

## BIOLOGY OF VACCINIA VIRUS ACYLPROTEINS

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### 1. ABSTRACT

Posttranslational processing of vaccinia virus proteins has proven to be a common mechanism for exerting regulatory control of function or targeting to subcellular and/or subviral structures. Fatty acylation, most commonly observed as the addition of myristate or palmitate, occurs on numerous vaccinia proteins and affects each in a distinct manner. Labeling of vaccinia-infected cells with tritiated myristic or palmitic acids demonstrates that vaccinia encodes at least six myristylproteins and six palmitylproteins. Where investigated, each of these proteins have been demonstrated to play important roles in the virus life cycle. Likewise, in each case studied, the fatty acyl modification greatly influences the function and/or biological activity of the protein.

### 2. INTRODUCTION

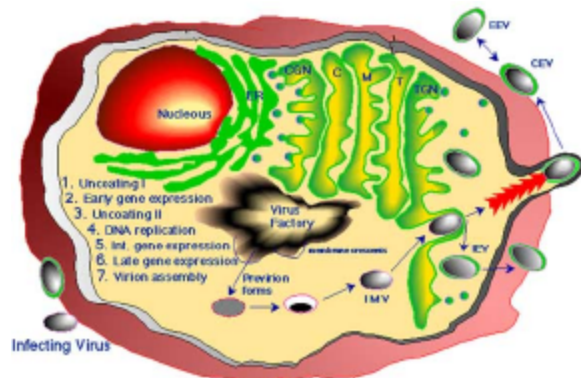
#### 2.1 Overview of the Vaccinia Life Cycle

Vaccinia virus (VV) is the most extensively characterized member of the Orthopoxvirus family of the *Poxviridae* (1). Its role as a vaccine in the eradication of smallpox represents one of the major achievements in medicine. Although smallpox vaccines are no longer

necessary, VV is widely used as a molecular and cellular biology tool as well as in the development of recombinant vaccines. In order to enhance or optimize VV for these purposes, basic research on the virus itself remains a major area of interest. Current research topics include structure/morphogenesis, replication /resolution of the genome, transcriptional regulation, enzymology, immune modulation, and protein processing.

VV is a large double-stranded DNA virus with a broad mammalian host range. Its entire life cycle occurs in the cytoplasm of the host with little or no requirement for the host cell nucleus (figure 1). Upon entry into the cell, the virion core is partially uncoated allowing transcription of the early class of genes. Their products are involved in genome replication and as transcription factors for the intermediate class of genes. The VV genome is composed of a nearly 200 kilobasepair linear double-stranded DNA molecule with potential to encode more than 200 polypeptides (2). Host protein synthesis is rapidly and efficiently inhibited upon infection so that nearly the entire translational capacity of a cell is harnessed by the virus. Following intermediate gene expression and genome

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**Figure 1.** The VV replication cycle. A diagram of the infected cell is shown with an exaggerated view of the endoplasmic reticulum (ER), *cis*, *medial*, *trans* Golgi and the *trans*-Golgi network (C, M, T, and TGN respectively). The major stages of the virus life cycle are listed. Following late gene expression, previrion forms assemble to form intracellular mature virus (IMV). The IMV is targeted to the TGN and following envelopment, intracellular enveloped virus (IEV) is formed. IEV are propelled to the cell surface by the polymerization of actin filaments. Once released the virus may remain attached to the membrane as cell-associated enveloped virus (CEV) or be released into the medium as extracellular enveloped virus (EEV).

replication the late class of genes are expressed. The majority of the late gene products are involved in the late stages of virion development serving as structural components or scaffolding for nascent virions. Virion morphogenesis occurs in perinuclear macromolecular clusters containing numerous copies of the viral genome and virus encoded proteins referred to as virus factories, viroplasm or virosomes. Immature virions form by packaging viroplasm within membrane crescents most likely derived from the membranes of the intermediate compartment (3). The genome is packaged within these membrane crescents in the proteinaceous core with the virus-encoded RNA polymerase and numerous cofactors involved in the early stages of the virus life cycle. Concomitant with the proteolytic processing of the three major core proteins (4), the core condenses, producing the first infectious form of the virus which is referred to as intracellular mature virus (IMV). At this stage the core is wrapped with two proteolipid envelopes.

The IMV particle is targeted to the *trans*-Golgi network (TGN) and by budding through the compartment acquires two additional membranes (5). After envelopment by TGN membranes, the quadruple membrane-bound particle referred to as intracellular enveloped virus (IEV), is propelled to the cell surface by the formation of thick actin filaments behind it (6). At the cell surface the outermost virion membrane may be lost by fusion with the cell membrane resulting in the release of a triple membrane-bound particle referred to as extracellular enveloped virus

(EEV). If the virion remains attached or reattaches to the cell surface it is referred to as cell-associated enveloped virus (CEV). Some poxviruses also package virions in cytoplasmic inclusion bodies referred to as A-type inclusions (ATI) which are primarily composed of a single protein, the ATI protein (7). The ATI protein is truncated and correspondingly nonfunctional in VV so that no inclusion bodies form although the truncated form of the ATI protein is still expressed at high levels (8).

