AN ABSTRACT OF THE THESIS OF

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Dennis E. Hruby

Vaccinia virus (VV) encodes at least six proteins that are modified by the addition of a 14-carbon saturated fatty acid through an amide linkage and at least eight proteins that are modified post-translationally by the addition of a 16-carbon saturated fatty acid through linkage to cysteine residues. These post-translational modifications are referred to as myristylation and palmitylation, respectively. The purpose of this work was to further characterize the known myristylproteins and to define a consensus motif for the palmitylation of a protein so that we could identify and begin the characterization of new palmitylproteins.

Through this work we have identified a loosely conserved consensus motif that directs the palmitylation of a protein. Using the VV palmitylprotein p37, we characterized this motif and then used it in the identification three new VV palmitylproteins. We have also determined the membrane orientation of the VV myristylproteins, L1R, within the intracellular mature virus (IMV) particle. Hydrophobicity plot analysis identified two possible membrane orientations based on two putative transmembrane domains. Through transient expression data, L1R

was determined to span the IMV membrane twice, with both the amino and carboxy termini being on the lumen side.

Three *lac* recombinant viruses which are inducible for the A16L, E7R, and G9R open reading frames were created and analyzed using a newly developed vector system that fuses the green fluorescent protein to the neomycin resistance gene. Propagation of these viruses in the absence of the inducer IPTG determined that these genes are essential to VV replication.

We have identified and characterized the primary structural determinants specifying the modification of a protein by palmitate, and have identified three new VV palmitylproteins. In addition, the membrane orientation of the VV myristylprotein L1R was deduced, which can enable the construction of better recombinant vaccines through efficient antigen presentation. Lastly, we have developed a technique and vector system to easily create and isolate VV recombinants for characterization. This system enabled us to further characterize the A16L, E7R, and G9R myristylproteins.

Identification and Analysis of Vaccinia Virus Acylproteins

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CONTRIBUTION OF AUTHORS

Doug Grosenbach provided scientific assistance, intellectual input for all of the work on palmitylproteins. Torrey Cope was an undergraduate researcher who performed some of the experiments presented in chapter 4. All of the work described in this thesis was performed in the laboratory of Dr. Dennis Hruby. Dennis advised me on the experimental design, analysis, and the writing presented here.

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Chapter 1

Introduction: Biology of Vaccinia Virus Myristylproteins

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1.1 Introduction

Post-translation modification of vaccinia virus (VV) proteins has proven to be an important process in the control of protein function or protein targeting to subcellular and/or sub-viral structures. Acylation of a protein with a fatty acid such as myristate or palmitate is the most common of these modifications. Radio-labeling of VV infected cells with tritiated myristic acid or palmitic acids demonstrates that vaccinia encodes six myristylproteins and as many as eight palmitylproteins. The modification of these proteins affects the function and/or biological activity of each protein in a distinct manner, but always seems to be important in the virus life cycle.

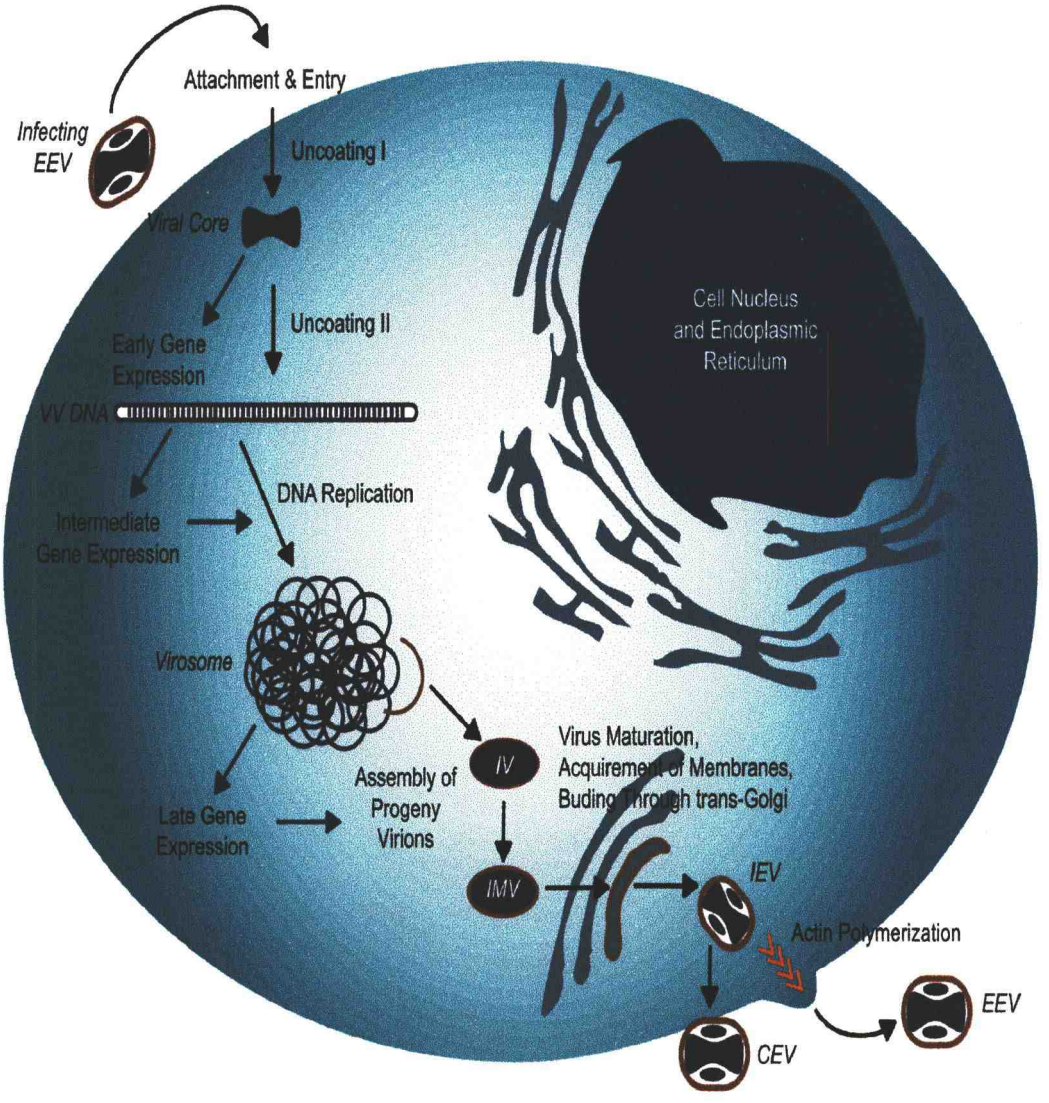
1.1.1 Overview of the VV Replication Cycle

VV is the prototypic member of the *Poxviridae*, a class of large, complex DNA viruses that replicate entirely within the cytoplasm of susceptible animal cells (Dales and Pogo, 1981). The VV genome is about 187 kilobase pairs (kbp) and encodes more than 200 viral proteins, enabling the virus to control its own replication and propagation (Moss, 1990). The nomenclature for VV open reading frames is alphanumerically. By digestion of the VV genomic DNA with the restriction enzyme *Hind III* and resolving this digest on an agarose gel one can visualize 16 fragments with the largest one being the A fragment and the smallest

fragment being the P fragment. Open reading frames within each fragment are number from left to right, and depending on which way the open reading is transcribed it is give an "R" or "L" designation. For example, the L1R open reading frame is the 12th fragment "L" and it is the 1 open reading frame from the left end of the fragment and is transcribed toward the left end of the fragment.

A typical VV virion consists of a biconcave core particle, containing the viral genomic double stranded DNA molecule, which is flanked by two-inverted terminal repeats. The viral particle is surrounded by a multi-layered lipid envelope. The VV life cycle is outlined in Figure 1.1. Entry into susceptible cells is not completely understood and is complicated due to the fact that intracellular mature virus and extra-cellular enveloped virus are both infectious. Antibody neutralization studies suggest that the process involves several unique viral proteins and/or cell surface receptors, including the products of the A27L open reading frame (ORF), the A17L ORF, and the 54 -kilodalton (kDa) tubule component (Oie and Ichihashi, 1987; Rodriguez and Smith, 1990; Stern and Dales, 1976; Wolffe *et al.*, 1995). Upon entry into the cell the viral envelope is degraded by host enzymes (un-coating I), which initiates the expression of viral immediate-early genes. The process of early transcription is carried out by several viral enzymes including VV DNA-dependent RNA polymerase, poly (A) polymerase, capping and methylating activities, and *trans*-acting transcription factors, all of which are packaged with the virus core particle (Moss, 1990). Approximately one hundred genes are transcribed

Figure 1.1. Summary of the VV Life Cycle. A summary of VV replication in eukaryotic cells highlights several important features of infection. Firstly, all transcription, translation, and DNA replication occurs in the cytoplasm of infected cells. Also depicted are the three distinct phases of protein expression, mediated by transcription factors and differential preferences of the VV RNA polymerase for specific promoter sequences over the course of infection. Finally, the four forms of infectious virus are shown: Intracellular Mature Virus (IMV), Intracellular Enveloped Virus (IEV), Cell-associated Enveloped Virus (CEV), and Extra-cellular Enveloped Virus (EEV).


Attachment & Entry
Infecting EEV
Uncoating I
Viral Core
Uncoating II
Early Gene Expression
VV DNA
DNA Replication
Intermediate Gene Expression
Virosome
Late Gene Expression
Assembly of Progeny Virions
IV
Virus Maturation, Acquirement of Membranes, Buding Through trans-Golgi
IMV
IEV
Actin Polymerization
EEV
CEV
Cell Nucleus and Endoplasmic Reticulum

during immediate-early gene expression, accounting for about half of the total genetic potential of VV. Immediate-early gene products are responsible for catalyzing the breakdown of the viral core particle (un-coating II), and also include enzymes required for viral DNA replication. Immediately following un-coating II, but before DNA replication, several VV genes are transcribed that are essential for DNA replication and are known as the delayed-early genes (Moss, 1990). Approximately three hours post infection, viral DNA replication begins and uses viral enzymes contained within the cytoplasm of infected cells. Once DNA replication begins viral transcription and translation of VV early genes are terminated and late transcription begins. Late gene products are primarily involved in the assembly and processing of new VV virions. Some genes are not classified as early or late, but are constitutively expressed (early/late proteins) throughout the VV life cycle. One such protein is the VV 7.5 - kDa gene product (Bernards *et al.*, 1987).

The replicating viral DNA is present in large viral factories that serve as a site for the assembly of progeny virion particles. This process begins about five hours post infection with the appearance of characteristic membrane crescents that envelopes a portion of the viroplasm, and results in the formation of an immature virion (IV). The IV particles will then migrate away from the virus factory and buds through the intermediate compartment, a structure between the endoplasmic reticulum and the Golgi stacks, and acquires a double-layered membrane (Dietze *et al.*, 1995). At this point the virion particle is termed intracellular mature virus

(IMV). The IMV particles are targeted to the *trans*-Golgi, by a process that is poorly understood. Once at the *trans*-Golgi the IMV particles will proceed to bud through the membrane, becoming enwrapped with yet another double-layered membrane. This multi-layered virus particle is now termed intracellular enveloped virus (IEV) (Hiller and Weber, 1985; Schmelz *et al.*, 1994). Actin nucleation from one side of the IEV particle will cause the particle to "rocket" from the cell in a unidirectional manner (a process that is also observed for *Shigella, Listeria, Salmonella,* and recently the *Baculoviridae*) and thus propelling it from the cell. The viral particles either remains fused to the outer surface of the cell, which is cell associated envelope virus (CEV), or are extruded into the media as extra-cellular enveloped virus (EEV). Numerous VV-encoded proteins mediate the process (Blasco and Moss, 1991; Engelstad and Smith, 1993; Isaacs *et al.*, 1992; Parkinson and Smith, 1994; Ravanello and Hruby, 1994b; Rodriguez and Smith, 1990; Rodriguez *et al.*, 1998; Roper *et al.*, 1996), including the proteins encoded by F13L, B5R, L1R, A33R, and A14L.

1.1.2 VV: The Model System

In many ways VV represents an ideal expression system in which to investigate protein function and modifications, and a variety of other phenomena pertaining to the field of molecular genetics. The above discussion on VV's life cycle provides many insights as to why VV is an excellent system for the study of

both foreign and wild type genes. First, VV's genome has been completely sequenced (Goebel *et al.*, 1990) allowing one to compare and identify regions of homology with other viral or eukaryotic proteins and to search for known motifs that direct a specific modification. Second, DNA replication and transcription take place within the cytoplasm of infected cells, and VV specific enzymes that recognize and act on specific sequences control these events. Cytoplasmic replication facilitates the introduction of foreign genes into the viral genome by marker transfer. Radio-labeling of DNA and proteins allows for convenient detection, and avoids potential complication of integration into host cell genome. Third, the large double-stranded genome can accept large and/or multiple foreign DNA inserts. The insertion of an exogenous expression cassette such as the neomycin gene allows for the selection of recombinant virus on the basis of resistance to the antibiotic G418. Using the preceding technique with the insertion of a gene that encodes a specific antigen enables the construction of a recombinant vaccine. Insertion of foreign genes is made relatively easy in that DNA can be directly abutted to a VV promoter, resulting in expression of that gene characteristic to that promoter. Fourth, transient expression vectors can be constructed that enable the expression of genes without the construction of recombinant virus. Transient expression vectors are plasmids that contain a specific gene (wild type, mutant, or foreign) downstream of a native VV promoter. Currently, the most common means of transient expression is by lipofection, protocols were first described by Rose *et al.* 1991 and later modified by Campbell

1995. Transient expression is relatively straightforward; it involves incubating plasmid DNA in the presence of liposomes in which a DNA/liposome complex will form. Adding both virus and the DNA/liposome complex to susceptible cells results in virus infecting the cell, and the DNA brought into the cytoplasm of the cells. The virus transcriptional apparatus will recognize the promoter and express the desired gene. Promoters are chosen based on the kind of expression one desires. Often a VV early/late promoter is used in expressing native genes or the T7 promoter, (from the T7 phage) transfected with a recombinant virus that expresses the T7 RNA polymerase, when one is looking to express a foreign protein. No VV gene contains splice junctions or requires special enhancers or promoters, unique polyadenylation sites, or RNA transport signals. Thus, any mutated or foreign gene can be expressed without the concern of providing the above consensus sequences.

1.1.3 Post-Translational Modifications of Proteins by Lipids

The covalent linkage of a variety of different prosthetic groups modifies numerous viral and eukaryotic proteins during or after their translation. The modification generally confers upon the protein new structural features and/or biochemical properties that are essential for maturation and function. The post-translational modification of proteins can serve various roles during a viral infection or in the life cycle of a cell. Protein modification enables proper targeting

of a protein to a specific intracellular location, can regulate a proteins enzymatic activity, or mediate folding and assembly of a protein into a three-dimensional structure. Protein modifications may be either reversible or irreversible, such as phosphorylation and proteolytic cleavage, respectively. Covalent modifications may be obvious, such as the attachment of multiple oligosaccharide side chains, or subtler with the addition of hydroxyl-groups to prolines.

The covalent addition of a lipid group to a protein causes a local alteration in its hydrophobic properties. The change in a proteins hydrophobic property may facilitate its interaction with a membrane or another protein, or may stabilize the structure of the protein by forming a hydrophobic pocket in the protein's core (Resh, 1996). Currently, there are four major types of lipid modifications described for eukaryotic proteins: glypiation, isoprenylation, palmitylation, and myristylation (Figure 1.2).

1.1.4 Glypiation

Perhaps the most unusual and structurally complex type of lipid modification is glypiation. The lipid-protein structure is a complex composed of the lipid, glycosylphosphatidylinositol (GPI), and a sugar moiety attached via ethanolamine to the carboxyl-terminus of selected membrane associated proteins. To date there has been no consensus sequence described for glypiation, but it has been recognized that there is a stretch of hydrophobic amino acids at the carboxyl-

terminus of the protein that display a recognizable pattern. However, this hydrophobic sequence is not precise enough to accurately predict that a protein will be glypiated. Glypiation appears to occur shortly after translation of the polypeptide chain and is preceded by a carboxy-terminal proteolytic processing

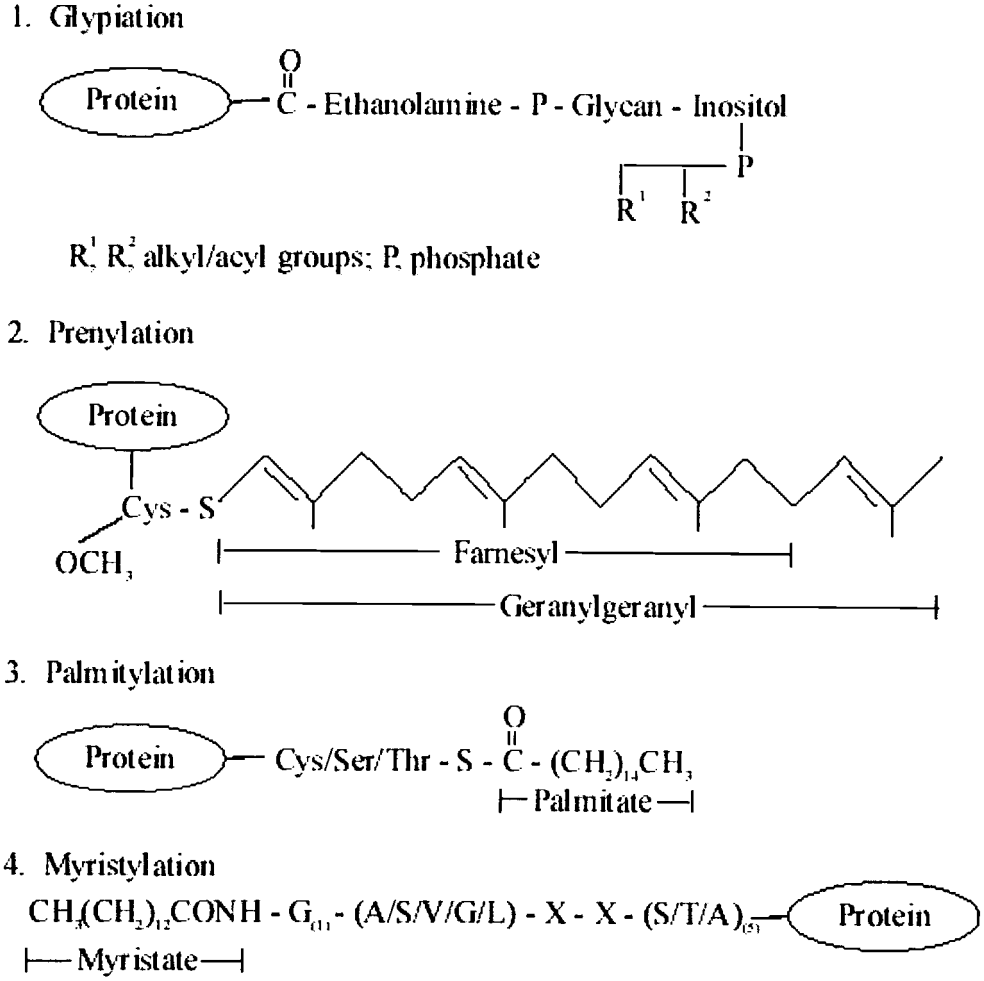


Figure 1.2. Structure of lipid modification of eukaryotic and viral proteins.

event, both mediated by the stretch of hydrophobic amino acids (Resh, 1996). The addition of GPI occurs very rapidly, suggesting that the GPI group is pre-assembled and then added to the protein (Caras *et al.*, 1987).

The primary function of glypiation is to attach a protein to a membrane. The hydrophobic GPI chains can be inserted into membranes, serving as "anchors", with the glycan-linked polypeptide extending outward from the cell membrane (Ferguson and Williams, 1988). This type of orientation allows for the glypiated protein to be anchored to, but not in, the membrane. It is strongly believed that this orientation enables the protein to interact with other molecules outside the cell and may play a role in cell activation (Stahl *et al.*, 1990).

1.1.5 Prenylation

Prenylation appears to occur immediately after translation, in which a particular fatty acid moiety is added to a cysteine at or near the carboxy-terminus via a thioester bond. The fatty acid moiety can be either a 15-carbon farnesyl or a 20-carbon geranylgeranyl group. A "C-*A*-*A*-*X* box" motif has been identified as the consensus sequence directing the prenylation of a protein (where C = is the modified cysteine, *A* = any aliphatic amino acid, and *X* = any amino acid) (Moores *et al.*, 1991). The fourth position of this box motif determines whether a farnesyl or geranylgeranyl group is added to the protein. If the *X* in the fourth position is a methionine or serine then the cysteine will serve as the substrate for farnesyl

transferase (FTase) (Cox and Der, 1992), whereas a leucine in this position enables geranylgeranyl transferase (GGTase I) to interact (Brown and Goldstein, 1993). Following prenylation, the C-*A*-*A*-*X* sequence is proteolytically cleaved, leaving only the prenylated C that is subsequently methylated to generate a new C terminal (Clarke, 1992).

It is still unclear as to the precise function of a prenyl-group attached to a protein. The properties of a prenylated protein are dependent upon whether a farnesyl-group or a geranylgeranyl-group is attached. Farnesyl groups may assist in membrane interaction and association, but it is believed that additional factors are required for stable binding to occur. This conclusion arises from analysis using Gibbs free energy for binding, which predicts that the farnesyl group alone is insufficient to anchor a protein to a membrane (Silvius and l'Heureux, 1994). However, geranylgeranyl groups are more hydrophobic than farnesyl groups and are sufficient to elicit binding to a membrane. Gibbs free energy predictions indicate that there is a 10 to 40-fold increase in binding affinity when geranylgeranyl groups are compared with farnesyl groups (Shahinian and Silvius, 1995).

1.1.6 Palmitylation

A third type of lipid modification is the addition of 16-carbon saturated fatty acid to eukaryotic and viral membranes. Palmitylation is a post-translational

event that results in the covalent attachment of the fatty acid moiety to a cysteine, threonine or serine via a thioester, or ester linkage respectively (Hruby and Franke, 1993). Palmitylation is unlike other lipid modifications in that it is both a dynamic and reversible process. Palmitylation serves one of three functions, which include activating a protein, mediating protein/protein interactions, and enabling binding to a membrane. Recently, a new consensus sequence for the palmitylation of VV palmitylproteins has bee identified, but it remains to be seen if the motif can be applied to other viral or eukaryotic proteins. The motif is written as follows, $TMD_{X1\text{-}12}A\ A$ C (C) *A*, where TMD is a transmembrane domain, *X* is spacer of any 1-12 amino acids, *A* is an aliphatic amino acid, and C is a cysteine or a cysteine doublet (Grosenbach *et al.*, 1997). Little is known about the enzymology of palmitylation due to there being several different types of palmitylated proteins. Currently three acyl-transferases have been identified. All three of these transferases are membrane-bound and include enzymes that catalyze palmitylation of mucin glycoproteins (Kasinathan *et al.*, 1990), an acyl transferase that palmitylates viral acyl-glycoproteins and Ras proteins (Gutierrez and Magee, 1991; Schmidt and Burns, 1989), and a palmityl-acyl -transferase that palmitylates members of the Src family of proteins (Berthiaume and Resh, 1995) and Gα subunits (Dunphy *et al.*, 1996).

Several different acyl-transferases may exist due to there being different types of palmitylproteins. There are four distinct types of palmitylated proteins (Resh, 1996). Type 1 palmitylproteins are palmitylated on a cysteine residue at or

near the transmembrane domain (e.g. G protein-coupled receptors). Type 2 palmitylproteins are prenylated on the cysteine residue within the C-*A*-*A*-*X* box prior to palmitylation within the C-terminal region of the protein (e.g. the Ras family of proteins). Type 3 palmityl proteins are palmitylated at one or more cysteines within the first 10-20 amino acids at the amino terminus of the protein (e.g. GAP43). Type 4 palmitylproteins are both myristylated and palmitylated at the amino-terminus (e.g. the Src family of proteins).

1.1.7 Myristylation

The fourth major type of lipid modification is myristylation. Myristylation is a co-translational attachment of myristic acid, a 14-carbon saturated fatty acid, via amide linkage to the α-amino group of the penultimate N-terminal glycine residue (Deichaite *et al.*, 1988; Wilcox *et al.*, 1987). More recently a second type of myristylation event has been observed and involves myristic acid being bound to a protein via an amide linkage (not ester) with an internal lysine residue (Stevenson *et al.*, 1993; Stevenson *et al.*, 1992).

Most of the work done in the field of myristylation has centered on the addition of myristic acid to the amino-terminal glycine via the enzyme myristyl-CoA: protein N-myristyl-transferase (NMT). This modification has been termed N-myristylation, which is distinct from internal myristylation in which no enzyme has yet been identified to catalyze this event. It appears that both types of

myristylation events are irreversible (Dohlman *et al.*, 1993), but de-myristylation activity has been isolated from the cytoplasmic fraction of bovine brain synaptosomes (Manenti *et al.*, 1994). This de-myristylation event has been shown to cause dissociation of protein kinase C from the membrane.

The enzyme NMT that catalyzes myristylation of proteins has been purified and the gene cloned from the yeast *Saccharomyces cerevisiae* (Towler *et al.*, 1987a). The gene encodes a 455-residue monomeric protein, which localizes to the cytosol (Knoll *et al.*, 1992). NMT lacks methionylaminopeptidase activity, indicating that the initiating methionine is removed by a cellular methionylaminopeptidase prior to myristylation of the glycine in the adjacent position (Towler *et al.*, 1987b). NMT has been extensively studied and appears kinetically to be a bi-bi reaction mechanism (Rudnick *et al.*, 1991). The first part of this reaction involves myristyl-CoA binding to the apo-enzyme complex by associating with Cys-169 of NMT, thereby forming an intermediate. This intermediate complex then binds the growing peptide chain, forming a ternary complex. Next, myristate is transferred from CoA to the peptide and CoA is released, followed by release of the myristyl-peptide (Gordon *et al.*, 1991).

