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Assembly of Infectious HIV-1 in Human Epithelial and T-Lymphoblastic Cell Lines

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The canonical view of the ultimate steps of HIV-1 replication is that virus assembly and budding are taking place at the plasma membrane of infected cells. Surprisingly, recent studies revealed that these steps also occur on endosomal membranes in the interior of infected cells, such as macrophages. This prompted us to revisit the site of HIV-1 assembly in human epithelial-like cells and in infected human T-lymphoblastic cells. To address this question, we investigated the intracellular location of the major viral structural components of HIV-1, namely Gag, Env and the genomic RNA. Using a sub-cellular fractionation method, as well as immuno-confocal and electron microscopy, we show that Gag, the Env glycoproteins and the genomic RNA accumulate in late endosomes that contain infectious HIV-1 particles. In epithelial-like 293T cells, HIV-1 assembles and buds both at the plasma membrane and in endosomes, while in chronically infected human T lymphocytes, viral assembly mostly occurs within the cell where large amounts of infectious virions accumulate in endosomal compartments. In addition, HIV-1 release could be enhanced by ionomycin, a drug stimulating calcium-dependent exocytosis. These results favour the view that newly made Gag molecules associate with the genomic RNA in the cytosol, then viral core complexes can be targeted to late endosomes together with Env, where infectious HIV-1 are made and subsequently released by exocytosis.

Keywords: HIV-1 assembly; late endosomes; 293T and MOLT cell lines

Introduction

In cells infected by human immunodeficiency virus type-1 (HIV-1) the current view is that virus budding occurs at the plasma membrane (PM). However, recent findings revealed that HIV-1 and other retroviruses, as well as other enveloped RNA viruses, can hijack the cellular machinery normally used for vesicle formation and trafficking, allowing the late steps of virion assembly and budding to take place at the level of endosomal vesicles.[1,2]

Assembly of an infectious retroviral particle requires three essential viral components, namely the structural Gag polyprotein, the envelope glycoproteins and the genomic RNA, and cellular components, such as cellular membranes and the budding machinery.[2–5] The Gag polyprotein contains all the determinants required for correct assembly and release of viral particles from mammalian cells.[6] The HIV-1 Gag determinants are matrix (MA), capsid (CA) and nucleocapsid (NC) corresponding to, respectively, MAp17, CAp24 and NCp7 in the mature virus. MA is myristylated, allowing Gag anchoring into the cellular membrane while NC is composed of two zinc fingers flanked by basic residues both of which direct specific genomic RNA recognition and packaging. In the mature virus, NC molecules extensively coat the genomic RNA, thus forming the viral ribonucleoparticle structure known as nucleocapsid where the viral enzymes are also found.[7] The envelope proteins (Env), the surface (SUgp120) and the transmembrane (TMgp41) glycoproteins, are anchored in the cellular membrane and thus present in the cell-derived membrane surrounding the viral core.[8] The Gag

Abbreviations used: PM, plasma membrane; LE, late endosomes; MVB, multi-vesicular body; Env, envelope glycoproteins; PNS, post-nuclear supernatant; NC, nucleocapsid; CA, capsid; RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1.

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polyprotein also contains other domains at variable position such as the late L domain that interacts with proteins of the cellular vacuolar sorting pathway and is required for retroviral budding.[1,9,10]

How and where HIV-1 Gag recruits the genomic RNA and Env during assembly in the host cell are poorly understood. Yet, the NC domain of Gag is believed to promote specific and tight interactions with the genomic RNA, but where this is taking place is a matter of debate.[7,11] Env glycoprotein recruitment is thought to occur at the plasma membrane where, according to the canonical view, the ultimate steps of retrovirus assembly and budding are taking place.[8] However, HIV-1 particles have been observed in intracellular cytoplasmic compartments[12] and recent data show that HIV-1 can bud and accumulate into intracellular late endosomes,[13–18] called multi-vesicular bodies (MVB),[19] notably in macrophages.[17,18] Viral particles would then exit the cell by fusion of the MVB with the plasma membrane.[13,17,20] In support of this, HIV-1 Gag was found to traffic through the endosomal–lysosomal pathway in different cell types,[14–16] and it was recently shown that HIV-1 Gag is addressed to late endosomes *via* the cellular transporter AP-3 through its interaction with the MA domain.[21] Another recent study also shows that HIV-1 Gag can interact with the transport AP-2, driving Gag from the PM into the endocytic pathway.[22] However, the localization of HIV-1 Gag together with Env and the genomic RNA in late endosomes, and the infectivity of HIV-1 virions in these compartments were not addressed in different cell types, such as human epithelial cells and lymphocytic CD4+ T cells, with the exception of macrophages.[18]

Here, we analysed the assembly of HIV-1 in the model human 293T cell line and in HIV-1 infected T lymphocytes, with the aim to determine whether intracellular compartments, such as late endosomes, contain Gag, the genomic RNA and Env, and thus infectious HIV-1. We find that indeed virion-associated Gag, Env and genomic RNA accumulate in late endosomes/multi-vesicular bodies (LE/MVB) in these cells and that HIV-1 particles associated to these intracellular vesicles are mature and infectious.

Results

Presence of intracellular infectious HIV-1 in 293T cells and in MOLT T lymphocytes expressing HIV-1

To investigate the presence of intracellular infectious HIV-1 in human cells, HIV-1 was expressed by DNA transfection in human epithelial 293T cells or in infected CD4(+) T lymphocytes (named MOLT/HIV-1 cells). The virus-containing supernatants were collected representing the "extracellular" virus. Cells were washed, lysed by homogenisation and the resulting post-nuclear supernatant (PNS) corresponded to the "intracellular" virus. The infectivity of the PNS (intracellular) and of the viral supernatant (extracellular) were assessed on HeLaP4 cells (Figure 1(a)). In 293T cells, 13% of infectious HIV-1 were intracellular and 29% in MOLT/HIV-1 cells, indicating that part of the infectious virus produced by these cells remained intracellular. To confirm that these infectious viruses were indeed intracellular and not cell surface-associated, the cells were washed, resuspended in the homogenisation buffer and either disrupted or not. After low-speed centrifugation, the pellets, containing either the cell debris (homogenized cells) or the intact cells (non-homogenized cells), were discarded and the resulting post-nuclear supernatant (PNS) or the cell supernatant (cells) were collected. Their infectivity was assessed on HeLaP4 cells. As shown in Figure 1(b), in the absence of cell lysis, there was only small amounts of infectious HIV-1 in the supernatants taken from intact cells (293T or MOLT). Following cell lysis and removal of the cell debris by centrifugation, there

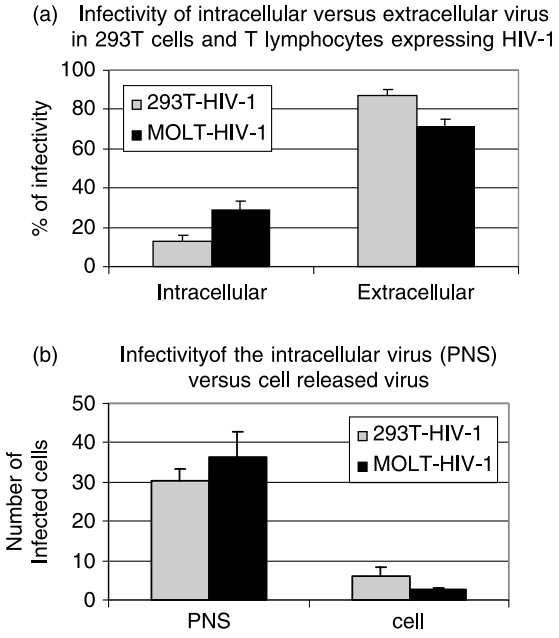

Figure 1. Presence of intracellular infectious HIV-1 in epithelial 293T and MOLT cells. (a) Total cell lysate (intracellular) and viral supernatant (extracellular) from 293T/HIV-1 and MOLT/HIV-1 cells were collected and their infectivity was assessed on HeLaP4 cells. The value is the percentage of blue cells counted in each sample reported to the sum of blue cells observed for the intra- plus extracellular viruses corresponding to 4×10^6 HIV-1 transfected 293T cells or of MOLT/HIV-1 cells. (b) 293T/HIV-1 and infected MOLT/HIV-1 cells were resuspended in homogenized buffer and proceeded to homogenization or not. The resulting PNS or intact cells were submitted to low-speed centrifugation, the pellets were discarded and the supernatants were recovered. The supernatants were then tested for infectivity on HeLaP4. The number of infected "blue" cells of two independent experiments was counted and reported in the graph as indicated. PNS, homogenized cells; cell, non-homogenized cells.

was at least six- to tenfold increase in infectious HIV-1 in the PNS of 293T and MOLT cells, respectively. These data show the presence of intracellular infectious HIV-1 in 293T/HIV-1 and MOLT/HIV-1 cells that can be released upon cell disruption.

