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POST-TRANSLATIONAL MODIFICATIONS OF POLIOVIRUS PROTEINS

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The post-translational modifications of poliovirus proteins have been investigated by analysis of glycosylation, sulphation, phosphorylation and acylation of the proteins made in the infected HeLa cells. No glycosylation or sulphation of proteins specific for virus-infected cells was apparent. A number of changes in the pattern of phosphorylated proteins took place. The specific myristylation of the structural protein VP4 and its precursors was clearly apparent. Acylation of viral proteins with oleic or palmitic acid was not detected. Myristylation took place in the presence of the protease inhibitor $ZnCl_2$, but not in the presence of inhibitors of translation, such as cycloheximide and anysomycin.

We have previously analyzed in detail the polypeptides synthesized in poliovirus-infected HeLa cells by two-dimensional gel electrophoresis (1). Some of the viral proteins migrated as multiple spots, whereas others appeared as smears, suggesting that some poliovirus-coded proteins can undergo post-translational modifications. A number of cellular and viral proteins suffer post-translational modifications such as glycosylation, acylation, sulphation, phosphorylation, etc. An increasing number of cellular and viral proteins are known to contain covalently bound lipid. Some of these proteins contain myristic acid, a 14 carbon saturated fatty acid, at the amino-termini (2-4). For picornaviruses no glycosylated proteins have been described (5-7). However, myristylation of the capsid protein VP4 has been documented for poliovirus and other members of the Picornaviridae family (8-10). We have now analysed by 2-D gel elec-

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trophoresis the post-translational modifications of poliovirus proteins.

MATERIALS AND METHODS

Cells and virus.- HeLa cells, mycoplasma free, were grown as monolayers in Dulbecco modified Eagle's medium containing 10% newborn calf serum and antibiotics (10,000 IU of penicillin per ml; 50 mg of streptomycin per ml). Poliovirus type 1 Mahoney strain was grown on HeLa cells.

Conditions of infection and protein labeling.- HeLa cells were placed in 96-well Linbro plates. Before the cells reached confluency, they were infected with poliovirus (50 pfu per cell). After 1h at 37°C, the medium was removed and replaced by fresh medium containing 2% newborn calf serum.

Labeling with [^{32}P]orthophosphate.- At 2 h post-infection, the medium was removed and replaced by 50 µl of [$PO_4^{\equiv}$] per ml [(^{32}P) sodium phosphate; 370 MBq/ml]. After 4 h of incubation at 37°C, the cell monolayer was dissolved in 75 µl of lysis buffer (9 M urea; 0,1 M dithiotheitol; 2,8% Nonidet P-40; 2% ampholytes, pH 7 to 9).
Labeling with (^{3}H)palmitic acid, (^{3}H)oleic acid or (^{3}H)myristic acid.- The cells were incubated at 37°C with 50 µl of Dulbecco modified Eagle's medium containing 2% newborn calf serum and 30 µCi of the corresponding labeled fatty acid per ml. [^{3}H] palmitic acid; 40 Ci/mmol. [^{3}H] oleic acid; 46 Ci/mmol. [^{3}H] myristic acid (60 Ci/mmol). Then, the medium was removed and the cells were dissolved in 75 µl of lysis buffer or 75 µl of 0,1 M Tris HCl, pH 6,5 - 1% SDS, 10% glycerol, 0,1 M 2-mercaptoethanol.

Analysis of proteins by two-dimensional gel electrophoresis.- The procedure for two-dimensional gel electrophoresis has been described in detail elsewhere (1,11).

RESULTS

Glycosylation and sulphation of poliovirus proteins

The glycosylation of poliovirus proteins was investigated by labelling the cells for two, or four hours with several sugars including (^{3}H)mannose, (^{3}H)galactose and (^{3}H) glucosamine. We did not detect any labelled protein specific for virus-infected cells. The sulphation of proteins was assayed by labelling during two hours (3-5 h.p.i.) or five hours (0-5 h.p.i.) with (^{35}S)SO_4^{2}. Again no labelling of viral proteins was evident (results not shown).