By using a dual plasmid system which encodes large synthetic peptides as model substrates and NMT, the sequence controlling the attachment of myristic acid at the amino-terminus has been determined and may be summarized as the following: (M-) G- (A/S/V/G/L)-*X*-*X*-(S/T/A)-. N-myristylation is absolutely dependent upon a glycine reside being present in the second position. The third

position is less stringent, but uncharged amino acids are preferred, and bulky hydrophobic or aromatic residues are excluded for they inhibit the cleavage of the initiator methionine by methionylaminopeptidase. The fourth and fifth positions are less constrained in that any amino acid can be put into this position, although, uncharged residues are preferred. A serine or threonine is the preferred residues in the sixth position. However, an alanine, glycine, cysteine or asparigine will function sub-optimally in this position. All NMTs appear to be highly specific for myristate, unlike the palmityl-transferases described previously which can substitute other fatty acids for palmitate (Towler *et al.*, 1987a).

Myristyl-proteins are diverse, both in function and in intracellular localization. Myristylation of proteins serve several functions including assisting the protein in membrane association, stabilizing protein conformation, and even aiding in protein-protein interactions. Numerous viral proteins are myristylated with the modification serving to assist in membrane association. Non-myristylating mutants of these proteins have shown to interfere with protein function and viral infectivity. Although, there are numerous membrane-bound myristylated proteins, myristic acid alone is insufficient for anchorage to a membrane. An additional co-factor is required for proper membrane association. Two such co-factors have been identified and include a stretch of basic residues within the protein (e.g. HIV-1 Gag protein) (Zhou *et al.*, 1994), and the addition of a second hydrophobic group, such as palmitic acid (Resh, 1996).

1.2 Identification of VV Acylproteins

Given the large number of proteins, multiple virion forms of VV, and the number of distinct intracellular sites used during viral maturation, it seemed likely that VV must employ several different targeting mechanisms, including protein acylation to direct viral proteins to their proper intracellular destination. Radiolabeling experiments validated this prediction that approximately 10% of VV late proteins are acylated. These experiments have revealed at least six palmitylproteins, four N-myristyl-proteins, and two internally myristylated proteins. Most of these proteins have been subjected to chemical and physical analyses by a number of labs.

1.2.1 Identification of VV Palmitylproteins

The first report of a VV-encoded acyl-protein was by Hiller *et al.* 1985. They described a 37 -kDa palmitylated protein (p37) encoded by the F13L ORF that was present in Golgi membrane fractions of infected cells and in the outer envelope of EEV. After this initial report Child and Hruby, 1992, demonstrated the existence of five more palmitylproteins encoded by VV. By addition of [^{3}H] palmitate to infected cells and SDS-PAGE resolution of the labeled proteins, they confirmed that VV encodes proteins that incorporate the label with electrophoretic motilities of 92, 42, 37, 26, 17, and 14 kDa.

Isaacs *et al* 1992 confirmed that addition of [^{3}H] palmitate to VV-infected cells resulted in the specific labeling of a 42-kDa glycoprotein (gp42). Antibodies that react with the gp42 were found to immunoprecipitate the protein, confirming the protein's identity. It is a member of the Type 1 palmityl-protein 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 having a very short carboxy-terminal tail exposed to the cytoplasm. The protein transits the cell using the normal protein export pathway, acquiring glycosyl-moieties in the process, and eventually resides in the TGN and plasma membranes. Due to the location of gp42 in the TGN and plasma membrane, it has been found on the outer envelopes of IEV, CEV and EEV. A large amino-terminal region of the protein is exposed to the extra-cellular environment and contains 4 short consensus repeats characteristic of complement control factors. Although gp42 shares this homology, it has not been demonstrated that the protein is capable of binding complement. Deletion mutants of the B5R ORF demonstrate the significance of this protein in the virus life cycle. Viruses lacking gp42 form small plaques in tissue culture resulting from the inability of the virus to produce normal amounts of EEV. Deletion of this ORF also has an attenuating affect on the virus *in vivo*. Transient expression of the transmembrane region of gp42 results in rescue of the virus, suggesting that the extra-cellular domain is dispensable for the formation of EEV in tissue culture.

The 26 - kDa palmityl-protein identified was present as four distinct species (55, 28, 23-kDa) in reducing SDS-PAGE gels. It has been demonstrated that

glycosylation inhibitors block the appearance of the slower migrating species, suggesting a complex glycosylation pattern. By using a monoclonal antibody generated to EEV-specific proteins, a palmityl-protein exhibiting similar characteristics to the 26-kDa palmityl-protein was immunoprecipitated from infected cell extracts. The monoclonal antibody was specific for the protein product of the A33R ORF. The A33R protein is predicted to be a type II transmembrane protein with a short amino-terminal cytoplasmic tail. Data has shown that the A33R protein is targeted to the membranes of the TGN. After envelopment of IMV by TGN membranes, the protein is present in the outer envelopes of IEV, CEV and EEV. An A33R deletion virus was created to study the function of the protein encoded by this ORF. In tissue culture it was found that the deletion virus was incapable of forming normal sized plaques similar to the B5R deletion virus. Also, it was demonstrated by immunoelectron microscopy that the deletion viruses were unable to form significant quantities of IEV, CEV, and EEV.

Since the initial discovery that the F13L ORF encoded p37 and that it was a palmityl-protein our lab has focused on characterizing the protein and ways to identify the palmitylation site. It was once thought that p37 was a transmembrane protein, as it is known to be membrane-associated within infected cells. However, it was discovered that the membrane association is peripheral and is mediated by the palmityl moiety alone. It is thought that p37 is a member of the phospholipase D super-family based on conserved sequences. 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. Mutation of the phospholipase domains resulted in the inability of VV to form IMV, a different phenotype from when the whole gene was deleted and the virus lacked the ability to form normal sized plaques due to the reduced amount of EEV.

1.2.2 Identification of VV Myristyl-proteins

The discovery of the myristylation motif allowed workers in our lab to undertake a systematic approach to identify VV encoded myristyl-proteins. Vaccinia virus-infected cells cultured in the presence of [^{3}H] myristic acid, resulted in the incorporation of radiolabel into six proteins with apparent molecular weights of 92, 41, 38, 25, 18, and 14 kDa. The entire sequence of the Copenhagen strain of VV is known, and by deduction, the amino acid sequences of the proteins it encodes. Four of the potential peptide sequences contain the amino-terminal motif MG*XXX*(S/T/A/C/N). They are encoded by the A16L, E7R, G9R, and L1R open reading frames, producing proteins with predicted masses of 43.6, 19.5, 38.8, 25 kDa, respectively. The initial labeling experiments indicated that two of these proteins predominated, the 25 and 38 kDa proteins. Solubilization of purified virions suggested that the 25 kDa protein was a constituent of the virion envelope, while the 38 kDa protein was associated with the virion core. It has been observed that myristate can be converted to palmitate, which is then utilized by palmityl-transferases to palmitate a protein. To test whether these proteins were in fact

myristyl-proteins, the cell lysates were treated with hydroxylamine, which cleaves thioester bonds. By these experiments it was shown that the fatty acid group was resistant to this treatment suggesting an amide linkage, and thus a myristyl-protein. However, to confirm this a chromatographic analysis was done. It was shown that greater than 75% of the protein-bound myristate was rapidly (<4 h) elongated to palmitate in these cells, and the fatty acid moieties analyzed from purified intracellular virions (prepared at >24 h) contained predominantly myristate (>75%). These results proved that these proteins were modified by myristate (Franke *et al.*, 1989).

To identify the genes that encoded the myristylated proteins, protein sequences derived from the known genomic sequence were scanned for the N-myristylation consensus motif, and their predicted molecular weight. Possible candidates were selected and then cloned, so that they could be expressed in a cell-free transcription/translation system. The myristylated late vaccinia virion protein (25 kDa protein) was identified as the gene product of the L1R ORF. To study this protein, it was attempted to insertionally-inactivate the gene using an exogenous expression cassette that mediates antibiotic resistance to G418, with no success. This result suggested that the gene product was essential for virus replication *in vivo*. These results were further verified *in vivo* by utilizing poly-clonal antiserum generated against a *trpE*:L1R fusion protein. Using site-directed mutagenesis it was possible to target the penultimate glycine codon of L1R. In these experiments the glycine residue was mutated to an alanine, which abolished myristylation,

providing evidence that the site of modification was the amino-terminal glycine residue of L1R (Franke *et al.*, 1990).

To test whether the modification of the penultimate glycine residue was myristylated in a typical fashion by cellular NMT, a family of chimeric reporter genes containing different parts of the first thirteen codons from the amino-terminus of the L1R open reading frame fused in-frame to the bacterial chloramphenicol acetyl-transferase gene was constructed. These fusion proteins would not only test how the proteins are myristylated, but also would enable a determination of whether there was a minimal signal directing myristylation of L1R, and if myristylation was necessary for proper intracellular targeting. Tests were conducted both *in vitro* in cell free systems, and *in vivo* with VV transfected cells. The results from these experiments suggested that although the first 5 amino acids from L1R protein were the minimum signal required to observe modification of the substrate by myristate, 12 amino acids were necessary to obtain wild-type levels of myristylation. Also, sub-cellular fractionation of infected cells expressing the fusion proteins indicated that a myristylated amino-terminus of the L1R protein was capable of targeting the fusion proteins to membrane-containing fractions, while non-myristylating mutants were found in the soluble fractions. Immunoblots of *in vivo* fractions containing intracellular vaccinia virus particles revealed that the first 12 amino acid residues from the L1R ORF abutted to the chloramphenicol acetyl-transferase protein were enough to target the fusion protein to the VV membrane. These results suggest a possibility that L1R:antigen gene fusions could

be used to direct chimeric proteins to the envelope of recombinant VV strains (Ravanello and Hruby, 1994b).

A large number of viral acyl-proteins have been found to localize to specific cellular and viral membrane and to be necessary for virus morphogenesis and/or egress from the infected cell. It was of interest to determine whether the VV L1R protein is specifically associated with one or more of the membranes enveloping various forms of VV virions. To this end two different forms of the VV virion were purified from VV-infected cell lysates, IMV, which contains a single-membrane, and EEV, which contains at least two distinct membranes. Immunoblot analyses of the IMV- and EEV- containing fractions using α-L1R antibodies revealed that both forms of VV contained L1R. It was later determined, by Proteinase K digestion of the different virion forms, that L1R is primarily associated with the membranes of the IMV; a result later confirmed by immunoelectron microscopy.

Previous attempts to isolate an insertionally inactivated L1R recombinant VV failed, suggesting that the L1R protein may play an essential role in virion morphogenesis. To test this hypothesis, a recombinant VV in which transcription of the L1R gene can be conditionally repressed was constructed. Phenotypic analysis of this recombinant under repressed conditions revealed that viral plaque formation was inhibited, viral DNA incorporation into virions was substantially reduced, and the proteolysis of the major virus core proteins was severely impaired. In the absence of L1R gene expression, only immature virions could be detected by

electron microscopy. Transient expression of a plasmid that contained L1R behind its native promoter was able to rescue the defective phenotype. When a non-myristylating mutant was transfected into infected cells, the defective phenotype was not rescued. Collectively, this data suggests that myristic acid modified L1R protein mediated essential interactions with viral, and/or cellular membranes and/or other virion components that lead to the assembly, maturation and release of virion particles (Ravanello and Hruby, 1994a).

Aside from the L1R protein, there still remained five other myristyl-proteins and/or open reading frames to identify. SDS polyacrylamide gel electrophoresis of [^{3}H] myristic acid labeled proteins demonstrated that each of the six proteins ran as distinct bands with apparent molecular weights of 92, 41, 38, 25, 18, 14 kDa (suggesting they were not glycoproteins). Hydroxylamine treatment of these proteins revealed that they were all resistant to cleavage suggesting that the fatty acid moiety was attached via an amide linkage, as observed with L1R, and are myristylated proteins. To identify the parental loci of the other proteins, the predicted protein sequence of the Copenhagen strain of VV was examined for the presence of the canonical amino-terminus myristylation motif (G-X-X-X-S/T/A/G/N/C). In addition to L1R (G-T-A-A-T), three other potential myristyl-protein genes were noted, A16L (G-A-A-V-T), E7R (G-T-A-A-T) and G9R (G-G-G-V-S) which were predicted to encode 43.6, 19.5, and 38.8 kDa proteins, respectively. Mono-specific polyclonal antiserum was prepared to each of these proteins and used it in concert with an *in vitro* transcription/translation system and

in vitro transient expression to confirm that each of these ORF's did indeed encode the predicted myristyl-protein. Analysis of the promoter structure in addition to time course labeling experiments showed each of these proteins were expressed at late times.

Several VV acylated proteins (L1R, p37, gp42) have been demonstrated to play essential roles in viral morphogenesis and egress (12,54,55). One would expect that other late VV acyl-proteins, such as A16L, G9R, and E7R, are also involved in virion maturation. *In vitro* transcription/translation in a cell free system of the A16L, G9R, and E7R ORF's demonstrated that the penultimate glycine is the target site for addition of myristic acid by NMT. To characterize these proteins further, it was important to determine whether they associate with membranes or remain soluble in the cytoplasm. Association would suggest a role in morphogenesis or egress, while solubility might indicate an association with another protein, some enzymatic activity, or play a role in assembly of the viral core. Both A16L and E7R were found only in the soluble sub-cellular fractions, while the solubility of G9R remained unclear, due to being detected only in the total cell extract. A hydrophobicity plot was done for each protein, revealing that each protein had near identical profiles. These data, together with the sub-cellular fractionation data, provides strong evidence that these proteins are not membrane associated. Since almost all viral myristylated proteins are components of the capsid or viral envelope, it was of interest to determine if A16L, E7R, and G9R are part of the VV virion. Using the mono-specific antibodies previously generated,

purified virions were probed for the presence of the three myristyl-proteins. It was determined that E7R, and G9R reside within mature infectious virions, while A16L was not found to be associated with the virion and found only within the total cell extract (Martin *et al.*, 1997).

The identity of the viral genes encoding the 92 and 14 kDa myristyl-proteins remained a mystery, as no VV open reading frames were of the appropriate size and contained an amino-terminal myristylation motif. This suggested that the last two remaining myristyl-proteins were internally myristylated. When labeling VV-infected cells at late times (12-24h) post-infection, a 92 kDa protein species becomes heavily labeled, suggesting that it is a major late viral protein. Due to the large size of this protein two candidate ORF's were identified, the 92 kDa P4a core protein precursor (gene product of A10L) and the 92 kDa Western Reserve (WR) VV homologue of the 160kDa A-type inclusion (ATI) protein expressed by the cowpox virus. The VV WR ATI protein shows significant homology with approximately 60% of amino-terminus of the cowpox ATI protein, but is truncated at that point which makes protein unable to form inclusions. Using mono-specific antiserum directed against P4a to attempt to immunoprecipitate a 92-kDa protein from [^{3}H] myristic acid labeled VV-infected cell extracts, met with no success. The same experiments were done with anti-cowpox ATI serum, in which the serum specifically reacted with the radiolabeled a 92-kDa protein. To confirm this result a plasmid vector was made that contained the VV WR ATI protein behind its native promoter. This plasmid was transiently expressed in Copenhagen strain of VV.

The Copenhagen strain of VV contains a fragmented and non- detectable ATI protein. Using this system, a 92-kDa myristyl-protein was observed in transfected Copenhagen VV infected cell lysates. Together these results strongly indicate that the 92-kDa protein is the VV WR ATI protein.

Unlike the previously described myristyl-proteins, the ATI protein does not contain an amino-terminal myristylation motif. To positively identify the fatty acid moiety as myristate both the identity of the attached acyl-group and the nature of the protein-acyl group bond were examined. To identify the fatty acid moiety as myristate [^{3}H] myristic acid labeled VV ATI was subjected to acid methanolysis and the released label was analyzed by reverse-phase HPLC. It was found that the radioactive fatty acid co-eluted with the unlabeled myristic acid standard, confirming that the acyl group attached to the VV ATI is myristic acid. To test the nature of the bond the labeled ATI protein was subjected to hydroxylamine treatment. These results indicated that the fatty acid linkage was hydroxylamine resistant, suggesting that the fatty acid was attached via an amide bond characteristic of myristylated proteins (Martin, 1997).

1.3 Discussion

It is abundantly clear that VV is an ideal system for the study of both viral and foreign proteins. It is also clear that VV encodes numerous acylproteins, most of which are essential for the virus's life cycle. The focus of this thesis is to

examine VV acylproteins in more depth with a goal to discover and characterize a palmitylation motif that enables the prediction of VV and other palmitylproteins. Once a consensus motif has been defined we would like to characterize it so that we would apply it to the numerous proteins encoded by VV.

The second focus of this thesis will be on the further characterization of the VV protein L1R. As described above the L1R protein has been extensively characterized, but it is still not known how this protein is oriented in the membrane of the IMV particle. It is of interest to know the orientation of the protein for several reasons. One being that it is not know how the IMV particle travels from the intermediate compartment to the TNG. It is believed that L1R plays a major role in this trafficking and it is thought that it may have both cellular and viral protein partners that assists in this translocation. L1R has also been implemented in binding and entry in to susceptible cells, thus knowing how L1R is oriented in the viral envelope one can possibly determine which part of the protein place in the receptor /ligand interaction. Lastly, since L1R is a envelope protein it is of interest to determine if L1R fusion proteins can be constructed to specifically deliver a foreign epitope to the outside surface of the viral particle.

It was also the goal of this thesis to design and create recombinant pox viruses of the A16L, E7R, and G9R open reading frames that place the lac repressor upstream of the transcriptional start site of these genes. Previous work with the L1R protein and others have determined that this method of transcriptional regulation is an effective means for turning on and off the expression of the protein

for characterization during the VV life cycle. Once recombinant viruses have been constructed it will be possible to characterize these proteins.

Chapter 2

Analysis of the Site Occupancy Constraints of Primary Amino Acid Sequences in the Motif Directing Palmitylation of the Vaccinia Virus 37-kdA Envelope Protein

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2.1 Introduction

In the current study we used the VV protein encoded by the F13L ORF, p37. The 37-kDa protein is the major palmitylprotein produced by VV. The protein is 372 amino acids in length and has been shown to be palmitylated on both cysteine residues in positions 185 and 186 (Grosenbach *et al.*, 1997). p37 is expressed at late times ~ 4 h p.i. until cell lysis and has been found to interact with the membranes of the *trans*-Golgi network (Schmelz *et al.*, 1994). When virion associated, p37 is found exclusively on the outer envelope of EEV. It has been demonstrated by the use of a F13L deletion mutant virus, vRB10, that p37 is essential for proper virus envelopment and release (Blasco and Moss, 1991). Recently, our laboratory has expanded on these findings to show that palmitylation of p37 is necessary for appropriate targeting to the *trans*-Golgi network and protein function (Grosenbach *et al.*, 1997).

Several of the VV acylproteins have been shown to play important roles in the VV life cycle. Five VV myristylproteins have been identified as the gene products of the A25L, A16L, E7R, G9R, and L1R open reading frames (Martin *et al.*, 1997). Four of these proteins contain the canonical amino-terminal myristylation motif consisting of M-G-*X*-*X*-*X*-S/T/A/C/N) (Duronio *et al.*, 1991), where *X* is any amino acid and glycine in the second position serves as the modification site. A25L, which encodes the A-type inclusion (ATI) protein, is not myristylated at the N-terminus, but is internally modified possibly on an arginine or

lysine residue as described previously (Martin, 1997). There are at least six VV palmitylproteins. Three species are derived from unidentified ORFs and the remaining are encoded by the A33R (Roper *et al.*, 1996), B5R (Isaacs *et al.*, 1992), and F13L (Hirt *et al.*, 1986) open reading frames.

Palmitylation is a reversible process that involves the covalent attachment of a 16-carbon saturated fatty acid via a thioester or ester bond to a cysteine or serine/threonine residue respectively (Hruby and Franke, 1993). The palmitylation of proteins facilitates one of three known functions: increasing specific protein-protein interactions, targeting proteins to membranes, and structural activation of proteins (Shenoy-Scaria *et al.*, 1994; Wedegaertner *et al.*, 1995). Unlike myristylation, little is known about the enzymology of palmitylation. It is likely that several palmityltransferases exist due to the wide diversity of protein sequences that are palmitylated. Currently there are two palmityltransferases activities known: a membrane-associated acyltransferase, which is capable of palmitylating mucin glycoproteins (Kasinathan *et al.*, 1990), and another membrane bound palmitylprotein acyltransferase (PAT) that transfers palmitate to members of the *Src* protein family (Berthiaume and Resh, 1995), and Gα subunits (Dunphy *et al.*, 1996).

Many of the known viral membrane bound palmitylproteins are essential for viral replication or assembly (Schmidt and Burns, 1989; Hruby and Franke, 1993; Ponimaskin and Schmidt, 1995; Resh, 1996, Veit *et al.*, 1996 Grosenbach *et al.*, 1997). In each case the palmityl moiety is necessary for some aspect of protein

function. With palmitylproteins playing such critical roles in many viral replication cycles, it has been of interest to predict potential palmitylproteins and their site of modification as a means to identify important viral gene products. This was first done by examining the contextual constraints for palmitylation, which includes two criteria: cysteine residues no more than 10 amino acids away from the transmembrane domain can serve as the acceptor site for palmitylation, and the palmitylation of cysteines within a transmembrane domain can only occur if located within six amino acids of the cytoplasmic borders (Ponimaskin and Schmidt, 1995). It was also realized that cysteines are not enough to direct the palmitylation of a protein, as most cysteine-containing proteins are not modified. Recently, palmitylproteins have been classified into four types (Resh, 1996). These classes are based on the site of modification, whether they are multiply modified by myristylation, palmitylation, and/or isoprenylation, and whether they interact with membranes. Type 1 palmitylproteins include transmembrane proteins that are palmitylated on cysteines close to a transmembrane spanning region. Type 2 palmitylproteins are palmitylated at the carboxy-terminus and requires prior prenylation of a cysteine within a C*AAX* box motif. Type 3 palmitylproteins are palmitylated multiple times within the first 10-20 amino acids. Last, Type 4 palmitylproteins are both myristylated and palmitylated within the amino-terminal motif: M-G-C, in which the glycine in position two serves as the myristylation site and the cysteine in position three serves as the palmitylation site. In this latter case, palmitylation is dependent on prior myristylation.

From these classifications, it would appear, that the loosely conserved motif defined by TMD$X_{1\text{-}12}$*AA*C(C)*A* would only predict Type 1 palmitylproteins (Grosenbach *et al.*, 1997). However, p37 does not fit any of the above classifications (p37 is not a transmembrane protein) but the motif was useful in identifying the correct site of palmitylation. This suggests that the TMD portion of the motif may only represent a region of hydrophobic sequence that may or may not be a transmembrane domain, or in the case of a dually modified protein, another hydrophobic molecule (myristate, palmitate, or prenyl group). Thus, a motif that can predict the palmitylation site of many palmitylproteins can be defined as Hydro**AA*C(C)*A* (where Hydro* represents a hydrophobic portion of a protein determined by any one of the following: a hydrophobic sequence, a transmembrane domain 1 to 12 amino acids away from the modification site, or the prior addition of a hydrophobic molecule). In order to determine the validity of the motif site, directed mutagenesis was performed on the F13L ORF at positions upstream and downstream of the palmitate acceptor sites. Amino acids located within the motif were mutated to different residues to determine the site occupancy constraints and the requirements for a hydrophobic region to direct palmitylation. The results presented here suggest that while any amino acid within the motif is capable of directing palmitylation, residues that have hydrophobic properties are most efficient. Also, the hydrophobic region upstream of the p37 palmitylation site is absolutely required for protein palmitylation. The motif Hydro**AA*C(C)*A,* may be useful in predicting the modification site of most palmitylproteins.

2.2 Materials and Methods

2.2.1 Cells and Virus

African green monkey kidney cells (BSC_{40}) were grown and maintained in modified Eagles minimal essential medium (MEM-E, Sigma) supplemented with 10% (vol/vol) heat inactivated fetal bovine serum (Summit Biotechnology), 2mM L-Glutamine (LG), 10 μg per ml gentamicin sulfate (GS), at 37°C, 95% humidity, and 5% CO_2. The VV IHD-J strain was grown and titered by plaque assay in BSC_{40} cells as described previously (Blasco and Moss, 1991). The vRB10 strain, also described previously, was grown using a low multiplicity infection in BSC_{40} cells in the presence of mycophenolic acid, xanthine, and hypoxanthine. Since vRB10 has 93% of the F13L ORF deleted it produces minute plaques, so titers were obtained by inoculating serial dilutions of virus onto a confluent monolayer of BSC_{40} cells followed by transfection of a plasmid born rescuing copy of the F13L gene. After 72 h p.i., the infected monolayer was stained with crystal violet to visualize plaques.

