JOURNAL OF VIROLOGY, Oct. 1989, p. 4285–4291
0022-538X/89/104285-07$02.00/0

Vol. 63, No. 10

Fatty Acid Acylation of Vaccinia Virus Proteins†

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Received 27 March 1989/Accepted 9 June 1989

Labeling of vaccinia virus-infected cells with [^{3}H]myristic acid resulted in the incorporation of label into two viral proteins with apparent molecular weights of 35,000 and 25,000 (designated M35 and M25, respectively). M35 and M25 were expressed in infected cells after the onset of viral DNA replication, and both proteins were present in purified intracellular virus particles. Virion localization experiments determined M25 to be a constituent of the virion envelope, while M35 appeared to be peripherally associated with the virion core. M35 and M25 labeled by [^{3}H]myristic acid were stable to treatment with neutral hydroxylamine, suggesting an amide-linked acylation of the proteins. Chromatographic identification of the protein-bound fatty acid moieties liberated after acid methanolysis of M25, isolated from infected cells labeled during a 4-h pulse, resulted in the recovery of 25% of the protein-bound fatty acid as myristate-associated label and 75% as palmitate, indicating that interconversion of myristate to palmitate had occurred during the labeling period. Similar analyses of M25 and M35, isolated from infected cells labeled during a 0.5-h pulse, determined that 46 and 43%, respectively, of the protein-bound label had been elongated to palmitate even during this brief labeling period. In contrast, M25 and M35 isolated from purified intracellular virions labeled continuously during 24 h of growth contained 75 and 70%, respectively, myristate-associated label, suggesting greater stability of these proteins or a favored interaction of the proteins containing myristate with the maturing or intracellular virion.

The covalent attachment of fatty acid residues to nascent polypeptides has become widely recognized as an important, and in some cases essential, protein modification reaction which occurs in eucaryotic organisms (28; for a review, see references 29, 33, and 41). Although the functional significance of this type of protein modification is not clearly understood, the attachment of the acyl prosthetic groups is likely to influence the structure, activity, and possibly the subcellular localization of the acceptor proteins (29, 33, 41). Several different fatty acid moieties have thus far been found attached to proteins, including palmitic acid (for a review, see references 29 and 33), myristic acid (1, 6, 8, 9, 11, 15, 17, 20–24, 26, 34, 35, 37), and, to a lesser extent, stearic acid, linoleic acid, and oleic acid (33). In the more common palmitation and myristylation modifications, distinct differences exist with regard to how the acyl groups are attached to proteins, which amino acids are modified, and where the modified proteins are targeted. For example, palmitic acid is posttranslationally added via an ester or thioester bond to internal threonine or cysteine residues of proteins which are primarily destined to be membrane associated (16–19, 21, 22). In contrast, myristylation of proteins occurs cotranslationally via an amide linkage to the N-terminal glycine residue of acceptor proteins which may or may not be membrane associated (21, 23, 33, 41). Little is known about what protein structural or sequence parameters influence the selection of acylation sites or determine which fatty acid will be added. In order to address these questions and to study what role protein acylation may play in the regulation of gene expression, the use of viral systems which are amenable to directed genetic manipulations would seem to offer an attractive experimental approach.

Acylated viral proteins have been reported, identified, and studied in several different families of RNA viruses, including the *Togaviridae*, the *Retroviridae*, and the *Picornaviridae* (30, 32, 33, 41). In sharp contrast, little is known about the status of fatty acid acylation reactions during the replicative cycles of some of the large DNA viruses, such as the *Poxviridae*. With regard to vaccinia virus (VV), which is the prototype of the *Poxviridae*, a single major palmitated protein (P37) has thus far been reported (12). The status of other types of potential acylation modifications on VV-encoded proteins has not been investigated. The function of the P37 protein is unknown, but it is found in the envelope of extracellular but not intracellular virus particles and has been suggested to be nonessential for virus replication in tissue culture cells (12, 13). Since VV completes its replicative cycle within the cytoplasm of infected cells and has already been shown to encode many of the *cis*- and *trans*-acting factors necessary to modulate the expression of its genetic program (36), this virus provides an excellent model system for studying transcriptional and translational regulatory mechanisms. Therefore, as part of an effort to begin to elucidate the various posttranslational regulatory strategies which may be used by VV (and perhaps the host cell), a study was undertaken to characterize the nature, specificity, and extent of fatty acid acylation of VV proteins.

We report here that myristic acid (as well as palmitic acid) is covalently linked to two VV proteins (M_r ~25,000 and ~35,000) by a hydroxylamine-resistant (i.e., presumably amide) linkage. Both proteins are expressed at late times during the virus replicative cycle and are present in purified intracellular virus particles. We also confirmed and extended the previous observations with regard to P37 (12) by identifying the attached acyl group and exploring the nature of its protein linkage. Experiments were carried out to examine the synthesis, maturation, and subvirion localization of the VV acyl proteins. The results obtained are discussed in the context of the potential biological roles of these modified proteins during the viral replicative cycle.

MATERIALS AND METHODS

Cells, virus, and labeling conditions. The WR strain of VV was grown in monolayers of BSC_{40} (African green monkey)

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† Oregon Agricultural Experiment Station technical paper no. 8823.

cells maintained in Eagle minimal essential medium with Earle salts (MEM-E) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 10 μg of gentamycin sulfate per ml as previously described (14).