Mature Gag, Env and the genomic RNA cofractionate with late endosomes

To investigate the location of intracellular infectious HIV-1 in 293T cells, HIV-1 was expressed by DNA transfection, and analyses were done 24 h later. Fractionation of the PNS was carried out by iodixanol gradients and fractions were analysed for their contents in Gag proteins and Env glycoproteins by immunoblotting, and in genomic RNA by RT-PCR (Figure 2(a)–(d)). Gag was found in different subcellular fractions (Figure 2(a)). Firstly, immature Pr55Gag was found at the bottom of the gradient (lanes 20–21) in a soluble cytoplasmic state non-associated with membranes. When the HIV-1-containing PNS was treated with a membrane detergent prior to fractionation, all Gag proteins sedimented at the bottom of the gradient (Figure 2(c), lanes 20–21), indicating that only Gag from those fractions was not associated with membranes. Secondly, partially processed Gag sedimented in the middle part of the gradient, most probably bound to membranes (Figure 2(a), lanes 13–17), since Gag disappeared from those fractions after detergent treatment (see Figure 2(c), lanes 13–17). Ti-Vamp (vesicle-associated membrane protein), Rab27a and Cellubrevin that are markers for the late endosomes,[23] small secretory vesicles[24,25] and recycling endosomes,[26] respectively, were found in the same fractions, indicating that Gag can be associated with recycling vesicles (lanes 18–19), or late endosomal vesicles soon after its synthesis. Thirdly, fully processed Gag was present in the middle–top fractions of the gradient (see CAp24 Figure 2(a), lanes 7–10) together with Lamp2, Ti-Vamp and Cellubrevin (Figure 2(a), lanes 7–10), and with mature Env TMgp41 (Figure 2(b), lanes 7–10). The genomic RNA was also present in the very same fractions (Figure 2(d), lanes 8–10). These results suggested that mature Gag could be associated with the genomic RNA and the mature Env glycoprotein (TMgp41) in the form of viral particles located in late endosomes/MVB (lanes 8–12). Lastly, a minor fraction of Gag was present at the top of the gradient together with mature Env and the genomic RNA (Figure 2(b) and (d), respectively, lane 1). This fraction was labelled with the Cellubrevin marker that recycles from the plasma membrane (PM),[26] indicating that at least fractions 1 and 2 correspond to the PM, and suggesting that formation of HIV-1 particles also occurs at the cell surface of 293T cells.

However, it was possible that LE-enriched fractions were contaminated by either Golgi/ER or PM containing Gag. Thus, to evaluate the possibility of PM contamination, cell surface biotinylation was performed on 293T cells expressing HIV-1 (Supplementary Data Figure 1). The results showed that after 10 min of cell surface biotinylation at 4 °C, and for equivalent amounts of total proteins in each fraction, the majority of biotinylated proteins were at the cell surface (Supplementary Data Figure 1A, fractions 1 and 2) and in fractions that should correspond to small (recycling) vesicles in the presence of HIV-1 (Supplementary Data Figure 1B, fractions 14–15), indicating that PM contamination in LE-enriched fractions was minimal. Endocytosis is probably activated in the case of cells producing HIV-1. To evaluate if LE fractions contained either Golgi or ER, a trans-median Golgi and ER-grp78 markers were used, respectively. As shown in Figure 2(a), the Golgi marker was only found in fractions 14–20, and not associated with LE fractions. The ER marker, which was not strongly associated with the LE fractions (7–10), was present in fraction 8, indicating that some Env could originate from the ER. However, since the majority of the ER marker was found in the fractions 14–15, it suggested that the mature Env gp41 resulted more from the LE-associated virions (fractions 7–10) than the ER/Golgi compartments. Overall, the data showed that mature Gag, Env and the genomic RNA have co-fractionated with late endosomes, suggesting that either mature virions and late endosomes sedimented at the same density or that HIV-1 could assemble within this compartment.

Intracellular HIV-1 virions are infectious

As shown in Figure 2, intracellular Gag together with mature Env and the genomic RNA were found associated with late endosomes. In addition, functional Gag-Pol must be present as evidenced by protease-mediated Gag cleavage to generate CAp24 (Figure 2(a), lanes 8–10). This prompted us to investigate the presence of intracellular infectious virions by testing each fraction for infectivity (it was first checked that iodixanol had no effect on HIV-1 infectivity, data not shown). Data show that the LE-associated fractions (Figure 2(a) and (e), lanes 7–10) represent 60% of the total amount of infectious virus found within cells (i.e. in the PNS), while the PM-associated fractions (lanes 1–3) represent 20%. These results suggested that infectious HIV-1 virions could accumulate in late endosomal/MVB compartments of 293T cells. In addition, the virions associated with the PM (lanes 1–2) showed an infectivity relative to Gag clearly higher than virions found in LE/MVB (lanes 8–10). These results suggest that production of infectious virus can occur at two locations in 293T cells, at the plasma membrane and in intracellular compartments associated with late endosomes.

Newly made Gag molecules reach both the plasma membrane and endosomal vesicles in 293T cells

In order to examine if after its synthesis Gag would first reach the plasma membrane or the endosomal vesicles, pulse-chase experiments