2.2.2 Plasmid Construction

The p37 transient expression vector pDG4.0, described previously (Grosenbach *et al.*, 1997), is derived from the pUC118 cloning vector in which the

F13L ORF is inserted downstream of a VV 7.5K promoter directing constitutive expression of p37. The Kunkel method of oligonucleotide (oligo)-directed mutagenesis (Kunkel *et al.*, 1991) was used to mutate the F13L gene within pDG4.0 to generate multiple amino acid mutants in positions 183, 184, and 187 of p37, immediately preceding and following the palmitate-acceptor cysteines. The following oligo was used to generate the degenerative mutants in position 183 while introducing an unique restriction site *Sph*I: 5' CTGGCAGACAGCATGCNNNAGAGCATAAATTCAACC 3', where NNN is any nucleotide. For position 184 the degenerate oligo 5'GTGCTAACCGGTA GACAACANNNCGCAG 3' was able to introduce the unique restriction site *Age*I. The oligo 5'GCTAACTGGNNNACAACAAGCCGCGGAGC 3' introduced a degeneracy in position 187 while introducing a *Sac*II site. Four transmembrane deletion mutants were constructed: L Δ10 using oligo 5'GGCAGACAACAA GCTTTTGCGCTGCTATTAAAGGC 3', deleted ten amino acids upstream of the modified cysteines and introduced an unique *Hind*III site. LΔ5 using oligo 5' GGCAGACAACAAGCATTCAACCATGAATTCTTTG CGC 3', deleted five amino acids upstream of the modified cysteines and introduced an unique *Eco*RI site. R Δ10 using oligo 5' CTCCACCAATTGGATTCTTCAGACAACAA GCCG 3', deleted ten amino acids downstream of the modified cysteines and introduced an unique *Mun*I site. R Δ5 using oligo 5'CTTAATATGATA

CGCCAGACAGCATGCCGC 3', deleted five amino acids downstream of the modified cysteines and introduced an unique *Sph*I site. All mutations were confirmed by DNA sequencing.

2.2.3 Computer-Assisted Analysis and Alignment of Palmitylproteins

The computer program Net Entrez, was used to search for and retrieve the amino acid sequences of numerous known palmitylproteins including the p37 sequence. Each sequence was then analyzed using the computer program TMPred (Hofmann and Stoffel, 1993) to identify potential transmembrane spanning domains and possible orientations in a membrane. Amino acid sequences of each protein were aligned using the palmitylated cysteines as a reference point. Amino acids were then analyzed upstream and downstream of the acceptor site for primary structural consistencies.

2.2.4 Transient Expression and Analysis of Palmitate Incorporation/Efficiency

IHD-J and vRB10 were used to infect monolayers of cells (2.5×10^6 cells) contained within a 35 x 10 mm dish at a multiplicity of infection (m.o.i.) of 10. vRB10 infected cells were transfected with 10μg of plasmid DNA using DMRIE-C reagent to enable the transient expression of both wild type (wt) and mutated forms of p37. The inoculum was prepared by adding 1ml of MEM-E containing LG,

and GS to a polystyrene tube. Ten micrograms of DNA was added to each tube along with 25µl of liposomes (DMRIE-C, supplied by Gibco). After 15 min at room temperature, 10^7 pfu of virus was added to each tube and then added to the monolayer of cells from which the culture media had been aspirated. The cells were then incubated at 37°C for 4 hours, after which the transfection inoculum was aspirated and replaced with 1ml of MEM-E containing 200 µCi of [^{3}H]-palmitic acid (^{3}HPA, supplied by DuPont NEN). At 24 h p.i., infected cells were harvested in the culture supernatant. Cells were pelleted by centrifugation at 15,000 x g for 30 minutes at 4°C and then resuspended in 100 µl of 1x phosphate buffered saline (PBS). The resuspended pellet was froze and then thawed three times after which 50 µl of 3x reducing sample buffer was added. The sample was then boiled for 3 minutes and then centrifuged at 15,000 x g for 1 minute to pellet the insoluble material. Each sample (13 µl) was loaded on two identical 12% polyacrylamide gels and resolved by discontinuous gel electrophoresis (SDS-PAGE) as described previously (Studier, 1973). After electrophoresis one gel from each sample was impregnated with 22.2% Diphenyloxazole (PPO) in Me_2SO (DMSO), dried and then exposed to Kodak BIOMAX MR film at -70°C for fluorography. The second gel was subjected to immunoblot analysis using anti-p37 antiserum (αp37). The antigen-antibody complex was then incubated with goat anti-rabbit antiserum conjugated to horseradish peroxidase (supplied by Pierce). The blot was developed by incubation with chemiluminescent substrate and exposed to Kodak BIOMAX MR film. The fluorograph of palmitate labeled proteins as well as the film of the

chemiluminescent blots were analyzed by densitometry, so that relative amounts of protein produced and label incorporated could be determined.

2.3 Results

2.3.1 Analysis and Alignment of Palmitylproteins

The amino acid sequence of p37 was deduced from the nucleotide sequence of the VV strain IHD-J F13L gene (Blasco and Moss, 1991). Grosenbach and Hruby (Grosenbach *et al.*, 1997) have previously demonstrated that the cysteines in positions 185 and 186 of p37 serve as the palmitate acceptor sites. Computer-assisted analysis of p37 was used to predict protein hydrophobicity and putative transmembrane spanning domains, and protein secondary structure. Hydrophobicity plots revealed that the central portion of p37 contains two hydrophobic regions (data not shown). The region consisting of the amino acids from positions 172 to 198 (encompassing the palmitylation sites) is predicted to be a transmembrane domain. Secondary structure predictions showed p37 to consist of 36% α–helix, 30% β–sheet, 21% turns, and the remaining 13% other structures.

Identification of the Palmitylation Consensus Motif

Palmitylated proteins		References
FLU-HA	MGLVF[C*]V KNGNMRC*TIC*	Veit *et al.* (1991); Veit *et al.* (1996)
VSV-G	HGLFLVL RVGIHLC*IK_KA*KKQ	Rose *et al.* (1984); Veit *et al.* (1996)
CD4	GIFFC*V RC*RHRRRQAERMSQIKR	Cruse and Rose (1992); Veit *et al.* (1996)
Rhodopsin	VIYIMMN KQFRNCMVTTLC*C*GKNPLGDDEA	Ovchinnikov Yu *et al.* (1988); Veit *et al.* (1996)
Trans.-rec	YC*ISGSC* RKPKTVNAKTNA DANEEEDVALKM	Omary and Trowbridge (1981); Veit *et al.* (1996)
Marbur virus GP	LSC*IC* RIFTKYIG	Volchkov *et al.* (1998); Veit *et al.* (1996)
F-MuLV env	LFGPC*ILN RLVQFVKDRISVVQAL	Hensel *et al.* (1995); Veit *et al.* (1996)
HEF/HA chim	GIAIC*V KNGNMRC*TIC*	Veit *et al.* (1990); Veit *et al.* (1996)
Sindbis E2	TVAVLC*AC* KARREC*LTPYALAPNAVIPTSLALLC*C*VRSA	Ivanova and Schlesinger (1993); Veit *et al.* (1996)
SFV E2	SC*YMLVAA RSKC*LTPYALTPGAAVPWTLGILC*C*APRAH	Ivanova and Schlesinger (1993); Veit *et al.* (1996)
AdenoDP	FVCLIIWL IC*C*LKRK	Hausmann *et al.* (1998)
Hum B2AR	TMD(−5) IAFQELLC*LRRSSLKAYG	O. Dowd *et al.* (1989)
Hum B1R	TMD(−6) KAFQGLLC*C*ARRAARRRH	Frielle *et al.* (1987)
Hum α1AR	TMD(−7) AFMRILGC*QCRSGRRRR	Schwinn *et al.* (1995)
Lyk	MGC*VCSSNPED	Sherroy-Scaria *et al.* (1993)
BovineNOS3	GPPC*GLGLGLGLGLC*GKQGPAS	Robinson and Michel (1995)
band 4.1	FTGIQIIC*LAVLWVVKST	Okubo *et al.* (1991)
RasK	KEEKTPGC*VKIKKCIIM	Barbacid (1987)
Mouse SSR2	KKSFQNVLC*LVKVSGT	Yamada *et al.* (1992)
p37 (IHD-I)	LNLCSAAC*C*LPVSTAYHIK	Grosenbach *et al.* (1997)
Consensus	$TMDX_{1-12}$*AAC(C)A* Hydro**AAC(C)A*	Grosenbach *et al.* (1997)

Table 2.1. The computer program Nentrez was used to compile the protein sequences of numerous known palmitylproteins. For each palmitylprotein analyzed, the site of modification and various protein properties were determined. This information allowed each protein to be aligned with the site of modification serving as the reference point. Similarities in sequence were determined by physical properties and mathematically by percent occupancy for each residue individually and then by amino acid class. From this analysis, the consensus motif, $TMDX_{1-12}$*AA*C(C)*A* was identified. Comparing the motif to p37's amino acid sequence allowed the determination of the site of palmitylation. The site of modification corresponds to the amino acids in positions 185 and 186, with aliphatic residues occupying positions 183, 184, and 187. C* represents putative or actual cysteine modification sites. Gapped sequences represent the putative boundary between a transmembrane domain and the cytoplasmic tail of the protein. The above motif has since been changed to Hydro**AA*C(C)*A*, so that it can be used to predict palmitylproteins of different types (types 1-4).

Ponamaskin and Schmidt (1995) have previously described criteria for palmitylation of viral glycoproteins. However, using this criteria we could not predict the modification site of p37. To facilitate the identification of the modified residues in p37 numerous palmitylproteins were examined of which the sequence and site(s) of modification had already been determined. Each known

palmitylprotein was subjected to the same type of analysis that p37 was subjected to, considering secondary structures, and membrane topologies (data not shown). An alignment was made in which the sequence of each palmitylprotein was aligned using the site(s) of modification as the reference point (Table 2.1). Primary structural properties were determined for each protein considering specific amino acid, amino acid properties, and amino acid classes. Based on this data, it appeared that palmitylation occurs most frequently 1-12 amino acids downstream (on the cytoplasmic side) of a transmembrane spanning region, downstream of a hydrophobic sequence, or immediately following the modification of the protein by another fatty acid. Also the modified cysteines are preceded by two aliphatic residues and followed by another aliphatic residue. This motif was thus defined as $TMDX_{1\text{-}12}AAC(C)A$, where TMD is a transmembrane domain, *X* is any amino acid, *A* is any aliphatic amino acid, and C is the palmitylated cysteine(s). This motif enabled the identification of a palmitylation site in p37, in which two alanines precede the cysteine doublet in positions 185 and 186, which is then followed by a leucine. However, the modification site of p37 is predicted to be within a transmembrane spanning domain, which does not agree structurally with the predicted motif. However, Ponamaskin and Schmidt have also reported that palmitylation of cysteines can occur within a transmembrane domain. In any case, the motif had enabled the prediction of palmitylation sites in p37. To this end, it was of interest to determine the requirements for a hydrophobic region, the amino

acid site occupancy constraints, and if the motif could be expanded to include palmitylproteins of different types.

2.3.2 Plasmid Construction and Oligonucleotide-directed Mutagenesis

A F13L deletion mutant virus, vRB10, has had 93% of the F13L open reading frame deleted by insertional-inactivation using an expression cassette mediating mycophenolic acid resistance. Even though vRB10 does not express p37, it is still viable in tissue culture, but the virus is unable to efficiently form plaques on susceptible monolayers of cells as a result of producing very little CEV or EEV. Reinsertion of the F13L gene into the vRB10 genome or transient expression of p37 in vRB10-infected cells restores the wild-type plaque-forming phenotype confirming the essentiality of p37 to this process (Grosenbach *et al.*, 1997).

Use of the transient expression vector pDG4.0, enabled the expression of both wild-type and mutant forms of p37. A nonpalmitylated form of p37 was also constructed in which the cysteine doublet had been mutated to a serine doublet, and two other mutants that are less efficient at incorporating palmitate due to a mutation of one of the cysteines to a serine (pCC, pSC, and pCS, respectively). To build on these earlier findings, oligo-directed mutagenesis was used to mutate amino acids at positions within the consensus motif and within the predicted hydrophobic region (Figure 2.1). By the use of three separate degenerate oligonucleotide

primers amino acids in positions 183, 184, and 187 of p37 were mutated to different amino acids. The goal for each position was to obtain mutants that represented the different classes of amino acids.

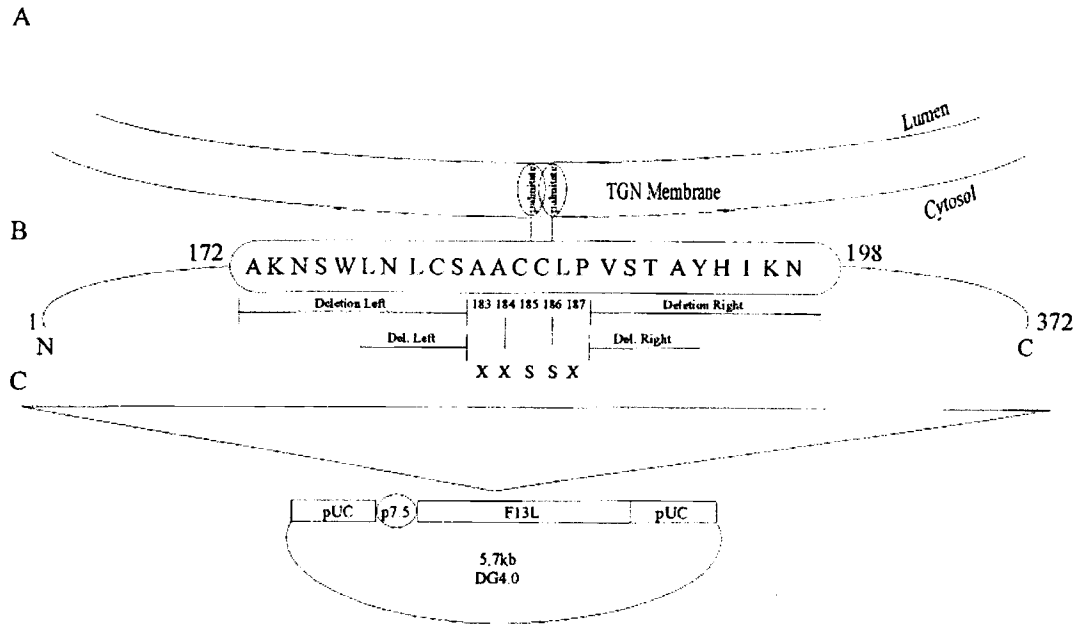


Figure 2.1. Design and construction of the transient expression vector DG4.0 and mutated derivatives. (A) Diagram of the hypothetical p37:membrane interaction mediated by its palmityl moieties. (B) The hydrophobic region of p37 from residues 172-198 are depicted using the single –letter amino acid code. The wild-type amino acids in position 183, 184, and 187 were targeted for mutagenesis. (C) An 1110-bp DNA fragment containing the wild-type VV F13L open reading frame was inserted downstream of the 7.5 early/late promoter to allow for constitutive expression throughout the viral infection.

Computer predictions indicate that there are 10 hydrophobic amino acids upstream and downstream of the palmitylation site (positions 185/186). To determine the requirement for the hydrophobic sequence surrounding the palmitate acceptor site of p37, four deletion mutants were constructed. Two deletion mutants

deleted all or half of the ten amino acids upstream of the palmitylation site (LΔ10 and LΔ5, respectively), while the other two deletion mutants eliminated all or half of the ten amino acids downstream of the palmitylation site (RΔ10 and RΔ5, respectively). In position 183 the wild-type alanine was changed to glycine (A183G), valine (A183V), serine (A183S), threonine (A183T), proline (A183P), tryptophan (A183W), lysine (A183K), and aspartic acid (A183D). The alanine is position 184 was changed to valine (A184V), serine (A184S), threonine (A184T), proline (A184P), phenylalanine (A184F), tyrosine (A184Y), tryptophan (A184W), lysine (A184K), and aspartic acid (A184D). In position 187 the wild-type leucine was mutated to glycine (L187G), valine (L187V), serine (L187S), cysteine (L187C), threonine (L187T), methionine (L187M), proline (L187P), phenylalanine (L187F), tryptophan (L187W), lysine (L187K), and glutamic acid (L187E). These mutants made used to determine the site occupancy constraints upstream and downstream of the palmitate acceptor site.

2.3.3 Analysis of Palmitate Incorporation/Efficiency

Confluent monolayers of cells were infected with the F13L deletion mutant, vRB10 , and then transfected with a wild type p37 transient expression vector (DG4.0), and the mutant p37 transient expression vectors for positions 183, 184, 187, and the deletion mutants. Transfected cell extracts were harvested and processed to first measure the expression of p37, and secondly for modification of p37. The samples

were treated with reducing sample buffer, which may cleave the thioester bond between p37 and palmitate, but all samples were treated identically and were analyzed immediately. To quantitate protein expression, gel electrophoresis together with immunoblotting were employed (Figures 2.2A, 2.3A, 2.4A, 2.5A). Protein production was detected for each transiently expressed mutant gene. An equivalent faction of the same total cell extracts were examined next for incorporation of [^{3}H]-palmitic acid into p37 using gel electrophoresis followed by fluorography (Figures 2.2B, 2.3B, 2.4B, 2.5B). Mutations in positions 183, 184, and 187 did not block palmitylation of p37, however, the majority of the mutated proteins were less efficient at incorporating label. For mutants of each position within the motif, palmitylation of p37 could be detected, while only three of the four deletion mutants retained activity. Film densitometry was used to quantify the amount of signal obtained on the blots. Densitometry has been used previously to quantify relative amounts of protein and signal (Hancock *et al.*, 1991). The amounts determined through film densitometry were used to obtain a ratio of protein production to [^{3}H]-palmitate incorporation (data not shown). The wild-type expression from plasmid DG4.0 was used as the standard and the percent for the mutants were determined based on this value (Figures 2.2C, 2.3C, 2.4C, 2.5C). Three other control plasmids were used in these experiments pCS, pSC, and pSS. Plasmids pCS, pSC, and pSS are DG4.0 derivatives that have had one or both cysteines at positions 185 and 186 (palmitate acceptor sites in p37) mutated to serine. Previous transfection experiments with these plasmids have demonstrated

that palmitylation is abolished with the double cysteine mutant (pSS) and less efficient with the single cysteine mutants (pCS and pSC). A minor palmitylprotein with an apparent molecular weight of 37-kDa is still observed in pSS lanes (Grosenbach, Ulaeto, and Hruby, 1997). The same palmitylprotein can be seen in vRB10 infected cells, which does not produce any p37. The protein observed in this position was considered background and subtracted away from the densitometry readings (value set to 0% incorporation of palmitate). Most of the mutants tested were less efficient at incorporating label, however, the results do indicate preferences for different types of amino acid. The palmitylation efficiency for the four deletion mutants was significantly less than DG4.0. Palmitylation was not observed when the entire 10 upstream amino acid sequence was deleted from p37 (LΔ10). Whereas, LΔ5 (42%), RΔ10 (26%), and RΔ5 (31%) still incorporated label but less efficiently (2.2C). In position 183 the results show a preference for the wild-type alanine. In general, hydrophobic residues or residues with a small side chain (glycine) in this position were more efficient at incorporating label as seen with A183G (47%), A183V (43%), A183P (43%), and A183W (39%) compared with A183S (33%), A183T (35%), A183K (38%), and A183D (19%) (Figure 2.3C). Results obtained for position 184 were similar to those observed in position 183 in that the wild-type alanine was the preferred residue. Although, the hydrophobic amino acids represented by A184P (32%), A184F (37%), A184Y (42%), and A184W (31%) were more efficient at directing palmitylation than A184S (28%), A184T (11%), A184K (22%), and A184D (5%), the mutant A184V

(48%) which has an aliphatic residue was the most efficient (Figure 2.4C). In position 187 the mutants L187G (120%), L187V (93%), L187S (69%), L187C (89%), L187T (112%), L187M (116%), L187F (72%), and L187E (67%) allowed for incorporation of label equal to, or in slightly greater amounts than the wild type leucine. In comparison the mutants L187P (11%), L187W (28%), and L187K (44%) were not preferred in position 187 (Figure 2.5C).

Figure 2.2. Analysis of palmitylation efficiency for transmembrane deletion mutants. BSC_{40} cells were infected with vRB10, and then transfected both with the wild type p37 transient expression vector (DG4.0), and mutated versions of this plasmid. Tritiated palmitic acid was added to the culture medium after 6 h post-infection. Total cell extracts were harvested at 24 h post-infection. (A) A fraction of the extract was subjected to SDS-PAGE and chemiluminescence using anti-p37 as the primary antibody. (B) An equivalent amount of total cell extract was subjected to SDS-PAGE and fluorography to detect incorporation of label, a measure of palmitylation. (C) Protein-antibody complexes were quantitated by application of chemiluminescent substrate and exposure to film followed by film densitometry. The fluorograph of palmitylation, was also quantified by film densitometry. The percent of incorporation of label vs. protein production is depicted by the bar graph. Results were determined from an average of three separate experiments.

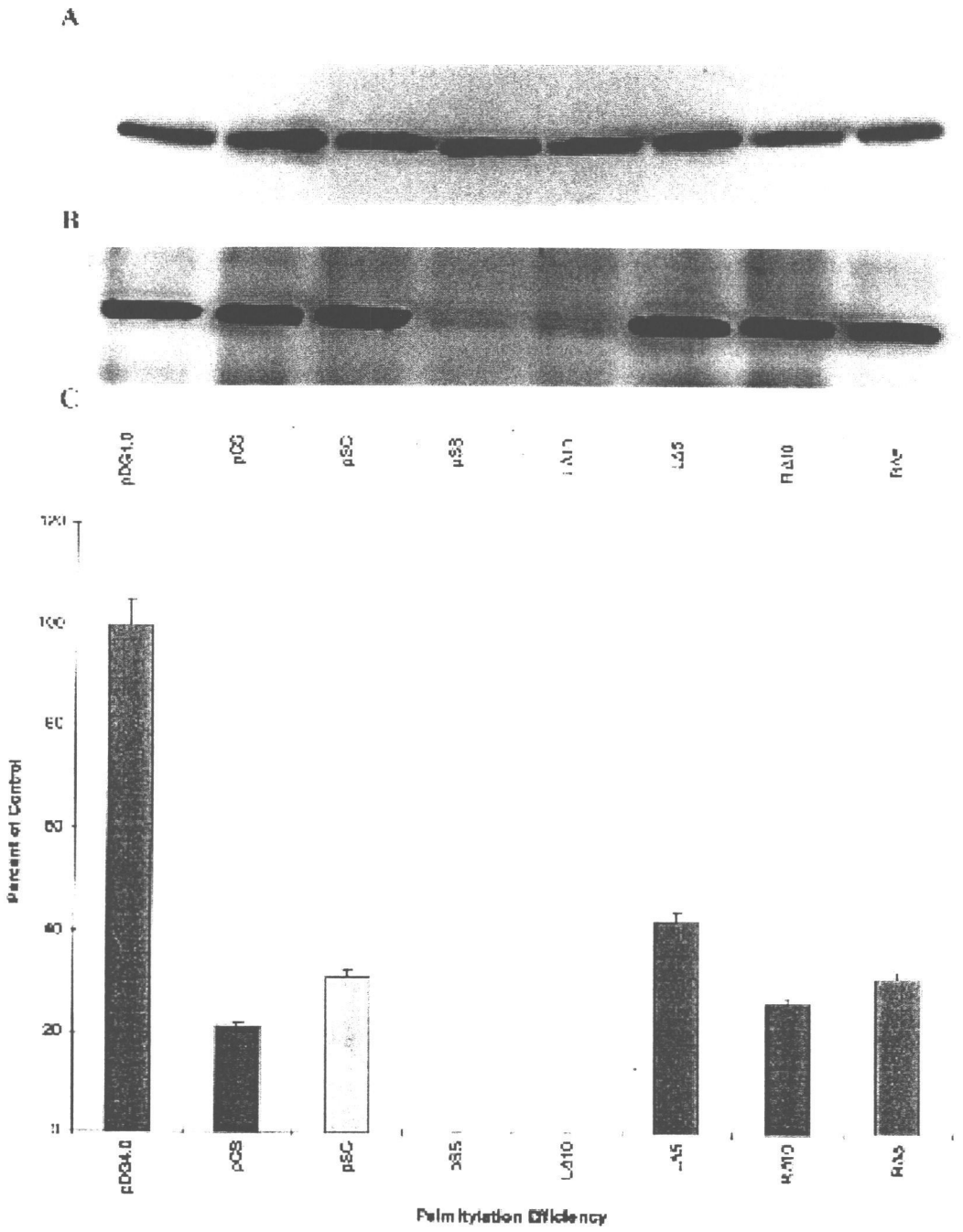

A
B
C
pDG4.0
pDS
pSC
Percent of Control
Palmitylation Efficiency

Figure 2.3. Analysis of palmitylation efficiency for position 183. BSC_{40} cells were infected with vRB10, and then transfected both with the wild type p37 transient expression vector (DG4.0), and mutated versions of this plasmid. Tritiated palmitic acid was added to the culture medium after 6 h post-infection. Total cell extracts were harvested at 24 h post-infection. (A) A fraction of the extract was subjected to SDS-PAGE and chemiluminescence using anti-p37 as the primary antibody. (B) An equivalent amount of total cell extract was subjected to SDS-PAGE and fluorography to detect incorporation of label, a measure of palmitylation. (C) Protein-antibody complexes were quantitated by application of chemiluminescent substrate and exposure to film followed by film densitometry. The fluorograph of palmitylation, was also quantified by film densitometry. The percent of incorporation of label vs. protein production is depicted by the bar graph. Results were determined from an average of three separate experiments.

