THE ROLE OF VIRAL AND CELLULAR PROTEINS IN THE BUDDING OF HUMAN IMMUNODEFICIENCY VIRUS

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Summary. – For over two decades, research on Human immunodeficiency virus (HIV), which is responsible for AIDS, has aimed at understanding of the molecular mechanisms used by this virus during its life cycle. An essential step in the HIV life cycle is the budding, which promotes the release of viral particles from the host cell. It has recently been revealed that HIV in the process of budding uses besides one viral protein also the machinery of the infected cell, in particular the proteins Tsg101 and ubiquitin. The viral protein is the p6 domain of the Gag precursor polyprotein. In normal cells, Tsg101 functions as a regulator of endocytic trafficking that recognizes ubiquitinated cargo and directs its delivery to degradative compartments. In HIV-infected cells, Tsg101 and ubiquitin interact with Gag p6 to promote the release of new viral particles from the host cell. Molecular mechanisms underlying the process of HIV budding from infected cells suggests a whole new range of drug targets that could prove useful in AIDS suppression in HIV-positive patients.

Key words: Gag p6 protein; human immunodeficiency virus; budding, multivesicular bodies; Tsg101; ubiquitin

Contents:

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Abbreviations: HIV = Human immunodeficiency virus; MA = matrix protein; MVB = multivesicular bodies; CA = capsid protein; NC = nucleocapsid protein; SU = surface protein or HIV gp120; TM = transmembrane protein or HIV gp41; PR = protease; IN = integrase; PTAP motif = Pro-Thr-Ala-Pro motif; UEV domain = the ubiquitin-conjugating E2 variant domain; Vps protein = vacuolar protein sorting protein

1. INTRODUCTION

Although HIV, the agent responsible for AIDS, was first described more than two decades ago, AIDS is still a disease of colossal proportions (LaBranche *et al*., 2001; Scarlata and Carter, 2003). Currently, more than 40 million people are infected with HIV worldwide.

The search for a vaccine against HIV infection and/or disease progression has been the focus of intense research and investments, but so far it has been unsuccessful. Effective antiviral therapies have been developed but these only succeed in prolonging the life of the individuals infected with this virus (LaBranche *et al*., 2001). Moreover, a steaily increasing number of HIV isolates resistant to approved drugs has provoked a significant adverse impact on both treatment options and disease outcome. It has been determined that in areas where antiretroviral therapy is widely used, 25% of the new HIV infections involve viruses resistant to one or more drugs (LaBranche *et al*., 2001). It is estimated that 40–45% of HIV-infected individuals harbor drug-resistant viruses. A rapidly growing subgroup (5–10%) of individuals carries HIV that exhibits resistance to all classes of inhibitors. These recent trends highlight the urgent need for new HIV therapies that could address the growing problem of drug resistance.

In principle, the virus budding represents an attractive target for therapeutic intervention, because this phase of virus life cycle seems to be both essential and general, and cellular targets might have better drug resistance profiles compared to conventional viral targets (Pornillos *et al*., 2002a). However, many questions concerning this phase remain unanswered (Scarlata and Carter, 2003), such as which cellular factors are required for HIV budding and how viral factors cooperate with cellular factors. It is hoped that answers to these questions will lead to the discovery of new classes of effective HIV therapeutics.

2. HIV AND ITS LIFE CYCLE

The genome of HIV (the species *Human immunodeficiency virus 1 and Human immunodeficiency virus 2*, the genus *Lentivirus,* the subfamily *Orthoretrovirinae*, the family *Retroviridae*) consists of two molecules of single-stranded positive-sense RNA of 9.3 kb. *gag, pol* and *env* genes direct the synthesis of polyprotein precursors Gag, Gag-Pol and Env, respectively, which are proteolytically cleaved into final proteins. Gag yields matrix protein (MA), capsid protein (CA), nucleocapsid protein (NC), and p6, Gag-Pol leads to protease (PR), reverse transcriptase (RT), and integrase (IN), and Env is cleaved into gp120 (surface protein, SU) and gp41 (transmembrane protein, TM). (Frankel and Young, 1998; von Schwedler *et al*., 2003). This type of structure of *gag* gene is common for all retroviruses except for spumaviruses (Frankel and Young, 1998). There are another 6 genes encoding accessory proteins with regulatory or unknown function: Vif, Vpr, and Nef (all found in the virion), and Tat, Rev, and Vpu (non-structural proteins) (Fig. 1).

The MA domain of Gag polyprotein (the Gag MA domain) contains a plasma membrane-targeting sequence and, in mature virions, lines the inner face of the host cell-derived membrane. The Gag CA domain participates in protein-protein interactions in virion assembly and forms the core shell in mature virions. The Gag NC domain recognizes the packaging signal in the genomic RNA and, in mature virions, coats and condenses genomic RNA. In addition to these well-characterized domains, which are invariably ordered as MA-CA-NC (from the N- to C-terminus) in the Gag precursor polyprotein, a variety of additional spacer peptides occur in individual retroviruses. In HIV-1, they are ordered as follows: MA, CA, spacer p2, NC, spacer p1, and p6 (Frankel and Young, 1998).

