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Release of Virus-Like Particles from Cells Infected with Poliovirus Replicons Which Express Human Immunodeficiency Virus Type 1 Gag

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The effectiveness of attenuated poliovirus vaccines when given orally to induce both systemic and mucosal immune responses against poliovirus has resulted in an effort to develop poliovirus-based vectors to express foreign proteins. We have previously described the construction of poliovirus genomes (referred to as replicons) in which the complete human immunodeficiency virus type 1 (HIV-1) *gag* gene was substituted for the capsid gene (P1) (D. C. Porter, D. C. Ansardi, and C. D. Morrow, J. Virol. 69:1548–1555, 1995). Infection of cells with encapsidated replicons resulted in the expression of a 55-kDa protein. To further characterize the biological features of the HIV-1 Gag proteins expressed in cells infected with encapsidated replicons, we utilized biochemical analysis and electron microscopy. Expression of the 55-kDa protein in cells infected with encapsidated replicons resulted in myristylation of the $Pr55^{gag}$ protein. The Gag precursor protein was released from infected cells; analysis on sucrose density gradients revealed that the precursor sedimented at a density consistent with that of an HIV-1 virus-like particle. Analysis of replicon-infected cells by electron microscopy demonstrated the presence of condensed structures at the plasma membrane and the release of virus-like particles. These studies demonstrate that poliovirus-based vectors can be used to express foreign proteins which require posttranslational modifications, such as myristylation, and assemble into higher-order structures, providing a foundation for the future use of poliovirus replicons as vaccine vectors.

The genome of poliovirus is a single-stranded RNA molecule which encodes a single open reading frame that is translated into a large precursor polyprotein (30, 32). The genome has a virus-encoded protein, VPg, covalently attached at the 5′ terminus and a poly(A) tract at the 3′ terminus (30, 32). The precursor polyprotein has been divided into three regions: P1, encoding the structural proteins, and P2 and P3, encoding the nonstructural proteins such as viral proteases 2A, 3C, and 3CD, as well as viral polymerase $3D^{pol}$ (21, 33, 56, 70). Following translation of 2A, the protease processes the polyprotein precursor at specific tyrosine-glycine amino acid pairs, resulting in the release of the P1 capsid precursor protein (41, 45, 64). The P1 protein is then processed in *trans* by 3CD (6, 26, 71, 72). 3C processes the P2 and P3 regions of the polyprotein to generate the nonstructural proteins involved in replication of the viral RNA (21, 32, 33, 52).

In recent years, several groups have reported on attempts to develop poliovirus as an expression system that could ultimately be used as a vaccine vector (3, 4, 13, 35, 36, 50, 51). Poliovirus is attractive for use as a vector for several reasons. The availability of an infectious cDNA clone for poliovirus has allowed the use of molecular genetics to modify the viral genome (53). Administration of the attenuated strains to humans results in the stimulation of antibodies to poliovirus in both the serum and secretions (37, 42, 43, 57, 61). Since poliovirus is an enterovirus, vectors based on poliovirus could be administered orally to deliver antigens that would stimulate both the systemic and mucosal immune systems. Studies have found that defective poliovirus genomes (8, 20), as well as genomes which contain foreign genes inserted into the P1 region, maintained the capacity of the RNA to replicate (3, 12, 49). Poliovirus "replicons" which contain gene fragments of the human immunodeficiency virus type 1 (HIV-1) *gag*, *pol*, and *env* genes substituted for the VP2 and VP3 genes have been previously constructed in our laboratory; transfection of the replicon genomes into cells resulted in expression of the HIV-1 proteins as fusion proteins with the poliovirus P1 protein (i.e., VP1) (13). Poliovirus replicons, which contain foreign gene sequences substituted for the P1 region, are similar to naturally occurring defective-interfering genomes of poliovirus in that both do not encode their own capsid proteins. For defective-interfering genomes to be propagated, coinfection with wild-type poliovirus must occur to provide the P1 precursor protein in *trans* (20). Previous studies in our laboratory have demonstrated that P1 protein expressed from a recombinant vaccinia virus (VV-P1) (6) can be used to complement defective genomes (8) or replicons (2, 5, 50, 51). Serial passage of the encapsidated genomes in the presence of VV-P1 resulted in the generation of virus stocks composed entirely of encapsidated replicons. Infection of cells with the replicons results in viral protein expression and synthesis of genomic RNA.

One of the drawbacks in the development of poliovirus as a vector has been the size limitation for expression of foreign genes. Recently, we have reported on the construction and characterization of replicons which contain the complete HIV-1 *gag* gene substituted for the P1 gene sequences (51). By using VV-P1 to provide the capsid proteins in *trans*, we demonstrated the encapsidation of these replicon genomes and the intracellular expression of a 55-kDa protein in cells infected with the encapsidated replicons. Previous studies have demonstrated that expression of the HIV-1 *gag* gene in the absence of the viral proteinase results in modification of the $Pr55^{gag}$ protein by the addition of myristic acid and transport of Gag protein to the plasma membrane, where immature virus particles were assembled and released from the cells (17–19, 24).

