

Effects of calcium ions on proteolytic processing of HIV-1 gp160 precursor and on cell fusion

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Abstract

Complete activation of human immunodeficiency virus type 1 (HIV-1) requires the endoproteolytic cleavage by cellular protease of the envelope glycoprotein precursor (gp160) into the external glycoprotein gp120, and the transmembrane glycoprotein gp41. We report here the effect of depletion of cellular calcium ions on maturation of precursor gp160 and its concomitant effect on syncytium formation. We show that the cellular endoprotease activity responsible for gp160 maturation and the capacity for HIV-1 to induce syncytium formation are calcium-dependent. In addition, we show that endoproteolytic maturation is a key step in syncytium formation induced by HIV-1.

Key words: HIV-1, Processing, Glycoprotein, Endoprotease, Calcium ion

1. Introduction

One of the consequences of HIV-1 infection *in vitro* is the formation of multinucleated giant cells resulting from cell-to-cell fusion [1,2]. Envelope glycoprotein gp120 has been shown to interact with the CD4 molecule of the target cells to initiate the process of infection [3–5], while gp41 mediates the fusion of viral and cellular membranes [6–8]. Endoproteolytic cleavage of gp160 into gp120 and gp41 occurs inside the cell, probably in the Golgi complex [9,10]. The nature and intracellular localization of the host cell endoprotease involved in the processing of gp160 remain speculative. Maturation of gp160 has been shown to be an essential step for HIV infectivity [11–13]. The cleavage site has been identified in a cluster of basic amino acids localized between Arg⁵¹¹ and Ala⁵¹². McCune et al. [11] reported that removing the trypsin-like endoproteolytic cleavage site by site-directed mutagenesis (Arg-Glu-Lys-Arg⁵¹¹ to Gly-Glu-Glu-Phe) generated biologically inactive particles, morphologically similar to infectious particles as shown by electron microscopy. More recently, Hallenberger et al. [14] demonstrated that the proteolytic processing of HIV-1 gp160, and consequently the formation of infectious virus, can be abolished by using specific peptide inhibitors. These results indicate that the endoproteolytic cleavage of gp160 is an essential activation step for the biological

activity of HIV-1. The consensus cleavage recognition sequence site, Arg-X-Lys/Arg-Arg, is shared with a number of viral glycoprotein precursors. This cleavage site is present in the specific substrate of furin, a Kex2-like protease [15,16]. Recent studies suggest that the calcium-dependent mammalian furin [17] correctly cleaves influenza virus hemagglutinin precursor [18], Newcastle disease virus glycoprotein [19], and precursor gp160 of HIV-1 [14]. In light of the work of Dimitrov et al. [20], who showed that calcium ions are required for cell-to-cell fusion, and in order to explain this effect at the molecular level, we show that cleavage of the precursor envelope glycoprotein (gp160) into gp120 and gp41 in HIV-1 infected cells is inhibited by calcium ion depletion, leading to the formation of immature viral particles unable to induce multinucleated giant cells.

2. Materials and methods

2.1. Virus and cells lines

Wild type HIV-1 LAV_{LAI} was obtained from Diagnostics Pasteur (Marnes-La-Coquette, France) as a supernatant from infected CEM cells containing 10⁵ tissue culture infective doses (TCID₅₀) per ml. Human cells of the CEM clone 13 (CEM13), derived from the CEM line, and MOLT-4 (American Type Culture Collection, Rockville, Md) were grown at 37°C in a humidified atmosphere with 5% CO₂ in RPMI 1640 culture medium (Flow Laboratories, Irvine, Scotland) containing 10% fetal calf serum supplemented with 1% glutamine and 1% penicillin-streptomycin (GIBCO Laboratories, Paisley, Scotland).

2.2. HIV-1 infectivity assay

CEM cells were infected with HIV-1 LAV_{LAI} 10⁴ TCID₅₀ of virus in

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100 μ l were added to 10^7 CEM cells suspended in 1 ml of RPMI 1640 and incubated for 2 h at 37°C. The cells were then washed twice in culture medium and cultured at 0.5×10^6 /ml in complete RPMI 1640 medium. After five days, infected CEM cells were radiolabelled or tested for syncytium formation with fresh MOLT-4 cells.