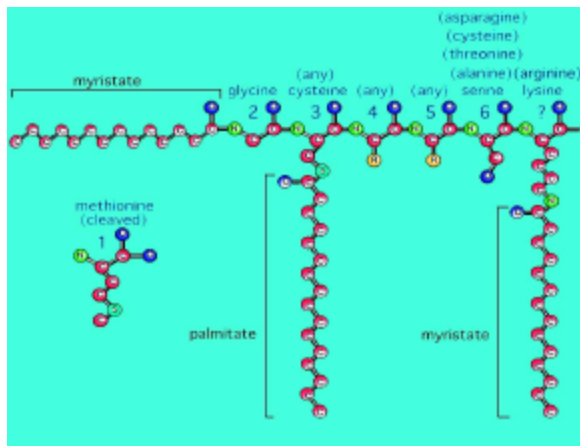
The enveloped forms of the virus (IEV, EEV, and CEV) are antigenically distinct from the IMV particle in that they contain at least six proteins in their outer envelope(s) that are not present on IMV. They are encoded by the A33R (9), A34R (10), A36R (11), A56R (12), B5R (13, 14) and F13L (15) open reading frames (ORFs) of VV. All of these proteins have been demonstrated to play important roles in the formation, release and/or infectivity of EEV with the exception of the A56R gene which encodes the viral hemagglutinin. None of these proteins affects the formation of IMV. Preliminary studies suggest that the IMV particle also contains proteins not found on the multiply enveloped forms of the virus. The ATI protein and the 4c protein are unique to IMV and although their biological relevance is not known they may represent an evolutionary relic. While VV does not occlude virions in ATIs, the closely related cowpox virus does (7). In that system, it has been demonstrated that the 4c protein is required for occlusion of virions in ATIs. It may be that the association of the ATI and 4c proteins with the VV IMV particle is an abortive attempt at ATI formation.

The virus and its life cycle are complex and not completely understood. Throughout its life cycle the virus uses numerous cellular protein processing pathways to include proteolytic processing, phosphorylation, sulfation, glycosylation, and ADP-ribosylation (16). Here we review the acylation of VV proteins and discuss the significance of these proteins and their respective acyl modifications in the biology of VV.

### 2.2 Overview of Protein Acylation

Two classes of fatty acylated proteins exist in eukaryotic cells (17) and by extension are present in VV-infected cells as well. Labeling of cultured cells with [<sup>3</sup>H]-myristic or [<sup>3</sup>H]-palmitic acid identifies distinct subsets of proteins. The distinction between the two classes may be determined in two ways. First, myristylation of proteins is a cotranslational event and is inhibited by the addition of reagents which block translation. Under these conditions, palmitylation of previously translated proteins occurs normally while myristylation does not. Second, the palmitate-protein bond is labile in the presence of mild alkali or neutral hydroxylamine due to the thioester linkage while the amide-linked myristate is stable under the same conditions. Many palmitylproteins are membrane associated either directly through the palmitate moiety or as transmembrane proteins anchored by the fatty acid although a few are actually secreted from cells. Cell-associated palmitylproteins are distributed throughout the

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**Figure 2.** Structure of a hypothetical acylated peptide. A peptide consisting of the canonical amino-terminal myristylation motif is shown. The amino-terminal methionine is cleaved and the penultimate glycine residue is modified by the amide-bound 14 carbon fatty acid myristate. Cysteine, in position 3 is modified by the thioester-linked 16 carbon fatty acid palmitate as in Type 4 palmitylproteins (see text). An internal lysine residue is modified by myristate as well. Alternate acceptable amino acids for each position are indicated in parentheses above the peptide backbone.

cell with the greatest concentration at the cell surface. Myristylproteins, on the other hand, may be cytoplasmic or membrane-associated. Some proteins are modified by both myristic and palmitic acid with both acyl moieties contributing to protein function and localization.

Myristylation of proteins involves the transfer of myristate from myristyl-Coenzyme A to the amino-terminal motif MGXXX(S/T/A/C/N) (using the single-letter amino acid code) of proteins by the enzyme N-myristoyl transferase (NMT) (18). This motif bestows substrate specificity for the *Saccharomyces cerevisiae*-encoded enzyme and although the human homologue has similar specificity, it may not be entirely the same. The initiating methionine is removed by methionine amino peptidase during translation and NMT recognizes the newly generated amino-terminal glycine of the nascent peptide after approximately twenty residues are free of the ribosome. NMT transfers myristate to the glycine residue after which the enzyme releases the peptide and translation proceeds normally. Mutations which change any of the conserved residues of the motif inhibit myristylation, with the greatest inhibition achieved by replacing the penultimate glycine with any residue (19). The residues at position six of the motif are less important with low levels of myristylation occurring even if they are changed. It should be noted though that not all proteins containing the amino-terminal motif are myristylated indicating that there are additional requirements. See figure 2 for the "ball and stick" model of a multiply fatty acylated peptide.

