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Human Immunodeficiency Virus Type 2 Glycoprotein Enhancement of Particle Budding: Role of the Cytoplasmic Domain

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Previous studies have shown that the glycoprotein cytoplasmic domains of human immunodeficiency virus type 2 (HIV-2) or simian immunodeficiency virus of macaques modulate biological activities of the viral glycoprotein complex, including syncytium formation, exterior glycoprotein conformation, and glycoprotein incorporation into budding virus particles. We have now utilized a recombinant expression system to study interactions of full-length or truncated HIV-2 glycoproteins with coexpressed HIV-2 Gag proteins which self-assemble and bud as virus-like particles. Interestingly, budding of HIV-2 virus-like particles from cells was enhanced 5- to 24-fold when Gag was coexpressed with the full-length HIV-2 glycoprotein, compared with Gag expressed either alone or with a truncated HIV-2 glycoprotein. The results obtained in this model system indicate that an additional effect of the lengthy cytoplasmic domain of the glycoprotein of HIV-2 is enhancement of particle budding. We speculate that the cytoplasmic domain of the viral glycoprotein of HIV-2 enhances budding by (i) potentiation of Gag structure or function or (ii) membrane modulation.

The glycoproteins of lentiviruses are unique in that the cytoplasmic domain is extraordinarily lengthy in primary structure (~150 to 220 amino acids) relative to those of all other retroviruses (~15 to 30 amino acids) (13, 43). Conservation of this lengthy cytoplasmic domain exclusively among all lentiviruses suggests that it fulfills an important and unique function in the life cycle of these viruses. Prior studies have indicated a role of the cytoplasmic domain of primate lentivirus glycoproteins in (i) the conformation, processing, and functions of glycoprotein ectodomains important for virus entry (20, 25, 41, 42, 54, 55, 66); (ii) the level of glycoprotein incorporation into virions (9, 65, 66); (iii) interactions with the matrix component of Gag during virion morphogenesis (12, 34, 35, 45); and (iv) down-regulating the level of cell surface expression of the simian immunodeficiency virus (SIV) glycoprotein (33).

The precise structure and functions of the lengthy cytoplasmic tails of lentiviral glycoproteins have remained unclear. Although spontaneous truncations of the cytoplasmic domains of the glycoproteins of SIV of macaques (SIV_{mac}) (3, 22, 29, 30, 48) or human immunodeficiency virus type (HIV-2) (1, 11, 18, 21, 31, 32) during in vitro passage were described, the advantages provided by truncation were poorly understood. In certain cell types syncytium formation by the HIV-2 or SIV_{mac} glycoprotein was enhanced by truncation of the cytoplasmic domain (42, 49, 54, 66). Previous investigations of the effects of truncations or alterations of the cytoplasmic domain on incorporation of lentiviral glycoproteins into budding virions have yielded seemingly conflicting results: decreased incorporation with HIV-1 (9, 65), increased incorporation with HIV-1 containing matrix mutations (12, 35), no effect on incorporation with HIV-1 (50, 63), or increased incorporation for SIV_{mac} (24, 66).

While HIV-2 clearly produces AIDS, the rates of disease progression and virus transmission reported for HIV-2 indicate that its pathogenic potential in human populations is significantly less than that of HIV-1 (6, 14, 26, 36). The novel molecular, pathogenic, and epidemiologic properties of HIV-2 have produced ongoing research efforts targeting this second human lentivirus (60). The unique properties of the glycoprotein cytoplasmic tail of HIV-2 relative to that of HIV-1 led us to further investigate the interactions of HIV-2 Env and Gag by utilizing recombinant vaccinia virus (rVV) expression of wild-type or mutant glycoproteins or Gag polyproteins. The objective was to investigate the importance of the lengthy cytoplasmic domain of HIV-2 glycoproteins in the budding of HIV-2 viral particles from cells. We therefore established for HIV-2 an experimental methodology for studying the assembly, budding, and maturation of virus-like particles (VLPs) similar to the methodology used in prior studies for HIV-1 or SIV (7, 19).

