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Myristylation of Poliovirus Capsid Precursor P1 Is Required for Assembly of Subviral Particles

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The poliovirus capsid precursor polyprotein, P1, is cotranslationally modified by the addition of myristic acid. We have examined the importance of myristylation of the P1 capsid precursor during the poliovirus assembly process by using a recently described recombinant vaccinia virus expression system which allows the independent production of the poliovirus P1 protein and the poliovirus 3CD proteinase (D. C. Ansardi, D. C. Porter, and C. D. Morrow, J. Virol. 65:2088–2092, 1991). We constructed a site-directed mutation in the poliovirus cDNA encoding an alanine at the second amino acid position of P1 in place of the glycine residue required for the myristic acid addition and isolated a recombinant vaccinia virus (VVP1myr−) that expressed a nonmyristylated form of the P1 capsid precursor. The 3CD proteinase expressed by a coinfecting vaccinia virus, VVP3, proteolytically processed the nonmyristylated precursor P1 expressed by VVP1myr−. However, the processed capsid proteins, VP0, VP3, and VP1, did not assemble into 14S or 75S subviral particles, in contrast to the VP0, VP3, and VP1 proteins derived from the myristylated P1 precursor. When cells were coinfected with VVP1myr− and poliovirus type 1, the nonmyristylated P1 precursor expressed by VVP1myr− was processed by 3CD expressed by poliovirus, and the nonmyristylated VP0-VP3-VP1 (VP0-3-1) protomers were incorporated into capsid particles and virions which sedimented through a 30% sucrose cushion. Thus, the nonmyristylated P1 precursor and VP0-3-1 protomers were not excluded from sites of virion assembly, and the assembly defects observed for the nonmyristylated protomers were overcome in the presence of myristylated capsid protomers expressed by poliovirus. We conclude that myristylation of the poliovirus P1 capsid precursor plays an important role during poliovirus assembly by facilitating the appropriate interactions required between 5S protomer subunits to form stable 14S pentamers. The results of these studies demonstrate that the independent expression of the poliovirus P1 and 3CD proteins by using recombinant vaccinia viruses provides a unique experimental tool for analyzing the dynamics of the poliovirus assembly process.

Poliovirus is a member of the family *Picornaviridae*, a group of small, nonenveloped, icosahedral viruses which encapsidate a single molecule of their positive-sense RNA genomes. The initial translation product of the poliovirus genome is a polyprotein, and virus-encoded proteinases release the individual proteins from the precursor by proteolytic cleavages (9, 32). The viral proteinase 2A releases the capsid precursor polyprotein, P1, from the genomic polyprotein by an intramolecular cleavage (32). The viral proteinase 3CD cleaves the P1 precursor into three individual capsid proteins, VP0, VP3, and VP1 (34). These three capsid proteins do not exist in a free form in the cytoplasm but remain associated together as a 5S capsid protomer (the VP0-3-1 protomer) (4), the smallest identical subunit of the icosahedral capsid. The 5S protomers are the building blocks for two well-characterized subviral particles present in poliovirus-infected cells: 14S pentamers, which consist of five copies each of VP0, VP3, and VP1, and 75S empty capsids or procapsids, which contain 60 copies each of VP0, VP3, and VP1 (24, 27). Upon encapsidation of the RNA genome, the proteolytic cleavage of VP0 produces VP2 and the small internal capsid protein VP4 (2, 11).

The amino-terminal glycine residue of the P1 polyprotein is covalently linked to a single molecule of the 14-carbon fatty acid myristic acid (*n*-tetradecanoic acid) (8, 21). The host cell enzyme *N*-myristoyltransferase cotranslationally modifies *N*-myristylproteins by the addition of a myristic acid molecule to the α-amino group of a glycine residue at the amino terminus (30, 33). The myristylated glycine residue of P1 is subsequently the amino terminus of VP0 and VP4, which are products of the proteolytic cleavages of P1. The three-dimensional structure of poliovirus revealed that the myristate moieties in the mature virion are clustered near the fivefold vertices on the capsid interior and are intimately associated with a twisted beta tube structure formed by five intertwining VP3 amino termini (8). This location suggests that the myristate hydrocarbon chains participate in stabilizing interactions between the five protomer subunits that form a pentamer. For many other *N*-myristylproteins, the presence of the myristate moiety is important for their targeting to and/or their association with cellular membranes (5, 6, 10, 13, 25, 28, 29, 31); however, this association is not characteristic of all myristylproteins, since several are found primarily in the cytosol (28, 31).

Previous studies have examined the role of myristylation of P1 and its cleavage products in the replication of the poliovirus RNA genome and the assembly of virions (14, 17–20). Myristylation of the poliovirus capsid precursor, P1, is required for virus infectivity (14, 17) and has been suggested to be important at different points during the assembly process, including the proteolytic processing of P1 by 3CD and the subsequent formation of subviral particles and virions. The poliovirus proteinase 3CD, provided by an extract of poliovirus-infected cells, processed in vitro-translated nonmyristylated P1 less efficiently than myristylated P1, especially at the cleavage site between VP0 and VP3 (14, 17). Further investigations of the role of myristylation of P1 and VP0 during the assembly of subviral intermediates and virions made use of mutant poliovirus genomes encoding P1

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proteins with altered myristylation signals that were created by site-directed mutagenesis of poliovirus cDNA (18–20). The P1 capsid precursors produced by these mutants were either not myristylated or myristylated less efficiently, depending on which amino acids within the myristylation signal were altered. Studies of the kinetics of virus assembly using the capsid proteins expressed upon transfection of mutant RNA genomes transcribed in vitro (18, 19) or from viruses generated by expression of mutant transcripts encoding a less efficiently myristylated P1 (20) concluded that myristylation of the capsid subunits was important for efficient poliovirus assembly at multiple stages. Those studies reported a less efficient assembly of all capsid protein structures, including 14S pentamers. Although replication of the mutant RNA genomes within the transfected or infected cells occurred normally, infectious virions did not assemble when the capsid proteins were unmyristylated.