Recently, myristylation has been demonstrated to occur on internal residues of proteins (20). Although the

motif directing this modification has not been discovered, the myristate acceptor residues appear to be arginine or lysine (figure 2). This may be due to the fact that these residues have free amines in their side-chains and is supported by experiments demonstrating their insensitivity to neutral hydroxylamine suggesting amide linkage for this modification as well. The enzyme(s) responsible for internal myristylation is unknown.

Palmitylation of proteins remains an enigma for researchers. Palmitylproteins are more aptly described as ester-type fatty acylated (21) proteins or even S-acylated proteins (22), in reference to the sulfur atom in the side-chain of the acceptor cysteine. Some proteins are preferably modified by stearic or oleic acids over palmitic acid but for the most part palmitate is the predominant fatty acid on these proteins with small percentages of them being modified by stearate and oleate or even arachidonate (23). While cysteine is the most common acceptor residue, serine or threonine may also serve as palmitate acceptors (24), so even the description of these proteins as S-acylated is not totally accurate.

To date, a consensus motif directing palmitylation of proteins as well as the enzyme(s) responsible for the modification are unknown. Palmitylprotein acyltransferase has been described as a membrane-bound component of cells with *in vitro* activity detected in endoplasmic reticulum, Golgi and plasma membrane fractions of cells. The enzyme has a requirement for the activated form of palmitate, palmityl-CoA, but little else is known about it. An enzyme capable of removing palmitate from proteins, palmityl-protein thioesterase, has been discovered, although it is secreted from cells and most likely is specific for secreted palmityl-proteins.

Some general characteristics of palmitylproteins are known and have allowed us to classify them into four subclasses (25). Type I palmitylproteins are transmembrane proteins that are modified on cysteines at or near the cytoplasmic membrane face. This group is typified by the G-protein coupled receptors and includes the vesicular stomatitis virus G and the influenza virus hemagglutinin proteins. The palmitylation of Type II proteins occurs in the carboxy-terminal region and is dependent on prior prenylation of cysteine in the CAAX motif at the extreme carboxy-terminus. Members of this group include the ras proteins. Types III and IV are dually fatty acylated in the amino-terminal region. Both groups are myristylated on glycine of the motif MGXXX(S/T/A/C/N). Type III palmitylproteins are modified on one or more cysteines within the first 10 to 20 amino acids while Type IV palmitylproteins are modified on cysteine immediately following the myristylated glycine residue (figure 2). Efficient palmitylation of Types III and IV are dependent on prior myristylation. The alpha subunits of the heterotrimeric G-proteins are grouped as Type III or IV.

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**Table 1.** Vaccinia acylproteins

PROTEIN/GENE	MODIFICATION	SITE OF MODIFICATION	LOCALIZATION	REFERENCE
15K/A14L	Myristate	Unknown	ERGIC <sup>c</sup> , IMV <sup>d</sup> envelope	(31)
Unnamed <sup>a</sup> /A16L	Myristate	MG*XXX(S/T/A/C/N) <sup>b</sup>	Cytosol	(29)
ATI protein/A25L	Myristate	Unknown	Cytosol, IMV	(30)
	Palmitate	Unknown		
Unnamed/A33R	Palmitate	Unknown	TGN <sup>e</sup> , IEV <sup>f</sup> , CEV <sup>g</sup> , EEV <sup>h</sup> outer envelope	(9)
gp42/B5R	Palmitate	Unknown	TGN, IEV, CEV, EEV outer envelope, plasma membrane	(13, 14)
Unnamed/E7R	Myristate	MG*XXX(S/T/A/C/N)	cytosol	(29)
p37/F13L	Palmitate	Cysteine 185 cysteine 186	TGN, IEV, CEV, EEV outer envelope	(15)
Unnamed/G9R	Myristate	MG*XXX(S/T/A/C/N)	unknown	(29)
M25/L1R	Myristate	MG*XXX(S/T/A/C/N)	IMV envelope	(38)
p14/unknown	Palmitate	Unknown	Virion associated	(27)
p17/unknown	Palmitate	Unknown	Virion associated	(27)

<sup>a</sup> Unnamed VV proteins are referred to by open reading frame in the text., <sup>b</sup> amino-terminal myristylation motif. \* indicates the myristyl acceptor glycine residue after cleavage of the initiating methionine., <sup>c</sup> endoplasmic reticulum-Golgi intermediate compartment, <sup>d</sup> intracellular mature virus, <sup>e</sup> *trans*-Golgi network, <sup>f</sup> intracellular enveloped virus, <sup>g</sup> cell-associated enveloped virus, <sup>h</sup> extracellular enveloped virus

### 3. IDENTIFICATION OF VV ACYLPROTEINS

Since the discovery that VV encoded a palmitylated protein localized to the TGN in infected cells and present on the surface of virions (5), a great deal of work has been performed to discover other VV acylproteins, their role in the VV life cycle and the significance of the modification. Workers in our laboratory have confirmed the existence of at least 6 myristylproteins (26) and 6 palmitylproteins (27) encoded by VV. Their salient features are summarized in table 1.