For cloning of the HIV-2 *gag-pro* gene, the HIV-2/ST.JSP427 proviral clone in plasmid pSXB-1 (23, 32) (provided by B. Hahn) was used as the template. Oligonucleotides 5′-CGGGTCGACGATTGTGGGAGATGGGCGCG and 5′-GCAGCTTCCCATTTAGTTGTCGACAGTTCCTAGCG were used as primers for PCR amplification and to introduce a translational termination codon and *Sal*I sites. The resulting 2,550-bp fragment was ligated into the *Sal*I-linearized plasmid pSC11-Sal1 (pSC11 provided by B. Moss and modified by C. Morrow), yielding pSC11-2GagPro. To allow expression of a full-length Gag-Pol polyprotein, a 2,483-bp *Bgl*II fragment from pSXB-1 (nucleotides 2486 to 4969) was ligated into the unique *Bgl*II site of pSC11-2GagPro, yielding plasmid pSC11-2GagPol. Construction (4) of a control vaccinia virus recombinant expressing only β-galactosidase (rVV-SC11) was described previously (44). The resulting vaccinia virus vectors rVV-*gpro*, rVV-*gpol*, and rVV-SC11 were inoculated onto SupT1 cells, and the cultures were metabolically labeled with [^{35}S]methionine-cysteine or [^{3}H]myristic acid. Preliminary experiments indicated that the rVV vectors expressing HIV-2 *gag-pro* or *gag-pol* in SupT1 cells had similar capacities for the expression, assembly into particles, and release of myristylated Gag proteins (data not shown). Furthermore, the enzymatic activities of HIV-2 protease and reverse transcriptase were

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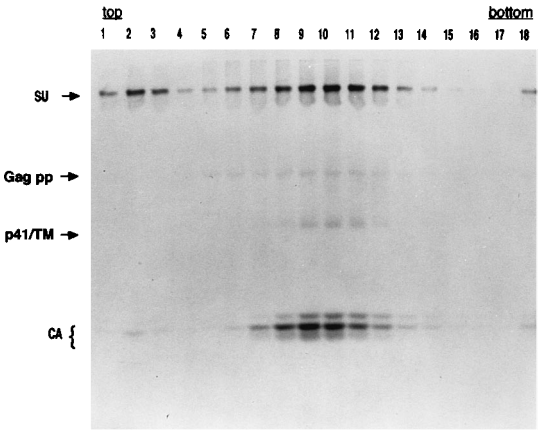

FIG. 1. Density gradient centrifugation demonstrated incorporation of HIV-2 glycoproteins into virus particles. Culture media from SupT1 cells coexpressing Gag and wild-type HIV-2 glycoprotein were pelleted through sucrose, resuspended, and then ultracentrifuged (170,000 × *g* for 3 h) over a 20 to 60% continuous sucrose gradient. Gradient fractions were then collected from the top, diluted with lysis buffer, and immunoprecipitated with antisera from HIV-2-infected humans. Fractions 9 and 10 contained the peaks of capsid protein (CA) and glycoprotein (SU and TM). Additional peaks of SU glycoprotein represented soluble glycoprotein that failed to enter the gradient (fraction 2) or aggregated VLPs which sedimented to the bottom of the gradient (fraction 18). Faint bands representing the Gag polyprotein (Gag pp) or its large proteolytic intermediate (p41) were also seen in the peak fractions. The electrophoretic mobilities of p41 and TM overlap. The human CD4-positive T-lymphoid cell line SupT1 was grown in RPMI 1640 medium supplemented with 15% fetal bovine serum.