A drawback of the previous studies was their dependence upon replication of the RNA genome for expression of the viral proteins required for assembly. In many cases, replication of viral genomes results in the reversion of mutations, which can complicate interpretation of experimental results (18, 19). We have overcome this limitation by using a recombinant vaccinia virus expression system for the production of authentic poliovirus capsid proteins that are competent for assembly into 75S empty capsids (1). This system makes use of two recombinant vaccinia viruses, one which expresses the P1 capsid protein precursor (VVP1) and a second which expresses the poliovirus proteinase 3CD (VVP3) (23). Upon coinfection of cells with both of these recombinant vaccinia viruses, 3CD cleaves the P1 polyprotein, producing VP0, VP3, and VP1; subsequently, the processed proteins assemble into readily detectable 75S empty capsids. In this report, we describe the use of this recombinant vaccinia virus expression system to further define the importance of the myristylation of P1 and VP0 for processing by the 3CD proteinase and the assembly of VP0-3-1 protomers into subviral particles.

Previous studies demonstrated that the glycine residue at position 2 of the P1 capsid precursor polyprotein is required for the cotranslational addition of myristic acid, and an alanine residue in place of glycine at this position abolished P1 myristylation (14, 18). We mutated P1-encoding DNA sequences by oligonucleotide site-directed mutagenesis so that an alanine residue was encoded at position 2 and subcloned the mutated sequences into vaccinia virus recombination plasmid pSC11-*SalI*, creating plasmid pSC11-P1myr− (Fig. 1) (1, 7). Transfection of plasmid pSC11-P1myr− into wild-type vaccinia virus-infected cells allowed the generation of recombinant vaccinia viruses by homologous recombination. The plaque-purified virus preparations were screened for expression of the P1 polyprotein by immunoprecipitation of metabolically radiolabeled proteins present in extracts of the infected cells using an antiserum to poliovirus type 1 (1), and several recombinant vaccinia viruses that expressed a protein which comigrated on sodium dodecyl sulfate (SDS)-polyacrylamide gels with P1 synthesized in poliovirus-infected cells were isolated. One of these purified virus stocks was designated VVP1myr−, and the P1 protein expressed by this virus was further analyzed to confirm that it was not modified by the addition of myristic acid. When HeLa cells infected with wild-type vaccinia virus, VVP1, VVP1myr−, or poliovirus type 1 were metabolically radiolabeled with [^{35}S]Translabel (Met-Cys), we readily detected P1 by immunoprecipitation from lysates of either VVP1- or VVP1myr−-infected cells but not from lysates of wild-type vaccinia virus-infected cells (Fig. 2A). As expected, when [^{3}H]myristic acid was used as a radiolabel, the P1 protein was detected by immunoprecipitation from lysates of VVP1-infected cells but not from VVP1myr−-infected cells (Fig. 2B). When poliovirus-infected cells were labeled with [^{3}H]myristic acid, the P1 precursor protein, as well as VP0, 1ABC (uncleaved VP0-3), and VP4, the products of capsid protein proteolytic processing that contain the amino-terminal myristylated glycine residue, was detected by immunoprecipitation. These results demonstrated the expression of a nonmyristylated form of the P1 polyprotein by using the recombinant vaccinia virus system.

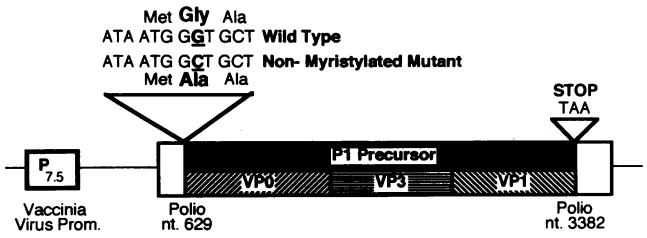

FIG. 1. Poliovirus cDNA sequences in plasmid pSC11-P1myr−. Plasmid pSC11-P1 contains the poliovirus cDNA sequences from nucleotides (nt.) 629 to 3382, encoding the complete P1 region, followed by an engineered termination codon and has been previously described (1). Plasmid pSC11-P1myr− was created by oligonucleotide-directed mutagenesis of cDNA encoding the amino-terminal portion of P1 which changed the glycine codon at position 2 to an alanine codon. The mutated sequences were subcloned into the vaccinia virus recombination plasmid pSC11-*SalI* (1, 7), generating pSC11-P1myr−. Prom., promoter.

Previously, other laboratories reported that 3CD proteinase provided by an extract of poliovirus-infected cells did not process in vitro translated nonmyristylated P1 as efficiently as myristylated P1, especially at the cleavage site between VP0 and VP3 (14, 17). Since we had previously observed that the 3CD proteinase processed P1 to VP0, VP3,

