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Lack of Myristoylation of Poliovirus Capsid Polypeptide VP0 Prevents the Formation of Virions or Results in the Assembly of Noninfectious Virus Particles

DANIEL MARC,[1] GÉRARD MASSON,[2] MARC GIRARD,[1] AND SYLVIE VAN DER WERF[1]*

Unité de Virologie Moléculaire (Centre National de la Recherche Scientifique UA 545)[1] and Unité d'Informatique Scientifique,[2] Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

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We previously described the generation of a set of mutations into a cDNA of poliovirus type 1 in the myristoylation signal of the capsid polypeptide VP4 (D. Marc, G. Drugeon, A.-L. Haenni, M. Girard, and S. van der Werf, EMBO J. 8:2661, 1989). Genomic transcripts synthesized in vitro from the mutated cDNAs were found to be noninfectious upon transfection of permissive cells, and this property correlated with the lack of VP0 myristoylation in vivo. In the study presented here, we analyzed the assembly intermediates that could be recovered from cells transfected with the mutated transcripts. We found that 14S pentamers could still assemble to a certain extent with an unmyristoylated VP0. Furthermore, viral particles sedimenting at 150S and containing capsid polypeptides VP1 to VP4 and virus-specific RNA were detected in the transfected cells. However, these mature virions were less abundant than those recovered after transfection with an infectious transcript, and they were devoid of infectivity. The results suggest that VP0 myristoylation plays a role in the late steps of poliovirus assembly and that the myristate moiety of VP4 may be required in the early steps of poliovirus infection.

Poliovirus belongs to the genus *Enterovirus* of the family *Picornaviridae*. It is a nonenveloped virus of icosahedral symmetry. Its capsid, which is made up of 60 copies of each of four capsid polypeptides (VP1, VP2, VP3, and VP4), contains a 7.5-kilobase single-stranded RNA genome of positive polarity (for a review, see reference 35). In culture, poliovirus grows on primate cells (e.g., HeLa and Vero), which possess the receptor for the virus (26, 27). Once adsorbed on its receptor, the virion enters the cell via the endosomic pathway (23, 42), and its decapsidation releases the genomic RNA into the cytoplasm. The viral RNA encodes a 247,000-dalton polyprotein which is rapidly cleaved into three precursors, P1, P2, and P3 (18). P1 is then processed to capsid polypeptides VP0, VP3, and VP1, which form the 5S protomer (3). Viral assembly begins with the pentamerization of these protomers to generate 14S pentamers $(VP0, VP1, VP3)_5$. Twelve of these pentamers then assemble into 74S empty capsids and, while including the genomic RNA, generate the short-lived 125S provirion (31, 35). During maturation to the 150S virion, which is the last step of virus assembly, VP0 is processed to VP4 and VP2, possibly by an autocatalytic process (2, 31).

The three-dimensional structure of the poliovirion capsid has been determined at 2.9-Å (0.29-nm) resolution (16). The 60 copies of VP1 are clustered around the fivefold axes of the icosahedron, whereas VP2 and VP3 alternate around its threefold axes. VP4, cleaved from VP2 during viral maturation, lies at the inner face of the virus shell, where its 60 copies are ordered as an internal network. It has been shown that VP4 as well as its precursors VP0 and P1 are myristoylated at their N-terminal glycine by an amide linkage (7, 29). The myristate moiety of VP4 is clustered with its fivefold-related counterparts around the fivefold axes of the icosahedron, building up a β annulus together with the amino termini of VP3 and VP4 (7, 10).

The covalent linkage of tetradecanoate (myristate) to the amino terminus of proteins is a cotranslational event (39). It is achieved by a cellular enzyme, *N*-myristoyltransferase, and depends on the N-terminal sequence (myristoylation signal) of the protein. Several eucaryotic proteins of cellular or viral origin have now been shown to be myristoylated (for reviews, see references 36 and 37). In certain cases (retroviral *gag* proteins and $pp60^{src}$), the myristate constitutes the hydrophobic anchor for the membrane-associated protein (4, 15, 30, 32, 33). However, this is not a general feature, since some myristoylated proteins, such as cyclic AMP-dependent protein kinase or calcineurin B, are known to be cytosolic (1, 6).

In a previous paper (25), we described the generation of a set of mutations into the myristoylation signal of VP4 and its precursors by site-directed mutagenesis of poliovirus type 1 (PV-1) cDNA. We showed that mutations which prevented myristoylation abolished the infectivity of the genomic RNA transcripts, and we demonstrated that the block was between the replication of viral RNA and the infection of neighboring cells. A negative effect of the mutations on the processing of P1 was evidenced in an in vitro translation assay, and our results suggested a possible role of the myristate moiety for the virus assembly or decapsidation processes.

Here we show that the myristoylation-negative mutations alter but do not totally prevent the late steps of poliovirus assembly and that the resulting mature virions are not infectious.

(A preliminary account to this work was reported at the Second International Symposium on Positive Strand RNA Viruses, Vienna, Austria, June 1989 [see reference 13].)

* Corresponding author.

