Purification and Characterization of a Ca^{2+}-Independent Endoprotease Activity from Peripheral Blood Lymphocytes: Involvement in HIV-1 gp160 Maturation†

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ABSTRACT: We have analyzed the calcium requirement for HIV-1 gp160 processing in cultured nonlymphoid (CV-1 and HeLa-CD4) and human-lymphoid [Jurkat, Molt-4 and peripheral blood lymphocytes (PBMCs)] cells. The processing of gp160 in these cells, infected with recombinant vaccinia virus encoding the gp160 gene, was only partially affected by intracellular calcium depletion induced by the calcium ionophore A23187 and calcium chelator EGTA. These observations prompted us to purify the Ca^{2+}-independent gp160 processing enzyme from natural targets of HIV-1 PBMCs. The endoprotease was purified to homogeneity by the use of four chromatography fractionation steps and the constant detection of the Ca^{2+}-independent activity at each one of them. The enzyme was believed to be a membrane-associated heteromeric 120-kDa protein composed of three subunits of 66, 32, and 24 kDa. It was found to be specifically inhibited by substrate analogues, decanoyl-RVKR-chloromethyl ketone, and serine protease inhibitors including diisopropyl fluorophosphate (DFP) and TLCK. In contrast, no effect was observed with reducing agents including 2-β-mercaptoethanol, *N*-ethylmaleimide, L-cysteine, and dithiothreitol. There were significant similarities between inhibition profiles of the purified enzyme in vitro and those of the endogenous endoprotease(s) in cell culture experiments. Therefore, the selectivity of purified endoprotease for the gp160 cleavage site, its requirement for additional residues around this consensus sequence, and its isolation from natural targets of HIV-1, made it a good candidate in the gp160 maturation process. We provide more direct and supporting evidence that HIV-1 gp160 maturation may involve at least two families of divergent endoproteases according to calcium dependence.

The envelope glycoproteins of HIV viruses are synthesized as precursors that are cleaved at the trans-Golgi level by cellular endoprotease(s), yielding mature external and transmembrane glycoproteins (*1*, *2*). Both subunits are present on the surface of viral particles and infected cells as oligomers linked by noncovalent bonds (*3*). External glycoproteins including gp120 for HIV-1 and gp125 for HIV-2 are responsible for the tropism of the virus for CD4 and for CCR5 and CXCR4 co-receptors present on the surface of host cells (*4*, *5*). This interaction is followed by a conformational change of viral envelope glycoproteins that results in the exposure of the hydrophobic N-terminal domain of the transmembrane glycoprotein (gp41 for HIV-1 and gp36 for HIV-2) that mediates fusion between the virus envelope and the host cell membrane (*6*, *7*). The cleavage of these envelope glycoprotein precursors by the cellular endoprotease(s) is a required step for the acquisition of fusogenic potential and thus infectious capacity by the newly produced viral particles (*8*). This has been shown for HIV-1 and HIV-2 viruses as well as for *Sendai* virus (*9*), *Sindbis* virus (*10*), *Influenza* viruses (*11*, *12*), *Newcastle* virus (*13*), *Rous sarcoma* virus (*14*), *Measles* virus (*15*), and Ebola virus (*16*).

Several endoproteases have been implicated in the maturation of the HIV-1 gp160 precursor including furin, PC1/3, PC2, PACE4, isoforms of PC5/6 and PC7, members of the family of *Subtilisin*-like protoxin prohormone convertases (SPC) (*17*). The activity of these SPCs is calcium-dependent and results in the specific cleavage of precursors at the C-terminal extremity of the consensus sequence Lys/Arg−X−Lys/Arg−Arg (*18*) within the constitutive secretory pathway where SPCs such as furin, PC5/6-B, and PC7 have been localized (*19*, *20*). The PC1/3, PC2 and in some cases PC5/6-A enzymes which exert their maximal activities in acidic compartments such as immature secretory granules cleave gp160 within the regulated pathway (*19*, *21*). Specific cleavage of the HIV-1 gp160 precursor into gp120 and gp41 has been demonstrated by assays in vitro with furin, PC1/3, PACE4, and PC5/6 and in overexpression experiments with furin, PC1/3, PACE4, PC5/6, and PC7 (*22*−*24*). Nevertheless, only furin and PC7 have been reported to fulfill the requirements of endoproteases responsible for activation of HIV glycoprotein precursors in vivo. These two SPCs were in fact the only *Subtilisin-like* enzymes reported to be expressed in HIV lymphoid host cells (*22*, *25*). These findings, however, cannot rule out the possibility that protease(s) divergent from the SPC family can play a significant role in gp160 processing. This hypothesis is supported by work of Kido et al. (*26*) who purified a calcium-independent endoprotease named viral envelope glycoprotein maturase (VEM) from Molt-4 lymphoid cell line and that can correctly mature HIV-1 gp160 in vitro. It nevertheless

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remains unclear whether other Ca^{2+}-independent endoprotease(s), either closely related or different from VEM are active in the natural targets of HIV viruses.

The hypothesis according to which protease(s) other than SPCs can contribute to the maturation of HIV viral glycoprotein precursors is further supported by several observations: (i) furin-deficient cells including LoVo human colon carcinoma and CHO-RPE.40 Chinese hamster ovary cell lines can specifically process HIV-1 gp160 ex vivo (*27*, *28*), while they fail to process *Pseudomonas* exotoxin A as well as viral glycoproteins of *Sindbis* virus, *Newcastle* virus, and the insulin- and lipoprotein proreceptors that are well documented to be cleaved by furin (*29*−*31*). (ii) HIV-1 and HIV-2 viruses can still replicate at a lower level despite the stable expression of α-1-antitrypsin Portland variant (α1-PDX), which has been reported to be a specific inhibitor of SPC (*20*, *24*, *32*−*34*). A significant inhibition of HIV-1 and HIV-2 *env* precursors processing and syncytium induction has been observed in the presence of stably expressed α1-PDX protein (*35*, *36*). In these studies, the effect of α1-PDX was investigated using a recently developped CD4 positive Jurkat lymphoid cell line stably overexpressing the inhibitor, in which it had been previously shown that α1-PDX effectively blocks intracellular Notch-1 receptor processing by furin-like enzymes (*37*). (iii) Although overexpressed furin-like SPC can process gp160, this ability does not seem physiologically relevant because gp160 is highly resistant to cleavage by purified furin and PC7. The gp160 precursor is about 1000-fold less sensitive in vitro to furin than anthrax toxin A, a substrate known to be cleaved by this SPC (*28*), and is insensitive to PC7 processing in vitro (*22*).

The present work was undertaken to clarify whether protease(s) different from SPC can be candidate(s) for HIV-1 gp160 processing in target lymphoid cells. One aim of this work was to directly characterize an enzymatic activity from peripheral blood lymphocytes (PBMCs) and divergent from SPC. We report the purification and characterization of a Ca^{2+}-independent enzyme that can convert gp160 to gp120 and gp41, thus supporting the concept that at least two different protease families can participate in HIV-1 gp160 maturation.

MATERIALS AND METHODS

Materials. Prepacked columns of Superdex 75 HR 10/30, Superdex 200 HR 10/30, metal chelating Superose FF gel, and protein A-Sepharose were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Molecular weight markers for SDS−PAGE were from Bio-Rad (Richmond, Calif.). Molecular weight markers for gel filtration, neomycin (G418), leupeptin, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin, pepstatin, aprotinin, EDTA, EGTA, dithiothreitol, various Boc-X_n-MCA fluorogenic substrates, Triton X-100, β-D-octylglucoside, CHAPS (3-(-cholamidopropyl)-dimethylammonio) 1-propano-sulfonate), *Galanthus nivalis* lectin, brefeldin A, TLCK, diisopropyl fluorophosphate, E64c, benzamidin, bestatin, A23187, tunicamycin, and bicinchroninic acid assay kit were purchased from Sigma (St. Louis, MO). DecRVKRcmk peptide was obtained from Bachem (Torrance, CA). Penicillin and streptomycin were purchased from Gibco/BRL (Grand Island, NY). RPMI 1640 and DMEM media, L-glutamine, and fetal calf serum were from Eurobio. Peroxidase-coupled anti-Immunoglobulin G gamma chain was from Dako.