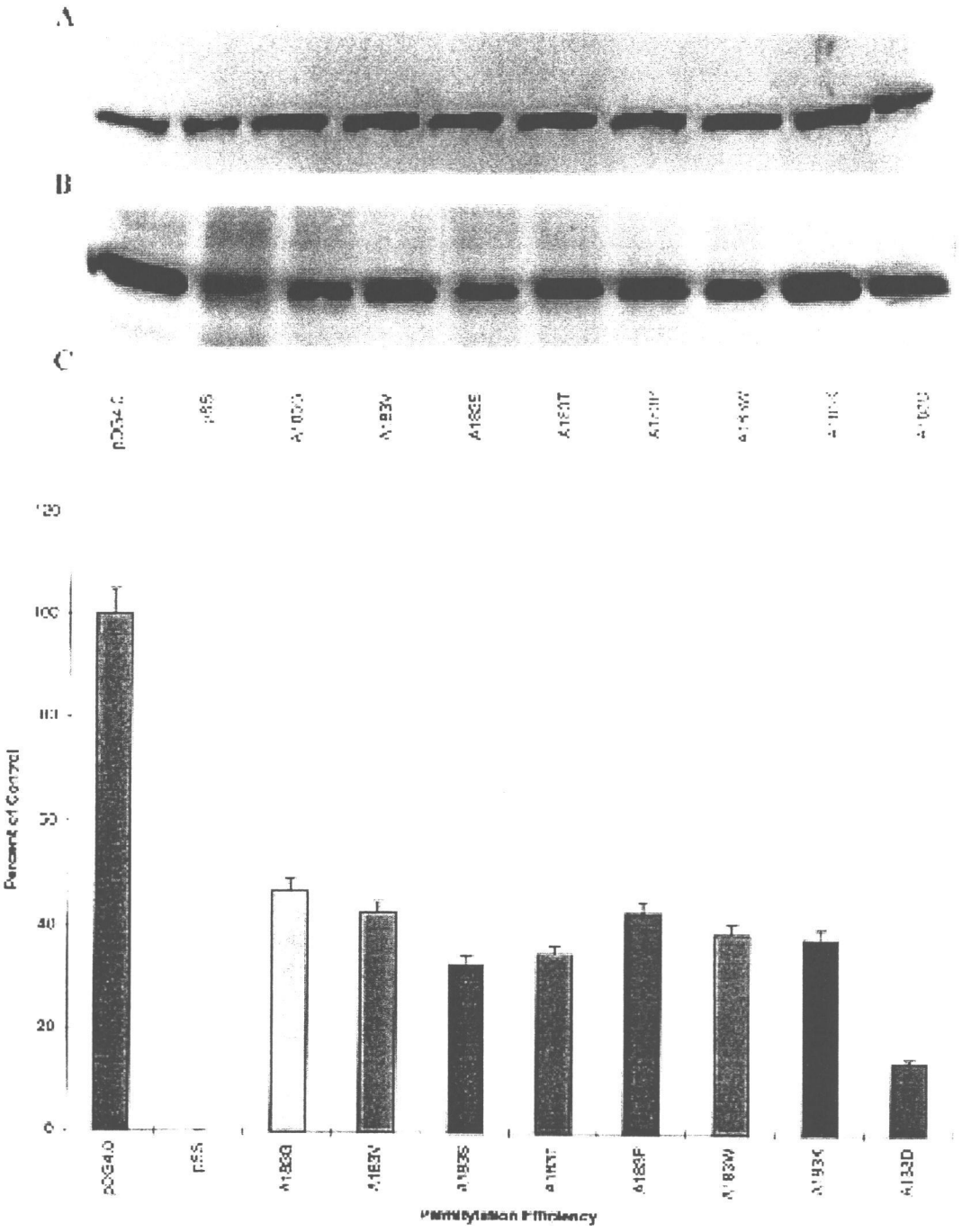

A
B
C
Percent of Control
Palmitoylation Efficiency

Figure 2.4. Analysis of palmitylation efficiency for position 184. BSC_{40} cells were infected with vRB10, and then transfected both with the wild type p37 transient expression vector (DG4.0), and mutated versions of this plasmid. Tritiated palmitic acid was added to the culture medium after 6 h post-infection. Total cell extracts were harvested at 24 h post-infection. (A) A fraction of the extract was subjected to SDS-PAGE and chemiluminescence using anti-p37 as the primary antibody. (B) An equivalent amount of total cell extract was subjected to SDS-PAGE and fluorography to detect incorporation of label, a measure of palmitylation. (C) Protein-antibody complexes were quantitated by application of chemiluminescent substrate and exposure to film followed by film densitometry. The fluorograph of palmitylation, was also quantified by film densitometry. The percent of incorporation of label vs. protein production is depicted by the bar graph. Results were determined from an average of three separate experiments.

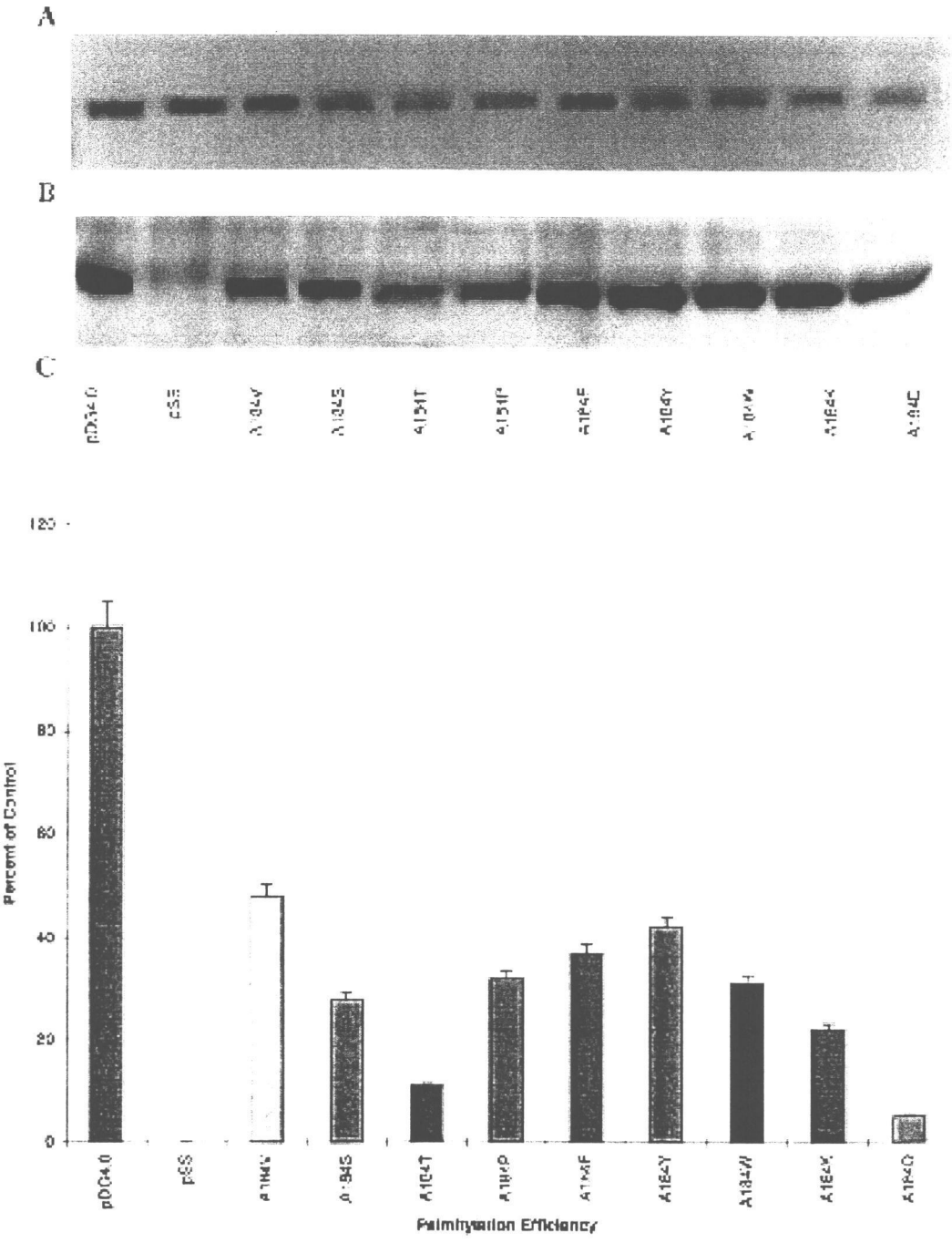
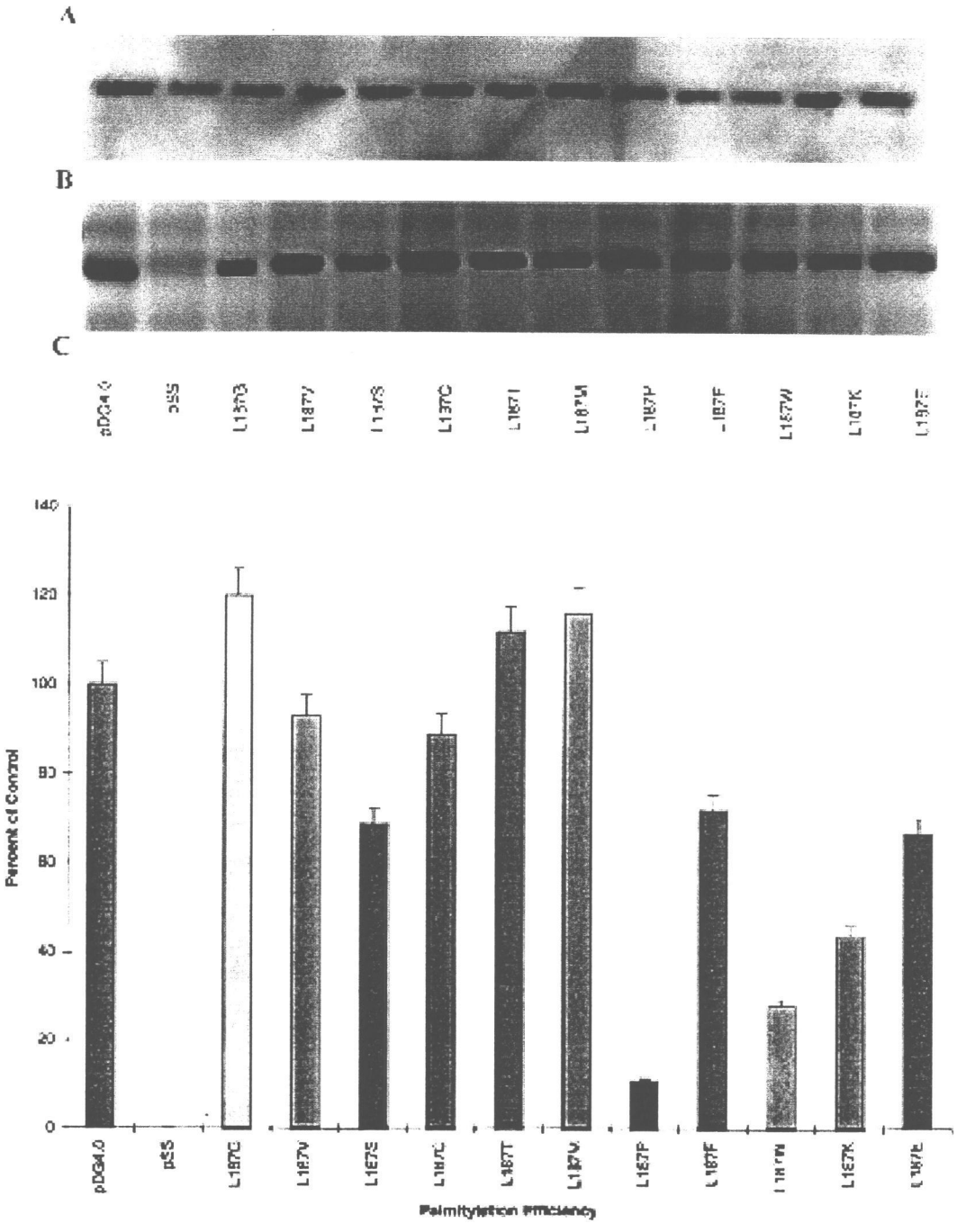

A
B
C
pDC410
pSS
A184V
A184S
A184T
A184P
A184F
A184Y
A184W
A184K
A184C
Percent of Control
Palmitoylation Efficiency

Figure 2.5. Analysis of palmitylation efficiency for position 187. BSC_{40} cells were infected with vRB10, and then transfected both with the wild type p37 transient expression vector (DG4.0), and mutated versions of this plasmid. Tritiated palmitic acid was added to the culture medium after 6 h post-infection. Total cell extracts were harvested at 24 h post-infection. (A) A fraction of the extract was subjected to SDS-PAGE and chemiluminescence using anti-p37 as the primary antibody. (B) An equivalent amount of total cell extract was subjected to SDS-PAGE and fluorography to detect incorporation of label, a measure of palmitylation. (C) Protein-antibody complexes were quantitated by application of chemiluminescent substrate and exposure to film followed by film densitometry. The fluorograph of palmitylation, was also quantified by film densitometry. The percent of incorporation of label vs. protein production is depicted by the bar graph. Results were determined from an average of three separate experiments.


A
B
C
pDG4.0
pSS
L187C
L187V
L187S
L187C
L187T
L187M
L187P
L187F
L187W
L187K
L187E
140
120
100
80
60
40
20
0
Percent of Control
Palmitylation Efficiency

2.4 Discussion

By analysis of numerous palmitylproteins a loosely conserved palmitylation motif, TMD$X_{1\text{-}12}AA$C(C)A, in which the palmitylated cysteines (C) are preceded by two aliphatic residues (A), and followed by another was identified (Grosenbach *et al.*, 1997). Several proteins studied were palmitylated within or proximal to a transmembrane domain (TMD). This motif is based on criteria that have been observed for palmitylated viral glycoproteins, and numerous cellular palmitylproteins (Schmidt, and Burns, 1989). This motif aided in the identification of the palmitylation site in the VV 37-kDa envelope protein, p37 (Grosenbach *et al.*, 1997). In this report, the validity of this motif was examined by testing the requirements for a transmembrane spanning domain, and determining what amino acid occupancy restrictions exist if any, in the residues that immediately preceding or following the palmitylated cysteines. To study the components of the motif a derivative of the transient expression vector pDG4.0, which has the intact F13L gene behind the VV 7.5k promoter was used. Use of a VV F13L deletion mutant, vRB10, which does not express p37, together with pDG4.0 derivatives, enabled the examination by transient expression of p37 and palmitylation efficiency.

2.4.1 Significance of the Hydrophobic Domain Surrounding the Palmitylation Site

Chou-Fasman and Hopp-Woods analyses predict that p37 contains two hydrophobic domains, one of which is predicted to be a transmembrane spanning domain. However, there is data to indicate that p37 does not span the membrane. Detergent partitioning studies were done using native fully palmitylated p37 and p37 treated with hydroxylamine which strips away the acyl-group by hydrolyzing the labile thioester bond between palmitate and p37. For the treated sample in which there was no palmitate attached to p37, it was found to partition to the aqueous phase (Schmutz *et al.*, 1995). These results were later confirmed *in vivo* by differential centrifugation subcellular fractionation and immunofluorescent microscopy on both wild type and nonpalmitylated mutants of p37 (Grosenbach *et al.*, 1997). This suggests that the palmitate moiety mediates membrane interaction, much as has been observed for the human immunodeficiency virus and simian immunodeficiency virus gp41 protein (Yang *et al.*, 1995). It is postulated that a palmitylated gp41 protein stabilizes the interaction of an amphipathic region with the membrane.

To test whether the hydrophobic sequence surrounding the palmitylation site affected palmitylation four deletion mutants were constructed, only one of which completely eliminated palmitylation. The data suggests that the amino acids preceding the palmitylation site were necessary for palmitylation. The three other

deletion mutants affected palmitylation by reducing the efficiency of label incorporation by more than half in some cases. The reason for this is unknown, but possibly the deletion interfered with the secondary structure or hydrophobic properties of p37 making it difficult or impossible for the protein to associate with the membrane, and thus preventing palmitylation. The results obtained in these experiments together with the alignments presented in Table 2.1 would suggest that the requirement for the upstream sequence is not sequence specific, but property (hydrophobic and secondary structure) specific. This specificity is seen with all types of palmitylproteins. Different hydrophobic/secondary structure elements are present depending on which type of palmitylprotein one is studying, but are generally one of the following: a hydrophobic sequence, a transmembrane domain 1-12 upstream of the palmitate acceptor site, or another hydrophobic moiety such as myristate. It can be concluded that the TMD portion of the motif is not required for palmitylation rather a hydrophobic element is needed or a specific secondary structure of the protein.

2.4.2 Significance of Residues Contained Within the Consensus Motif

From the above conclusion one would expect that hydrophobic amino acids would perform better within the consensus motif. To test this hypothesis, numerous mutants were constructed for each site within the motif using a

degenerative oligonucleotide, with intention of obtaining at least one residue from each amino acid class (Table 2.2).

Summary of the TMDX_{1-12}*AA*C(C)*A* Mutants

Mutant	Amino acid property	Side chain characteristics	Palmitylation efficiency (percentage of wild-type)
A183G	Aliphatic	Small side chain	[illegible]
A183V	Aliphatic	Hydrophobic, branched	[illegible]
A183P	Cyclic	Hydrophobic, branched	[illegible]
A183N	Amide	Hydrophobic, branched	[illegible]
A183S	Hydroxyl, sulfur	Polar	[illegible]
A183T	Hydroxyl, sulfur	Polar	[illegible]
A183K	Basic	Charged	[illegible]
A183D	Acidic	Charged	[illegible]
A184V	Aliphatic	Hydrophobic	[illegible]
A184P	Cyclic	Hydrophobic	[illegible]
A184F	Aromatic	Hydrophobic	[illegible]
A184Y	Aromatic	Hydrophobic	[illegible]
A184W	Aromatic	Hydrophobic	[illegible]
A184S	Hydroxyl, sulfur	Polar	[illegible]
A184T	Hydroxyl, sulfur	Polar	[illegible]
A184K	Basic	Charged	[illegible]
A184D	Acidic	Charged	[illegible]
I187G	Aliphatic	Small side chain	[illegible]
I187V	Aliphatic	Hydrophobic	[illegible]
I187S	Hydroxyl, sulfur	Polar	[illegible]
I187C	Hydroxyl, sulfur	Polar	[illegible]
I187T	Hydroxyl, sulfur	Polar	[illegible]
I187M	Hydroxyl, sulfur	Polar	[illegible]
I187F	Hydroxyl, sulfur	Hydrophobic	[illegible]
I187E	Acid	Charged	[illegible]

Table 2.2. Summary of the TMDX_{1-12}*AA*C(C)*A* mutants. The table represents the data obtained by mutating the amino acids in positions 183, 184, and 187 of p37. Mutants have been defined in the text and in Figures 2.2-2.5.

Position 183 was analyzed first by testing several amino acids in this position and comparing their palmitylation efficiency to that of the wild-type protein (Figure 2.3). For all amino acids analyzed, palmitylation efficiency was reduced by more than 50%, and dropped to as low as 20% when aspartic acid was in this position. As expected the hydrophobic amino acids were more efficient in directing palmitylation. The aliphatic residue glycine in position 183 gave the best results, although efficiency was significantly less than the wild-type alanine. These results

suggest a preference for amino acids with hydrophobic properties, or for amino acids with small side chains (glycine). Position 184, the second aliphatic amino acid in the motif, was analyzed next. Numerous amino acids were tested in this position and again the preferred residue was the wild type alanine (Figure 2.4). However, this time the graph revealed a different overall activity profile. Although, palmitylation was significantly reduced with every residue, the mutants with greater than 30% efficiency were amino acids with hydrophobic properties. Again these results suggest a preference for hydrophobic amino acids, with aliphatic residues giving the best results. The results obtained by mutating position 187, the third aliphatic residue within the motif, were quite different. The site occupancy for position 187 was considerably less stringent compared to the other positions analyzed (Figure 2.5). Several amino acids with varying properties (hydroxyl-sulfur containing, acidic, aromatic, and basic) allowed for palmitylation efficiency comparable to that of the wild-type leucine. Surprisingly, a glycine in position 187 seemed to drastically increase palmitylation efficiency, for reasons, which are unknown. The only residues that gave poor efficiency of palmitylation were proline, possibly as a result of an unfavorable change in secondary structure. The results obtained for 187 suggest that essentially any amino acid can be placed in this position except proline.

From these results, several conclusions can be reached: (i) The aliphatic amino acids preceding the palmitylation site are required for efficient palmitylation of p37. (ii) Alanine is the preferred residue in position 183 and 184. (iii) Site

occupancy in position 187 is permissive. (iv) Last the motif can be defined as Hydro**AA*C(C)*A* (where Hydro* represents a hydrophobic portion of a protein determined by any one of the following: a hydrophobic sequence, a transmembrane domain 1 to 12 amino acids away from the modification site, or the prior addition of a hydrophobic molecule; C, palmitate acceptor cysteines; *A*, aliphatic residue). Although, the aliphatic alanines were the preferred residues preceding the palmitate acceptor site, another aliphatic residue following the cysteines actually enhances modification.

These results suggest that the motif may require further refinement in order to predict the palmitylation sites in other proteins. However, the motif was sufficient in predicting the correct palmitylation site for p37. Other VV palmitylproteins will be analyzed using this motif to test its utility in predicting modification sites. VV has proved to be a useful tool in the study and analysis of eukaryotic protein modification and processing. Continued studies of VV will hopefully lead to a better understanding of the many processes that govern other viral systems, and more importantly give us new insights to protein palmitylation.

Chapter 3

Identification and Analysis of Vaccinia Virus Palmitylproteins

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3.1 Introduction

VV serves as an ideal model system for the study of protein processing for several reasons: VV proteins (or exogenous proteins expressed either transiently from transfected plasmids or from recombinant VV) are subject to modifications typical of mammalian systems, to include sulfation (Payne, 1992), phosphorylation, ADP-ribosylation, glycosylation and as highlighted here, acylation (VanSlyke and Hruby, 1990). Its genes are expressed in the cytoplasm without mRNA processing, allowing prediction of gene products directly from genomic sequence. We have previously observed that many VV proteins are acylated, providing numerous subjects for study (Child and Hruby, 1992; Franke *et al.*, 1989; Ravanello and Hruby, 1994a). Acylation of VV polypeptides have been demonstrated to play a major role in the assembly of virions.

At least four VV proteins are myristylated on glycine of the amino-terminal motif, composed of the initiator methionine, followed by a glycine residue, a three-amino acid spacer with no specific residue requirement, and then a serine, threonine, alanine, cysteine or asparagine in the last position (MGXXXS/T/A/C/N) (Martin *et al.*, 1997). The most studied of these is the 25 kDa protein product of the L1R open reading frame.

It has also been previously demonstrated that there are at least six VV-induced palmitylproteins present in infected cells (Child and Hruby, 1992). They are all localized to the membrane fraction of cells and when virion associated, are present in the outer membrane fraction of CEV and EEV. Three VV

palmitylproteins are encoded by open reading frames A33R (Roper *et al.*, 1996), B5R (Isaacs *et al.*, 1992), and F13L (Hirt *et al.*, 1986) while unknown open reading frames encode the remaining VV palmitylproteins.

Although many cellular and viral palmitylproteins have been characterized with regard to site(s) of modification, a definitive structural motif specifying palmitylation of proteins has not been identified. Palmitylproteins have been separated into four separate classes based on membrane topology, prior modification by myristate or isoprenyl moieties, and relative to the termini, which region of the protein is modified (Resh, 1996). We have previously reported that the palmitate acceptor residue(s) of proteins may predicted by the consensus motif, Hydro*$_{1\text{-}12}$*AACA* (Grosenbach *et al.*, 1997; Hansen *et al.*, 1999). The hydrophobic element (Hydro*) may be a transmembrane domain, a hydrophobic sequence of amino acids or a glycine residue that has been co-translationally myristylated. It is followed by a 1-12 amino acid spacer that precedes aliphatic residues (*A*) surrounding the palmitylated cysteine (C). This motif is based on work in our laboratory as well as the work of others (Bizzozero *et al.*, 1994; Ivanova and Schlesinger, 1993; Ponimaskin and Schmidt, 1995; Schmidt and Burns, 1989; Schmidt *et al.*, 1995; Veit *et al.*, 1996).

Here we report on the identification of previously unknown palmitylproteins encoded by the A22R, A36R and A56R (Copenhagen strain) open reading frames of VV. We also tested our ability to predict the palmitylated residue

by site-directed mutagenesis and analysis of palmitate incorporation by the mutated proteins.

3.2 Materials and Methods

3.2.1 Cells and Viruses

BSC_{40} (African green monkey kidney) and RK13 (rabbit kidney) cell lines were cultured in Eagle's minimal essential medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 10 μg/ml gentamycin-sulfate (MEM-10 LG/GS) at 37°C in a 5% CO_2 humidified atmosphere. Upon infection with VV, the cells were then cultured in Eagle's minimal essential medium supplemented with 2 mM L-glutamine, 10 μg/ml gentamycin-sulfate (MEM LG/GS) at 37°C in a 5% CO_2 humidified atmosphere. The Copenhagen (COP), IHD-J, and WR strains of VV were routinely propagated and titered in BSC_{40} cells as previously described. The recombinant VV, vTF7-3 (Fuerst *et al.*, 1986), was used for transient expression of genes cloned into the plasmid vector, pTM1:6xHis (described below). This virus contains of copy of T7 *gene 1* inserted at the thymidine kinase locus of the VV genome and expresses T7 RNA polymerase nearly constitutively during infection. Genes downstream of a T7 promoter are expressed to high levels when present in the cytoplasm of vTF7-3-infected cells. Standard methods were used to propagate and titer this virus as well.