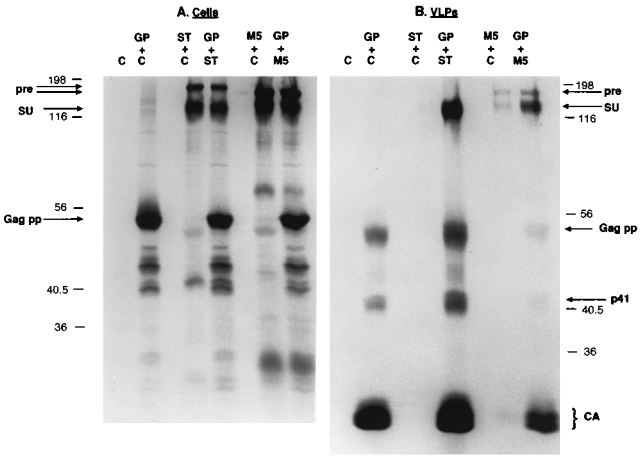

FIG. 2. Wild-type HIV-2 glycoprotein caused enhancement of particle budding. Immunoreactive HIV-2 bands are identified as follows: pre, the HIV-2 precursor glycoprotein; SU, the surface glycoprotein gp120; Gag pp, Gag polyprotein; p41, the Gag intermediate; and CA, capsid protein. (A) SupT1 cells were coinfected with pairs of vaccinia virus vectors indicated as follows: C, rVV-SC11 (control); GP, rVV-*gpol*; ST, rVV-ST*env*; and M5, rVV-M5*env*. Similar levels of cellular Gag pp were observed when Gag was coexpressed with the control vector, ST glycoprotein, and M5 glycoprotein. (B) Visual inspection revealed that within the corresponding viral pellets (VLPs), coexpression of Gag with wild-type glycoprotein (GP+ST lane) caused greater release of Gag pp, p41, and CA than coexpression of Gag with the control vector or the truncated glycoprotein (GP+C and GP+M5, respectively). The final MOI was a constant 3 PFU per cell in all experiments (1.5 PFU per cell per vector). The viral proteins were analyzed by radioimmunoprecipitation as described previously (40–42). The data presented are representative of multiple independent experiments yielding similar results.

demonstrated. Previous reports described the cloning, expression, processing, transport, and function of the full-length or carboxy-terminus-truncated recombinant HIV-2 glycoproteins by rVV-ST*env* or rVV-M5*env*, respectively (40–42). The glycoprotein expressed by rVV-M5*env* possessed a truncated cytoplasmic domain of only 17 amino acids. To establish that the Gag proteins isolated from the viral pellets truly represented assembled virus particles, and to determine whether such particles could coassemble viral glycoproteins, SupT1 cells were coinfected with rVV-*gpol* and rVV-ST*env*. After metabolic labeling, the culture medium was clarified, filtered, and pelleted over a sucrose cushion. The viral pellet was resuspended, loaded onto a continuous sucrose gradient, and centrifuged. Gradient fractions 9 and 10 contained the peak of HIV-2 capsid antigen present as a triplet with one dominant band (Fig. 1). In the same gradient fractions, less-intense bands representing the Gag polyprotein or the intermediate p41 were detected. The peak level of SU glycoprotein gp120 was present in fraction 10. A second less-intense peak of SU protein observed in fraction 2 represented soluble SU not entering into the top of the gradient. Fraction 18, at the bottom of the gradient, contained faint SU and CA bands, presumably representing aggregated virus particles. The measured sucrose density of gradient fraction 10 containing the VLP peak was 1.15 g/ml, consistent with the density of infectious HIV-1 particles. These results clearly established that the recombinant HIV-2 Gag proteins self-assembled, budded from the plasma membrane into the culture medium, and underwent proteolysis by the viral protease. Furthermore, coexpressed wild-type HIV-2 glycoproteins were coassembled into glycoprotein-bearing HIV-2 particles of an appropriate density. Electron microscopic studies further confirmed that coexpression of the recombinant Gag and Env proteins resulted in the assembly and release of large numbers of glycoprotein-bearing HIV-2 VLPs (data not shown).