Cell Culture and Viruses. Jurkat and Molt-4 lymphoid cell lines (American type culture collection, Rockville, MD) were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1% penicillin−streptomycin antibiotic stock solutions. The HeLa-CD4 cell line that constitutively expresses the CD4 receptor (from Dr. P. Charneau, Pasteur institute, Paris, France), and monkey kidney CV-1 cell line were maintained in Dubelco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine, and 1% penicillin−streptomycin antibiotic stock solutions, and also with 0.1 mg/mL of G418 for HeLa-CD4 cells. Peripheral blood mononuclear cells (PBMCs) from healthy donors were prepared by Ficoll-Hypaque gradient separation and cultured for 2 days in complete RPMI medium. All cells were taken during the exponential growth phase for the experiments.

The recombinant vaccinia virus carrying the complete HIV-1 LAV_{Lai} *env* gene (VV-TG-9.1-1139) (VV-gp160) was a gift from Transgène (Strasbourg, France). The virus was titered by phase assay on monolayers of CV-1 cells.

Viral Infection and Syncytia Formation Assay. Confluent monolayer of CV-1 or HeLa-CD4 cells in tissue culture plates (30 mm) were infected with VV-gp160 at a multiplicity of infection (MOI) of 5 plaque forming units (PFU)/cell for 1 h. The inoculum was removed, 2 mL of growth medium were added, and cells were further incubated for 24 h at 37 °C. 10^7 Jurkat, Molt-4 or PBMCs cells were infected with VV-gp160 at 20 PFU/cell for 1 h and then harvested for removing unbound virus particles. The cells were then incubated at 37 °C for 24 h in 3 mL of growth medium. The number of multinucleated CD4 positive cells in each well was determined by microscopic examination and photomicrographs were taken for presentation.

Cell Lysis and Immunoblotting. After infection and incubation of infected cells at 37 °C, the media were collected and cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8), 1% Triton X-100, 1% β-D-octylglucoside, 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM aprotinin] for 15 min at 4 °C and, then centrifuged at 16000*g* for 15 min at 4 °C in a microcentrifuge. Supernatants were transferred to clean tubes and precleared with 25 μL of 50% protein A-Sepharose beads by shaking for 2 h at 4 °C, and centrifuging at 3000*g* for 5 min. Appropriate amounts of rabbit polyclonal anti-gp160 serum were added to the protein A-precleared media and incubated overnight at 4 °C. Protein A-Sepharose beads were then added, and the samples were incubated for 3 h at 4 °C. Cell lysates and immunoprecipitated glycoproteins were analyzed by 8 and 7% SDS−polyacrylamide gel electrophoresis (PAGE), respectively, and electrotransferred to nitrocellulose membranes. The membranes were incubated with serum from an HIV-1 seropositive patient to detect gp160-related glycoproteins, and antibody binding was determined using peroxidase-coupled rabbit anti-human antibodies. Bands of gp160 and gp120 were scanned with a densitometer and percent cleavage was calculated with the formula: 100 × (intensity of gp120 produced)/[(intensity of uncleaved gp160) + (intensity of gp120 produced)].

Purification of env Glycoproteins. HIV-1 gp160 was purified from Jurkat cells infected with VV-gp160 in the

presence of 5×10^{-7} M of the calcium-specific ionophore A23187, as described by Gilljam (*38*), on a Snowdrop (*Galanthus nivalis*) bulbs lectin (GNA-lectin). This GNA-lectin has been shown to purify HIV-1 glycoproteins in a one-step procedure to a high level of purity (*38*). Adsorbed HIV-1 glycoproteins were eluted from the GNA-lectin with 1 M methyl-α-D-mannopyranose.

Peptide Substrates. The synthetic peptides used that do or do not mimic *env* sequences around the processing cleavage sites of envelope glycoprotein precursors of HIV-1, HIV-2, and SIV viruses are listed in Table 4. They were synthesized with a solid-phase procedure according to Merrifield (*39*). Stepwise elongation was carried out with an automated peptide synthesizer (Applied Biosystems Inc., model 430A) utilizing NMP-HOBt (*N*-methylpyrrolidone-2 hydroxybenzotriazole) as adapted by Applied Biosystems. After anhydrous hydrogen fluoride cleavage, the crude peptides were purified by C18 reversed-phase medium-pressure liquid chromatography and characterized by analytical RP-HPLC, amino acid analysis and mass spectrometry.

Preparation of Membrane Protein Fractions. All purification procedures were performed at 0−4 °C. 10^9 PBMCs grown for 2 days were washed twice with phosphate-buffered saline and lysed by hypotonic shock using the modified method of Wira and Munck (*40*). Nuclei and undisrupted cells were pelleted by centrifugation at 270*g* for 10 min. The pellet was washed once with 1.5 mM $MgCl_2$, and the supernatant was pooled with the first. The pooled supernatants were centrifuged at 100000*g* for 3 h and the microsomal fraction pellet was solubilized for 2 h with 25 mM Tris-HCl (pH 8) buffer containing 0.5% CHAPS and 0.5% β-D-octylglucoside detergents. The homogenate was then centrifuged at 100000*g* for 3 h, and the supernatant was transferred to clean tubes for subsequent analysis.

Endoproteolytic Cleavage Assays in Vitro. Enzyme preparations were tested for amidolytic activity as follows. (i) Fifty microliters of samples were mixed with 10 μL (20 mM) of individual 7-amino-4-methylcoumarin (MCA) substrates in 440 μL of 50 mM Tris-HCl (pH 7) in the presence or absence of 5 mM $CaCl_2$. The reaction was started by adding the enzyme preparation. It was incubated at 37 °C for 12 h and stopped by adding 200 μL of glacial acetic acid. The amount of MCA released was determined fluorometrically with excitation and emission wavelengths of 380 and 460 nm. One unit of enzyme activity was defined as the amount degrading 1 mmol of substrate/min. (ii) 10 μL (20 nmol) of synthetic peptides that mimic the processing cleavage sites of envelope glycoprotein precursors were subjected to proteolysis in 100 μL of reaction mixture containing 20 μL of enzyme preparations in 50 mM Tris-HCl (pH 7) in the presence or absence of 5 mM $CaCl_2$ at 37 °C for 12 h. Following the incubation period, the digests were acidified with 5 μL of glacial acetic acid and analyzed by RP-HPLC using a 5 mm RP C18 endcapped column (5 × 250 mm). Elution was carried out with linear gradient of 0−45% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow rate of 1 mL/min. Products from emerging peaks were identified by determining the amino acid composition. (iii) 500 ng of gp160 from VV-gp160 infected Jurkat cells purified with GNA-lectin were incubated with 20 μL of enzyme preparations at 37 °C for 12 h in 50 mM Tris-HCl (pH 7) in the presence or absence of 5 mM $CaCl_2$. The processing of gp160 was analyzed by Western blotting as described above.

Protein Measurement and Cytotoxicity Assay. Protein concentrations were determined with the bicinchoninic acid protein assay kit using bovine serum albumin as a standard. Cell viability was assessed with the trypan blue dye exclusion test by incubating uninfected cells in the indicated experimental conditions. Survival was calculated as the percentage of unstained cells. Percent cytotoxicity is the difference between controls (100%) and the percent survival.

