Cellular aspartyl proteases promote the unconventional secretion of biologically active HIV-1 matrix protein p17

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The human immune deficiency virus type 1 (HIV-1) matrix protein p17 (p17), although devoid of a signal sequence, is released by infected cells and detected in blood and in different organs and tissues even in HIV-1-infected patients undergoing successful combined antiretroviral therapy (cART). Extracellularly, p17 deregulates the function of different cells involved in AIDS pathogenesis. The mechanism of p17 secretion, particularly during HIV-1 latency, still remains to be elucidated. A recent study showed that HIV-1-infected cells can produce Gag without spreading infection in a model of viral latency. Here we show that in Gag-expressing cells, secretion of biologically active p17 takes place at the plasma membrane and occurs following its interaction with phosphatidylinositol-(4,5)-bisphosphate and its subsequent cleavage from the precursor Gag (Pr55Gag) operated by cellular aspartyl proteases. These enzymes operate a more complex Gag polypeptide proteolysis than the HIV-1 protease, thus hypothetically generating slightly truncated or elongated p17s in their C-terminus. A 17 C-terminal residues excised p17 was found to be structurally and functionally identical to the full-length p17 demonstrating that the final C-terminal region of p17 is irrelevant for the protein's biological activity. These findings offer new opportunities to identify treatment strategies for inhibiting p17 release in the extracellular microenvironment.

The human immunodeficiency virus type 1 (HIV-1) matrix protein p17 (p17) is a small (17 kDa) basic protein that is cleaved from the precursor Gag (Pr55Gag) by the viral protease during particle release from the cell1. The three-dimensional structure of p17 has been determined by nuclear magnetic resonance (NMR) and X-ray crystallography. Individual folded p17 molecules are composed of five major α-helixes and a highly basic platform consisting of three β-strands2,3. This partially globular protein presents four helixes centrally organized to form a compact globular domain capped by the β-sheets. The fifth helix (H5) in the C-terminus of the protein projects away from the packed bundle of helixes and, according to NMR, p17 conformation in this region may be partially unfolded4. The NH2-terminal region of p17 is modified by myristoylation, which is important for membrane binding5–7. In addition, the p17 highly basic region, which is comprised of residues 17 to 31, interacts with acidic phospholipids in the inner leaflet of the membrane and is required for targeting of Gag to the plasma membrane during viral assembly8,9. The ability of Gag, through p17, to co-localize at specific subcellular membranes, is essential for viral replication and for establishing intracellular viral reservoirs that are protected from the immune system10–12.

Besides its well established role in the virus life cycle, increasing evidences suggest a role for exogenous p17 in deregulating the biological activity of different immune cells13–16, which are relevant in the context of viral pathogenesis. More recently, p17 was also found to exert chemokine and pro-angiogenic activities. These are mediated

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by p17 binding to CXCR1 and CXCR2, also termed IL-8RA and IL-8RB, and indeed, p17 was found to mimic some of the biological activities exerted by IL-817-19.

Significant amounts of virion-free p17 are released from cells during active HIV-1 replication1. Moreover, p17 was found to be released in the course of combined antiretroviral therapy (cART) and during viral latency6,20. Several reports have shown that HIV-1 transcription can be efficiently induced by different stimuli21 even in the presence of protease inhibitors22, further suggesting the possibility that p17 synthesis and release may occur under cART. Moreover, latently infected resting CD4+ T cells were found to transcribe and translate Gag proteins without stimulation while in a latent state23, supporting the hypothesis that resting CD4+ T cells can synthesize HIV-1 Gag proteins without contributing to the spread of infection. All these findings strongly suggest that p17 may be chronically present in the infected microenvironment, even during pharmacological control of viral replication, thus in the absence of any HIV-1 protease activity.

Although p17 is devoid of signal sequence, the fact that nanomolar p17 concentrations are present in the plasma of HIV-1-infected patients16 indicates that this secretion process should be efficient. Nevertheless, if the production and release of p17 is conceivable during HIV-1 replication, the mechanism of p17 secretion during HIV-1 latency, in the absence of any viral protease activity, remains to be elucidated. Some cell proteins characterized by small dimensions, including numerous cytokines, are secreted despite their lack of signal sequence by unconventional export routes24-26. Previous studies showed that the highly basic region at the p17 NH2-terminus interacts with PI(4,5)P₂27-29. PI(4,5)P₂ is a phospholipid that is specifically concentrated within the inner leaflet of the plasma membrane wherein it recruits proteins involved in several important cell activities such as endocytosis, phagocytosis, exocytosis and cell adhesion28,29. Interestingly, the HIV-1 transactivating regulatory protein Tat has been recently found to be secreted in an unconventional manner after binding to PI(4,5)P₂30. This prompted us to investigate possible unconventional routes of p17 secretion via PI(4,5)P₂ interaction.

Herein we report that p17 is released in an active form by Gag-expressing cells. We show that secretion of p17 takes place at the plasma membrane and involves the interaction of p17 with PI(4,5)P₂. The release of p17 occurs following its cleavage by Pr55Gag by cellular aspartyl proteases.

**Results**

**A cellular ELISA to quantify p17 secretion by T cells.** Our first aim was to set up a sensitive ELISA to quantify p17 in culture medium. A standard curve was generated using different concentrations of recombinant p17 diluted in complete medium. Capture of recombinant p17 was performed by the anti-p17 mAb MBS-15 coated on 96-well plates, whereas detection was obtained by adding biotin-conjugated anti-p17 mAb MBS-34 and peroxidase-labeled streptavidin. As shown in Fig. 1A, the limit of quantitation for the p17 capture ELISA was 0.125 nM of p17 since measurements of p17 detection at this concentration was the lowest with a p-value still significant (p < 0.001) as compared to the background signal. Specificity of the assay was demonstrated using HIV-1 p24 as unrelated protein in the ELISA, whose signal never significantly exceeded the level of background noise. Indeed, the HIV-1 capsid protein was not detected by the anti-p24 mAbs MBS-15 even when it was added to p24 as unrelated protein in the ELISA, whose signal never significantly exceeded the level of background noise.