The HIV life cycle consists of six major events: attachment of the virion to the host cell, reverse transcription of genomic RNA, integration of proviral DNA into the host cell,

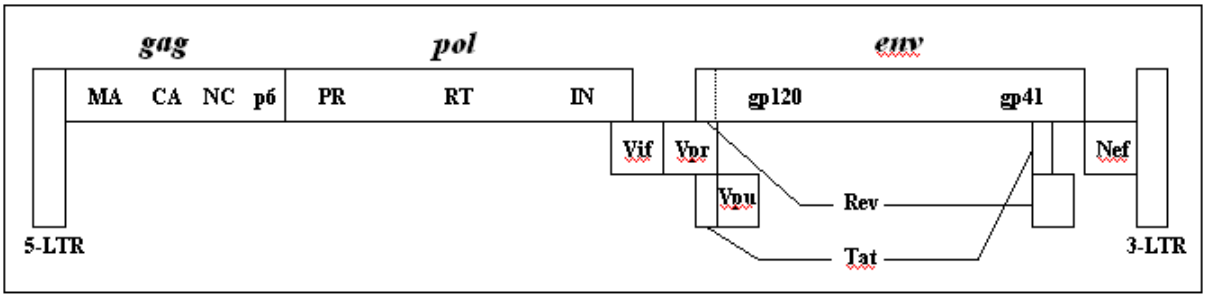

Fig. 1

HIV genome structure

transcription of proviral DNA, translation of mRNAs and post-translational processing of precursor polyproteins, assembly of viral nucleocapsids, and final maturation and budding of virions (LaBranche *et al*., 2001; Frankel and Young, 1998).

An essential step in the HIV life cycle, identical for all enveloped viruses, is the budding that occurs at the plasma membrane. The separation of the nascent virion from the host cell requires a membrane fusion event that could, in principle, be either spontaneous or mediated by viral and/or cellular factors (Göttlinger *et al*., 1991). Studies on the morphogenesis of retroviral particles have indicated that the Gag protein(s) are required for budding (Göttlinger *et al*., 1991). Because retroviral Gag protein(s) can assemble and bud in the absence of other viral proteins, any additional machinery necessary for viral budding and membrane fision must be supplied by the cell and recruited by Gag protein(s) (Pornillos *et al*., 2002a). All the cellular systems that HIV must exploit to realize its budding are so far unknown.

The knowledge on this subject acquired to date is summarized in this review in three parts: the proteins involved in HIV budding, the interactions between these proteins, and the cellular machinery recruited by HIV during its life cycle.

3. THE PROTEINS INVOLVED IN HIV BUDDING

In HIV budding, three proteins play a crucial role, Gag p6 (viral protein), Tsg101 and ubiquitin (both cellular proteins).

3.1. Gag p6

3.1.1. General characteristics

The *gag* gene is primarily translated into a precursor 55 K polyprotein whose N-terminus is myristylated (Göttlinger, 2001). According to Göttlinger *et al*. (1991), deletion of the 6 K C-terminal domain of HIV Gag caused a marked defect in virus production in transfected cells. Mutant particles failed to detach from transfected cells but rather remained attached to the plasma membrane by a thin tether. These observations suggested that the Gag p6 domain plays a critical role in virus release. Subsequent studies, using a variety of cell lines and Gag expression systems, failed to observe a virus release defect upon a p6 truncation (Hockley *et al*., 1994; Spearman *et al*., 1994). However, by using a full-length HIV genome clone expressed in human cells, the requirement for Gag p6 in virus release was eventually confirmed (Huang *et al*., 1995). A highly conserved Pro-Thr-Ala-Pro (PTAP) motif was identified as playing an essential role in the p6 late (L) domain activity (Göttlinger *et al*., 1991).

3.1.2. The Gag p6 L-domain and its involvement in HIV budding

Late (L) domains are short amino acid sequences that are essential for efficient release of virions from cytoplasmic membrane (Ott *et al*., 2003; Freed, 2002). These domains play a late role in the budding process (Parent *et al*., 1995). Three types of motifs in L-domains have been found in retroviruses (Freed, 2002). In HIV, the core element of the L-domain, the PTAP motif is located in the C-terminal portion of Gag p6. In Equine infectious anemia virus, a YPDL motif occurs in Gag p9. A PPPPY motif is located within Gag p2b of avian retroviruses, Gag p12 of Murine leukemia virus, Gag p16 of Mason-Pfizer monkey virus, and the matrix protein of rhabdoviruses.

Type C retroviruses and lentiviruses assemble at the plasma membrane, while types B and D retroviruses assemble in the cytoplasm. Both assembly schemes appear to require L-domains for efficient budding. Mutations in these sequences cause virions to remain attached to the cell by a membrane tether, or to appear as a series of trapped particles (Ott *et al*., 2003). Interestingly, in several cases, viral L-domains are functionally interchangeable and can exert their activity when positioned at different locations within Gag (Parent *et al*., 1995). These observations strongly suggest that L-domains act by recruiting host cell factors to facilitate virion egress and by playing a role in mediating protein-protein interactions among cellular proteins (Senior, 2001), rather than by directly influencing virion morphogenesis (Freed, 2002). The first clues that L-domains are docking sites for cellular proteins that function in virion release were provided when the substitution of a given L-domain motif by another preserved the ability of promoting virion release, indicating that different motifs share a common function (Parent *et al*., 1995). For example (Serrano *et al*., 2001), the Ebola virus L-domain not only substitutes for the HIV L-domain in the egress function, but it also permits production of virus particles that are competent for infection.