#### 3.1. Identification of VV myristylproteins

The discovery of the myristylation motif allowed workers in our lab to undertake a systematic approach to identify VV-encoded myristylproteins. The entire sequence of the Copenhagen strain of VV is known (2), and by deduction, the amino acid sequences of the proteins it encodes. Four of the potential peptide sequences contain the amino-terminal motif MGXXX(S/T/A/C/N). They are encoded by the A16L, E7R, G9R and L1R ORFs producing proteins with predicted masses of 43.6-, 19.5-, 38.8- and 25-kDa respectively. Labeling VV-infected cells with [<sup>3</sup>H]-myristic acid and resolution by SDS-PAGE demonstrates that six polypeptides incorporate the label with electrophoretic mobilities indicating masses of 92-, 39-, 36-, 25-, 17-, and 14-kDa. By *in vitro* transcription and translation, the identities of the 39-, 36-, 25-, and 17-kDa proteins were demonstrated to be encoded by the A16L, G9R, L1R and E7R ORFs respectively as predicted (28, 29). In this system, the ORFs were cloned into plasmid vectors downstream of T7 promoters. By transcription using T7 polymerase and translation in the presence of [<sup>3</sup>H]-myristic acid using wheat germ extracts the proteins were demonstrated to incorporate the label. Mutants of these proteins in which the penultimate glycine was

changed to alanine did not incorporate label in this same system. Antibodies directed against these proteins immunoprecipitated [<sup>3</sup>H]-myristic acid labeled proteins from infected cells which co-migrated with the *in vitro* translated proteins as well as proteins from whole cell extracts confirming their modification by myristate *in vivo* as well. High-performance liquid chromatography (HPLC) of fatty acids extracted from these proteins demonstrated that myristate was the predominant modifying moiety but palmitate was also present. This is most likely the result of interconversion of the myristate post-modification as the addition of [<sup>3</sup>H]-palmitic acid to infected cells did not label these same proteins efficiently.

The identity of the 92-kDa myristylprotein was deduced by Martin *et al*(30) Although a number of VV-encoded proteins are known to migrate with similar electrophoretic mobility, only one matched the observed expression kinetics. Pulse-chase labeling of infected cells with [<sup>3</sup>H]-myristic acid demonstrated that the label was incorporated most efficiently at very late times post-infection. The only protein known to be expressed by VV in this manner was the ATI protein. To confirm that the ATI protein was indeed myristylated, cells were infected with the Western Reserve (VV-WR) or Copenhagen (VV-CH) strains of VV or the closely related cowpox virus and cultured in the presence of [<sup>3</sup>H]-myristic acid. Cowpox is known to encode the full-length (160-kDa) ATI protein while VV-WR encodes a truncated 92-kDa form. VV-CH does not encode the ATI protein due to a frameshift mutation which introduces a stop codon near the initiating methionine. Resolution of the labeled infected cell extracts by SDS-PAGE demonstrated that VV-CH did not encode a high molecular weight myristylprotein while cowpox and VV-WR encoded 160-kDa and 92-kDa myristylproteins respectively. Confirmation of these findings was obtained by the development of antiserum to the ATI protein which

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was used to immunoprecipitate the [<sup>3</sup>H]-myristic acid labeled protein from infected cell extracts.

The ATI protein does not contain the amino-terminal myristylation consensus motif suggesting that the modification occurred either at an internal site or that the label was incorporated as result of interconversion of myristate to palmitate. It turns out that both scenarios are probably correct. HPLC analysis of fatty acids extracted from the ATI protein confirmed that myristate and palmitate were both present on the protein, a finding that is not uncommon for myristylproteins. It was also found that translational inhibitors reduced the efficiency of label incorporation but did not block it entirely suggesting the protein was palmitylated while at the same time, treatment with neutral hydroxylamine did not entirely remove the label suggesting stable amide linkage for the fatty acid. Addition of [<sup>3</sup>H]-palmitic acid to infected cell cultures also resulted in label incorporation by the ATI protein. These findings taken together provide evidence for dual modification of the ATI protein by both amide-linked myristate at an internal site and by ester-linked palmitate. The sites of both modifications are presently unknown.