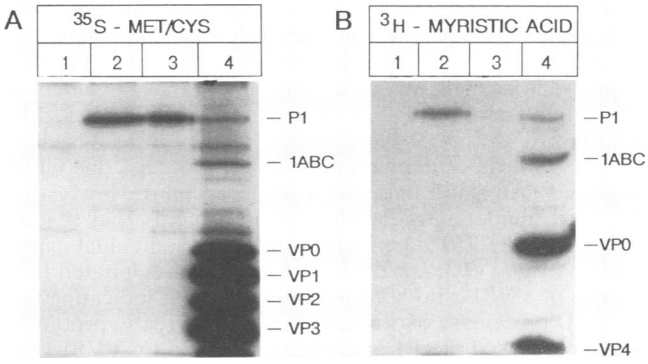

FIG. 2. Expression of myristylated and nonmyristylated P1 by recombinant vaccinia viruses VVP1 and VVP1myr−. Fluorograms of SDS-polyacrylamide gel separation of radiolabeled proteins immunoprecipitated from lysates of infected HeLa cells with an antiserum to poliovirus type 1 are shown. Infected HeLa cells were radiolabeled with either [^{35}S]Translabel (Met-Cys) (A) or [^{3}H]myristic acid (B). The samples are from cells infected as follows: lanes 1, 20 PFU of wild-type vaccinia virus per cell; lanes 2, 20 PFU of VVP1 per cell; lanes 3, 20 PFU of VVP1myr− per cell; lanes 4, 30 PFU of poliovirus type 1 per cell. The relevant viral proteins are indicated.

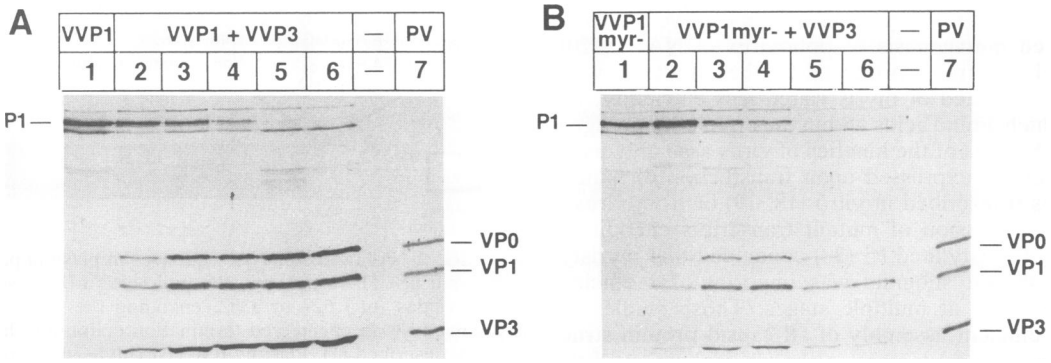

FIG. 3. Processing of myristylated and nonmyristylated P1 expressed by VVP1 or VVP1myr− by proteinase 3CD expressed by VVP3. Fluorograms of SDS-polyacrylamide gel separation of radiolabeled proteins immunoprecipitated from lysates of infected HeLa cells with an antiserum to poliovirus type 1 are shown. (A) HeLa cell monolayers were infected with 10 PFU of VVP1 (lane 1) per cell or 10 PFU each of VVP1 and VVP3 per cell (lanes 2 to 6). (B) HeLa cell monolayers were infected with 10 PFU of VVP1myr− per cell (lane 1) or 10 PFU each of VVP1myr− and VVP3 per cell (lanes 2 to 6). In both experimental sets, the infected cells were incubated with [^{35}S]Translabel (Met-Cys) for 1 h beginning 4 h postinfection. Cells singly infected with VVP1 alone or VVP1myr− alone (lanes 1) were further incubated in complete medium for 3 h after being labeled. After being labeled, one set of the coinfected cells was immediately harvested (0-h chase [lanes 2]), and the remaining sets of infected cells were further incubated in complete medium for chase times of 0.5 h (lanes 3), 1 h (lanes 4), 2 h (lanes 5), or 3 h (lanes 6). The poliovirus (PV) marker proteins (lanes 7) were immunoprecipitated from lysates of poliovirus-infected cells that had been incubated with [^{35}S]Translabel. The relevant viral proteins are indicated. Lanes —, empty lanes on the gels.

and VP1 in cells coinfected with VVP1 and VVP3 (1), we used a similar coinfection strategy to determine whether 3CD would process nonmyristylated P1 expressed by VVP1myr−. We infected one set of HeLa cells with VVP1 or VVP1myr− alone and several sets with either VVP1 and VVP3 or VVP1myr− and VVP3 and metabolically labeled the infected cells with [^{35}S]Translabel for 1 h beginning 4 h postinfection. After the labeling period, one set each of the coinfected cells was harvested (0-h chase) in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), while the remaining sets of infected cells were further incubated in complete medium for a chase time of 30 min, or 1, 2, or 3 h. The cells infected with either VVP1 alone or VVP1myr− alone were incubated for the full chase time of 3 h. The poliovirus capsid-related proteins were then immunoprecipitated by poliovirus type 1 antiserum from the cell lysates and analyzed on SDS-polyacrylamide gels. Unprocessed P1 was the only poliovirus capsid-related protein expressed in cells infected with either VVP1 alone or VVP1myr− alone (Fig. 3). The P1 proteins expressed by VVP1 or VVP1myr− were stable for the entire 3-h chase period. Two other immunoprecipitated proteins, one which migrated faster and one which migrated more slowly than P1, were unidentified vaccinia virus proteins that reacted with the poliovirus type 1 antiserum. The individual capsid proteins VP0, VP3, and VP1 were immunoprecipitated from lysates of VVP1- and VVP3-coinfected cells, indicating that the 3CD proteinase expressed by VVP3 correctly proteolytically processed the P1 precursor expressed by VVP1 (Fig. 3A). The 3CD proteinase expressed by VVP3 also processed the nonmyristylated P1 precursor in cells coinfected with VVP1myr− and VVP3 (Fig. 3B). Although the nonmyristylated P1 precursor was processed to VP0, VP3, and VP1, the processed proteins did not appear to accumulate during the extended chase periods. In fact, after longer chase periods, we detected less VP0, VP3, and VP1 than after shorter chase periods, suggesting that the processed proteins were unstable. In contrast, the VP0, VP3, and VP1 proteins produced in VVP1- and VVP3-coinfected cells appeared to accumulate as chase times were extended. Interestingly, more VP1 than VP0 or VP3 was immunoprecipitated from the lysates of cells coinfected with VVP1myr− and VVP3. We do not believe this observation indicates that the P1 protein was cleaved more efficiently at the VP3-VP1 site, since we did not observe a coordinate appearance of an unprocessed VP0-VP3 protein (1ABC).