To determine whether glycoprotein coexpression had an effect on the ability of HIV-2 Gag proteins to assemble and bud, we performed coinfections of SupT1 cells with rVV-*gpol* and either rVV-ST*env*, rVV-M5*env*, or the control vector. Vaccinia virus vector stocks were carefully titered just prior to these experiments to ensure equal levels of infection with each vector. High levels of Gag or Env proteins were expressed in the cells and were unaffected by coinfections (Fig. 2A). The viral pellets from cultures which coexpressed envelope glycoproteins with Gag released VLPs bearing glycoproteins as expected (Fig. 2B). The truncated glycoprotein but not the full-length glycoprotein, also incorporated low levels of precursor glycoprotein in addition to the cleaved subunits. The viral pellets were free of unincorporated wild-type glycoprotein as determined by expressing ST glycoprotein in the absence of Gag and evaluating the pellets. However, low levels of Env precursor and SU of the truncated glycoprotein M5 expressed in the absence of Gag were detected in the pellets in some, but not all, experiments (Fig. 2B; compare lanes ST+C and M5+C). Presumably this was due to a greater tendency of the polyprotein and SU derived from the truncated glycoprotein to form aggregates which entered the pellets. Interestingly, visual inspection of several initial gels suggested that cultures in which Gag was coexpressed with wild-type HIV-2 glycoproteins released higher levels of virus particles than did cultures in which Gag was coexpressed with either the control vector or the truncated glycoprotein (Fig. 2B; shown by comparison of the denser bands of Gag polyprotein, p41, and CA in the GP+ST lane with the less-dense bands in the GP+C and GP+M5 lanes).

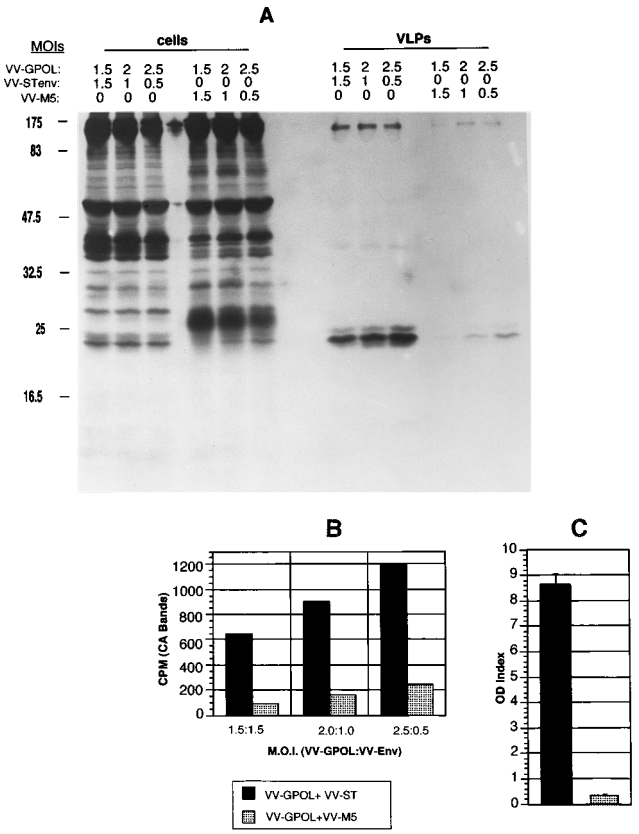

FIG. 3. Enhanced budding of virus particles demonstrated by two quantitative methods. (A) Radioimmunoprecipitation-polyacrylamide gel electrophoresis of cell lysate supernatants (Cells) or the lysed viral pellets (VLPs) derived from SupT1 cells coexpressing HIV-2 Gag with either full-length or truncated glycoproteins. The MOIs of the vaccinia virus vectors used to express Gag (VV-GPOL) with either wild-type glycoprotein (VV-STenv) or truncated glycoprotein (VV-M5) were as indicated above the autoradiograph. At 3 h after infection, cultures underwent metabolic labeling with [^{35}S]methionine-cysteine for 2 h, followed by a 5-h incubation. Culture media were clarified, filtered, and pelleted over a 25% sucrose cushion. The viral pellet was lysed, and the proteins were separated by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Visual inspection revealed greater particle release with coexpression of the wild-type glycoprotein than with coexpression of the truncated glycoprotein at all MOI ratios (see the VLPs). In the viral pellet lanes (VLPs), the SU band is at ~120 kDa and the CA bands are at ~24 kDa. (B) Particle release was quantitated by direct measurement on gels of radioactive counts present in the capsid bands (AMBIS Systems, Inc., San Diego, Calif.). At the three different MOI ratios, the full-length glycoprotein (VV-ST) was associated with CA counts per minute eight-, five-, and fivefold higher, respectively, than those observed with the truncated glycoprotein (VV-M5). (C) A second method used to quantitate CA release was a capsid antigen ELISA (SIV_{mac} p27 capsid antigen ELISA; Coulter, Inc., Hialeah, Fla.) performed on precleared culture media. SupT1 cells were coinfected with rVV-*gpol* and either rVV-ST*env* or rVV-M5*env* at MOIs of 1.5 each. The data are presented as optical density (OD) indexes ± standard deviations derived by multiplying the dilution of medium required to obtain an on-scale OD reading (25 for VV-ST; undiluted [given a value of 1] for VV-M5) by the OD value. The resulting OD index for VV-ST is 24-fold higher than the OD index for VV-M5. The results of three experiments, each done in triplicate, are shown as means ± standard deviations.