Phosphorylation

To investigate this possibility in poliovirus-infected cells the proteins were labelled during four hours (2-6 h.p.i.) with (^{32}P)PO_4^{3-}

and they were analyzed by 2-D gel electrophoresis. Fig. 1A shows the pattern of proteins phosphorylated in mock- infected HeLa cells. Fig. 1B shows the polypeptides phosphorylated in poliovirus-infected cells. Previous studies have analyzed protein phosphorylation in ribosomal fractions from poliovirus- infected cells. In agreement with those studies, we found a number of new phosphorylated proteins not present in uninfected cells. In addition, changes in the phosphorylation of some of these proteins is also evident. However, comparison of this pattern with the (^{35}S)methionine-labelled gels (1) indicates that none of the identified viral protein is phosphorylated.

Acylation

The discovery that poliovirus protein VP4 (1A) has its amino-terminal myristylated (8-10) indicates that acylation occurs in this poliovirus structural protein. In preliminary studies, we analyzed the acylation of poliovirus proteins with (^{3}H)myristic acid, (^{3}H)-oleic acid or (^{3}H)palmitic acid (results not shown). Only myristic acid labelled proteins specifically appeared in poliovirus-infected cells. These proteins migrated with the same coordinates as proteins 1ABCD, 1ABC, 1AB and 1A, further indicating that this modification took place in the precursor proteins, before cleavage and assembly of VP4 into virions. These proteins were further analyzed by 2-D gel electrophoresis. Fig. 2 shows the myristylated proteins from HeLa cells. Two major labelled spots appear in uninfected cells in the IEF gels in the region of 65,000 and 45,000 respectively. In poliovirus-infected cells, the proteins 1ABC, 1AB and 1A are clearly visualized. This result also corroborates the positions that we assigned previously to these polypeptides (1).

The time course of the myristylation of poliovirus proteins under various labelling conditions, indicates that this modification does not correlate exactly with the synthesis of viral proteins, but it is slightly delayed (Fig. 3). The myristylated cellular proteins are very stable after poliovirus infection. However, de novo myris-