In summary, although the Gag polyprotein or just its p6 L-domain is essential for virion assembly, HIV does not have the genes to code for cell membrane fusion and fission and therefore recruits cellular proteins for this job (Senior, 2001). Their small size, interchangeability, and positional independence are consistent with L-domains functioning as modules that bind and recruit host factors to the budding site (Freed, 2002). For example, a variety of studies have indicated that the L-domain with the HIV PTAP motif recruits Tsg101 (VerPlank *et al*., 2001).

3.2. Tsg101

3.2.1. General characteristics

Originally, the mouse *tsg101* gene (*mtsg101)* was discovered as a tumor susceptibility gene whose functional knockout in mouse fibroblasts led to loss of protection of nude mice against metastatic tumors (Li and Cohen, 1996). Sequence analysis of *mtsg101* cDNA indicated that the gene encoded a 43 K protein containing a proline-rich domain and DNA-binding motifs characteristic of transcriptional factors.

The cloning and mapping of the human *tsg101* gene localized it in the chromosome 11, the subbands p15.1-15.2, a region showing loss of heterozygosity in a variety of human malignancies (Li *et al*., 1997). As *tsg101* mutates at high frequency in human breast cancer, its defects occur apparently during breast cancer tumorigenesis and/or progression (Li *et al*., 1997). The mechanism(s) by which the interference with *tsg101* expression leads to neoplastic transformation and phenotypic alterations that persist after restoration of the *tsg101* function are unknown (Li and Cohen, 1996; Li *et al*., 1997). According to more recent studies (Krempler *et al.*, 2002; Wagner *et al.*, 2003), a null *tsg101* mutation (a mutation that completely abolishes the activity of the gene) did not result in neoplastic transformation, suggesting that it is not a primary tumor suppressor gene. Moreover, a *htsg101* deletion was not able to establish tumorigenic cell lines but promoted cell cycle arrest and rapid cell death. Taken together, it has been demonstrated that *tsg101* is an important factor controlling cell cycle regulation and acting as a cell survival factor, but its involvement in neoplastic transformation and tumorigenesis is still elusive (Krempler *et al*., 2002).

tsg101 gene encodes a protein of 380 amino acids, Tsg101 with 86% homology to the mouse analog, mTsg101 (Li *et al*., 1997). Tsg101 contains a N-terminal region of homology to ubiquitin-conjugating (E2) enzymes, a proline-rich sequence with the features of a transcription transactivation domain, a leucine zipper, and a central coiled-coil region (Li and Cohen, 1996; Koonin and Abagyan, 1997). The Tsg101 N-terminal region (the E2 variant domain or the ubiquitin-conjugating E2 variant domain (UEV domain), amino acids 1–150) is structurally similar to the catalytic domain of E2 enzymes, suggesting a potential role of Tsg101 in the regulation of ubiquitin-mediated protein degradation (Koonin and Abagyan, 1997). The C-terminal region of Tsg101 has coiled-coil and proline-rich domains that can function in transcriptional repression and transactivation (Krempler *et al*., 2002).

On the basis of its structural features, Tsg101 has been speculated to be: (i) a dominant-negative ubiquitin regulator (Koonin and Abagyan, 1997), (ii) a transcriptional regulator (Krempler *et al*., 2002), (iii) a cell cycle regulator (Zhong *et al*., 1998), and (iv) a membrane protein trafficking regulator (Perez and Nolan, 2001; Babst *et al*., 2000; Lemmon and Traub, 2000). It is not clear whether these apparently diverse roles reflect independent or related functions of the protein.

One of the several cellular Tsg101 functions mentioned above is the participation in the endocytic trafficking pathway (Perez and Nolan, 2001; Babst *et al*., 2000; Lemmon and Traub, 2000; Katzmann *et al*., 2001). This mosaic of structural and functional domains coordinates protein transport either to recycling routes where cargo proteins are brought back to the plasma membrane, or to degradation pathways where proteins are carried through several compartments to the lysosome or proteasome in mammalian cells, or to the vacuole in yeast (Gruenberg, 2001). Central to the functioning of the endosomal trafficking system is the sorting that takes place in early endosomes, where it is determined which pathway will be taken by a given protein cargo. As part of the process, two structures emanate from the early endosomes: tubular structures, which mediate recycling to the cell surface, and large transport intermediates or multivesicular bodies (MVB), which are derived from late endosomes and eventually fuse with lysosomes. The proteins required for cargo delivery to MVB in yeast, the class E vacuolar protein sorting (Vps) proteins, have orthologs in broadly diverse organisms, including humans (Babst *et al*., 2000; Lemmon and Traub, 2000). This indicates that the pertinent molecular mechanism is highly conserved (Carter, 2002).

3.2.2. Similarities between the Tsg101 UEV domain and E2 enzymes

Tsg101 is a homolog of ubiquitin conjugase (E2) enzymes (Pornillos *et al*., 2002b). All E2 enzymes contain a conserved core sequence composed of approximately the first 150 amino acids from the N-terminus, of which at least 25% are identical. The amino acids at the N-terminus may play a critical role in the interaction with ubiquitin and/or other enzymes of the ubiquitin-conjugation pathway (Cook *et al*., 1992). The cysteine necessary for formation of the thioester bond with the ubiquitin COOH terminus is also present in this region. Some E2 enzymes have small internal insertions and/or C-terminal extensions; the latter may serve ubiquitin ligase (E3)-like functions, whereas shorter E2 enzymes require E3 to transfer ubiquitin to a protein acceptor (Cook *et al*., 1992).