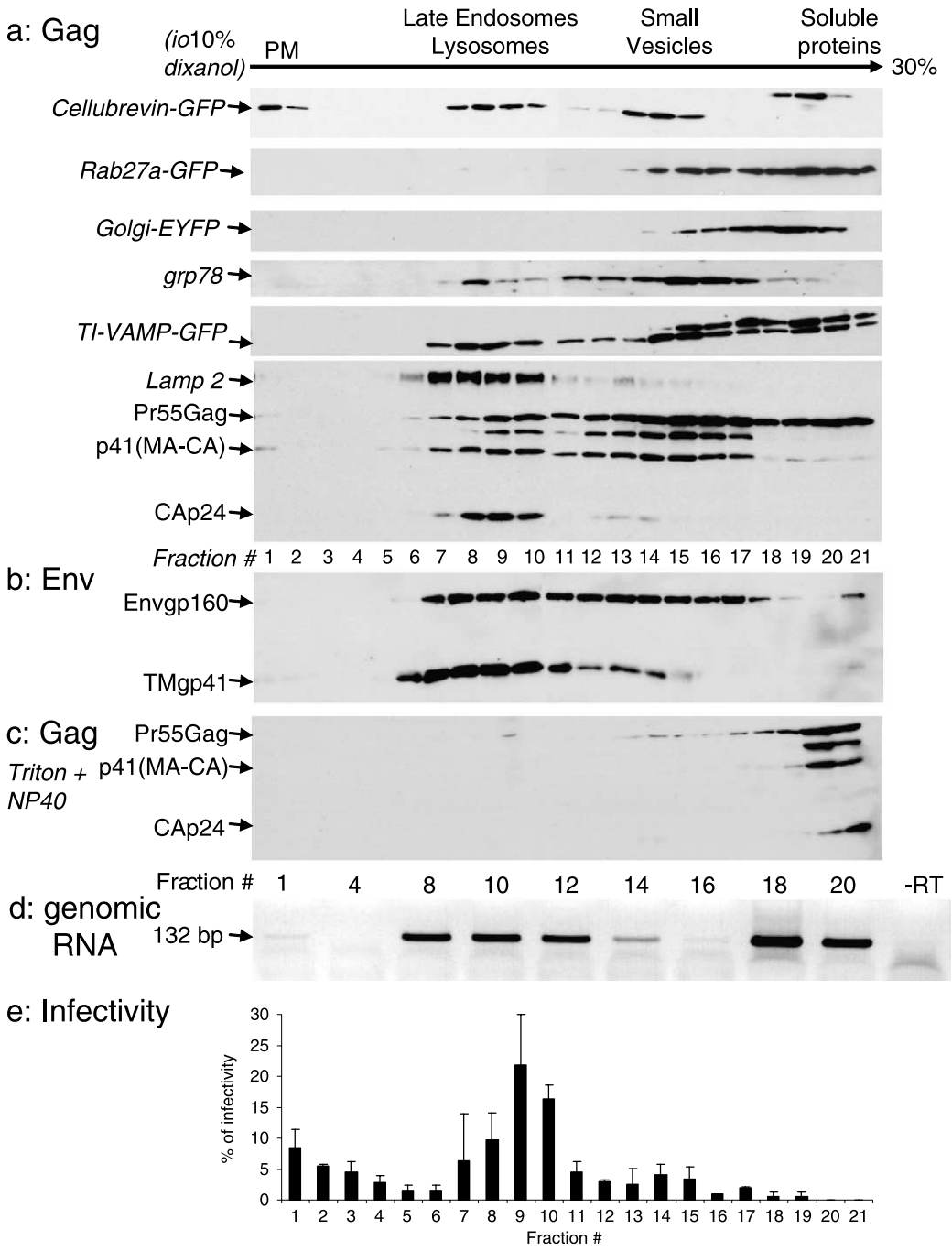

Figure 2. Accumulation of mature infectious HIV-1 virions in late endosomes. (a) and (b) 293T cells were transfected with pNL43 only, or together with pCellubrevin-GFP, pGolgi-EYFP, pTi-Vamp-GFP, or pRab27a-GFP as markers for recycling vesicles, trans-median Golgi, late endosomes or secretory vesicles, respectively. Cells were treated as described in Materials and Methods and the post-nuclear supernatant (PNS) was fractionated by Optiprep gradient centrifugation. 15 µl of each fraction was loaded on a SDS-PAGE and Gag, Lamp2, grp78 (a) and Env (b) were analysed by immunoblotting using anti-CAp24, anti-Lamp2, anti-grp78 and anti-gp41 antibodies, respectively. The cellular Cellubrevin, Rab27a, Ti-Vamp and Golgi markers were detected using an anti-GFP antibody. (c) PNS, as in (a), from 293T cells expressing wild-type HIV-1 was fractionated by Optiprep gradient centrifugation with detergent (1% Triton + 1% NP40). Aliquots of each fraction were analysed by immunoblotting using the anti-CAp24 antibody. (d) Each fraction of the gradient in (a) was tested for the presence of the genomic RNA by RT-PCR (see Materials and Methods). The expected 132 bp DNA fragment was visualized by 2% agarose gel electrophoresis and ethidium bromide staining. (−)RT indicates the control lane where the RT-PCR reaction was performed on the PNS in the absence of reverse transcriptase (RT). (e) Each fraction of the gradient in (a) was tested for infectivity on HeLaP4 cells. Numbers of positive infected cells were counted and the percentage relative to the number of infected cells found in the PNS was calculated. The average of two independent experiments is reported here.

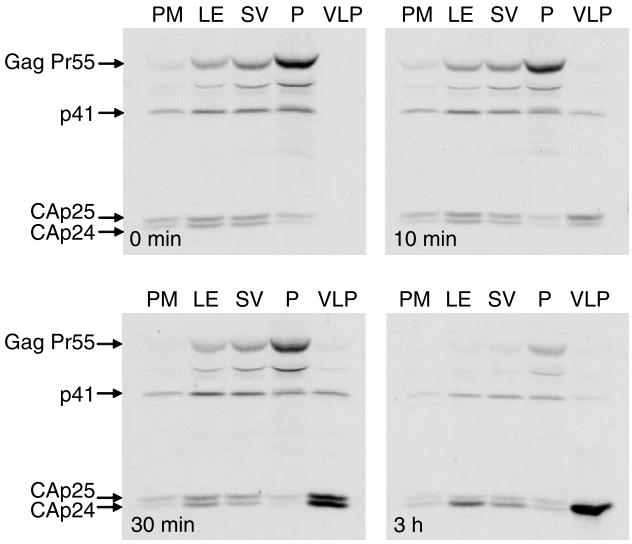

Figure 3. Pulse-chase and biochemical fractionation analysis of newly made Gag. 293T cells expressing HIV-1 were metabolically labelled with [^{35}S]methionine for 15 min and chased with unlabelled medium for 0 min, 10 min, 30 min and for 3 h, as indicated. Virions were purified by ultracentrifugation through a 20% sucrose cushion. Cells were homogenized and analysed as described in the legend to Figure 2. Virions and cell-associated proteins in homogenization buffer were immunoprecipitated using a cocktail of anti-CAp24 and anti-MAp17 antibodies and analysed by SDS-PAGE. The mobilities of Pr55Gag, incompletely processed Gag (p41 and p25), and mature CAp24 are indicated. VLP represents the extracellular virus released from the labelled cells during the time indicated. As referred to in Figure 2, the plasma membrane (PM) corresponds to a pool of fractions 1 and 2; the late endosomes (LE), fractions 8 and 9; the small vesicles (SV), fractions 14 and 15; the cytoplasmic proteins (P), fractions 20 and 21.

followed by biochemical subcellular fractionation were performed on 293T cells expressing HIV-1 (Figure 3). After 15 min of metabolic labelling, HIV-1 Gag was found mainly in the bottom fraction with the cytosolic proteins (P, time 0), and some Gag already appeared associated with endosomal vesicles (SV and LE) and at the PM. From 10 min to 3 h, the P fraction of Gag disappeared, and the extracellular mature virions increased (VLP, time 3 h). We also noticed over time an increase of mature CAp24 associated with the LE fraction and extracellular VLPs, suggesting that mature HIV-1 virions could accumulate in LE and as extracellular VLPs, then get released from the PM as well as from the endosomal compartments. These data suggest that in the model 293T cells, HIV-1 could be produced from both plasma and endosomal membranes.

Endosomal localisation of Gag, Env and genomic RNA by confocal microscopy

We then examined by immunofluorescence staining and confocal laser scanning microscopy (CLSM) whether Gag, the genomic RNA and the Env glycoproteins accumulate in LE/MVB. We found HIV-1 Gag located either in patches at the plasma membrane or in cytoplasmic vesicles (Figure 4 and Supplementary Data Figure 2B). Gag-containing vesicles were labelled with Lamp3 (Figure 4(a)) and CD81 (Figure 4(b)), which are markers for the late endosomal compartments. As observed on CLSM images and assessed by the Metamorph software, 35% of Gag accumulated in LE. Furthermore, we examined these Gag-labelled vesicles for the presence of Env and the genomic RNA in 293T cells expressing HIV-1 (Figure 4). Env was found associated with Gag either in intracellular vesicles or in large endosomal vesicles located close to the plasma membrane: these vesicles exhibited strong labelling signals for Gag, Env and the LE markers, Lamp3 (Figure 4(a), white arrows on the merge image) and CD81 (Figure 4(b)). To confirm that these vesicles containing Gag, Env and the late endosomal markers (Lamp3 or CD81) were indeed intracellular, we performed a Z-stack sectioning of the cell presented in Figure 4(a): only images in the middle section of the cell showed vesicles with the intracellular labelling for Gag, Env and Lamp3 (see white arrows in Supplementary Data Figure 2A), indicating the presence of HIV-1 Gag and Env together associated with late endosomes. We also found, by FISH coupled to immunofluorescence staining, that the genomic RNA was associated with Gag-containing vesicles and colocalized with Lamp3 (Figure 4(c), white arrows on the merge image). Altogether, these results strongly suggest that HIV-1 particles containing Gag, Env and the genomic RNA accumulate in Lamp3/CD81-positive endosomal vesicles, i.e. in late endosomes/MVB. In agreement with our results, it was recently reported that HIV-1 Gag preferentially accumulates in Rab7- and Lamp3/CD63-labelled vesicles in other cell lines.[14–16]

Infectious HIV-1 is found in late endosomes of chronically infected MOLT T lymphocytes

Since CD4+ T lymphocytes are the natural host cells for HIV-1 replication, we investigated if HIV-1 could be found in intracellular vesicles in HIV-1 infected T lymphocytes (MOLT/HIV-1). We analysed Gag and Env localization in these cells (Figure 5). The same protocols as for 293T cells were used to process the lymphocytes for gradient analysis (Figure 5(a)). HIV-1 Gag was mainly found in three fractions corresponding to immature soluble Gag (bottom of the gradient; lanes 19–21)

