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Control of human immunodeficiency virus type-1 protease activity in insect cells expressing Gag-Pol rescues assembly of immature but not mature virus-like particles

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Abstract

Expression of human immunodeficiency virus type 1 (HIV-1) Gag protein in insect cells using baculovirus vectors leads to the abundant production of virus-like particles (VLPs) that represent the immature form of the virus. When Gag-Pol is included, however, VLP production is abolished, a result attributed to premature protease activation degrading the intracellular pool of Gag precursor before particle assembly can occur. As large-scale synthesis of mature noninfectious VLPs would be useful, we have sought to control HIV protease activity in insect cells to give a balance of Gag and Gag-Pol that is compatible with mature particle formation. We show here that intermediate levels of protease activity in insect cells can be attained through site-directed mutagenesis of the protease and through antiprotease drug treatment. However, despite Gag cleavage patterns that mimicked those seen in mammalian cells, VLP synthesis exhibited an essentially all-or-none response in which VLP synthesis occurred but was immature or failed completely. Our data are consistent with a requirement for specific cellular factors in addition to the correct ratio of Gag and Gag-Pol for assembly of mature retrovirus particles in heterologous cell types.

Introduction

An essential step in the morphogenesis of infectious Human Immunodeficiency Virus Type 1 (HIV-1) is the proteolytic maturation of immature viruses (Hoshikawa et al., 1991; Kohl et al., 1988). Immature particles assemble on the cytoplasmic face of the plasma membrane by oligomerization of the viral structural protein Gag (p55) and the co-incorporation of about 5% Gag-Pol. Maturation is thought to occur during or shortly after the immature virus particle buds from the host cell and is mediated by the virion-encoded protease. Protease and the other viral enzymatic proteins are encoded by the *pol* domain and are expressed as the Gag-Pol fusion protein via a −1 translational frameshift (Jacks et al., 1988). The level of frameshifting, approximately 5% of translation events, regulates the expression of Pol and the enzymatic activities it contains. During maturation, the protease cleaves Gag p55 into four major proteins; matrix protein (p17), capsid protein (p24), nucleocapsid protein (p7), and a proline-rich protein (p6). Cleavage of Gag results in a structural rearrangement of the particle, producing a central condensed conical capsid core and a virion that is infectious (for reviews of HIV assembly and maturation see Doms and Trono, 2000; Freed, 1998; Kräusslich, 1996).

The expression of Gag using heterologous expression systems has been an important factor in understanding the innate properties of Gag in the assembly of virus-like particles (Jones and Morikawa, 1998; Nermut et al., 1994; Royer et al., 1991; Zhang et al., 1996). Using recombinant baculoviruses, expression of Gag in insect cells results in the assembly of virus-like particles (VLPs), which are morphologically indistinguishable from immature particles assembled in human cells (Gheysen et al., 1989; Jones and Morikawa, 1998; Nermut et al., 1994). However coexpression of Gag with HIV-1 protease, either as part of the whole *pol* domain or as a truncated version encoding only pro-

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tease, results in the abolition of VLP assembly (Gheysen et al., 1989; Wagner et al., 1992). In mammalian cells, tight regulation of protease activity is also essential for correct virion morphogenesis as premature or accelerated proteolytic processing prevents particle assembly (Hoshikawa et al., 1991; Karacostas et al., 1993; Kräusslich, 1991, 1992; Mergener et al., 1992). If protease activity is too low, however, virus maturation is limited and virus infectivity is reduced (Kaplan et al., 1993; Kohl et al., 1988; Kräusslich, 1992; Rose et al., 1995). In accordance with these observations, the failure of VLPs to assemble in insect cells in the presence of protease has been proposed to be due to protease activity resulting in premature intracellular cleavage of Gag (Gheysen et al., 1989; Wagner et al., 1992). Assembly of HIV-1 VLPs in insect cells expressing Gag-Pol can be rescued by inactivation of the protease, providing support for this proposal (Hughes et al., 1993). However, purposeful modulation of protease activity at intermediate levels similar to those required for the generation of mature virions has not been previously investigated.