MATERIALS AND METHODS

Plasmids, recombinant DNA procedures, and in vitro transcriptions. Plasmids pT7 PV1-51, p1A1 to p1A4, and pSW3002 have been described elsewhere (25). Plasmids were propagated in *Escherichia coli* 1106 (28) and purified essentially as described previously (24). Restriction enzymes (Boehringer Mannheim Biochemicals, Mannheim, Federal Republic of Germany; Bethesda Research Laboratories, Inc., Gaithersburg, Md.; or New England BioLabs, Inc., Beverly, Mass.) were used as instructed by the manufacturer. Genome-length RNAs were transcribed with T7 RNA polymerase (Pharmacia, Uppsala, Sweden) from purified plasmids pT7 PV1-51 and p1A1 to p1A4, which were linearized with *Eco*RI as previously described (38). A ^{32}P-labeled riboprobe complementary to nucleotides 3417 to 4830 of the viral RNA was transcribed from plasmid pSW3002 in the presence of [α-^{32}P]UTP as described previously (25).

Virus growth and titrations. Poliovirus type 1 (PV-1), Mahoney strain, was grown on HeLa cell monolayers in Dulbecco modified Eagle medium supplemented with 2% fetal calf serum. Virus was titrated on HeLa cell monolayers, using a standard plaque assay.

In vivo labeling of viral proteins. Subconfluent HeLa cell monolayers (2×10^6 cells per 60-mm-diameter plate) were transfected in the presence of DEAE-dextran with 60 μl of transcription mixture (approximately 6 μg of RNA) as described previously (38). Transfected cells were incubated for 2.5 h in Dulbecco modified Eagle medium containing 2% fetal calf serum and 5 μg of dactinomycin per ml. They were then washed twice and incubated for 45 min in methionine-free Dulbecco modified Eagle medium containing 2 μg of dactinomycin per ml before labeling with [^{35}S]methionine (30 μCi/ml; >1,000 Ci/mmol; Amersham Corp., Buckinghamshire, England). At 8 h posttransfection, cells were washed twice with ice-cold phosphate-buffered saline and lysed by a 15-min incubation at 0°C with TNE (10 mM Tris [pH 7.4], 0.1 M NaCl, 1 mM EDTA) containing 0.5% Nonidet P-40 and 25 μg of aprotinin (Boehringer) per ml. After removal of the nuclei, the cytoplasmic extracts were kept frozen until further analysis.

Fractionation of poliovirus assembly intermediates. [^{35}S]methionine-labeled cytoplasmic extracts prepared from 10^7 transfected HeLa cells (see above) were fractionated through 5 to 20% sucrose gradients prepared in TNE buffer by centrifugation for 17 h at 37,000 rpm in an SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C. Fractions (≈0.4 ml) were collected from the bottom of the gradients. The pellet of each gradient was carefully resuspended in 0.4 ml of TNE buffer containing 0.5% Nonidet P-40 and 25 μg of aprotinin per ml and constituted fraction zero. Fractions zero were analyzed by centrifugation through a 10 to 30% sucrose gradient prepared in TNE buffer. Centrifugation was for 110 min at 40,000 rpm in an SW41 rotor (Beckman) at 4°C.

Immunoprecipitations. Samples (0.2 ml) of the even fractions of the gradients were brought to 0.4 ml in 1× RIPA (10 mM Tris [pH 7.2], 0.15 M NaCl, 1% Triton-X100, 1% sodium deoxycholate, 25 μg of aprotinin per ml), incubated for 10 min at 4°C with activated *Staphylococcus aureus* (immunoglobulin G sorbent; The Enzyme Center Inc.), and centrifuged for 2 min. The supernatant was then incubated for 1 h at 37°C with 7.5 μl of a rabbit immune serum directed against PV-1 C particles, after which the immune complexes were adsorbed on protein A by overnight incubation at 4°C in the presence of activated *S. aureus*. The immunoprecipitates were washed twice with RIPA and once with TNE, boiled in Laemmli buffer, and analyzed by 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (20). The gels were fixed, treated with Amplify (Amersham), dried, and subjected to autoradiography.

TABLE 1. Characterization of myristoylation-negative transcripts

RNA	N-terminal sequence of given polyprotein[a]							Myristoylation of VP0	Infectivity
	0	1	2	3	4	5	6		
PV-1	Met	Gly	Ala	Gln	Val	Ser	Ser	+++	+
1A1	Met	**Arg**	Ala	Gln	Val	Ser	Ser	−	−[b]
1A2	Met	Gly	Ala	Gln	Val	**Thr**	Ser	+++	+
1A3	Met	Gly	Ala	Gln	Val	**Pro**	Ser	+/−[c]	−[b]
1A4	Met	Gly	**Pro**	Gln	Val	Ser	Ser	+/−[c]	−[b]

[a] Substituted amino acids are in boldface.
[b] Spontaneous reverse mutations occurred at a detectable frequency.
[c] Very low levels of VP0 myristoylation could be detected in cells transfected with transcripts 1A3 and 1A4 (25).