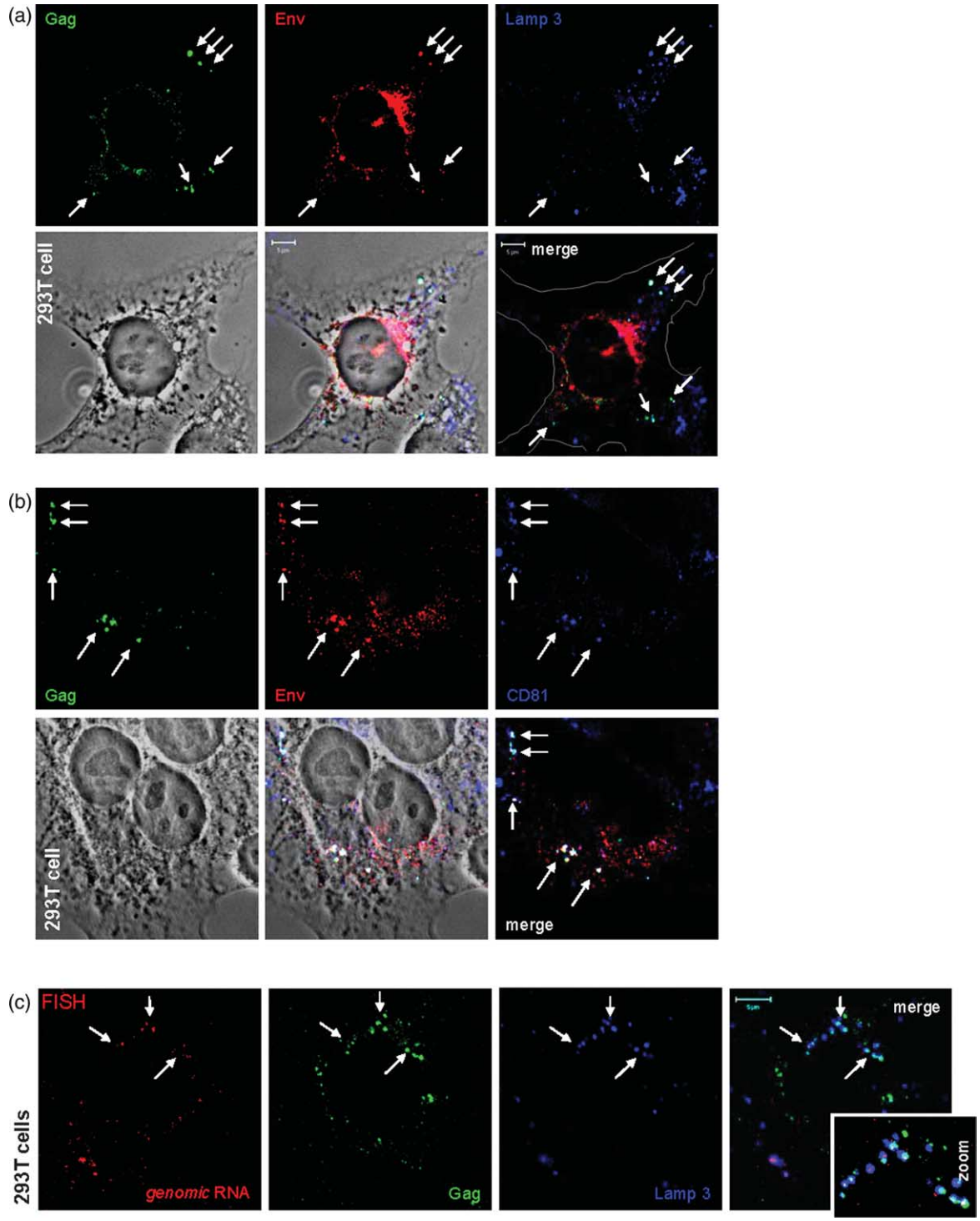

Figure 4. Localization of HIV-1 Gag, Env and genomic RNA in late endosomes. (a) 293T cells expressing HIV-1 were immuno-stained and triple imaging was carried out by CLSM to reveal HIV-1 Gag (anti-MAp17), Env (anti-gp120) and Lamp3 (also known as CD63), a marker for LE. Many intracellular large triply labelled vesicles were found close to the plasma membrane (PM), as indicated by the white arrows in the merge picture. (b) 293T cells expressing HIV-1 were also stained for CD81, another marker for LE/MVB. A significant colocalization was observed between Env, Gag and CD81. (c) 293T cells expressing HIV-1 were treated for FISH with a probe specific for HIV-1 *gag* labelled by nick-translation with dCTP-Alexa546 (a), then cells were double labelled by immuno-staining for Gag (b, anti-MAp17) and Lamp3 (c). On the merge image, white spots are vesicles labelled for the genomic RNA, Gag and Lamp3, as shown by the arrows.

that associated with small vesicles and immature Env (gp160) (lanes 13–16), and mature Gag together with mature Env (gp41) in the LE/lysosomal fractions (LE Fractions, lanes 8–10). Gag proved to be hardly detectable at the top of the gradient, corresponding to the PM fraction (lanes 1–2). The genomic RNA was also present in the fractions enriched in Gag/CAp24 and Env (gp41)

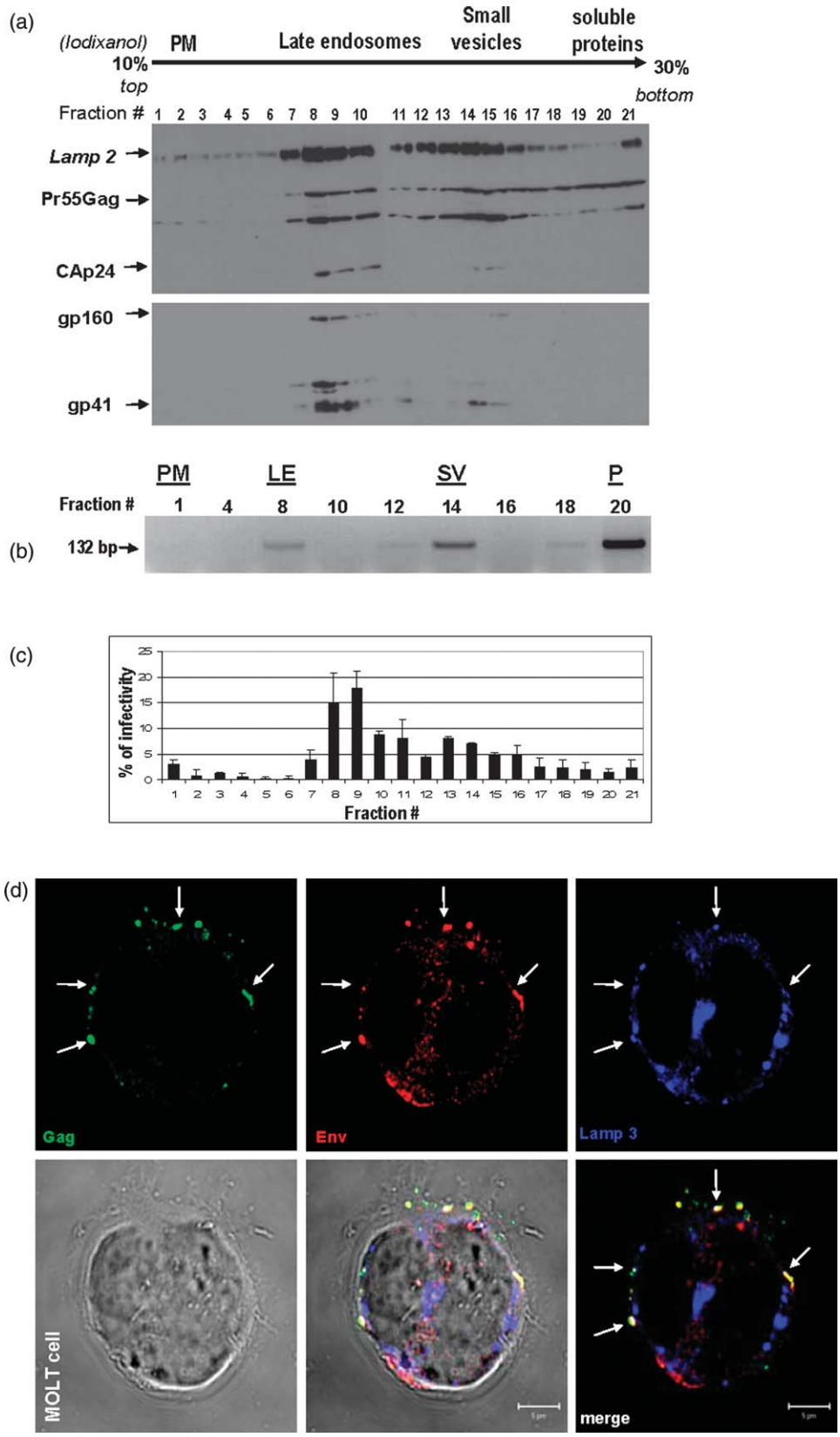

Figure 5. Accumulation of infectious virions in late endosomes of HIV-1 infected T lymphocytes. (a) Subcellular fractionation of HIV-1-infected MOLT cells. Cells were treated as described in Materials and Methods and the PNS wet fractionated by Optiprep gradient centrifugation. Fifteen microlitres of each fraction were loaded on a SDS-PAGE and Gag, Lamp2 and Env (gp160 and gp41) were analysed by immunoblotting using anti-CAp24, anti-Lamp2 and anti-gp41 antibodies, respectively, as indicated by arrows. (b) Fractions, numbers of which are indicated, of the gradient in (a) were tested for the presence of the genomic RNA by RT-PCR (see Materials and Methods). The expected 132 bp DNA

(Figure 5(b), lanes 8 and 14). In contrast to 293T cells expressing HIV-1, the majority of the genomic RNA was found associated with vesicles of smaller size (lane 14). The results indicate that in T lymphocytes, mature Gag is most probably in the form of viral particles associated with the genomic RNA and the mature Env glycoprotein (TMgp41) in intracellular vesicles such as late endosomes of different sizes, large (lanes 8–9) and small (lanes 14–15). The infection profile of the gradient fractions showed that late endosomal fractions contained infectious HIV-1, corresponding to 55% of all infectious viral particles (Figure 5(c), fractions 7–11). Small vesicles containing mature Gag, mature Env (gp41) (Figure 5(a), fractions 14–15) and the genomic RNA (Figure 5(b)) were also found to contain infectious HIV-1 (Figure 5(c)). Fractions corresponding to the plasma membrane only contained a small amount of infectious HIV-1 (~3%) (Figure 5(b), fractions 1–2) and CAp24, Env (gp41) and the genomic RNA were under the level of detection (Figure 5(a) and (b), fraction 1).