2.3 Syncytium formation assay

This was done in 12-well plates by cocultivating 0.2×10^6 HIV-1 infected CEM cells with 0.8×10^6 MOLT-4 cells in 1 ml of complete RPMI 1640 medium or in calcium-free RPMI 1640 medium (J. Bio. Paris, France) containing ionophore A23187 at various concentrations. Cells were incubated in a CO₂ incubator at 37°C for 24 h, and the number of multinucleated giant cells in each well was scored by microscopic examination.

2.4 Ionophore studies

Ionophore A23187 was purchased from Calbiochem (Meudon, France) and stored as a 1 mM stock solution in dimethyl sulfoxide (DMSO). The processing of gp160 was examined by analyzing viral radiolabelled proteins produced in HIV-1 infected CEM cells cultured in RPMI 1640 medium lacking calcium chloride and in presence of the ionophore at various concentrations.

2.5 Radioimmunoprecipitation assay (RIPA)

The processing of gp160 in the presence or absence of calcium ions was examined by growing cells in complete culture medium or in calcium-free RPMI 1640 medium and with ionophore A23187 at the indicated concentrations. The cells were pulsed for 12 h with 100 μ Ci per ml of radiolabelled [³⁵S]methionine-cysteine (Trans Label, ICN, Costa Mesa, CA) and incubated in unlabelled medium for 4 h. Cells and supernatants were separated by centrifugation at 12,000 rpm for 10 min. The cell pellet was washed three times in ice-cold phosphate-buffered saline (PBS). A soluble cellular lysate was prepared by disrupting the cells with 200 μ l of RIPA buffer (0.15 M NaCl, 20 mM Tris HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). The lysate was clarified by centrifuging at 15,000 rpm for 15 min. Viral glycoproteins obtained from the lysates of cells or cell-free virus (present in the supernatant of cell culture) were immunoprecipitated with rabbit anti-gp160 prepared in our laboratory as described by Benjouad et al. [21]. Immune complexes were separated with protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) after an overnight incubation at 4°C. Viral glycoproteins were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). Before loading on the gel, samples were boiled for 5 min at 110°C with SDS (2%) and β -mercaptoethanol (2%). Gels were fluorographed to visualize [³⁵S]methionine/cysteine-labelled proteins.

2.6 Measure of viral production in the supernatants of infected cells

Reverse transcriptase assay (RT) activity was measured by the incorporation of [³H] thymidine (Amersham) as previously described [22].

3. Results

3.1 Effect of cell calcium depletion on proteolytic processing of precursor gp160

The effect of calcium ions on the synthesis of HIV-1 glycoproteins in infected cells was determined as follows. HIV-1 infected CEM cells were radiolabelled with [³⁵S]methionine-cysteine for 12 h in complete culture medium or in calcium-free RPMI 1640 medium containing the calcium specific ionophore A23187 at 100 nM. After radiolabelling, proteins were immunoprecipitated with a polyclonal anti-gp160, which also cross-reacts with gp120, and were separated by SDS-PAGE. In normal conditions (presence of calcium ions and absence of ionophore A23187), both precursor gp160 and its cleavage product gp120 were specifically immunoprecipitated

in the cell lysate (Fig. 1, lane 1). In these conditions, and taking into account label intensity, the bands of precursor gp160 and gp120 are present in the same ratio (gp160/gp120 $\approx$ 1). In these normal conditions and as expected, only a band corresponding to gp120 was detected in the viral lysate, while the band corresponding to precursor gp160 was not detected (Fig. 1, lane 2). The specificity of immunoprecipitated viral glycoproteins was assessed with cellular and viral lysates by using a rabbit preimmune serum. In these conditions, no bands corresponding to gp160 and gp120 were detected (Fig. 1, lanes 3 and 4).

When infected CEM cells were labelled with [³⁵S]methionine-cysteine for 12 h in calcium-free culture medium in the presence of 100 nM of ionophore A23187, however, simple visual inspection (Fig. 1, lane 5) shows a significant increase of the gp160/gp120 ratio (gp160/gp120 > 1) in the cell lysate as judged by the intensity of the corresponding bands. Moreover under these conditions, the analysis of the cell-free culture supernatant, containing viral particles, clearly shows the presence of uncleaved gp160, which appears to be associated with viral particles (Fig. 1, lane 6). On the other hand, in the presence of calcium ions, precursor gp160 was undetectable (Fig. 1, lane 2). Furthermore, we found that addition of calcium ions to the medium completely restored cleavage efficiency (data not shown).