Quantitation of virus-specific proteins in SDS-polyacrylamide gels. A computerized image from the autoradiographs of the gels corresponding to the 5 to 20% or 10 to 30% sucrose gradients was created by using a CCD camera (Pulnix) connected to a PC-AT computer (IBM) through a Matrox PIP 1024 interface. The program uses VISILOG functions (Noesis, Jouy en Josas, France) and converts the image into a set of pixels, to each of which a level of gray comprising between 0 and 255 is associated. Briefly, for analysis by image processing, bands were selected by "top hat" transformation to eliminate the background. Tracks were delineated on the screen in a rectangular graph. Pixels were projected perpendicularly to the axis of protein migration, and their intensities were summed. Each band is therefore represented as a peak on a two-dimensional graph, and its intensity is defined as the integration value of the peak (G. Masson and A. Ermine, unpublished data).

Slot blot hybridization. Subconfluent HeLa cell monolayers (8×10^5 cells per 35-mm-diameter plate) were infected with 15 to 100 μl of appropriate fractions of the 10 to 30% sucrose gradients. Cytoplasmic RNAs (10 μg), prepared at various times postinfection, were hybridized with a ^{32}P-labeled riboprobe complementary to nucleotides 3417 to 4830 of the PV-1 genome essentially as previously described (25).

RESULTS

Using oligonucleotide-directed site-specific mutagenesis of plasmid pT7 PV1-51, a series of four mutations was introduced into the region of the PV-1 cDNA corresponding to the myristoylation signal (Met-0–Gly-1–Ala-2–Gln-3–Val-4–Ser-5–Ser-6) of poliovirus capsid protein VP4 and of its precursors VP0 and P1 (25). The resulting cDNAs were transcribed in vitro with T7 RNA polymerase into genome-length transcripts, and the properties of the mutated RNAs were then analyzed after transfection of primate cells.

We found that mutation Ser-5 to Thr (1A2) still allowed myristoylation of VP0, whereas mutation Gly-1 to Arg (1A1), Ser-5 to Pro (1A3), or Ala-2 to Pro (1A4) prevented myristoylation (25) (Table 1). The mutated myristoylation-negative transcripts replicated normally in the transfected host cell but failed to initiate a second cycle of virus multiplication. This finding suggests that the different steps

that could involve the myristate moiety are (i) processing of precursor P1, (ii) viral assembly, and (iii) viral adsorption and decapsidation. Processing of P1 in a cell-free translation assay occurred efficiently in the case of the infectious transcript harboring the 1A2 mutation (Ser-5→Thr), whereas it was impaired, particularly at the VP0-VP3 cleavage, in the case of the myristoylation-negative mutants (25). While this manuscript was in preparation, essentially the same results were reported by others (19).

To address the question of whether the myristate also plays a role in viral assembly, we analyzed the assembly intermediates that could be recovered from cells transfected with the myristoylation-negative transcripts.