By immuno-staining coupled to confocal microscopy, we observed large vesicles labelled with Gag, Env and Lamp3, that were located close to or in limited regions of the plasma membrane (Figure 5(d), white merge signals). These results suggested that in chronically infected T lymphocytes, infectious HIV-1 particles could also accumulate in intracellular vesicles, such as late endosomes located close to the plasma membrane.

Visualisation of HIV-1 in endosomal structures by electron microscopy

In order to determine if virions were budding from the plasma membrane, endosomal structures, or both, we performed thin-sections of 293T cells and MOLT lymphocytes expressing HIV-1, for electron microscopy analyses. In 293T cells, we observed several membranes from which HIV-1 was budding (Figure 6(a)) as mature (Figure 6(a), a) or immature particles at the plasma membrane (Figure 6(a), a and b), as well as viral particles budding into intracellular vesicles close to the PM (b, arrow), or in "clear" endosomal structures (or in PM invagination) (c). Furthermore, we observed mature virions inside endosomal vesicles close to the PM (d), supporting the idea that HIV-1 virions can bud and reach full maturation in endosomal structures, and subsequently be transported to the plasma membrane for virion release.

In MOLT/HIV-1 lymphocytes, we were not able to detect viral particles budding from the plasma membrane; instead large amounts of mature virions were seen within intracellular vacuoles (Figure 6(b), a and b) or being released at the PM most probably by exocytosis (Figure 6(b), c and d).

Last, we investigated if the virus envelope was carrying markers from late endosomes. MOLT/HIV-1 cells were fixed, permeabilized and labelled in solution with anti-Lamp3 as the primary antibody and a 5 nm-Gold secondary antibody, and treated as for TEM (see Materials and Methods). In Figure 6(c), the arrows show the 5 nm-Gold/Lamp3 antibody complexes labelling HIV-1 virions produced by MOLT/HIV-1 T cells, indicating that the envelope of HIV-1 particles partly derived from late endosomal membranes. Extracellular virions from 293T/HIV-1 and MOLT/HIV-1 cell supernatants (or from mock-transfected cells or uninfected MOLT cells) were collected and doubly purified before immunoblot analysis for their content in Gag/CAp24 and Lamp3 (Figure 6(d), upper). Lamp3 was found in 293T cells (lanes 1 and 2), and in MOLT/HIV-1 cells (lane 4), barely in non-infected MOLT cells (lane 3). The Lamp3 marker was found associated with purified HIV-1 particles produced by 293T cells (Figure 6(d), lane 5) and MOLT/HIV-1 cells (Figure 6(d), lane 7), but not in mock-cell supernatant (lanes 6 and 8). The same immunoblot membrane was probed with the anti-CAp24 antibody, and the corresponding Gag and CA were found in cells and virions, as expected (Figure 6(d), below).

These results strongly suggest that HIV-1 assembly can occur on late endosomal membranes.

Stimulation of calcium-dependent exocytosis increases HIV-1 release

Since some endosomal vesicles located close to the plasma membrane contained HIV-1 virions, we hypothesized that viral particles could be released from these internal structures (LE/MVB) by exocytosis. Endosomal vesicles can fuse with the plasma membrane and release their material into the extracellular lumen *via* exocytosis, which is dependent upon incoming calcium.[27] This prompted us to examine the effect of ionomycin, a calcium ionophore, on HIV-1 release in the presence of extracellular calcium. Treatment of 293T cells expressing HIV-1 with increasing amounts of ionomycin in the presence of extracellular calcium resulted in an increase of virus production by 293T cells (Figure 7, A1) and infected T lymphocytes (MOLT/HIV-1) (Figure 7, B1), while the amount of the intracellular Gag remained unchanged (Figure 7, A2 and B2, respectively). As an internal control, we probed for a non-exosomal cellular protein, the ribosomal S6 protein: we observed that the

fragment was analysed by 2% agarose gel electrophoresis and ethidium bromide staining, in the plasma membrane (PM), late endosomes (LE), small vesicles (SV) and soluble protein (P) fractions, as indicated. (c) Each fraction of the gradient in (a) was tested for infectivity on HeLaP4 cells. Numbers of positive infected cells were counted and the percentage relative to the number of infected cells found in the PNS was calculated. The average of two independent experiments is reported here. (d) Gag and Env localization by CSLM. MOLT/HIV-1 T cells were immuno-stained and triple imaged by CLSM for HIV-1 Gag (anti-MAp17), Env (anti-gp120) and Lamp3. Triple labelled large compartments were found close to the plasma membrane and in the cell interior as indicated by the arrows.