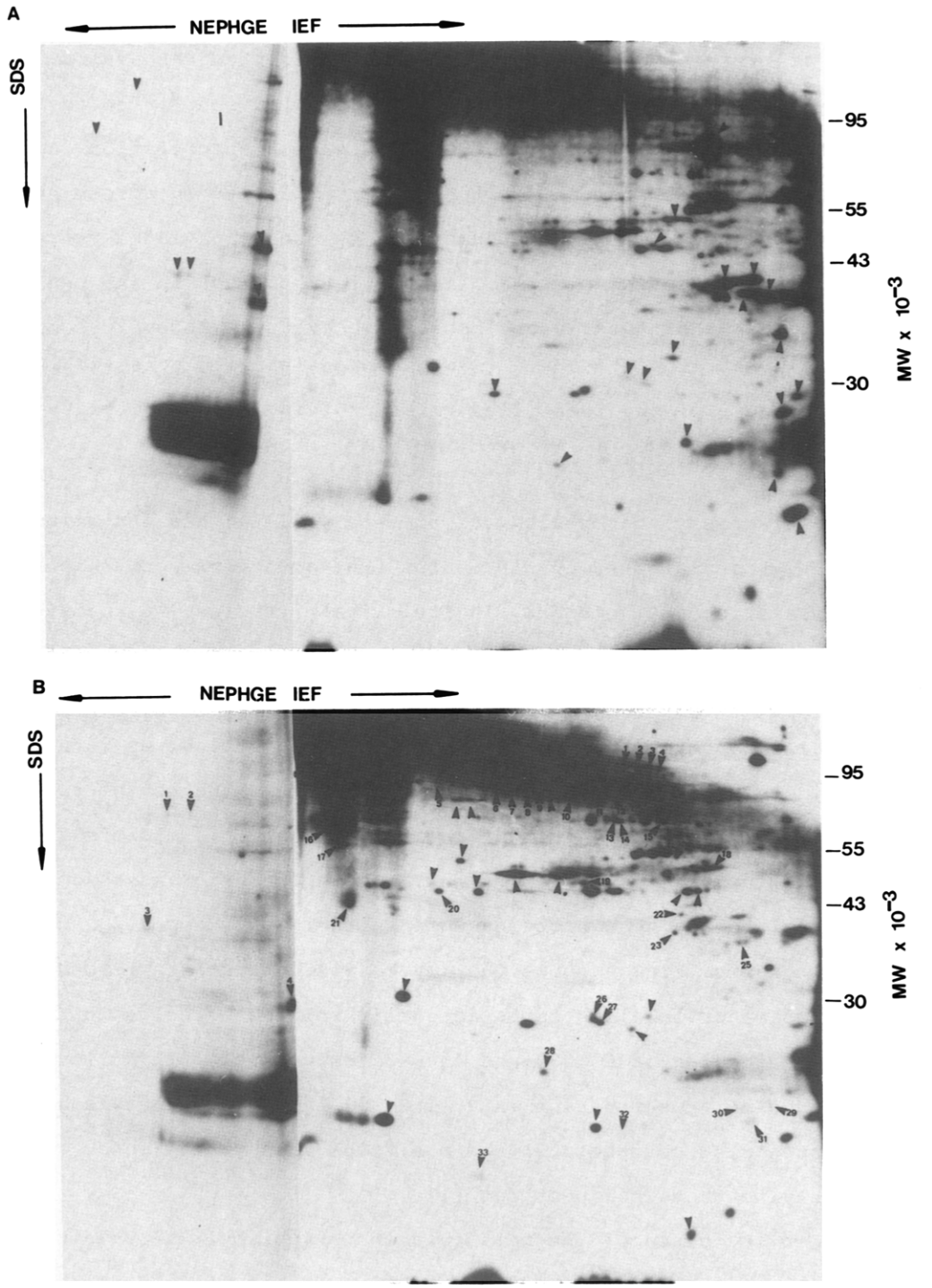

Fig. 1.- A) Two-dimensional gel electrophoresis of HeLa cell proteins labeled for 10 h with (^{32}P)orthophosphate (600 μCi/ml). B) Two-dimensional gel electrophoresis of poliovirus-infected HeLa cell proteins labeled from 2 to 6 hours postinfection with (^{32}P)orthophosphate. Arrows show the proteins that are modified after poliovirus infection.