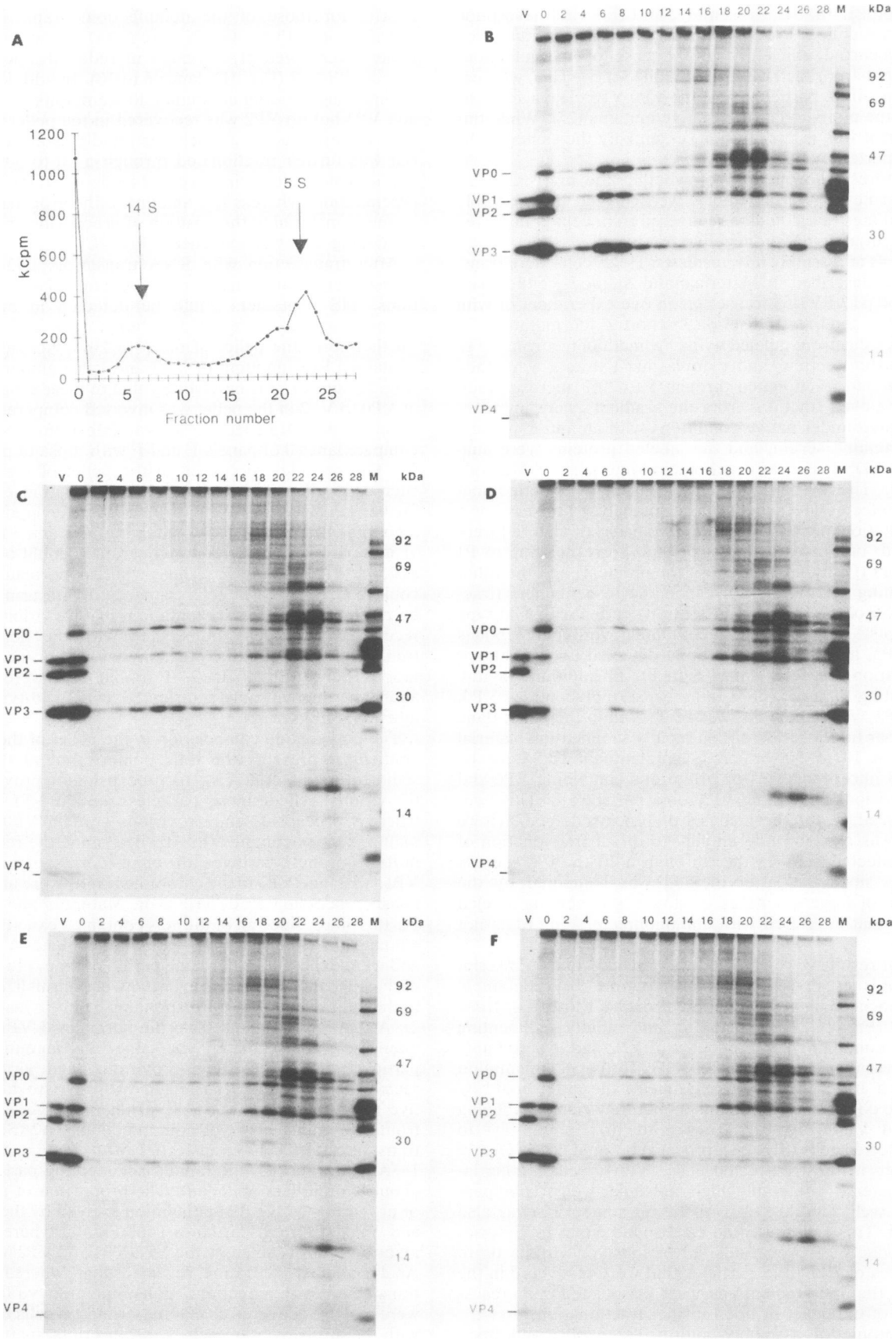
Analysis of assembly intermediates. HeLa cells were transfected as described in Materials and Methods, either with wild-type pT7 PV1-51 transcripts in one experiment or with one of the genomic transcripts harboring the mutations in another. Cells were labeled with [^{35}S]methionine from 3.5 to 8 h posttransfection, and cytoplasmic extracts were then prepared and fractionated through 5 to 20% sucrose gradients. The even fractions from the gradients were immunoprecipitated under native conditions with an anti-C-particle rabbit immune serum, and the labeled proteins were analyzed by SDS-PAGE as described in Materials and Methods (Fig. 1). The cytoplasmic extract from [^{35}S]methionine-labeled infected HeLa cells was also analyzed to serve as a sedimentation marker (Fig. 1A). Two peaks of material were identified: one sedimenting at 5S and corresponding to P1 protomers (fractions 21 to 25 of the gradient) and the other sedimenting at 14S and corresponding to pentamers (fractions 5 to 8 of the gradient). After transfection with wild-type transcripts, 14S pentamers, containing viral polypeptides VP0, VP1, and VP3, were readily detected upon immunoprecipitation (Fig. 1B, lanes 6 to 8). In addition, rapidly sedimenting material, containing capsid polypeptides VP0, VP1, VP2, VP3, and VP4, was recovered in the pellet (lane 0). Further fractionation of this rapidly sedimenting material through a 10 to 30% sucrose gradient allowed it to be resolved into procapsids or provirions containing VP0 and mature virions containing VP2 (data not shown). This two-step procedure for the analysis of procapsids and mature virions was chosen preferentially to direct fractionation of the transfected cells extract through a 10 to 30% sucrose gradient, since the latter method was hampered by the presence of large quantities of slowly sedimenting labeled material that interfered with immunoprecipitation (data not shown).

Similar results were obtained with the infectious transcript harboring the Ser-5→Thr (1A2) mutation: 14S pentamers were evidenced by sedimentation through a 5 to 20% sucrose gradient (Fig. 1C, lanes 6 to 10), and rapidly sedimenting material containing large amounts of VP2 and low amounts of VP0 was recovered in the pellet. By further sedimentation through a 10 to 30% sucrose gradient, the latter could be shown to contain large amounts of mature virions containing VP2 and VP4 (Fig. 2B, lanes 6 to 10). In contrast to PV-1-infected cells (Fig. 2A), procapsids could not be clearly evidenced in the transfected cells. Slowly sedimenting labeled material containing high-molecular-weight polypeptides as well as VP0, VP1, VP3, and some VP2 was also detected. This material could correspond either to dissociation products of procapsids, provirions, or virions or to aggregates of 5S or 14S particles that were recovered in the pellet of the first gradient and were dissociated upon resuspension of the pellet or upon further fractionation.

The profile of assembly intermediates observed upon transfection of 1A2 (Fig. 1C and 2B) served as the positive control for those of the noninfectious transcripts. After transfection with the Gly-1→Arg (1A1) transcript, 14S pentamers were present, although in reduced amounts compared with 1A2 (Fig. 1D, lanes 8 to 10). In addition, rapidly sedimenting material containing low amounts of VP0, VP1, and VP3 but no VP2 was recovered in the pellet of the 5 to 20% sucrose gradient. When this rapidly sedimenting material was further fractionated through a 10 to 30% sucrose gradient, no mature virions could be detected after immunoprecipitation, whereas low amounts of heterogeneously sedimenting material at the position of procapsids or provirions containing VP0 was evidenced (Fig. 2C).

