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Myristoylation of the Poliovirus Polyprotein Is Required for Proteolytic Processing of the Capsid and for Viral Infectivity

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The poliovirus polyprotein is cotranslationally linked to myristic acid at its amino-terminal glycine residue. We investigated the role of myristoylation in the viral replication cycle by site-directed mutagenesis of this glycine codon. Synthetic full-length RNA transcripts carrying a Gly-to-Ala mutation (G4002A) gave no infectious virus on transfection into permissive cells (HeLa). However, mutant viral RNA was replicated in the transfected cells, albeit at a reduced level. The virus-specific polypeptide P1, the precursor for the capsid proteins, was found in HeLa cells transfected with wild-type or mutant RNA, but only the wild-type P1 was myristoylated; the G4002A mutant P1 was not myristoylated. We also introduced the G4002A mutation into an in vitro transcription-translation vector encoding poliovirus P1 precursor. Processing of the mutant precursor by poliovirus-infected cell lysate (providing 3C^{pro} and 3CD^{pro} activities) was severely inhibited, whereas the normally inefficient cleavage by purified 3C^{pro} was not affected. These results suggest that the myristic acid moiety of the P1 precursor may be required for efficient processing by 3CD^{pro}.

Poliovirus, a member of the *Picornaviridae*, is a small nonenveloped icosahedral virus containing a single-stranded RNA genome of positive polarity. The poliovirus capsid is composed of essentially equimolar amounts of four nonidentical polypeptides (VP1 to VP4). These polypeptides are synthesized as a precursor which is severed from the nascent polypeptide chain by the action of the virus-encoded proteinase 2A^{pro}. The structural precursor (P1) is then further processed to VP0, VP3, and VP1 in a reaction catalyzed by the virus-encoded proteinase 3CD^{pro} (6, 13, 23). During maturation VP0 is processed to VP4 and VP2, probably by an autocatalytic process involving viral RNA (reviewed in reference 8). Polypeptides P1, VP0, and VP4 have common amino termini, and their N-terminal glycine residue is blocked (3) by covalently linked myristic acid (2, 16, 21). Myristoylation of polypeptide P1 appears to occur cotranslationally, immediately after removal of the initiator methionine (3). A number of other viral (primarily retroviral) and cellular proteins are also myristoylated at their N-terminal glycine residues, and it is thought that the main function of myristoylation is to direct these proteins to the membrane (reviewed in reference 20). However, there are also soluble proteins that are myristoylated (e.g., cyclic AMP-dependent kinase). The substrate specificity for *N*-myristoyl transferase has been determined, and it has been shown that the N-terminal glycine is absolutely required (19).

Nicklin et al. (13) found that the processing of the P1 precursor by the addition of extracts from poliovirus-infected HeLa cells is sensitive to nonionic detergent, whereas processing by purified 3C^{pro} is not. Purified 3C^{pro} catalyzes the cleavage of P1 only when the proteinase is present at a very high concentration and then preferentially cleaves the VP3-VP1 bond; the VP0-VP3 bond is almost completely resistant to 3C^{pro} (13). In infected cells, processing of P1 is normally catalyzed by 3CD^{pro}, an intermediate precursor containing the sequences of the poliovirus proteinase 3C^{pro} and the viral polymerase 3D^{pol} (6, 23). On the basis of these results and the observation of proteolytic cleavage in the presence of detergent, Nicklin et al. (13) concluded that processing of P1 requires a hydrophobic interaction between the P1 precursor and the 3D domain of 3CD^{pro}. To investigate the role of myristoylation in the poliovirus life cycle, particularly with regard to the processing of polypeptide P1, we wanted to abolish the myristoylation signal by site-directed mutagenesis of the codon specifying the second amino acid of P1, a glycine (which is also the second amino acid of VP4; for this reason we designated this residue G4002). This was facilitated by cloning the 5′-terminal sequences of the poliovirus cDNA into a vector suitable for mutagenesis. The *Kpn*I-*Nru*I fragment (nucleotides [nt] 70 to 1174) of the cDNA of poliovirus type 1 (Mahoney) was inserted into the polylinker region of plasmid pBS(KS$^+$) (Stratagene), which contains an M13 origin as well as a ColE1 origin and a *bla* gene for amplification in *Escherichia coli*. Single-stranded DNA substituted with uracil was prepared by passage through *E. coli* BW313 by using the method of Kunkel (9). A synthetic oligonucleotide complementary to nt 740 to 759 of the poliovirus cDNA introducing two point mutations at nt 747 (guanine to cytosine) and nt 751 (thymine to guanine) was annealed to the DNA, and second-strand synthesis and transformation into *E. coli* C600 were performed as previously described (9). The first mutation changed the ATG GGT (Met-Gly) sequence at the N terminus of the poliovirus polyprotein to ATG GCT (Met-Ala); the second mutation was a silent third-base change that introduced a novel *Fsp*I restriction site. Double-stranded DNA was analyzed by restriction enzyme digestion with *Fsp*I, and the mutant sequence was confirmed by sequence analysis, using the dideoxynucleotide chain termination method (17). The mutant cDNA sequence was then cloned into a full-length poliovirus transcription vector by inserting the *Kpn*I-*Aat*II fragment of pBS (*myr*) (corresponding to nt 70 to 1122 of the poliovirus cDNA) into the *Kpn*I-*Aat*II fragment of plasmid pT7XL derived from plasmid pT7 PV1-5 (22), and the mutations were verified by sequence analysis.

