysis. Acid precipitation of proteins was performed to monitor the total incorporation of [^{35}S]methionine. It was found that no significant additional [^{35}S]methionine was incorporated during the chase period.

2.5. Cell lysis and immunoprecipitation

Cellular lysates were made and immunoprecipitation was conducted as described [3]. Both polyclonal and monoclonal antibodies reactive with the envelope glycoproteins were titrated to immunoprecipitate all the glycoproteins present in the cellular lysates (1:100 final dilution of human serum; 10 μg/ml of ADP330). The precipitates were resolved for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography as described previously [3]. To calculate relative protein concentrations, the fluorographs were scanned and analyzed with an imaging densitometer (Model GS-670; BioRad). The optical density per mm^2 determined for each band was normalized to the specific density determined at time 0.

To monitor the amount of gp160 lost intracellularly by n h of chase ($gp160_{nloss}$), the densitometric data were fitted with the following equation: $gp160_{nloss} = gp160_{syn} - gp160_n - (gp120_n + gp120_{nloss}) - (gp41_n + gp41_{nloss}) - sgp120_n$. $gp160_{syn}$ is the amount of gp160 synthesized during the pulse period and was determined as $gp160_{syn} = gp160_{1\,h} + gp120_{1\,h} + gp41_{1\,h}$. gp_n indicates the amount of each envelope glycoprotein present intracellularly at n h of chase. gp120 and $gp41_{nloss}$ are the amounts of gp120 and gp41 lost intracellularly by n h of chase, and were determined according to the following equations: $gp120_{nloss} = gp120_{4\,h} - gp120_n - sgp120_n$, and $gp41_{nloss} = gp41_{4\,h} - gp41_n$.

2.6. Inhibition of lysosomal proteolysis with bafilomycin A_1

The inhibition of lysosomal proteolysis was monitored by measuring the percent reduction of the intracellular degradation of ^{125}I-labelled human epidermal growth factor (^{125}I-hEGF, more than 750 Ci/mmol; Amersham) in the presence of bafilomycin A_1 (Sigma). pTK19-transfected cells were first starved of EGF by culturing in Eagle's MEM containing 0.1% BSA (MEM/BSA). The cells were then incubated in the same medium containing 0.5 μCi of ^{125}I-hEGF at 4°C for 2 h. After washing with ice-cold MEM/BSA, the cells were incubated in either the presence (7.8–125 nM) or absence of bafilomycin A_1 at 37°C for 4 h. The culture medium was then collected, and the cells were washed and treated with 1 mg per ml of pronase (Calbiochem) at 4°C for 40 min to remove uninternalized ligand. The cells were then either counted directly to measure cell-associated total radioactivity or solubilized with a 0.5% NP-40 and 1% Triton X-100 mixture. Cellular lysates as well as the culture medium were treated with trichloroacetic acid to determine acid-precipitable and acid-soluble radioactivity, as described by Gollins and Porterfield [13]. Lysosomal degradation of endocytosed ^{125}I-hEGF [14] was calculated as follows: (trichloroacetic acid-soluble radioactivity in medium and lysate)/(medium, uninternalized ligand and cell-associated total radioactivity) × 100. The inhibition of lysosmal degradation by bafilomycin A_1 at each concentration tested was then determined by calculating the percent reduction of the lysosomal degradation.

2.7. Inhibition of gp160 degradation

pTK19-transfected cells were starved, pulse-labelled and then chased (up to 4 h) as described above, either without an addition (control) or in the presence of leupeptin (0.4–2.1 mM). Alternatively, bafilomycin A_1 (15.6 nM) was only included in the complete medium during the chase period. *env* gp160 was then isolated from the cellular lysates by immunoprecipitation and resolved by SDS-PAGE. The amount of gp160 remaining at each time point was determined as described above.

2.8. Immunofluorescence microscopy

Cells grown on Lab-Tek tissue culture chamber slides were fixed, and then incubated with primary and secondary antibodies as described [3]. The primary antibodies used were as above. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit IgG, rabbit anti-human IgG (both from Dakopatts), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) were used, at the concentrations recommended by the manufacturers, as secondary antibodies. TRITC-conjugated wheat germ agglutinin (WGA, 10 μg/ml; E-Y Laboratories) was used to label the Golgi region [15]. For dual labelling, sequential antibody incubations were performed. For the morphological chase experiment [3], transfected cells were treated at 37°C with 500 μM CHX for 8 h prior to processing for the immunofluorescence assay. Images were recorded using an Olympus LSM-GB200 confocal microscope with an objective of 60× at zoom 1.5.