Previously we demonstrated that the processed capsid proteins VP0, VP3, and VP1 produced in cells coinfected with VVP1 and VVP3 assembled into particles that cosedimented on sucrose density gradients with 75S procapsids or empty capsids produced in poliovirus-infected cells (1). To determine whether myristylation of the poliovirus capsid subunit protomers is required for the assembly of empty capsids, the capacity of nonmyristylated VP0-3-1 protomers to assemble into empty capsids was determined by sucrose density gradient fractionation of extracts made from radiolabeled infected cells. HeLa cells were coinfected with either VVP1 and VVP3 or VVP1myr− and VVP3 (20 PFU of each virus per cell) and incubated with [^{35}S]methionine-cysteine (0.25 mCi/ml in methionine- and cysteine-free Dulbecco modified Eagle medium) for 2 h, beginning 4 h postinfection. The cells were incubated in complete medium for an additional 2 h after being labeled, and cell extracts were then prepared in RSBK buffer (10 mM Tris-HCl [pH 6.8], 10 mM KCl, 1.5 mM $MgCl_2$) as previously described (1). After centrifugation of the extracts through linear, continuous 10 to 30% sucrose–RSBK gradients at 35,000 rpm for 3.5 h at 4°C in an SW41 rotor, fractions were collected from the bottom of the tube, 4× RIPA buffer was added to a final concentration of 1×, and the poliovirus capsid-related proteins present in the individual odd-numbered fractions were immunoprecipitated with a poliovirus type 1 antiserum. The immunoprecipitated proteins were then separated on SDS-polyacrylamide gels (Fig. 4). From this analysis, we found that significant portions of the processed capsid proteins VP0, VP3, and VP1 produced in VVP1- and VVP3-coinfected cells sedimented deeply into the 10 to 30% sucrose–RSBK gradient and were immunoprecipitated from fractions 9 and 11 (Fig. 4B). These fractions corresponded to those

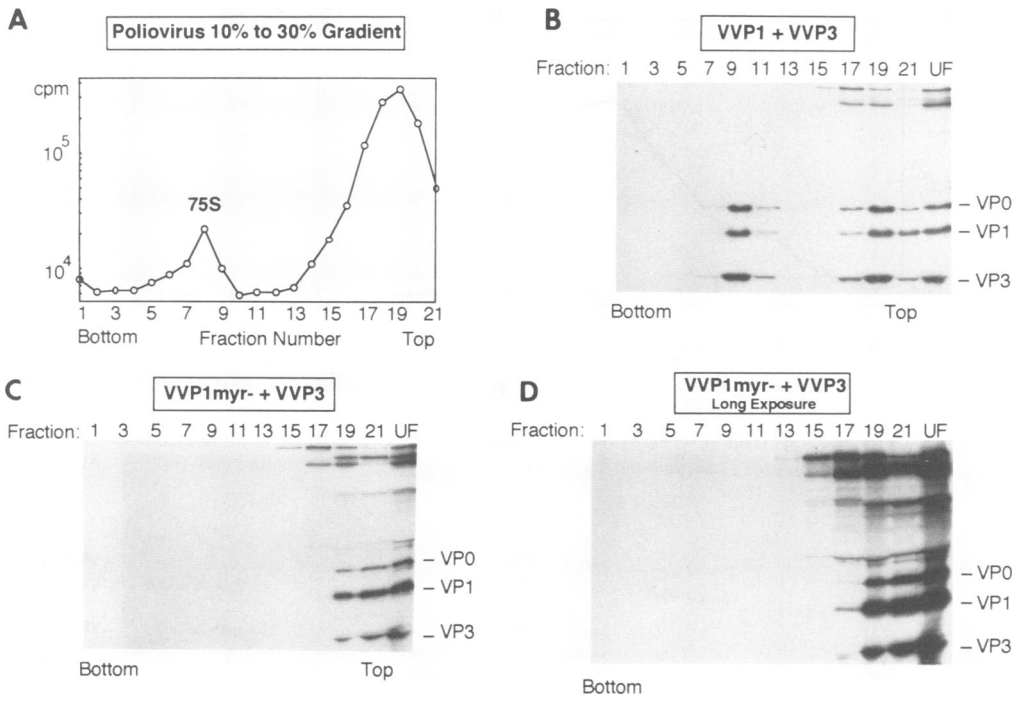

FIG. 4. Sucrose density gradient (10 to 30%) analysis of infected cell extracts. Infected HeLa cells were metabolically radiolabeled with [^{35}S]Translabel (Met-Cys) for 2 h beginning 4 h postinfection and then further incubated in complete medium for 2 h. Extracts were made in RSBK and fractionated on linear continuous 10 to 30% sucrose density gradients. Poliovirus capsid-related proteins were immunoprecipitated from the odd-numbered fractions with antiserum to poliovirus type 1 and separated on SDS-polyacrylamide gels. (A) To standardize the 10 to 30% sucrose density gradients for the 75S procapsid or empty capsid fractions, an RSBK extract of radiolabeled poliovirus-infected cells was fractionated on a 10 to 30% sucrose gradient, fractions were collected from the bottom of the tube, and the radiolabeled trichloroacetic acid-precipitable material from equal portions of each fraction was quantitated with a scintillation counter. The fractions containing the 75S procapsids are indicated. (B) Cells were coinfected with VVP1 and VVP3 (20 PFU of each virus per cell). The lanes of the gel contain proteins immunoprecipitated from gradient fractions from the bottom (left, fraction 1) to the top (right, fraction 21) of the gradient. (C) Cells were coinfected with VVP1myr− and VVP3 and analyzed in the same manner as in panel B. (D) Fluorogram exposed six times as long as the fluorogram in panel C to the same gel. Lane UF in panels B, C, and D contains immunoprecipitated proteins from an aliquot of unfractionated RSBK extract.