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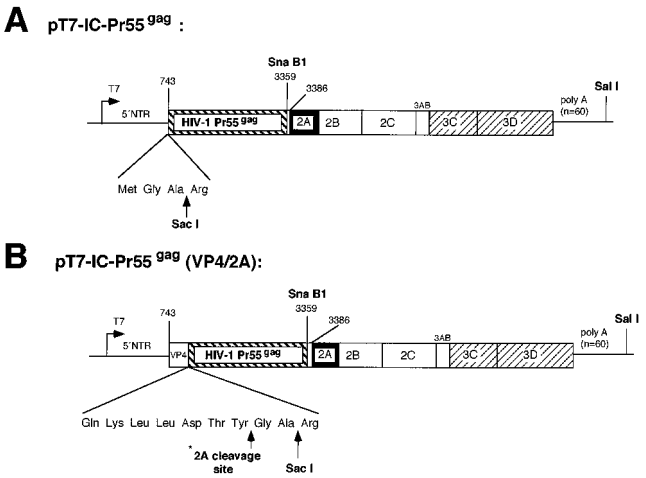

FIG. 1. Schematics of poliovirus replicons which contain the complete HIV-1 *gag* gene. The construction and characterization of pT7-IC-Pr55^{gag} and pT7-IC-Pr55^{gag}(VP4/2A) have been previously described (51). Each replicon genome contains promoter sequences for the T7 RNA polymerase promoter positioned 5′ to the poliovirus cDNA and a unique *Sal*I restriction site after the poly(A) tract for linearization of the plasmid before in vitro transcription. (A) Replicon pT7-IC-Pr55^{gag} contains the entire *gag* gene (nucleotides 345 to 1837, PCR amplified from the HXB2 genome [54]) of HIV-1 substituted in frame for the P1 coding region (nucleotides 748 to 3359) between unique restriction sites located at nucleotides 748 (*Sac*I) and 3359 (*Sna*BI). Amino acids constituting a cleavage site for 2A are positioned 3′ to the complete *gag* gene (22). (B) Replicon pT7-IC-Pr55^{gag}(VP4/2A) contains the complete *gag* gene of HIV-1 substituted in frame for the VP2, VP3, and VP1 capsid sequences (nucleotides 949 to 3359) between unique *Sac*I and *Sna*BI restriction sites. The gene sequences encoding the VP4 capsid gene (nucleotides 743 to 949) are positioned 5′ to the *gag* gene. An additional cleavage site for protease 2A was introduced between the VP4 coding region and the *gag* gene for processing of the Gag protein from the precursor polyprotein. NTR, nontranslated region.

Since poliovirus induces profound alterations in the physiology of infected cells, including shutoff of host protein synthesis and redistribution of intracellular membranes (9, 10, 32), it was possible that proteins expressed from the encapsidated replicons would not undergo posttranslational modifications or be released from the infected cell. To address this possibility, we analyzed the expression of the HIV-1 Gag protein from cells infected with encapsidated replicons by using biochemical methods and electron microscopy. The results of our study establish that poliovirus vectors can be used to express an HIV-1 Pr55^{gag} core precursor protein that assembles into virus-like particles.

The construction of poliovirus replicons which contain the 1.5-kb *gag* gene (nucleotides 345 to 1837 of the HXB2 genome [54] obtained by PCR amplification) of HIV-1 substituted in frame for the P1 region has been previously described (51). These replicons, referred to as pT7-IC-Pr55^{gag} (Fig. 1A) and pT7-IC-Pr55^{gag}(VP4/2A) (Fig. 1B), contain the complete *gag* gene substituted in frame for the P1 region between unique *Sac*I and *Sna*BI sites, followed by the amino acid sequences constituting a cleavage site for 2A (22) positioned 3′ to the *gag* gene. Since VP4 has been proposed to contain an encapsidation signal for poliovirus (20, 27), replicon pT7-IC-Pr55^{gag}(VP4/2A) contains the VP4 capsid sequences (nucleotides 743 to 949), followed by the amino acids constituting a 2A cleavage site, positioned 5′ to the *gag* gene (Fig. 1B). RNA generated by in vitro transcription of each of the replicon genomes was replication competent, resulting in the expression of a 55-kDa protein (51). Furthermore, the RNA could be encapsidated in *trans* by using recombinant vaccinia virus, VV-P1, to generate virus stocks of the replicons, referred to as vIC-Pr55^{gag} and vIC-Pr55^{gag}(VP4/2A) (51).