3. Results

3.1. Uncleaved gp160 is degraded intracellularly

To examine HIV-1 envelope glycoprotein biosynthesis and cleavage, an *env* expression system (pTK19 [3]) was used to transfect COS-1 cells. The kinetics of glycoprotein synthesis were then monitored by metabolic labelling with [^{35}S]methionine, followed by immunoprecipitation with pooled sera from asymptomatic HIV-1-positive donors. As shown in Fig. 1A, after reaching a maximal level during the first hour or two (see also Fig. 4B), gp160 decayed exponentially with a half-life of approx. 7 h. Cell-associated gp120 and gp41 were considerably more stable than gp160, exhibiting half-lives of approx. 16 h (Fig. 1B). Relatively little of the gp120 produced was secreted (Fig. 1A,B).

Densitometry tracing of envelope glycoproteins present during the chase period showed that while 169.2 ($gp160_{1\,h} + gp120_{1\,h} + gp41_{1\,h}$) arbitrary units of gp160 was initially synthesized during the pulse period, only 10% remained after 21 h (Fig. 1B,C). Possible explanations for this loss include: (i) processing to the gp120-gp41 complex, followed by sgp120 release into the medium; or (ii) intracellular proteolysis of the uncleaved form. However, when one adds the amount of gp120 secreted over 21 h to the amounts of gp160, gp120 and gp41 remaining in the cells after 21 h of chase, the results of a representative experiment indicated that 76% (129.5 units) of the initial radioactivity incorporated into gp160 was lost during the chase. Since less than 40% (65.6 units after 4 h of chase) of the initial labelled gp160 was processed into the gp120-gp41 complex (Fig. 1B), the most likely explanation for this loss is that uncleaved gp160 and/or some of the complex formed were degraded within the cells. Panel C shows the kinetics of gp160 loss during the chase period and that the majority of envelope glycoproteins degraded during the chase (70% at 21 h) is derived through the turnover of uncleaved gp160.

3.2. gp160 is found exclusively in the Golgi region

We subsequently aimed to establish the site where gp160 is degraded. A morphological chase experiment involving CHX was then conducted to monitor the membrane trafficking of gp160 (Fig. 2). As previously described [3], pTK19-transfected control COS-1 cells showed that in the steady state all the gp160 resided in the WGA-stained perinuclear Golgi region

and the associated vesicles (Fig. 2A). Although WGA binds to the sialic acid and outer chain *N*-acetylglucosamine residues of complex-type oligosaccharides in the Golgi complex, it also allows detection of lysosomal proteins carrying similar sugar moieties being transported from the Golgi cisternae [9,15]. After 8 h of chase in the presence of CHX, some of the Golgi proteins labelled with WGA appeared to be transported to cytoplasmic non-Golgi vesicles, as shown by the increased cytoplasmic punctate staining as well as the concomitant reduction in the intensity of staining of the perinuclear Golgi region (Fig. 2B, right). In contrast, identical cells demonstrated that gp160 selectively accumulates in the Golgi region during the same chase period (Fig. 2B, left), implying that the envelope precursor remains associated with the Golgi appara-