After transfection with RNA transcripts 1A3 and 1A4, respectively, harboring Ser-5→Pro and Ala-2→Pro mutations, 14S pentamers could be detected in both cases, although in slightly reduced amounts compared with 1A2. Furthermore, the pellet of the 5 to 20% sucrose gradients contained low amounts of VP2 and large amounts of VP0 (Fig. 1E and F). It is noteworthy that in these cases the ratio of VP0 to VP2 in the pellet was inverted compared with that observed after transfection with an infectious RNA (Fig. 1, compare lanes 0 of panels E and F with those of panels B or C). The particles recovered in the pellet of the 5 to 20% sucrose gradients from the 1A3 and 1A4 transcripts were further resolved on a 10 to 30% sucrose gradient. Low amounts of mature virions containing VP2 and VP4 (clearly visible on overexposed autoradiographs) could be detected at 150S (Fig. 2D and E). Thus, whereas no mature virions could be evidenced in cells transfected with transcript 1A1 harboring the Gly-1→Arg mutation, mature virions could be recovered from cells transfected with the noninfectious transcripts harboring the other myristoylation-negative mutations (Ser-5→Pro or Ala-2→Pro), but these were much less abundant than in cells transfected with wild-type or 1A2 infectious transcripts.

The polypeptide composition in the pellet of the 5 to 20% gradients represents the total composition of the rapidly sedimenting material, i.e., (i) procapsids or provirions and (ii) virions. All of these particles contain VP1 and VP3, whereas procapsids and provirions contain VP0 and only mature virions contain VP2. To determine the relative proportions of these particles, the bands corresponding to VP0, VP1, VP2, and VP3 in the autoradiographs were analyzed by image processing as described in Materials and Methods. Furthermore, since all of the gels contained exactly the same amount of ^{35}S-labeled virus as a marker (lanes V in Fig. 1) and were exposed for the same period of time, the intensities of the VP1 and VP3 bands in lane 0 were normalized relative to the intensities of the corresponding bands in lane V (Table 2). As can be estimated from the normalized VP1 and VP3 intensities, approximately the same total amounts of viral particles (i.e., procapsids, provirions, and virions) were recovered from cells transfected with myristoylation-negative transcripts 1A3 (Ser-5→Pro) and 1A4 (Ala-2→Pro) as from cells transfected with the infectious 1A2 (Ser-5→Thr) transcripts. In cells transfected with mutant 1A1 (Gly-1→Arg) transcripts, the rapidly sedimenting particles were about twofold less abundant. The proportion of procapsids or provirions within that population is given by the VP0/VP1 and VP0/VP3 ratios in lanes 0 of Fig. 1, whereas that of mature virions is given by the VP2/VP1 and VP2/VP3 ratios. Analysis of these ratios revealed that whereas in cells transfected with infectious transcripts the viral particles were mostly recovered as mature virions, in cells transfected with the myristoylation-negative transcripts, mature virions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400
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0 5 10 15 20 25
Fraction number
B
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kDa
92
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VP0
VP1
VP2
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VP4
C
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TABLE 2. Quantitation of viral particles recovered from cells transfected with myristoylation-negative transcripts

RNA	Viral particles in pellets of 5–20% gradients[a]							VP3 of virions in 10–30% gradients	
	VP1	VP3	VP0/VP1	VP0/VP3	VP2/VP1	VP2/VP3	VP0/VP2	% cpm[b]	% Intensity[c]
1A1	0.5	0.4	4.2	4.4	<0.05	<0.05	>84	6	1
1A2	1	1	1	1	1	1	1	100	100
1A3	1.1	1.1	2.6	2.7	0.3	0.3	9	21	18
1A4	0.8	0.8	2.9	2.8	0.2	0.2	14	18	10

[a] Data for VP1 and VP3 represent normalized intensities of the indicated bands from lane 0 of the 5 to 20% sucrose gradients relative to the 1A2 values, as determined by image processing of the autoradiographs. Data for other proteins represent ratio of intensity of the bands from lane 0 of the 5 to 20% sucrose gradient relative to the 1A2 values, as determined by image processing.

[b] Relative to the 1A2 value, as determined by scintillation counting after excision of the indicated band from the gel.

[c] Relative to the 1A2 value, as determined by image processing.

represented only a minority of the total amount of rapidly sedimenting viral particles (Table 2).

Infectivity of viral particles from myristoylation-negative mutants. The mature virions evidenced in the pellet of the 5 to 20% gradients (Fig. 1) and in the 10 to 30% gradients (Fig. 2) by immunoprecipitation were tested for infectivity. The infectivity of the pellet of the 5 to 20% gradient from cells transfected with transcript 1A2 was 6.6 10^6 PFU/ml. In contrast, the infectivity of the pellets from cells transfected with the myristoylation-negative transcripts 1A1, 1A3, and 1A4 was less than 100 PFU/ml. Fractions 0 to 15 of the 1A2 10 to 30% gradient were also titrated, and the peak of infectivity was found in fractions 6 and 7 (10^6 and 6.3 10^5 PFU/ml, respectively). As expected, fractions 6 and 7 of the 10 to 30% gradients from 1A1, 1A3, and 1A4 were not infectious. These results indicate that in cells transfected with mutant transcripts 1A3 (Ser-5→Pro) and 1A4 (Ala-2→Pro), viral particles with a normal VP1-VP4 capsid polypeptide content could assemble but were not infectious.