Plasmids pT7XL and pT7XL (*myr*) were transcribed in vitro with T7 RNA polymerase, and the synthetic RNA was transfected into HeLa cells by using the DEAE-dextran

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method described previously (22). Transfection of wild-type (wt) RNA resulted in complete cytopathic effects within 24 to 48 h, whereas in the case of pT7XL (*myr*) RNA or mock transfection, no cytopathic effects were observed in five independent transfections. Cell lysates obtained 3 days after transfection with pT7XL (*myr*) RNA were used for infection of fresh HeLa cell monolayers, but no cytopathic effects were observed.

Since transfection of the mutant RNA did not yield infectious virus, we analyzed the poliovirus-specific RNA in transfected HeLa cells. Cell monolayers in 6-cm-diameter dishes were transfected with pT7XL or pT7XL (*myr*) RNA or were mock transfected. At various times posttransfection, the growth medium was aspirated and the monolayers were rinsed twice with cold phosphate-buffered saline. Cells were lysed in 0.4 ml of cold lysis buffer (10 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.2% [vol/vol] Nonidet P-40). Lysates were clarified by centrifugation at 5,000 × *g* for 5 min, and RNA was denatured by adding 0.12 ml of 1X SSC (0.15 M NaCl plus 0.015 sodium citrate [pH 7.0]) (11) and 0.08 ml of 37% (wt/vol) formaldehyde and incubating the preparation at 60°C for 15 min. RNA was spotted onto Zeta-Probe nylon membranes (Bio-Rad Laboratories) by using a filtration manifold (Schleicher & Schuell, Inc.). Prehybridization was performed in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.7]) (11)–10× Denhardt reagent (11)–0.75% sodium dodecyl sulfate (SDS)–0.05 mg of tRNA per ml for 2 h at 42°C. For hybridization the membranes were incubated overnight at 48°C (positive-strand-specific probe) or 58°C (negative-strand-specific probe) in 6× SSPE–0.75% SDS–50% (wt/vol) formamide–0.05 mg of tRNA per ml–7 × 10^6 cpm of radiolabeled oligonucleotide. After two 15-min washes in 6× SSPE–0.3% SDS at room temperature and two 15-min washes in 1× SSPE–0.75% SDS at 37°C, the membranes were exposed to Kodak X-OMAT AR film.

Replication of both positive- and negative-strand RNAs occurred in cells transfected with pT7XL RNA, whereas RNA from mock-transfected cells did not react with the poliovirus-specific probes (Fig. 1). The amount of poliovirus-specific RNA increased for up to 18 h and decreased only slightly over the following 12 h. On the other hand, in cells transfected with pT7XL (*myr*) RNA there was considerably less poliovirus RNA synthesized during the first 12 h posttransfection. The amount of positive- and negative-strand RNAs increased up to 18 h. However, the overall level of RNA synthesis was considerably lower than in wt-RNA-transfected cells. The level of total poliovirus-specific RNA in pT7XL (*myr*)-transfected cells never reached the level observed in pT7XL RNA-transfected cells at 6 h posttransfection, well before release of virus and second-round infections would be expected.