Tsg101 is inactive as an ubiquitin conjugase because its UEV domain lacks the catalytic cysteine required for thioester bond formation with ubiquitin (Pornillos *et al*., 2002b). Consequently, Tsg101 is presumably incapable of directly conjugating ubiquitin. Nevertheless, it has been reported that the Tsg101 can bind to ubiquitin (Sundquist

et al., 2004). Although the function of these enzymatically inactive proteins is not fully understood, they appear to play a regulatory role in the modulation of the ubiquitination reaction (Babst *et al*., 2000; Katzmann *et al*., 2001). The Tsg101 UEV domain also differs from canonical E2 enzymes in that it displays a hydrophobic groove that specifically recognizes the PTAP motif (Pornillos *et al*., 2002b, 2003).

3.2.3. The involvement of Tsg101 in HIV budding

Studies from several laboratories (VerPlank *et al*., 2001; Serrano *et al*., 2001; Garrus *et al*., 2001; Demirov *et al*., 2002) have demonstrated that the ability of Gag p6 to stimulate HIV budding requires a direct interaction between the Gag p6 L-domain and the host Tsg101 endosomal sorting protein (Freed, 2003).

VerPlank *et al.* (2001) have shown that Tsg101 specifically interacts with the HIV-1 Gag p6 both *in vitro* and in the cytoplasm of transfected cells. The interaction was much more efficient in cytoplasmic extracts than *in vitro*, suggesting the existence of other stabilizing cellular factors or of a particular Gag assembly state. Two highly conserved prolines in the Gag p6 L-domain were critical for Tsg101 binding.because the deletion of a L-domain motif, a Pro-rich sequence highly conserved in HIV (Freed, 2002), prevented the interaction of Gag p6 with Tsg101. Moreover, alterations in the ubiquitin-binding site in the UEV domain or other regions of Tsg101 influenced its interaction with Gag p6. These results implicated a specific component of the cellular trafficking machinery in virus budding and maturation (VerPlank *et al*., 2001). However, how the Gag participation in these functions might be related to the Tsg101 role in the ubiquitination process was until now unknown. The observation of Tsg101 interaction with HIV Gag in mammalian cells suggested that the interaction was relevant to the viral life cycle.

The co-expression of the Tsg101 N-terminal region with the full-length HIV genome clone markedly inhibited HIV particle production by blocking efficient virus budding from the plasma membrane (Demirov *et al*., 2002). This effect was specific for HIV and was dependent on an intact Gag p6 L-domain. Furthermore, it was demonstrated that Tsg101 was incorporated into the wild-type but not the L-domain-deficient virus particles. These results have significant implications for the understanding of HIV budding because they strongly supported the hypothesis that Tsg101 plays a physiologically relevant role in HIV budding. The consideration of the roles Tsg101 and other E2-like proteins in the cell might offer some clues. Two general classes of models seem most likely: Tsg101 could influence the ubiquitination of Gag or a host factor, or, Tsg101 could alter the localization or the sorting of a ubiquitinated host protein.

Table 1. Gag p6, Tsg101 and ubiquitin, the main proteins involved in HIV budding

Protein	Characteristics
Gag p6	Viral protein that contains the necessary information for HIV budding. The PTAP motif-containig L-domain of viral Gag p6 protein interacts directly with the UEV domain of cellular protein Tsg101. Lysines near the L-domain interact with ubiquitin, a cellular protein.
Tsg101	Cellular protein functions as a dominant negative ubiquitin regulator, a transcriptional regulator, a cell cycle regulator and a membrane protein trafficking regulator. The UEV domain of Tsg101 is involved in HIV budding that is a process similar to MVB formation through the endocytic trafficking pathway.
Ubiquitin	Cellular protein responsible for many diverse cellular functions, specially in the protein degradation mechanisms. Ubiquitin binds to cellular and viral proteins. Ubiquination precedes the formation of MVB (degradation of cellular proteins) and release of new viral particles (HIV budding).

The reported ability of Tsg101 and other E2-like proteins to modulate the target specificity of ubiquitination (Hofmann and Pickart, 1999) raises the possibility that the binding of Tsg101 by Gag p6 can alter the extent to which Gag becomes ubiquitinated (Demirov *et al*., 2002).

Other experiments (Garrus *et al*., 2001) have also demonstrated the requirement for Tsg101 in HIV budding. The Tsg101 UEV specifically bound to the PTAP motif within Gag p6 (VerPlank *et al*., 2001; Garrus *et al*., 2001), and there was excellent correlation between mutations inhibiting Tsg101 binding and those inhibiting virus release (Demirov *et al*., 2002; Huang *et al*., 1995). Tsg101 depletion, caused by small interfering RNAs, decreased viral titers 10–50 times and strongly inhibited viral release. Reintroduction of a recombinant Tsg101 protein, which was resistant to the action of these RNAs, phenotypically rescued HIV budding (Perez and Nolan, 2001). Depleting Tsg101 or inhibiting endosomal trafficking also arrested HIV release at a late stage, indicating that

Tsg101 and the Vps pathway perform essential functions in viral budding. Moreover (Serrano *et al*., 2001), a mutation of the Tsg101 recognition signal in HIV Gag PTAP motif was accompanied by a viral processing defect in mammalian cells that included the accumulation of late Gag processing intermediates, a characteristics of L-domain-defective HIV mutants. In addition, proviruses encoding each of the mutant Gag proteins that did not bind Tsg101 generated very small amounts of virions. In contrast, alteration of amino acids immediately flanking the PTAP motif resulted in only minor defects in both Tsg101 binding and virion production.