To analyze protein expression from the replicons, cells were infected with normalized amounts of either vIC-Pr55^{gag} or vIC-Pr55^{gag}(VP4/2A) and metabolically labeled with [^{35}S] Translabel (ICN) or [^{3}H]Myristic Acid (NEN Dupont) (Fig. 2). Upon labeling with [^{35}S]Translabel and immunoprecipitation with anti-p24 antibodies (62), a 55-kDa protein from cells infected with vIC-Pr55^{gag} or vIC-Pr55^{gag}(VP4/2A) was detected (Fig. 2A, lanes 2 and 3); this protein comigrated with the Pr55^{gag} protein expressed from cells infected with recombinant vaccinia virus vVK1 (lane 4) (28). A protein with a higher molecular mass was also immunoprecipitated from cells infected with vIC-Pr55^{gag}(VP4/2A) (lane 3). The molecular mass of this protein is consistent with the predicted size of a VP4-Pr55^{gag} fusion protein.

Previous studies have established that addition of myristic acid to the amino-terminal glycine residue of the HIV-1 Gag protein is a prerequisite for virus assembly and release of

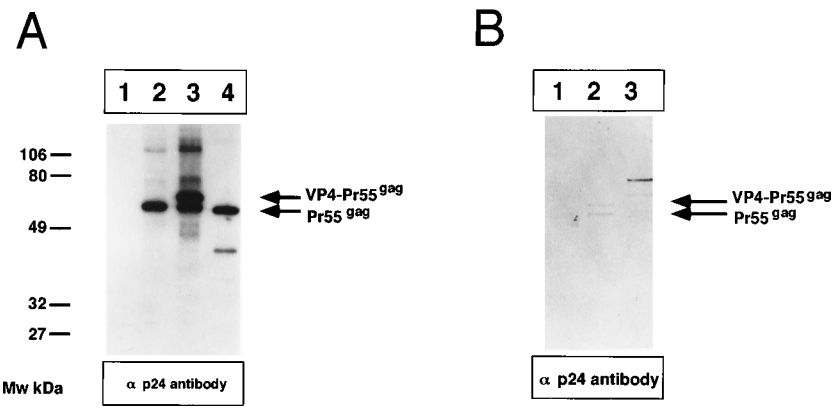

FIG. 2. Analysis of HIV-1-specific protein expression from cells infected with poliovirus replicons which contain the HIV-1 *gag* gene. (A) Cells were infected with normalized amounts of encapsidated replicon virus stocks and metabolically labeled with [^{35}S]Translabel. Cell lysates were incubated with anti-p24 antibodies, and immunoreactive proteins were analyzed on a sodium dodecyl sulfate-polyacrylamide gel. Lanes: 1, mock-infected cells; 2, cells infected with vIC-Pr55^{gag}; 3, cells infected with vIC-Pr55^{gag}(VP4/2A); 4, cells infected with vVK1. Molecular mass (Mw) standards and positions of relevant proteins are indicated. (B) [^{3}H]Myristic Acid labeling of cells infected with encapsidated replicons. Cells were infected with the encapsidated replicon virus stocks and metabolically labeled with [^{3}H]Myristic Acid, and cell lysates were incubated with anti-p24 antibodies. Immunoreactive proteins were analyzed on a sodium dodecyl sulfate-polyacrylamide gel. Lanes: 1, mock-infected cells; 2, cells infected with vIC-Pr55^{gag}(VP4/2A); 3, cells infected with vIC-Gag1 (50) as a control for myristylation. The positions of relevant proteins are indicated.