For pulse-labeling experiments, BSC_{40} cell monolayers (60-mm dishes) infected with VV (20 PFU/cell) were incubated in MEM-E (1/10 the normal methionine concentration was used when [^{35}S]methionine was used for labeling) containing 5% delipidated fetal calf serum (7), L-glutamine (2 mM), and either L-[^{35}S]methionine (25 μCi/ml, 1,129 Ci/mmol; New England Nuclear Corp.), [9,10-^{3}H]myristic acid (250 μCi/ml, 22.4 Ci/mmol; New England Nuclear), or [9,10-^{3}H]palmitic acid (250 μCi/ml, 60 Ci/mmol; New England Nuclear) at 37°C for the times specified in each experiment. At the end of the labeling period, the medium was removed, and the cell monolayer was washed three times with ice-cold phosphate-buffered saline and lysed in gel sample buffer (68 mM Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10 mM dithiothreitol containing 10% glycerol and 0.1% bromophenol blue). DNA was sheared by repeated pipetting through a 22-gauge needle, and lysates were boiled for 3 to 5 min prior to analysis on SDS-polyacrylamide gels.

Radiolabeled virus was isolated from two 150-mm dishes of BSC_{40} cells infected with 10 PFU of virus per cell and incubated in the presence of either L-[^{35}S]methionine (12.5 μCi/ml), [^{3}H]myristic acid (125 μCi/ml), or [^{3}H]palmitic acid (125 μCi/ml) for 24 h at 37°C and purified by sucrose gradient sedimentation as described previously (14).

Separation of viral envelope and core fractions. Purified radiolabeled virions were separated into Nonidet P-40 (NP-40)-soluble (envelope) and NP-40-insoluble (core) fractions as described previously (42). SDS-polyacrylamide gel electrophoresis and fluorography were used to analyze the protein composition of both fractions of each labeled virus preparation.

Gel electrophoresis and protein isolation. Radiolabeled proteins were analyzed on 10% polyacrylamide slab gels containing SDS (39). The gels were then impregnated with 2,5-diphenyloxazole and fluorographed at −70°C on Kodak XAR-5 film (5).

To isolate individual protein species, radiolabeled proteins were separated by SDS-polyacrylamide gel electrophoresis, and the region of the gel that contained the ^{3}H-labeled acyl protein was located by interpolation from adjoining lanes that contained [^{35}S]methionine-labeled virion proteins. The labeled proteins were recovered from the excised gel strip by electrodialysis in a volatile buffer system (3).

Analysis of protein-bound fatty acid. For hydroxylamine treatment of labeled proteins following SDS-polyacrylamide gel electrophoresis, duplicate sets of protein extracts were separated on a 10% polyacrylamide gel, and the gel was cut in half and soaked in either 1.0 M hydroxylamine (pH 7.0) or 1.0 M Tris hydrochloride (pH 7.0) for 2 h at room temperature (18, 22). Gels were then fluorographed as described above.

Identification of protein-bound lipids was carried out essentially as described previously (22). Briefly, individual ^{3}H-labeled VV acyl proteins were subjected to acid methanolysis as follows. Labeled protein (20 μl) was treated with 1 ml of 2 N HCl–83% methanol in vacuo at 95°C for 60 h. The reaction products were extracted three times with 1 ml of petroleum ether at 4°C. The combined ether extracts were evaporated to dryness under a stream of nitrogen and redissolved in 150 μl of high-pressure liquid chromatography (HPLC)-grade methanol containing 6.25 mg each of myristic acid, palmitic acid, myristic acid methyl ester, and palmitic acid methyl ester (Sigma Chemical Co.) per ml as standards. The ether-extractable radioactivity recovered after acid methanolysis was separated and identified by reverse-phase HPLC with a μBondapak C_{18} column (3.9 mm by 30 cm; Waters Associates) eluted isocratically with 80% acetonitrile as the mobile phase at a flow rate of 1.0 ml/min. Fractions eluting from the column were collected in 0.5-min intervals and counted in 3 ml of 3a70 scintillation fluor (Research Products International, Inc.). The absorbance elution profile (A_{254}) of the internal standards was compared with the elution of labeled species to identify each lipid.