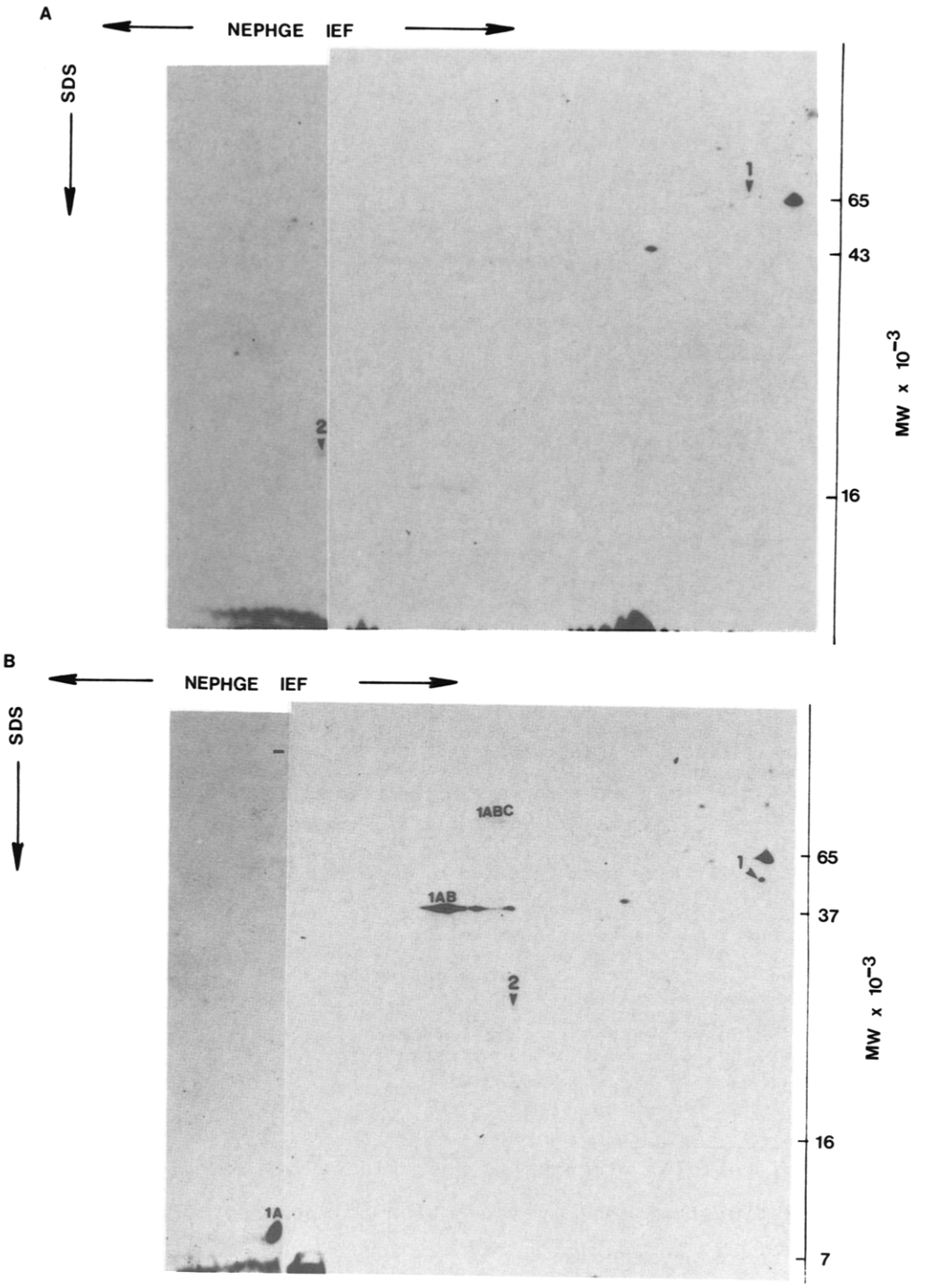

Fig. 2.- Two-dimensional gel electrophoresis of mock-infected (A) and poliovirus infected HeLa cell proteins (B) labeled with (^{3}H)myristic acid. Cells were labeled for 12 h with the fatty acid and then, were infected or mock-infected with poliovirus. After virus adsorption, more radioactive precursor was added and cells were harvested at 5 h.p.i. The viral proteins 1ABC, 1AB and 1A are indicated.