The ability to assemble mature HIV-1 VLPs using heterologous expression systems would greatly facilitate functional studies into the poorly understood structural role (if any) of Gag-Pol in virus assembly and the precise mechanism of virus maturation. It would also provide a basis for the production of high-titer infectious HIV-1 from a nonmammalian cell background with potential safety advantages for HIV-1-based gene delivery vectors (Kim et al., 1998; Kotsopoulou et al., 2000). Here, we investigate the consequence on VLP morphology of the expression of Gag-Pol containing protease active site mutants that reduce inherent protease activity (Rose et al., 1995). We also modulate intracellular proteolytic activity within insect cells expressing Gag-Pol through the addition of the HIV-1 protease inhibitor saquinavir (Roberts et al., 1990). We show that while both of these strategies reduce intracellular protease activity in insect cells to levels normally associated with virus maturation, neither strategy resulted in an intermediate rate of proteolytic cleavage that was compatible with the efficient assembly of mature VLPs.

Results

Expression of Gag-Pol containing T26S and A28S protease mutations

Studies of the expression of HIV protease in mammalian cells have shown that mutations T26S and A28S reduce intrinsic catalytic activity by 4- and 50-fold, respectively (Rose et al., 1995). A 4-fold reduction in activity resulted in virions very similar to wild-type, with only a slight reduction in infectivity, while a 50-fold reduction in activity resulted in immature noninfectious viruses (Rose et al., 1995). As HIV protease activation appears excessive in insect cells (Gheysen et al., 1989; Wagner et al., 1992), we

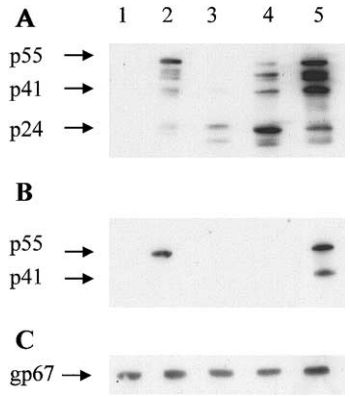

Fig. 1. Western blot analysis of Gag proteins expressed in *Sf*9 cells. Cell lysates and supernatants were harvested and adjusted to an equivalent protein concentration. Total loading of cellular extracts was 40 μg of total protein per lane except for AcGag, where the loading was reduced to 10 μg. Similarly, while the supernatants were adjusted to 800-μl equivalents of infected cell media, the AcGag supernatant was 20-fold less to compensate for an increased Gag signal. The samples analyzed were cells infected with BacPAK6 (control) (lane1); AcGag (lane 2); AcGag-Pol (lane 3); AcGag-Pol$_{T26S}$ (lane 4); and AcGag-Pol$_{A28S}$ (lane 5). Infected cell lysates (A and C) or supernatants (B) were collected 48 h postinfection, resolved by 12% SDS–PAGE, and Western blotted using anti-p24 antibody (A and B) or anti-gp67 (C). The positions of uncleaved Gag p55, intermediate p41, cleaved p24, and the baculovirus expression control gp67 are indicated.

hypothesized that the expression of Gag-Pol incorporating these two mutations in insect cells may result in an intermediate rate of proteolysis compatible with VLP assembly and maturation. Accordingly, recombinant baculoviruses expressing HIV-1 Gag (AcGag), Gag-Pol (AcGag-Pol), Gag-Pol$_{T26S}$ (AcGag-Pol$_{T26S}$), and Gag-Pol$_{A28S}$ (AcGag-Pol$_{A28S}$) were constructed and their expression profiles were evaluated by SDS–PAGE and Western blotting as described (Fig. 1). Expression of Gag alone resulted in predominantly uncleaved p55 precursor protein, while expression of wild-type Gag-Pol resulted in extensive intracellular proteolytic processing of Gag, with p24 capsid protein the predominant protein detected (Fig. 1A, lanes 2 and 3). Expression of Gag-Pol$_{T26S}$ resulted in increased levels of Gag, while a marginally less extensive intracellular proteolytic processing was observed with the predominant protein product still p24, but with detectable amounts of the processing intermediate p41 and the uncleaved precursor p55 (Fig. 1A, lane 4). Expression of Gag-Pol$_{A28S}$, however, increased further the level of Gag antigen, with significantly reduced intracellular processing in insect cells with the predominant bands Gag p55 and p41 (Fig. 1A, lane 5).