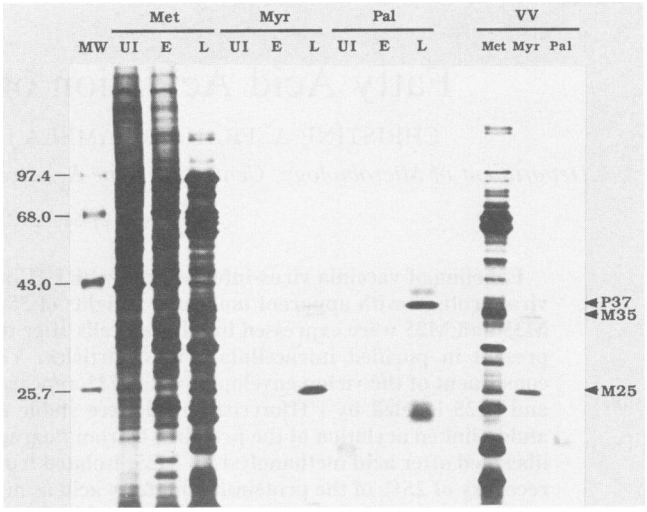

FIG. 1. SDS-polyacrylamide gel electrophoresis of metabolically labeled proteins. Uninfected (UI) and vaccinia virus-infected cell monolayers were labeled with [^{35}S]methionine (Met), [^{3}H]myristic acid (Myr), or [^{3}H]palmitic acid (Pal) at early (E, 0.5 to 2.5 h) or late (L, 4 to 8 h) times after infection. Purified intracellular virus (VV) was prepared from infected cells labeled continuously for 24 h after infection. Labeled proteins from total-cell lysates and purified intracellular virions were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and visualized by fluorography. Arrows indicate the positions of the major protein labeled with [^{3}H]palmitic acid (P37) and the major (M25) and minor (M35) proteins labeled with [^{3}H]myristic acid. Radioactive molecular size markers (MW) are phosphorylase B (97.4 kilodaltons [kDa]), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and α-chymotrypsin (25.7 kDa).

RESULTS

Acylated proteins of VV. In order to identify VV acyl proteins, uninfected or VV-infected BSC_{40} cells were pulse-labeled with either [^{35}S]methionine, [^{3}H]palmitic acid, or [^{3}H]myristic acid at early (0.5 to 2.5 h) or late (4 to 8 h) times postinfection, and total-cell lysates were prepared. Similarly, intracellular virus particles were purified from infected cells labeled continuously for 24 h. The pattern of proteins which were labeled with the fatty acids was examined by electrophoresis on 10% polyacrylamide gels, followed by fluorography. Equivalent amounts of fatty acid-labeled extracts were analyzed along with 0.02% of the amount of [^{35}S]methionine-labeled extracts. The radiolabeled palmitate and myristate were incorporated into distinct sets of proteins which comigrated with methionine-labeled polypeptides

from VV-infected cell lysates (Fig. 1). The protein which was most highly labeled with palmitate had an apparent M_r of ~37,000 (designated protein P37). P37 has been reported previously by other investigators to be the major antigen present on the envelope of extracellular VV particles, but absent from intracellular virus particles (12). P37 has been included in this study as a control and to extend the information known about the nature of the fatty acid moiety and its linkage to this acyl protein. The protein which was most highly labeled with myristate had an apparent M_r of ~25,000 (designated M25) and did not comigrate electrophoretically with any of the palmitate-labeled VV proteins. In addition to M25, a minor myristate-labeled protein of M_r ~35,000 (designated M35) was also detected. In some extracts, an additional minor myristate-labeled protein was seen that had a slightly slower electrophoretic mobility than M35 and appeared to comigrate with P37. The relative amounts of the two species varied from almost equivalent (lane Myr/L) to exclusively the faster-migrating M35 (lane VV/Myr). It is unclear at this time whether the myristate-labeled polypeptide which comigrated with P37 is a myristylated protein distinct from M35, a modified form of M35, or perhaps P37 which has become labeled by metabolic interconversion of the myristate label.

The data shown in Fig. 1 also established that the myristate-labeled proteins M25 and M35 were present in purified intracellular VV particles (lane VV/Myr). None of the proteins observed in infected cells labeled with palmitate were detected in an equivalent amount of protein from purified intracellular virus particles (lane VV/Pal). Other investigators have demonstrated that the major palmitate-labeled protein, P37, was present in extracellular virus particles (but not intracellular virus particles) and that the gene encoding this protein maps within the viral genome (12, 13). Although the major myristate- and palmitate-labeled proteins differed in their apparent M_r and presence in or absence from intracellular virus particles, both sets of labeled proteins belonged to the late temporal class of virus gene products, since they were expressed in infected cells at times after the onset of viral DNA replication. Little or no evidence of these proteins was observed when pulse-labeling with the fatty acids was performed at early times during infection (lanes Myr/E and Pal/E). This kinetic class was assigned to P37 in the characterization of this protein described previously (12).

The subvirion location of M35 and M25 was investigated by experiments in which purified [^{35}S]methionine- or [^{3}H]myristic acid-labeled intracellular virus particles were treated with 0.5% NP-40 and 50 mM dithiothreitol and then separated into viral envelope (detergent soluble) and core (detergent insoluble) fractions prior to SDS-polyacrylamide gel electrophoresis and fluorography. M25 was present entirely in the NP-40-soluble fraction, suggesting that it resides within the virus envelope, while the majority of M35 was seen in the NP-40-insoluble fraction, with a lesser amount in the envelope fraction, indicating a peripheral or weak association of M35 with the virion core (data not shown). The biological function of the major (M25) and minor (M35) proteins labeled with myristate has not yet been determined.

The covalent attachment of myristate and palmitate to the proteins was investigated by experiments in which fatty acid-labeled cell extracts were delipidated by extraction with chloroform-methanol (2:1) prior to SDS-polyacrylamide gel electrophoresis. The pattern of labeled proteins was unaffected by this treatment except that the non-protein-bound radioactive lipid which migrated to the dye front during electrophoresis was absent after treatment (data not shown). The possibility that the fatty acids are metabolically interconverted cannot be ruled out from these experiments. However, the specificity of fatty acid labeling of these proteins appears to minimize the possibility that the fatty acid labeling of the proteins was due solely to metabolic conversion of fatty acids to amino acids which were reincorporated into protein. Likewise, the fatty acid-labeling profiles were substantially distinct from each other and from those of proteins labeled with methionine.