3.2.2 Metabolic Labeling of VV Proteins

[9,10 ^{3}H(N)]-myristic acid (Dupont NEN research products [^{3}H]-MA) and [9, 10 ^{3}H(N)]-palmitic acid (Dupont NEN research products [^{3}H]-PA), purchased as ethanolic solutions, were dried by nitrogen overflow. The lipids were then dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 μCi/μl.

3.2.3 Labeling of COP, IHD-J and WR Acylproteins

BSC_{40} cells were grown to confluency in 35 mM wells (10^{6} cells/well). The cells were synchronously infected with either COP, IHD-J or WR strains of VV at a multiplicity of infection (moi) of 10. At 4 hours post-infection (hpi), [^{3}H]-MA or [^{3}H]-PA was added to a final concentration of 100 μCi/ml in no more than 10 μl of DMSO. At 24 hpi the cells were harvested and resuspended in reducing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and frozen until needed.

3.2.4 Time Course Labeling

BSC_{40} cells were grown to confluency in 35 mM wells. The cells were synchronously co-infected with COP and WR strains of VV at a moi of 10 each

(final moi = 20) so that both COP-specific and WR-specific palmitylproteins could be observed in the same context. Labeled lipids were added to a final concentration of 100 μCi/ml in no more than 10 μl of DMSO. The labeling periods included the 0-1, 1-2, 2-4, 4-8, and 8-24 hpi time-points. Infected cells were immediately harvested at the end of the labeling periods and resuspended in reducing SDS-PAGE sample buffer and frozen until needed.

3.2.5 Subcellular Fractionation and Purification of Labeled IMV and EEV

BSc_{40} cell were grown to confluency on 150 mM tissue culture plates ($2x10^7$ cells). The cells were co-infected with the COP and WR strains of VV at a moi of 5 each (final moi = 10). At 4 hpi [^{3}H]-PA was added to a final concentration of 100 μCi/ml. At 24 hpi both the culture medium and the infected cells were collected for purification of IMV and EEV and for subcellular fractionation of VV palmitylproteins. The culture supernatant was clarified of cellular debris by centrifugation at 700 x *g* for 20 minutes. Labeled EEV was then pelleted from the clarified culture medium by centrifugation at 100,000 x *g* for 30 minutes. The EEV pellet was resuspended in 500 μl of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4; PBS), titered, and then frozen until needed. The infected cells were resuspended in 6 ml of PBS, rapidly frozen, and then thawed to lyse cells. To ensure cell lysis the cellular extracts were vortexed vigorously while still partially frozen using the ice crystals to homogenize

the cell extracts. One milliliter of the infected cell lysate was set aside for subcellular fractionation. IMV was purified from the remaining 5 ml of cell lysate using CsCl gradients following techniques standard for VV (Payne, 1980).

3.2.6 Differential Centrifugal Subcellular Fractionation

From the 1 ml fraction of infected cell lysates (approx. $3x10^6$ cells), 100 µl was removed and set aside as the total cell extracts (TCE). The remaining 900 µl were centrifuged at 700 x *g* for 10 min to pellet nuclei. The pellet was resuspended in 900 µl of PBS and frozen until needed. The supernatant was centrifuged at 20,000 x *g* for 30 min to pellet cytosolic membranous components and whole virions. The pellet (P20) was resuspended in 900 µl of PBS then frozen until needed. The supernatant containing the soluble components of the cytoplasm (S20) was transferred to another microfuge tube and then frozen as well.

3.2.7 SDS-PAGE Affinity Blots and Fluorography of VV Proteins

For resolution of VV proteins by SDS-PAGE, all samples were resuspended in 1X reducing SDS-PAGE sample buffer. To minimize reduction of the thio-ester bonded palmitate, samples were heated to 70°C for 1 min then rapidly cooled to 4°C prior to loading the gels. All gels used were 12.5% acrylamide:bis-acrylamide (30:0.8). For affinity blots, SDS-PAGE gels were electroblotted to

nitrocellulose by standard western blotting techniques (Towbin *et al.*, 1992) and then probed using Ni^{+}-activated horse-radish peroxidase, which binds histidine-tagged proteins (see below). The blots were developed using chemiluminescent reagents and exposure to film. To detect [^{3}H]-PA or [^{3}H]-MA labeled proteins in gels, the gels were first dehydrated by immersion in DMSO for 30 minutes. The gels were then impregnated with diphenyloxazole (PPO) by immersion in a 22.2% PPO:DMSO solution for 1 hour (Bonner and Laskey, 1974). The PPO was precipitated in the gel by immersion in water for 30 minutes. The gels were then dried and exposed to film.

3.2.8 Identification of Candidate Palmitylproteins

The identification of candidate VV-encoded palmitylproteins was made utilizing a number of criteria: We have observed (see Results) that VV palmitylproteins are primarily "late" proteins, have relative molecular masses of 14, 17, 23-28 (a single protein), 37, 42, 46, 86, and 94 kDa, as determined by SDS-PAGE, and are specific for EEV. Focusing then, on VV gene products that matched these criteria, we then scanned the predicted gene products expressed by the COP strain of VV for sequences which fit with our previously reported palmitylation motif, Hydro*1-12*AACA*. "Hydro" can be a previously myristylated glycine, a transmembrane domain, or a hydrophobic stretch of amino acids usually at least 10 residues in length. The hydrophobic element is usually followed by a 1-12 amino

acid spacer then a cysteine (C) residue (or residues) surrounded by aliphatic amino acids (*A*).

3.2.9 Cloning and Transient Expression of Candidate Palmitylproteins

All of the candidate palmitylprotein genes were cloned into pTM1:6xHis, constructed as follows: The L5R gene of VV was amplified from IHD-J genomic DNA by polymerase chain reaction (PCR) using primers L5R-5'-(5'CCGGAA TTCATATGGAGAATGTT CCTAATG-3') and L5R-3'6xHis (5'-CCAATGCATT GGTTCTGCAGTTTCATCAGTGGTGGTGGTGGTGGGATCCTCTGCGA AGAACATCG-3'). The PCR product was treated with restriction enzyme *Eco*RI, generating an overhang at the 5' end of the product and leaving the 3' end blunt, and then ligated into *Eco*RI/*Stu*I-restricted pTM1 producing the plasmid pTM1:L5R-6xHis. This placed the 5' end of the L5R gene downstream of the T7 promoter and encephalomyocarditis virus 5'-untranslated region/internal ribosomal entry site (EMC-leader) on pTM1. The normal L5R stop codon was not included in the PCR primer and was instead, replaced by sequences that following transcription and translation resulted in the addition of a glycine and a serine followed by six histidines at the carboxy terminus of the protein. The remaining candidate palmitylprotein genes were cloned into pTM1:L5R-6xHis. In order to do this, the L5R coding sequence was excised by restriction with *Nde*I and *Bam*HI leaving the 6-histidine tag sequence intact on the plasmid. All other candidate palmitylprotein

genes were PCR amplified from IHD-J genomic DNA with the exception of the A56R gene that was amplified from COP and WR genomic DNA as well as IHD-J. The A14L gene was amplified using primers A14L-5' (5'-CCGGAATTCATATGG ACATG ATGCTTATG-3') and A14L-3' (5'-GCGGGATCCGTTCATGGAAATAT CGCTATG-3'); the A22R gene was amplified using primers A22R-5' (5'-CCGGAATTC ATATGTCGTCACCG AT G-3') and A22R-3' (5'-GCGGGATCCCATTTTTTTTATGT AATTCC TAG-3'); the A33R gene was amplified using primers A33R-5'(5'CCGGAA TTCATATGATGACACCAG-3') and A33R-3' (5'-GCGGGATCCGTTCATTGT TTTA ACAC-3'); the A34R gene was amplified using primers A34R-5' (5'-GCATCAGCCCCATATGAAATCGCTTAATAGACAAACTGTAAG-3') and A34R-3' (5'-GCGGGATCCCTTGTAGAATTTTT TAACAC-3'); the A36R gene was amplified using primers A36R-5' (5'-CCGGAATTCATATGATGCTGG TACC-3') and A36R-3' (5'-GCGGGATCCCACCAATG ATACG-3'); the A56R gene was amplified using primers A56R-5' (5'-CCGGAATTCATATGACACGA TTACC-3') and A56R-3' (5'-GCGGGATCCGACTTTGTTC TCTG-3'); the B5R gene was amplified using primers B5R-5' (5'GCATCAGCCCCATATGAAA ACGATTTCCGTT GTTACGTT-3') and B5R-3' (5'-GCGGGATCCCGGTAGCA ATTTATGG-3'); the F13L gene was amplified using primers F13L-5' (5'GCATCAGCCCC ATATG TGGCCATTTGC-3') and F13L-3' (5'-GCGGGATCCAAT TTTTAACG-3'). All of the PCR products were digested with restriction enzymes *Nde*I and *Bam*HI and ligated into *Nde*I/*Bam*HI-digested pTM1:L5R-6xHis producing

plasmids pTM1:A14L-6xHis, pTM1:A22R-6xHis, pTM1:A33R-6xHis, pTM1:A34R-6xHis, pTM1:A36R-6xHis, pTM1:A56R(COP)-6xHis, pTM1:A56R(IHD-J)-6xHis, pTM1:A56R(WR)-6xHis, pTM1:B5R-6xHis, and pTM1:F13L-6xHis.

Transient expression of the candidate palmitylproteins was facilitated by use of the vTF7-3 recombinant VV. BS_{40} cells were grown to confluency on 35 mM wells in 6-well tissue culture plates (10^6 cell/well). The cells were infected with vTF7-3 at a moi of 10. Following a 1 h virus adsorption period, the inoculum was aspirated and replaced with 500 µl of MEM-E LG/GS containing 10 µg of plasmid DNA complexed with the transfection reagent DMRIE-C (Gibco-BRL). At 4 hpi (also 4 h post-transfection) an additional 500µl of MEM-E, LG/GS was added. If label was to be added, it was added with the 500 µl of MEM-E, LG/GS at 4 hpi. At 24 hpi the cells were washed free of the tissue culture plates using the culture medium. The cells were pelleted from the medium by centrifugation at 700 x *g* for 10 min. The cells were resuspended in 100 µl of PBS containing 5 U of DNase I to reduce the viscosity of the cell lysate, following lysis by 3 cycles of freezing and thawing, to facilitate gel loading. Eighty µl of each of the cell lysates were set aside for immunoprecipitation. The remaining 20 µl of cell lysates were made 1X reducing SDS-PAGE sample buffer by addition of 4 µl of 6X reducing SDS-PAGE sample buffer prior to loading gels.

3.2.10 Immunoprecipitation of Proteins

Eighty microliter samples of infected cell lysates (approx. $8.0x10^5$ cells), labeled during infection with [^{3}H]-PA, were diluted to 1 ml with radio-immunoprecipitation assay (RIPA) buffer (1% w/v sodium deoxycholate, 1% v/v Triton X-100, 0.2% w/v SDS, 150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4). Histidine tagged proteins were immunoprecipitated by addition of an anti-6-histidine monoclonal antibody (Qiagen) and Protein A-coated sepharose beads. The reactions were performed on a rotary mixer at 4°C for 18 h. The immunoprecipitates were washed numerous times with RIPA buffer then resolved by SDS-PAGE. The gels were fluorographed in PPO:DMSO and exposed to film for detection.

3.2.11 Mutagenesis of Palmitate Acceptor Residues in VV Palmitylproteins

Oligonucleotide (oligo)-directed mutagenesis of VV palmitylprotein genes on pTM1:6xHis plasmids followed the method of Kunkel (Kunkel *et al.*, 1987). All plasmids resulting from the mutagenesis reactions were identical to their parental plasmids with the exception of the mutated codon and novel restriction sites engineered for preliminary screening. The restriction sites did not alter the coding potential of the genes. All mutations were confirmed by DNA sequencing. The codon encoding cysteine 99 (C99, numbering from the initiating methionine) of

A22R was mutated to encode serine using oligo A22R-C99S (5'GCCAAAGTGATCAGCGTC TCGCCTGTC-3') producing plasmid pTM1:A22R-C99S. The codon encoding C36 of A33R was mutated to encode serine using oligo A33R-C36S (5'-GTGATTGGTCTATCGATTAGAATAT CTATGG-3') producing plasmid pTM1:A33R-C36S. The codon encoding C19 of A36R was mutated to encode serine using oligo A36R-C19S (5'-GGAACAAT ACTAGTATCTTATATATTATATATTTGTAGG -3') producing plasmid pTM1:A36R-C19S. The codon encoding C25 of A36R was mutated to encode serine using oligo A36R-C25S (5'-GTTATATATTATATATCTCGAGGAAAA AGATACG-3') producing plasmid pTM1:A36R-C25S. The codon encoding C301 of B5R was mutated to encode serine using oligo B5R-C301S (5'-GTTATAGTAT TAGTCTCGAGTTGTGACAAAAATAATGAC -3') producing plasmid pTM1:B5R-C301S. The codon encoding C303 OF B5R was mutated to encode serine using oligo B5R-C303S (5'-GTTATAGTATTAGTTTGCTCGAGTGACAA AAATAATGAC-3') producing plasmid pTM1:B5R-C303S. An internal region of the F13L open reading frame on pTM1:F13L-6xHis containing codons for C185 and C186 was replaced with the homologous sequence from pDG5.3 (Grosenbach and Hruby, 1998). This plasmid contains a copy of F13L but harbors mutations leading to the replacement of C185/C186 with serines without altering the otherwise wild-type sequence. This produced plasmid pTM1:F13L-C185/6S. No mutations were made to the A56R plasmid. Instead clones of A56R from COP, IHD-J and WR DNA were made. The COP and WR sequences of the A56R gene

are known (Goebel *et al.*, 1990; Smith *et al.*, 1991). The IHD-J A56R DNA was sequenced for comparison to WR and COP. All plasmids were sequenced through the cloned genes to ensure accuracy of the sequence.

3.3 Results

3.3.1 Analysis of VV Acylproteins

VV proteins are expressed in three distinct temporal classes, early, intermediate and late. In an effort to limit the number of proteins to target for our study we performed a time-course labeling experiment with [9,10 ^{3}H(N)]-myristic acid ([^{3}H]-MA) and [9, 10 ^{3}H(N)]-palmitic acid ([^{3}H]-PA) to determine their temporal class. BSC_{40} cells were synchronously co-infected with the Copenhagen (COP) and Western Reserve (WR) strains of VV at a combined multiplicity of infection of 10. During the course of this study, we observed strain-specific acylproteins (both myristyl- and palmitylproteins) (Figure 3.2). Co-infection with COP and WR allowed the analysis of all VV-encoded acylproteins within a single set of experiments. The infected cells were labeled by addition of [^{3}H]-PA or [^{3}H]-MA from 0 to 1, 1 to 2, 2 to 4, 4 to 8 and 8 to 24 hours post-infection (hpi). The infected cells were harvested at the end of each labeling period. The total cell extracts were resolved by SDS-PAGE, followed by fluorography, and then exposed to film. With the exception of a 14-kDa protein that incorporated label from both

myristic and palmitic acid in the 1 to 2 hpi labeling period, all other acylproteins encoded by VV appear to be "late" proteins (Figure 3.1).

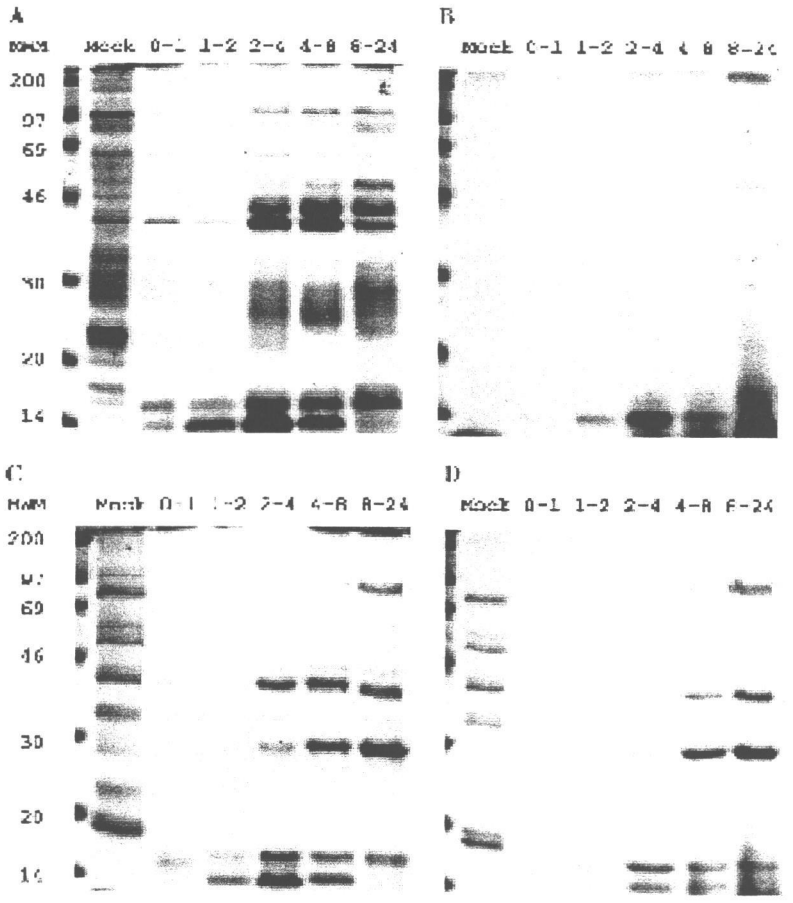


Figure 3.1. SDS-PAGE analysis of time-course labeled VV acylproteins. BSC40 cells were co-infected with WR and COP strains of VV at a combined moi of 10. Cells were labeled with [^{3}H]-PA (A and B) or [^{3}H]-MA (C and D) for time periods as indicated above the gel lanes (hours post-infection). Identical gels were treated with neutral hydroxylamine to cleave thioester-bonded fatty acids (B and D). All gels were fluorographed by impregnation with PPO prior to film exposure. Note the 14-kDa acylprotein recalcitrant to hydroxylamine treatment in B and D.

The 14-kDa acylprotein continued to incorporate label from 2 to 8 hpi but was nearly absent in the 8 to 24 hpi labeling period. All other VV acylproteins incorporated label in the 2 to 4, 4 to 8 and 8 to 24 hpi labeling periods. To distinguish between authentic myristylproteins and those that were labeled via interconversion to palmitic or other long-chain fatty acids, identical gels were treated with neutral hydroxylamine prior to fluorography (Figure 3.1B and D). Vaccinia encodes palmitylproteins with apparent molecular masses of 14, 17, 23-28 (a single glycoprotein with a common peptide backbone migrating as three distinct species), 37, 42, 50-55, and 94 kDa (Figure 3.1A).

Additionally, the COP strain produced an 86-kDa protein that was not detectable in the WR- or International Health Department strain J (IHD-J)- infected cell extracts (Figure 3.2A). The 23-28-, 37-, and 42-kDa proteins have previously been reported to be encoded by the A33R (Roper *et al.*, 1996), F13L (Hirt *et al.*, 1986) and B5R (Isaacs *et al.*, 1992) open reading frames respectively. The identities of the 14-, 17-, 50-55-, 86-, and 94-kDa palmitylproteins are unknown and represent the topic of this report.

The myristylproteins migrated with apparent molecular masses of 14, 17, 25, 36, 39, and 92 kDa (Figure 3.1C). The 92-kDa myristylprotein was not produced by the COP strain (Figure 3.2A) and most likely is the ATI protein encoded by the A25L open reading frame of WR and IHD-J as previously reported (Meyer and Rziha, 1993). The 25-kDa myristylprotein is encoded by the L1R open reading frame and has been the subject of numerous studies (Franke *et al.*, 1990;

Ravanello and Hruby, 1994a; Ravanello and Hruby, 1994b; Wolffe *et al.*, 1995). We have recently reported that the 17-, 36-, and 39-kDa proteins are encoded by the E7R, A16L and G9R open reading frames respectively (Martin *et al.*, 1997). In a single report, the 14-kDa myristylprotein was identified as the product of the A14L open reading frame (Rodriguez *et al.*, 1997) - a finding that is not supported by our work (see below).

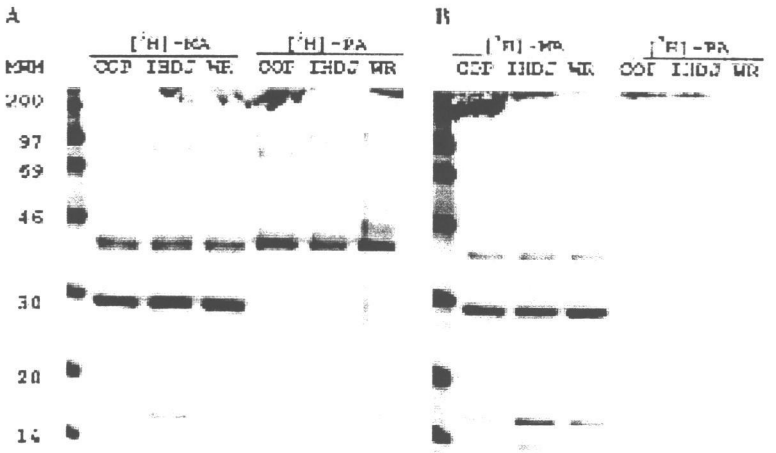


Figure 3.2. Comparison of acylproteins encoded by COP, IHD-J and WR strains of VV. BSC_{40} cells were infected with COP, IHD-J or WR strains of VV. Cells were labeled by addition of [^{3}H]-PA or [^{3}H]-MA. Total cellular extracts were resolved by SDS-PAGE and acylproteins were detected by exposure of PPO impregnated gels to film (A). An identical gel was treated with neutral hydroxylamine to cleave thioester-bonded fatty acids (B). Note the absence of a 92-kDa myristylprotein ([H]-MA) from COP extracts. Also note the presence of a 86-kDa palmitylprotein in COP extracts that is absent from IHD-J and WR extracts.

3.3.2 Subcellular Fractionation and Virion Association of VV Acylproteins

The L1R myristylprotein is known to be a component of both IMV and EEV (Ravanello *et al.*, 1993; Ravanello and Hruby, 1994b). The A33R (Roper *et al.*, 1996), B5R (Isaacs *et al.*, 1992) and F13L (Blasco and Moss, 1991) palmitylproteins are known to be specific for TGN membrane-enveloped virions (IEV, CEV and EEV) and are found in TGN membranes prior to envelopment (Schmelz *et al.*, 1994). To determine the intracellular location of the other acylproteins, we fractionated infected, labeled cells by differential centrifugation to yield a nuclear fraction, particulate cytosolic fraction (containing whole virions and cytosolic membranous components) and soluble cytoplasmic fraction. We also purified IMV and EEV from labeled cells. The L1R myristylprotein was present in the nuclear fraction and the membrane fraction, which also contains whole virions (Figure 3.3B). It was also present on IMV and EEV. All other myristylproteins were found in the soluble fraction of cells although a small amount of the G9R protein is also present in IMV also. All VV palmitylproteins found in total cell extracts were present in the nuclear fraction, the membrane fraction and were specific for EEV (Figure 3.3A). Two additional palmitylproteins with apparent molecular masses of 20 and 22 kDa that were not obvious in the total cell extracts were present on EEV as well. No VV palmitylproteins were found to be soluble or on IMV.

3.3.3 Identification of Candidate Palmitylproteins

To identify candidate palmitylproteins, we considered that VV palmitylproteins are of the late temporal class, are specific for IEV, CEV or EEV, and most likely contain hydrophobic sequences mediating membrane association. We then scanned the sequence of the COP strain (which is entirely sequenced)(Goebel *et al.*, 1990) for open reading frames preceded by late promoters and encoding proteins whose predicted mass corresponded to SDS-PAGE observed mobility of VV palmitylproteins. These sequences were then analyzed for hydrophobic regions and for cysteines that were within the consensus, Hydro*1-12*AACA*. By these criteria the following open reading frames were identified as potentially encoding palmitylproteins: A14L, A22R, A33R, A34R, A36R, A56R, B5R and F13L (Table 3.1). It has been previously reported that the products of the A33R (Roper *et al.*, 1996), B5R (Isaacs *et al.*, 1992) and F13L (Hiller and Weber, 1985; Hirt *et al.*, 1986)open reading frames are palmitylproteins and led us to conclude that our criteria were sufficient. Additionally, the products of the A33R (Roper *et al.*, 1996), A34R (Duncan and Smith, 1992), A36R (Parkinson and Smith, 1994), A56R (Payne and Norrby, 1976), B5R (Engelstad, Howard, and Smith, 1992; Isaacs *et al.*, 1992), and F13L (Hiller and Weber, 1985; Hirt *et al.*, 1986) open reading frames are specific for either IEV, CEV or EEV further supporting these as candidate palmitylproteins.

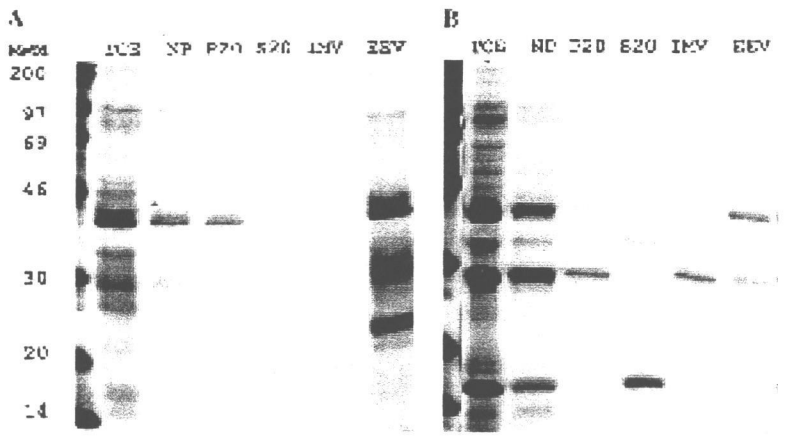


Figure 3.3. Differential centrifugal subcellular fractionation of VV acylproteins. BSC_{40} cells were co-infected with COP and WR strains of VV. These infected cells were labeled with [^{3}H]-PA (A) or [^{3}H]-MA (B. EEV was purified from the culture medium. A portion of the total cell extracts (TCE) was fractionated to yield a nuclear fraction (NP), a particulate cytosolic fraction (P20), containing intracellular membranes and whole virions, and a soluble cytosolic fraction (S20). IMV was purified from infected cells. Subcellular fractions and equal titers of IMV and EEV were resolved by SDS-PAGE and fluorographed to detect [^{3}H]-PA- or [^{3}H]-MA-labeled proteins.