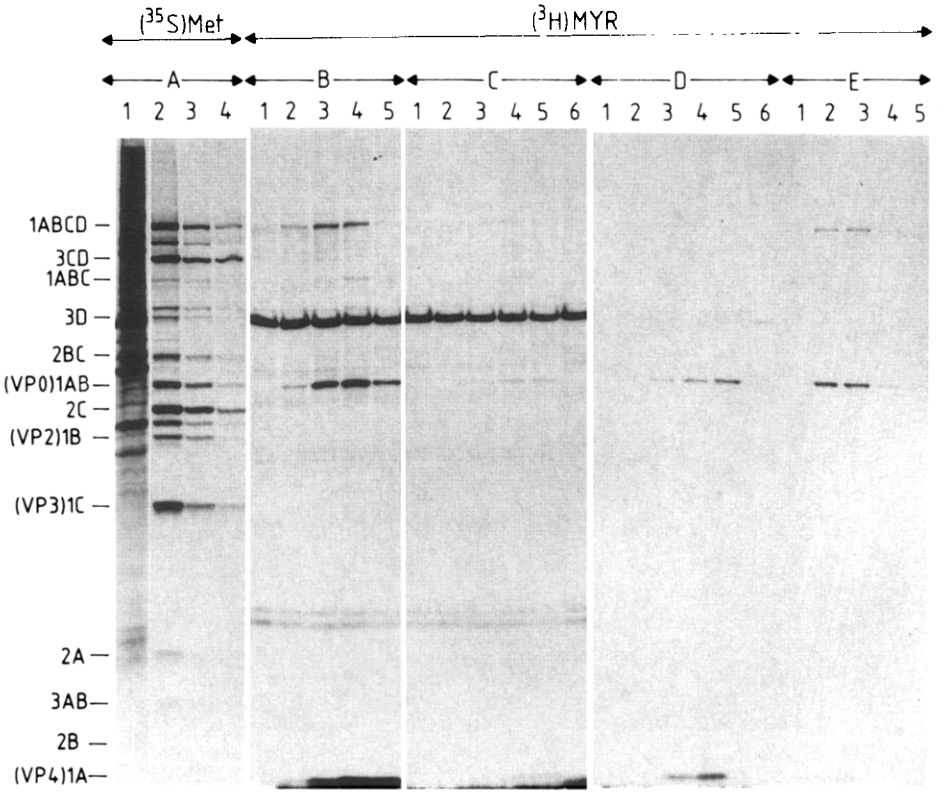

Fig. 3.- A) Analysis by SDS/PAGE of the proteins synthesized during 1 hour in mock-infected (1) and poliovirus infected HeLa cells. (^{35}S)-methionine (100 μCi/ml) was added at $2^{1/2}$ (2), $3^{1/2}$ (3) and $4^{1/2}$ (4) hours post-infection. B) HeLa cells were labeled for 12 h with myristic acid (30 μCi/ml) and then were mock-infected (1) or infected with poliovirus. At 0.h.p.i., more labeled fatty acid was added, and the cells were harvested at $2^{1/2}$ (2), $3^{1/2}$ (3), $4^{1/2}$ (4) and $5^{1/2}$ (5) hours post-infection. C) After 12 h of labelling with (^{3}H)myristic acid, HeLa cells were mock-infected or infected with poliovirus. Mock-infected cells were harvested at 0 (1) or $5^{1/2}$ (6) h.p.i., and poliovirus-infected HeLa cells were harvested at $2^{1/2}$ (2), $3^{1/2}$ (3), $4^{1/2}$ (4) and $5^{1/2}$ (5) hours post-infection. D) HeLa cells were mock-infected or infected with poliovirus. At 0.h.p.i., (^{3}H)myristic acid was added; mock-infected cells were harvested at $2^{1/2}$ (1) and $5^{1/2}$ (6) h.p.i. and poliovirus infected cells were harvested at $2^{1/2}$ (2), $3^{1/2}$ (3), $4^{1/2}$ (4) and $5^{1/2}$ (5) h.p.i. E) Proteins labeled with ^{3}H)-myristic acid synthesized in mock-infected (1) or poliovirus-infected HeLa cells, during 1 h. Labeled myristic acid was added at $2^{1/2}$ (2), $3^{1/2}$ (3), $4^{1/2}$ (4) and $5^{1/2}$ (5) h.p.i.

tylation of cellular proteins is not evident in poliovirus-infected cells suggesting that the myristylation of these cellular proteins is coupled to its synthesis.