No VV-encoded protein is predicted to match the size of the 14-kDa myristylprotein and contain the amino-terminal myristylation motif, suggesting that this protein represented the second example of an internally myristylated VV protein. In a report by Rodriguez *et al* (31) this protein was identified as being encoded by the A14L ORF. In arriving at this conclusion, they labeled infected cells with [<sup>3</sup>H]-myristic acid and immunoprecipitated the A14L protein (which migrated as a 15-kDa protein in their system) demonstrating that it incorporated label. They did not, however, demonstrate that the label was incorporated as myristate and not some other long chain fatty acid arising from interconversion. In addition, they suggested a number of internal glycine residues as possible sites of modification. This is not likely to be the case as glycines do not have free amines to react with the carboxyl moiety of the fatty acid. It is more likely that the protein is modified by myristate on lysine(s) or arginine(s) (which have free amines) at internal sites or that the fatty acid was converted to palmitate or other long-chain fatty acid and added through an ester-type linkage to cysteine, serine or threonine. Further analysis of this protein needs to be performed to confirm the exact nature of the acyl modification.

### 3.2 Identification of VV palmitylproteins

The first report of a VV-encoded fatty acylated protein was by Hiller *et al* (5). In it they described a 37-kDa palmitylated protein (p37) present in Golgi membrane fractions of infected cells and in the outer envelope of EEV. Since this initial finding the protein has been confirmed to be the product of the F13L ORF in numerous reports (15, 32, 33). Additionally, Child and Hruby have demonstrated the existence of at least five more palmitylproteins induced

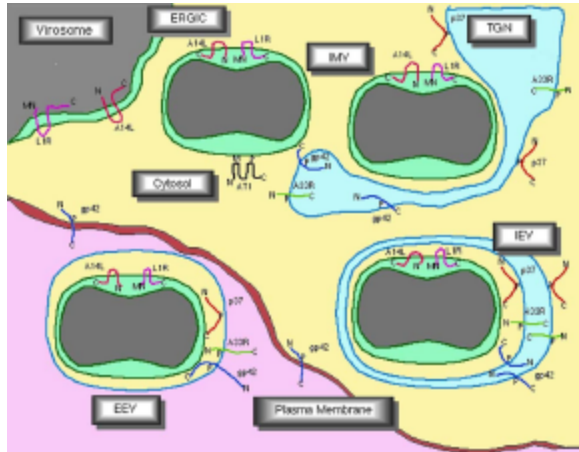
by VV (27). By addition of [<sup>3</sup>H]-palmitic acid to infected cells and SDS-PAGE resolution of the labeled proteins, they confirmed VV encodes proteins that incorporate the label with electrophoretic mobilities of 92-, 42-, 37-, 26-, 17-, and 14-kDa. To date, no laboratory has undertaken a directed effort to identify these proteins although the identities of some have been obtained as an aside in other efforts to characterize VV proteins.

Isaacs *et al* (14) confirmed that addition of [<sup>3</sup>H]-palmitic acid to VV-infected cells resulted in the specific labeling of a 42-kDa glycoprotein (gp42). Antibodies known to react with the product of the B5R ORF immunoprecipitated gp42 confirming its identity. It most likely is a member of the Type I palmitylprotein subclass. Following cleavage of the signal sequence the protein is oriented in the membrane as a type I transmembrane protein, spanning it only once and has a very short carboxy-terminal tail exposed to the cytoplasm. The protein transits the cell utilizing the normal protein export pathway, acquiring glycosyl moieties in the process, and eventually resides in the plasma membrane. The palmitate modification site is unknown although it is likely that it is in the carboxy-terminal cytoplasmic tail which contains a number of cysteine residues.

The 26-kDa palmitylprotein that we identified actually migrates as four distinct species in reducing SDS-PAGE gels; as a 55-kDa species and as 3 species of 23- to 28-kDa. Glycosylation inhibitors block the appearance of the three slower migrating species suggesting a complex glycosylation pattern. This protein was once thought to be the product of the A34R ORF (34) but has since been demonstrated to be encoded by the A33R ORF (9). The misidentification is an understandable one. At the time that the protein was first identified as the product of the A34R ORF, it was known that the A34R protein (gp22) was a glycoprotein migrating in SDS-PAGE gels with electrophoretic mobilities of 22- to 24-kDa and was unique to the outer envelope of EEV (10). Using a monoclonal antibody generated to EEV specific proteins, a palmitylprotein exhibiting similar characteristics was immunoprecipitated from [<sup>3</sup>H]-palmitic acid labeled infected cell extracts. It has since been determined that the monoclonal antibody used is specific for the product of the A33R ORF. The A33R protein is predicted to be a type II transmembrane protein with a short amino-terminal cytoplasmic tail. Based on this prediction, the A33R protein is most likely a member of the Type I palmitylprotein subclass.