RESULTS

Role of Calcium in Syncytium Induction. The involvement of Ca^{2+}-independent enzyme(s) in HIV *env* processing was first examined by determining the effect of Ca^{2+} depletion on gp160 processing in different cell lines, including nonlymphoid HeLa-CD4 and lymphoid Jurkat and Molt-4 cell lines, and PBMCs. For this purpose, cells were infected with VV-gp160 and incubated in conditions that should have depleted them of Ca^{2+} ions. After adsorption of recombinant vaccinia virus, cells were washed and incubated in Ca^{2+}- and FCS-free medium with or without increasing concentrations of A23187, a Ca^{2+}-specific ionophore, or EGTA, a Ca^{2+}-chelator. Cells were then microscopically examined for syncytium induction and immunologically examined by Western blotting for gp160 processing.

All cells incubated in Ca^{2+}- and FCS-free medium alone continued to form syncytia, whose size and number were comparable to controls (2% FCS medium) (Figure 1, fields B and C). Using A23187 and EGTA, we found that syncytium induction could occur in stringent conditions of Ca^{2+} depletion (10^{-7} M A23187 and 10 mM EGTA in Figure 1, fields E and G, respectively). This clearly indicates adequate gp160 processing and thus expression of the gp120/gp41 functional subunits on cell surfaces. Nevertheless, a strong reduction in syncytium induction could be seen at 10^{-6} M A23187 (Figure 1, fields F) and at 20 mM EGTA (Figure 1, fields H and I). This was probably due to the direct cytotoxic effect of the inhibitors as revealed by the trypan blue dye exclusion assay (Table 1, percent cytotoxicity). These results led to the conclusion that decreases in intracellular Ca^{2+} levels did not impair gp160 expression, its transport along the secretory pathway, or its enzymatic cleavage into functional gp120 and gp41.

Since syncytium induction occurred despite Ca^{2+} in both nonlymphoid cells including Hela-CD4 and lymphoid cells including Jurkat, Molt4 cells, and PBMCs, it can be argued that Ca^{2+}-independent gp160 pocessing activity is ubiquitous as is the case of the SPC family.

Role of Ca^{2+} in gp160 Processing. Cleavage of gp160 in conditions of Ca^{2+}depletion was analyzed in cell lysates and supernatants of VV-gp160 infected cells by Western blotting. Anti-gp160 polyclonal antibodies were used to detect gp160 cleavage products. The results (Figure 2) clearly show that despite the presence of 10^{-7} M A23187 or 10 mM EGTA, gp160 continued to be cleaved to gp120, gp41, and additional products in all the cell lines tested (Figure 2, lanes 5 and 6, respectively) and in PBMCs (data not shown). The quantification of the percent of cleaved gp160 according to the formula 100 × intensity of gp120 produced/[(intensity of gp120 produced + intensity of uncleaved gp160 precursor)]

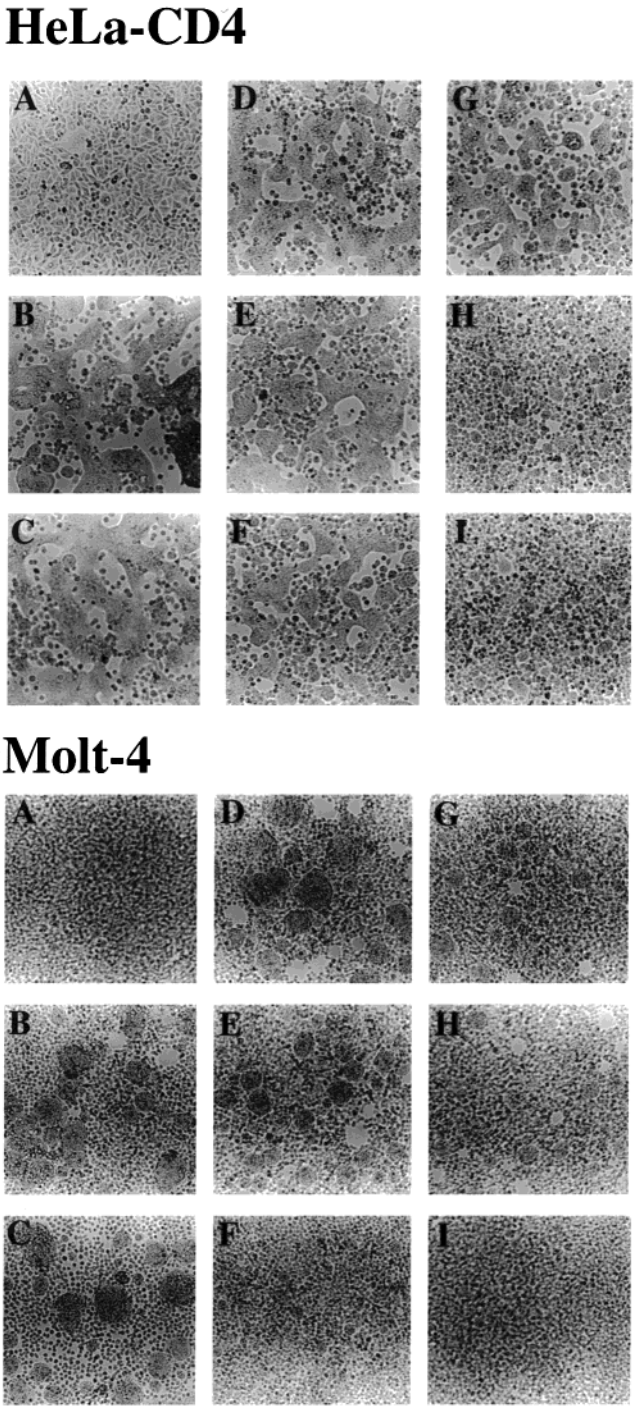

FIGURE 1: Effect of calcium depletion of VV-gp160 infected cells on syncytium induction. HeLa-CD4 and Molt-4 cells were depleted of calcium by cultivating in Ca^{2+}-free medium in the presence of A23187 or EGTA for 3 h at the indicated concentrations and were then infected with VV-gp160 and incubated at 37 °C for 24 h in the Ca^{2+}-free medium plus A23187 or EGTA. (A) Uninfected cells in 0% FCS; (B and C) infected cells in 2 and 0% FCS; (D, E, and F) infected cells in 0% FCS and 10^{-8}, 10^{-7}, and 10^{-6} M A23187; (G, H, and I) infected cells in 0% FCS and 10, 20, and 50 mM EGTA.

clearly shows a decrease of this ratio in the presence of A23187 and EGTA (Table 1). This could be due to inhibition of the entire pool of Ca^{2+}-dependent proteases that are responsible for gp160 degradation or incorrect maturation. This interpretation is in agreement with both the detection of additional products in CV-1 cells in the presence of Ca^{2+} ions (Figure 2, CV-1 cells, top panel, lanes 1 and 2) and previous reports showing that a high proportion of gp160-related molecules (nearly 50%) are degraded in the endoplasmic reticulum by metalloproteases and proteasome (*41*, *42*). In contrast, the transmembrane hydrophobic gp41 seems to be more stable than gp120, since it contains far fewer cleavage sites. In our experiments, gp41 was found to be highly stable in vitro and persisted for longer incubation times in vitro than gp120 (data not shwon). This can explain why gp41 accumulates in parallel to gp160 in conditions of Ca^{2+} depletion (Figure 2, CV-1 and Hela-CD4, top panels). On the other hand, it is noteworthy that gp120 production was more important in the presence of FCS (Figure 2, down panels, lanes 1 vs 2). This observation suggests that an extracellular endoprotease(s) present in the serum, such as the recently proposed plasmin (*43*), can cleave the gp160 molecules that have reached cell surface in uncleaved form.