Finally, to analyze the myristylation of poliovirus proteins in the presence of inhibitors of protein synthesis or polyprotein processing, the proteins were labelled with myristic acid in the presence of cycloheximide, or cycloheximide plus anysomycin, or $ZnCl_2$. The

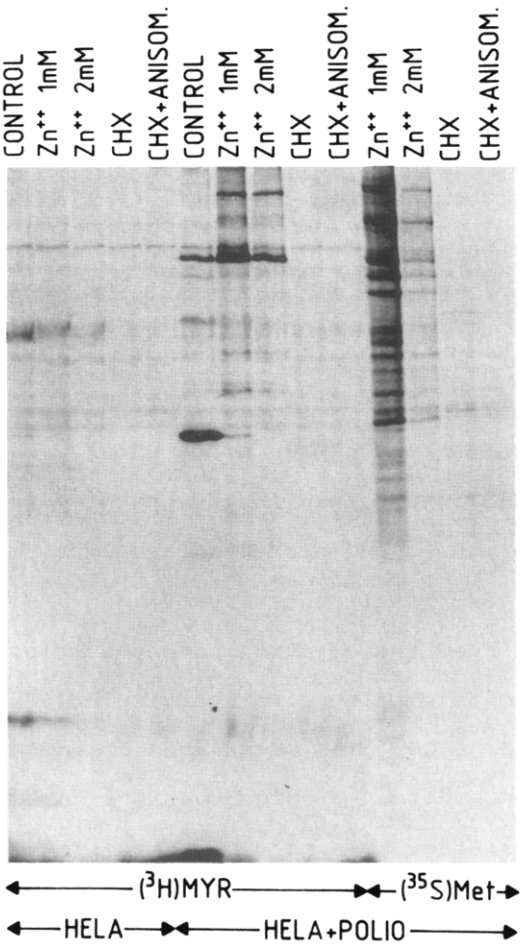

Fig. 4.- Analysis by SDS/PAGE of the proteins from mock-infected or poliovirus-infected HeLa cells labeled with (^{3}H)myristic acid (30 μCi/ml), or (^{35}S)methionine from 4 to 6 h. p.i. Labelling was carried out in the absence or in the presence of $ZnCl_2$ (1 or 2 mM), cycloheximide CHX (10^{-4} M) or cycloheximide CHX (10^{-4} M) and anisomycin (10^{-5} M).

translation inhibitors also block the myristoylation of cellular proteins in uninfected HeLa cells. A similar result is obtained in the infected cells with the virus- coded proteins (Fig. 4). This result reinforces the idea that myristylation of poliovirus proteins is tightly coupled to its synthesis. The finding that the precursor polypeptides are labelled with (^{3}H)myristic acid in the presence of $ZnCl_2$ also agrees well with this conclusion. Therefore, the myristylation of poliovirus amino-terminus most probably takes place on polyribosomes and does not necessitate the cleavage of the polyprotein to take place.

DISCUSSION

The modification of proteins during or after translation is an important step to render a native protein possessing all the biological activity (2). The reversible modification of many proteins is a biological device often used to regulate its activity. In an attempt to get further insight into the characterization of poliovirus-coded proteins, we analyzed the modifications of these proteins by glycosylation, sulphation, phosphorylation and acylation. Previous reports suggested that none of the capsid proteins of picornaviruses were glycosylated (6-8). In agreement with these reports we found that none of the poliovirus-coded proteins was glycosylated or sulphated. Also it does not seem likely that any of them are phosphorylated. These results led open the question of why some poliovirus polypeptides do not migrate as single spots, particularly striking is protein 2C. This protein migrates as a smear when extracts from the infected cells are analyzed, whereas it migrates as a neat spot when it is synthesized in cell-free systems. The possibility that other modifications hitherto unidentified remains open.

The number of myristylated proteins discovered increases every day (2), but the exact function of this modification still remains unclear. For some of them the myristic acid can serve to anchor the protein to the membrane, but many myristylated proteins are cytoplasmic. For poliovirus, the analysis of the virion structure at high resolution suggested that VP4 could be myristylated (8). This prompted more detailed biochemical studies about this modification (9,10).

The exact role that the myristic acid residue may play in poliovirus remains unclear. It has been speculated that it might play a part after virion attachment to the receptor, to facilitate virus entry, or perhaps it might serve during virus assembly to get the exact folding and interactions of viral proteins (8, 9). Our results suggest that the modification of VP4 occurs before its proteolytic processing, and is tightly coupled to translation. Experiments direc-

ted to block the myristylation of VP4 will shed some light about its biological role.

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