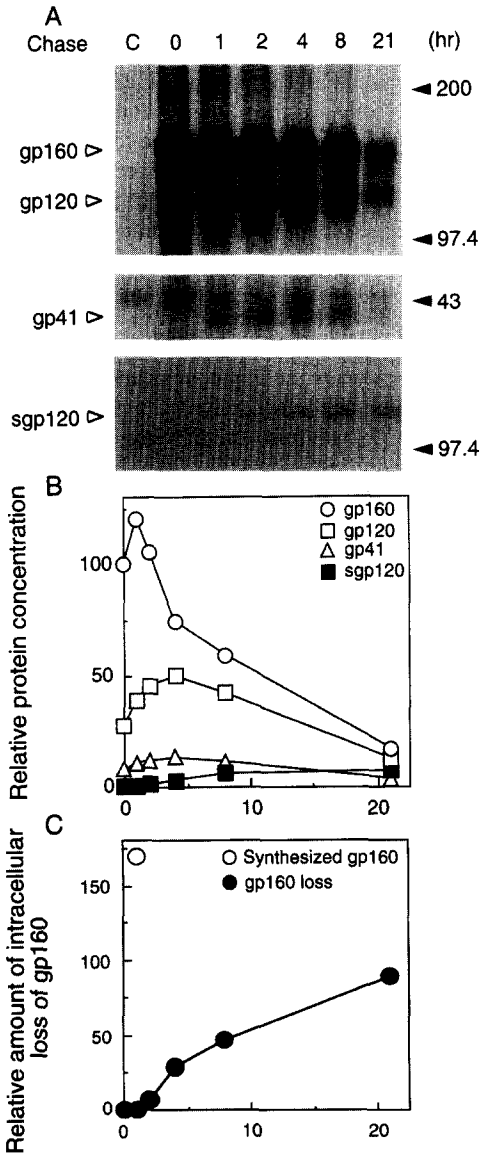

Fig. 1. Biosynthesis, processing and turnover of HIV-1 envelope glycoproteins. (A) Pulse-labelled HIV-1 envelope glycoproteins were chased for various periods up to 21 h. The proteins were recovered from transfected COS-1 cells and the culture medium. Open arrowheads indicate the envelope precursor, gp160, and the mature gp120/gp41 cleavage products, as well as secreted (s) gp120. The mobilities of the molecular mass standards are indicated on the right. (B) The relative concentration of each envelope glycoprotein was determined as described in Section 2, using the samples shown in A. The optical density of each protein band was normalized to gp160 present at time 0 (=end of 30 min pulse). (C) The relative amount of intracellular gp160 loss was determined as described in Section 2, by calculation from the data shown in B. gp160syn = 169.2.