that contained radiolabeled 75S procapsids when we performed an identical sucrose gradient sedimentation analysis on extracts from poliovirus-infected cells (Fig. 4A). Fractionation of extracts from radiolabeled cells coinfected with VVP1myr− and VVP3, however, revealed that VP0, VP3, and VP1 remained in the upper fractions of the gradient (Fig. 4C). Even upon exposure of the gel to X-ray film for a period six times as long as the first exposure (Fig. 4D), we could not detect VP0, VP3, and VP1 in the 75S fractions. On the longer exposure of the gel, though, we did detect three faint protein bands in fractions 5 and 7 which did not comigrate exactly with VP0, VP3, and VP1; we also detected similar protein bands in fractions 5 and 7 on the longer exposure of the gel from the gradient fractionation of the VVP1- and VVP3-coinfected-cell extract (data not shown). In addition, we analyzed fractions 8 and 10 from the VVP1myr−/VVP3 gradient by immunoprecipitation to ensure that nonmyristylated 75S capsid proteins had not sedimented primarily into one fraction of the gradient, but those fractions were also devoid of VP0, VP3, and VP1 (data not shown). These results demonstrate that VP0, VP3, and VP1 derived from nonmyristylated P1 do not assemble into empty capsids under conditions in which VP0, VP3, and VP1 generated from myristylated P1 readily form these subviral particles.

Empty capsids are preceded in the assembly pathway by a 14S pentamer subviral intermediate (24, 27). The 14S pentamer consists of five copies each of VP0, VP3, and VP1 and forms when five 5S protomers associate together. In order to determine whether myristylation of VP0 is required for the assembly of 14S pentamers from 5S protomers, we coinfected cells and metabolically radiolabeled them as described for the previous experiment, except that we harvested the cells immediately after the 2-h radiolabeling period. We then fractionated RSBK extracts of the metabolically ^{35}S-labeled coinfected cells on 5 to 20% sucrose–RSBK density gradients by centrifugation at 35,000 rpm for 12.5 h at 4°C in an SW41 rotor. The VP0, VP3, and VP1 proteins present in the extracts from VVP1- and VVP3-coinfected cells sedimented deeply into the 5 to 20% gradient (Fig. 5B). This position was consistent with the peak of radioactivity detected for 14S pentamers from poliovirus-infected cells analyzed under identical centrifugation conditions (Fig. 5A). In fact, the intensity of the capsid protein bands present in the gel lanes corresponding to the 14S fractions was much greater than the intensity of the bands present in lanes containing proteins immunoprecipitated from the top fractions of the gradient, indicating that VP0, VP3, and VP1 derived from myristylated P1 readily assembled into 14S pentamers. In contrast, VP0, VP3, and VP1 present in extracts from cells coinfected with VVP1myr− and VVP3 remained in the top fractions of the gradient (Fig. 5C). These fractions corresponded to those in which 5S VP0,