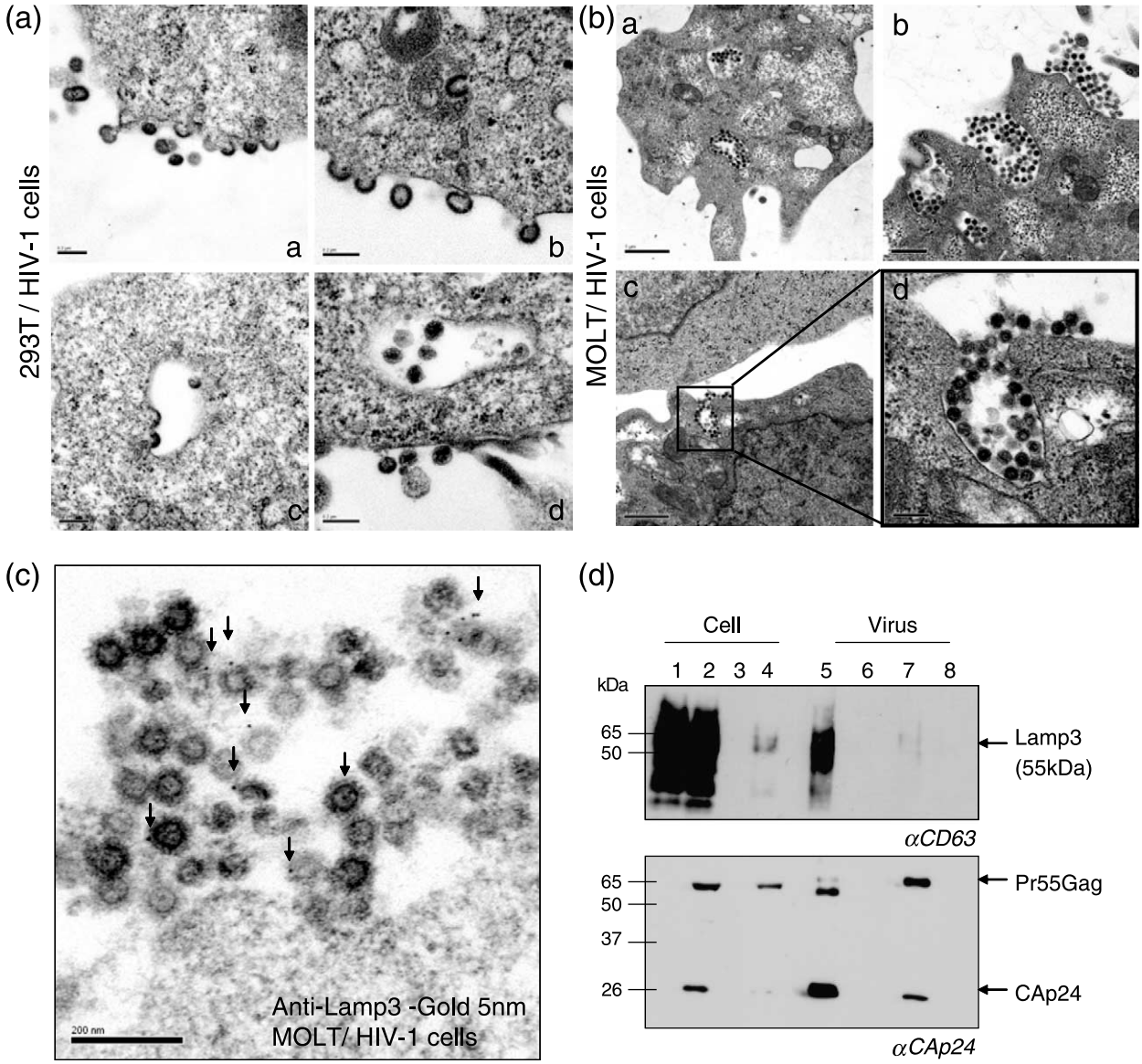

Figure 6. HIV-1 accumulates at the plasma membrane and on endosomal vesicles as viewed by electron microscopy. Thin-sections of 293T cells expressing HIV-1 (a) and MOLT/HIV-1 (b) were observed by transmission electron microscopy (TEM). Budding immature virus particles were detected at the plasma membrane (PM) ((a): a and b) and in endosomal structures ((a): b and c). Mature HIV-1 virions were apparently released from the PM ((a): a) and in endosomal vesicles close to the PM ((a): d). For MOLT/HIV-1 T lymphocytes, virions could not be detected at the PM, but all were located in intracellular compartments ((b), a and b). HIV-1 particles were released in groups by apparent fusion of the virion-containing vesicles with the PM ((b), c and zoomed image d). (c) Thin-section of MOLT/HIV-1 cells labelled with anti-Lamp3 and Gold 5 nm-anti-mouse antibodies were observed by TEM. Released HIV-1 virions are strongly labelled with anti-Lamp3 antibody, as indicated by the arrows. (d) Cell lysates of mock-transfected 293T cells (lane 1), HIV-1 transfected 293T cells (lane 2), MOLT (lane 3), MOLT/HIV-1 T cells (lane 4) and virion-associated proteins were analysed by immunoblots with anti-Lamp3 (upper panel) and anti-CAp24 (lower panel). The same amount of proteins was loaded on each lane. Virion-associated proteins from 293T/HIV-1 (lane 5) and from MOLT/HIV-1 (lane 7) were purified by ultracentrifugation through a 25%–45% double sucrose-TNE cushion and a second one through a 25% sucrose-TNE. The same amount of virions (lanes 5 and 7) or mock-associated proteins (lane 6 for 293T and lane 8 for MOLT cell lines) were loaded on the gel, corresponding to the same volume of cell supernatant.

increased amount of ionomycin had no effect on S6 intracellular level in 293T or MOLT cells (Figure 7, A2 and B2, lower immunoblots, respectively). These results indicate that, at least, part of HIV-1 virions could be released upon stimulation through a calcium-dependent exocytosis in human cell lines such as 293T cells and MOLT T lymphocytes.

Discussion

The aim of this study was to investigate whether HIV-1 virion assembly occurs at sites other than the plasma membrane in model 293T cells and in infected CD4(+) T lymphocytes that are a natural host for HIV-1.To this end, we analysed the

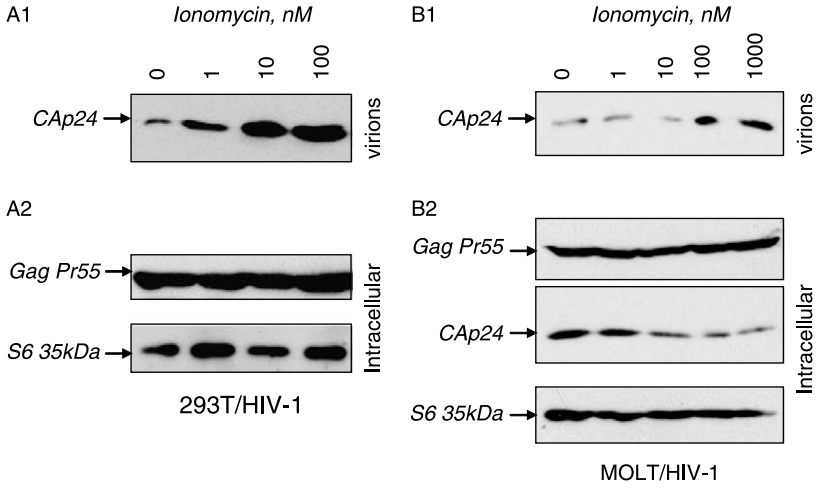

Figure 7. Effect of ionomycin on HIV-1 production. (a) 293T cells expressing HIV-1 were treated with increasing concentrations of ionomycin (0 nM, 1 nM, 10 nM, 100 nM) in the presence of 2.5 nMmM $CaCl_2$ for 4 h. The virus-containing supernatant was ultracentrifuged to recover viral particles and analysed by immunoblot using an anti-CAp24 antibody (A1). The cells were washed and lysed to evaluate the intracellular amounts of HIV-1 Gag precursor (Gag Pr55) (A2). Equivalent amounts of viral pellets and cell lysates were loaded on SDS/PAGE to evaluate by immunoblotting, the viral production by measuring CAp24 released (A1) and the intracellular Gag (A2). The cellular ribosomal protein S6 was also analysed on the same immunoblot with an anti-S6 antibody to evaluate if ionomycin treatment had an effect on a non-secreted cellular protein (A2). (b) HIV-1 infected MOLT T cells were treated for 4 h with increasing concentrations of ionomycin (0 nM, 1 nM, 10 nM, 100 nM and 1000 nM) in the presence of 2.5 mM $CaCl_2$. Viral production (B1), intracellular amounts of Gag and the ribosomal S6 protein (B2) were examined by immunoblots with anti-CAp24 and anti-S6 antibodies, respectively, as described in (a).

intracellular location of the structural components of HIV-1, namely Gag, Env and the genomic RNA in such cells. Indeed, we show that these cell lines contained intracellular infectious HIV-1 (Figure 1), as it was reported for macrophages.[18]

Using an original method preserving the diverse cellular membranes,[28] we proceeded to the subcellular fractionation of 293T cells expressing HIV-1 (Figure 2(a) and (b)), or HIV-1-infected MOLT lymphocytes (Figure 5(a)). Mature Gag and Env were found associated with LE/lysosomes as evidenced by colocalization with Lamp2 and Ti-Vamp markers, and to a lesser extent with recycling vesicles (Cellubrevin) and endoplasmic reticulum, but not with secretory ones (Rab27a) or Golgi. At the same time, it was surprising to find only small amounts of mature Gag, Env and genomic RNA at the PM. These results suggest that in 293T cells, HIV-1 assembly is taking place both at the plasma membrane and on late endosomal membranes. Yet, it was difficult to exclude that part of the Gag-containing fractions was not contaminated by PM-associated Gag spreading in the LE fractions. However, the results obtained with cell surface biotinylation on 293T cells expressing HIV-1 argue against this possibility (Supplementary Data Figure 1). Furthermore, pulse-chase experiments were performed in an attempt to localize Gag after its synthesis (Figure 3). In 293T cells expressing HIV-1, Gag reaches simultaneously the plasma membrane and the endosomal membranes. In other cell types, such as HeLa cells and lymphocytic Jurkat cells, HIV-1 Gag location was recently evaluated by dynamic fluorescent imaging: it appears that nascent Gag proteins primarily reach the PM.[29] This apparent contradiction with our results would either depend on the cell type or on the fluorescent tagged construct expressing HIV-1 Gag.

As shown by immuno-confocal microscopy, Gag, Env and the genomic RNA mainly accumulated in LE/MVB localized in the cytoplasm or very close to the plasma membrane in 293T cells and in MOLT/HIV-1 cells (Figures 4 and 5(d)). Taken together these results favour the notion that complete HIV-1 virions associated with LE/MVB membranes are routed to the extracellular space by fusion of the LE/MVB with the plasma membrane.

This prompted us to reconsider how and where the Gag core recruits the Env glycoproteins before leaving the cell as infectious particles. HIV-1 Env does not appear to strongly stimulate particle release, in contrast to other retroviruses, but it probably plays a role in Gag targeting in polarized cells and in lymphocytes.[30] In addition, rather than trafficking from the Golgi to the cell surface, Env is directed to intracellular vesicles.[31] One possibility is that Env translocation to the PM is dependent upon the recruitment of Gag on Env-containing vesicles. All together, this indicates that Gag and Env, or at least part of them, could meet first at the level of intracellular vesicles such as late endosomes, as previously shown for MuLV using MuLV-derived vectors,[32] and then traffic to the cell surface.

The genomic RNA is a major actor in virus assembly since it is involved in the viral core structure[33,34] and in the efficient Gag assembly and trafficking to cellular membranes following RNA export.[35] In a previous work, the unspliced *gag* RNA was reported to be present in the cytoplasm of HeLa cells transiently expressing HIV-1 and in HIV-1-infected T lymphocytes.[36] In the present work, the genomic RNA was found in the nucleus, the cytoplasm and within vesicles enriched in

Gag/CAp24 and Lamp (Figures 2(d) and 5(b) by RT-PCR) (Figure 4(c) by FISH), showing that the HIV-1 genomic RNA and Gag are most probably in the form of core complexes trafficking on endosomal vesicles, as suggested by recent reports on MuLV and HIV-1.[37,38] In agreement with this interpretation, deleting the Gag-NC domain, which is critical for genomic RNA selection and packaging[7,11] and assembly,[33,39] drastically alters the cellular distribution of GagΔNC, found evenly distributed at the cell surface and diffuse within the cell cytoplasm (data not shown).