This lack of infectivity may simply be due to the fact that the virions contained a noninfectious RNA. Alternatively, it may reflect their inability to release their RNA into the cytoplasm of the infected cells. To address this question, HeLa cells were infected with equal amounts of viral particles recovered from fractions 6 and 7 of the 10 to 30% gradients. From analysis of the bands corresponding to VP3 in fraction 6 and 8 of the gradients, it could be estimated that 5- to 10-fold fewer viral particles were recovered with noninfectious transcripts 1A3 and 1A4 than from infectious transcript 1A2 (Table 2). Therefore, each HeLa cell monolayer (8×10^5 cells) was infected with 15 μl of fractions 6 and 7 from the 1A2 sucrose gradient or with 100 μl of the same fractions from the 1A1, 1A3, or 1A4 gradients. At various times postinfection, cytoplasmic RNAs were extracted, immobilized on a nylon filter, and hybridized with a ^{32}P-labeled riboprobe complementary to the viral RNA (Fig. 3). Viral RNA replication was readily evidenced from 5 to 12 h postinfection in cells infected with mature virions recovered from the 10 to 30% gradient after transfection with 1A2. As expected, no viral RNA could be detected in cells infected with the material from the 1A1 gradient. In cells infected with the noninfectious virions recovered from cells transfected with the myristoylation-negative transcripts 1A3 and 1A4, a very low level of viral RNA replication could be evidenced from 5 to 7.5 h postinfection, i.e., during the first viral replication cycle (Fig. 3). Since RNA molecules harboring myristoylation-negative mutations have been shown to replicate normally but failed to initiate a second cycle of viral replication (25), these results indicate that only a very minor proportion of the noninfectious viral particles was able to release a replication-competent RNA into the target cells. In cells transfected with transcripts 1A3 and 1A4, a residual VP0 myristoylation has been shown to occur, which could be due, at least in part, to the presence of some revertant RNA molecules from which precursor P1 with a functional myristoylation signal is translated (25). Furthermore, slight amounts of [^{3}H]myristate label could be evidenced in VP0 and VP4 of the noninfectious viral particles recovered from cells transfected with the 1A3 and 1A4 transcripts (data not shown). This observation supports the idea that the viral particles that were able to release their RNA into the target cells contained myristoylated VP0 or VP4 molecules, whereas viral particles that were not, or were incompletely, myristoylated were unable to release their RNA. However, to confirm this hypothesis, it will be necessary to precisely quantitate the extent of myristoylation of the noninfectious mutant viral particles.

DISCUSSION

The precise location of the myristate moiety of poliovirus VP4 according to the three-dimensional structure of the capsid (7, 10) suggests that it could play a central role in the assembly of the 14S pentamers. Indeed, the fivefold-related myristates constitute a hydrophobic cluster of 65 methyl groups, which could account for the stability of the β tube and hence of the whole pentamer. The results presented here show that 14S pentamers could be recovered from cells transfected with noninfectious RNAs harboring myristoylation-negative mutations but that they were present in slightly lower amounts than in cells transfected with the infectious RNA 1A2. This finding could be due to the fact that, as previously reported (19, 25), processing of the P1 precursor

FIG. 1. Analysis of assembly intermediates on 5 to 20% sucrose gradients. HeLa cells were infected at a multiplicity of infection of 40 PFU/cell with PV-1(Mahoney) (A) or transfected with each of the wild-type (B), 1A2 (C), 1A1 (D), 1A3 (E), and 1A4 (F) transcripts and labeled from 3.5 to 8 h with [^{35}S]methionine. The cytoplasmic extracts were then prepared and fractionated through 5 to 20% sucrose gradients as described in Materials and Methods. (A) The acid-precipitable material was counted in each fraction; (B to F) one-half (0.2 ml) of the even fractions and 1/10 of the pellet were immunoprecipitated with an anti-C-particle immune serum. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Lane V, [^{35}S]methionine-labeled proteins from purified PV-1; lane M, [^{35}S]methionine-labeled extract from PV-1-infected HeLa cells. kcpm, 10^3 cpm.