Table 1: The Percent Cytotoxicity of A23187 and EGTA on Uninfected Cells and Percent Processing of gpl6O in VV-gpl6O Infected Cells in Figures 1 and 2[a]

fields/lanes	A/1	B/2	C/3	D/4	E/5	F	G/6	H/7	1
				Cytotoxicity					
CV-1	0	0	4	5	9	100	5	60	100
HeLa-CD4	0	0	5	7	8	70	8	57	100
Jurkat	0	0	6	18	42	100	16	56	100
Molt-4	0	4	15	20	45	100	25	80	100
				gpl60 processing					
CV-1	ND	60	52	38	42	ND	44	5	ND
HeLa-CD4	ND	58	48	43	55	ND	38	6	ND
Jurkat	ND	53	44	43	42	ND	40	42	ND
Molt-4	ND	51	43	41	43	ND	42	40	ND

[a] The cytotoxicity was determined using the trypan blue dye exclusion assay, and the gpl6O processing were assessed as described in Material and Methods. (ND, not determined).

The persistence of both syncytium formation and gp160 processing in the absence of Ca^{2+} ions suggests that Ca^{2+}-independent enzyme(s) can play a significant role in HIV-1 gp160 maturation.

Purification of Ca^{2+}-Independent gp160 Processing Activity from PBMCs. Enzymes involved in HIV-1 *env* processing are expected to be expressed in lymphocytes, the principal natural targets of HIV-1. To identify the Ca^{2+}-independent enzyme(s) implicated in gp160 maturation, we thus carried out enzyme purification using PBMCs of healthy donors. To localize the Ca^{2+}-independent gp160 processing endoproteases in PBMCs, cells were solubilized with Triton X114 and treated as described (*44*). After partitioning Triton X114 solubilized proteins, activity was found only in the detergent phase that contained membrane proteins.

To design inhibitors of HIV *env* processing, we synthesized peptides mimicking the sequence surrounding the maturation site and tested their activities. When a panel of these peptides was tested, $_{499}$TKAKRRVVEREKRV$_{512}$, composed of D-amino acids (14D peptide) exhibited remarkable inhibition ex vivo toward the processing of HIV-1 and HIV-2 *env* precursors in HeLa-CD4, Jurkat, and Molt-4 cells, and toward SIVmac virus in CEM X174 cells, as well as an inhibition of syncytium induction (*45*).

The lack of cleavage of the 14D peptide was tested by incubating in vitro various amounts of the peptide with total solubilized membrane proteins. The same peptide composed

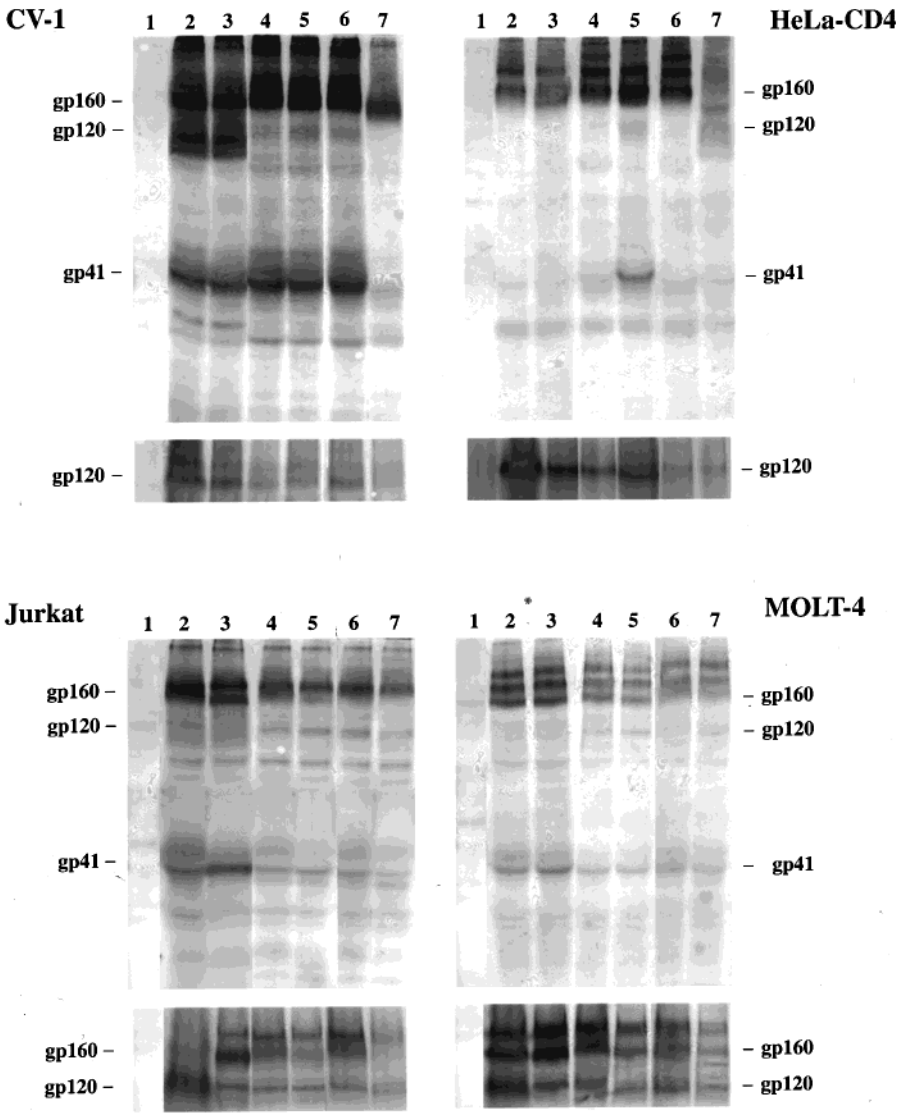

FIGURE 2: Effect of calcium depletion in VV-gp160 infected cells on gp160 processing. CV-1, HeLa-CD4, Jurkat, and Molt-4 cells were infected with VV-gp160 as described in the legend of Figure 1. They were then lysed and analyzed by Western blotting using a polyclonal human anti-HIV-1 serum. This serum is highly specific since it does not react with proteins of uninfected cells or those infected with wild-type vaccinia virus (see lanes 1). Top panels, cell lysates. Bottom panels, immunoprecipitated gp120 from supernatants. Lane 1, cells infected with wild-type vaccinia virus. Lanes 2 and 3, infected cells in 2 and 0% FCS. Lanes 4 and 5, infected cells in 0% FCS and 10^{-8} and 10^{-7} M A23187. Lanes 6 and 7, infected cells in 0% FCS and 10 and 20 mM EGTA.

of L-amino acids (14L peptide) was tested in the same conditions. HPLC fractionation showed that the 14L peptide was completely degraded, while the 14D peptide exhibited a remarkable resistance to proteolysis (data not shown). Since the 14D peptide inhibits *env* precursor processing ex vivo, these data suggest that the peptide may interact with the processing endoprotease(s), but the resulting complex did not yield any products. The 14D peptide was therefore used as a ligand in affinity chromatography to isolate the putative gp160 cleavage enzyme(s). After incubation, the specifically adsorbed proteins were eluted with 1 M L-arginine in Tris-CHAPS buffer (Figure 3, panel A). Collected fractions were tested for their capacities to cleave the fluorogenic substrates REKR-MCA, EKR-MCA, and CER-MCA, longer nonfluorogenic peptides mimicking the cleavage sites of HIV-1, HIV-2, and SIV *env* precursors (Table 4), and HIV-1 gp160 purified by GNA-lectin.