To quantitatively measure the effects of glycoprotein coexpression on HIV-2 particle budding, we next utilized a radiometric detection system. Viral pellets were collected from labeled cultures coexpressing Gag with either full-length or truncated HIV-2 glycoproteins. In this experiment, the ratio of Gag expression to Env expression was varied by altering the multiplicity of infection (MOI) ratio of rVV-*gpol* to rVV-*env* utilized in the coinfections (Fig. 3A). Visual inspection of the autoradiograph again indicated that greater particle release occurred when Gag was coexpressed with the full-length glycoprotein than when it was coexpressed with the truncated glycoprotein (Fig. 3A; see the VLPs). The levels of capsid protein radioactivity were directly quantitated from the gel. Interestingly, whether the relative expression ratio of Gag to Env was 1:1, 2:1, or 5:1, coexpression of Gag with full-length glycoprotein compared with truncated glycoprotein consistently caused a five- to eightfold increase in the level of radiolabeled capsid protein measured in viral pellets (Fig. 3B). Since the result was consistent over a range of Gag/Env expression ratios, this experiment indicated that the observed enhancement of particle release was not simply a function of recombinant glycoprotein overexpression. Furthermore, this result was not caused by an artifactual increase in release of particles due to glycoprotein-induced cytopathic effect, since (i) in our previous work the full-length glycoprotein caused significantly less syncytium formation and cytopathic effect than the truncated glycoprotein (42) and (ii) we observed no differences between the cytopathic effect in cell cultures expressing full-length glycoproteins and that in cultures expressing truncated glycoproteins at the time of harvest. We also performed cell surface biotinylation experiments and observed similar levels of biotinylated SU glycoprotein expressed on the plasma membranes of cells expressing full-length glycoproteins and on those of cells expressing truncated glycoproteins (data not shown).

In separate experiments, we obtained independent quantitative measurements of the relative levels of HIV-2 capsid release resulting from coexpression of HIV-2 Gag with either full-length or truncated glycoproteins. The levels of capsid antigen released into culture media were analyzed by an antigen capture enzyme-linked immunosorbent assay (ELISA). After 26 h of incubation, cultures coexpressing Gag with full-length glycoprotein released 24 times as much HIV-2 capsid antigen as cultures in which Gag was coexpressed with truncated glycoprotein (Fig. 3C). After 7 h of incubation, similar results were obtained, while at the earliest time point, 2 h, low levels of released capsid were detected with the wild-type glycoprotein but not with the truncated glycoprotein (data not shown). These results confirmed that the magnitude of particle release was enhanced by the wild-type glycoprotein and also indicated that the rate of particle release was enhanced.

Therefore, several independent experiments employing visual inspection of autoradiographs or two quantitative methods clearly demonstrated that the full-length glycoprotein of HIV-2 enhanced release of virus particles by 5- to 24-fold above the level of release achieved by either Gag expression alone or Gag coexpression with a truncated glycoprotein. These findings describe an additional effect of the lengthy cytoplasmic domain of the glycoprotein of HIV-2, i.e., enhancement of the release of virus particles. A general feature of the morphogenesis of retroviruses is the inherent capacity of Gag proteins to assemble and release virus particles (27). However, enhancement of particle release has previously been ascribed to the HIV-1 ancillary protein Vpu (16, 28, 47, 51, 57, 59) and to the HIV-1 carboxy-terminal Gag component p6 (17, 46). The molecular mechanisms responsible for the enhancement of HIV-1 budding by Vpu or p6 remain unknown. A novel finding of the present study was the enhancement of viral budding mediated by the wild-type HIV-2 glycoprotein. The effect was partially mapped to the region of the long cytosolic domain carboxy terminal to the membrane-proximal 17 amino acids. Two mechanistic models for this glycoprotein effect on budding will be discussed: Gag potentiation and membrane modulation.