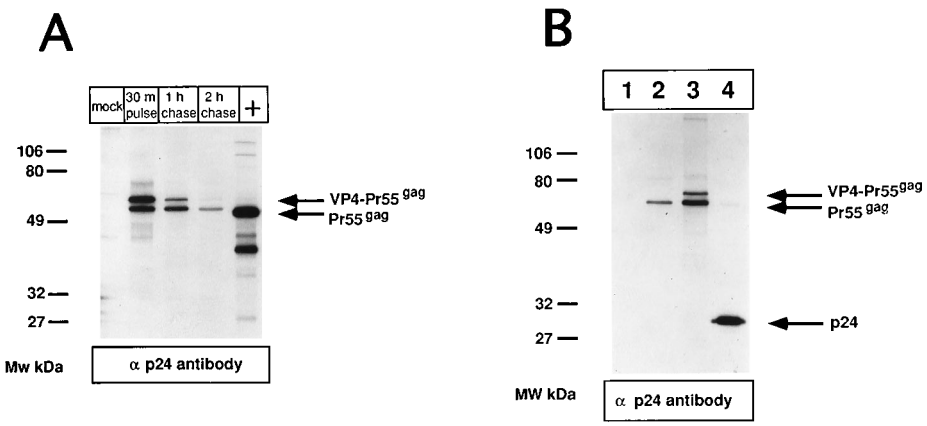

FIG. 3. Analysis of HIV-1-specific protein expression from cells infected with encapsidated replicons. (A) Cells were infected with a vIC-Pr55^{gag}(VP4/2A) replicon stock and pulse-labeled for 30 min (m), and complete medium was added for 1- and 2-h chase periods. Cell lysates were incubated with anti-p24 antibodies, and the immunoreactive proteins were analyzed on a sodium dodecyl sulfate-polyacrylamide gel. The order of the samples is indicated. Lane +, cells infected with vVK1. Molecular mass (Mw) standards and positions of relevant proteins are indicated. (B) Detection of a 55-kDa protein in the medium from cells infected with replicons which contain the Pr55^{gag} gene. The culture supernatant from cells infected with vIC-Pr55^{gag} or vIC-Pr55^{gag}(VP4/2A) replicon stocks or vVK1 was pelleted, and the samples were analyzed on a sodium dodecyl sulfate-polyacrylamide gel. Lanes: 1, mock-infected cells; 2, pelleted material from the medium of cells infected with vIC-Pr55^{gag}; 3, pelleted material from the medium of cells infected with vIC-Pr55^{gag}(VP4/2A); 4, pelleted material from the medium of cells infected with vVK1. Molecular mass standards and positions of relevant proteins are indicated.

particles from cells (11, 18, 44, 47). In the present study, since expression of the Pr55^{gag} protein from vIC-Pr55^{gag}(VP4/2A) requires posttranslational processing by poliovirus 2A to release the Pr55^{gag} protein from the polyprotein and expose the amino-terminal glycine residue, we wanted to analyze the myristylation of the 55-kDa protein expressed in cells infected with vIC-Pr55^{gag}(VP4/2A). Infected cells were labeled with [^{3}H]Myristic Acid, and upon incubation of cell lysates with anti-p24 antibodies, we immunoprecipitated the Pr55^{gag} protein, as well as the VP4-Pr55^{gag} precursor protein (Fig. 2B, lane 2). From cells infected with replicon vIC-Gag1, we immunoprecipitated an 80-kDa VP4-*gag*-VP1 fusion protein, which was used as a positive control for myristylation (Fig. 2B, lane 3) (50). Since studies have demonstrated that the poliovirus P1 protein and cleavage products VP0 and VP4 are modified by addition of myristic acid, the myristylation of the VP4-Pr55^{gag} protein is probably a result of the presence of the poliovirus VP4 protein at the amino terminus (7, 14, 48). However, myristylation of the 55-kDa protein expressed from vIC-Pr55^{gag}(VP4/2A) suggested that after processing of the VP4-Pr55^{gag} fusion protein by poliovirus 2A, the Gag protein maintains the appropriate signals for addition of myristic acid in a posttranslational reaction (68).

To further investigate the origins of the higher-molecular-mass protein expressed from vIC-Pr55^{gag}(VP4/2A)-infected cells, cells were infected with vIC-Pr55^{gag}(VP4/2A) and metabolically labeled with [^{35}S]Translabel for 30 min (pulse); complete medium was added, and the mixture was incubated for 1 and 2 h. HIV-1 Gag proteins were immunoprecipitated from lysates from infected cells by using anti-p24 antibodies (62) (Fig. 3A). The higher-molecular-mass precursor protein and a protein which migrated at 55 kDa were detected in cells pulse-labeled for 30 min; at this time, increased levels of the proposed precursor protein were detected relative to the 55-kDa protein. After a 1-h chase, the levels of the precursor protein had decreased relative to levels of the 55-kDa protein; this was most likely due to processing of the intracellular precursor protein and release of the 55-kDa protein into the medium of infected cells. Following the 2-h chase period, only the 55-kDa protein was detected in infected-cell lysates. Since previous studies have shown that expression of the HIV-1 Pr55^{gag} precursor protein results in assembly of HIV-1 virus-like particles that are released into the culture medium of infected cells, we also wanted to examine whether the Gag-containing proteins expressed from the replicons were released into the supernatants of infected cells (17, 19, 23, 28, 55, 58, 60, 66). Following established methods (19, 28), cells were infected with vIC-Pr55^{gag} or vIC-Pr55^{gag}(VP4/2A) and pulse-labeled, complete medium was added, and the mixture was incubated for 16 h. The supernatants were clarified by low-speed centrifugation, filtered, layered on a 20% sucrose cushion, and ultracentrifuged (19, 28). The pelleted material was analyzed by immunoprecipitation with anti-p24 antibodies (Fig. 3B) (62). A protein that migrated at 55 kDa was detected in the supernatants of cells infected with vIC-Pr55^{gag} and vIC-Pr55^{gag}(VP4/2A) replicon virus stocks. Interestingly, the VP4-Pr55^{gag} precursor protein was also detected in the supernatant of cells infected with vIC-Pr55^{gag}(VP4/2A). The appearance of the VP4-Pr55^{gag} protein in the supernatant suggested the possibility that this protein assembled into particles and was released from cells.