In the absence of calcium ions, the first pools eluted (fractions 1 and 2) not adsorbed to 14D peptide could not cleave any of the above-mentioned substrates. In contrast, fraction 3 that was adsorbed to 14D peptide and eluted by L-arginine 1 M could cleave in vitro not only MCA-coupled peptides, but also the longer peptides including peptides 511 (Figure 3, panel B), 14L, and 491 (data not shown), and gp160 into gp120 and gp41 (Figure 6, panel A, lane 1). In the presence of 5 mM $CaCl_2$, however, the adsorbed fraction exhibited nonspecific endoprotease activity on both longer peptides (Figure 3, panel B) and the gp160 molecule (Figure 6, panel A, lane 2). The gp160 precursor was in fact rapidly converted to 80- and 30-kDa products, indicating that the adsorbed 14D peptide fraction contained at least two types of cleavage proteases, depending on Ca^{2+}-dependence. Proteins in this fraction were then separated using the principle that enzymes requiring divalent ions for activity can adsorb to metal-chelating column (Zn^{2+}-form, Figure 4, panel A). This assay had previously been used to purify a Ca^{2+}/Mg^{2+}-dependent enzyme (*44*). As expected, the results showed that in the absence of Ca^{2+} ions, nonadsorbed

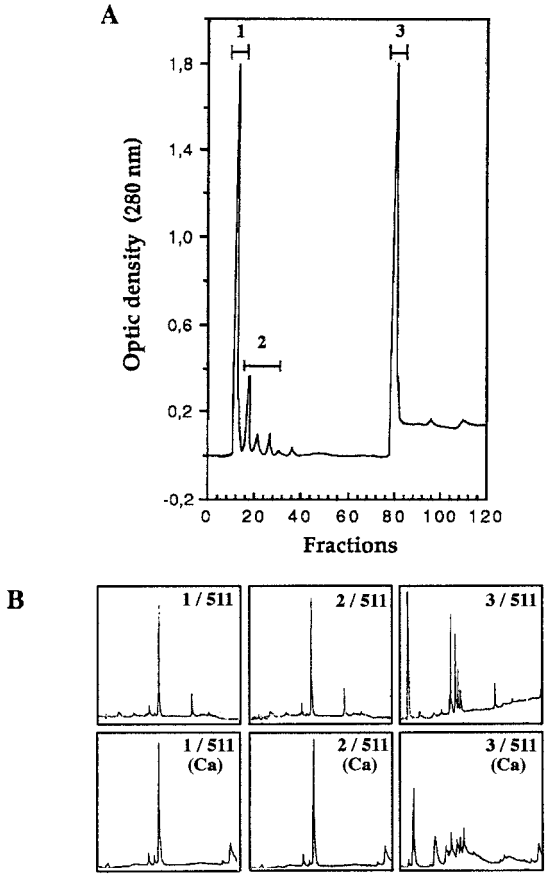

FIGURE 3: 14D peptide affinity chromatography. (A) solubilized membrane proteins were equilibrated in TNC buffer (25 mM Tris-HCl, 0.15 M NaCl, 1% CHAPS, pH 7) and applied at a flow-rate of 0.5 mL/min to a packed 14D-Sepharose CL-4B column (10 mg of 14D peptide/5 mL of solid phase). After washing with 10 volumes of TNC buffer (fractions 1 and 2), 14D peptide-adsorbed proteins (fractions 3) were eluted with TNC buffer supplemented with 1 M L-arginine. (B) Proteolytic cleavage by pooled fractions of a 511 synthetic peptide mimicking the SIV *env* consensus sequence. The reactions were run in the presence or absence of 5 mM $CaCl_2$ as described in Materials and Methods.

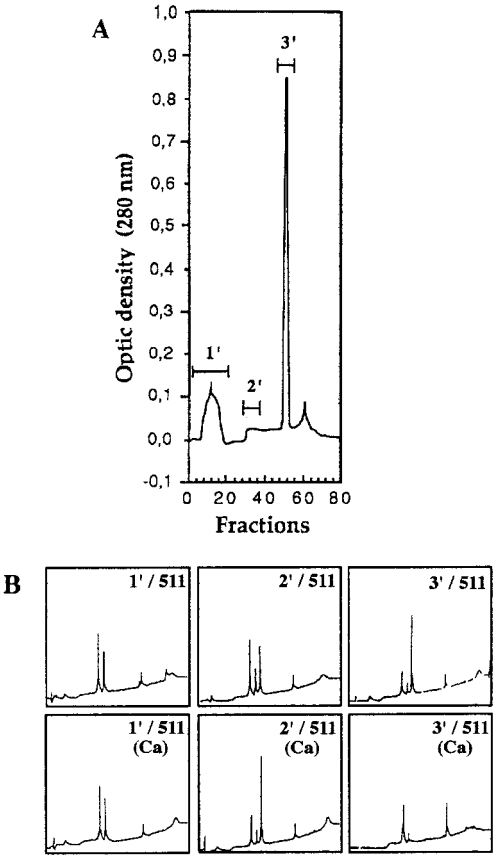

FIGURE 4: Zn^{2+}-chelating Superose chromatography. (A) the 14D peptide adsorbed proteins with Ca^{2+}-independent gp160 processing activity were equilibrated in TNC buffer and applied to a Zn^{2+}-chelating column (5 mL) at a flow-rate of 0.5 mL/min (fractions 1′ is nonadsorbed proteins). The column was then eluted with 1 M ammonium chloride in TNC buffer (fractions 2′). Finally, Zn^{2+}-adsorbed proteins (fractions 3′) were eluted with 10 mM EDTA in TNC buffer and extensively dialyzed to remove EDTA. (B) proteolytic cleavage of 511 peptide by collected pools as described in Materials and Methods.

proteins in fraction 1′ could specifically cleave peptides including 511 (Figure 4, panel B) and the gp160 precursor (data not shown). Furthermore, the addition of Ca^{2+} or Mg^{2+} ions had no effect on cleavage (Figure 4, panel B). These findings indicate that this pool contained primarily divalent ion-independent activity, and thus was analytically homogeneous. The addition of 1 M ammonium chloride led to the elution of another endoprotease activity (fraction 2′), Ca^{2+}-independent and that could cleave peptides including 511 (Figure 4, panel B) and the gp160 precursor (data not shown) albeit with a very low affinity. Furthermore, this enzyme(s) was apparently inhibited by divalent ions, including Ca^{2+} (Figure 4, panel B) and Mg^{2+} (data not shown). The examination of this activity was not further pursued. Finally, elution with 10 mM EDTA resulted in a third fraction 3′ in which Ca^{2+}-dependent enzymes were the majority. They could nonspecifically digest peptide 511 (Figure 4, panel B), gp160 to 80 and 30 kDa products (data not shown) only in the presence of Ca^{2+} ions.

The degree of heterogeneity of the pooled fractions as analyzed by SDS−PAGE and silver nitrate staining revealed the selection of specific proteins in fraction 1′, although several bands were present (Figure 7, panel A, lane 1′). To further ensure homogeneity of the partially purified gp160-processing enzyme(s), gel filtration fractionation was carried out on Superdex 75 and 200 columns. After Superdex 75 filtration, the peptides and gp160 specific cleaving pool was obtained in the exclusion limit of the gel (>75 kDa) (Figure 5, panels A and B). When the Superdex 75 excluded active fraction was chromatographed on Superdex 200 filtration, it was eluted as a single peak of gp160 specific processing activity at 120 ± 5 kDa (Figure 5, panel C). Conversion of gp160 to gp120 and gp41 by the final Ca^{2+}-independent enzyme preparation is shown in Figure 6, panel B, lanes 3 and 4. It is noteworthy that while gp120 produced was processed further, as reported for Ca^{2+}-independent gp160 processing VEM (*26*), no further digestion of gp41 was observed despite the presence of calcium ions in comparison to the 14D-adsorbed active fraction (Figure 6, panel A, lane 2). On the other hand, thrombin and trypsin proteases yielded several nonspecific products, in contrast to the purified activity of gp160 processing (Figure 6, panel B, lanes 11 and 12, respectively). We could not identify Ca^{2+}-dependent enzymes with detectable gp160 processing activity, although there were several that hydrolyzed the synthetic MCA-coupled monobasic and dibasic substrates.