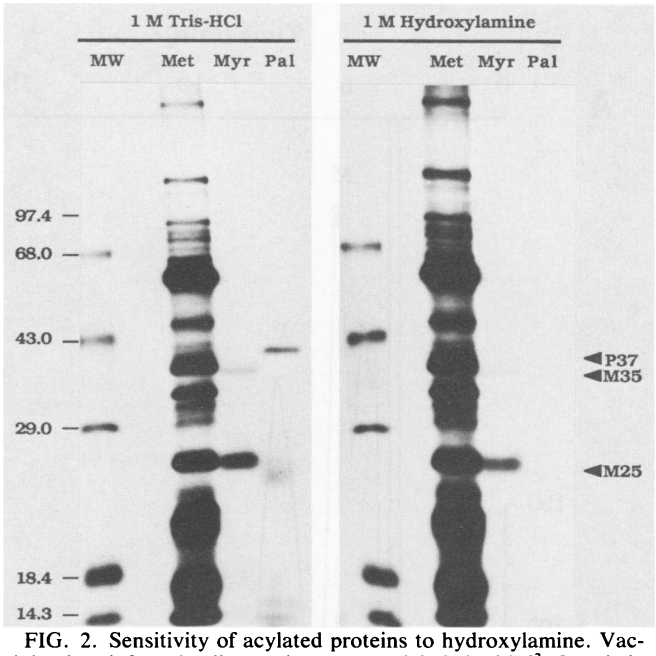

FIG. 2. Sensitivity of acylated proteins to hydroxylamine. Vaccinia virus-infected cell monolayers were labeled with [^{3}H]myristic acid (Myr) or [^{3}H]palmitic acid (Pal) at late (4 to 8 h) times after infection. Purified intracellular virus (Met) was prepared from infected cells labeled continuously with [^{35}S]methionine for 24 h after infection. After separation of labeled proteins by SDS-polyacrylamide gel electrophoresis on a 10% gel, the gel was cut in half and treated for 2 h at room temperature with either 1 M Tris hydrochloride (pH 7.0) or 1 M hydroxylamine (pH 7.0), after which they were treated for fluorography. Arrows indicate the positions of the major protein labeled with [^{3}H]palmitic acid (P37) and the major (M25) and minor (M35) proteins labeled with [^{3}H]myristic acid. Radioactive molecular size markers (MW) are phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa).

Analysis of protein-bound fatty acids. Fatty acids have been shown to be attached to proteins via both ester and amide linkages, and in general, some fatty acyl chain specificity of protein-lipid linkages has been observed. The linkage of the palmitic acid moiety to the majority of acyl proteins studied has been identified as an ester or highly reactive thioester bond from its lability to alkaline methanolysis and sensitivity to the nucleophile hydroxylamine (4, 16, 19, 22, 43). In contrast, the bond through which myristate is linked to proteins is resistant to treatment with hydroxylamine and alkaline methanolysis, suggesting amide linkage of this fatty acid to the acyl protein (22). In order to investigate the nature of the fatty acid linkage to P37, M25, and M35, the sensitivity of the myristate- and palmitate-labeled proteins to cleavage by hydroxylamine was examined. As shown in Fig. 2, treatment of the proteins with