Expression of the HIV-1 *gag* gene in the absence of the viral protease results in the assembly and release of virus-like particles (19). Detection of HIV-1 Gag-containing proteins in the supernatants of cells infected with either of the encapsidated replicons encoding Pr55^{gag} suggests that the proteins had assembled into HIV-1 virus-like particles. To address this possibility, the supernatants from cells infected with the replicons were analyzed by using a continuous 20 to 60% sucrose gradient (Fig. 4). The 55-kDa protein was detected in fractions 8 to 16 from the samples derived from the supernatants of cells infected with vIC-Pr55^{gag} (Fig. 4A) and vIC-Pr55^{gag}(VP4/2A) (Fig. 4B). The amount of Pr55^{gag} protein immunoprecipitated by fractionation of cells infected with vIC-Pr55^{gag}(VP4/2A) was consistently less than that obtained from samples from cells infected with vIC-Pr55^{gag} replicons. The density of the fractions, obtained by refractometer, containing predominant levels of the 55-kDa protein in each of the gradients (Fig. 4A and B) was approximately 1.16 g/cm^3. The capsid (p24) protein was also predominant in fractions 8 to 16 from the supernatant of cells infected with vVK1, corresponding to a density of 1.16 g/cm^3, which is consistent with the results of a previous study (28) (Fig. 4C). The VP4-Pr55^{gag} fusion protein was immuno-

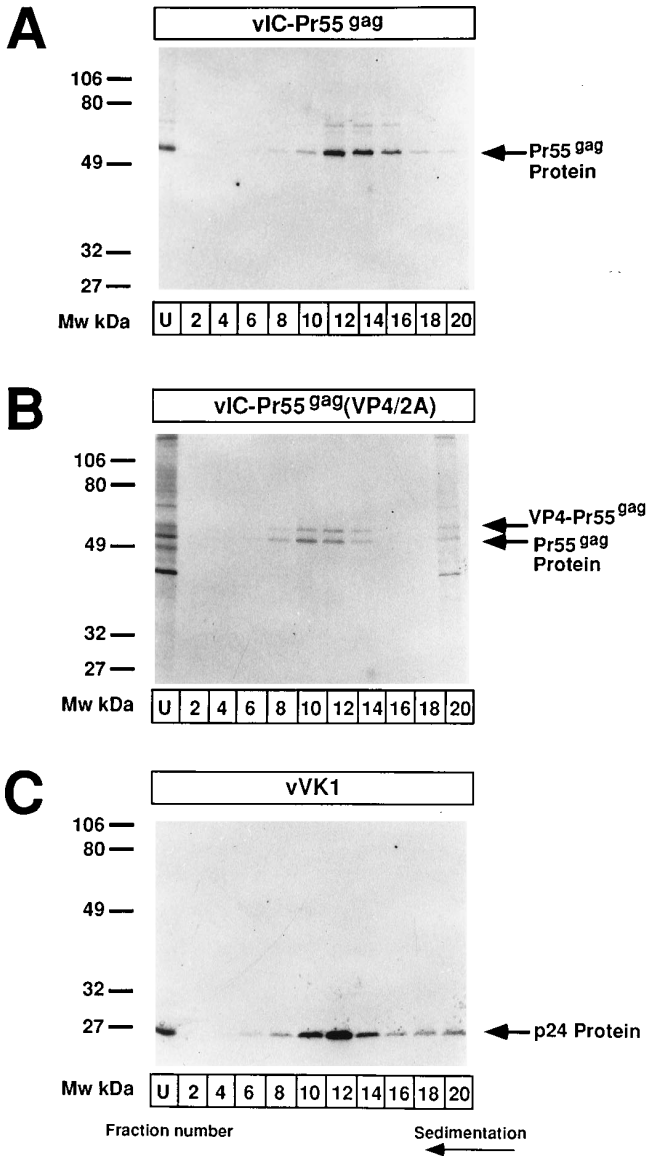

FIG. 4. Sucrose density gradient analysis of virus-like particles released from cells infected with replicons which contain the Pr55^{gag} gene. Supernatants from cells infected with the encapsidated replicons for 5 h were metabolically labeled with [^{35}S]Translabel for 1 h, complete medium was added, and the mixture was incubated for 16 h. After this time, the medium was clarified, filtered, and fractionated by ultracentrifugation as described previously (47). Even-numbered fractions were collected and incubated with anti-p24 antibodies and are depicted on the autoradiographs from the bottom (left) to the top (right) of the gradients. U, unfractionated. (A) Autoradiogram depicting sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of HIV-1 Gag-specific proteins immunoprecipitated by fractionation of cells infected with vIC-Pr55^{gag} encapsidated replicons. (B) Autoradiogram depicting sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of HIV-1 Gag-specific proteins immunoprecipitated by fractionation of cells infected with vIC-Pr55^{gag}(VP4/2A) encapsidated replicons. (C) Autoradiogram depicting sodium dodecyl sulfate-polyacrylamide gel electrophoresis of HIV-1 Gag-specific proteins immunoprecipitated by fractionation of cells infected with recombinant vaccinia virus vVK1. Positions of relevant proteins and molecular mass (Mw) standards are indicated.

precipitated from the same fractions in the gradient which contained predominant levels of the Pr55^{gag} protein (Fig. 4B). These results suggest that particles formed by infection of cells with vIC-Pr55^{gag}(VP4/2A) might contain both the Pr55^{gag} and VP4-Pr55^{gag} proteins or that the VP4-Pr55^{gag} precursor protein has the capacity to form particles. Further studies are required to address this question.