The results of purification of the gp160 precursor processing enzyme are summarized in Table 2. The final fraction

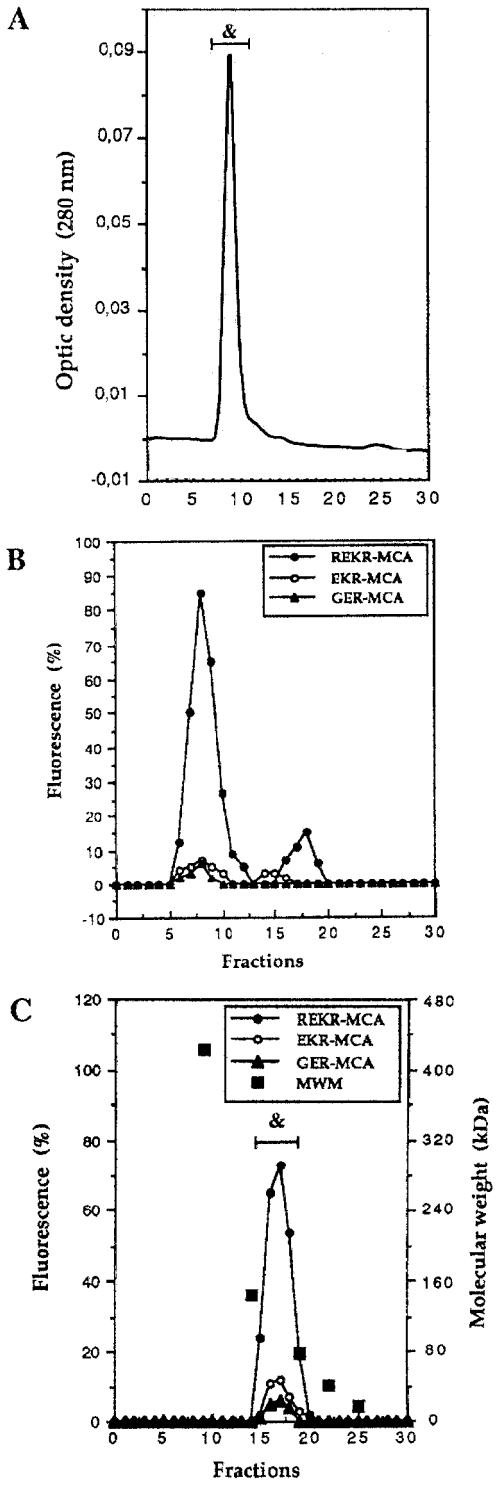

FIGURE 5: Superdex 75 and 200 column filtrations. (A and B) the Zn^{2+}-column nonadsorbed proteins with Ca^{2+}-independent gp160 processing activity were equilibrated in TNC buffer and applied to prepacked Superdex 75 HR 10/30 column (1 × 30 cm; 24 mL) at a flow-rate of 0.5 mL/min. For Superdex 200 filtration (C), fractions with gp160 processing activity were concentrated and then applied to the prepacked HR 10/30 column (1 × 30 cm; 24 mL) at a flow-rate of 0.5 mL/min. Fractions were tested for amidolytic activity as described in Materials and Methods. The molecular weight markers used in panel C in the same chromatographic conditions are ferritin (440 kDa), human IgG (150 kDa), human transferrin (81 kDa), ovalbumin (43 kDa), and myoglobin (17.6 kDa).

was analyzed in reducing and nonreducing conditions by SDS−PAGE and silver nitrate staining (Figure 7, panel B). Nonreducing conditions revealed two bands around 66 and 52 kDa, while reducing conditions revealed bands at 66, 32, and 24 kDa (Figure 7, panel B, lane 1 vs 2). The 32- and 24-kDa peptides are likely products of the 52 kDa band detected under nonreducing conditions and leads to the suggestion that this 52-kDa form is a dimer maintained by SDS-resistant interactions. This heteromeric enzyme was designed as VLP (VEM-like protease).

Physicochemical Characterization of VEM-Like Protease. This was assessed by testing the effects of different inhibitors on gp160 processing in vitro. Only 14D and decRVKRcmk peptides, TLCK, DFP, PMSF, and aprotinin (Figure 6, panel C, lanes 5 to 10, respectively) could inhibit gp160 processing to gp120 and gp41 by the purified enzyme in vitro and showed that this activity was due to a serine endoprotease. To determine if there was an association between the purified processing activity of gp160 and that/those observed ex vivo in conditions of Ca^{2+} depletion, we tested VLP inhibitors on gp160 processing in vitro, on syncytium induction and on gp160 processing ex vivo using gp160-recombinant vaccinia virus (Table 3) in the same conditions of Ca^{2+} depletion described above. It was found that the Ca^{2+}-independent gp160 processing enzyme(s) observed ex vivo was/were closely related to the purified VLP enzyme, even though aprotinin, less permeable than DFP or TLCK, had little effect on processing activity ex vivo. Interestingly and, in contrast to VEM that was inhibited by reducing agents such as L-cysteine, *N*-acetyl-L-cysteine, β-mercaptoethanol, and dithiothreitol (DTT) (*46*), we detected no effect on our enzyme either in vitro or ex vivo (Table 3). Furthermore, ammonium chloride inhibited gp160 processing ex vivo, resulting from the likely involvement of an acidic enzyme as previously reported (*2*).

It is noteworthy that the pH optimum of VLP activity was 6.5 to 7 (data not shown). The effect of the *N*-glycosylation inhibitor tunicamycine, and that of brefeldin A that causes the redistribution of the cis and medial Golgi to the ER on gp160 maturation, also agreed with previous reports indicating that gp160 must reach the Golgi apparatus for processing to gp120 and gp41 (Table 3).

Studies of the structure−function relationship in VLP processing of gp160 was investigated using the different peptides that did or did not mimic the cleavage site of HIV-1, HIV-2, and SIV viruses (Table 4). It was found that the peptides genuinely cleaved by the purified activity all contained the second basic site $RAKRR_{504}$ for HIV-1 and KR_{515} for SIV, in addition to the maturation site. Therefore, these data would seem to be consistent with previous findings showing that more than the maturation site is required for correct gp160 conversion to gp120 and gp41 (*47*, *48*).