To assess the possibility that reduced protease activity allowed rescue of VLP particle formation, the supernatant fractions from recombinant baculovirus-infected cultures expressing Gag-Pol was also analyzed (Fig. 1B). Confirming numerous reports, expression of Gag p55 led to abundant Gag antigen in the supernatant of infected cells (Fig. 1B, lane 2), while expression of Gag-Pol showed no evidence of Gag antigen release (Fig. 1B, lane 3). Expression

of Gag-Pol$_{T26S}$ also showed no evidence of Gag antigen release (Fig. 1B, lane 4), although cell lysates showed weak evidence of uncleaved and partially cleaved Gag antigen (Fig. 1A). Expression of Gag-Pol$_{A28S}$, however, lead to release of Gag p55 in the supernatant of infected cells, although levels were lower than those by expression of Gag only (Fig. 1B, lane 5). In addition, significant levels of Gag p41 were present in supernatants from Gag-Pol$_{A28S}$-infected cells. The differential Gag expression observed was not the result of nonequivalent infection by any of the recombinant baculoviruses as blotting with a monoclonal antibody to the major baculovirus glycoprotein gp67 showed equivalent infection by all recombinants (Fig. 1C).

These data are consistent with the reported mutant protease activities in mammalian cells (Rose et al., 1995) and show that adjustment of protease activity through modifications *in cis* does allow intermediate levels of Gag proteolytic processing in heterologous expression systems. Furthermore, the reduction in activity associated with the A28S, though not the T26S, mutation was sufficient to rescue Gag release into the supernatant of expressing cells.

Nature of the Gag antigen secreted by expression of Gag-Pol$_{A28S}$

The release of Gag antigen following expression of HIV *gag* is the result of VLP assembly at the plasma membrane (Boulanger and Jones, 1996; Gay et al., 1998; Gheysen et al., 1989), a diagnostic for which is the sedimentation profile of the antigen to band in sucrose gradients. Soluble Gag antigen, as released by cell lysis, remains at the top of the gradient (Royer et al., 1992). Accordingly, to assess the nature of Gag antigen released by AcGag-Pol$_{A28S}$-infected cells Gag antigen in the supernatant was collected by ultracentifugation and analyzed on a 20–60% sucrose velocity gradient (Jowett et al., 1992). Following centrifugation, the gradient was fractionated and analyzed by SDS–PAGE and Western blotting. In addition, as AcGag-Pol$_{A28S}$ encoded the *pol* gene and there was evidence of Gag processing in the supernatant of infected cells, peak fractions were also tested for reverse transcriptase activity to assess the possibility of copackaged Gag-Pol, a prerequisite for mature VLP formation. Gag-reactive antigen expressed by AcGag was detected in the middle fractions of the gradient, with a peak at ~1.16 g/ml as reported (Jowett et al., 1992), whereas Gag antigen expressed by AcGag-Pol$_{A28S}$ exhibited a broader band of reactivity, with the peak fraction marginally lighter at around 1.13 g/ml (Figs. 2A and B). Such changes in sedimentation rates have been previously associated with aberrant particle synthesis by a variety of mutations, including deletion of the p2 sequence (Jowett et al., 1992; Wang et al., 1998) and matrix only particles (Giddings et al., 1998), and would be consistent with particles of altered size or composition. Reverse transcriptase assay of the peak fractions from control, AcGag, and AcGag-Pol$_{A28S}$ gradients revealed detectable RT activity associated with AcGag-Pol$_{A28S}$, but not AcGag-infected cell supernatants (Fig. 2C). However, concentration of the supernatant was required before detection was possible and total activity equated to ~10 mU/ml compared to a typical productive mammalian vector system of >3000 mU/ml (e.g., see Kim et al., 1998).