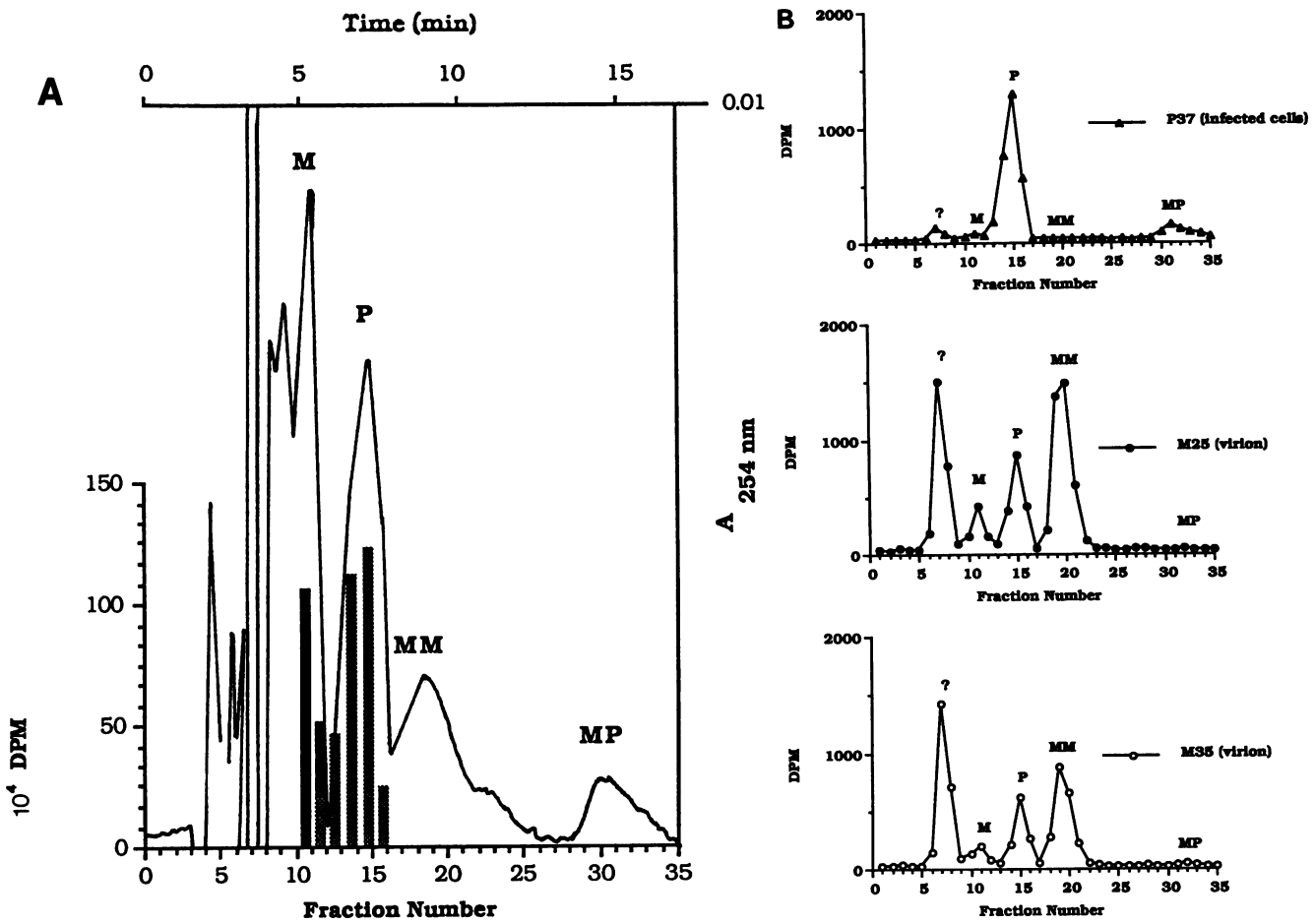

FIG. 3. Analysis of fatty acids. (A) HPLC of unlabeled fatty acid and fatty acid methyl ester standards and labeled fatty acid standards. HPLC separations were performed as described in Materials and Methods. The elution of radioactivity after chromatography of [^{3}H]myristic acid (solid bars) or [^{3}H]palmitic acid (stippled bars) is superimposed on a tracing of the absorbance elution profile of internal fatty acid and fatty acid methyl ester standards (M, myristic acid; P, palmitic acid; MM, myristic acid methyl ester; and MP, palmitic acid methyl ester). (B) HPLC of labeled protein-bound fatty acids. Purified labeled proteins were subjected to acid methanolysis, and the released fatty acids and fatty acid methyl esters were separated by HPLC as described in Materials and Methods. The distribution of radioactivity is compared with the elution of fatty acid and fatty acid methyl ester internal standards (see above) to identify the radioactive acid methanolysis products. Top panel, Fatty acids released from gel-isolated P37 (infected cells); center panel, fatty acids released from gel-isolated M25 (virion) protein; bottom panel, fatty acids released from gel-isolated M35 (virion) protein.

hydroxylamine resulted in the removal of label from the palmitate-labeled proteins, but did not substantially alter the myristate or methionine labeling patterns. The release of the palmitate-bound label from P37 by this treatment is consistent with an ester or thioester linkage of the palmitate to the protein. Conversely, the stability of the protein-bound myristate label to hydroxylamine suggests that the fatty acid linked to these proteins (M25 and M35) is attached via an amide bond. However, this type of experiment still does not rule out labeling of the proteins by metabolic interconversion of the labeled fatty acids into the amino acid pool.

To determine whether the label associated with the proteins was present as fatty acid rather than lipid metabolites, and to identify the moiety covalently bound to the proteins, individual fatty acid-labeled proteins were isolated by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The identity and purity of the recovered proteins were confirmed by gel electrophoresis (data not shown). The chromatographic separation of unlabeled fatty acid and fatty acid methyl ester standards which were cochromatographed with the labeled fatty acids ([^{3}H]palmitic acid and [^{3}H]myristic acid) used for labeling in these studies is shown in Fig. 3A. From these data it can be seen that the fatty acid (myristic acid [M] and palmitic acid [P]) and fatty acid methyl ester (myristic acid methyl ester [MM] and palmitic acid methyl ester [MP]) standards were well separated by this chromatographic system into peaks (A_{254}) which eluted at 5.2 (M), 7.4 (P), 9.5 (MM), and 15.5 (MP) min. The purity of the [^{3}H]palmitic acid and [^{3}H]myristic acid used for labeling in these studies was also demonstrated by the coelution of the labeled fatty acids with the peak of absorbance for either myristic acid or palmitic acid standards.