Previous studies have demonstrated that immature HIV-1 particles released from the plasma membrane of infected cells do not contain the electron-dense cores characteristic of mature HIV-1 virions (19, 28, 60). To confirm that cells infected with vIC-Pr55^{gag} or vIC-Pr55^{gag}(VP4/2A) resulted in the assembly and release of virus-like particles, we examined HeLa cells infected with each of the replicon virus stocks at a multiplicity of 10 by electron microscopy at 8 to 24 h postinfection. Cells infected with vIC-Pr55^{gag} encapsidated replicons exhibited numerous virus-like particles in the process of budding at the cell surface, as well as extracellular enveloped particles about 100 to 110 nm in diameter which had been apparently released from cells (Fig. 5). A subpopulation of smaller particles about 50 to 60 nm in diameter was also observed. Virus particle assembly was observed exclusively at the plasma membrane, and no evidence of intracellular assembly of core particles was observed, nor were any particles seen budding at intracellular membranes. At a higher magnification (Fig. 5, inset), the budding particles were seen to exhibit crescent-shaped core structures and the released particles showed an immature core morphology, as expected for particles containing uncleaved Gag precursor proteins (29). In contrast to the cells expressing only the Pr55^{gag} protein, cells infected with vIC-Pr55^{gag}(VP4/2A), which expresses the VP4-Pr55^{gag} fusion protein, exhibited lower levels of assembly of virus-like particles. Both intracytoplasmic assembly and release of enveloped, immature particles at the cell surface were observed in low numbers (Fig. 6). These results indicate that although each of the poliovirus replicons which express HIV-1 Gag precursor proteins result in the assembly and release of viruslike particles, the pattern of virus release differs between the vIC-Pr55^{gag} and vIC-Pr55^{gag}(VP4/2A) encapsidated replicon viruses.

In this study, we examined the expression of the complete *gag* gene of HIV-1 from encapsidated poliovirus replicons. Infection of cells with the replicons containing the *gag* gene resulted in the expression of a 55-kDa protein which comigrated with HIV-1 Pr55^{gag}; a second protein, probably a VP4-Pr55^{gag} fusion protein, was also expressed and released from cells infected with the replicon which maintained the VP4 coding sequences. One of the unique features of this study was the expression of the HIV-1 Pr55^{gag} protein from the replicon containing the VP4 coding sequences. A cleavage site, within this replicon, for poliovirus proteinase 2A was positioned immediately preceding the HIV-1 *gag* gene. Analysis of the intracellular expression of Gag-containing proteins from these replicons indicated the generation of a VP4-Pr55^{gag} precursor protein which was then processed in *trans* by poliovirus 2A posttranslationally to generate the Pr55^{gag} protein. Therefore, proteinase 2A must have the capacity to process at tyrosine-glycine dipeptide bonds positioned within the context of foreign proteins. The cleavage of a 2A site within the context of a foreign protein in the poliovirus cDNA is unique, since other studies have described only cleavage at 3C sites (4, 35).

An important result of the expression of the HIV-1 Gag protein from cells infected with vIC-Pr55^{gag}(VP4/2A) was that both the Pr55^{gag} protein and the VP4-Pr55^{gag} precursor protein were myristylated and would have the capacity to form stable associations with membrane components (11, 17, 18, 44, 69). Myristylation of the VP4-Pr55^{gag} protein was not surprising, since previous studies in this laboratory and others have demonstrated that the poliovirus VP4 protein is myristylated (7, 14, 48). However, myristylation of the Pr55^{gag} protein released from the VP4-Pr55^{gag} precursor protein after processing by 2A