Although the precise mechanism by which Tsg101 mediates viral budding remains unclear, recent findings provide some hints. Tsg101, also known as Vps23, is a component of a 350 K complex, ESCRT-1, which is

essential for the sorting of ubiquitinated proteins into the MVB in yeast and for endosomal targeting in mammalian cells (Babst *et al*., 2000; Katzmann *et al*., 2001). The budding and membrane-fusion events that lead to the formation of a vesicle within the MVB are topologically equivalent to the budding of enveloped viral particle, differing only in the contents of the vesicle and cellular location. A speculative, but entirely plausible hypothesis is that viral proteins recruit the machinery that normally mediates MVB formation to virus budding sites at the plasma membrane. It is in a good agreement with the fact that the Tsg101 UEV is a multifunctional domain that can simultaneously bind ubiquitin and the PTAP motif, suggesting that Tsg101 may detect, or possibly participate, in the ubiquitin-transfer event involved in HIV budding. An attractive model is that ubiquitination of HIV Gag during viral assembly creates high affinity binding sites that recruit Tsg101 to assist in final stages of budding. Alternatively, Tsg101 could bind Gag p6 and recruit a ubiquitinated cellular factor to the site of viral assembly.

Taking into account that mentioned above, Tsg101 could play a key role in bringing cargo into the MVB. Some observations indicate that the Vps pathway is critical for HIV release (Garrus *et al*., 2001; Nydegger *et al*., 2003; Carter, 2002). Thus, at present, the possibility that Gag trafficking occurs through pathways other than, or in addition to, those involving sorting to the MVB cannot be eliminated. Consistent with this model, emerging studies have indicated that HIV budding also requires E proteins of several classes. For example, HIV budding also seems to require an interaction between Tsg101 and Vps28, suggesting that the whole ESCRT-1 complex probably participates in the budding process (Serrano *et al*., 2003a). Whether viral budding is mediated by the Vps-MVB pathway or through a distinct trafficking route, Tsg101 is clearly indicated to play an essential role. As viral budding and MVB vesicle formation appear to be topologically equivalent, that is, both entail membrane-fusion events and vesicular pinching off away from the cytoplasm, Tsg101 could facilitate HIV budding through the use of the sorting machinery involved in the genesis of MVB. Tsg101 would serve as the ticket for HIV and ubiquitin would be a key regulator (Carter, 2002), although whether ubiquitin is directly involved or is just a bystander is currently unclear.

3.3. Ubiquitin

3.3.1. General characteristics

Ubiquitin is a well known small cellular protein consisting of 76 highly conserved amino acids (Vogt, 2000). Inside the cell, most of ubiquitin is found either as free molecule or as long polymeric chains that are covalently attached to internal lysines of a wide range of cellular proteins (Ciechanover, 1994; Hochstrasser, 1995). The ubiquitin pathway has been found to be involved in many diverse cellular functions as cell cycle control, antigen presentation, heat-shock response, receptor signaling, transcriptional activation, and DNA repair (Ott *et al*., 1998).

The ubiquitin/proteasome pathway is the principal mechanism of turnover of normal short-lived proteins in mammalian cells (Pickart, 2004). In a protein targeted for degradation, ubiquitin couples to the α-amino group of its lysines via its own C-terminal glycine, forming an isopeptide bond. Other ubiquitin molecules are similarly coupled to lysines residues of the first ubiquitin, and thus the target protein ends up carrying a ramified polyubiquitin chain that acts as a signal recognized by the proteasome (Pickart, 2004). This modification appears to be specific and is thought to mark a protein for degradation by the 26S proteasome. The ubiquitination process occurs through sequential steps catalyzed by activating (E1), conjugating (E2), and ligating (E3) enzymes (Ciechanover, 1994; Hochstrasser, 1995).

3.3.2. The involvement of ubiquitin in HIV budding

The hint that ubiquitin might have something to do with retrovirus assembly came over a decade ago from the observation that a Rous sarcoma virus particle contains some 100 molecules of this protein in an unconjugated form, which represents an enrichment over the concentration of free ubiquitin in the cell cytoplasm (Putterman *et al*., 1990). Ubiquitin was found in virions in approximately 10% of the amount of Gag proteins (Ott *et al.*, 1998). According to Ott *et al*. (2003), proteins can be monoubiquitinated at several sites and not serve as proteasome degradation signals. Some studies have established a putative causal link between budding and ubiquitin, by showing that depletion of the intracellular pool of free ubiquitin inhibits budding. (Patnaik *et al*., 2000; Strack *et al*., 2000).

Monoubiquitination is often a signal for sorting proteins from either the biosynthetic or endocytic pathway to MVB and, ultimately, to the lysosome.