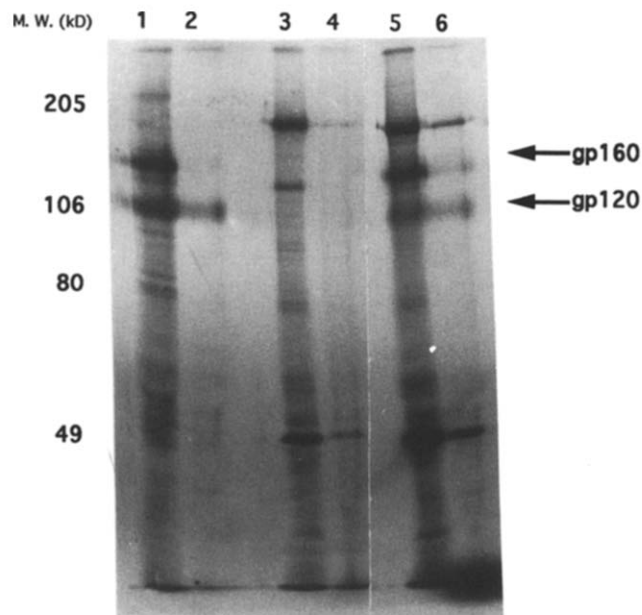


Fig. 1 Effect of calcium depletion of infected CEM cells on gp160 proteolysis. Profile of viral glycoproteins from HIV-1 infected cells in presence of A23187. HIV-1 infected CEM cells were radiolabelled with 100 μ Ci per ml of [³⁵S]methionine/cysteine for 12 h in RPMI 1640 medium (lanes 1, 2, 3 and 4) or in RPMI 1640 medium without calcium and with 100 nM A23187 (lanes 5 and 6). The labelled cells and the culture medium were collected and lysed with lysis buffer. Culture supernatants and cell lysates were immunoprecipitated with anti-gp160 (lanes 1, 2, 5 and 6) or with preimmune rabbit serum (lanes 3 and 4).

3.2 Calcium is required for syncytium formation

Calcium-free RPMI 1640 medium containing A23187 at various concentrations was tested in order to examine the effect of calcium ions in syncytium formation and virus replication. In standard conditions, HIV-1 infected CEM cells formed syncytia within 24 h when co-cultured with MOLT-4 cells (Table 1). After treating HIV-1 infected cells with A23187 concentrations equal to or higher than 10 nM, however, the number of syncytia decreased in a dose-dependent manner (Table 1). This shows that depletion of calcium ions abolishes neither the synthesis nor the expression of precursor gp160, which continues to interact with the CD4 receptor as judged by formation of cell aggregates (Fig. 2). Furthermore, viral-associated reverse transcriptase activity from HIV-1-infected CEM cells propagated in calcium-free RPMI 1640 supplemented with the ionophore A23187 (10, 25, 50 and 100 nM) was similar to that in infected cells cultured in normal medium (Table 2). This indicates that calcium depletion did not seem to affect virions production.

4. Discussion

The analysis of radiolabelled viral proteins from HIV-1 infected CEM cells by one-dimensional gel electrophoresis, has shown that gp160 is cleaved into gp120 and gp41 in the presence of calcium ions. In contrast, when radiolabelling was in calcium-free medium and in the presence of the calcium-specific ionophore A23187 at 100 nM, on the other hand, the endoproteolytic processing of HIV-1 gp160 was significantly reduced. More interestingly, depletion of calcium ions affected the ability of HIV-1 to induce cell-to-cell fusion.