The amount of p17 secreted by Jurkat-Gag, as measured by the anti-p17 mAb MBS-12, even when it was added to the well culture at a concentration as high as 16 nM. Then, we developed a protocol for quantitating p17 secretion by living HIV-1-infected or by Gag-expressing cells.Jurkat cells, which are similar to most HIV-1 infected CD4+ T-cells31, were first labeled with CFSE (used as a control for cell lysis), then transiently nucleofected with pNL4-3 (Jurkat-HIV-1), with AG49CMV-Gag-RTEn26EGFP plasmid (Jurkat-Gag) or with pEGFP-N3 (Jurkat-EGFP; used as negative control and for evaluating nucleofection efficiency). Nucleofection efficiency was always higher than 80% as assessed by flow cytometric analysis. Twenty-four hours after nucleofection, 7 × 10⁶ Jurkat-HIV-1, Jurkat-Gag or Jurkat-EGFP cells were cultured for 24 h in complete medium in anti-p17 mAb MBS-15-coated wells of ELISA plates. Plate-bound p17, secreted by seeded Jurkat-HIV-1 or by Jurkat-Gag cells, was then quantified by ELISA. As previously reported1, p17 was easily detected in the supernatant of Jurkat-HIV-1 and the amount of secreted p17 ranged from 2.39 to 5.77 nM (mean ± SD:4.11 ± 0.75 nM). At the same time, p17 was also secreted by Jurkat-Gag cells and this occurred in the absence of any viral protease. The amount of p17 secreted by Jurkat-Gag ranged from 1.54 to 5.14 nM (mean ± SD: 3.47 ± 0.44 nM) (Fig. 1B, left panel). CFSE was not released in tissue culture supernatants of nucleofected cells, attesting to their viability during the whole period of cell culture (Fig. 1B, right panel).

In order to confirm the intracellular expression of Pr55Gag, as well as the release of extracellular p17 protein dissociated from Pr55Gag, we immunoprecipitated the two viral proteins from the cell lysate and from the supernatant of Jurkat-Gag cells, using a rabbit anti-p17 polyclonal antibody as specific reagent. As shown in Fig. 1C (left panel), Jurkat-Gag cell lysate showed the expression of Pr55Gag. On the other hand, when rabbit anti-p17 polyclonal antibody was used to precipitate p17 from the supernatant of Jurkat-Gag cells, p17 was the only protein detected in the immunoprecipitated sample (Fig. 1C, right panel). The proper molecular size of the viral protein suggests the presence of a specific cellular protease at work in cleaving p17 from Pr55Gag. At the same time, p24 was not detected in the immunoprecipitated material, thus excluding the presence of Pr55Gag in the cell supernatant (Fig. 1C, right panel).

**Secreted p17 is biologically active.** To evaluate whether secreted p17 was biologically active, we took advantage of the ability of the protein to promote angiogenesis at a concentration as low as 0.58 nM20. In particular, we investigated the effect of p17 on human endothelial cell migratory activity using the wound healing assay23. This method highlights the ability of p17 to promote endothelial cell migration by sealing a confluent cell monolayer after a mechanical injury. HUVECs were grown on collagen-coated plates and starved for 24 h, then co-cultured with 1 × 10⁶ non-transfected Jurkat, Jurkat-Gag or Jurkat-EGFP cells. After an additional 24 h of incubation Jurkat cells were removed, confluent HUVEC monolayers were scratched with a 200-μl tip, then recorded over a period of 12 h. As shown in Fig. 2A, control HUVECs reached approximately 35% sealing (range from 28% to 44%) after 12 h of culture. The same level of sealing was reached when HUVECs were co-cultured in the
presence of Jurkat-EGFP. On the other hand, HUVECs co-cultured with Jurkat-Gag cells reached 100% sealing, showing a considerable improvement in scrape wound repair ability. In order to confirm the direct involvement of p17 in promoting HUVEC migration, cell monolayers were cultured with Jurkat-Gag cells in the presence or absence of the p17 neutralizing mAb MBS-3 (2.5 μg/ml). Again, HUVECs reached 100% of wound sealing after 12 h of culture in p17-containing medium, even in the presence of an isotype-matched mAb. At the same time, the anti-p17 neutralizing mAb MBS-3 was strongly inhibitory (range from 77.2% to 88.8%) (Fig. 2B), thus confirming that enhancement of HUVEC migratory activity has to be ascribed to Jurkat-Gag secreted p17.

Pr55Gag binding to PI(4,5)P2 is required for p17 secretion. Pr55Gag binding to PI(4,5)P2 is required for p17 secretion. PI(4,5)P2 is a phospholipid that is specifically concentrated within the inner leaflet of the plasma membrane. Several groups have examined the interaction between p17 and PI(4,5)P2 and their studies revealed that the highly basic residues (HBR) in the p17 NH2-terminal region are responsible for such interaction. Therefore, it is likely that precursor Pr55Gag processing has to occur after p17 binding to PI(4,5)P2. To assess the role of p17/PI(4,5)P2 interaction in the process of p17 secretion, we evaluated the presence of viral protein in the supernatant of Jurkat-Gag treated with neomycin, a polycationic aminoglycoside antibiotic which binds to PI(4,5)P2 ligands by steric hindrance. Neomycin, in concurrence with previous results, was deprived of any cell toxicity at concentrations ranging from 100 to 500 μM. At both concentrations, neomycin was found to completely inhibit p17 secretion (Fig. 3A). Evaluation of the intracellular localization of p17 in the presence of neomycin was then carried out by confocal microscopy. HeLa cells were nucleofected with AG49CMV-Gag-RTEm26CTE (HeLa-Gag), then stained with the anti-p17 mAb MBS-3. Representative confocal images of untreated cells showed p17 as a punctate staining at the plasma membrane, whereas cells treated with neomycin displayed a...
diffuse staining in the cytoplasm (Fig. 3B). These results confirm that displacement of Pr55Gag/PI(4,5)P2 interaction by neomycin prevents its localization to the plasma membrane and p17 secretion. To further confirm a key role of PI(4,5)P2 in the p17 secretion mechanism, we reduced cellular PI(4,5)P2 by overexpressing 5ptaseIV, an enzyme that depletes cellular PI(4,5)P2, and examined the impact of PI(4,5)P2 depletion on p17 secretion. As shown in Fig. 3C, p17 secretion was strongly impaired in Jurkat cells co-nucleofected with AG49CMV Gag-RTEm26CTE and the 5ptaseIV expression vector (Jurkat-Gag-5ptaseIV). At the same time, Jurkat cells co-nucleofected with AG49CMV Gag-RTEm26CTE and a vector expressing the mutant form of 5ptaseIV (Jurkat-Gag-5ptaseIV-Δ) lacking the 5-phosphatase signature domain, released p17 at concentrations not significantly different from those detected in the supernatant of Jurkat-Gag cells. We next examined whether localization of Gag to the plasma membrane was affected by PI(4,5)P2 depletion. HeLa cells were co-nucleofected with AG49CMV Gag-RTEm26CTE and the 5ptaseIV expression vector (HeLa-Gag-5ptaseIV) and analysed by confocal microscopy. As shown in Fig. 3D, HeLa-Gag overexpressing 5ptaseIV displayed a punctate staining at the plasma membrane resembling that observed in HeLa-Gag.