The notion that ubiquitination and endosomal sorting pathways are intimately involved in HIV budding is derived from several key observations: (i) lysines in HIV Gag p6 are ubiquitinated (Ott *et al*., 1998), (ii) proteasome inhibitors, which deplete intracellular pools of free ubiquitin and disrupt the endosomal sorting and degradation of several cell-surface receptors (Longva *et al*., 2002), induce defects in HIV budding (Strack *et al*., 2000), and (iii) the cellular endosomal sorting factor Tsg101 that binds HIV Gag p6 is a ubiquitin-binding protein (VerPlank *et al*., 2001; Garrus *et al*., 2001).

Both retroviral budding (Patnaik *et al*., 2000; Strack *et al*., 2000) and MVB protein sorting (Katzmann *et al*., 2001) involve ubiquitin, providing two more examples of biological pathways where ubiquitin functions otherwise than targeting proteins for proteasomal degradation. While, in the latter case, ubiquitin is required for correct sorting, its role in the former case is still uncertain. It is clear that further studies will be needed to fully elucidate the relationship between ubiquitin and virus budding.

4. THE INTERACTIONS BETWEEN Gag P6, Tsg101 AND UBIQUITIN

It has been shown (Garrus *et al*., 2001) that the Tsg101 UEV domain contains a region that binds the HIV-1 Gag p6 PTAP motif. The lysines near this motif can be monoubiquitinated *in vitro* and this modification can enhance the affinity of Tsg101 to Gag p6 *in vitro*. It has been proposed that there might be a role for ubiquitination in luring the machinery that communicates with vesicular trafficking.

4.1. Interaction between Tsg101 and Gag p6

The structures of the Tsg101 UEV domain and of Gag p6 PTAP motif that bind each to other have recently been solved (Pornillos *et al*., 2002b,c), providing valuable insights into this crucial protein-protein interaction. In general, the overall folding of the Tsg101 UEV domain resembles that of E2 enzymes. The C-terminus of the PTAP motif, which adopts a left-handed polyproline helical conformation II, interacts with two distinct pockets in the peptide-binding groove of Tsg101 distinct from the ubiquitin-binding surface. Extensive hydrophobic and hydrogen bonding contacts occur between Tsg101 amino acids and each of the four PTAP motifs. The first proline of the PTAP motif Pro lies in a shallow groove (the first pocket) created by the Tsg101 amino acids Thr58, Pro71, Thr92 and Met95. A deeper pocket (the second), formed by several Tsg101 amino acids including Tyr63 and Tyr68, accommodates the alanine-proline dipeptide of the PTAP motif. Mutation of either the PTAP motif or Tsg101 UEV domain amino acids that are in direct contact disrupts their interaction (Freed, 2003). The apparent importance of all four amino acids of the PTAP motif is consistent with the observation that mutations at each of these positions disrupt both the L-domain and Tsg101 binding activity of Gag p6 (Freed, 2003).

Interestingly, in these interactions between Gag p6 PTAP motif (L-domain) and Tsg101, the Tsg101 C-terminal half, which contains binding site(s) for other components of the ESCRT-1 complex, is sufficient to stimulate virus release if covalently fused to Gag protein to produce a chimeric Gag-Tsg101 protein (Serrano *et al*., 2001, 2003a,b). Taken together, these studies support a direct role for Tsg101 in HIV budding and suggest that the Tsg101 function in this regard is to bind the Gag protein and to recruit additional cellular factors (e.g. ubiquitin) to the budding site (Freed, 2003).

4.2. Interaction between Tsg101 and ubiquitin

Recent evidence has indicated that Tsg101 plays a role in the sorting of ubiquitinated proteins into the endosomal pathway (Pornillos *et al*., 2002a; Demirov *et al*., 2002). The crystal structure of a complex between ubiquitin and the Tsg101 UEV domain has been determined (Strack *et al*., 2002; Pornillos *et al*., 2002c; Sundquist *et al*., 2004).

The Tsg101 UEV domain-ubiquitin complex structure has relevance for numerous cellular functions owing to the fundamental importance of the receptor downregulation (Katzmann *et al*., 2001) and the budding of enveloped viruses (Pornillos *et al*., 2002c). Several observations confirm that the Tsg101 UEV domain and ubiquitin form the same complex in solution and in crystalline state (Sundquist *et al*., 2004): (i) there is an excellent agreement between the crystal structure and maps of the Tsg101 UEV domain-ubiquitin interaction surfaces (Pornillos *et al*., 2002b); (ii) alanine substitution mutations in the Tsg101 UEV domain that diminish its binding affinity (mutations in Val43, Asn45, Asp46 and Phe88) all map to the ubiquitin binding interface seen in the complex structure (Pornillos *et al*., 2002b); (iii) alanine substitutions in ubiquitin that diminish its binding affinity to Vps23 (yeast Tsg101) (mutations Ile44, Gln6 and Val70) also map to the interface (Bilodeau *et al*., 2003). Taken together, the mutations in ubiquitin or the Tsg101 UEV domain that diminish their binding affinity cover almost the entire interface seen in the crystal structure. Moreover, the hypothesis on the function of the Tsg101 UEV domain-ubiquitin interface in HIV is supported by the observation that viral trafficking and release were disrupted by expression of a Tsg101 construct lacking amino acids 41–43 and hence unable to bind ubiquitin (Goff *et al*., 2003). This kind of deletion mutation apparently inhibited ubiquitin binding.