3.3.4 Transient Expression and Palmitylation of Candidate Palmitylproteins

The open reading frames for each candidate palmitylprotein were PCR amplified from VV genomic DNA and cloned into a modified pTM1 vector, pTM1:6xHis. This vector contains sequences that, following expression, result in the addition of a carboxy-terminal 6-histidine tag on each of the candidate palmitylproteins. The T7 promoter and encephalomyocarditis virus 5' untranslated region (EMC leader) are left intact as well. Following infection of cells with vTF73 (a T7 polymerase expressing recombinant vaccinia virus) the T7 promoter is

Gene	Promoter	Peptide Sequence	Size
A[illegible]L	[illegible]**ATG**	[illegible]	18 kDa (15)
A22R	[illegible]**ATG**	[illegible]	21 kDa (21)
A33R*	[illegible]**ATG**	[illegible]	21 kDa (23-28)
A34R	[illegible]**ATG**	[illegible]	28 kDa ([illegible])
A36R	[illegible]**ATG**	[illegible]	[illegible] kDa ([illegible])
A56R	[illegible]**ATG**	[illegible]	[illegible] kDa ([illegible])
B5R*	[illegible]**ATG**	[illegible]	[illegible] kDa ([illegible])
B13L*	[illegible]**ATG**	[illegible]	[illegible] kDa ([illegible])

Table 3.1. Identification of candidate VV palmitylproteins. VV proteins (predicted from gene sequences) were evaluated as candidates for palmitylation. Only proteins whose promoters contain the consensus "late" transcription start sequence upstream of the start codon ATG (bolded) were considered for further evaluation. Late promoter elements are underlined. Numbers in parenthesis indicate the number of nucleotides that separate the nucleotides shown. The A56R gene does not contain a consensus "late" promoter but is included for additional reasons (see text). The peptide sequences of VV late proteins were inspected for homology to the palmitylation motif Hydro*$_{1\text{-}12}$*AACA*. Proteins containing hydrophobic regions (underlined) with cysteine residues in an aliphatic environment (C*) were considered for further analysis. The predicted size of the protein is compared to the SDS-PAGE observed mobility (numbers in parenthesis under "Size").

recognized by the virus-produced T7 polymerase, mediating high-level transcription. The EMC leader present on the transcript serves as an internal ribosomal entry site mediating cap-independent translation. For these experiments, BSC40 cells were infected with vTF7-3, followed by transfection of each of the plasmid DNAs in separate 35-mm wells. At 4 hpi [^{3}H]-PA was added to the culture medium. Infected cells were harvested at 24 hpi and then two equal portions of the total cell extract were resolved by SDS-PAGE on two different gels. The first gel was electroblotted to nitrocellulose and probed with Ni^+-activated horseradish peroxidase, which binds to the 6-histidine tag on the transiently expressed proteins.

The blot was developed using chemiluminescent reagents and exposed to film. The other gel was impregnated with diphenyloxazole: dimethylsulfoxide (PPO: DMSO), dried and exposed to film for detection of all [^{3}H]-PA-labeled proteins. The remaining portions of the total cell extracts were immunoprecipitated. The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed as described above.

The affinity blot (referring to the Ni^+-activated horseradish peroxidase probed blot) demonstrated that all of the proteins were efficiently expressed by the vTF7-3/pTM1:6xHis system (Figure 3.4A). We observed that; A14L encodes a protein of 14 kDa with minor species migrating at approximately 16 and 23 kDa; A22R encodes a 22-kDa protein; A33R encodes three proteins migrating at 23, 25, and 28 kDa; A34R encodes two proteins migrating at 22 and 24 kDa; A36R encodes a protein poorly resolving between 50 and 55 kDa; A56R encodes possibly three proteins resolving between 72 and 86 kDa; B5R encodes a 42-kDa protein and is present in a higher molecular weight complex of approximately 94-kDa; and F13L encodes a 37-kDa protein. The multiple banding seen by SDS-PAGE for A33R, A34R, A36R and A56R is most likely the result of a complex glycosylation pattern for these proteins. The fluorograph of the same cell extracts labeled with [^{3}H]-PA, demonstrates that the A14L, A34R and A56R (WR strain) proteins did not incorporate the label above background levels suggesting that they are not palmitylproteins (Figure 3.4B and C). Proteins expressed from A22R, A33R, A36R, A56R (COP strain), B5R, and F13L incorporated the label above

background levels suggesting that these open reading frames do encode palmitylproteins. Although sequence analysis of the cloned genes demonstrated that the histidine tag was intact on all the plasmid constructs, we were unable to immunoprecipitate A22R and B5R using anti-6-histidine monoclonal antibodies (Figure 3.4C). This may be due to protein conformations that deny the antibody access to the histidine tag during the immunoprecipitation reaction. It has been previously demonstrated that the A33R, B5R and F13L genes encode palmitylproteins while the modification status of A22R, A36R and A56R (COP strain) has not been reported previously.

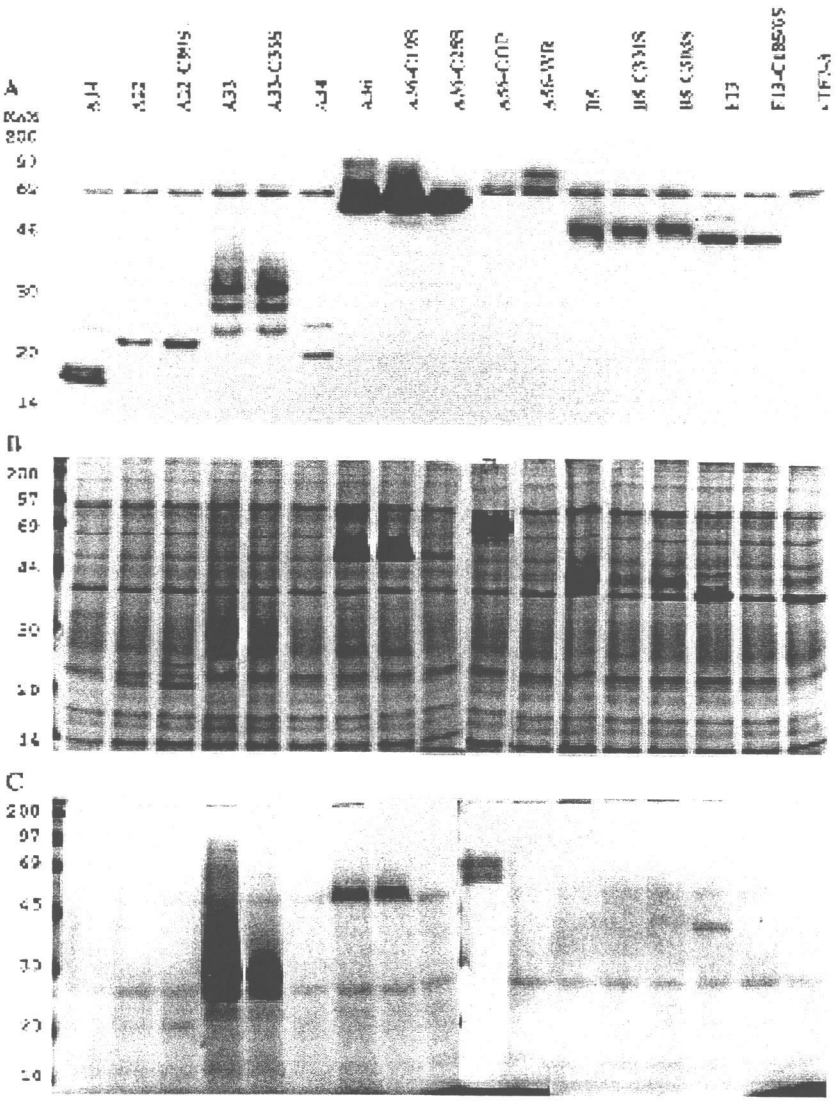
3.3.5 Identification of Palmitate-Modified Residues of VV Palmitylproteins

In order to narrow the number of residues that might be subject to palmitylation, each of the VV palmitylprotein sequences was examined for cysteine residues that fell within the context of the palmitylation motif, Hydro*1-12*AA*C*A*. We also considered that those regions of proteins exposed to the lumenal compartment of intracellular organelles or the extracellular environment were unlikely to be palmitylated. By these analyses it was predicted that the following residues (denoted by the single-letter amino acid code and residue number starting from the initiating methionine) were palmitylated: C99 of A22R, C36 of A33R, C19 or C25 of A36R, C296 or C301 of A56R, C301 or C303 of B5R, and C185 and/or C186 of F13L. The codons for these residues were mutated, by oligonucleotide (oligo)-directed mutagenesis, to encode serine instead of cysteine. In the case of F13L,

both cysteine 185 and 186 were mutated to serine since it has been previously demonstrated to be palmitylated on both residues (Grosenbach *et al.*, 1997). Also, since we have observed that the COP-encoded A56R is palmitylated but is not palmitylated as expressed from the WR or IHD-J strains (data not shown for IHD-J), their amino acid sequences were analyzed for differences occurring in the region predicted to be palmitylated. The only difference noted was that a tyrosine residue in both WR and IHD-J replaced C301 from COP A56R. Tyrosine is not known to be an efficient substrate for palmitylation suggesting that C301 was the palmitate acceptor residue in that protein.

Each of the mutated proteins were transiently expressed and analyzed by SDS-PAGE as above (Figure 3.4). The C99 mutant of A22R still incorporated label as efficiently as the wild-type protein, perhaps even more efficiently. It was found that the C36 mutant of A33R also incorporated label but at a slightly lower level than the wild-type protein. This may be due to differences in expression levels though and as such C36 may not be the palmitate acceptor residue. The C19 mutant of A36R incorporated label as efficiently as the wild-type protein while the C25 mutant of A36R incorporated very little, suggesting that C25 is the primary palmitate-acceptor residue for A36R. The C301 mutant of B5R incorporated very

Figure 3.4. Transient expression and [^{3}H]-PA labeling of VV candidate palmitylproteins. Candidate palmitylproteins were transiently expressed in BSC_{40} cells using the vTF7-3/pTM1:6xHis system. Proteins were labeled by addition of [^{3}H]-PA to infected cells. Total cellular extracts were resolved on two identical gels (A and B). One gel (A) was blotted to nitrocellulose and probed with Ni^+-activated horseradish peroxidase that binds histidine-tagged proteins. The blot was developed using chemiluminescent reagents and exposed to film. The other gel (B) was fluorographed to detect all palmitylproteins in the cellular extracts. Histidine-tagged proteins were immunoprecipitated from the same cellular extracts using an anti-6-histidine monoclonal antibody. Immunoprecipitates were resolved by SDS-PAGE and was also fluorographed to detect immunoprecipitated palmitylproteins (C). Gel lane assignments are identical for all three gels and are indicated above gel "A".


A
B
C

little label, suggesting it is the primary palmitate-acceptor residue. The C303 mutant of B5R incorporated less than the wild-type protein but more than the C301 mutant, suggesting possibly a secondary modification site. In previous reports we have demonstrated that F13L is palmitylated on C185 and C186. This is supported by the data presented here. The C185/186 mutant of F13L did not incorporate label at detectable levels above background.

3.3.6 Myristic Acid Labeling of the A14L Protein

It has been previously reported that the A14L protein is myristylated (Rodriguez *et al.*, 1997). We attempted to confirm this by transient expression of

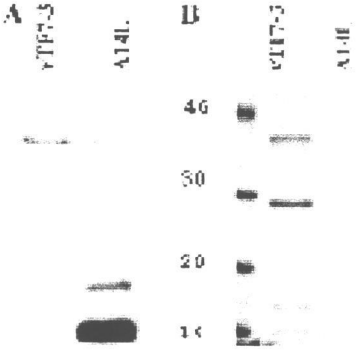


Figure 3.5. [^{3}H]-MA labeling of the VV A14L protein. BSC_{40} cells were infected with vTF7-3 and transfected with pTM1:A14L-6xHis. Cells were labeled by addition of [^{3}H]-MA. Total cellular extracts were resolved by SDS-PAGE on two identical gels. One gel was blotted to nitrocellulose and probed with Ni^+-activated horseradish peroxidase (A). The other gel was developed using chemiluminescent reagents and exposed to film. The other gel was fluorographed to detect [^{3}H]-MA-labeled proteins (B).

the A14L protein using the vTF7-3/pTM1:6xHis system. Although the protein was efficiently expressed (Figure 3.5A), it did not incorporate the label (Figure 3.5B). We noticed though that over expression of the A14L protein inhibited the production of other late VV myristylproteins while the intermediate 14-kDa myristylprotein was still expressed at a low level (Figure 3.5B).

3.4 Discussion

In identifying candidates for this study, a number of criteria were utilized. First, we considered the work of others to identify both viral and cellular palmitylproteins and their sites of modification (Bizzozero, Tetzloff, and Bharadwaj, 1994; Dietzen *et al.*, 1995; Ivanova and Schlesinger, 1993; Koegl *et al.*, 1994; Magee and Courtneidge, 1985; Ponimaskin and Schmidt, 1995; Schmidt *et al.*, 1995). In doing so, we observed a loosely conserved motif Hydro*1-12*AACA* (described in the introduction) (Grosenbach *et al.*, 1997; Hansen, Grosenbach, and Hruby, 1999).

This motif by itself did not allow the identification of VV palmitylproteins. Therefore, as a second means of identifying VV palmitylproteins, we undertook a biochemical characterization of palmitylated proteins expressed in VV-infected cells. Table 3.2 summarizes the results described here and elsewhere. We first made an effort

Summary of VV Acylproteins[a]

Protein (gene)	Modification	Site of modification	Localization
ATI(A25L)	Myristate	Unknown (internal)	Cytosol, IMV
Unnamed (A16L)[b]	Myristate	MG*XXX(S,T,A,C,N)	Cytosol
Unnamed (G9R)[b]	Myristate	MG*XXX(S,T,A,C,N)	Cytosol, IMV
M65 (L1R)[b]	Myristate	MG*XXX(S,T,A,C,N)	MV, EEV
Unnamed (E7R)[b]	Myristate	MG*XXX(S,T,A,C,N)	Cytosol
M14 (unknown)	Myristate	Unknown (internal)	Unknown
Hemagglutinin (A56R)	Palmitate	Cysteine 32[c]	EEV
Unnamed (A36R)	Palmitate	Cysteine 25[c]	EV
gp42 (B5R)	Palmitate	Cysteines 301/303	TGN, EV, CEV, EEV
P37 (F13L)	Palmitate	Cysteines 185/189	TGN, EV, CEV, EEV
Unnamed (A33R)[b]	Palmitate	Cysteine 36	TGN, EV, CEV, EEV
Unnamed (A22R)	Palmitate	Unknown	EEV
P17 (unknown)	Palmitate	Unknown	Unknown
Unnamed (A14L)	Nonacylated	Nonacylated	MV, EEV
Unnamed (A34R)	Nonacylated	Nonacylated	EV, CEV, EEV

Table 3.2. Summary of VV Acylproteins. Labeling experiments with [^{3}H]-MA or [^{3}H]-PA have shown that VV encodes numerous acylated proteins. Depicted in the table are the known VV myristyl- and palmitylproteins. Modification with myristate can occur at an internal site on a arginine or lysine, or at the amino terminus on a glycine within the consensus sequence MG*XXX(S,T,A,C,N). VV palmitylation occurs mostly on cysteine residues within a loosely conserved consensus sequence Hydro*$AACA$. The ORF that encodes the 14 kDa myristylprotein (M14) has yet to be identified, therefore, the modification site has not been discovered. The ORFs encoding for the palmitylproteins have all been identified except for the 17 kDa species (P17). Known modification sites and localization of each protein are listed in the table.

to distinguish between VV myristylproteins and VV palmitylproteins, many of which co-migrate when resolved by SDS-PAGE. One of our first discoveries was that the ATI protein, which we previously thought to be modified by both myristate and palmitate (Manenti *et al.*, 1994), is in fact only myristylated. This was determined by the analysis of [^{3}H]-MA or [^{3}H]-PA labeled extracts from cells infected with the COP, IHD-J or WR strains of vaccinia. We observed that an 86-kDa palmitylprotein was expressed by COP that was absent from the extracts from IHD-J or WR-infected cells (Figure 3.2). A 94-kDa palmitylprotein was present in

the extracts from all three viruses. In the [^{3}H]-MA-labeled extracts we observed a 92-kDa myristylprotein encoded by IHD-J and WR but not COP. It is known that the COP strain contains a mutation in the A25L gene, encoding the ATI protein, which results in the premature termination of translation, giving rise to a truncated peptide (Goebel *et al.*, 1990; Patel, Pickup, and Joklik, 1986). Considering though, that COP encodes palmitylproteins of 86- and 94-kDa it is likely that due to the similar migration rates in SDS-PAGE gels that the 94-kDa palmitylprotein (also expressed by WR and IHD-J) and the 92-kDa myristylprotein were considered to be one and the same. The finding further supports our conclusion that the ATI protein is not palmitylated, that with the exception of L1R, all VV myristylproteins are soluble in the cytoplasm of infected cells while all VV palmitylproteins are found in the particulate fraction of infected cells. A remote possibility exists that the ATI protein is palmitylated and once it is becomes a component of the particulate fraction of cells. This does not seem likely though due to the fact that a 92-kDa palmitylprotein present in extracts from WR and IHD-J but not COP has not been observed.

Also, in comparing the acylproteins expressed by COP, IHD-J and WR strains of VV, we noted that COP encoded an 86-kDa palmitylprotein whose migration pattern in SDS-PAGE gels suggested that it was a glycoprotein. We also observed what appeared to be the same protein present on EEV. It is known that the VV hemagglutinin protein (encoded by gene A56R) exhibits similar characteristics so we considered it a candidate for palmitylation. Analysis of the protein expressed

by the COP A56R gene suggested that either C296 or C301 would be possible palmitate acceptor residues. C296, although surrounded by aliphatic residues, is within the predicted transmembrane region. C301 is at the membrane/cytosolic boundary but is followed by the amide of aspartic acid, asparagine. We sequenced the IHD-J A56R gene and compared it to the WR and COP sequences. The amino acid sequences from the transmembrane domain through the cytoplasmic tails for all three proteins were identical with position 301 being the exception. While COP had a cysteine at position 301, both IHD-J and WR had a tyrosine residue. Considering that we have observed the COP hemagglutinin to be palmitylated while the IHD-J and WR hemagglutinin are not we must conclude that C301 is the palmitate acceptor residue. Payne (Payne, 1992) first demonstrated that the IHD-J hemagglutinin was not palmitylated but did not analyze the WR- or COP-encoded hemagglutinin.

As part of our biochemical characterization of VV acylproteins, we performed a time-course labeling experiment to determine the temporal class to which the VV acylproteins belong. All VV myristyl- and palmitylproteins are primarily expressed at late times suggesting a role in assembly. The one exception is the 14-kDa acylprotein that incorporated both myristic and palmitic acid label. Identical expression characteristics and size suggest that they are the same protein. Both the myristic and palmitic acid labeled 14-kDa acylproteins commenced expression in the 1 to 2 hpi labeling period, continued expression through the 2 to 8 hpi labeling period and were not detectable in the 8 to 24 hpi labeling period. The

possibility that both the myristylated and palmitylated proteins are the same is supported by the fact that a fraction of the label, whether added as myristic or palmitic acid, incorporated by the 14-kDa protein was recalcitrant to treatment by neutral hydroxylamine (Figure 3.1B and D), which is known to cleave thioester linked moieties such as palmitate (Schlesinger *et al.*, 1980). This suggests that at least part of the label incorporation was due to the formation of neutral hydroxylamine-stable amide linkage as is typical of myristylation. Vaccinia is not predicted to encode a 14-kDa protein containing the amino-terminal myristylation motif MG*XXX*S/T/A/C/N and therefore suggests that this 14-kDa protein is the second example of a VV-encoded, internally myristylated protein, the first being the ATI protein. It has been previously reported that the A14L gene encodes a 14-kDa myristylprotein (Rodriguez *et al.*, 1997). We attempted to confirm this and to determine if this same protein was palmitylated as well. Over expression of the A14L gene in the presence of [^{3}H]-PA or [^{3}H]-MA did not result in the incorporation of either label by the protein although the protein was expressed at a high level (Figure 3.4 and 3.5). We did note though that the high-level expression of A14L inhibited other late protein production.

We have not only identified novel palmitylproteins encoded by the A22R, A36R and A56R (COP strain) genes of VV, but we have been able to accurately predict the palmitate acceptor residue for A36R, A56R, B5R and F13L encoded proteins. Although, by our analyses, VV proteins appear to be palmitylated in a predictable manner, the misidentification of the A14L and A34R proteins as

candidate palmitylproteins and the inability to predict the modification sites on the A22R and A33R proteins suggest shortcomings to our system for identification of VV palmitylproteins. Perhaps additional criteria should be considered. The A14L protein is known to be a component of the IMV membrane and the inner membrane of EEV. Although the primary amino acid sequence indicates that it could be a substrate for palmitylation, its presence in the IMV membrane may sequester it from the palmitylation machinery. It is not clear to us why a protein in the membrane of IMV would not be palmitylated while a protein in the membrane of EEV would be.

Another factor that must be considered is membrane orientation. The A36R protein is predicted to be a type Ib membrane protein and as such, the most likely palmitate acceptor residue would be exposed to the lumenal environment of intracellular membrane-bound compartments or to the extracellular environment if it were exported to the plasma membrane. Palmitylation most commonly occurs on regions of proteins exposed to the cytoplasm. The finding that A36R is palmitylated on C25 and data generated from Röttger *et al* and van Eijl *et al* conclusively demonstrate that this protein has a type Ib membrane orientation, with a short amino-terminal lumenal tail and a long carboxy-terminal cytoplasmic tail (Rottger *et al.*, 1999). Membrane orientation may explain why the A34R protein is not palmitylated. The A34R protein is predicted to be a type II transmembrane protein with a long, lumenally exposed carboxy-terminal tail. The predicted orientation appears to be correct based on the findings of Röttger *et al.* Even though

the primary amino acid sequence is an exact match for what we define as an optimal consensus for palmitylation, the presence of the motif in the lumenal compartment may preclude palmitylation.

In this same report by Röttger *et al*, the type II membrane orientation for the A33R protein was confirmed. Only one cysteine residue (C36) is present in the cytoplasmic domain of the protein. When C36 is changed to serine by oligo-directed mutagenesis the A33R protein still incorporated [^{3}H]-PA label, although at slightly reduced efficiency. Serine was chosen to replace cysteine due to similar biochemical characteristics such as size and the polar side chain. In retrospect, this may not have been the best choice. Serine and threonine are also substrates for palmitylation, but only rarely (Schmidt *et al.*, 1995). It may be that replacement of a palmitate-acceptor cysteine with a serine in an optimal environment results in palmitylation of the serine residue through ester linkage.

Many VV palmitylproteins are thought to interact either through disulfide bonds or noncovalently (Payne, 1992). The exact nature of these interactions or their biological significance is not known. These same proteins are involved in the formation of IEV, the precursor of EEV, the propulsion of the IEV particle through and out of cells by actin polymers, or in the infectivity of released virions. They are all specific for the TGN-enveloped virions and prior to virion envelopment are found associated with the membranes of the TGN (Schmelz *et al.*, 1994). It is not likely a coincidence that many of these same proteins are palmitylated. It may be that the palmitate moiety targets these proteins to their respective intracellular

location, i.e. the TGN. Alternatively, it may be that the palmitate moiety stabilizes hydrophobic protein/protein interactions. We have previously reported that at least for the F13L protein (p37), the palmitate modification is necessary for TGN association (Grosenbach and Hruby, 1998; Grosenbach *et al.*, 1997). A mutant of p37 that is not palmitylated is soluble in the cytoplasm of infected cells and cannot function efficiently in regard to envelopment of IMV. It will be of interest to determine the biological significance of the palmitylation of the other VV palmitylproteins.

VV continues to stand out as an excellent system for the study of mammalian protein processing. VV proteins (or exogenous proteins expressed either transiently from transfected plasmids or from recombinant VV) are subject to modifications typical of mammalian systems, to include sulfation, phosphorylation, ADP-ribosylation, glycosylation and as highlighted here, acylation. The ease with which VV is genetically manipulated has facilitated the study of numerous VV gene products leading to the identification of three novel VV palmitylproteins.

Chapter 4

Transmembrane Topology of the Vaccinia Virus 25-kDa Myristylprotein, L1R

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4.1 Introduction

Viruses as obligate intracellular parasites, must use (and in some cases redirect) their hosts' metabolic pathways in order to replicate their genomes and synthesize the constituents needed to form progeny virions. What is perhaps less well appreciated, are the logistical problems encountered by viruses during the replicative process. Once viral proteins have been synthesized, how does the virus ensure that these proteins assume active configurations (at the proper time), interact with the correct protein partners and find their way to specific intracellular locations in quantities sufficient to catalyze the efficient assembly of infectious virions? One of the major ways that viruses solve these problems is by taking advantage of protein modification pathways (such as phosphorylation, glycosylation, proteolytic cleavage, and acylation), which are normally used to assist with intracellular trafficking of cellular proteins (VanSlyke, 1990).