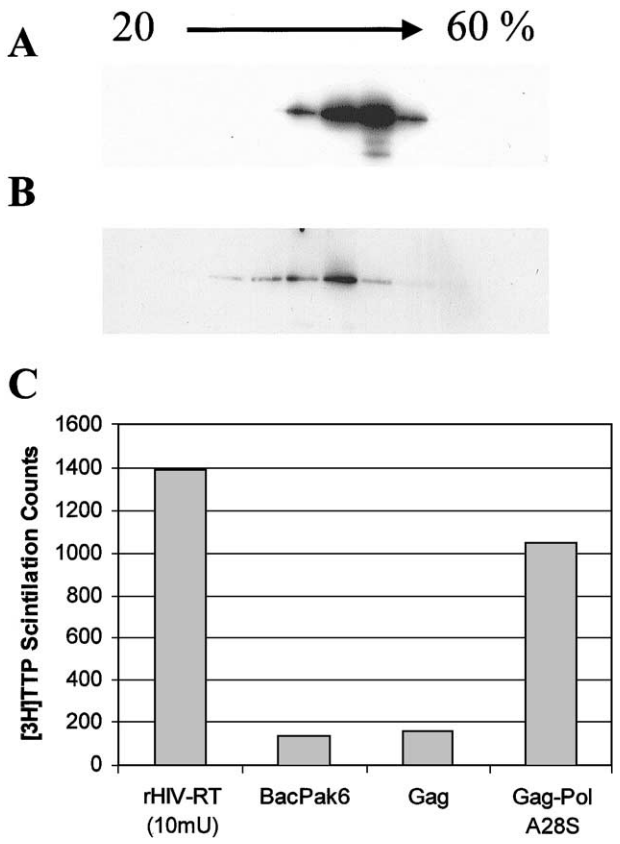

Fig. 2. Sucrose gradient analysis and RT activity of VLPs assembled in *Sf*9 cells. VLPs present in the supernatant of infected *Sf*9 cells were sedimented through a 20–60% step sucrose gradient (30K rpm, 1.5 h, 4°C, Beckman SW41 rotor) in PBS. VLPs produced by AcGag (A) or AcGag-Pol$_{A28S}$ (B) were detected by 12% SDS–PAGE and Western blotting using anti-p24 antibody following gradient fractionation from the top. (C) Reverse transcriptase activity detected in supernatants from cells infected with BacPAK6 (control), AcGag, or AcGag-Pol$_{A28S}$ is shown. The VLPs present in 0.65 ml of infected cell supernatant were pelleted, resuspended in 10 μl of H_20, and assayed using the Quant-T-RT kit, which utilizes the scintillation proximity assay (SPA) principle to measure reverse transcriptase activity by incorporation of [^{3}H]TTP by reverse transcriptase into a DNA/RNA primer/template bound to SPA beads (Amersham Pharmacia). Purified recombinant HIV-reverse transcriptase (rHIV-RT, Amersham Pharmacia) was used a positive control.

These data suggest that modulation of HIV-1 protease activity in insect cells, through mutation of the protease active site, can lead to a change in the ratios of different Gag cleavage products that is sufficient to rescue the expression of Gag antigen into the supernatant of expressing cells. However, the predominant secreted Gag band was at 55 kDa, the Gag precursor protein, there was no discernable further processing by residual protease incorporated into the VLPs, and the detected RT activity was very low. Thus, the moderation of protease activity afforded by mutation A28S

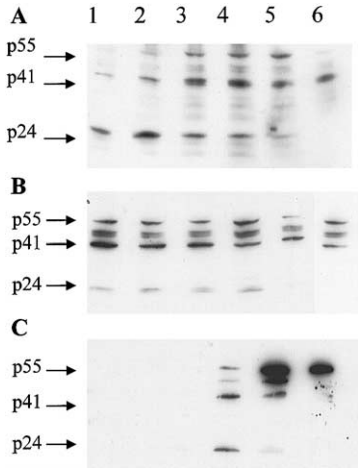

Fig. 3. Expression of Gag proteins in the presence of saquinavir. Gag proteins expressed in *Sf*9 cells infected with recombinant baculoviruses expressing AcGag-Pol (A) and AcGag-Pol_{T26S} (B and C) and treated with the protease inhibitor saquinavir are shown: lane 1, no drug; lanes 2–6, 10-fold increases in drug concentration from 0.001 to 10 μM, respectively. (A and B) Shown are cell lysates harvested 48 h postinfection, while C represents soluble protein (800 μl of supernatant). Protein samples were separated by 12% SDS–PAGE and followed by Western blotting using anti-Gag-p24 antibody. The positions of uncleaved Gag p55, the partial Gag cleavage intermediate p41, and cleaved p24 are indicated.

was incompatible with the assembly of mature VLPs at a level consistent with the intracellular Gag ratios observed.