Lastly, in order to differentiate between HIV-1 assembly at the PM and/or in endosomes, electron microscopy examinations were carried out. Immature virions budding at the PM and in endosomal vesicles were found in 293T cells expressing HIV-1 (Figure 6(a)). This is in agreement with a recent study showing HIV-1 Gag budding at the PM and in intracellular vesicles of 293T cells.[29] Mature virions were also seen in endosomal structures located next to the PM, suggesting that viral particles can form both at the plasma membrane and in endosomal vesicles (Figure 6(a)). To rule out the possibility that these virions were submitted to an endocytic process, 293T cells expressing HIV-1 were subjected to an immuno-staining for Gag and early endosomal markers such as EEA1, caveolin-1 or transferrin internalisation, and observed by confocal microscopy. Only 4% of Gag was labelled with EEA1 or transferrin, and none with caveolin-1 (data not shown). In addition, we investigated whether naïve 293T cells were capable of incorporating HIV-1 virions by endocytosis (Supplementary Data Figure 3A). No virus was detected and very little infectivity (less than 5%) was observed in comparison to the PNS of 293T cells expressing HIV-1. Finally, immuno-staining and triple labelling of 293T cells incubated with free HIV-1 virions, in the presence of AZT to prevent virus replication, were performed to localize Gag, Lamp3 and EEA1 (Supplementary Data Figure 3B). It was found that a small number of cells had incorporated Gag and less than 5% of intracellular HIV-1 Gag was labelled with EEA1 and none with Lamp3. Studies on HIV-1-infected MOLT cells further strengthen the point that virions can be generated from endosomal vesicles, since a high number of viral particles was only seen in large intracellular vesicles and none budding from the plasma membrane (Figure 6(b)). These results clearly show that intracellular HIV-1 virions are located in late endosomes of producing cells rather than in early/recycling endosomes that would have trapped free virions.

Another possibility is that HIV-1 particles subjected to the LE pathway are directed for degradation rather than release.[40] Indeed, we observed a cofractionation of mature HIV-1 with Lamp2, also a marker for the lysosomes, and Gag processing in those fractions (Figures 1(a) and 3(a)), but only 10% of Gag-containing vesicles were labelled with Lamp1 or Lamp2 markers by immuno-staining and CLSM analysis (data not shown). Rab7, a marker for LE/lysosome fusion, was found to colocalize with vesicles containing Gag,[15] suggesting that HIV-1 in LE/MVB is either directed for degradation in lysosomes, or for extracellular release, as proposed in the Trojan horse exosome hypothesis.[13,20] We favoured the latter hypothesis since LE-associated fractions contain mature infectious HIV-1 virions (HIV subjected to degradation would not be infectious) (Figures 2(e) and 5(b)). Furthermore, if HIV-1 virions have been formed on late endosomal membranes, markers from such membranes would be present in the virus envelope. Indeed, the late endosomal Lamp3/CD63 marker was found in purified virions, as shown by immunoblot and immunoEM (Figure 6(c) and (d)), strongly suggesting that HIV-1 egresses through the late endosomal membranes of 293T and MOLT cell lines, as shown in macrophages.[18,41] Similarly, the presence of HIV-1 particles in large vacuoles was reported in chronically infected T cells by immunoelectron microscopy, thought to be in a degradative process.[42] However, another interpretation would propose that it is a virus reservoir, as it is suggested by our results on HIV-1 infected MOLT T cells (Figures 6 and 7).

It is worth noticing that there is an apparent difference in HIV-1 localization between chronically and acutely infected T cells. It was long reported that HIV-1 budding in T lymphocytes occurs at the PM.[43] However we, and others,[42] reported the intracellular accumulation of infectious HIV-1 virions in chronically infected T cells, suggesting a selective advantage for intracellular budding during long-term culture. Another possibility would be that HIV Gag buds from endosomal-like domains located at the T cell plasma membrane, as reported recently.[44]

Finally, if part of Gag uses the LE/MVB pathway for the production of infectious HIV-1 particles, these virions would be released by a mechanism resembling exocytosis and HIV-1 production would be sensitive to drugs interfering with Ca^{2+} signalling often required in membrane fusion events.[27] In agreement with this view, we report that stimulation of regulated exocytosis by the calcium ionophore ionomycin increased HIV-1 virion release (Figure 7). These results suggest that infectious HIV-1 virions formed in late endosomes are probably released by fusion of the LE/MVB with the PM. Such a mechanism of HIV-1 virion formation and release has been reported in macrophages and HeLa cells.[16–18] Therefore, this appears to be a general mechanism in various cell types whereby HIV-1 hijacks the endosomal pathway for virion production rather than for degradation.

Further investigations are needed to determine what are the vesicular/molecular signals and proteins exploited by HIV-1 to exit the cell and what is the original route followed by the HIV-1 core complexes before recruiting the Env glycoproteins. Indeed, it was recently reported that a particular phosphoinositide can influence intracellular HIV-1 Gag targeting and thus particle production.[45]

In conclusion, the accumulation of infectious HIV-1 within infected cells, on intracellular late endosomes, as shown here in two human cell lines, seems to be a powerful strategy for HIV-1 to assemble hidden from, and thus possibly protected from, the immune system. Then under a cellular signal, such as increased intracellular calcium, that triggers exocytosis, massive transmission of the viruses could occur in a regulated manner. In addition, this endosomal production of infectious HIV-1 may favour a mechanism for virus propagation *via* cell–cell contacts through the virological synapse.[2,46] These cellular contacts between infected cells and CD4+T cells, that do not require coreceptors or cell membrane fusion, may induce the transfer of high amounts of HIV-1 particles.[47] This mechanism and HIV-1 assembly in general could thus be a target for the development of new antiretroviral drugs.[4,48]

Materials and Methods

Cell culture and transfection

Human epithelial-like embryonic kidney 293T cells, HeLa P4 cells expressing the CD4 receptor and the *lacZ* gene under the control of the HIV-1 long terminal repeat (LTR), and chronically HIV-1_{NL4-3} infected MOLT T lymphocytes were used in this study. Parental MOLT-4 cells are prototype lymphoid T cells received from NIH AIDS reagent program (NIH, USA) and infected with HIV-1_{NL4-3} to generate MOLT/HIV-1 cells. MOLT/HIV-1 cells are CD14(−), CD16(−), CD20(−) and also CD4(−) at the cell surface because HIV infection down-regulates CD4, as tested by fluorescence-activated cell sorting (FACS) analysis (data not shown). Cells were grown in Dulbecco's modified essential medium (DMEM), except for MOLT/HIV-1 that were grown in RPMI, all supplemented with 10% (v/v) fetal calf serum and antibiotics. 293T cells were transfected with DNA using the calcium phosphate method as described elsewhere.[34]

For drug treatment, 293T cells were transfected with HIV-1 pNL4-3 (0.5 μg of DNA per 3×10^5 cells in a 1 cm^2 culture dish) and 24 h post-transfection, the cells were washed once with phosphate buffered saline (PBS). Then, fresh medium containing increasing amount of drugs such as Ionomycin (Sigma) in the presence of 2.5 mM $CaCl_2$, was added on HIV-1 transfected 293T cells or MOLT/HIV-1 cells for 4 h. The cell supernatant was then collected, clarified by low speed centrifugation and viruses were pelleted by ultracentrifugation at 4 °C for 1 h at 75,000 rpm in a Beckman TL100. The cells were lysed in 0.5% Triton-X100 (Sigma) in PBS. Virion production and intracellular HIV-1 Gag precursor were analysed by immunoblotting, as described below.

Plasmid DNA

HIV-1 (NL4-3) DNA was provided by the National Institute of Health, USA. The HIV-1 Gag DNA construct[49] was provided by A. Cimarelli. Plasmids expressing Cellubrevin-GFP, Ti-Vamp-GFP, and Rab27a-GFP were a kind gift from T. Galli (France) and H. Knapton (UK), respectively. The plasmid expressing a trans-medial Golgi marker was pEYFP-Golgi vector purchased from BD Biosciences. The pcDNA3.1 plasmid (Clonetech) was used as a control DNA vector.

Subcellular fractionation and infectivity assays

At 24 h post transfection, 293T cells were removed from the plate in PBS-1 mM EDTA, pelleted by centrifugation at 600*g*, resuspended in 1 ml of a homogenisation buffer containing 10 mM Tris (pH 7.5), 0.25 M sucrose, 1 mM EDTA and protease inhibitors (Complete Mini EDTA-free from Roche), and then fragmented using a glass homogeniser. Nuclei were eliminated by centrifugation at 600*g* for 10 min at 4 °C. The resulting post-nuclear supernatant (PNS) was subjected to subcellular fractionation for the separation of the different membrane compartments.[28] Equal volumes of these three solutions were layered in centrifuge tubes subsequently centrifuged at 50,000 rpm for 3 h at 4 °C in a Beckman SW60Ti rotor. Fractions (180 μl) were collected and proteins were analysed by SDS-PAGE and immunoblotting. The same experiment was carried out with MOLT/HIV-1 cells.