Acylation refers to a series of either co-translational or posttranslational modifications that result in the covalent addition of a fatty acid prosthetic group onto an acceptor protein. Although the precise role(s) of protein acylation is in the process of being experimentally defined, depending on the type of prosthetic group added and the unique characteristics of each acceptor protein, in general acylation appears to increase protein hydrophobicity and affinity for membranes and/or may facilitate protein-protein interactions (Hruby and Franke, 1993). The two major

types of acyl modifications that occur on nascent proteins are myristylation and palmitylation.

Myristylation is a co-translational modification that occurs at the amino terminus of a nascent protein by the time the growing peptide has reached 50 amino acids in length. A 14-carbon saturated fatty acid (myristic acid) is covalently attached to a penultimate amino-terminal glycine residue via a hydroxylamine-resistant amide. The role of the myristate moiety on acceptor proteins remains an enigma as some are found associated with membranes (e.g. $pp60^{src}$, (Buss *et al.*, 1985)) while others are cytosolic (e.g., cAMP-dependent protein kinase, (Clegg *et al.*, 1989)), some are structural proteins (e.g., poliovirus VP4,(Chow *et al.*, 1987)) whereas others are enzymes (e.g., cytochrome b_5 reductase, (Pietrini, 1992). Furthermore, myristylation can also serve as a reversible regulatory switch in signaling pathways such as those involving tyrosine kinases or heterotrimeric G proteins (Bano, 1998).

Unlike myristylation, the second major type of fatty acyl modification, palmitylation, occurs internally within a protein and can occur at multiple sites within the same protein. In this case, palmitic acid is a 16-carbon saturated chain fatty acid that is added following translation via a hydroxylamine-sensitive thioester or ester bond to acceptor proteins (Schmidt, 1989; Bizzozero *et al.*, 1994). The enzyme(s) catalyzing this reaction are believed to be membrane-associated, and thus modification occurs in the context of a membrane environment. This is in agreement with the observation that many palmitylated proteins are also

glycosylated. Most palmitylproteins contain the palmitate attached to a cysteine residue, with serine and threonine residues less commonly serving as modification sites. Palmitylproteins are typically membrane associated and this modification is thought to play a major role in modulating membrane affinity and intracellular signaling. Hence, many palmitylproteins are either receptors (e.g., transferrin receptor, (Alvarez, 1990; Magee, 1985) or associated with the cytoskeleton (e.g., actin, (Caron, 1997).

It appears that many animal viruses have taken advantage of cellular acylation pathways to enhance the hydrophobic characteristics of virus-encoded proteins for their correct localization within the infected cell and/or to facilitate the assembly/disassembly of infectious virions (Zhang, 2000; Peisajovich, 2000; Kim, 2000; Yang, 1996; Roper, 1999). Acylproteins have been reported in a wide range of animal virus families ranging from simple RNA viruses such as the *Picornaviridae* (Urzainqui, 1989) to complex DNA viruses such as the *Herpesviridae* (Brynestad, 1990). Of the major animal virus families, only the *Caliciviridae*, *Filoviridae*, *Arenaviridae* and *Parvoviridae* have not yet been shown to express at least one acylprotein. Most of the reported examples of virus acylproteins contain myristate or palmitate. Regardless of the type of acyl modification, most viral acylproteins (but not all) are destined to be incorporated into assembling progeny virions, either as capsid components or envelope constituents. Since the structure and composition of a virus particle are both

important in determining tissue tropism and host range, viral acylproteins play a central role in pathogenesis in many viral systems.

Poxviruses, such as vaccinia virus (VV), are amongst the largest and most complex of the eukaryotic DNA viruses and are distinguished by replicating exclusively within the cytoplasmic compartment of infected cells (Moss, 1996). VV regulates the expression of its' more than 250 gene products in a temporal fashion during the viral replicative cycle that begins with entry of the virus into the host cell and terminates with the assembly of complex macromolecular structures to form an infectious particle. Unlike many other viruses, VV produces multiple virion forms, all of which appear to be infectious. Although the molecular details of poxvirus assembly and differentiation remain an area of active investigation, the most widely accepted scenario of events that transpire is as follows. After (or concurrent with) viral DNA replication, assemblages of progeny DNA molecules, virion enzymes and structural proteins coalesce at the periphery of perinuclear virosomes to form immature pre-virion particles (Senkevich, 2000; Vanslyke, 1991). These particles acquire two membranes by budding through the intermediate compartment (between the endoplasmic reticulum and the Golgi) to become immature virus (IV) that undergoes a series of morphogenic condensations to become infectious intracellular mature virus (IMV). A portion of the IMV then becomes enveloped by two additional membranes derived from the *trans*-Golgi network to form intracellular enveloped virus (IEV). Following migration towards the cell surface along the cell's cytoskeletal matrix, the IEV particles are propelled

to and though the plasma membrane by rapidly polymerizing "actin rockets" (Cudmore, 1995). During this process, the outermost IEV membrane fuses with the plasma membrane to liberate extracellular enveloped virus (EV) (Moss, 1996). The EV can either remain associated with the cell (cell-associated enveloped virus, CEV) or be released into the external medium as extracellular enveloped virus (EEV) (Moss, 1996). The relative proportion of progeny that are destined to become IMV, IEV, CEV or EEV depends on the strain of virus, cell type and growth conditions. Some poxviruses, such as cowpox virus (CPV), produce yet another virion form. In CPV-infected cells, large inclusion bodies are produced which are composed primarily of a single 160 KDa viral protein (Patel, 1986). Within these A-type inclusions are occluded (and infectious) virions. The phenotype (IMV, IEV, or "other") of the occluded virions has not been determined.

Thus, given the large number of viral encoded proteins, the multiplicity of VV virion forms and the number of distinct intracellular sites used during the viral assembly and morphogenesis process, we hypothesized that VV must employ a number of targeting mechanisms, including protein acylation, to direct viral proteins to the proper location. Experiments carried out over the last decade have verified this prediction in a resounding and surprising fashion. It has been discovered that approximately 10% of VV late proteins are acylproteins. To date, no early VV proteins have been shown to be acylated. Amongst the late VV acylproteins are eight palmitylproteins, four N-myristylproteins and two internally myristylated proteins. Of these, our colleagues and we have discovered and

verified the identity of six of the palmitylproteins (A33R, B5R, F13L, A22R, A36R and A56R), all of the N-myristylproteins (L1R, A16L, G9R and E7R) and one of the internal myristylproteins (A25L). Many of these proteins have been subjected to chemical, physical and functional analyses. In all cases, it has been demonstrated that the fatty acid moiety is required for both targeting and protein function.

Of the VV acylproteins, the L1R protein has received the most attention. The VV L1R gene product is a multi-functional late protein that appears to be involved in both assembly and maturation of infectious IMV particles (Ravanello *et al*, 1994) as well as penetration and disassembly of the virion during the initiation of the subsequent round of infection (Ravanello, 1994). We have previously shown that the protein is N-myristylated and that myristylation together with a N-terminal targeting signal within the L1R protein is necessary for targeting to the correct face of the IMV envelope (Ravanello, 1994). The L1R protein has also been shown to target to the membranes of the intermediate compartment (Ravanello, 1994). Since this is the site of IMV envelopment, this suggests that the L1R protein may be intimately involved in this process. The L1R protein also appears to the terminal acceptor of a intracellular redox pathway (catalyzed by the G4L, O2L and perhaps other VV proteins) that introduces intramolecular disulfide bonds that may contribute to some or all of these activities (Senkevich, 2000).

Due to its intimate association with the IMV membrane and its' multiple biological roles, we have been interested in obtaining the details of how LIR

interacts with a membrane. A hydropathic analysis of the L1R protein predicts one probable and a second possible transmembrane domain (TMD). The predicted orientation of the L1R protein is distinctly different depending on if one or both of the TMDs span the IMV membrane. Our first approach to this problem was to examine *in vitro* the phase partitioning of L1R protein and a series of engineered mutants in Triton X-100. The results of these experiments indicated that L1R behaves like a integral membrane protein and that the myristic acid, TMD1 and TMD2 all had additive effects for increasing affinity for the non-aqueous phase (Eskenassis and Hruby, unpublished data). Recently we have extended these experiments *in vivo*. In the work reported here, reporter signals have been engineered at various locations within the L1R protein. Analysis of the mutant proteins expressed in VV infected cells has allowed the membrane orientation of the L1R protein to be determined and suggests potential roles for various sub-domains of the L1R protein in the assembly and/or disassembly of infectious virions.

4.2 Materials and Methods

4.2.1 Cells and Virus

The recombinant VV, vlacIlacO-L1R, was propagated on confluent monolayers of BSC_{40} (African green monkey kidney) cells were maintained in

modified Eagle's medium (MEM-E; Sigma cell culture reagents; Sigma, St. Louis, MO.), supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Pierce, Santa Fe, CA.), 2 mM L-glutamine (LG), and 10 μg of gentamicin sulfate (GS) per ml at 37°C, 5% CO_2, and 95% humidity. Recombinant virus vlacIlacO-L1R was replicated in the continuous presence of 5 mM isopropylthioglactopyranoside (IPTG). vlacIlacO-L1R (a virus that has the L1R open reading frame transcriptionally regulated by the *lac* operator) was purified from infected BSC_{40} cells by two successive cycles of sucrose gradient centrifugation and titrated by plaque assay as described previously (Ravanello, 1994; Hruby, 1979).

4.2.2 Computer Analysis

The gene and amino acid sequence to L1R (VV Copenhagen strain) were retrieved from GenBank. The amino acid sequence was then submitted for analysis by TMPred to identify potential transmembrane-spanning domains of the protein.

4.2.3 Plasmid Vector Construction

The L1R open reading frame (ORF) was amplified using the polymerase chain reaction (PCR) using the following oligonucleotides: L1R5' (CATATGGGTGCCGCAGC), and L1R3' (GGATCCCTTTTGCATATCCG)

which incorporate a *Nde*I site and *Bam*HI respectively. The amplified gene was cloned into pSH1.0 which contains a multiple cloning site upstream of the VV synthetic early late promoter, using the *Nde*I and *Bam*HI restriction endonucleases found on the plasmid and the amplified gene product to make pL1R. The nucleotide sequence that encodes for the restriction endonuclease Nhe I was inserted into pL1R at three different locations upstream or downstream of the computer predicted transmembrane domain by the Kunkel method of oligonucleotide mutagenesis. The pL1R-1 clone used the oligonucleotide Site 1 (ATGTGCTCTGCGGCTAGCGGATTAACACCG) to insert the *Nhe*I site upstream of the first predicted transmembrane domain (nucleotides 42-85). PL1R-2 was constructed using the oligonucleotide Site2 (CAAATTAAAGAGCTAGCGATACAAAGGTAC) which inserts a *Nhe*I site downstream of the transmembrane 1 and upstream of transmembrane 2. PL1R-3 was generated by using the oligonucleotide Site 3 (CGCTTCCATTGGAGCTAGCCTACTTACTAGGAC), which inserts the last *Nhe*I site downstream of transmembrane 2. The c-Myc epitope was inserted into the Nhe I site of pL1R-1, 2, and 3 by using the restriction endonuclease *Nhe*I found on the respective plasmids and the c-Myc epitope that was generated using the two complementary oligonucleotides Myc5' and Myc3' (AAAGCATCGGCGGATCGGACT and AGTCCGATCATGCTTT respectively). Myc5' and Myc3' were annealed prior to digestion by combining 80 picamoles of each oligonucleotide with annealing buffer, boiled for 1 minute and allowed to cool

to room temperature (25°C). Insertion of the c-Myc epitope into pL1R-1, 2, and 3 generated the clones pMyc-1, 2, and 3. Using the same process as described above a sequence encoding for a synthetic N-glycosylation site were inserted into the pL1R-1, 2, and 3, to generate pGly-1, 2, and 3. The complementary oligonucleotides used to generate the synthetic glycosylation sites were Gly5' and Gly3' (AAAAACGCACTCTACGATCTA and TAGATCGTAGAGTGCGTTTTT respectively). For expression in an *in vitro* transcription and translation system the mutated L1R gene was subcloned (*Nde*I and *Bam*HI digestion) from pMyc/Gly 1, 2, and 3, into the T7 expression vector pET23a (Clonetech) that generated the clones pET23a-Myc/Gly 1, 2, and 3. Clones were confirmed by DNA sequencing.

4.2.4 *In vitro* Analysis of L1R Mutants

Transcription and Translation (TNT) experiments were performed using the TNT T7 quick coupled transcription/translation system from Promega. Experiments were conducted according to supplier's recommendations. Briefly, 0.5 μg of plasmid DNA was added to a microcentrifuge tube containing the TNT T7 quick master mix, [^{35}S]methionine, and canine pancreatic microsomal membranes. The DNA containing mixture was incubated at 30°C for 90 minutes after which the translated products were visualized by autoradiography or used for proteolytic digestion experiments.

4.2.5 *In vivo* Analysis of L1R Mutants

Monolayers (90% confluent) of BSC_{40} cells in 35 mm wells were infected with vLacILacO-L1R at a multiplicity of infection of 10. Concurrent with infection, cells were transfected with plasmids pMyc/Gly-1, 2, and 3 using DMRIE-C reagent (Gibco Life Technologies). Inocula was prepared in polystyrene tubes to which was added 1 milliliter (ml) of MEM-E, the appropriate amount of virus, 25 µl of liposomes, and 10 µg of plasmid DNA. As controls some inocula lacked both virus and DNA (cells alone) an/or virus (virus alone). Cells transfected with pGly 1, 2, 3 were done +/- tunicamycin (glycosylation inhibitor). After 15 minutes had elapsed the inoculum was added to the cell monolayer from which the culture medium had been aspirated. The viral infected cells were placed at 37°C for 4 hours, after which the inoculum was aspirated and replaced with 1 ml of MEM-E with 5% FCS, LG, and GS. At 24 hours post infection (HPI) the cells were harvested in the culture supernatant and transferred to microcentrifuge tubes. The samples were centrifuged in a microcentrifuge 4°C at 15,000 x *g* for 30 minutes, to pellet cells and any virus released during the infection. The supernatant was aspirated and the pellet was resuspended in 80 µl of phosphate-buffered saline (PBS) and freeze-thawed three times for cell lysis. Each sample was then prepared for discontinuous gel electrophoreses (SDS-PAGE) by adding an equal amount of reducing sample buffer and boiled for 3 minutes. The proteins were resolved by utilizing 12% polyacrylamide gels as described previously. Following

electrophoresis the gels were blotted to nitrocellulose filter and subjected to immunoblotting using either α-L1R or α-c-Myc antibodies. L1R antigen-antibody complexes on the filter were detected by incubation with goat anti-rabbit antiserum conjugated to horseradish peroxidase (HRP) and developed with chemiluminescent reagents (Pierce). Anti-cMyc antibodies conjugated to HRP were used, thus only one additional antibody incubation was required.

4.2.6 Proteinase K Treatment

Transcription and translation (TNT) experiments were performed using the TNT T7 quick-coupled transcription/translation system from Promega. Experiments were conducted according to supplier's recommendations. Briefly, 0.5 μg of plasmid DNA was added to a microcentrifuge tube containing the TNT T7 master mix, [^{35}S]methionine, and canine pancreatic microsomal membranes. The DNA containing mixture was incubated at 30°C for 90 minutes after which the translated products were visualized by autoradiography or used for proteolytic digestion experiments. For the proteinase K experiments, TNT extracts were centrifuged at 900 *g* for 10 minutes at 4°C to obtain a pellet. The pellets were resuspended in 20 μl of PBS and then incubated with proteinase K (Boehringer Mannheim) at 10 μg/ml on ice for 30 or 60 minutes after which the digestion was terminated by addition of 1 mM-PMSF for 30 seconds on ice and then boiling for 5 minutes. Treated extracts were then ran on a SDS-PAGE gel and immunoblotted

using the c-Myc monoclonal antibody (Clonetech) following manufacture's recommendations.

4.3 Results

4.3.1 Bioinformatic Analysis

Previous data has suggested that the L1R protein resides within the intermediate compartment of infected cells and is an integral component of the membrane surrounding the IMV particle. It was therefore of interest to determine whether L1R was a bonafide transmembrane protein or associated with these membranes simply by virtue of a hydrophobic interaction between the N-terminal L1R myristate and the corresponding membrane. The predicted amino acid sequence of the L1R protein (Copenhagen strain) was analyzed using the computer program Topology Predictor III, scanning for stretches of hydrophobicity. Regions of 10 or more amino acids that have an overall hydrophobic character are considered possible transmembrane domains. From this analysis, L1R contains two potential transmembrane spanning domains located at residues 85-105 (TMD1) and 185-205 (TMD2). Based on the strong hydrophobic character, TMD2 is highly likely to span the membrane whereas the weaker hydrophobic character of TMD1 makes it less obvious it this region is membrane associated (Figure 4.1A). Based on these predictions, two possible membrane orientations of L1R are

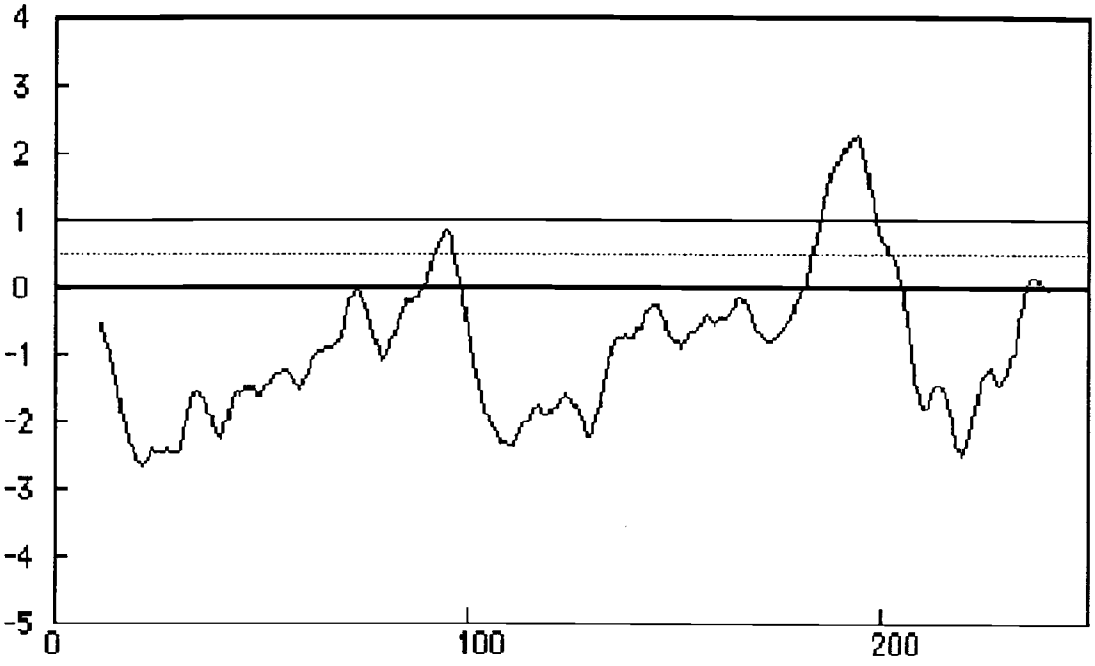
possible (Figures 4.1B, and C). Figure 4.1B represents the L1R protein with the N- and C-termini on different sides of the membrane if only one transmembrane domain exists. Figure 4.1C shows L1R spanning the membrane twice with the N- and C-termini on the same side of the membrane if both predicted transmembrane domains are utilized.

4.3.2 Directed Genetics

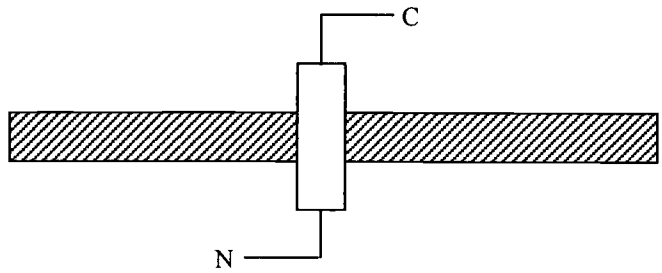
To determine which model of L1R membrane association was correct, site-directed mutagenesis was used to insert a Nhe I restriction site upstream, between and downstream of the two predicted transmembrane domains, TMD1 and TMD2 (Figure 4.3.2.1) of the L1R open reading frame to generate novel in-frame insertion sites. The Nhe I sites were used to insert two different reporter signals. The first was an oligonucleotide sequence encoding nine amino acids with a Asn – Ala – Thr tri-peptide in the middle, thereby creating engineered glycosylation sites pGly1, pGly2, and pGly3 (Figure 4.3.2.2). As an alternative approach, an oligonucleotide sequence encoding nine amino acids containing a well-characterized *c-myc* epitope was inserted into each NheI site generating pMyc1, pMyc2, and pMyc3 (Figure 4.3.2.2). The identity of both inserted peptide signals is shown in Figure 4.3.2.1c. Following sequencing to confirm in-frame insertions and the lack of secondary mutations, both series of engineered L1R mutants (pGly1-3 and pMyc1-3) were

Figure 4.1. Computer analysis of L1R's the predicted amino acid sequence of L1R. A. Hydrophobicity plot generated by the computer program Topology Predictor III. Topology Predictor analyzed L1R's amino acid sequence from the Copenhagen strain of VV and thus generated a hydrophobicity plot. Sections of 10 amino acids or more that have an overall hydrophobic property are seen as a peek in the plot and considered putative transmembrane domains (TMD). L1R has two putative TMD and they are represented by TMD1 and TMD2 on the plot. B. Schematic representation of how L1R would look in a membrane if there were only one TMD. C. Schematic representation of how L1R would look in a membrane if there were two TMD

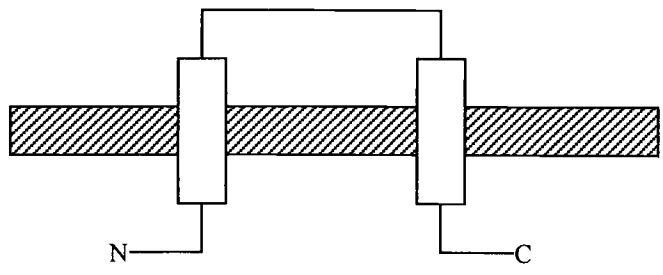
A



B



C



MGAAASIQTTVNTLSERISSKLEQEANASAQTKCDIEIGNGYIRQN

HGCNLTV(^1)KNMCSADADAQLDAVLSAATETYSGLTPEQKAYV

PAMFTAALNIQTSVNTVVRDFENYVQTCNSSAVVDNKLKIQNVIID

ECYGAPGSPTN(^2)LEFINTGSSKGNCAIKALMQLTTKATTQIAPRQ

VAGTGVQFYMIVIGVIILAALFMYYAKRMLFTSTNDKIKLILAKEN

VHW(^3)TTYMDTFFRTSPMVIATTDMQN

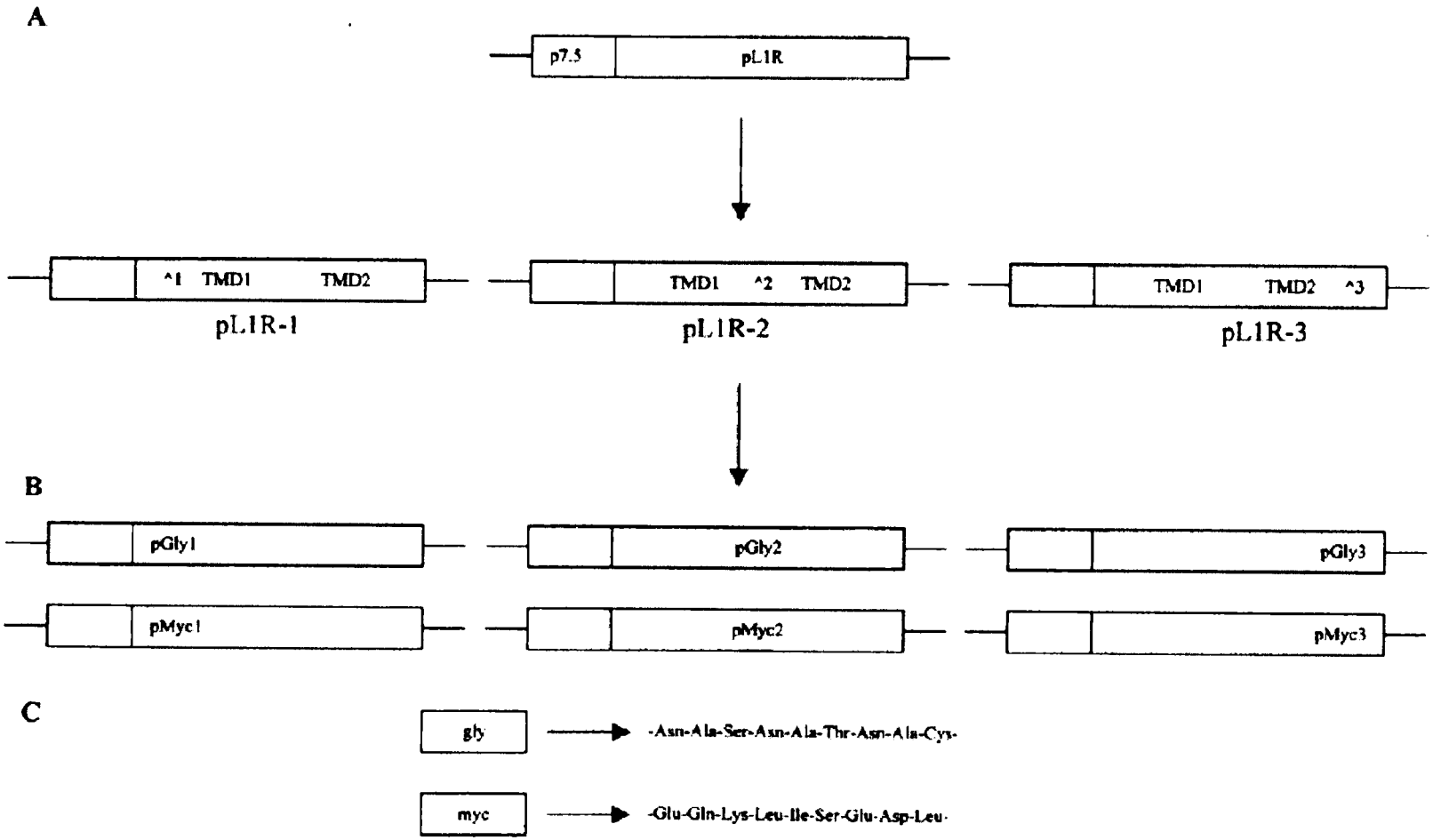
Figure 4.2. L1R mutagenesis strategy. Amino acid sequence is represented by the single letter code. The underlined sections represent TMD1 and TMD2, respectively. Insertion sites for synthetic glycosylation mutation and for the c-Myc epitope are represented by ^1, ^2, and ^3. These mutations were done at the DNA level to insert foreign sequence upstream or downstream of the putative transmembrane domains.

subcloned into a pET23a plasmid expression vector containing a T7 promoter to facilitate subsequent *in vitro* transcription and translation experiments.