A combination of protease mutation T26S and saquinavir rescues the assembly of immature but not mature VLPs

While protease mutation A28S was sufficient to recover Gag expression in the supernatant of expressing cells, protease activity was essentially inactivated. By contrast protease mutation T26S failed to recover Gag release of any sort. This suggested that a protease activity between that shown by A28S and T26S might accomplish Gag VLP assembly and maturation in the baculovirus heterologous expression system. To do this, the activity of the protease inhibitor saquinavir on Gag processing by Pro_{T26S} in insect cells was investigated. The addition of saquinavir over a range from 10 to 0.001 μM to insect cells infected with wild-type AcGag-Pol inhibited intracellular Gag proteolytic processing, as shown by decreased band intensity associated with the cleaved p24 product, at levels similar to those previously described (Overton et al., 1990) (Fig. 3A). However, no Gag antigen was detected in supernatants of expressing cells (not shown), suggesting that protease inhibition was insufficient to rescue Gag VLP assembly. Similar concentrations of saquinavir also caused inhibition of Gag processing in cells infected with AcGag-Pol_{T26S} (Fig. 3B) but in this case Gag antigen was evident in the supernatant of expressing cells at the higher saquinavir concentrations (Fig. 3C, lanes 4–6), with the highest concentration (10 μM) showing essentially only uncleaved Gag p55 (Fig. 3C, lane 6). As the intermediate Gag processing observed in AcGag-Pol_{T26S}-infected cells in the presence of 0.1–1 μM saquinavir resembled the pattern observed in HIV-infected cells (Speck et al., 2000), electron microscopy was used to assess the form of Gag released in the supernatant of expressing cells. VLP assembly with an immature morphology typical of that produced by uncleaved p55 Gag was observed in infected cells expressing Gag-Pol T26S supplemented with 10 μM saquinavir (Figs. 4A and B), consistent with the essential inhibition of protease activity. Some VLPs with an immature morphology were also observed in cells expressing Gag-Pol_{T26S} supplemented with 1 μM saquinavir (Fig. 4C) but the yield of VLPs was very low when compared to the overall level of Gag antigen expression. No VLPs were observed in cultures in the presence of 0.1 μM saquinavir despite the presence of cleaved Gag antigen in the supernatant. In addition, cells expressing Gag-Pol_{T26S} in the presence of 1 and 0.1 μM saquinavir had an increased level of cell disruption typical of the cytotoxicity associated with cells expressing active HIV protease (Konvalinka et al., 1995).

Discussion

Expression of the HIV *gag* and *gag-pol* genes in heterologous expression systems was investigated as early as 1989 (Gheysen et al., 1989; Karacostas et al., 1989; Overton et al., 1989). Expression of the HIV-1 *gag* gene in insect cells led to the production of abundant VLP with an immature morphology, while expression of *gag-pol* abolished VLP synthesis due to premature activation of the protease (Gheysen et al., 1989), an explanation also supported by later work linking particle assembly with protease activity (Karacostas et al., 1993). The cytotoxicity of the protease has also been suggested to play a role in the necessary balance between Gag cleavage and cell integrity needed for the formation of budded particles that are able to mature (Konvalinka et al., 1995). Stable expression of single copy HIV *gag-pol* in human cell lines or expression of the structural proteins through plasmid-based transfection results in the production of mature retrovirus particles (Kim et al., 1998; Wang et al., 1999), suggesting that no other viral factor is required. Thus, baculovirus expression of Gag and Gag-Pol at levels that mimic the level of Gag cleavage observed during human cell expression should give rise to virion particles with a mature phenotype. We used site-directed mutagenesis of the HIV protease within a *gag-pol* cassette to modify HIV protease activity to investigate an intermediate level low enough to allow virus particle assembly but with sufficient residual activity to carry out particle maturation inside the assembled VLP.

Baculovirus expression of Gag-Pol encoding protease active site mutations T26S and A28S (Rose et al., 1995) showed that mutation T26S had a marginal effect on the Gag cleavage pattern, while A28S significantly reduced