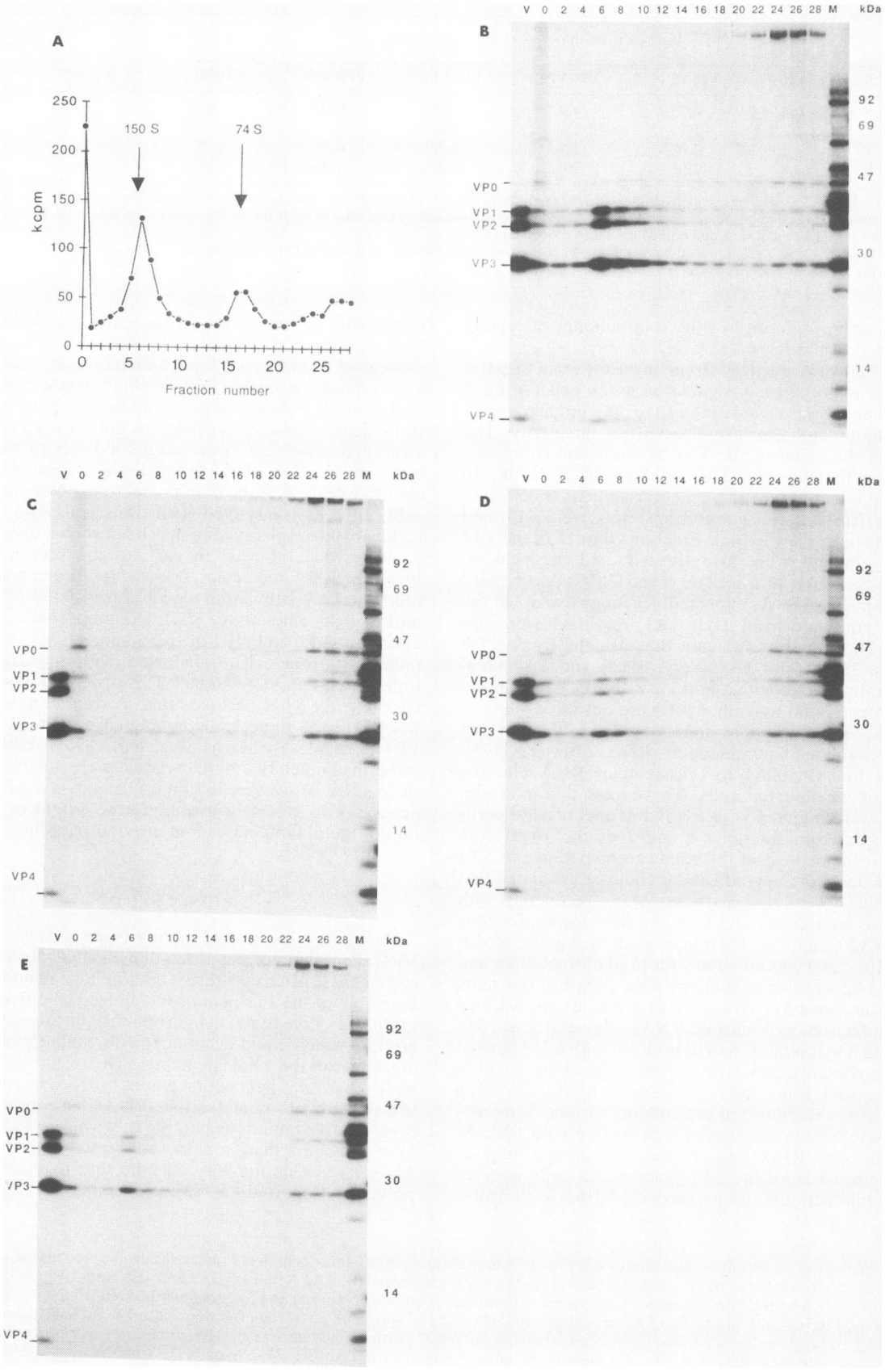
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FIG. 2. Analysis of virions on 10 to 30% sucrose gradients. The pellets from the 5 to 20% sucrose gradients in Fig. 1 were fractionated again through 10 to 30% sucrose gradients. (A) The acid-precipitable material was counted in each fraction of the gradient from PV-1-infected cells; (B to E) one-half (0.2 ml) of the even fractions from the gradients from cells transfected with 1A2 (B), 1A1 (C), 1A3 (D), and 1A4 (E) was analyzed as described in the legend to Fig. 1. Lane V, [^{35}S]methionine-labeled proteins from purified PV-1; lane M, [^{35}S]methionine labeled extract from PV-1-infected HeLa cells. kcpm, 10^3 cpm.

of VP0, VP3, and VP1 is impaired in the case of the myristoylation-negative mutants. Furthermore, in cells transfected with mutant transcripts, rapidly sedimenting viral particles, including mature virions sedimenting at 150S, were detected (Fig. 2). The relative amount of mature virions within the rapidly sedimenting particles was repeatedly found to be lower in cells transfected with the transcripts harboring the 1A3 (Ser-5→Pro) and 1A4 (Ala-2→Pro) mutations than in cells transfected with infectious transcripts, and 150S virions were never observed after transfection with the 1A1 transcript which harbors the Gly-1→Arg mutation. This finding may be correlated with the fact that myristoylation of VP0 was totally abolished in the case of the 1A1 (Gly-1→Arg) mutant, whereas a very low level of myristoylation of VP0 could be evidenced in the case of the 1A3 (Ser-5→Pro) and 1A4 (Ala-2→Pro) mutants (25). The mature virions recovered from cells transfected with mutants 1A3 and 1A4 contain some myristoylated VP0 and VP4 molecules, but it remains to be determined in what proportion.

In the case of mutant 1A1 (Gly-1→Arg), viral assembly is blocked at the stage of either the procapsid or the provirion. We have observed in one instance (13) the occurrence of capsid polypeptide VP2 together with VP1, VP3, and some VP0 in material sedimenting as a broad peak spanning the 14S region of the 5 to 20% gradient from a 1A1-transfected cell extract. No VP2 was found in the pellet of the gradient. This result suggests that in this particular experiment, cleavage of VP0 to VP4 and VP2 had occurred but was followed by disruption of the virions. The lack of formation, or the lack of stability, of the 150S viral particles of mutant 1A1 may be due either to the total lack of myristoylated VP0 in the transfected cells or, alternatively, to the abnormal presence of the charged Arg residue at the N terminus of VP0. Experiments are in progress to address this question by making other substitutions of the Gly-1 residue.