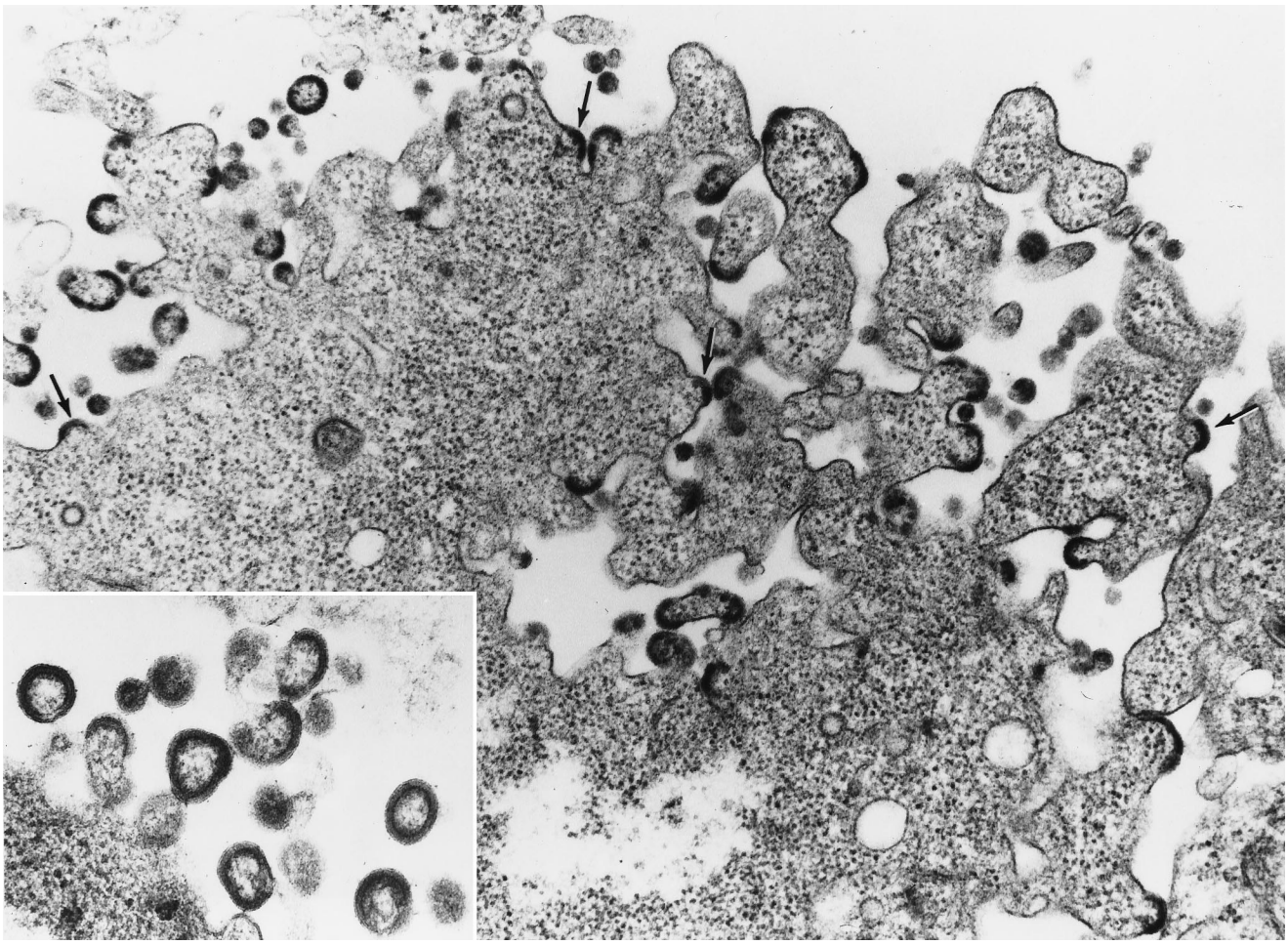

FIG. 5. Budding and release of HIV-1 virus-like particles expressed from a recombinant poliovirus. For preparation, infected cells were scraped from dishes and collected by pelleting before fixation in 1% glutaraldehyde in phosphate-buffered saline (pH 7.0; 330 mOs). The samples were then washed and dehydrated with ethyl alcohol, infiltrated with epoxy resin, and embedded. Sections were stained with uranyl acetate and lead citrate and examined at 80 kV in a Philips CM10 electron microscope. In these samples, numerous crescent-shaped budding structures are visible (arrows). The released virus particles (inset) retain an immature core morphology consisting of an electron-dense layer beneath the viral envelope. Magnification, ×47,120 (inset, ×76,000).

was unexpected. Previous studies have suggested that addition of myristic acid occurs after cleavage of the amino-terminal methionine from proteins in a reaction which occurs rapidly after protein synthesis, many times before the ribosome has finished complete translation of the protein (68). The results of pulse-chase analysis of the cleavage of the VP4-Pr55^{gag} precursor protein indicated that processing of the intracellular precursor protein by 2A occurred over a 1-h period, which argues against the notion that cleavage of the VP4-Pr55^{gag} protein occurs strictly in a cotranslational reaction. Thus, signals inherent within the protein are sufficient to target it to the appropriate location within the cell to be myristylated post-translationally. This idea is consistent with previous studies which have found that amino acids other than the penultimate glycine are essential for the myristylation of a protein (18, 63). Furthermore, in agreement with our results, Mattion et al. have demonstrated myristylation of the poliovirus VP0 and VP4 proteins after the processing of foreign gene sequences positioned 5′ to the P1 region (35).