Different authors have demonstrated that Gag binding to PI(4,5)P2 triggers exposure of the amino-terminal myristic acid moiety that inserts into the membrane, anchoring and stabilizing Gag-membrane interaction. Therefore, we asked whether elimination of the myristic acid moiety within p17 was impairing its secretion. As shown in Fig. 3C, cells expressing a not myristoylated G2A mutated Gag (GagG2A) were impaired in their capability to secrete p17. At the same time, GagG2A-expressing cells showed a lower p17 signal at the cell surface compared to cells expressing Gag, but instead typically displayed a greater accumulation of p17 throughout the cytoplasm.

To further support the hypothesis that p17 secretion relies on anchorage of Pr55Gag to plasma membrane through PI(4,5)P2 interaction, we evaluated the role of p17 HBR in p17 secretion by using different mutated Pr55Gag polyproteins. We first generated a Gag mutant with reduced PI(4,5)P2 binding, based on previous reports showing that mutation of lysine 30 and 32 to threonine (K30T/K32T) within the p17 HBR impairs Gag/PI(4,5)P2 interaction and relocalizes Gag from the plasma membrane to intracellular compartments. As shown in Fig. 3C, secretion of p17 by Jurkat cells expressing the GagK30T/K32T was significantly reduced — but not completely abolished — compared to cells expressing Gag. Moreover, as expected, confocal images of cells expressing the GagK30T/K32T showed a diffuse staining in the cytoplasm (Fig. 3B). These results confirm that displacement of Pr55Gag/PI(4,5)P2 interaction by neomycin prevents its localization to the plasma membrane and p17 secretion. To further confirm a key role of PI(4,5)P2 in the p17 secretion mechanism, we reduced cellular PI(4,5)P2 by overexpressing 5ptaseIV, an enzyme that depletes cellular PI(4,5)P2, and examined the impact of PI(4,5)P2 depletion on p17 secretion. As shown in Fig. 3C, p17 secretion was strongly impaired in Jurkat cells co-nucleofected with AG49CMV Gag-RTEm26CTE and the 5ptaseIV expression vector (Jurkat-Gag-5ptaseIV). At the same time, Jurkat cells co-nucleofected with AG49CMV Gag-RTEm26CTE and a vector expressing the mutant form of 5ptaseIV (Jurkat-Gag-5ptaseIV-Δ) lacking the 5-phosphatase signature domain, released p17 at concentrations not significantly different from those detected in the supernatant of Jurkat-Gag cells. We next examined whether localization of Gag to the plasma membrane was affected by PI(4,5)P2 depletion. HeLa cells were co-nucleofected with AG49CMV Gag-RTEm26CTE and the 5ptaseIV expression vector (HeLa-Gag-5ptaseIV) and analysed by confocal microscopy. As shown in Fig. 3D, HeLa-Gag overexpressing 5ptaseIV displayed a punctate staining at the plasma membrane resembling that observed in HeLa-Gag.

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redistribution of p17 in the cytoplasm (Fig. 3D), as compared to the plasma membrane-associated expression in cells nucleofected with Gag (Fig. 3B). At the same time, a GagK26T/K27T mutant with elevated PI(4,5)P2-independent binding to plasma membrane36 showed a dramatic impairment of p17 secretion compared to Gag (Fig. 3C) and a marked p17 intracellular accumulation (Fig. 3D). This result indicates that promiscuous Gag binding to membrane is not sufficient to promote p17 cleavage and secretion and highlights the role of specific amino acid residues within the