4.3. Interaction between Gag p6 and ubiquitin

The significance of high levels of free ubiquitin, up to 10% of Gag proteins in viral particles (Ott *et al*., 1998) began to gain clarity when it was realized that (i) ubiquitin is involved in retrovirus budding, (ii) L-domains were important to allow correlated ubiquitination of viral Gag proteins, and (iii) interestingly, ubiquitination of the Gag

proteins on target lysines was proximal to the L-domains in some viruses (Perez and Nolan, 2001). The studies indicating that the Gag p6 PTAP motif recruits the ubiquitin machinery and that certain proteasome inhibitors cause alterations in viral particle budding similar to defects resulting from mutations in the PTAP motif support the possibility that the cells ubiquitination machinery is linked to viral assembly (Patnaik *et al*., 2000; Strack *et al*., 2000).

However, at present, it is unclear whether this link reflects a direct or indirect involvement of ubiquitin. Virus replication and release were not affected by a mutation of the two HIV Gag p6 lysines (Lys27 and Lys33), the major sites for Gag ubiquitination (Garrus *et al*., 2001). However, since readily detectable amounts of ubiquitin were incorporated by both the mutant and the wild-type viruses, it was concluded that the ubiquitination did not require the lysines in Gag p6 (Ott *et al*., 2000). Although the ubiquitination was not required for virion assembly and budding, and thes processes appeared to occur normally in the absence of ubiquinqtion, the presence of mono- and di-ubiquitinated forms were detected. Considering these data, the ubiquitination of Gag p6 does not appear to play a direct role in virion assembly and budding but this modification might be a consequence of an interaction between the cellular ubiquitinating complex and the assembling Gag, rather than a result of an event required for viral function. The Gag ubiquitination could facilitate budding either by targeting defective Gag molecules for proteolytic degradation and thereby preventing them from interfering with the budding, or by creating docking sites for cellular factors that actively participate in viral budding (Garrus *et al*., 2001). Indeed, recent studies strongly support the concept that L-domains in general and Gag p6 L-domain in particular interact with the host ubiquitination and endosomal sorting machinery (Freed, 2003; Strack *et al*., 2002). These results might assist in the discovery of the host proteins and complexes that interact with HIV in its assembly and budding (Ott *et al*., 2000).

5. THE CELLULAR MACHINERY RECRUITED BY HIV DURING ITS LIFE CYCLE

Tsg101 appears to be essential for efficient HIV virion egress. Although the precise mechanism by which Tsg101 mediates viral budding is unclear, it has been proposed that the budding of enveloped viruses from the cell plasma membrane is topologically equivalent to the budding of vesicles into the lumen of late endosomes (Babst *et al*., 2000; Lemmon and Traub, 2000). In support of this, the ubiquitination of the HIV-1 Gag p6 does not destine it for proteolytic degradation by packaging it into endosomal compartments, contrasting with the effect ubiquitination has on several mammalian cell surface receptors (Seto *et al*., 2002).

5.1. General characteristics of MVB

Regulated degradation of cell surface molecules is essential for the control of many biological processes (Seto *et al*., 2002). Endocytic membrane cargo can be partitioned either into recycling vesicles, for delivery back to the cell surface, or into late endosomes, for delivery to degradative compartments (Gruenberg, 2001). Cargo recognition at the limiting membrane of the late endosome results in bilayer invagination, cargo budding into intraluminal vesicles, and MVB formation. The vesicles and their contents are then degraded by proteases and hydrolases after fusion of MVB with lysosomes (Katzmann *et al*., 2001).

MVB are created when the cargo in endosomes is sorted for lysosomal degradation via formation of small vesicles. A mature MVB that can be constituted by a variable number of small vesicles, then fuses with the lysosome, releasing the vesicles for degradation in this hydrolytic organelle.

Extensive characterization of these vesicles has identified a set of multiprotein complexes (endosomal-sorting complex required for transport, ESCRT-1, -2 and -3) comprised of Vps proteins that participate in a vectorial chain of recruitment and association at the prevacuolar membrane necessary for MVB formation (Katzmann *et al*., 2002). ESCRT-1, comprising Vps23, Vps28, and Vps37 in yeast, activates ESCRT-2, which in turn recruits ESCRT-3. Beyond this, the actual function of these complexes is unclear.

5.2. MVB and HIV budding

Since specific host cell factors (Tsg101 UEV domain and ubiquitin) have been implicated in mediating the activity of HIV L-domain in viral budding and since these factors are involved in the MVB formation, it will be interesting to learn whether the host machinery involved in the MVB formation overlaps with that required for retrovirus budding (Patnaik *et al*., 2000**;** Pornillos *et al*., 2002a).

What advantage would HIV have by being sequestered in MVB? Pelchen-Matthews *et al*. (2003) have proposed that HIV particles are assembled within secretory vesicles in macrophages and are then released by exocytosis upon specific signals. HIV, like exosomes, may undergo regulated release in a T helper cell-enriched environment, thus facilitating viral spread. Unlike macrophages, dendritic cells are not readily infected with HIV. However, interaction of HIV with dendritic cells results in substantial enhancement of infectivity of adjacent T cells, and this requires HIV endocytosis into a so far undefined vesicular compartment within dendritic cells.