Previous studies established that expression of the HIV-1 Gag protein, in the absence of other HIV proteins, results in the intracellular expression of a 55-kDa protein which is transported to the plasma membrane, where immature virus parti-

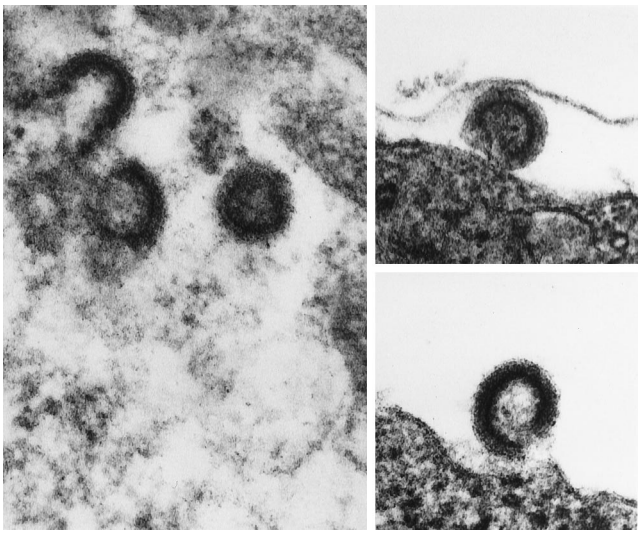

FIG. 6. Virus-like particles in cells expressing the VP4-Pr55^{gag} precursor protein. Left panel, apparent intracellular assembly of virus-like particles. Magnification, ×96,000. Right panels, particles budding at the cell surface. Magnification, ×107,000.

cles are assembled and subsequently released from the infected cell (19, 28, 60). Since infection of cells with poliovirus induces substantial intracellular alterations, which include the induction of virus-specific membrane structures in the cytoplasm, it was possible that these structures would interfere with and prevent the assembly of foreign proteins at cellular membranes expressed from poliovirus vectors (9, 10, 15, 32). The results of our study demonstrate that this is not the case, since sucrose density gradient analysis of the supernatants from infected cells revealed the presence of particles which had a density consistent with that of immature HIV-1 particles. In addition, analysis of infected cells by electron microscopy demonstrated the appropriate assembly and release of viruslike particles 100 to 110 nm in diameter from cells infected with vIC-Pr55^{gag} and vIC-Pr55^{gag}(VP4/2A). From cells infected with vIC-Pr55^{gag} encapsidated replicons, we also detected a subpopulation of particles 50 to 60 nm in diameter, although the exact nature of these particles is not known and is under investigation. Even though our studies demonstrated that both VP4-Pr55^{gag} and Pr55^{gag} proteins expressed from cells infected with vIC-Pr55^{gag}(VP4/2A) were released into the medium and detected in fractions from gradients which had a density consistent with that of immature virus-like particles, whether VP4-Pr55^{gag} has the capacity to form virus-like particles is not clear. Previous studies have reported that virus-like particles were formed when regions of HIV-1 Gag were replaced with different amino acids at the carboxy terminus of the protein (34, 67). To our knowledge, there have been no reports describing the fusion of heterologous amino acids at the amino terminus of the HIV-1 Gag protein. An alternative to assembly of the VP4-Pr55^{gag} protein into virus particles is that VP4-Pr55^{gag} could be incorporated into particles by interactions with Pr55^{gag}, similar to the Pr160$^{gag\text{-}pol}$ precursor protein, which does not alone have the capacity to form particles (38, 46, 47, 58). The decreased levels of released particles from cells infected with vIC-Pr55^{gag}(VP4/2A) by immunoprecipitation of gradient fractions and visual inspection of cells by electron microscopy suggest that VP4-Pr55^{gag} could act in a manner similar to dominant-negative mutations in the HIV-1 *gag* gene (65). It is also possible that the presence of the VP4 protein could affect the assembly and budding of immature particles, since the assembly of immature viruslike particles was observed both at the plasma membrane and within the cell's cytoplasm.

Finally, in recent years, there have been considerable efforts to develop poliovirus as a vector because of the potential uses in recombinant vaccines. Oral administration of the attenuated vaccines results in production of antibodies to the virus in the serum and secretions. The vaccines also stimulate cell-mediated immune responses, as evidenced by the determination that peripheral blood lymphocytes from immunized individuals proliferate if exposed to poliovirus (59). Several experimental strategies have previously been employed to develop poliovirus as a vaccine vector, including recombinant polioviruses which displayed foreign epitopes on the outer surface of the virus (16, 25, 31) and chimeric polioviruses which increase the size of the genome by insertion of foreign genes (1, 4, 35, 36). Potential drawbacks of these approaches include size limitations and instability of the foreign genes. The results of our studies establish that poliovirus replicons, with the P1 gene replaced with foreign genes such as the HIV-1 *gag* gene, can be used to express proteins which retain the features of the native proteins. Encapsidation of these replicons by the P1 capsid protein in *trans* provides a strategy for oral or parenteral gene delivery that takes advantage of the novel aspects of poliovirus. In preliminary studies, we have demonstrated that replicons delivered parenterally are immunogenic (5, 39, 40). Studies are ongoing to analyze the immunogenicity of replicons given orally.

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