Figure 3. PI(4,5)P2 is involved in p17 secretion. (A) Quantification of extracellular p17 released by neomycin-treated Jurkat-Gag cells. Jurkat cells were transfected with AG49CMVGag-RTEm26CTE and cultured for 24h in the absence or in the presence of neomycin, at the concentration of 100μM and 500μM. After washing, cells were added to anti-p17 mAb MBS15-coated wells for 16h at 37°C in the absence or in the presence of 100μM or 500μM of neomycin. Plate-bound p17, secreted by seeded Jurkat-Gag cells, was then quantified by ELISA. Amount of secreted p17 was calculated as mean ± SD of three independent experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA, and the Bonferroni’s post-test was used to compare data (**P < 0.01). (B) Intracellular localization of p17 in Gag-expressing cells. Gag-expressing HeLa (HeLa-Gag) cells, soon after nucleofection with AG49CMVGag-RTEm26CTE, were cultured for 16h at 37°C in the absence (NT) or in the presence of neomycin (100μM). Cells were then fixed and permeabilized as described in the materials and methods section. Cells were then stained with biotinylated anti-p17 mAb MBS-3 followed by Alexa488-streptavidin and 4′,6-diamidino-2-phenylindole. Analysis was performed by confocal fluorescence microscopy. Images display mAb MBS-3 signals in green and cell nuclei in blue. z-Stack sections and orthogonal z reconstitution are also shown. Scale bar, 10μM. (C) Quantification of p17 released by Jurkat cells co-nucleofected with AG49CMVGag-RTEm26CTE and 5ptaseIV or 5ptaseIV-Δ1 mutant expression plasmids, or by Jurkat cells expressing G2A, K30T/K32T or K26T/K27T Gag mutants. Amount of secreted p17 was calculated as mean ± SD of three independent experiments performed in triplicate. Statistical analysis was preformed by one-way ANOVA, and the Bonferroni’s post-test was used to compare data (***P < 0.001). (D) Intracellular localization of p17 in HeLa cells co-expressing Gag and 5ptaseIV or 5ptaseIV-Δ1 and in HeLa cells expressing G2A, K30T/K32T or K26T/K27T Gag mutants. Cells were stained with biotinylated anti-p17 mAb MBS-3 followed by Alexa488-streptavidin (green) and 4′,6-diamidino-2-phenylindole (in blue). Representative median confocal sections of at least 20 cells per conditions are shown. z-Stack sections and orthogonal z reconstitution are also shown. Scale bar, 10μM.
p17 HBR in ensuring specific Gag/PI(4,5)P2 interaction, thus allowing p17 proteolytic cleavage from Pr55Gag and its release in the extracellular microenvironment. Altogether, our data show that targeting Gag to the plasma membrane through PI(4,5)P2 interaction is the only feasible mechanism for p17 secretion.

Molecular basis for p17 secretion. The structure of the p17/PI(4,5)P2 complex has been recently defined26. NMR data are consistent with a single binding mode which involves, in addition to the p17 highly basic domain, the participation of other parts of the molecule. Among hydrophobic residues that could be involved in PI(4,5)P2 interaction, p17 contains a well-preserved tryptophan at position 36 (W36). W36 was found to be important in interacting with both the glycerol moiety and the phosphoinositide head group of PI(4,5)P2 and in participating to the p17/PI(4,5)P2 complex stabilization26. Another tryptophan at position 16 (W16) is also located near the p17 highly basic domain. Binding of p17 to p17/PI(4,5)P2 results in small local conformational changes that reposition hydrophobic residues such as W16 26. To examine whether W16 and/or W36 were important in participating to the mechanism of p17 secretion, we determined the presence of p17 in the supernatant of Jurkat cells nucleofected with AG49CMV GagW16A-RTEm26CTE (Jurkat-Gag-W16A) or AG49CMV GagW36A-RTEm26CTE (Jurkat-Gag-W36A) constructs carrying respectively a W16Alanin (A) and a W36A mutation in the Gag sequence. As shown in Fig. 4A, W36A mutation strongly impaired p17 secretion,

Figure 4. Secretion of p17 does not occur if its W36 residue is mutated or when it is expressed alone. (A) Quantification of extracellular p17 released by Jurkat cells expressing W16A or W36A Gag mutants. Cells were nucleofected with construct expressing wild type Gag or Gag mutants containing W16A and W36A amino acid changes. After 24 h later cells were cultured for 16h onto ELISA plates and secreted p17 was quantified by cellular ELISA. Box plots represent statistical analyses of five independent experiments. Significance was assessed using one-way ANOVA and the Bonferroni’s post-test was used to compare data; ***P < 0.001. (B) Confocal analysis of Gag localization. HeLa nucleofected with the same constructs as in (A) were stained with biotinylated anti-p17 mAb MBS-3 followed by Alexa488-streptavidin (green) and 4′,6-diamidino-2-phenylindole (in blue). Representative median confocal sections of at least 20 cells per conditions are shown. Images highlight that W36A mutation leads to protein accumulation in proximity of the plasma membrane. z-Stack sections and orthogonal z reconstitution are also shown. Scale bar, 10μM. (C) Quantification of extracellular p17 released by Jurkat cells expressing wild type p17 (myr⁺) or its G2A mutant (myr⁻). Cells were nucleofected with the construct that expresses wild type Gag or with constructs that express p17 or a mutant containing the G2A amino acid change (p17 G2A). Cells were recovered 24 h later, then cultured for 16h onto ELISA plates and secreted p17 was quantified by cellular ELISA as in Fig. 1. Box plots represent statistical analyses of five independent experiments. Significance was assessed using one-way ANOVA and the Bonferroni’s post-test was used to compare data; ***P < 0.001. (D) Confocal analysis of p17 localization. HeLa nucleofected with the same constructs as in (C) were stained with biotinylated anti-p17 mAb MBS-3 followed by Alexa488-streptavidin (green) and 4′,6-diamidino-2-phenylindole (in blue). Representative median confocal sections of at least 20 cells per conditions are shown. Images highlight that p17 alone is trapped in the cell cytosolic compartment regardless of whether or not it is myristoylated. z-Stack sections and orthogonal z reconstitution are also shown. Scale bar, 10μM.
as assessed by the low amount of extracellular p17 detected in Jurkat-GagW36A supernatants (0.08–0.26 nM). On the other hand, Jurkat-GagW16A secreted approximately the same amount of p17 (1.2–2.35 nM) as those nucleofected with AG49CMV-Gag-RTEm26CTE (1.37–2.64 nM). This finding indicates the importance of W36 but not of W16 in the p17 secretion mechanism. Confocal analysis performed on nucleofected HeLa cells revealed that differently to HeLa-Gag, HeLa cells expressing GagW36A displayed a uniform distribution of the viral protein in the cytoplasm and at near all areas of the plasma membrane (Fig. 4B). This evidence suggests that mutation in W36 does not affect the ability of p17 to reach the plasma membrane.