Since the initial discovery that p37 was encoded by the F13L ORF, we have made a systematic effort to identify the site of modification and understand the significance of the acyl moiety in the biology of VV. Although the primary amino acid sequence necessary for recognition by palmitylprotein acyltransferase and subsequent transfer of palmitate to proteins is not known, Ponimaskin and Schmidt have identified sequence constraints for numerous viral glycosylated palmitylproteins (35). Their observations

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**Figure 3.** Cellular and viral distribution of VV acylproteins. A close-up view of an infected cell is shown, highlighting membranes and structures involved in virion morphogenesis. Membrane crescents derived from the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) form in the vicinity of the virosome leading to the production of intracellular mature virus (IMV). IMV is enveloped by membranes from the *trans*-Golgi network (TGN) forming intracellular enveloped virus (IEV). Following fusion of the outermost IEV envelope with the plasma membrane, extracellular enveloped virus (EEV) is released into the extracellular environment. Hypothetical protein:membrane interactions are shown. The amino- and carboxy-terminal ends of the proteins are indicated by "N" and "C" respectively. Myristyl and palmyl modifications are indicated by "M" and "P", respectively. Analogous membranes and luminal environments are indicated by color.

indicate that transmembrane proteins containing cysteine residues from within six amino acids into the transmembrane region (on the cytoplasmic side) to ten amino acids away from the membrane:cytoplasm boundary are efficient substrates for palmylation. Cysteines located in the middle of the membrane, on the extracellular face, or more than 10 amino acids away from the membrane:cytoplasm boundary are not efficiently palmylated. Cysteines alone in close proximity to the membrane are not sufficient to direct palmylation, for insertion of cysteine residues at the membrane boundary of nonpalmylated proteins did not convert them to substrates for palmylation.

It was once thought that p37 was a transmembrane protein as it is known to be membrane-associated within infected cells, fractionate with membrane-bound components of cells and is associated with the EEV envelope. In fact, the membrane association is peripheral in nature and interestingly is mediated by the palmyl moiety

alone (36). Treatment of p37-containing membrane fractions of cells with neutral hydroxylamine results in the release of p37 from the membrane leaving it free-floating in the aqueous fraction. Type III and IV palmylproteins are peripherally associated with membranes but are also substrates for myristylation. p37 is not myristylated so it can not be classified as Type III or IV. It may be appropriate to suggest that p37 is a member of a novel subclass of palmylproteins; one in which palmylation is necessary and sufficient for peripheral membrane association.

In our analysis of the primary amino acid sequence of p37, using the guidelines of Ponimaskin and Schmidt, we were unable to predict potential modification sites in the protein. Therefore, we analyzed by sequence alignment, numerous palmylproteins whose sites of modification were known and discovered a loosely conserved motif. We found that cysteine (or a cysteine doublet) within a transmembrane or hydrophobic stretch of amino acids that was preceded by two aliphatic residues and followed by another served as a substrate for palmylation. Comparison of this motif to the p37 sequence yielded one possible site for modification—a cysteine doublet in the middle of a hydrophobic domain in the central region of the protein. Mutation of one or the other of these cysteines to serine reduced the efficiency of palmylation while mutation of both to serine inhibited it completely (37).

The identification of the 92-kDa palmylprotein as the ATI protein was made by Martin and Hruby as discussed above, but the 14- and 17-kDa palmylproteins have not yet been identified. We are currently investigating this matter utilizing the information that we have obtained in our study of p37.

## 4. BIOLOGICAL SIGNIFICANCE OF VV ACYLPROTEINS

### 4.1 Myristylproteins

#### 4.1.1 L1R

The L1R protein (also referred to as M25) is the best characterized VV myristylprotein with respect to protein function and significance of the myristyl modification. The protein is localized to the envelope of the IMV particle and is predicted to span the membrane twice (38). There, it presumably acts as a necessary structural component of the developing virion (see figure 3 for hypothetical membrane topologies and virion association of all VV acylproteins). A mutant virus in which transcription of the L1R ORF was inhibited by the *lac* repressor unless isopropyl-thio-galactopyranoside (IPTG) was added to the medium, could not progress to the virion morphogenetic

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stage at which the core condenses to form IMV (39). By electron microscopy, numerous immature virions were observed as membrane enclosed viroplasm. Analysis of the virion core proteins confirmed that proteolytic processing to their mature forms did not occur either, demonstrating the co-dependency of both processes. This virus could be rescued by addition of IPTG or by transient expression of the wild-type L1R from a transfected plasmid carrying the gene. Transfection with a plasmid encoding a mutant L1R in which the myristyl acceptor glycine had been changed to alanine, could not rescue the virus. It is clear from these findings that L1R plays a significant role in the maturation of virions. Additionally, the myristylation of L1R appears to be essential for protein function. Although the myristylation of L1R may assist in the correct folding of the protein, it is also involved in targeting the protein to virion membranes. A fusion protein in which the first twelve amino acids from L1R were fused to the entire bacterial chloramphenicol acetyl transferase gene, was myristylated and present in virions (40). The molecular mechanisms by which L1R functions are unknown.