Prior reports provide evidence that during lentiviral morphogenesis an interaction between the glycoprotein and the matrix protein occurs (8, 12, 34, 35, 45, 62, 64). The HIV-2 glycoprotein effect on budding might similarly be due to direct or indirect (i.e., via a cellular protein intermediary) interactions of the Env and Gag proteins. For alphaviruses, another group of enveloped viruses, budding is driven by a required interaction of glycoprotein spikes with core proteins (58). For HIV-2, the proposed Env-Gag interaction differs from the mechanism in alphaviruses in that it is not required for budding but rather potentiates the capacity of Gag to assemble and release particles. Glycoprotein potentiation of Gag might occur during an early step in particle morphogenesis, e.g., Gag multimerization, Gag protein trafficking, or membrane binding by Gag. In this model, mutant glycoproteins with truncated cytoplasmic domains are unable to potentiate Gag and therefore fail to enhance virus particle release. Alternatively, the glycoprotein tail might potentiate Gag at a later step in virion morphogenesis, e.g., the final condensation of Gag molecules at the plasma membrane into a core structure.

Membrane modulation by the lengthy tail of the HIV-2 glycoprotein is a second mechanistic model to explain the enhancement of virus budding. In this model, the integrity of the plasma membrane is perturbed by the glycoprotein in a way that favors virus budding. Evidence supporting this model is provided by reports that the glycoprotein cytoplasmic domains of HIV-2, HIV-1, and SIV contain two amphipathic helical segments (10, 56, 61). Amphipathic alpha-helices have been identified as structural motifs which interact with membranes. Recombinant HIV-1 glycoproteins (19a) or synthetic peptides representing the HIV-1 amphipathic helices (2, 5, 15, 37–39) were shown experimentally to interact with lipid bilayers or form membrane channels or pores which destabilize membranes. The possibility that membrane association by these amphipathic helical segments may modulate virus budding or other membrane-related events in the viral life cycle was raised previously (56). A related possibility is that the long glycoprotein tail might extend across the neck of a nearly completed viral bud to undergo a hydrophobic interaction with the opposing bilayer, thereby facilitating final release of the budding particle.

It is of interest that previous results for the HIV-2/SIV group of viruses have been divergent from those for HIV-1 with regard to the glycoprotein cytoplasmic tail. While HIV-2 and SIV_{mac} often select for glycoprotein truncations during passage in certain human cells (40–42, 49, 54, 66), HIV-1 apparently does this only very rarely (53). One possible inference is that HIV-2 and SIV are less adapted than HIV-1 for growth in certain human cells. It may be relevant that the HIV-1 protein Vpu was reported to contain an amphipathic helix in its membrane-spanning domain (52). Data correlating Vpu-mediated enhancement of virus release with the capacity of the Vpu membrane-spanning domain to form ion channels were previously reported (51). Since another way in which HIV-2 differs from HIV-1 is that HIV-2 lacks the *vpu* gene, one can speculate that the HIV-2 glycoprotein cytoplasmic domain with its amphipathic helices may have evolved to provide Vpu-like enhancement of HIV-2 budding. Biochemical and genetic experimental approaches can be employed to further explore the role of the HIV-2 glycoprotein cytoplasmic domain in the assembly and release of infectious virions. Such work may reveal new targets for inhibition of the lentiviral life cycle.

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ADDENDUM IN PROOF

After this article was accepted, glycoprotein enhancement of budding with a different strain of HIV-2 was reported (S. Bour, U. Schubert, K. Peden, and K. Strebel, J. Virol. **70:**820–829, 1996).

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