In order to assess if p17 needs to be part of Pr55Gag to reach plasma membrane and be subsequently cleaved and secreted, or if p17 can be also secreted after Pr55Gag enzymatic digestion in intracellular compartments other than plasma membrane, further experiments were conducted by expressing p17 alone in cells. There is considerable evidence that PI(4,5)P2 binds to a conserved cleft on p17 and promotes myristate exposure and tight membrane binding26. Therefore, we expressed myristoylated (myr+) and not myristoylated (myr−) p17 in Jurkat cells by nucleofecting them with AG49CMVp17-RTEm26CTE (Jurkat-p17) or AG49CMVp17G2A-RTEm26CTE (Jurkat-p17G2A) constructs, carrying respectively a wild type and a G2A mutation in the p17 sequence and checked for secretion of p17 in the supernatant. As shown in Fig. 4C, we found that both myr+ and myr− p17s are not secreted although, using confocal microscopy, they are well expressed in the cell cytosolic compartment (Fig. 4D). This result is in agreement with previous data showing that within full-length Pr55Gag, the membrane targeting signal is exposed and can direct Pr55Gag to the plasma membrane, whereas in the context of p17 alone, this signal is hidden and unable to confer plasma membrane binding40,41. This data suggests that p17 secretion occurs only after Pr55Gag targeting to PI(4,5)P2 on the plasma membrane.

Secretion of p17 is modulated by inhibitors of cellular aspartyl proteases. Cellular ELISA and Western blot confirmed the presence of properly sized and biologically active p17 in the supernatant of Jurkat-Gag cells, suggesting that Gag polyproteins are cleaved by cellular proteases. To confirm this hypothesis, a cocktail of different cellular protease inhibitors were added to Jurkat-Gag cells into the ELISA plate. Data obtained after 16 h of culture showed that the cocktail of inhibitors completely blocked p17 secretion (Fig. 5A). Therefore, we examined in detail which inhibitor(s) of the cocktail was responsible for this effect. Different amounts of each protease inhibitor included in the original cocktail were used according to the higher dose without any toxic effect.
on Jurkat-Gag. In particular, Pepstatin-A (an inhibitor of aspartyl proteases), Aprotinin (a serine protease inhibitor), and Bestatin (an aminopeptidase inhibitor) were used at 0.5 μM, whereas Leupeptin (an inhibitor of serine and cysteine protease) was used at the concentration of 10 μM. As shown in Fig. 5B, Pepstatin A almost completely abolished p17 secretion, whereas Bestatin, Aprotinin and Leupeptin did not show any significant effect in modulating p17 secretion. The capability of Pepstatin A to inhibit p17 secretion was found to be statistically significant even at a concentration as low as 0.007 μM (Fig. 5C). As a result of Pepstatin A activity, p17 expression was scattered within the cell cytoplasm but it resulted to be more pronounced at the plasma membrane (Fig. 5D). This result suggests that Pepstatin A favors p17 accumulation in Gag-expressing cells by interfering with its enzymatic cleavage from Pr55Gag.

Renin, Cathepsin-D and γ-secretase cleave p17 from the Gag polyprotein. Pepstatin-A is a highly selective inhibitor of aspartyl proteases, forming a complex with γ-secretase. Renin and Cathepsin-D, with the exception of an aspartyl protease with β-secretase activity named BACE-1, in order to evaluate the capability of γ-secretase to cleave p17 from the precursor Pr55Gag, we examined the effect of two specific inhibitors of γ-secretase, namely Avagacestat and LY411575, on p17 secretion. As shown in Fig. 6, Avagacestat and LY411575 were both able to interfere with p17 secretion from Jurkat-Gag cells. However, both inhibitors only partially impaired protein secretion, being able to reduce p17 detection of approximately 53.9% and 46.2% using Avagacestat and LY411575 at the not toxic concentration of 10 μM and 500 μM, respectively. Following the only partial decrease of p17 secretion observed by blocking γ-secretase activity, we asked whether other aspartyl proteases as Renin, Cathepsin-D or BACE-1 might be involved in the p17 cleavage from the Gag polyprotein. To this purpose, the enzymatic activity of these proteases was tested in vitro on a synthetic peptide representative of the precursor Pr55Gag region containing the cleavage site of p17 from p24 (p17/p24). As summarized in Fig. 7, HIV-1 protease cleaved specifically the p17-p24 peptide only between the tyrosine (Y) 132 of p17 and proline (P) 1 of p24 (see also Fig. S1). Interestingly, both Renin and Cathepsin-D were found to hydrolyze the p17-p24 peptide. Renin operated a complex proteolysis, by generating two different peptides: the first one resulted from the cleavage between asparagine (N) 126 of p17 and P1 of p24 and the second one between serine (S) 129 of p17 and glutamine (Q) 4 of p24 (see also Fig. S2). Therefore, Renin is likely to produce a 4 amino acid shorter p17 (p17Δ4) and a protein containing an additional amino acid (P) at its C-terminus belonging to the p24 molecule. Also Cathepsin-D was found to cleave the peptide in two sites, the first one is the canonical cleavage site for HIV-1 protease, located between Y132 of p17 and P1 of p24 and the second one between Q7 and glycine (G) 8 of p24. This finding attests that Cathepsin-D proteolysis of the Gag polyprotein is likely to result in the production of a full-length p17 and of a p24 devoid of seven amino acids at its N-terminus (see also Fig. S3). Our results show that the consensus primary sequence, at least for Renin, does not overlap with that of the HIV-1 protease. This finding is in agreement with previous observation showing that there is no consensus sequence for proteolysis among different substrates for aspartyl proteases. Interestingly, BACE-1 was totally unable to hydrolyze the peptide (see also Fig. S4). These results show that Cathepsin-D, Renin and γ-secretase are likely participating to p17 cleavage from the Gag polyprotein. The possibility that cellular aspartyl proteases may have more cleavage sites on p24 cannot be ruled out. Further studies using p24 peptides or the entire Gag polyprotein as specific substrates for cellular aspartyl proteases are necessary to test this hypothesis.