### 4.1.2 ATI protein

Although the ATI protein is known to function as a structural matrix for the formation of ATI bodies in other poxviruses (7), it has no demonstrable function in VV (41). As stated above, VV expresses a truncated form of the protein which is conserved across numerous strains with the exception of the CH strain which does not express the ATI protein. There has been some speculation that it may serve as an immune decoy or deterrent to phagocytosis but there is no supporting data for this hypothesis (8). Our analysis of the significance of the acyl modifications have been hindered by the lack of an observable phenotype attributable to this protein. Likewise, the large size of the protein has made mutational analysis of the protein a lengthy task.

### 4.1.3 A14L

The 15-kDa myristylprotein product of the A14L ORF has been demonstrated to be associated with membranes of the endoplasmic reticulum-Golgi intermediate compartment (31). In addition to acylation the protein is phosphorylated. The function of both modifications are unknown. The protein has two hydrophobic domains predicted to be alpha-helical in structure and is characteristic of transmembrane proteins. The protein exists as part of a complex with the A17L (21-kDa) and the A27L (14-kDa) proteins with unknown stoichiometry (31). These proteins collectively are involved in the formation of previrion membrane crescents at the site of virion development and are found associated with the outer envelope of immature virions and IMV. They are presumably a component of the inner membranes of IEV,

CEV and EEV. It has been recently reported that both the A14L and A17L gene products are necessary for the recruitment of intermediate compartment membranes to virus factories (42).

### 4.1.4 A16L, E7R, G9R

The functions of these three remaining myristylproteins have not yet been determined. Hydropathy and secondary structural predictions suggest that the A16L and G9R proteins might be targeted to membranes while the E7R protein is largely hydrophilic (29). Surprisingly, subcellular fractionation of infected cells into soluble and particulate fractions demonstrated that the A16L and E7R proteins were soluble. The G9R protein was not detected in this assay suggesting that it is made in low quantity, or that it is labile and was degraded in the purification process. Neither of these three proteins was detected in purified virions.

## 4.2 Palmitylproteins

The three VV palmitylproteins that have been identified all prove essential to the formation of IEV, virion egress and release of EEV/CEV from cells. This process has been the subject of intense scrutiny by numerous laboratories, all providing pieces to the puzzle. The IMV particle, while fully infectious and representing the majority of virus purified from tissue culture systems, most likely is an intermediate virion *in vivo*. IMV are targeted to the TGN and by budding through it acquire additional membranes, forming IEV (5). The formation of IEV is dependent on expression of the A27L gene encoding the 14-kDa protein found on the surface of IMV (43), as well as the products of the A33R (44), A36R (11), B5R (gp42) (14, 45) and F13L (p37) (32) genes, all specific for the multiply enveloped forms of virions. By a poorly understood mechanism, IEV are able to recruit actin, forming thick filaments which propel the virus unidirectionally to the cell surface, sometimes into neighboring uninfected cells (6). An additional requirement for this task is the A34R gene product, gp22 (46), which is also a component of IEV, CEV and EEV outer membranes. Deleting or inhibiting the expression of any of these genes results in the accumulation of IMV and/or inhibition of virus dissemination. The exact role each of these proteins plays is not clear.

### 4.2.1 p37

Recent data suggest that p37 is a member of the phospholipase D superfamily based on conserved sequences (47, 48). Although no phospholipase D activity could be detected in VV-infected cell extracts, the purified protein was demonstrated to possess phospholipase A and C activity (49). Mutation of the sequences in p37 that are conserved in the phospholipase family of proteins resulted in a protein that was no longer capable of functioning in

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regard to IMV envelopment, suggesting that phospholipase activity is a necessary protein function. Palmitoylation of p37 is also necessary for function (37). We have demonstrated that p37 is palmitoylated on both cysteines of a doublet in the central region of the protein. A mutant virus expressing a nonpalmitoylated form of p37 was defective for envelopment of IMV and subsequent release of virus from the cell. Wild-type p37 is normally a component of TGN membranes but the nonpalmitoylated p37 was soluble in the cytoplasm of infected cells. It would appear that VV requires the very specifically localized phospholipase activity of p37 in order to progress past the IMV morphogenetic stage. The substrates of this enzyme are unknown.