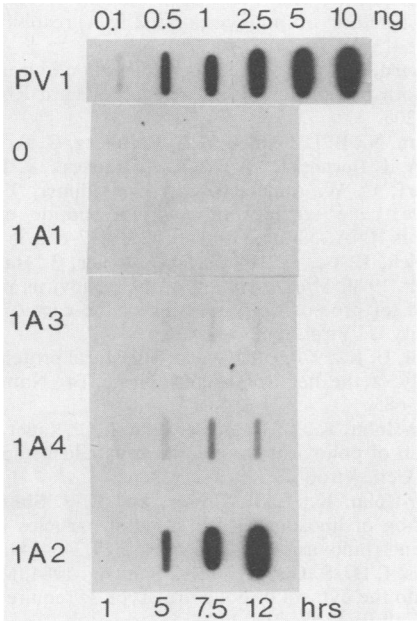

FIG. 3. Detection of viral RNA in cells infected with myristoylation-negative particles. HeLa cells were mock infected (0) or infected with 15 μl (1A2) or 100 μl (1A1, 1A3, and 1A4) of fractions 6 and 7 from the 10 to 30% sucrose gradients (see Fig. 2). At the indicated times postinfection, the cytoplasmic RNAs were extracted, blotted onto a nylon membrane, and hybridized with a PV-1-specific riboprobe as described in Materials and Methods. PV1, Increasing amounts of purified PV-1 RNA blotted in parallel.

Altogether, our observations suggest that myristoylation is required for poliovirion assembly and particularly that the late steps of assembly are much less efficient when VP0 is not myristoylated. As already discussed (25), the myristate moiety of VP0 could be required to maintain a high concentration of protomers and pentamers via membrane anchorage near the RNA replication site, where poliovirion assembly is thought to occur (5, 12). Alternatively, efficient RNA packaging may require a precise structural conformation of the pentamers. Finally, the cleavage of VP0 into VP4 and VP2, which occurs at the last maturation step in virion assembly, could have structural requirements that would involve the presence of myristate. This hypothesis is strengthened by the observation that the myristoylation-negative mutants showed a 10- to 15-fold-higher VP0/VP2 ratio in their viral particles than did wild-type or 1A2 virus (Fig. 2 and Table 2).

The 150S mature virions recovered after transfection with mutants 1A3 and 1A4 were not infectious. They contained capsid proteins VP0 to VP4, of which some VP0 and VP4 molecules were myristoylated, and viral RNA. RNA molecules harboring myristoylation-negative mutations have been shown to replicate normally in the transfected cells but failed to initiate a second cycle of viral replication (25). Therefore, the fact that only a very low level of virus-specific RNA replication could be detected during the first cycle of viral replication, but not at later times, in cells incubated with the 1A3 and 1A4 mutant virions strongly suggests that their lack of infectivity is not simply due to the fact that they contained a noninfectious RNA. This observation could be explained, for the most part, by the fact that they could not release their RNA into the target cells and were thus deficient in at least one of the early steps of infection: (i) adsorption of the virion onto its receptor, (ii) receptor-mediated endocytosis via the endosomic pathway (23, 42), and (iii) uncoating, which results in the release of the viral RNA in the cytosol.

It is tempting to speculate that the absence of the fivefold-related myristate moieties may induce some distortion in the structure of the entire capsid. Particularly, some alteration in the structure of the canyon, which is believed to be the receptor attachment site (8, 34), may hinder the binding of the virions to their cellular receptor.

Even if the mutant virions managed to bind properly to their receptor, the lack of myristate might prevent the release of the viral genome into the cytosol. Once adsorbed to receptors, virions are internalized in clathrin-coated vesicles as intact particles (40, 42). Molecular events leading to

the uncoating and release of viral RNA into the cytosol are largely unknown. Uncoating has been shown to be dependent on acidification of the endosomes (23, 41, 42), but it may require other factors such as endosomal proteins. Altered particles, sedimenting at a lower rate than intact virions, are usually found in the infected cells early after adsorption (21). Such particles could also be generated in vitro by heating the virions (22) or by incubating them with HeLa cell membranes or membrane extracts (9, 14). Both the altered particles found in vivo and those generated in vitro are devoid of capsid protein VP4.

All of these results strongly suggest that VP4 plays a crucial role in the early steps of poliovirus entry. The protrusion of VP4 outside the virion may be one of the most important conformational changes occurring during the uncoating process, and it is conceivable that the myristate moiety of VP4 is required in this process, for instance through the anchoring of VP4 in the membrane of the endosome, which would somehow facilitate the release of the viral RNA into the cytosol. While this manuscript was in preparation, Kirkegaard reported that small deletions in the amino terminus of VP1 critically affect uncoating and release of the viral RNA (17). More recently, Fricks and Hogle (11) reported that the N terminus of VP1 is externalized in cell-altered virions and is responsible for liposome binding. The N terminus of VP1, although not clearly resolved in the three-dimensional structure of the virus, is thought to project into the interior of the particle near the fivefold axis, where it could interact with VP4 (10, 11). Polypeptide VP4 and the N terminus of VP1 could thus be coordinately externalized during the cell-mediated structural alteration of poliovirus. Further experiments are needed to explore a possible role of myristate in the mechanism of cell entry, and in this respect the use of chemical analogs of myristate may prove to be a valuable approach.

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