Impact of the C-terminus on p17 structure and bioactivity. Proteolysis of the Gag polyprotein operated by cellular aspartyl proteases is likely to produce p17s slightly different in their C-terminus in terms of length. Since we found that secreted p17 is biologically active, further experiments were conducted to understand the role of the C-terminus in the p17 action. Recent data point to the C-terminus of p17 as partially α-helical
(H5 helix) ending in a disordered region which would have little impact on protein folding and stability. The disordered C-terminal region of p17 varies in length in all known solution and crystal structures, where the last H5 \(\alpha\)-helix terminates at residues 109–122 depending on experimental conditions \(^{47,48}\). We synthesized a C-terminally truncated p17 protein with 17 disordered C-terminal residues excised (p17\(\Delta\)17) on the basis of the crystal structure of p17"}\(^{48}\). As predicted, NH-NH NOEs of synthetic p17\(\Delta\)17 (red) nicely overlapped with those of the entire p17 molecule (black), suggesting that p17\(\Delta\)17 adopted an \(\alpha\)-helical conformation highly similar, if not identical, to p17 (Fig. S5). Notably, we found that both synthetic p17 and p17\(\Delta\)17 were functionally identical in sustaining HUVEC motility in a wound healing assay (Fig. 8), fully corroborating the structural findings by NMR spectroscopy. This finding demonstrates that the disordered region in the C-terminus of p17 is irrelevant for the viral protein’s biological activity.

**Discussion**

Several evidences, collected in the past by different investigators, were already highly suggestive of cellular mechanisms at work to operate p17 secretion. Immunofluorescence staining of HIV-1-infected cell lines showed that different antigenic regions of p17 were found to be randomly expressed on the outer leaflet of live cell plasma membrane during HIV-1 replication\(^{49,50}\). In particular, at least three regions of p17 (aa.12–29, 53–87, 87–115) were constantly detected on the cell surface by different anti-p17 mAbs, used as specific reagents, whereas other epitopes, even conformational, were occasionally expressed. Based on the evidence that these mAbs lacked HIV-1 neutralizing activity, the authors concluded that the reactive epitopes of p17 were not the portions of viral protein

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**Figure 7.** Summary of cleavage patterns of peptide p17-p24 by HIV protease, Renin, Cathepsin D and BACE 1. The residues formed following incubation with each enzyme are reported.

| Enzyme       | Cleavage Pattern                             |
|--------------|----------------------------------------------|
| HIV Protease | HNCOC-SNQVSNYPPIVQNLGQ-COONH \(\rightarrow\) 995 m/z |
| Renin        | HNCOC-SNQVSNYP-COONH \(\rightarrow\) 946 m/z    |
| Cathepsin D  | HNCOC-PIVQNLG-COONH \(\rightarrow\) 811 m/z    |
| BACE 1       | No cleavage sites                             |

**Figure 8.** A C-terminally truncated p17 protein with 17 disordered C-terminal residues excised (p17\(\Delta\)17) is biologically active. Ability of p17\(\Delta\)17 to promote endothelial cell migration. Confluent HUVEC monolayers were scratched using a 200 \(\mu\)l pipette tip and cultured for 12 h at 37 °C with medium alone (NT) or containing 10 ng/ml of p17 (used as a positive control) or p17\(\Delta\)17. Cell migration was recorded by light microscopy soon after (initial) and at 12 h after the wound. Images are representative of three independent experiments with similar results (magnification 10x). Graphs in the right panels represent quantitative analyses of wound-healing upon different culture conditions. Statistical analysis was performed by one-way ANOVA, and the Bonferroni’s post-test was used to compare data (\(*\)\(\ast\)\(\ast\)\(\ast\) \(P<0.001\)). NT indicates not treated cells.
in the virion itself, but more likely short polypeptide chains of p17 transported to the surface of HIV-1-infected cells. More interestingly, p17 secretion has been hypothesized also in patients undergoing successful cART, since the viral protein was easily detected at nM concentration in the blood of cART-treated HIV-1-seropositive patients. Moreover, p17 was detected in abundant amounts in the lymph node germinal centers and in the liver of aviremic cART-treated patients.

Although hypothesized, the process of p17 secretion has remained poorly characterized due to difficulty in discriminating whether the presence of viral protein in the extracellular microenvironment was resulting from a mechanism of active cellular secretion or a product of viral particle degradation during active HIV-1 replication. Meanwhile, several studies showed that extracellular p17 was indeed a viral toxin that could affect the biological activity of different cell types. A recent study shed new light on the possibility that even during HIV-1 latency, cells can produce Gag polyproteins without supporting virus assembly and therefore, spreading infection. According to the authors, this was due to the presence of sufficient unspliced RNA to result in nuclear export and translation of Gag, whereas the same could not be said for Env. This finding is in agreement with previous in vivo studies showing that a population of resting cells can transcribe and translate HIV-1 and SIV proteins. These findings prompted us to investigate whether Gag could be processed in the absence of HIV-1 protease and give rise to p17 proteins capable of being released, in a biologically active form, in the extracellular microenvironment. Evidence that cellular polyproteases may process the Gag polyprotein has been recently provided by Chandel et al. who showed that Renin is able to cleave Gag polyproteins to p24. This is not surprising since Renin and HIV-1 protease are both aspartyl proteases and similarly structured, so much so that HIV-1 protease inhibitors have been generally constructed on Renin structure. As summarized in Fig. 9, here we provide evidence, for the first time, that the matrix protein p17 is digested from the Gag polyprotein by cellular intra-membrane aspartyl proteases following its binding to PI(4,5)P₂. Thus, although p17 is devoid of signal sequence, it is secreted in a biologically active form by cells using an unconventional secretion pathway. These findings offer to explain why Gag proteins can be released also during HIV-1 latency, when the viral protease is not synthesized.