In the experiment shown in Fig. 3B, M25 and M35 (from myristate-labeled purified intracellular virus particles) and P37 (from virus-infected cell lysates labeled with palmitic acid from 4 to 8 h postinfection) were isolated and then subjected to acid methanolysis followed by ether extraction as described in Materials and Methods, and the liberated fatty acids were identified after separation by reverse-phase

TABLE 1. Fatty acid composition of acylated proteins[a]

Protein source and protein	Radioactivity as fatty acid (%)[b]	% of total protein-bound fatty acid[c]	
		Myristate	Palmitate
Virion (24-h pulse)			
M25	72	75	25
M35	60	70	30
Infected cells			
4-h pulse			
P37	96	2	98
M25	92	25	75
30-min pulse			
M25	99	54	46
M35	97	57	43

[a] The composition of the isolated, labeled protein-bound fatty acids was determined after acid methanolysis followed by HPLC.

[b] The percentage of the total radioactivity recovered that coeluted with the fatty acid and fatty acid methyl ester standards upon separation by HPLC.

[c] The percentage of the total protein-bound radioactivity that coeluted with either myristic acid and myristic acid methyl ester (myristate) or palmitic acid and palmitic acid methyl ester (palmitate) standards upon separation by HPLC.

HPLC. In each case, an unidentified peak of radioactivity eluted early in the chromatography (fraction 7, 3.2 min) that was minor in some instances (top panel) and considerable in others (center and bottom panels). The magnitude of this peak correlated directly with the length of the labeling period, suggesting that this peak represents amino acids or other metabolites which had become labeled by interconversion of the radioactive fatty acids during long labeling periods. When either unlabeled (20 amino acids) or labeled amino acids, [^{3}H]lysine or [^{35}S]methionine, were subjected to the same chromatographic analysis, greater than 97% of the peak of either absorbance or radioactivity eluted at 3.2 min (in fractions 7 and 8), corroborating the suspected identity of this peak (data not shown). Acid methanolysis of the palmitate-labeled protein P37 resulted in the recovery of palmitic acid and methyl palmitate (top panel). Essentially no myristic acid or methyl myristate was detected, and less than 5% of the label associated with the protein existed as labeled amino acids (Fig. 3B and Table 1). The majority (70 to 75%) of the labeled lipid bound to M25 and M35 (isolated from purified intracellular virus particles) was found to exist as myristate moieties (Fig. 3B and Table 1). These analyses indicate some elongation of myristate to palmitate during the prolonged labeling period (24 h), as well as substantial interconversion of the label into amino acids (28 to 40%). In an effort to circumvent the extensive metabolysis of the labeled myristate during prolonged labeling periods, M25 was isolated from infected cells that had been labeled with [^{3}H]myristic acid during a 4-h pulse (from 4 to 8 h postinfection). Analysis of the M25-bound radioactive species by HPLC after acid methanolysis revealed very little conversion of label into the amino acid component of the protein (4 to 8%) (Table 1). Strikingly, 75% of the acyl groups bound to M25 had been elongated from myristate to palmitate during the 4-h pulse-labeling period. Reduction of the pulse-labeling period to 30 min (6 to 6.5 h postinfection) still resulted in conversion of 43 to 46% of the labeled myristic acid to palmitic acid when the fatty acid content of M35 and M25 (isolated from infected cells) was analyzed (Table 1).

DISCUSSION

Fatty acid acylation has been reported for a variety of viral and cellular proteins; however, the specificity of this modification and the function of the attachment of fatty acids to these proteins are only beginning to be examined. The present study demonstrates that at least two types of fatty acylation reactions (palmitylation and myristylation) occur on distinct late protein species of VV. Two intracellular virion-associated proteins, designated M35 and M25, were found to contain primarily myristic acid. However, analysis of the fatty acid content of M25 and M35 isolated from infected cells revealed significant interconversion of myristic acid to palmitic acid, the amount depending on the length of the labeling period. The finding that fatty acids are readily converted to their longer-chain derivatives has been noted before by Schmidt (31), and the metabolic fate of myristic acid has been shown to differ in different cells (22). Whether the myristic acid is metabolized before or after it is utilized as the acyl donor, whether myristic acid and palmitic acid are attached to the same or different acceptor sites, and what role the host cell plays with respect to the interconversion have not yet been determined for M25 and M35. However, since *N*-myristyl transferase has been shown to be highly specific for myristate, this implies that elongation of myristate to palmitate is most likely occurring after protein acylation. There have been previous reports of protein-bound palmitate present in an amide linkage (40). The observation that normal cellular enzymes are fatty acylated indicates that acylation can be performed by cellular enzymes; however, there is still the possibility of participation by viral enzymes or regulatory factors. In contrast, the palmitate-labeled protein isolated from infected cells, P37, was demonstrated to contain almost exclusively palmitic acid bound by a hydroxylamine-sensitive, presumably thioester linkage. The labeled fatty acids bound to M25 and M35 were resistant to treatment with hydroxylamine, suggesting that they are attached to the proteins by an amide bond. With respect to the linkage of the lipid to M25 and M35, the fatty acid could be attached to the N-terminal amino acid of the protein or to the ε-NH_2 group of a lysine residue. In most instances of myristylation of viral proteins, the fatty acid has been attached to an N-terminal glycine residue (8, 24). Further studies will be needed to identify the residue(s) modified by the lipids in M35 and M25.