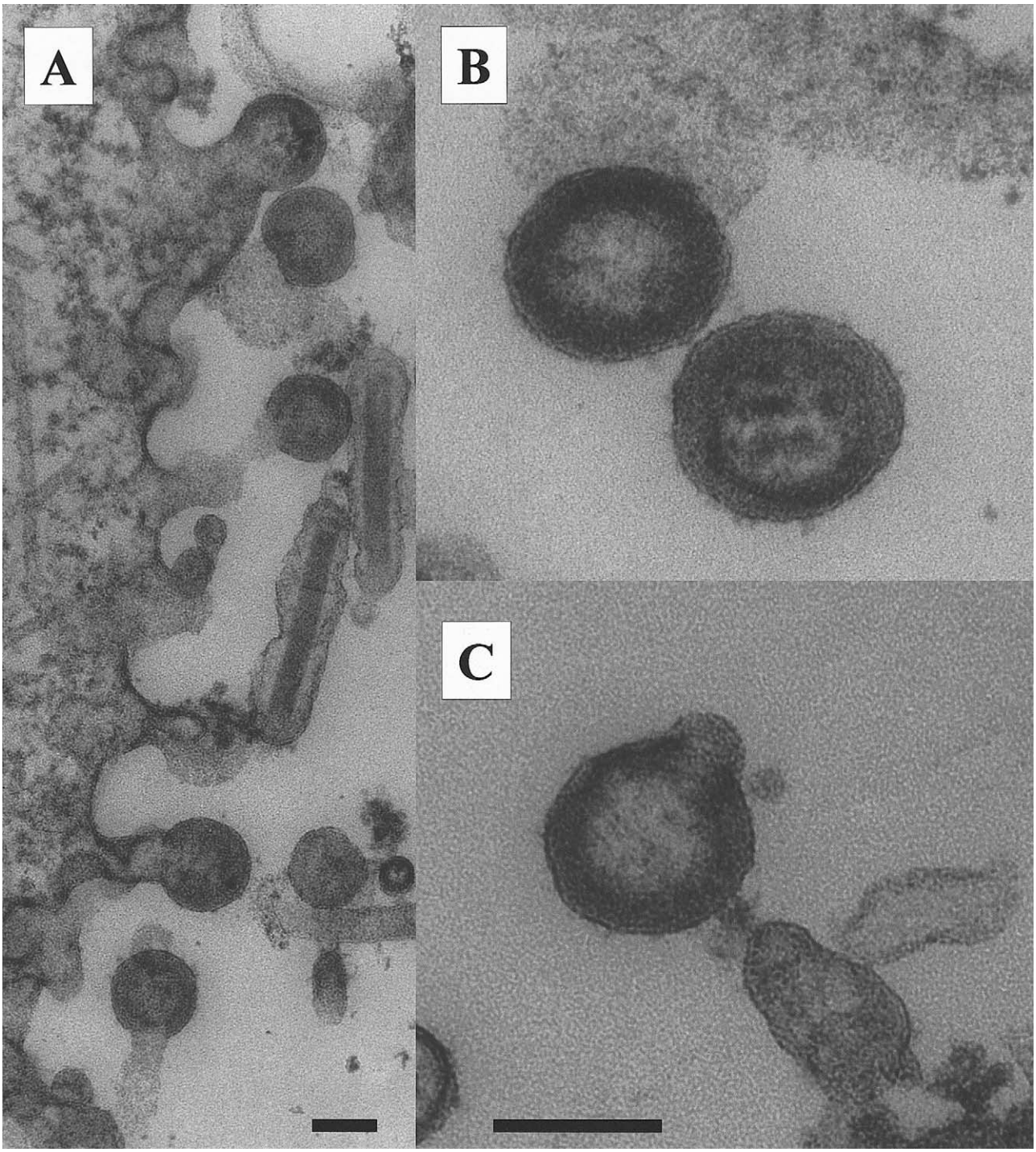

Fig. 4. Electron microscopy of assembled VLPs. Representative thin section electron micrographs of HIV VLPs assembled in *Sf* 9 cells infected with AcGag-Pol$_{T26S}$ and treated with saquinavir at 10 μM (A and B) and 1 μM (C). All the VLPs examined had a morphology typical of immature viruses. Rod-shaped baculoviruses are also present in A. Bars is 100 nm.

proteolytic processing to a level similar to that observed when Gag alone is expressed (i.e., to a level where only proteolysis by cellular proteases is observed (Jowett et al., 1992)). In addition, overall levels of Gag antigen were higher when protease activity was reduced, reflecting the reduced cytotoxicity, due to loss of nuclear integrity and proteolytic cleavage of host cytoskeletal cell proteins, normally observed following expression of active HIV-1 protease (Konvalinka et al., 1995; Kräusslich, 1991). VLP synthesis following expression of Gag-Pol$_{A28S}$, but not Gag-Pol$_{T26S}$, reflected the levels of precursor p55 protein present in cells and the morphology was immature, although

slight sedimentation differences on sucrose gradients could have been due to altered particle composition or size when compared to Gag-only-derived VLPs.