Virus infectivity was assessed on HeLaP4 cells (a gift from P. Charneau, IP France). Cells were infected with 50 μl of each fraction, cultured for 48 h and then fixed with 0.5% formaldehyde in PBS for 10 min. After two washes with PBS, X-Gal was added and the cells were incubated at 37 °C. Positive blue cells were counted. The percentage of infection was calculated as the ratio of infected blue cells for each fraction divided by the number of cells infected by the PNS fraction, normalized to 100%.

Virion purification and immunoblotting

HIV-1 virions from 293T transfected-cells or from MOLT/HIV-1 cell supernatants were purified by pelleting through a double layer of 25%–45% (w/v) sucrose cushion in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA) at 25,000 rpm for 1 h 30 min in a SW28 Beckman rotor. The 5 ml sucrose–cushion interfaces containing the virions were collected, diluted in PBS and the virions were finally purified by another ultracentrifugation through a 25% sucrose–TNE cushion, and resuspended in TNE.

Viral proteins, mock-cell supernatant or cell lysate (PNS) were separated on 10% (w/v) SDS-PAGE and detected by immunoblotting with primary and secondary antibodies as follows: mouse anti-CAp24 (BioMérieux or NIH), mouse anti-GFP (Zymed®), mouse anti-Lamp2, anti-Lamp3 and goat anti-Grp78 (sc-18822, sc-5275 and sc-1050, Santa Cruz Biotechnology Inc.), mouse anti-gp41 (Hybridolab, IP), mouse anti-S6 (Gibco). Corresponding immunoglobulins conjugated with horseradish peroxidase (HRP) (DakoCytomation) were used and the signal was detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

Pulse chase and immunoprecipitation

After 1 h starvation in methionine-free and cysteine-free medium, transfected 293T cells expressing HIV-1 were metabolically labelled for 15 min with 50 μCi of [^{35}S]methionine (Amersham) per ml. The cells were then washed once with PBS and lysed immediately (time 0) or chased in unlabelled cell culture media for 10 min, 30 min or 180 min. The supernatants were collected, filtered and ultracentrifuged to pellet virions. The viral pellets were lysed in McDougal buffer (20 mM Tris (pH 8), 120 mM NaCl, 0.2 mM EDTA, 0.2 M NaF, 0.2% (w/v) sodium

deoxycholate, 0.5% NP40) containing protease inhibitors (Complete Mini-Roche, Invitrogen). The cells were washed twice with PBS-1 mM EDTA and treated as described in Subcellular fractionation. One hundred microlitres of fractions of number 1 and 2, 8 and 9, 14 and 15, 20 and 21 were mixed and lysed with McDougal buffer. Cell lysate- and virion-associated fractions were incubated with protein-G Sepharose beads and a cocktail of precleared antibodies containing anti-MAp17 and anti-CAp24 (NIH) at 4 °C overnight. The antibodies were precleared for 4 h with cold 293T cell lysate prior to the incubation. Beads were washed twice with McDougal buffer, once with a buffer containing 25 mM Tris (pH 7.5), 500 mM NaCl, 0.05% sodium deoxycholate and 0.1% NP40 and once more without NaCl. Protein associated to the beads was analysed on SDS-PAGE and by autoradiography.

RT-PCR

Fractions from Optiprep® gradients were resuspended in equal volumes of a lysis buffer containing 100 mM Tris–HCl (pH 7.4), 20 mM EDTA, 2% SDS, 200 mM NaCl and 200 μg/ml proteinase K and incubated at 37 °C for 30 min. RNA extraction was performed using acidic phenol/chloroform. RNA was precipitated with ice-cold ethanol and pelleted by centrifugation at 4 °C, 14,000 rpm for 30 min. RNA pellets were resuspended in pure water. Contaminant DNA was eliminated by digestion with RQ1 Dnase. RNA aliquots from all samples were taken and reverse transcribed using the Invitrogene RT assay. The RT reaction was followed by PCR of the cDNA using primers for the cPPT as follows: up cPPT, nt 4775 GCGCGATCGATCCACAATTTTAAAAGAAAAGGGG GGATTG, and down cPPT, nt 4907 GCGCGATCGATTG TAATAAACCCGAAAATTTTG. The PCR DNA product of 132 bp was separated on a 2% (w/v) agarose gel and visualized by ethidium bromide staining.

Immunofluorescence staining and confocal microscopy imaging

HIV-1 transfected 293T cells were grown on poly-lysine coated coverslips and fixed 24 h post DNA transfection in 3% paraformaldehyde–PBS for 20 min. The fixative was then removed and free aldehydes were quenched with 50 mM NH_4Cl. Cells were then permeabilized using 0.2% Triton X-100 for 5 min and blocked in 1% (w/v) BSA. The fixed cells were incubated for 1 h at room temperature with primary antibodies: rabbit anti-MAp17 (NIH, USA), human anti-HIV-1 gp120 Mab(b12) (NIH, USA), mouse anti-Lamp3 and anti-CD81 (Santa Cruz Biotechnology Inc.). The corresponding fluorescent Alexa® 488, 546 and 633-conjugated secondary antibodies were used (Molecular probes). Coverslips were washed and mounted on microscope slides with Mowiol (Sigma). Images were acquired on Axioplan 2 Zeiss CLSM 510 confocal microscope with Argon 488/458, HeNe 543, HeNe 633 lasers and plan apochromat 63×1.4 oil objective, supplied with LSM 510 3.4 software. The percentage of colocalization (merge signals) was evaluated by the Metamorph software (UIC).

FISH (fluorescent *in situ* hybridization)

Transfected 293T cells were grown on poly-lysine treated coverslips and 24 h post DNA transfection were washed several times with PBS, fixed as described before, treated with Dnase RQ1 (Biolabs) at 37 °C for 2 h, washed again, and stored at 4 °C in 70% (v/v) ethanol. The pHIV-1 Gag plasmid was used as a probe to detect the HIV-1 genomic RNA by hybridisation. The probe was generated by nick-translation using the Invitrogen kit. Briefly, 1 μg of plasmid DNA was incubated in a buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 1 mM DTT; 0.05 mM dTTP, dATP, dGTP and dCTP-Label-Alexa546 (Molecular Probes), 11.8 units DNA polymerase I (USB), 0.01 unit Dnase Amplification grade (Invitrogen). Samples were incubated at 15 °C for 1 h and then precipitated with ethanol, 0.3 M sodium acetate and 50 μg denaturated salmon sperm DNA. The probe was redissolved in 120 μl of 83% formamide containing 0.83 mg/ml of tRNA and 0.83 μg/ml sheared salmon sperm DNA, heated at 70 °C for 10 min and immediately chilled on ice. The probe was mixed at a ratio of 1:1 with the hybridisation buffer as described by Maldarelli & Berthold,[36] then applied to each coverslip and incubated at 37 °C overnight in a humid chamber. The following day, cells were washed, then permeabilised using 0.2% Triton X-100 in PBS and immunofluorescence staining was performed as described above for the detection of MAp17 and Lamp3.

Transmission electron microscopy

Transfected 293T cells expressing HIV-1 and MOLT/HIV lymphocytes were washed in PBS and then fixed in TRUMP (4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2), for 48 h. Cells were washed again, harvested, and post-fixed with 1% osmium tetroxide for 1 h. Cells were dehydrated in a graded series of acetone solutions and the final cell pellets were embedded in Epon resin, which was allowed to polymerise for 24 h at 60 °C. Ultrathin sections were cut with a Reichert ultramicrotome (Heidelberg, Germany), collected on copper grids and stained with 1% uranyl acetate and 1% lead citrate. The grids were then observed with a Jeol 1010 XC electron microscope (Tokyo, Japan).

In the case of immunostaining with Lamp3 antibody, HIV-1 transfected 293T cells or MOLT/HIV lymphocytes were pelleted by low-speed centrifugation, washed and then fixed in 2% paraformaldehyde in PBS for 30 min. Cells were rinsed with PBS, permeabilized in PBS with 0.2% Triton X-100 and 1% BSA for 10 min, rinsed with PBS/1% BSA and stained with the primary antibody (mouse anti-Lamp3) for 1 h at room temperature. Cells were then rinsed again and stained with the secondary 5 nm Gold-conjugated anti-mouse antibody, overnight, at 4 °C. This protocol was adapted from Giepmans and collaborators.[50] Cells were then rinsed extensively and post-fixed with the TRUMP and treated as described above for transmission electron microscopy.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.04.017.

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