Several features involved in characterizing and identifying M25 and M35 should be noted. Both M25 and M35, like P37, can be provisionally classified as late proteins, in that they are expressed in cells after the onset of viral DNA replication. They appear to be relatively abundant proteins. Presuming that, as usual, myristylation occurs at only 1 mol per mol of protein and considering the low specific activity of the [^{3}H]myristic acid (22.4 Ci/mmol) used in these experiments, fluorography had to be done for only 2 to 3 days to visualize the labeled viral proteins. Again assuming one fatty acylated residue per protein, M25 was much more abundant or stable than M35. Both myristylated proteins were associated with the mature intracellular virus particle and were present in proportions similar to those seen in infected cells. In the cells commonly used for the propagation of VV, most of the progeny virus remain cell associated (intracellular virus), and only small amounts of antigenically distinct virus are naturally released from the cell (extracellular virus) (2). Both intracellular and extracellular viruses are infectious, but they differ markedly in their mode of adsorption and penetration in cell cultures (25). It will be of interest to determine whether M25 and M35 are present in purified extracellular virus particles or their destination is intracellular virus specifically. In contrast, distinct subvirion locations have been observed for each protein; M25 is released by NP-40

with the virion envelope, whereas M35 remains associated with the virion core, although somewhat loosely.

The finding that M35 and M25 are myristylated raises the question of the functional significance of the modification. A scaffolding role for the formation of virus particles has been proposed for the myristylated VP2 proteins of simian virus 40 and polyomaviruses (38) and for the myristylated VP4 proteins of the picornaviruses (8, 24). Mutations in either these proteins or in the glycine acceptor residue have resulted in either poor replication (10) or immature, noninfectious particles (27). It has also been suggested that myristate could be important in the early stages of infection, during the adsorption and penetration of virus particles into the cell, by directing the modified protein to the cell or vesicle membrane, where it could function in the passage of the virus across the membrane (8). In view of the differences observed in this regard between intracellular and extracellular VV noted above, this might provide a clue toward a possible biological function of the modification of one or both of these proteins. Directed genetic studies will be necessary to clarify the exact function of the myristate moiety of M35 and M25 of VV. To that end, genomic and peptide mapping procedures are currently in progress.

ACKNOWLEDGMENTS

We thank W. G. Dougherty for helpful comments on the manuscript.

This research was supported by a Public Health Service research grant (AI-21335) and a Research Career Development Award (AI-00666) from the National Institutes of Health.