The expression of Gag-Pol$_{T26S}$ did not lead to the rescue of VLP synthesis but the addition of the protease inhibitor saquinavir caused a reduction in the level of intracellular Gag processing to a level that rescued the assembly of particles, as shown by Gag antigen in the supernatant of infected cells and evidence of VLPs by electron microscopy. VLPs were only formed at saquinavir concentrations that essentially inhibited protease activity completely, resulting in high amounts of Gag p55 and an immature VLP morphology. Intermediate levels of saquinavir produced a level of Gag intracellular cleavage that was similar to the cleavage patterns seen in HIV-infected cells yet produced a very low level of particles, all with immature morphology. We cannot rule out that our strategy of partial protease activity results in small numbers of mature VLPs ($<0.1\%$ of total VLPs based on examined EMs) but in this case their assembly efficiency does not reflect the level of intracellular Gag cleavage products observed. These data therefore do not support a mechanism of VLP assembly that requires only the correct ratios of Gag and Gag-Pol, as inferred from studies in mammalian cells (Karacostas et al., 1993).

Two models can be envisaged for the failure of suitable ratios of Gag and Gag-Pol to produce mature VLP. In the first, a selection mechanism operates at the level of assembly in which only uncleaved molecules enter the VLP assembly pathway and that Gag-Pol molecules, even with a protease activity appropriately modified to prevent premature cleavage, are either excluded or included in an inactive state. In the second, no distinction is made between Gag and Gag-Pol molecules, but premature activity by the protease results in particle collapse and only uncleaved Gag-Pols, by default, complete the assembly and budding pathway. Our data do not discriminate clearly between these possibilities but the fact that no graded response in VLP release between immature and mature phenotypes is seen argues in support of the former model rather than the latter. Recently, host factors, specific to human cells, have been found to be involved in the assembly and budding of HIV (Demirov et al., 2002; Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001; Zimmerman et al., 2002). These factors have been shown to be involved in the trafficking and budding of virus more than in budding and maturation per se (Perez and Nolan, 2001). In our experiments budding of immature VLPs was very efficient, indicating no alteration in Gag trafficking, but when budding was combined with an active protease, particle assembly adopted an either/or pathway of VLP assembly or non assembly. These data suggest that host cell factors, including those above, may have a role in sorting the correct Gag and Gag-Pol ratios or creating a microenvironment necessary for the development of a particle that is, first, competent for assembly and, second, capable of onward maturation. A link between virus release and the initiation of protease action has been previously reported but the molecular basis of the interaction was ill-defined (Kaplan et al., 1994). A link between budding and maturation has been also shown through the use of proteasome inhibitors that have no direct effect on the viral protease, yet inhibit both processes (Schubert et al., 2000). Last, lack of a chaperone activity specific for protease containing Gag molecules has been suggested to be the cause of the failure to assemble RSV VLPs in insect cells (Johnson et al., 2001), suggesting that all retroviruses may share a similar requirement.

Our data would be consistent with the fact that there is an intricate yet ill-defined step in the retrovirus assembly pathway that demands host involvement only when an active viral protease is present. Producing VLPs that are able to mature, for structural study or use as gene vectors, from heterologous cells will require a precise definition of this step.

Materials and methods

Recombinant baculoviruses and cell culture

All recombinant baculoviruses produced were derived from *Autographa californica* multiple nuclear polyhedrosis virus. The baculoviruses were generated by cotransfecting *Spodoptera frugiperda* (*Sf* 9) cells with recombinant transfer vector and linear BacPAK6 viral DNA (Clontech). Cell supernatants, harvested after 5 days, were plaque assayed and plaques picked and grown for virus stocks as described (King and Possee, 1992). Recombinant baculoviruses were confirmed by the immunodectection of Gag at 2 days postinfection (pi). Serum-free adapted *Sf* 9 cells were propagated at 28°C as described (O'Reilly et al., 1992), except that Sf-900 II medium (GibcoBRL, Life Technologies) was used throughout.

Construction of recombinant transfer vectors

To produce the recombinant transfer vector expressing HIV-1 Gag-Pol, the *gag-pol* gene was amplified from HIV-1 proviral clone strain HXB2 (AIDS reagent repository (Holmes, 2000)) by the polymerase chain reaction using the following primers (*Not*I linkers underlined): 5′-GGCGCGGCCGCATGGGTGCGAGAGCGTCGG-3′ and 3′-GTTCATCTGTCCTACTCCTAATTCGCCGGCGCGG-5′.