To determine whether the G4002A substitution encoded by pT7XL (*myr*) prevented myristoylation of the P1 precursor in vivo, HeLa cells were infected with PV1 or transfected with pT7XL or pT7XL (*myr*) transcripts and labeled from 3 to 7.5 h posttransfection with [^{3}H]myristate. Cell lysates were harvested and immunoprecipitated with antibody specific for purified but denatured PV1 particles. [^{3}H]myristate-labeled VP0 and VP4 were found in cells infected with PV1 or transfected with pT7XL RNA but not in cells transfected with pT7XL (*myr*) RNA (Fig. 2). Therefore, replacement of the position-1 glycine with alanine blocks myristoylation of the N terminus of the P1 polypeptide.

To investigate whether the myristic acid moiety is essential for proper proteolytic processing in vitro, we performed proteolytic cleavage assays with mutant and wt P1 precursors. Poliovirus RNA is inefficiently translated in rabbit reticulocyte lysate, but deletion of the 5′-terminal 670 nt of the nontranslated sequence in a synthetic mRNA comprising the P1 coding region (derived from plasmid pMN22 [14]) has been shown to direct efficient translation of the P1 precursor. Therefore, we exchanged *Bam*HI-*Bsm*I fragments (nt 670 to 1519 of the poliovirus cDNA) between plasmids pT7XL (*myr*) and pMN22 to give plasmid pMN22 (*myr*). This plasmid contains the poliovirus cDNA sequence from nt 670 to 3381 followed by a termination codon under the control of the promotor of bacteriophage T7 gene 10 and also contains the two point mutations at nt 747 and 751. Synthetic RNAs were transcribed from plasmids pMN22 and pMN22 (*myr*) with T7 RNA polymerase and translated in rabbit reticulocyte lysate (Promega Biotec) in the presence of [^{35}S]methionine as previously described (7, 14). The results of the in vitro translation experiment are shown in Fig. 3. Synthetic RNAs derived from plasmids pMN22 and pMN22 (*myr*) both directed the synthesis of P1 precursor proteins which comigrated with the P1 polypeptide found in poliovirus-infected

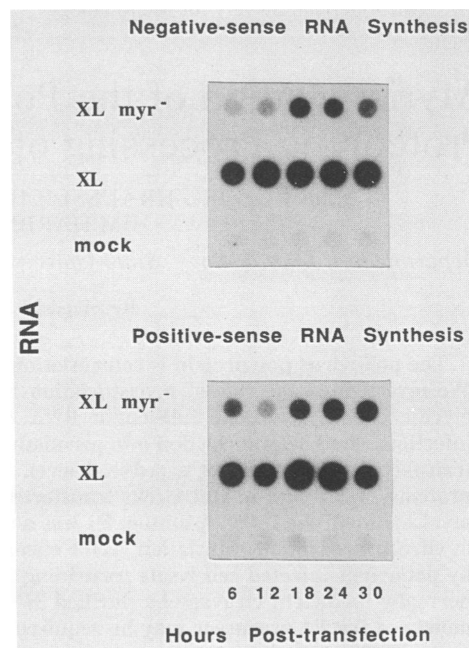

FIG. 1. Dot blot analysis of RNA replication in transfected cells. HeLa cell monolayers were transfected with wt (XL) or mutant (XL myr$^-$) RNA or were mock transfected, and total cellular RNA was extracted at various times and spotted onto Zeta-Probe membranes as described in the text. Radioactively labeled oligonucleotide probes specific for positive-sense RNA [corresponding to nt 5402 to 5421 of the cDNA of poliovirus type 1 (Mahoney) containing one mismatch] and negative-sense RNA (complementary to nt 5402 to 5424 of the poliovirus cDNA with one mismatch) were prepared by end labeling with T4 polynucleotide kinase. Reaction mixtures contained 200 pmol of dephosphorylated oligonucleotides in a solution containing 50 mM Tris hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 40 μCi of [^{32}P]ATP (specific activity, 4,500 Ci/mmol), and 10 U of T4 polynucleotide kinase. The reaction mixtures were incubated for 30 min at 37°C; 0.15 ml of 50 mM Tris hydrochloride (pH 8.0)–5 mM EDTA was added, and labeled oligonucleotides were purified by chromatography through Sephadex G-50 (Pharmacia). Prehybridization and hybridization were performed as described in the text.