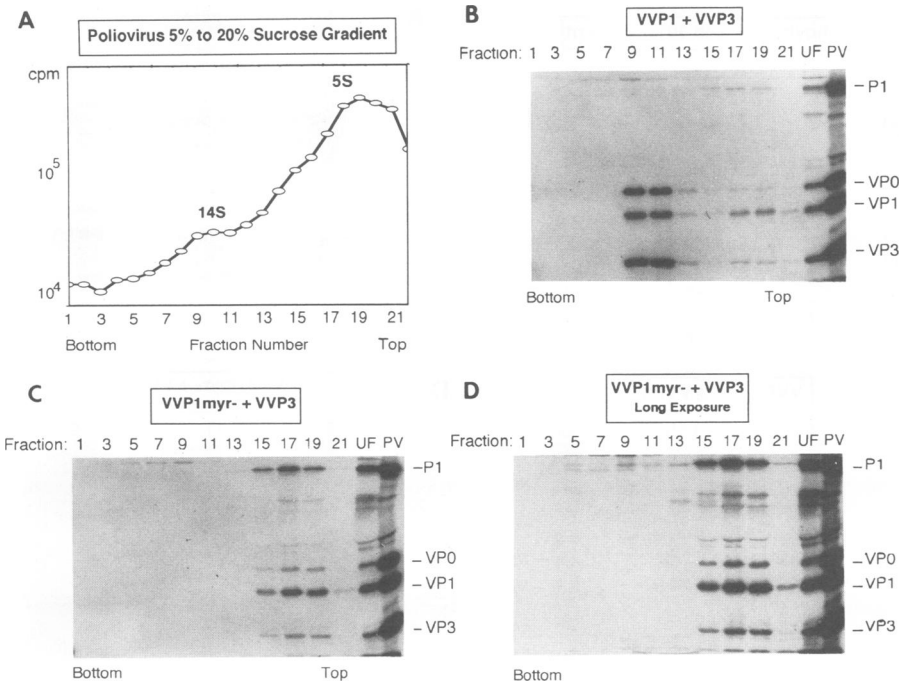

FIG. 5. Sucrose density gradient (5 to 20%) fractionation of infected cell extracts. Infected HeLa cells were metabolically radiolabeled with [^{35}S]Translabel (Met-Cys) for two hours beginning at 4 h postinfection. Extracts were prepared in RSBK and fractionated on linear, continuous 5 to 20% sucrose density gradients. Fractions were collected from the bottom of the gradient, and poliovirus capsid-related proteins were immunoprecipitated with antiserum to poliovirus type 1. (A) Radioactivity profile of radiolabeled proteins precipitated with trichloroacetic acid from fractions collected from the bottom (left) to top (right) of a 5 to 20% sucrose gradient separation of an RSBK extract of poliovirus-infected cells. The fractions of the gradient containing 14S and 5S poliovirus capsid proteins are indicated. (B) Fluorogram of proteins immunoprecipitated from fractions collected from bottom (left, fraction 1) to top (right, fraction 21) of a 5 to 20% sucrose gradient fractionation of an RSBK extract of VVP1- and VVP3-coinfected cells. (C) Fluorogram of proteins immunoprecipitated from an identical fractionation of an RSBK extract of cells coinfected with VVP1myr− and VVP3. (D) Fluorogram exposed eight times as long as the fluorogram in panel C to the same gel. In each panel, lane UF contains immunoprecipitated proteins from an aliquot of the unfractionated RSBK extract, and lane PV contains proteins immunoprecipitated with an antiserum to poliovirus type 1 from ^{35}S-labeled poliovirus-infected cells.

VP3, and VP1 generated in cells coinfected with VVP1 and VVP3 sedimented, indicating that myristylation of VP0 is probably not necessary for VP0, VP3, and VP1 to remain associated together as a 5S protomer. We found no VP0, VP3, or VP1 in the 14S fractions of the VVP1myr−/VVP3 gradient even upon exposure of the gel to X-ray film for a period eight times as long as the first exposure (Fig. 5D). We again noted that more VP1 than VP0 or VP3 was immunoprecipitated by the poliovirus type 1 antiserum from extracts of the cells coinfected with VVP1myr− and VVP3. Interestingly, more VP1 than VP0 or VP3 was also immunoprecipitated from the 5S fractions of the 5 to 20% sucrose gradient of VVP1- and VVP3-coinfected cell extracts. Thus, under the experimental conditions used, the poliovirus type 1 antiserum may have immunoprecipitated VP1 from 5S material more efficiently than VP0 or VP3.

It was possible that the nonmyristylated 5S protomers assembled into 14S pentamers more slowly than the myristylated protomers. We tested this possibility by conducting a new coinfection experiment during which we metabolically radiolabeled cells coinfected with VVP1myr− and VVP3 from 4 to 6 h postinfection and then allowed them to incubate for an additional 6 h in complete medium. Upon subsequent analysis of extracts of the coinfected cells by sedimentation through 5 to 20% sucrose density gradients, however, we still did not detect VP0, VP3, and VP1 in the 14S fractions (data not shown). In fact, we detected less VP0, VP3, and VP1 in the 5S fractions of the gradient than during the previous experiment, confirming our observation that VP0, VP3, and VP1 derived from nonmyristylated P1 were unstable. These results demonstrate that assembly of 14S pentamers from 5S VP0-3-1 protomer subunits is prevented when VP0 is unmyristylated.

To further explore the requirement for myristylation of P1 and VP0-3-1 protomers during poliovirus assembly, we determined whether the nonmyristylated P1 precursor or VP0-3-1 protomers were excluded from poliovirus assembly complexes in cells coinfected with VVP1myr− and poliovirus type 1. For these experiments, we relied on a previous study which reported that when cells were coinfected at the same time with a recombinant vaccinia virus and poliovirus type 1, the proteins labeled with [^{35}S]methionine provided in the growth medium early in infection (before 3 h postinfection) were those expressed by the recombinant vaccinia virus, while the proteins synthesized by poliovirus were not detectable by radiolabeling until 3 h postinfection and thereafter (12). Therefore, using cells coinfected with VVP1 or VVP1myr− and poliovirus at the same time, we were able to selectively label P1 proteins synthesized by the recombinant vaccinia virus by incubating the cells with [^{35}S]Translabel before 3 h postinfection. Upon removal of the radiolabel from the medium, the labeled proteins were chased in the