DISCUSSION

To our knowledge, only two reports have attempted to identify gp160 processing endoprotease(s) from natural targets of HIV infection. Kido et al. (*26*) purified a Ca^{2+}-independent enzyme called VEM from the Molt-4 lymphoid cell line that cleaves gp160 to gp120 and gp41 in vitro, and Hallenberger et al. (*25*) partially purified a furin enzyme from the Jurkat lymphoid cell line. We have extended these findings by purifying a Ca^{2+}-independent endoprotease activity from PBMCs that can specifically convert HIV-1

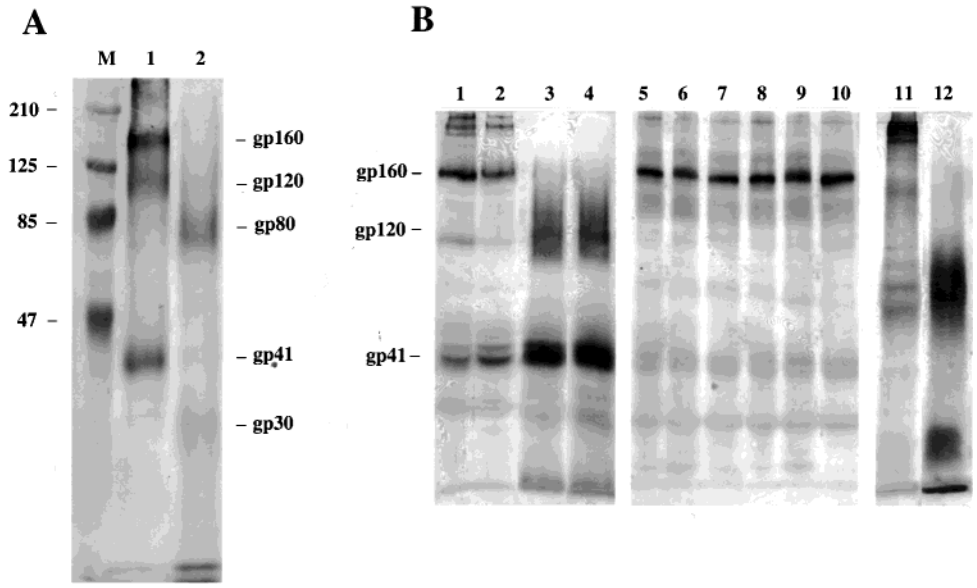

FIGURE 6: gp160 processing by the purifed activity and inhibitory spectrum. **(**A) GNA-lectin purified gp160 was incubated in vitro at 37 °C for 12 h with the 14D-adsorbed fraction in the absence (lane 1) or presence of 5 mM $CaCl_2$ (lane 2). (B) GNA-lectin purified gp160 was incubated at 37 °C for 12 h alone in the absence (lane 1) or presence of 5 mM $CaCl_2$ (lane 2), with the purified enzyme in the absence (lane 3) or presence of 5 mM $CaCl_2$ (lane 4). Lanes 5 to 10, GNA-lectin purified gp160 was incubated with the enzyme preparation as described above in the absence of 5 mM $CaCl_2$ and the presence of 70 μM 14D peptide, 70 μM decRVKRcmk, 100 μM TLCK, 1 mM DFP, 100 μM PMSF, and 10 μM aprotinin, respectively. Lanes 11 and 12, GNA-lectin purified gp160 was incubated with thrombin and trypsin enzymes, respectively, as controls.

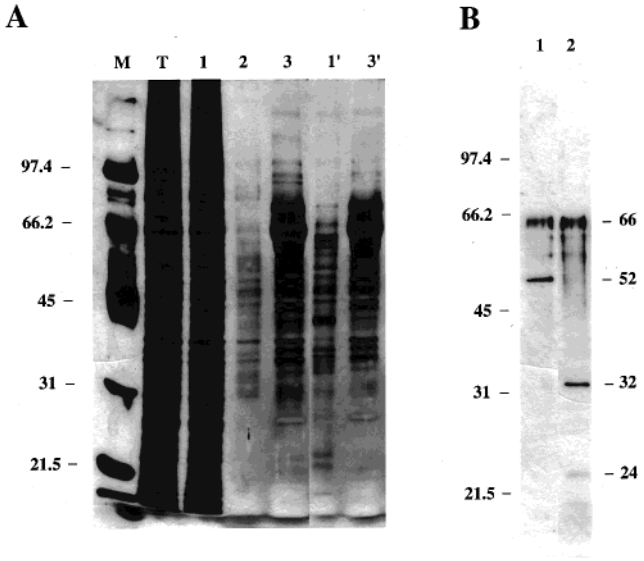

FIGURE 7: Electrophoretic analysis of the fractions collected at different enzyme purification steps. **(**A) Samples of total solubilized membrane proteins (lane T, 50 μg), 14D-nonadsorbed fractions (lane 1, 50 μg), 14D-column wash eluant (lane 2), L-arginine eluted 14D-adsorbed fractions (lane 3, 10 μg), Zn^{2+}-nonadsorbed proteins (lane 1′, 4 μg), and EDTA eluted Zn^{2+}-adsorbed fractions (lane 3′, 10 μg) were subjected to 12% SDS−PAGE in denaturing and reducing conditions and were stained with silver nitrate for proteins. (B) 12% SDS−PAGE of Superdex 200 active fractions in nonreducing (lane 1, 0.5 μg) and reducing conditions (lane 2, 0.5 μg). Lane M, molecular weight markers in kDa.

gp160 to gp120 and gp41. Several lines of evidence incited us to perform this study. First, the SPC selective inhibitor, α1-PDX, did not inhibit HIV-1 and HIV-2 replications completely or the maturation of *env* precursors (*35*, *36*). Inversely, the decRVKRcmk peptide, shown to have a broad inhibition spectrum toward all known gp160 converting enzymes, can block viral replication to a greater extent (*36*). Second, the drastic depletion of intracellular Ca^{2+} levels in both nonlymphoid and lymphoid cells including PBMCs did not cause the inhibition of syncytium induction or enzymatic gp160 cleavage to gp120 and gp41 ex vivo. Finally, there was no difference in the degree of syncytium induction or gp160 cleavage in stably transfected α1-PDX lymphoid Jurkat cells (J-PDX) and controls (J-pcDNA3) devoid of Ca^{2+} ions (M. Bendjennat, B. Bahbouhi and E. Bahraoui, unpublished observations). This indicates that Ca^{2+}-independent enzyme(s) could be responsible for persisting HIV-1 and HIV-2 replications in J-PDX cells. These data are a first proof that other endoprotease(s), presumably divergent from SPCs, could be implicated in the HIV *env* processing

We have characterized a potent Ca^{2+}-independent gp160 processing endoprotease from PBMCs. It was found that the totality of activity was membrane associated. The enzyme was purified to homogeneity by using four chromatography fractionation steps coupled with the continuous detection of the Ca^{2+}-independent activity of each. This positive result also resulted from the use of three types of substrates for screening gp160 cleaving endoprotease(s), including shorter MCA-coupled peptides, and longer peptides that mimic the consensus cleavage sites of HIV and SIV viruses and native gp160 precursor. Cleavage of the short and longer peptides by an endoprotease in fact does not necessarily mean efficient cleavage of the native gp160, as is the case of SPC, since residues distal from the natural site could be involved in interaction with the enzyme. SDS−PAGE analysis of the enzyme in nonreducing and reducing conditions revealed three products migrating at 66, 32, and 24 kDa. Despite protein disaggregating treatments with sonication and zwiterionic agents such as betain and taurin, purified activity was eluted at 120 kDa on Superdex 200 columns (data not shown). It can thus be argued that the 66-kDa subunit and

Table 2: Purification of the Ca^{2+}-Independent gpl60 Processing Enzyme from PBMCs

procedure	protein (mg)	total activity[a] (milliunits)	yield (%)	specific activity (microunits/mg)	purification (fold)
membrane fraction	150	25.77	100	171.8	1
14D-affinity column	4.5	8.50	32	1888.8	10.9
Zn^{2+}-affinity column	0.23	0.75	2.9	3260.5	18.9
Superdex 75	0.09	0.36	1.4	4000	23.2
Superdex 200	0.031	0.16	0.6	5161.2	30

[a] One unit of enzyme activity was defined as the amount degrading 1 mmol of Boc-REKR-MCA/min.