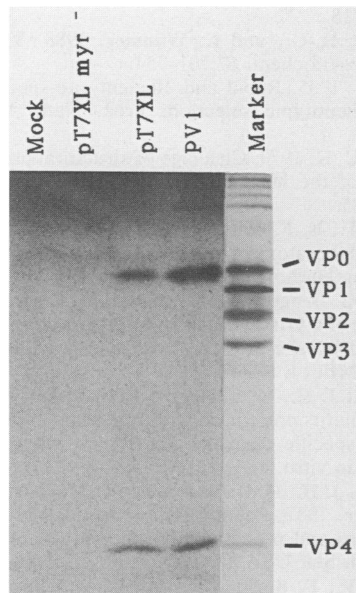

FIG. 2. Myristoylation of VP0 in vivo. A total of 10^6 HeLa cells were mock infected, transfected with Ca. 1 μg of pT7XL (*myr*) or pT7XL, or infected with PV1 at a multiplicity of infection of 50. The cells were labeled 3 h later with [9,10(*n*)-^{3}H]myristic acid (150 μCi/ml; 10 to 60 Ci/mmol; New England Nuclear Corp.). At 7 h postinfection or 7.5 h posttransfection, the cells were rinsed three times with cold phosphate-buffered saline and lysed in 100 μl of lysis buffer (see text). Samples were immunoprecipitated with anti-C polyclonal rabbit serum as described previously (4). Immunoprecipitated material was electrophoresed on a 13.5% SDS-polyacrylamide gel (10). The gel was fixed and treated with En^{3}Hance (New England Nuclear Corp.), and labeled protein bands were visualized with Kodak AR X-ray film after 18 days of exposure. The marker lane contained [^{35}S]methionine-labeled extract from PV1-infected HeLa cells. The positions of PV1 capsid polypeptides are indicated on the right.

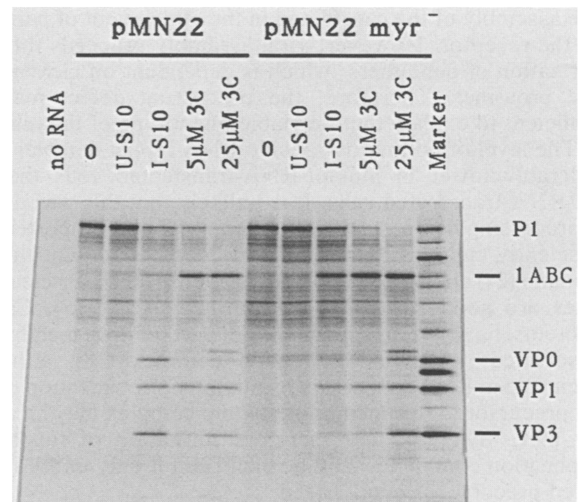

FIG. 3. Products of in vitro translation of synthetic RNAs and in vitro processing. Samples of translation mixtures were diluted 1:10 into 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 7.4)–100 mM NaCl–2 mM dithiothreitol–1 mM EDTA containing either no enzyme, 5 or 25 μM purified 3C^{pro}, or 5-μl portions of postmitochondrial extracts from uninfected (U-S10) or poliovirus-infected (I-S10) HeLa cells. HeLa cell extracts and purified 3C^{pro} were prepared as described previously (1, 13). Reaction mixtures were incubated for 1 h at 30°C, and this was followed by analysis on a 10 to 20% SDS-polyacrylamide gel and autoradiography. The no-RNA lane contained a translation reaction mixture without added template, and the marker lane contained a lysate of [^{35}S]methionine-labeled poliovirus-infected HeLa cells.