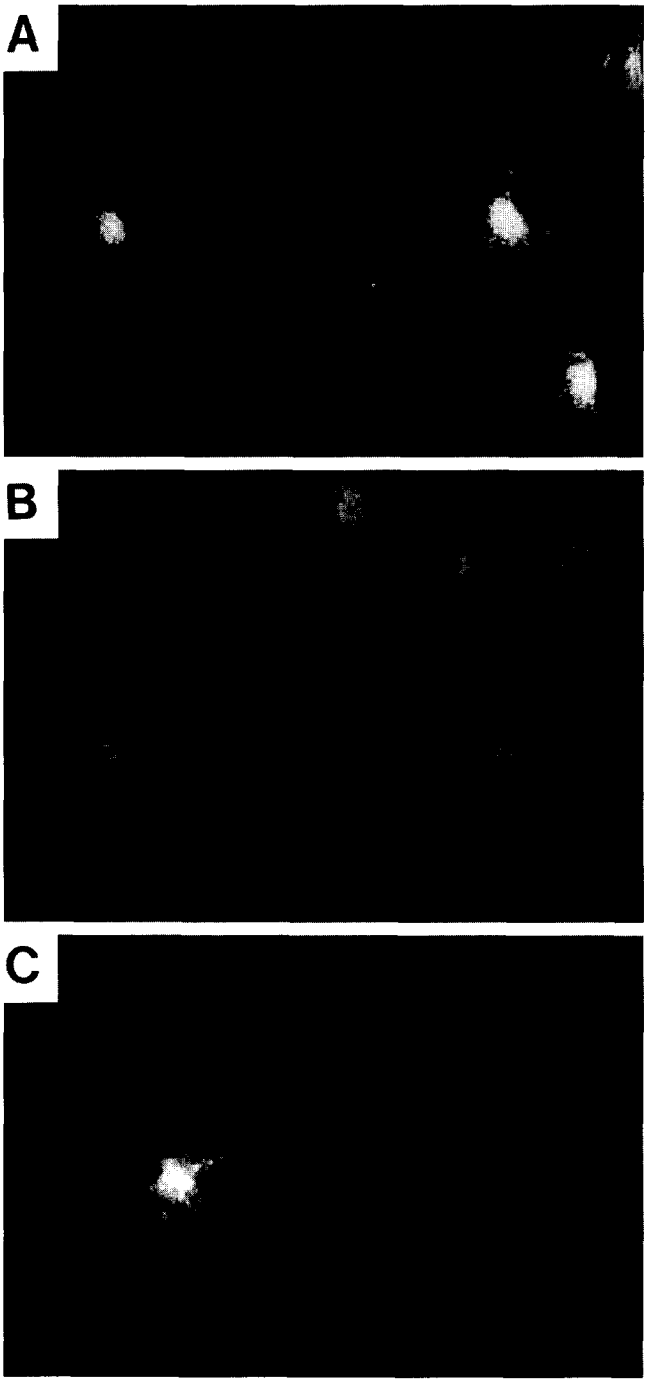

Fig. 2. Subcellular localization of HIV-1 envelope glycoproteins. (A) COS-1 cells transfected with pTK19 were stained with the ADP330 monoclonal antibody and FITC-conjugated goat anti-mouse IgG (left), and TRITC-conjugated WGA (right). (B) COS-1 cells from the same culture as for (A) were incubated with CHX as described in Section 2. After 8 h of chase, *env* gp160 (left) as well as the perinuclear Golgi region (right) were stained as above. (C) Cells from the same culture as for (A) were stained with rabbit anti-gp120-gp160 and TRITC-conjugated swine anti-rabbit IgG (left), and the 1B5 anti-lysosomal antibody and FITC-conjugated goat anti-mouse IgG (right).

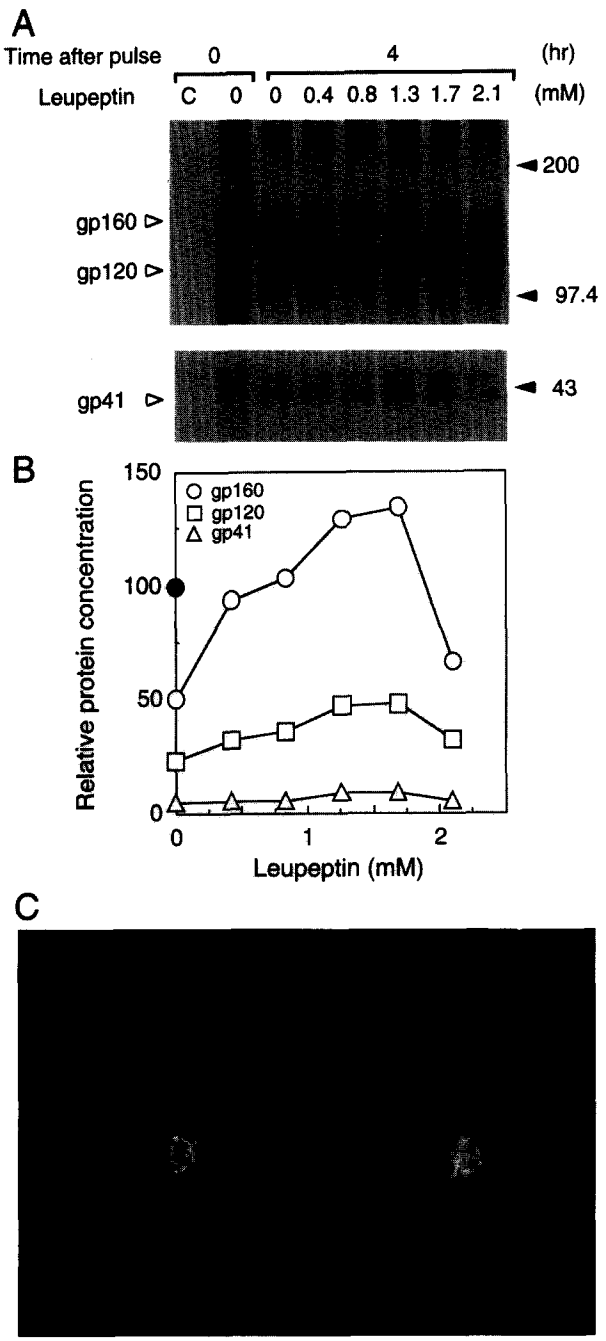

Fig. 3. Inhibition of *env* gp160 degradation by leupeptin. (A) In the presence of various concentrations of leupeptin, pTK19-transfected cells were pulse-labelled and chased as described in Section 2. After 4 h of chase, the envelope glycoproteins were recovered from the cells and resolved. Open arrows indicate the proteins. Molecular mass standards are shown on the right. C: mock-transfected control. (B) The relative concentration of each envelope glycoprotein was determined as described in the legend to Fig. 1, using the samples shown in (A). The closed circle indicates gp160 present at the end of the pulse period. (C) COS-1 cells, cotransfected with pTK19 and pHE22M, were treated with 1.7 mM leupeptin at 37°C for 5.5 h. The cells were then stained with the ADP330 monoclonal antibody and FITC-conjugated goat anti-mouse IgG (left), and rabbit anti-c-Myc tag antiserum and TRITC-conjugated swine anti-rabbit IgG (right).

tus for longer than the half-life of the molecule (see above). To further confirm the subcellular distribution of gp160, cells from the same culture were doubly stained with rabbit anti-envelope polyclonal serum, and a monoclonal antibody specific for prelysosomes and late endosomes (1B5). The immunofluorescence experiment revealed that the HIV envelope proteins that accumulated in the Golgi region are distinct from those in prelysosomes and late endosomes (Fig. 2C). It follows that at the steady state of expression, gp160 appears not to be translocated from the Golgi apparatus into these prelysosomal vesicular compartments.