### 4.2.2 gp42

The 42-kDa glycosylated palmitoylprotein is a type I transmembrane protein present on the surface of infected cells and on the outer envelopes of IEV, CEV and EEV (33). The majority of the amino terminus of the protein is exposed to the extracellular environment and contains 4 short consensus repeats characteristic of complement control factors. VV encodes a secreted homologue of gp42 that has been demonstrated to bind complement (50), but whether gp42 functions in an analogous manner is not known. Deletion mutants of the B5R ORF demonstrate the significance of this protein in the VV life cycle. Viruses that do not make gp42 are attenuated *in vivo* and form small plaques in tissue culture (51). This is a direct result of the inability of these viruses to produce normal amounts of EEV and not related to the loss of the extracellular short consensus repeats. Restoration of the transmembrane domain and short carboxy-terminal cytoplasmic tail rescues B5R deletion viruses suggesting that the extracellular domain is dispensable for the formation of EEV (52). Not only is the carboxy-terminal region all that is necessary for protein function it is also sufficient to target heterologous fusion proteins to EEV particles (52). This does not preclude the possibility of a role for the extracellular domain though. Based on our predictions, the carboxy-terminus of gp42 is also the site of palmitate modification. The significance of the modification is unknown but considering the importance of this region it will be of interest to investigate this possibility.

### 4.2.3 A33R

The A33R protein is targeted to the membranes of the TGN and is predicted to be oriented as a type II transmembrane protein with the amino-terminal region exposed to the cytoplasm (9). After envelopment of IMV by TGN membranes, the protein is present in the outer envelopes of IEV, CEV and EEV and is required for their formation (44). Deletion of the A33R gene results in a small

plaque phenotype which is indicative of reduced efficiency of dissemination. The exact role this protein plays is unknown as is the site of palmitate modification.

## 5. PERSPECTIVE AND FUTURE DIRECTIONS

### 5.1 Perspective

VV is a large virus with a complex life cycle. This complexity is due to more than 200 gene products expressed in VV-infected cells. To maintain regulatory control and correct targeting, the virus has adopted many cellular protein processing pathways for its own use. This in itself highlights the advantages of using VV as a transient mammalian expression system. Proteins expressed in VV-infected cells are modified in their native manner and are targeted intracellularly or transit the cell normally. Protein modifications known to occur in VV-infected cells includes proteolytic processing, phosphorylation, sulfation, glycosylation, ADP-ribosylation and as reviewed here, acylation (16). The acylation of VV polypeptides in many ways models cellular systems, having similar motifs specifying the modifications, and playing analogous roles in protein function and in targeting. Although we have learned much from these proteins, there is still a lot of work to be performed in completing their characterization.

### 5.2 Future Directions

#### 5.2.1 Further analysis of VV myristylproteins

Through our efforts to characterize the L1R protein we have demonstrated its essentiality to the virus as well as the significance of the myristyl modification to protein function. Our focus has now shifted to gaining a better understanding of the protein:membrane interaction. With a knowledge of the membrane topology of L1R it may be possible to dissect functional domains of the protein and gain insight into the molecular mechanism of action. Additionally, it is not known that L1R has any protein partners and will be of interest to determine if there are any.

The role that E7R, G9R and A16L play in the VV life cycle is unknown. Current efforts involve the construction of recombinant viruses in which these genes are under inducible control. This should allow us to ascertain the necessity of these proteins to the virus. If significant, we would then further the research by an analysis of the biological role the myristyl moieties play.

#### 5.2.2 Further analysis of VV palmitylproteins

Much is already known of p37 and the significance of its palmitoylation but based on recent reports of phospholipase activity, it will be of interest to discover the *in vivo* substrates for this enzyme. Considering the numerous protein:membrane interactions necessary for envelopment of IMV to form EEV, one can only speculate



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on possible roles that a phospholipase would play in this process.

Both gp42 (B5R) and the A33R gene product are essential for dissemination of enveloped virions, but why? No enzymatic activity has been proposed for either of these proteins, and although gp42 has homology to complement control proteins, that entire domain is dispensable for the formation of enveloped virus. The A33R protein does not share homology with any protein other than its homologues in other poxviruses so no function can be hypothesized as yet. While many laboratories are working to determine the function of these proteins, we are investigating the importance of the palmitate modification. Unlike p37 which requires palmitoylation for membrane association, these proteins are inserted into the membrane as a function of their primary amino acid sequence. In other systems, the palmitoylation of transmembrane proteins has been demonstrated to be dispensable for membrane association and in some cases, function (53), while in others it is necessary for both (54).

In addition to the discovery of the biological significance of the palmitoylation of gp42 and the A33R protein, we aim to identify their sites of modification, as well as the 14- and 17-kDa palmitoylproteins and their modification sites. An analysis of the primary amino acid sequences and secondary structures of all the VV palmitoylproteins should allow us to refine the consensus motif directing acylation of VV proteins and perhaps extend that to include cellular proteins. We also hope to identify the internal myristylation site of the ATI protein. Few proteins have been discovered that are modified in this manner. Although the protein may not have a biological role in the virus life cycle, the determination of its biochemical properties directing internal myristylation should be of general interest.

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