Table 3: Inhibitor Sensitivity of Purified VLM on gpl6O Processing in Vitro and on Syncytium Induction and gpl6O Processing ex Vivo after VV-gpl60 Infection of Jurkat Cells in the Conditions of Ca^{2+} Depletion[a]

addition	conc	in vitro inhibition of gp160 processing (%)	ex vivo inhibition of syncytia formation (%)	ex vivo inhibition of gp160 processing (%)
none		0	0	0
dec-REKR-cmk	70 μM	100	100	95
14D peptide	70 μM	100	85	91
aprotinin	10 μM	100	ND	ND
DFP	1 mM	100	100	97
PMSF	100 μM	100	ND	ND
TLCK	100 μM	100	100	98
leupeptin	50 μM	0	0	4
pepstatin	50 μM	0	ND	ND
E64c	50 μM	0	0	5
A23187	5×10^{-7} M	ND	0	11
EDTA	10 mM	0	ND	ND
EGTA	10 mM	0	0	9
$CaCl_2$	5 mM	0	ND	ND
$MgCl_2$	5 mM	0	ND	ND
$ZnCl_2$	5 mM	0	ND	ND
D′TT	1 mM	0	0	0
L-cysteine	10 mM	0	0	6
N-acetyl-L-cysteine	10 mM	0	ND	ND
NH_4Cl	20 mM	ND	85	78
tunicamycine	5 μg/mL	ND	100	100
BFA	2.5 μg/mL	ND	100	97
BSA	1 mg/mL	0	ND	ND

[a] All the tested inhibitors were added to culture medium after VV-gpl60 adsorption as descibed in Materials and Methods. (ND, not detemined).

Table 4: Substrate Specificity of Purified VLP[a]

		peptides	cleav
VIH-1			
	33	$_{33}$**K**LWVTVYYGVPVW**K**EATTTLFCA$_{55}$	−
	14L	$_{499}$T**K**A**KRR**VVQ**RE****KR**V$_{512}$	+
	491	$_{491}$**K**IEPLGVPT**K**A**KRR**VVQ**RE****KR**VGIG$_{515}$	+
VIH-2			
	Gp4	VEITPIGFAPT**K**E**KR**YSSAHG	−
SIV			
	511	$_{511}$PTDV**KR**YTTGGTS**RN****KR**GVF$_{530}$	+
	521	$_{521}$GTS**R**N**KR**GVFVLGFLGFLAT$_{540}$	−

[a] Synthetic peptides that mimic or not the cleavage consensus sequence of HIV-1, HIV-2, and SIV glycoprotein precursors, respectively, were incubated in vitro with the purified enzyme at 37 °C for 12 h and then analyzed by RP-HPLC as described in Materials and Methods. Products from emerging peaks were identified for specific processing by determining amino acid composition. (+) specific cleavage, (−) no cleavage.

52-kDa dimer form a heteromeric complex in the Ca^{2+}-independent purified enzyme (VLP). The fold purification is undoubtedly an underestimation of the actual increase in specific activity, since the presence of other protease activities was noted in the initial crude membrane fraction.

The VLP enzyme exhibited higher selectivity for the maturation site, since it cleaved the gp160 precursor predominantly into authentic gp120 and gp41, and required the second basic site in proximity to the maturation site for activity. In agreement with our observations, Bosch et al. (*48*) showed that mutations of all residues in the basic site ($KAKRR_{504}$) upstream from the maturation site abolished gp160 cleavage. In addition, even though LoVo cells cleave gp160 to gp120 and gp41, they cannot activate other substrates such as NDV and *Influenza* envelope glycoprotein precursors that have a homologous gp160 cleavage site (*27*, *28*). These data underline the importance of the three-dimensional structure in the gp160 cleavage reaction. The requirement for the $KAKRR_{504}$ sequence for purified gp160 processing enzyme thus reflects its specificity for gp160 maturation. The amino acid residues in the $KAKRR_{504}$ site may interact with the enzyme and/or contribute to adequately exposuring the maturation site in both mimicking peptides and gp160 molecules. On the other hand, the isolation of a Ca^{2+}-independent gp160 processing activity confirmed our ex vivo experiments by indicating that the observations recorded were not due to alterations in cell machinery by the use of the inhibitors A23187 and EGTA.

Using a panel of protease inhibitors, we showed that the purified Ca^{2+}-independent protease: (i) is a serine endoprotease; (ii) is not sensitive to reducing agents, in contrast to VEM previously described by Kido et al. (*26*); and (iii) is sensitive to NH4Cl suggesting the involvement of an acidic enzyme as previously reported (*2*); (iiii) shares the ability of tunicamycine and brefeldin A to interfere with the

transport of gp160 to the TGN to inhibit gp160 processing, suggesting that gp160 cleavage into gp120 and gp41 occurs at the TGN and/or post-TGN level.

Finally, it was also noteworthy that calcium ions activated nonspecific degradation of gp160 molecules, suggesting that cellular degradation of gp160 might be a Ca^{2+}-dependent event. The conditions of Ca^{2+} depletion may have contributed to stimulating Ca^{2+}-independent enzyme(s) by raising the proportion of gp160 molecules having the appropriate conformation.

Subtilisin-like convertases are believed to be involved in the proteolytic activation of a variety of cellular substrates, including viral envelope glycoprotein precursors, hormone precursors, receptors, cell adhesion molecules, and neurotransmitters (*18*). Furin-deficient LoVo cells express PACE4 and PC7 but not PC1/3, PC2, or PC5/6. It is possible that these SPCs expressed could serve as alternative enzymes for gp160 processing in these cells. These SPCs have in fact been shown to specifically convert gp160 to gp120 and gp41 in vitro and/or in a cell-type specific manner. Nevertheless, no evidence was provided concerning the ability of LoVo cells to process gp160 in the absence of Ca^{2+} ions, as is the case of the present work in PBMCs, and thus their role in these cells cannot be completely excluded out. Furthermore, although PACE4, PC7, PC5/6, and PC1/3 could process gp160 to gp120 and gp41 ex vivo, these enzymes were apparently less efficient than furin as shown by the level of unprocessed gp160 (*24*). This led us to question the idea whether furin-like enzymes would be the primary proteases involved in the cleavage of gp160 in vivo. A more recent study identified an enzymatic activity that can process HIV-1 gp160 in vitro (*49*). This enzyme(s), detected in the liver, was mapped to be active in endosomes and was shown to be distinct from furin and PC7. These enzymes were active in the presence of $CaCl_2$, but additional data are required to accurately determine their degree of Ca^{2+} dependence and their expression in lymphocytes.

Finally, the role of SPC as gp160 processing enzymes has been studied by co-overexpression experiments using genes encoding for SPCs and gp160, but cleavages attributed to the transfected proteinases could also occur indirectly via the activation of the endogenous endoprotease(s) that may be divergent from the SPC family (*28*). The use of specific inhibitors of SPCs including, α1-PDX, A23187, and EGTA, did not abolish gp160 maturation ex vivo; hence, the purification of at least two Ca^{2+}-independent gp160 processing proteases (VEM and VLP) from lymphocytes can provide a rational explanation for these observations. Although these enzymes appear to play a minor role as compared to those active in the presence of Ca^{2+}, they clearly triggered gp160 processing and syncytium induction, indicating that they could explain HIV-1-mediated cytopathic effects in vivo.

In all, this study has described the purification of a gp160 processing enzyme directly from natural host cells of HIV-1, PBMCs. It can thus likely play a significant role in HIV-1 infection and cytopathogenicity. We provide here more direct and supportive evidence that intracellular HIV-1 gp160 maturation may involve at least two families of divergent endoproteases, depending on calcium dependence. The selectivity of VLP for the cleavage site, its requirement for additional residues in the gp160 sequence around this cleavage site and its direct isolation from the natural target of HIV-1 make it a new potent candidate in the gp160 maturation process.

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