presence of replicating poliovirus RNA and unlabeled poliovirus proteins produced during the further incubation. This allowed us to determine whether the recombinant vaccinia virus-expressed P1 precursor proteins were processed in *trans* by 3CD provided by poliovirus and incorporated into assembling poliovirus virions. For these experiments, HeLa cells were infected with poliovirus alone (10 PFU per cell) or coinfected with poliovirus (10 PFU per cell) and either wild-type vaccinia virus, VVP1, or VVP1myr− (20 PFU per cell). After 1 h of infection, the cells were incubated in methionine- and cysteine-free medium for 30 min, and the medium was then replaced with methionine- and cysteine-free medium containing [^{35}S]Translabel (0.1 mCi/ml) for 1 h. Following removal of the label at 2.5 h after addition of virus, we immediately harvested one set of VVP1- and poliovirus-coinfected cells and further incubated the remaining sets of cells in complete medium for 6 h. The cells were lysed by the addition of Triton X-100 to the medium at a final concentration of 1%, and the lysates were clarified by low-speed centrifugation. The lysates were layered over a 30% sucrose cushion and centrifuged at 45,000 rpm for 1.5 h in an SW55Ti rotor to concentrate capsid protein particles and virions. The pelleted material was solubilized in RIPA buffer, the poliovirus capsid proteins were immunoprecipitated with an antiserum to poliovirus type 1, and the immunoprecipitated proteins were separated on SDS–10% polyacrylamide gels (Fig. 6). After the 6-h incubation period, low levels of capsid proteins (from residual label) were detected from cells infected with poliovirus alone or coinfected with wild-type vaccinia virus and poliovirus. Only unprocessed P1 was detected by immunoprecipitation from an unfractionated lysate of cells that had been coinfected with VVP1 and poliovirus and harvested immediately after the labeling period. We did not detect unprocessed P1 in the 30% sucrose cushion pellet; this result indicates that the unprocessed precursor was not contained in nonspecific protein aggregates capable of sedimenting through 30% sucrose. We immunoprecipitated radiolabeled processed proteins VP0, VP3, and VP1 from VVP1- and poliovirus-coinfected and VVP1myr−- and poliovirus-coinfected cell material which had sedimented through the 30% sucrose cushion; this indicates that the P1 polyproteins produced by the recombinant vaccinia viruses were accessible to 3CD provided by poliovirus and that the 5S protomers produced upon processing assembled into large particles. Furthermore, the appearance of the mature virion protein VP2 demonstrated that some of the VP0 generated from the P1 precursors provided by both VVP1 and VVP1myr− had been processed to the mature virion proteins VP2 and VP4 (2, 11, 27). We did observe less VP2 protein derived from the nonmyristylated precursor, however, than from the myristylated precursor. Nevertheless, in cells coinfected with the P1-expressing recombinant vaccinia viruses and poliovirus type 1, the capsid proteins expressed by the recombinant vaccinia viruses entered the poliovirus assembly pathway. Thus, the absence of myristate did not exclude capsid protomers from the assembly process in the presence of myristylated 5S protomers produced by poliovirus.

In this report, we have described the construction and characterization of a recombinant vaccinia virus which expresses a nonmyristylated form of the poliovirus capsid precursor protein P1 (VVP1myr−). The characteristics of the nonmyristylated poliovirus capsid-related proteins produced in cells coinfected with VVP1myr− and VVP3 were different from those of the capsid-related proteins produced in cells coinfected with VVP1 and VVP3. Although the 3CD

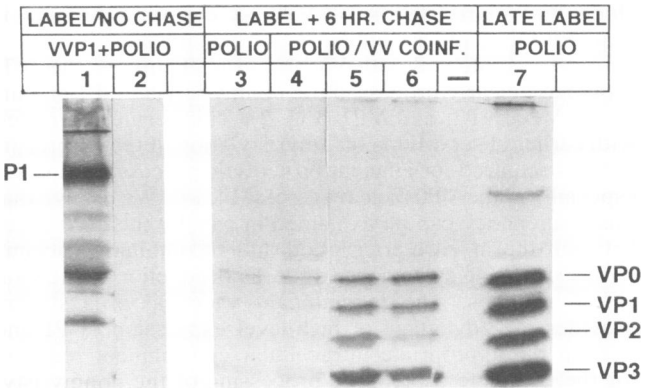

FIG. 6. Incorporation of myristylated and nonmyristylated capsid proteins expressed by VVP1 and VVP1myr− into poliovirus type 1 virions. A fluorogram of radiolabeled proteins immunoprecipitated with antiserum to poliovirus type 1 and separated on SDS-polyacrylamide gels is shown. The samples were generated as follows. For lane 1, cells were coinfected with VVP1 and poliovirus, metabolically labeled for 1.5 h beginning 1 h postinfection, and harvested immediately. Next, poliovirus capsid-related proteins were immunoprecipitated with antiserum to poliovirus type 1. This sample contains proteins immunoprecipitated from one-fifth of the cell lysate prior to centrifugation over a 30% sucrose cushion. For lane 2, the remainder of the lysate from lane 1 was layered over a 30% sucrose cushion and centrifuged at 45,000 rpm for 1.5 h in an SW55 Ti rotor. The pelleted material was solubilized in RIPA buffer, and poliovirus capsid-related proteins were immunoprecipitated with an antiserum to poliovirus type 1. For lane 3, cells were infected with poliovirus type 1 and labeled and processed as described for the sample in lane 2. For lane 4, cells were coinfected with wild-type vaccinia virus and poliovirus and labeled and processed as described for the sample in lane 2. For lane 5, cells were coinfected with VVP1 and poliovirus and labeled and processed as described for the sample in lane 2. For lane 6, cells were coinfected with VVP1myr− and poliovirus and labeled and processed as described for the sample in lane 2. For lane 7, cells were infected with poliovirus type 1, incubated with [^{35}S]Translabel for 1.5 h beginning 6 h postinfection, and processed as described for the sample in lane 2. The appropriate poliovirus capsid proteins are indicated.

proteinase correctly processed nonmyristylated P1, the nonmyristylated VP0-3-1 protomers did not assemble into either 14S pentamers or empty capsids at detectable levels. In contrast, we readily detected 14S pentamers and empty capsids in cells coinfected with VVP1 and VVP3. These studies have clearly demonstrated that myristylation of 5S VP0-3-1 protomers is critical for further assembly into 14S pentamers. Previous studies reported that the glycine-to-alanine change at the amino terminus of P1 resulted in a noninfectious virus (14, 18). For those studies, mutant RNA genomes were transcribed in vitro and transfected into cells; in one report, the subviral particles produced in cells transfected with the mutant RNA genomes were characterized (18). In contrast to our findings, that investigation did find low levels of 14S pentamers assembled from the capsid proteins expressed by the mutant RNA genomes. However, the low levels of 14S pentamer formation may have resulted from reversion of the mutation during the replication of the mutant RNA genomes (18, 19). By using the recombinant vaccinia virus expression system, we have reduced the likelihood of reversion mutations which complicate the interpretation of experimental results. On the basis of the results of the experiments reported in this article, we con-

clude that myristylation of VP0-3-1 capsid protomers is required for stable assembly of 14S pentamers.