Figure 9. Hypothetical model of p17 secretion from Gag-expressing cells. The cytosolic Pr55\(^{\text{Gag}}\), a polyprotein composed of the matrix protein p17 — whose NH\(_2\)-terminal myristic acid moiety in its quiescent conformation is in light green —, the capsid protein p24, the nucleocapsid protein p7, and the p6 domains (1), is recruited to the cellular membrane by PI(4,5)P₂, according to the model proposed by Saad \textit{et al.} (2). PI(4,5)P₂ association with the p17 highly basic domain induces a conformational change allowing the membrane-embedded aspartyl-protease to cleave p17 from the polyprotein Pr55\(^{\text{Gag}}\) (3). As a Pr55\(^{\text{Gag}}\)-free protein, p17 then moves to the extracellular space through an unconventional secretion mechanism (4).
and Cathepsin D. This finding called for further studies elucidating the role of p17 C-terminus in the viral protein biological activity. Data here presented show that the C-terminus of p17 is devoid of any biological activity and that deletion of as much as 17 amino acids in this region does not affect folding and biological activity of the viral protein. This finding is in agreement with several observations indicating that the functional region of p17 is located in a partially unfolded α-helix at the protein NH₂-terminal region. Moreover, the p17 C-terminus is highly flexible and with high level of predicted intrinsic disorders, which sub tends to lack of function for protein activity. Indeed, mutations in the p17 C-terminus generate proteins that do not affect viral replication and infectivity and do not compromise p17 binding to its cellular receptors.

In conclusion, the function of extracellular p17 in HIV-1 infection and pathogenesis might be more significant than previously thought. In fact, our data sustain the possible role of extracellular p17 in HIV-1-related diseases which detrimentally affect cART-treated patients. It is worth noting that recent data have already highlighted the possible contribution of extracellular p17 to support aberrant angiogenesis and promote vascular diseases. Moreover, it is now becoming evident that p17 and its variants, may play a role in B-cell lymphoma pathogenesis and that deletion of as much as 17 amino acids in this region does not affect folding and biological activity of the viral protein. This finding is in agreement with several observations indicating that the functional region of p17 is located in a partially unfolded α-helix at the protein NH₂-terminal region. Moreover, the p17 C-terminus is highly flexible and with high level of predicted intrinsic disorders, which sub tends to lack of function for protein activity. Indeed, mutations in the p17 C-terminus generate proteins that do not affect viral replication and infectivity and do not compromise p17 binding to its cellular receptors.

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Invitrogen, Carlsbad, CA). Briefly, cells were resuspended at a density of $10^6$ cell/ml in PBS with 0.1% bovine serum albumin (BSA, AppliChem, Missouri, USA) and CFSE was added at a final concentration of 2.5μM. After 10 min, the unbound CFSE was quenched by the addition of ice-cold complete medium (5/1 vol/vol) and by two additional washings. Cellular lysate and supernatants derived from ELISA plates were tested for CFSE content by fluorometric technique.

**p17 immunoprecipitation and Western Blot.** Cells (1.2 × 10^7) were nucleofected with AG49CMV Gag-RTEm26CTE as above. After 48h, cells and supernatants were harvested and subjected to p17 immunoprecipitation. Cells were lysed in 200μl of lysis buffer [10mM Hepes (pH7.9), 10mM KCl, 1.5mM MgCl2, 0.5mM EDTA, 0.5mM EDTA, 0.6% Nonidet P-40] containing a mixture of protease inhibitors (Complete Mini; Roche) and protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Cell supernatants were concentrated by Amicon® Ultra-4 Centrifugal Filter Units (3kDa) (Millipore, Darmstadt, Germany). Immunoprecipitation was performed using a rabbit polyclonal antibodies to p17 (PRIMM, Milan, Italy) and the Protein G PLUS-Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology, CA, USA) following the manufacturer’s suggestion. Immunoprecipitated proteins were then detected by Western blotting using mAb MBS-3 to p17 and peroxidase-conjugated goat anti-mouse IgG (Thermo Scientific, Waltham, MA). The immune complex was revealed using the ECL System (Santa Cruz Biotechnology, Dallas, TX).

**Wound Healing Assay.** HUVECs were plated into 24-well plates (1 × 10^5 cells/well) in EGM containing 10% FCS and confluent monolayers were starved for 24h by replacing medium with EGM containing 0.5% FCS. HUVECs were then co-cultured with Jurkat cells previously nucleofected or not with AG49CMV Gag-RTEm26CTE (Jurkat-Gag) and pEGFPN3 (Jurkat-EGFP). After 24h of culture, Jurkat cells were removed and HUVEC monolayers were scratched using a 200μL pipette tip. In some experiments starved HUVEC monolayers were scratched, washed with warm PBS and cultured in EGM containing 10% FCS in the presence or absence of synthetic p17s at different concentrations. HUVEC migration was evaluated at different time points using an inverted microscope (DM-IRB microscope system, Leica, Milan Italy). HUVECs migrating into the wounded area, or protruding from the border of the wound, were photographed using a CCD camera (Hitachi Inc., Krefeld, Germany) connected to a computer via a frame grabber (Matrox Meteor). Analysis of the images was performed using the QWin-lite software (Leica).

**Confocal microscopy.** HeLa cells were nucleofected with AG49CMV Gag-RTEm26CTE (HeLa-Gag), cultured for 24h in complete medium and then fixed with 3% paraformaldehyde/2% sucrose in PBS for 30 min, permeabilized with 0.5% Tween 20, and saturated with 1% BSA in PBS. For staining, the cells were incubated for 1 h with the anti-p17 mAb MBS-3 followed by Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR). Nuclei were counterstained with 4',6-diamidino,2-phenylindole (DAPI, Sigma). In some experiments, HeLa cells were nucleofected with AG49CMV Gag K26T/K27T-RTEm26CTE (HeLa-Gag K26T/K27T), a construct carrying K26T and K27T mutations in the Gag sequence, AG49CMV Gag K30T/K32T-RTEm26CTE (HeLa-Gag K30T/K32T), a construct carrying K30T and K32T mutations in the Gag sequence, AG49CMV Gag W36A-RTEm26CTE (HeLa-Gag W36A), a construct carrying a W36A mutation in the Gag sequence and with AG49CMV p17-RTEm26CTE (HeLa-p17) and AG49CMV p17G2A-RTEm26CTE (HeLa-p17G2A), two constructs carrying myr-p17 and myr-p17, respectively. HeLa-Gag were also treated with neomycin [as PI(4,5)P2 inhibitor] (Life Technologies, Monza, Italy), with a protease inhibitor cocktail (Sigma), and with the specific aspartyl protease inhibitor Pepstatin A (Sigma). In some experiments, HeLa cells were co-nucleofected with AG49CMV Gag-RTEm26CTE and the 5paseIV expression plasmid pcDNA4TO/Myc5paseIV or with AG49CMV Gag-RTEm26CTE and the mutant expression plasmid pCDNA4TO/Myc Δ15paseIV. Cells were analyzed using a Zeiss LSM510 confocal microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective. Orthogonal z-sections reconstitution were obtained through AxioVision 4D module.