HeLa cells. Both P1 precursors were stable to incubation with extracts from uninfected HeLa cells. Incubation with extracts from poliovirus-infected cells (providing 3C^{pro} and 3CD^{pro} activities) gave complete processing to VP0, VP3, and VP1 in the case of RNA derived from pMN22 (Fig. 3), whereas the P1 precursor containing the Gly-Ala change at the N terminus [referred to as P1(G4002A)] was partially resistant to processing, and a considerable amount of P1(G4002A) remained uncleaved (Fig. 3). Cleavage of P1(G4002A) was limited to partial processing at the VP3-VP1 site to give 1ABC and VP1, and the VP0-VP3 site was resistant to incubation with infected cell extracts. This situation is reminiscent of the incubation of wt P1 precursor with purified 3C^{pro}. A concentration of 5 μM 3C^{pro} gave partial processing of wt P1 precursor to yield primarily 1ABC and VP1 (Fig. 3). On the other hand, cleavage at the VP0-VP3 site was observed when P1 was incubated with 25 μM 3C^{pro} (Fig. 3) (the concentration of in vitro-translated substrate was approximately 1 to 10 nM). At this concentration of 3C^{pro} we also observed an aberrant cleavage product that migrated slightly slower than VP0 (Fig. 3). Incubation of P1(G4002A) with the two different concentrations of purified 3C^{pro} gave the same results as incubation with the wt P1 polypeptide (Fig. 3). No defect in processing of P1(G4002A) by purified 3C^{pro} was detected.

Previously, it had been shown that poliovirus polyprotein precursors synthesized in rabbit reticulocyte lysate were N-terminally blocked (3) and that in vitro-synthesized foot-and-mouth disease virus precursor could be labeled with [^{3}H]myristic acid (2), suggesting that myristoylation occurs quite efficiently in rabbit reticulocyte lysate. Therefore, it is likely that the in vitro-synthesized poliovirus wt P1 precursor was completely myristoylated, although the level of incorporation of [^{3}H]myristic acid was below the detection limit (data not shown). From our in vitro processing experiments we concluded that myristoylation of the capsid precursor is required for efficient processing by the viral 3CD proteinase. This conclusion is further supported by the observations that deletion of the N-terminal VP4 moiety abolished processing of an in vitro-synthesized ΔP1 precursor (14) and that 3CD-mediated processing of P1 was detergent sensitive (13). It is very likely that high-efficiency processing of P1 requires a hydrophobic interaction between the 3D domain of 3CD^{pro} and the myristic acid moiety of the P1 precursor. In this respect it would be interesting to study the effect of mutations in the 3D domain of 3CD^{pro} on the processing of wt and mutant P1 precursor. Mutational analysis should allow the identification of amino acid residues in 3D that mediate the interaction with P1.

The processing defect of P1(G4002A) could also be due to a misfolded substrate with partially occluded cleavage sites. Detailed structural analysis of poliovirus particles by X-ray crystallography (5) has shown that the myristate moiety interacts with the N terminus of VP3 and with VP1. However, structural studies of the uncleaved P1 precursor would be required to predict whether these interactions are a prerequisite for proper folding of the protomer. Clearly, both cleavage sites can still be recognized in P1(G4002A), since processing by purified 3C^{pro} was unaffected. Additional defects of the myristoylation-deficient mutant could occur in

the assembly of the capsid and in the attachment of particles to the receptor. However, viral assembly proceeds through formation of pentamers, which is dependent on cleavage of the protomer. Therefore, the processing defect may be sufficient to explain the nonviable phenotype of the mutant.

The level of poliovirus-specific RNA replication was considerably lower in mutant-RNA-transfected cells than in wt-RNA-transfected cells. It is unlikely that this was due to a processing defect, since cleavage of P2 and P3 proteins is efficiently catalyzed by $3C^{pro}$ and does not require the 3D domain (24) and since peptides modeled after P2-P3 cleavage sites are good substrates for purified $3C^{pro}$ (15). Capsid proteins have been shown to be part of the membrane-associated poliovirus replication complex (18), although their role in RNA replication is unknown. Localization of the P1 precursor to this membrane-bound complex may be aided by myristoylation, and therefore formation of functional replication complexes may be inefficient if only unmyristoylated precursor is synthesized.

During the preparation of our manuscript, a paper by Marc et al. (12) was published. Their data led to essentially the same conclusions as those reported here.

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