In cells coinfected with VVP3 and VVP1myr−, the 3CD proteinase clearly processed the nonmyristylated P1 precursor to VP0, VP3, and VP1 (Fig. 3). This result contrasted with earlier observations that myristylation of the P1 precursor was required for efficient proteolytic processing in vitro, especially at the VP0-3 cleavage site (14, 17). We believe that this discrepancy can be explained in part by the differences between the in vitro translation and recombinant vaccinia virus expression systems used for the production of the viral capsid proteins. The recombinant vaccinia virus system provides the advantage of high-level expression of P1 and 3CD in the appropriate intracellular environment required for the complete proteolytic processing of the nonmyristylated P1 protein.

We noted during several experiments that VP0, VP3, and VP1 generated from nonmyristylated P1 were more unstable in cells than VP0, VP3, and VP1 generated from myristylated P1. We do not believe that this instability explains the lack of assembly of nonmyristylated 5S capsid protomers into 14S pentamers and empty capsids. During the sucrose gradient experiments, we immunoprecipitated VP0, VP3, and VP1 from aliquots of unfractionated extracts of cells coinfected with VVP1myr− and VVP3 as well as from the upper fractions of the gradients, suggesting that sufficient pools of nonmyristylated 5S protomers were available for assembly into subviral particles (Fig. 4 and 5). The majority of the processed proteins generated in VVP1- and VVP3-coinfected cells over a 2-h metabolic labeling period sedimented at a rate of 14S, indicating that the assembly of VP0, VP3, and VP1 derived from myristylated P1 occurred rapidly (Fig. 5B). The instability of the nonmyristylated P1 products may be a result of their inability to assemble. In support of this idea, Macadam et. al. have recently reported that the assembly-defective 5S protomers synthesized by a temperature-sensitive mutant of poliovirus type 3 were unstable when not assembled into subviral particles at the nonpermissive temperature (16). These results suggest that the assembly-defective 5S protomers are targeted for degradation. Interestingly, emerging concepts about cellular chaperone proteins suggest that cells have mechanisms for recognizing proteins that are not yet maturely folded (3, 15, 22, 26). Since processed but unassembled poliovirus capsid proteins are not mature structurally, those that cannot assemble or reach their final conformational state may be targeted into a cellular degradation pathway. Further experiments are underway to explore this possibility.

The independent expression of P1 by recombinant vaccinia viruses provided the opportunity to study the interaction of nonmyristylated P1 with the poliovirus assembly complexes in cells coinfected with VVP1myr− and poliovirus. Since we had observed that unmyristylated 5S protomers did not assemble into 14S pentamers in cells coinfected with VVP1myr− and VVP3, we were surprised to find that, in cells coinfected with VVP1myr− and poliovirus, the nonmyristylated protomers were present in large particles that sedimented through 30% sucrose cushions. This result suggested that the nonmyristylated capsid protomers were still capable of making the protomer-protomer contacts required for assembly when the stabilizing forces of myristate were provided by other myristylated protomers within a common pentamer subunit. Furthermore, the nonmyristylated P1 precursor and nonmyristylated VP0-3-1 protomers were not excluded from sites of virion assembly. We did note that less of the nonmyristylated precursor P1 protein than the myristylated P1 protein was chased into the mature virion protein VP2. This observation might reflect an inefficient incorporation of the nonmyristylated VP0-3-1 protomers into the assembly pathway or a selection against the inclusion of capsid intermediates composed of a mixture of myristylated and nonmyristylated subunits during the final stages of virion formation. The second explanation is in agreement with previous studies which reported an enrichment for myristylated capsid subunits during the formation of mature poliovirus virions in cells infected with mutant viruses that expressed a mixture of myristylated and nonmyristylated capsid subunits (20). Another possibility might be that nonmyristylated VP0, and thus subsequently VP2 and VP4 upon proteolytic cleavage of VP0 after RNA encapsidation, was selectively excluded from entering the assembly pathway, whereas VP1 and VP3 generated from the processing of nonmyristylated P1 were still included. This possibility seems unlikely, however, given that VP0, VP3, and VP1 remain tightly associated as a 5S protomer capsid subunit (4). The reduced appearance of VP2 might also be the result of an inefficient maturation cleavage of nonmyristylated VP0 to VP2 and VP4. Further studies are required to address these possibilities.

In summary, the experimental results discussed in this report demonstrate that myristylation of the poliovirus capsid precursor protein P1 and the individual capsid protein VP0 is critical for the efficient assembly of poliovirus subviral particles. Furthermore, the experiments presented demonstrate the utility of the recombinant vaccinia virus expression system for studying the poliovirus assembly process. Although the first steps in the poliovirus assembly pathway involving the proteolytic processing of P1 to VP0, VP3, and VP1 by the 3CD proteinase occur when P1 is unmyristylated, the subsequent assembly of these processed proteins into 14S pentamers or 75S empty capsids is prevented. Furthermore, myristylation is not required for the intracellular localization of 5S capsid protomers to sites of virus assembly, and the conformation of nonmyristylated 5S capsid protomers appears to be compatible with assembly in the presence of excess myristylated protomers expressed by poliovirus. Since nonmyristylated capsid protomers, when not in the presence of myristylated protomers, do not efficiently associate into 14S pentamers, myristylation is likely to be critical for stabilization of the 14S pentamer. A stabilization function for the myristate moieties, which are clustered near the contact area between the five protomer subunits that form a pentamer, is completely compatible with information given by the crystal structure of the virus (8).

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