There is still much to be learned about the biochemical mechanisms of vesicle formation during both MVB

biogenesis and virus budding. Unresolved issues are: (i) the chemical and conformational changes that allow the sequential recruitment of ESCRT complexes and give directionality to the pathway; (ii) the mechanisms by which proteins are sorted into plasma and endosomal membrane microdomains; (iii) how membrane curvature is generated; and (iv) what actually catalyzes the membrane fission step that allows vesicle release. The unique topology of the virus and MVB budding requires a very different mechanism that must involve catalysis of membrane fission by molecules located inside or adjacent to the neck of the budding vesicle. Given these unusual topological requirements, it is perhaps not surprising that HIV and other viruses have evolved to appropriate the cellular machinery that can accomplish such vesiculation events (Pornillos *et al*., 2002a).

Recently, the involvement of Tsg101/Vps23 (Tsg101 is the human ortholog of Vps23 in yeast) in MVB formation has been described in more detail (Katzmann *et al*., 2001; Pornillos *et al*., 2002b; Bache *et al*., 2003). According to these reports, the cellular partner for Tsg101/Vps23 is the adaptor protein Hrs/Vps27 (hrs is the human ortholog of Vps27 in yeast), which has proline-rich motifs similar to those found in viral L-domains. Hrs/Vps27 serves the key function of delivering cargo from the early endosomal membrane to the late endosome limiting membrane for formation of MVB. These findings should be considered along with the observation that, in HIV infected macrophages, virions bud into MVBs rather than at the plasma membrane (Amara and Littman, 2003). The interactions of these proteins, resulting in the recruitment of ESCRT-1 to the late endosome limiting membrane, will be discussed below.

Hrs and ESCRT-1, -2, and -3, are involved in the endosomal sorting of membrane proteins into MVB, lysosomes or vacuoles. The ESCRT complexes are also required for formation of intraluminal endosomal vesicles and budding of certain enveloped RNA viruses such as HIV. It has been shown (Bache *et al*., 2003) that Hrs binds to the ESCRT-1 subunit Tsg101 via a PSAP motif that is conserved in Tsg101-binding viral proteins. Depletion of Hrs causes a reduction in membrane-associated ESCRT-1 subunits, a decrease in the number of MVB and an increase in the size of late endosomes. Overexpression of Hrs causes accumulation of Tsg101 on early endosomes and prevents its shift to late endosomes (Bache *et al*., 2003).

Hrs appears to act in a similar way at the early endosome and is critical for the formation of endosomal invaginations and vesicles.It has been reported (Pornillos *et al*., 2002b) that the Hrs PSAP motif-containing part can functionally replace the PTAP motif-containing part of the HIV Gag protein in budding of virus-like particles from the plasma membrane, suggesting that HIV Gag mimics the Hrs Tsg101-recruiting effect. These results argue that Hrs plays a dual role in endosomal protein sorting: (i) it recruits ubiquitinated proteins into clathrin-coated microdomains on early endosomes, and (ii) it recruits the ESCRT-1 complex, which is essential for further sorting and MVB formation (Bache *et al*., 2003).

Another report (Pornillos *et al*., 2003) has suggested that a full affinity Tsg101 binding requires at least two Hrs elements, one which contains the Hrs PSAP motif binding to the Tsg101 UEV domain, and another that includes the downstream Hrs element that spans the putative coiled-coil region and at least part of the proline/glycine-rich region. Both interactions are functionally important, although mutations that disrupt the Tsg101 UEV-PSAP motif interaction do not completely eliminate complex formation, indicating that the downstream Hrs elements may play the dominant role in Tsg101 recruitment. The use of multiple contact sites could simply serve to increase the affinity and specificity of the Hrs-Tsg101 interaction.

Summing up, both Tsg101 and Hrs can bind to ubiquitin, and most importantly, their ubiquitin-binding activities are required for their endocytic functions (Katzmann *et al*., 2001; Raiborg *et al*., 2002). The interaction between Hrs and Tsg101 apparently facilitates the trafficking of cargo-bearing early endosomes to late endosomes capable of incorporating the cargo in MVB. In this scenario, Hrs associated with both ubiquitinated proteins (L-domain-like proteins) and the membranes of early endosomes may recruit Tsg101, where Tsg101 can bind to the ubiquitin-tagged endosomal cargo and mediate subsequent trafficking.

6. CONCLUSIONS

HIV infection remains a significant issue in global health. Drug-resistant mutants and high cytotoxicity have limited the utility of antiviral therapies for an increasing number of people. Intensive research has been focused on vaccine strategies and targets, but novel therapeutic drugs are urgently needed. Novel antiviral drugs targeting all the steps of the HIV replicative cycle, specially the budding, are being developed. It has been reported that HIV recruits some cellular proteins, like Tsg101, to promote its release from host cell by mimicking the MVB pathway. This is especially true given that the Tsg101 pocket appears to be a site of catalytic action with the viral Gag p6 protein (L-domain) and ubiquitin. These findings indicate an interesting strategy by which HIV harnesses the endosomal protein machinery for the finalization of the budding process during virion maturation.

The mechanistic details of the virion-assimilated host process identify novel molecular targets for potential pharmacological intervention. Because Tsg101 is recruited by small, conserved viral sequence motifs (L-domains), the

agents that mimic these structures are potential inhibitors of virus replication. However, the possibility of cellular toxicity is a legitimate cause for concern, since the MVB pathway carries out important cellular functions. Although the budding step is still not completely understood, the clear need for new antivirals with novel targets in the HIV replication cycle will provide the impetus for studies aimed at developing virus budding inhibitors.

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