The amplified product was cloned into the *Not*I restriction site of the baculovirus transfer vector pBAC4x-1 (Cambridge Bioscience), placing the gene under the control of a polyhedrin promoter and the entire *gag-pol* coding region was verified by sequencing prior to expression studies.

Mutations $Thr_{26}\rightarrow$Ser (T26S) and $Ala_{28}\rightarrow$Ser (A28S) were introduced into the protease active site using the Quick Change Site-Direct Mutagenesis kit (Stratagene) using the mutagenic primers 5′-CTAAAGGAAGCTCTATTAGATTCTGGAGCAGATGATACAGTATTAG-3′ (T26S) and

5′-GAAGCTCTATTAGATACAGGATCCGATGATACA-GTATTAGAAGAAA TG-3′ (A28S). Clones containing the correct mutations were confirmed by DNA sequencing.

Expression and preparation of proteins

Monolayers of *Sf*9 cells were infected with baculovirus as described (King and Possee, 1992) using a multiplicity of infection (m.o.i) of 5 and harvested 48 h pi. Cell lysates were prepared by resuspension in cell lysis buffer (1 × PBS, 1% v/v NP-40) for 20 min at 4°C followed by clarification at 4K rpm for 10 min at 4°C. Protein concentrations were determined using a commercial protein assay kit (Bio–Rad).

Isolation and purification of virus-like particles

VLPs present in the supernatant of infected insect cells were collected by centrifugation at 24K rpm for 1 h at 4°C in a SW28 rotor (Beckman). The pellet was resuspended in PBS and loaded onto a 20−60% step sucrose gradient that had been allowed to stand overnight at 4°C before use and centrifuged for 1.5 h at 30K rpm, 4°C, in a SW41 rotor (Beckman). Fractions were collected from the top and a 10-μl aliquot of each fraction was analyzed by SDS–PAGE and Western blot. Fraction density, as a measure of sedimentation, not particle density at equilibrium, was measured by weight.

SDS–PAGE and Western blotting

Protein samples were prepared in Laemmli sample buffer (Laemmli, 1970), separated by 12% SDS–PAGE, and visualized by staining with Coomassie brilliant blue. For Western blots, proteins were transferred to PVDF membranes (Immobilon-P, Millipore) and blocked for 1 h at room temperature in PBS containing 0.1% Tween 20 (PBS-T) and 5% milk powder. Incubation with anti-HIV-1 Gag p24 antibody (Aalto BioReagents, Dublin at 1:1000 dilution) was for 1 h at room temperature followed by washing several times with PBS-T. Membranes were incubated for a further 1 h with peroxidase-conjugated donkey antisheep antibody (Sigma) and bound antibodies were detected by chemiluminescence (Roche).

Treatment with the protease inhibitor saquinavir

The antiprotease drug saquinavir (Ro 31-8959, Roche) was prepared as a 10 mM stock solution in methanol and stored at −80°C. Following infection of *Sf*9 cells with a Gag-Pol expressing baculovirus, drug was added to the cell media at concentrations ranging from 0.001 to 10 μM and expression profiles were analyzed by SDS–PAGE and Western blot as before.

Electron microscopy

The procedure followed was that described by Jowett et al., (1992). Briefly, infected cells dislodged by gentle tapping were collected by low-speed centrifugation at 1K rpm for 5 min at 21°C. The cells were resuspended in 0.1 M sodium cacodylate buffer, pH 7.4, containing 1% v/v glutaraldehyde (25% EM grade). Following fixation the cells were embedded in Araldite, sectioned, and poststained with uranyl acetate as described (Hockley et al., 1988). Specimens were examined using a Philips CM12 electron microscope operating at 80 kV.

Reverse transcription assay

Particles in the supernatants from infected Sf cells were concentrated by sedimentation as described and reverse transcriptase activity was assessed using the Quant-T-RT kit (Amersham Pharmacia). Purified HIV-1 reverse transcriptase at 1, 10, and 100 mU/ml, which gave an activity linearly related to dose, was used as a positive control. Only the positive sample approximating the activity found in the tests was included in the data shown.

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