LITERATURE CITED

1. **Aitkin, A., P. Cohen, S. Santikarn, D. H. Williams, A. G. Calder, A. Smith, and C. B. Klee.** 1982. Identification of the NH_2-terminal blocking group of calcineurin B as myristic acid. FEBS Lett. **150**:314–318.
2. **Appleyard, G., A. Hapel, and E. A. Boulter.** 1971. An antigenic difference between intracellular and extracellular rabbit-pox virus. J. Gen. Virol. **13**:9–17.
3. **Bhown, A. S., J. E. Mole, F. Hunter, and J. C. Bennett.** 1980. High-sensitivity sequence determination of proteins quantitatively recovered from sodium dodecyl sulfate gels using an improved electrodialysis procedure. Anal. Biochem. **103**:184–190.
4. **Bolanowski, M. A., B. J. Earles, and W. J. Lennarz.** 1984. Fatty acylation of proteins during development of sea urchin embryos. J. Biol. Chem. **259**:4934–4940.
5. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–91.
6. **Carr, S. A., K. Biemann, S. Shagi, D. C. Parmelee, and K. Titani.** 1982. *n*-Tetradecanyl is the NH_2-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. Proc. Natl. Acad. Sci. USA **79**:6128–6131.
7. **Cham, B. E., and B. R. Knowles.** 1976. A solvent system for delipidation of plasma or serum without protein precipitation. J. Lipid Res. **17**:176–181.
8. **Chow, M., J. E. Newman, D. Filman, J. M. Hogle, D. J. Rowlans, and F. Brown.** 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature (London) **327**:482–486.
9. **Clark, B., and U. Desselberger.** 1988. Myristylation of rotavirus proteins. J. Gen. Virol. **69**:2681–2686.
10. **Cole, C. N., T. Landers, S. P. Goff, S. Manteuil-Brutlag, and P. Berg.** 1977. Physical and genetic characterization of deletion mutants of simian virus 40 constructed in vitro. J. Virol. **24**:277–294.
11. **Henderson, L. E., H. C. Krutzsch, and S. Oroszlan.** 1983. Myristyl amino-terminal acylation of murine retrovirus proteins: an unusual post-translational protein modification. Proc. Natl. Acad. Sci. USA **80**:339–343.
12. **Hiller, G., and K. Weber.** 1985. Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. J. Virol. **55**:651–659.
13. **Hirt, P., G. Hiller, and R. Wittek.** 1986. Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. J. Virol. **58**:757–764.
14. **Hruby, D. E., L. A. Guarino, and J. R. Kates.** 1979. Vaccinia virus replication. I. Requirement for a host cell nucleus. J. Virol. **29**:705–715.
15. **Jorgensen, E. C., N. O. Kjeldgaard, F. S. Pedersen, and P. Jorgensen.** 1988. A nucleotide substitution in the *gag* N terminus of the endogenous ecotropic DBA/2 virus prevents $Pr65^{gag}$ myristylation and virus replication. J. Virol. **62**:3217–3223.
16. **Kaufman, J. F., M. S. Krangel, and J. L. Strominger.** 1984. Cysteines in the transmembrane region of major histocompatibility complex antigens are fatty acylated via thioester bonds. J. Biol. Chem. **259**:7230–7238.
17. **Magee, A. I., and S. A. Courtneidge.** 1985. Two classes of fatty acid acylated proteins exist in eukaryotic cells. EMBO J. **4**:1137–1144.
18. **Magee, A. I., A. H. Koyama, C. Malfer, D. Wen, and M. J. Schlesinger.** 1984. Release of fatty acids from virus glycoproteins by hydroxylamine. Biochim. Biophys. Acta **798**:156–166.
19. **Magee, A. I., and M. J. Schlesinger.** 1982. Fatty acid acylation of eucakyotic cell membrane proteins. Biochim. Biophys. Acta **694**:279–289.
20. **Marchildon, G. A., J. E. Casnelli, K. A. Walsh, and E. G. Krebs.** 1984. Covalently bound myristate in a lymphoma tyrosine protein kinase. Proc. Natl. Acad. Sci. USA **81**:7679–7682.
21. **Olson, E. N., and G. Spizz.** 1986. Fatty acylation of cellular proteins. J. Biol. Chem. **261**:2458–2466.
22. **Olson, E. N., D. A. Towler, and L. Glaser.** 1985. Specificity of fatty acid acylation of cellular proteins. J. Biol. Chem. **260**:3784–3790.
23. **Ozols, J., S. A. Carr, and P. Strittmatter.** 1984. Identification of the NH_2-terminal blocking group of NADH cytochrome b_5 reductase as myristic acid and the complete amino acid sequence of the membrane-binding domain. J. Biol. Chem. **259**:13349–13354.
24. **Paul, A. V., A. Schultz, S. E. Pincus, S. Oroszlan, and E. Wimmer.** 1987. Capsid protein VP4 of poliovirus is *N*-myristylated. Proc. Natl. Acad. Sci. USA **84**:7827–7831.
25. **Payne, L. G., and E. Norrby.** 1978. Adsorption and penetration of enveloped and naked vaccinia virus particles. J. Virol. **27**:19–27.
26. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1987. The pre-S1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. J. Virol. **61**:1672–1677.
27. **Rhee, S. S., and E. Hunter.** 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. J. Virol. **61**:1045–1053.
28. **Schlesinger, M. J., A. I. Magee, and M. F. Schmidt.** 1980. Fatty acid acylation of proteins in cultured cells. J. Biol. Chem. **255**:10021–10024.
29. **Schmidt, M. F.** 1983. Fatty acid binding: a new kind of post-translational modification of membrane proteins. Curr. Top. Microbiol. Immunol. **102**:101–129.
30. **Schmidt, M. F., M. Bracha, and M. J. Schlesinger.** 1979. Evidence for covalent attachment of fatty acids to Sindbis virus glycoproteins. Proc. Natl. Acad. Sci. USA **76**:1687–1691.
31. **Schmidt, M. F. G.** 1984. The transfer of myristic and other fatty acids on lipid and viral protein acceptors in cultured cells infected with Semliki Forest and influenza virus. EMBO J. **3**:2295–2300.
32. **Schmidt, M. F. G., and M. J. Schlesinger.** 1979. Fatty acid binding to vesicular stomatitis virus glycoprotein: a new type of posttranslational modification of the viral glycoprotein. Cell **17**:813–819.
33. **Schultz, A. M., L. E. Henderson, and S. Oroszlan.** 1988. Fatty acylation of proteins. Annu. Rev. Cell Biol. **4**:611–647.
34. **Schultz, A. M., and S. Oroszlan.** 1983. In vivo modification of

retroviral *gag* gene-encoded polyproteins by myristic acids. J. Virol. **46**:355–361.
35. **Sefton, B. M., and J. E. Buss.** 1987. The covalent modification of eukaryotic proteins with lipids. J. Cell Biol. **104**:1449–1453.
36. **Shuman, S., and B. Moss.** 1988. Factor-dependent transcription termination by vaccinia RNA polymerase: evidence that the *cis*-acting termination signal is in nascent RNA. J. Biol. Chem. **263**:6220–6225.
37. **Smith, A., and C. B. Klee.** 1982. Identification of the NH_2-terminal blocking group of calcineurin B as myristic acid. FEBS Lett. **150**:314–318.
38. **Streuli, C. H., and B. E. Griffen.** 1987. Myristic acid is coupled to a structural protein of polyoma virus and SV40. Nature (London) **326**:619–622.
39. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237–248.
40. **Towler, D., and L. Glaser.** 1986. Acylation of cellular proteins with endogenously synthesized fatty acids. Biochemistry **25**: 878–884.
41. **Towler, D. A., and J. I. Gordon.** 1988. The biology and enzymology of eukaryotic protein acylation. Annu. Rev. Biochem. **57**:69–99.
42. **Weir, J. P., and B. Moss.** 1985. Use of a bacterial expression vector to identify the gene encoding a major core protein of vaccinia virus. J. Virol. **56**:534–540.
43. **Wen, D., and M. J. Schlesinger.** 1984. Fatty acid-acylated proteins in secretory mutants of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:688–694.