3.3. *Leupeptin blocks gp160 degradation in a concentration-dependent manner*

The above biochemical and immunocytochemical results, when taken together, indicate that HIV-1 *env* gp160 is degraded in nonlysosomal compartments within the secretory pathway, most likely in the Golgi complex. To determine whether the gp160 turnover could be due to proteolysis within the Golgi cisternae, pTK19-transfected cells were incubated with a cysteine/serine proteinase inhibitor, leupeptin, for 60 min before the pulse period, and the inhibitor was included in the pulse and chase media. Although leupeptin showed no effect on gp160 degradation at concentrations up to 0.2 mM (data not shown), which were reported to be enough to block lysosomal degradation [16], the inhibitor effectively suppressed the degradation at higher concentrations (Fig. 3A). At 1.7 mM, the compound exerted the maximal inhibitory effect on gp160 degradation, there being a 2.6-fold increase in the protein concentration (Fig. 3B). Under these conditions, the subcellular localization of gp160 clearly matched that of a *cis*-Golgi resident protein, the KDEL receptor [17], indicating that undegraded gp160 was last detected in the Golgi apparatus, not in the lysosomal compartments of the cells (Fig. 3C).

3.4. *Interference with lysosomal activity does not affect gp160 degradation*

To further confirm that the degradation of gp160 takes place in the Golgi complex but not in lysosomes, we examined the effect on gp160 degradation of bafilomycin A_1, an inhibitor of vacuolar type H^+-ATPase (V-ATPase [14]). This compound is known to inhibit lysosomal proteolysis by directly blocking the acidification of lysosomes (i.e. by neutralizing low pH-dependent lysosomal enzymes; [14]) or, at higher concentrations in the exocytic route (> 100 nM in BHK-21 cells), by affecting vesicular transport into lysosomal compartments [18]. After incubation at 4°C with ^{125}I-EGF, pTK19-transfected COS-1 cells degraded 85% of the cell surface-bound EGF within 4 h after warming up to 37°C (data not shown). Degradation of EGF was nearly completely blocked by bafilomycin A_1, more than 90% inhibition being observed at 15.6 nM (Fig. 4A). In a similar experiment shown in panel B, however, this inhibitor of lysosomal proteolysis failed to prevent gp160 degradation (Fig. 4B; compare columns BMA/4 h and 4 h), while 1.7 mM leupeptin again successfully protected gp160 from nonlysosomal, Golgi degradation (Fig. 4B, column Leu/4 h; see also Fig. 3).

4. Discussion

Despite the processing of HIV-1 *env* gp160 into the gp120-gp41 complex, followed by transport to the plasma membrane, being an essential step for the production of an infectious progeny [1,2], the cleavage takes place in a very ineffi-

cient manner in both HIV-1-infected PBLs (10–20% [6]) and heterologous expression systems (13–24% [5,6]; < 40% in this report). HIV-1, therefore, should make host cells provide a system for preventing both the overaccumulation and surface expression of gp160, since uncleaved gp160 reaching the plasma membrane would almost certainly become incorporated into budding virions and, hence, the production of defective particles would occur [19,20].

In this study involving COS-1 cells we found a unique, nonlysosomal pathway for the selective catabolism of excess, uncleaved gp160. All the data obtained in the present series of biochemical and immunocytochemical experiments point to the failure of gp160 degraded in this pathway to reach the lysosomal compartments. These data include the inability to detect the movement of the protein out of the Golgi apparatus (Fig. 2), the Golgi association of undegraded gp160 (Fig. 3), and the failure of an inhibitor of lysosomal proteolysis, bafilomycin A_1, to block gp160 degradation (Fig. 4).