**Peptide synthesis.** A peptide (H$_2$COC-SNPQSVQYPIVQNLQGQ-CONH$_2$) containing the HIV-1 protease specific cleavage site (PI) between matrix protein p17 and core protein p24 (p17/p24) was synthesized using Fmoc-protected amino acid (Flamma, Bergamo, Italy) by solid-phase chemistry on a Rink Amide resin and used as a substrate for different enzymes. Synthesis were carried out using an Applied Biosystems 433 A peptide synthesizer (Life Technologies, Monza, Italy) at 0.1 mmol scale. Peptide was purified by reverse phase HPLC and characterized by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis, using a Bruker Reflex III TOF mass spectrometer operating in reflector mode (Bruker, Billerica, MA, USA). The purity was always >95%.

**Preparation of enzyme and p17/p24 peptide solutions.** Three μl of the stock solution of biologically active HIV-1 protease (Abcam, Cambridge, UK) (200 ng/μl, 25.59 μM) were diluted with HIV-1 protease solution (20% Glycerol, Tris-HCl 20 mM, KCl 500 mM, DTT 1 mM, EDTA 200 μM, pH 8) to obtain a final working solutions of 30 μl containing 100 ng of HIV-1 protease. Three μl of Renin (Anaspec, San Jose, CA, USA) stock solution of 50 ng/μl were diluted with 3-(N-morpholino) propanesulfonic acid (Mops) 50 mM buffer, pH 7, to obtain a final working solution of 30 μl (18 ng). Lyophilized Cathepsin D (Sigma) was dissolved in double distilled water (ddH$_2$O) to obtain a final working solution of 30 μl (100 ng). Stock solution of 50 μM of BACE1 (Sigma) was diluted in acetate buffer 50 mM, pH 4.5, to obtain a final working concentration of 420 pM in 30 μl. Aliquots of 100 μg of p17-p24 were solubilized in DMSO to obtain a concentration of 100 μM. This solution was then further diluted in acetate buffer 50 mM, pH 4.5, or in ddH$_2$O to obtain a standard working solution of 6.25 μM.
Enzymatic Cleavage and Mass Spectrometry Analysis. Twenty μl of standard working solution of p17-p24 (dissolved in H2O for Renin, Cathepsin D and HIV-1 protease or in acetate buffer (50 mM, pH 4.5) for BACE1) were added and incubated at 37 °C for 30 min. The determination of p17-p24 cleavage sites was carried out by MALDI-TOF. Briefly, 1 μl of sample was mixed with 1 μl of HCCA matrix [a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50/50/0.1% of water/acetonitrile/trifluoroacetic acid] and spotted on metal target plate (MTP 384 Ground Steel, Bruker). Mass spectra were acquired with 200 laser shots for sample at a constant laser energy in reflector mode and elaborate using Flex Analysis software.

Protein synthesis. Chemical synthesis of p17 and of a C-terminally truncated forms of p17 lacking the last 17 amino acids (p17Δ17) via native chemical ligation was performed as described previously87. Synthetic proteins, after HPLC purification to homogeneity, were folded by dissolving lyophilized polyprotein at 1 mg/ml in 0.2 M phosphate buffer saline (PBS) containing 6 M GuHCl, pH 7.4, followed by a six-fold dilution with 0.2 M PBS containing 10 mg/ml DTT and extensive dialysis against 0.2 M PBS. Proteins were quantified by UV absorbance measurements at 280 nm using calculated molar extinction coefficients as described86.

Nucleic magnetic resonance (NMR). NMR spectra were recorded at 25 °C on an 800 MHz (800.27 MHz for protons) Bruker Avance-series NMR spectrometer equipped with four frequency channels and a 5 mm triple-resonance z-axis gradient cryogenic probehead. A one-second relaxation delay was used, and quadrature detection in the indirect dimensions was obtained with states-TPII phase cycling; initial delays in the indirect dimensions were set to give zero- and first-order phase corrections of 90° and −180°, respectively69,70. Data were processed using nmrPipe on Mac OS X workstations. 2D NOESY experiments with a 150 ms mixing time were collected to monitor changes in the backbone and side-chain 1 H protein resonances71. Typical NMR samples contained 3 mg/ml protein in a 10 mM sodium phosphate and 25 mM sodium chloride buffer, pH 7.4, to which 10% D2O (v/v) was added.

Statistical Analysis. Data obtained from multiple independent experiments are expressed as the mean ± SD. Data were analyzed for statistical significance using the Student’s two-tailed t test or one-way ANOVA, when appropriate. Bonferroni’s post-test was used to compare data. Differences were considered significant at P < 0.05. Statistical tests were performed using GraphPad Prism 5 software.

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**Author Contributions**

F.C., M.L.I. and A.C. conceived and designed research; F.C., M.L.I. and S.F. developed plasmid constructs; F.C., M.L.I., P.M., C.G., A.B., and F. Campilongo performed *in vitro* experiments; S.M. performed confocal microscopy and image analysis; K.V. and W.L. performed protein synthesis and NMR studies; A.R., S.S. and M.S. performed peptide synthesis and mass spectrometry analysis; A.C., W.L. and M.S. wrote the manuscript. All authors reviewed and approved the manuscript.

**Additional Information**

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