The present results clearly show that calcium ions depletion results in a significant decrease of gp160 processing as judged by the increase of the gp160/gp120 ratio in the cell extract. Simultaneously, the analysis of viral lysates showed the presence of precursor gp160, probably associated with the viral particles as previously shown by McCune et al. [11] and by Hallenberger et al. [14]. These results show that calcium ions depletion abolishes nei-

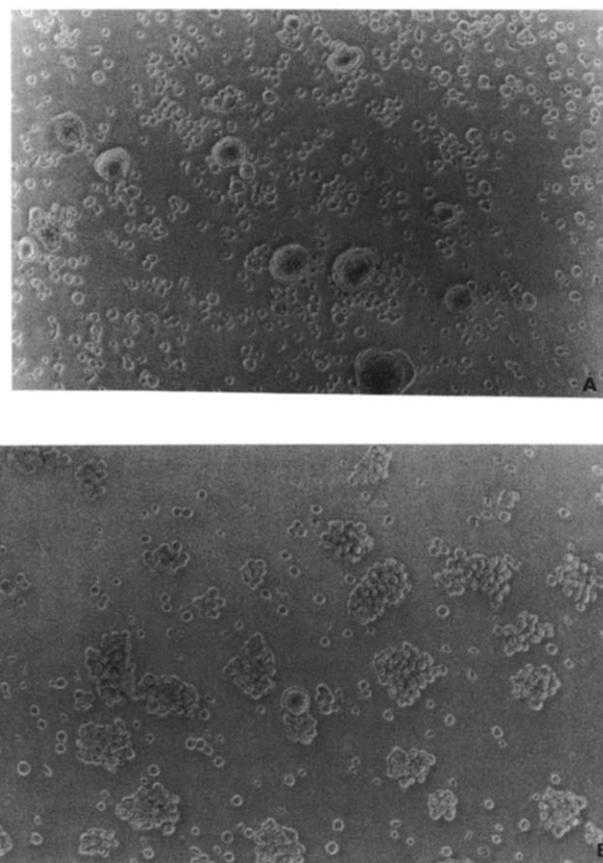
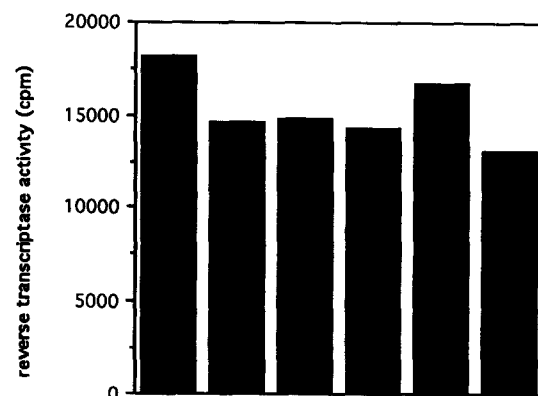


Fig. 2 Effect of calcium ions on syncytium formation. CEM cells were infected with HIV-1, washed with calcium-free RPMI 1640 medium and co-cultured with MOLT-4 cells in RPMI 1640 with calcium ions (A) or without calcium in the presence of ionophore 10 nM A23187 (B). The photomicrographs were taken 24 h after co-culture at 37°C in a CO₂ incubator.

Table 2

Reverse transcriptase activity detected in the cell-free culture supernatant after HIV-1 infection of CEM treated with DMSO or A23187 for 24 h



Ca	+	-	-	-	-	-
A23187 nM	-	-	10	25	50	100
DMSO	-	+	-	-	-	-

Table 1

Effect of calcium ionophore on syncytium formation

A23187	Concentration (nM)	Syncytia, No. per field
None (control)	–	160
DMSO alone	–	158
	10	15
	50	2
	100	0

The number of syncytia per field was scored by microscopic examination. In the control, the same volume of DMSO (10 µl) as used to solubilize the ionophore A23187 was added alone in the co-culture.

ther the synthesis nor the expression of precursor gp160, which continues to interact with the CD4 receptor, as judged by the formation of cell aggregates. This observation indicates that syncytium formation depends on fusion-competent envelope glycoprotein. Precursor gp160 must be cleaved into gp120 and gp41 in order to generate the fusion-active region included in the N-terminal part of gp41.

Our data are in good agreement with those of Hallenberger et al. [14], who clearly established that inhibition of gp160 cleavage by furin interfered with the formation of infectious particles. As postulated for the proteolytic activation of influenza virus hemagglutinin [23] and the precursor gB from human cytomegalovirus [24], we may assume that A23187 interferes with the proteolytic cleavage of gp160, probably directly by inactivating the processing enzyme via calcium depletion of the host cell, consequently blocking the initiation of cell-to-cell fusion mediated by the transmembrane envelope glycoprotein gp41.

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