It was reported recently that bafilomycin A_1 neutralizes not only lysosomal [14] but also Golgi compartments [18,21] by binding to the hydrophobic sector [22] of the respective resident V-ATPase. It follows that, in the exocytic route, the site at which bafilomycin A_1 inhibits protein degradation is determined in a concentration-dependent manner; firstly and primarily at the lysosomes by affecting the activity of resident proteases [14], and secondly, at higher concentrations, in the Golgi apparatus by blocking protein transport, including that of viral glycoproteins [18], to the *trans*-Golgi network, and then to lysosomes [18,21]. In either case, however, the degradation of gp160 observed in the presence of bafilomycin A1 (Fig. 4) suggests that the protein turnover occurred within the Golgi apparatus, where the viral protein had been retained.

The lack of sensitivity of this degradation pathway to the V-ATPase inhibitor further suggests that nonlysosomal proteases are involved. Since they were effectively inhibited by the cysteine/serine proteinase inhibitor, leupeptin, at concentrations higher than those required for suppressing lysosomal endopeptidases (Figs. 3 and 4 [16,23]), these enzymes would probably include Ca^{2+}-dependent proteinase II and a number of serine proteinases, which are nonlysosomal targets of the compound [16].

In COS-1 cells, uncleaved *env* gp160 is retained and degraded within the Golgi cisternae (Figs. 2–4). In addition, preliminary experiments involving HeLa cells showed gp160 being degraded in the Golgi apparatus, where the viral protein had accumulated (see [3]). These results are, however, in contrast to what has been observed in other systems [6]. Willey et al. [6] reported that in PBLs and a T cell line the majority of uncleaved gp160 was transported to lysosomes, where it was degraded. They reached this conclusion by using NH_4Cl and methionine methyl ester as lysosomotropic compounds to block the degradation of gp160. Although the employment of different degradation pathways could simply depend on the host cells used [24], it should be noted that NH_4Cl- and methionine methyl ester-insensitive, nonlysosomal degradation of gp160 was also observed in their systems [6]. While more detailed experiments to analyze the Golgi degradation of gp160 are required, using a variety of cell lines, these results indicate that the nonlysosomal degradation pathway reported above is not unique to COS-1 cells, i.e. it may be found in other cell lines.

Furthermore, the Golgi degradation, although relevant to the HIV-1 life cycle, might have other, intrinsic roles in the maintenance of cellular homeostasis. For example, protein degradation in the endoplasmic reticulum (ER), which is thought to remove defective proteins from the secretory pathway [25], has also been suggested to explain the turnover of an ER resident protein [26,27]. Such an example may account for its role in Golgi degradation with selective retention of resident enzymes [28] within the compartments.

Thus, it seems reasonable that the nonlysosomal, Golgi degradation of gp160 described above could both prevent further translocation of uncleaved gp160 through the secretory pathway and ensure that only the mature gp120-gp41 complex reaches the cell surface for the subsequent production of an infectious progeny. Inhibition of the enzymes responsible for gp160 cleavage would, therefore, result in the proteolysis of uncleaved gp160 and, hence, provide a basis for the design of antiviral strategies aimed at repression of virus production.

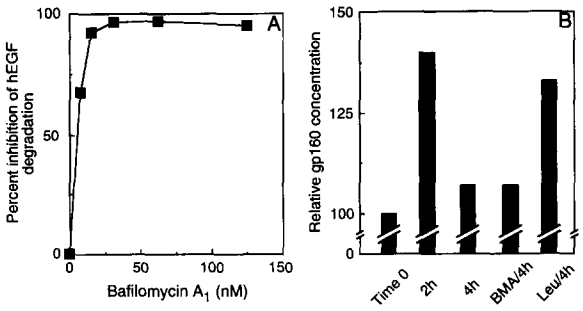

Fig. 4. Effect of bafilomycin A_1 on intracellular degradation of *env* gp160. (A) Inhibition of the degradation of endocytosed hEGF by bafilomycin A_1. ^{125}I-hEGF-adsorbed, pTK19-transfected COS-1 cells were incubated with various concentrations of bafilomycin A_1 at 37°C for 4 h. The percent degradation of internalized ^{125}I-hEGF was determined as described in Section 2. (B) Pulse-labelled, pTK19-transfected COS-1 cells were chased for the various periods indicated on the horizontal axis, either without an addition or in the presence of bafilomycin A_1 (BMA; 15.6 nM) or leupeptin (Leu; 1.7 mM). *env* gp160 was immunoprecipitated with ADP330 and the relative concentrations were determined with the samples shown on the horizontal axis, as described to the legend to Fig. 1.

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