4.3.3 Cell-free Transcription and Translation

It is well known that enzymes responsible for N-linked glycosylation are found exclusively on the lumenal side of the ER or Golgi membranes. Therefore, we hypothesized that if the L1R pGly1-3 mutant proteins were synthesized *in vitro* in the presence of canine pancreatic microsomal membranes, the newly synthesized

Figure 4.3. Construction of glycosylated and c-Myc expressing L1R. A. By Kunkel mutagenesis three separate plasmids were generated pL1R-1, pL1R-2, and pL1R-3 that have a unique restriction site inserted into L1R's open reading frame upstream and downstream of the putative transmembrane domains. B. Cloned into pL1R-1, pL1R-2, and pL1R-3 was sequence encoding for a synthetic glycosylation site or the c-Myc epitope (shown in C), to generate pGly1, pGly2, pGly3, pMyc1, pMyc2, and pMyc3.


A
p7.5
pL1R
^1 TMD1 TMD2
pL1R-1
TMD1 ^2 TMD2
pL1R-2
TMD1 TMD2 ^3
pL1R-3
B
pGly1
pGly2
pGly3
pMyc1
pMyc2
pMyc3
C
gly
-Asn-Ala-Ser-Asn-Ala-Thr-Asn-Ala-Cys-
myc
-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Asp-Leu-

proteins would be myristylated (Franke *et al*, 1990) and associate with the microsomal membranes. In doing so, the mutant proteins that intercalated into the membrane in such a manner so as to expose the artificial glycosylation site on the lumenal side would be glycosylated, whereas the mutant proteins with the glycosylation sites exposed on the cytoplasmic face would not be modified. Presence of sugar residues on the protein should be detectable by a concomitant increase in molecular mass when analyzed by SDS-polyacrylamide gel electrophoresis. The results of this experiment are shown in Figure 4.4. It can be seen that wild-type L1R protein migrates with an apparent molecular weight of 29kDa as does the Gly2:L1R protein, indicating that the addition of nine amino acids alone does not produce a detectable effect on protein migration. In contrast, both the Gly1:L1R and Gly3:L1R exhibited slightly reduced mobility, consistent with modification by glycosylation.

4.3.4 *In vivo* Transient Expression

To confirm and extend these *in vitro* observations, pGly1 pGly2 and pGly3 were transiently expressed in cells super-infected with a mutant virus (vLacILacO-L1R) in which the genomic copy of the L1R gene is transcriptionally regulated by the *lac* operator (Zhang, 1991; Ravanello, 1994). In the absence of an inducer such

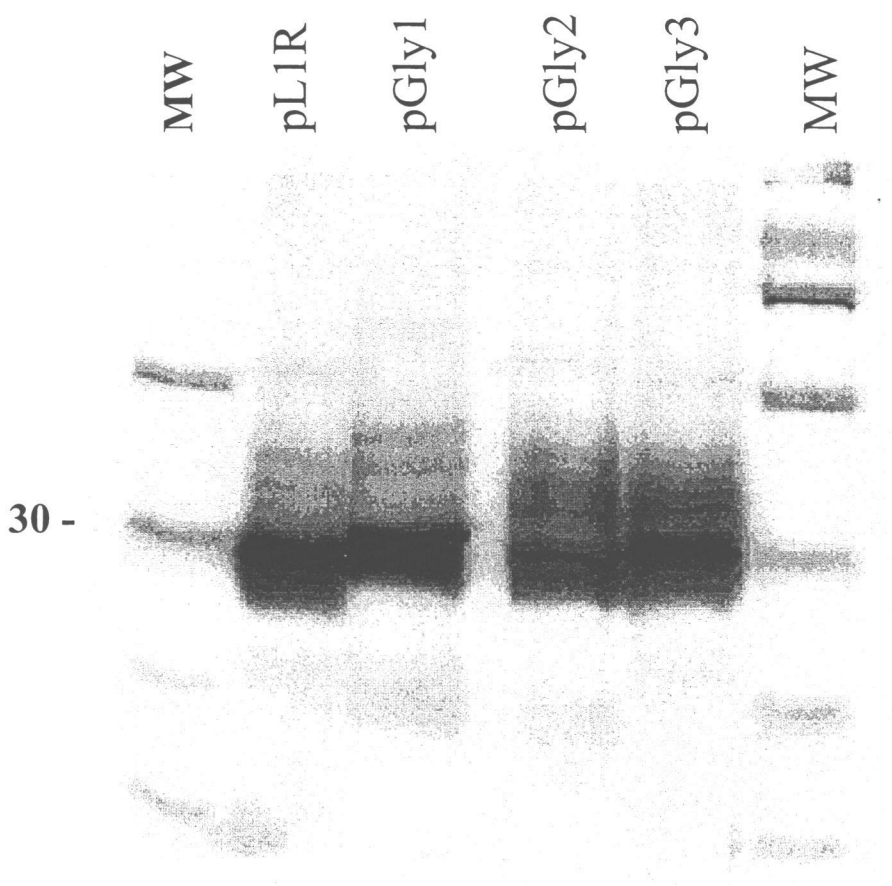


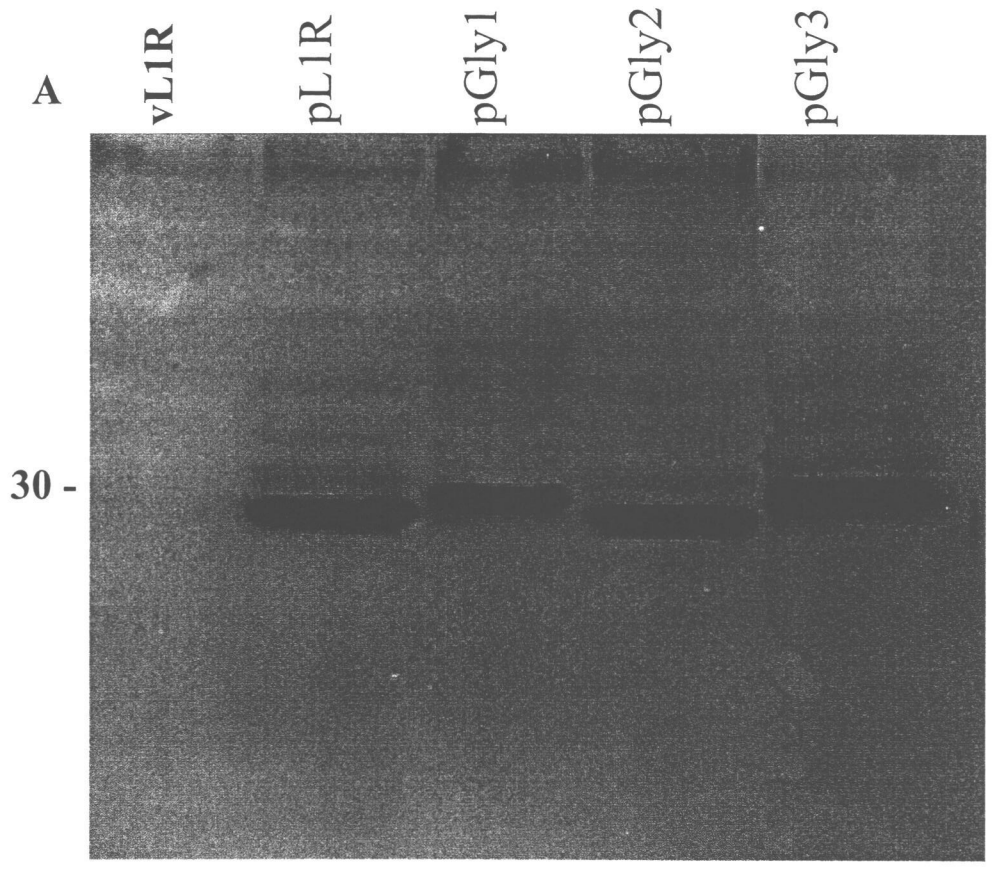
Figure 4.4. Transcription and translation expression of pGly1, pGly2, and pGly3. The glycosylation L1R mutants were cloned downstream of the T7 promoter in the pET23a vector and then subjected to TNT expression in the presence of canine pancreatic microsomal membranes and [^{35}S]methionine. TNT extracts were subjected to SDS-PAGE analyzed by autoradiography.

as isoprenylthioglactoside (IPTG), the native L1R gene cannot be expressed. Using vLacILacO-L1R in the absence of inducer in the transient expression protocol, ensures that the only L1R expressed will be that from the plasmid-derived copy, either wild-type or the engineered mutant forms of the L1R protein. Transient expression of the L1R glycosylation mutants (pGly1-3) was carried out in the absence and presence of the N-linked glycosylation inhibitor tunicamycin (Figure 4.5A and B, respectively). The total transfected cell extracts were analyzed by gel electrophoresis and subsequent immunoblotting using an anti-L1R polyclonal antisera (Ravanello, 1994). The results shown in Figure 4.5A are consistent with the previous *in vitro* experiment, namely that the migration of the pGly1 and pGly3 L1R variants was retarded (relative to wild-type or pGly2 L1R) suggesting that these proteins were being modified by glycosylation. This conclusion was substantiated by the results shown in Figure 4.5B where all the proteins run with the same apparent molecular weight when the transfections were carried out in the presence of tunicamycin.

4.3.5 Proteolytic Digestion

As an alternative approach to confirm the conclusions reached by the glycosylation experiments, the pMyc1-3 plasmids were also expressed in the *in vitro* coupled transcription-translation system in the presence of canine pancreatic

Figure 4.5. Transient expression of L1R glycosylation site mutants. The transient expression vectors pGly1, pGly2, and pGly3 were transfected with cells infected with vlacIlacO-L1R . At 24 hours post infection, virus infected cells were harvested and subjected to immunoblotting using α-L1R antibody. Panel A are extracts expressed tunicamycin, and panel B are extracts expressed in the presence of tunicamycin.


A
vL1R
pL1R
pGly1
pGly2
pGly3
30 -
B
30 -

microsomal membranes. Following protein synthesis, and after allowing time for intercalation of the newly synthesized proteins into the membrane, the reactions were treated with proteinase K for 0, 30, and 60 minutes. The protein extracts were then subjected to gel electrophoresis and immunoblotting using an α–c-Myc monoclonal antibody (Figure 4.6). The results in Figure 4.6 show that in the absence of proteinase K treatment, the pMyc1-3 L1R proteins all migrated with apparent molecular weights of 30 KDa, as expected. After 30 minutes of proteinase K treatment, immunoreactive truncated products of 17-kDa and 14 KDa were observed with pMyc1 L1R and pMyc3 L1R, respectively. No immunoreactive band was observed in the pMyc2 L1R sample that had been similarly treated. This result is consistent with the pGly1-3 data and suggests that the *myc* epitopes of pMyc1 and pMyc3 L1R proteins were oriented within the microsomes where they were protected from degradation, whereas the myc epitope of the pMyc2 L1R protein was accessible to the proteinase K on the cytoplasmic side of the membrane. It appeared that after prolonged incubation (60 minutes) the proteinase K was able to either break down the microsomes and/or cross the membrane and digest all of the immunoreactive epitopes of the pMyc L1R proteins.

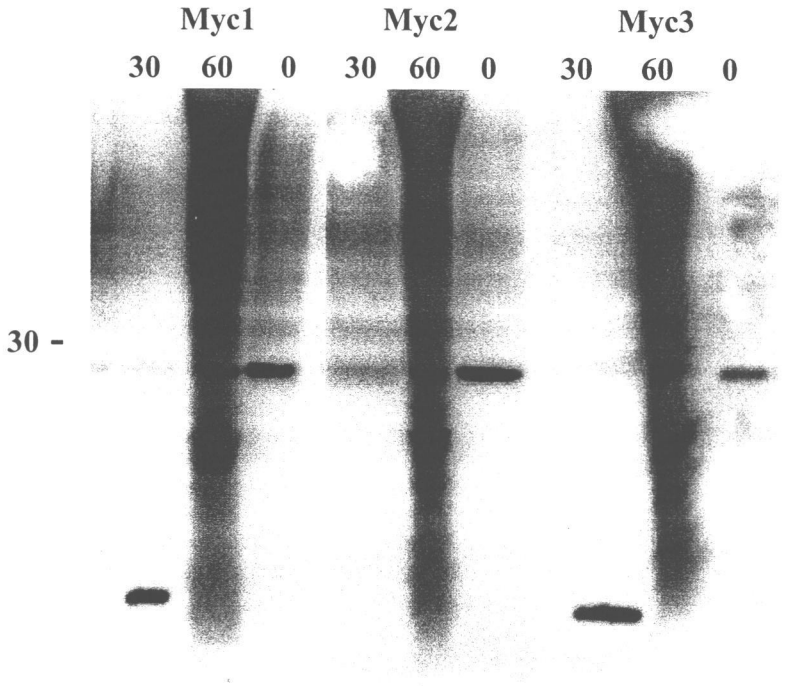


Figure 4.6. Proteinase K treatment of *myc*-containing L1R. The TNT plasmids pMyc1, pMyc2, and pMyc3 were expressed in the presence of canine pancreatic microsomal membranes and [^{35}S]methionine and then treated with proteinase K for 0, 30, and 60 minutes. After proteolytic digestion extracts were analyzed by SDS-PAGE and autoradiography.

4.4 Discussion

The VV myristylprotein L1R protein appears to play a central role both in the assembly of infectious progeny virions, and in the binding, penetration and disassembly of viral particles during the subsequent round of infection. Since all of these activities involve membranes, and since previous studies have demonstrated that the L1R is exclusively targeted to the IMV membrane, it was of interest to determine if L1R was a bonafide membrane protein, and if so, what the topology of

it's membrane interaction was. Computer analysis predicted two transmembrane domains, one (TMD1) with low probability and another (TMD2) with high probability. To determine if these putative membrane-spanning regions were employed, we used an approach of insertional mutagenesis to probe this question. Short peptide regions encoding either a synthetic glycosylation site or a *myc* epitope were inserted N-terminal, between or C-terminal to the two transmembrane domains. Although this approach carries the inherent risk that the introduced sequences will change the structure, stability, or targeting of the mutant proteins, all of the experiments conducted here indicated that the mutant proteins had an identical phenotype to wild-type L1R protein. Remarkably, analysis of the two series of mutant L1R proteins, both in vitro and in vivo, produced data that was consistent with only one conclusion: L1R is a integral membrane protein, both transmembrane domains span the membrane, and the N- and C-terminal regions of the L1R protein are oriented within the viral particle whereas the middle of the L1R protein is on the outside of the viral particle (Figure 4.7A).

Based on this result and in view of what is known about the L1R protein, we have developed the following working model to explain the many roles of the L1R protein during the VV replication cycle (Figure 4.7A). The protein is synthesized and co-translationally N-myristylated at late times during infection. The myristic acid and N-terminal L1R membrane targeting signal collaborate to translocate the L1R protein into the membranes of the intermediate compartment

(IC). The L1R protein accumulates there until a signal triggers glutaredoxin-like ("GTX") redox pathway to introduce intramolecular disulfide bonds into L1R.

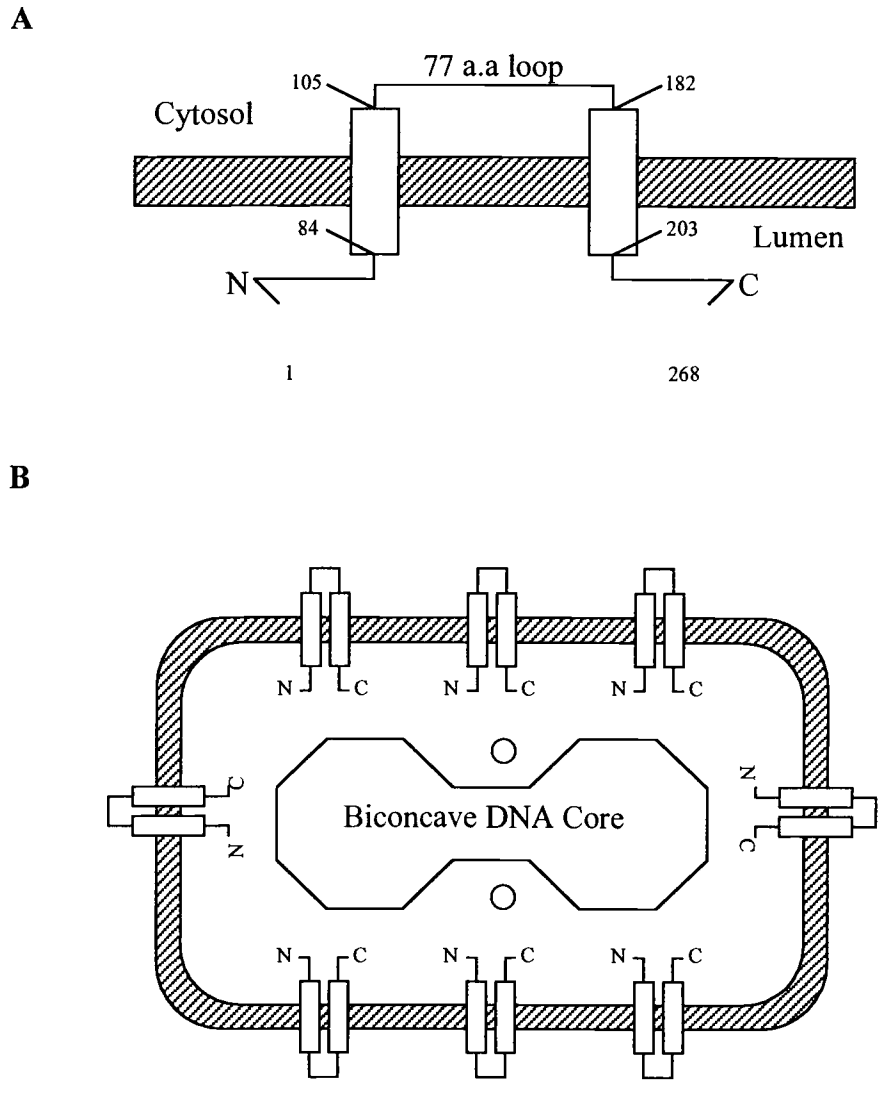


Figure 4.7. Model of L1R virion association. Data obtained in these experiments suggest that L1R is orientated in the membrane according to panel A. L1R thus contains two transmembrane domains and is positioned such that its amino- and carboxy-terminals are located on the lumen side of the membrane. B. Schematic representation of how L1R may be positioned in the IMV membrane as a result of budding through the intermediate compartment.

In doing so, the structure of the L1R protein becomes spatially constrained which causes the membrane to begin bending to form membrane crescents. In this configuration, the N- and C-termini of the L1R protein serve as a docking signal for a viroplasmic previrion to bud through the IC-L1R membrane to form an immature virion particle (IV) that can then undergo morphogenesis into infectious IMV. This model also serves as a basis for understanding the role of L1R during penetration. The binding of the extraviral loop of L1R to an appropriate cellular receptor could trigger the removal of the intramolecular L1R disulfide bonds and relaxation of the IMV envelope to participate in membrane fusion and liberation of the IMV core into the cytoplasm to initiate infection. This is an attractive model that accounts for much of what we know about L1R protein biology. These concepts will serve as a framework for future experiments that will be designed to test various aspects of the model and to identify regions of the L1R protein, which participate at various stages as well as attempting to identify the other viral proteins, which participate in these key replicative processes.

Chapter 5

BiZyme: A Novel Fusion Protein Mediating Selection of Vaccinia Virus Recombinants by Fluorescence and Antibiotic Resistance

Scott G. Hansen, Torrey A. Cope, and Dennis E. Hruby

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30 pages

5.1 Introduction

Vaccinia virus (VV) has played an important role in history as the first successful vaccine developed to combat a human disease. Global immunization efforts with VV resulted in the eradication of smallpox in the late 1970's, thereby terminating further need for this viral vaccine. The utility of VV has persisted however, as it is now widely employed as a valuable research tool. Specifically, VV is being used as an eukaryotic cloning and expression vector for the study of various gene systems, to over-express proteins of interest, and for the development of new vaccines and immunotherapeutics (Belyakov *et al.*, 1999; Moss, 1991; Moss, 1996a).

There are several aspects of the VV replication cycle that make this virus particularly amenable to laboratory manipulation. First, is the availability of the entire genome sequence (Goebel *et al.*, 1990; Johnson *et al.*, 1993). This information provides a quick means of comparison of predicted VV genes to similar viral, prokaryotic, or even eukaryotic sequences for the elucidation of gene function, and provides the basis for designing effective gene insertion strategies. Second, analysis of the VV genome has shown that many VV proteins are dedicated to nucleic acid metabolism and biosynthesis (Moss, 1996; Challberg and Englund, 1979; Spencer *et al.*, 1980; Kao *et al.*, 1981; Larder *et al.*, 1987), a feature contributing to the ability of VV to replicate entirely within the host cell cytoplasm. The cytoplasmic replication mode employed by VV facilitates homologous

recombination of plasmid-borne foreign genes. Third, proteins expressed by VV in the cytoplasm are subjected to a wide range of cellular post-translational modifications including N- and O-linked glycosylation, phosphorylation, acylation, proteolytic cleavage, polarized membrane and nuclear transport, and secretion, and are therefore expressed foreign gene products are usually indistinguishable in structure, location and activity to the bonafide protein (VanSlyke, 1990). In addition, due to the extranuclear VV replication strategy, expression of a foreign gene from the context of the VV genome does not require splice junctions enhancers, or RNA targeting signals. Recombinants expressing such diverse foreign proteins such as streptococcal M protein (Hruby *et al.*, 1988), HIV gp120 envelope glycoprotein, and a membrane-bound tyrosine kinase (Moss, 1991) have all been produced.

The large VV genome allows insertions of up to 26,000 base pairs of foreign DNA (Merchlinsky and Moss, 1992) and there are a number of non-essential loci that have been targeted for homologous recombination, such as sites within the *Hin*dIII fragments C, F, and K, and the hemagglutanin and thymidine kinase genes (Coupar *et al.*, 2000). Recombination vectors can be constructed where a gene of interest is abutted to a native VV promoter and flanked by sequences homologous to a specific region of the VV genome. These homologous sequences are able to catalyze a double reciprocal recombination event that results in the generation of recombinant virus (Ball, 1987 & 1995). While VV recombination occurs at a very high fidelity, with at least 99% accuracy, the event

occurs at a very low frequency, resulting in recombination in approximately 0.1% of the progeny virus genomes (Ball, 1987 & 1995). This makes selection and amplification an essential aspect of generating recombinants. Most of the techniques that are currently available rely on a specific phenotype of the recombinant virus such as antibiotic resistance or altered plaque formation.

Though effective, the recombination systems have a major disadvantage in the time required to isolate pure VV recombinants. Even at a low multiplicity of infection, several viruses can co-infect the same cell resulting in rescue of wild type virus by recombinants and heterogeneous viral populations. Due to the low frequency of recombination, the titer of recombinant virus must be significantly increased for isolation of pure recombinant stocks. This requires numerous passages of the virus by transferring the total cell extract of one viral-infected culture to a new cell monolayer in the presence of a selective agent such as G418. Though this process will eventually dilute out WT virus, it requires several weeks before recombinant virus can be isolated by plaque purification. Therefore, current methods are not well suited for rapid generation of recombinants required for a variety of purposes in molecular biology.

In this contribution, we describe a new selection methodology that was developed to overcome these limitations. The genes encoding Green Fluorescent Protein (GFP) and neomycin phosphotransferase have been fused together to generate a single protein that retains both biological activities. This bifunctional protein, BiZyme, serves to greatly reduce the time required to isolate and purify

recombinant virus by coupling fluorescence to antibiotic selection. A homologous recombination vector, pGNR, was constructed to quickly produce purified recombinants by insertional inactivation of the VV TK locus with the BiZyme gene. The versatility of the pGNR selection system was demonstrated by the production of recombinant virus capable of expressing chloramphenicol acetyltransferase and the chlamydial protein *Inc*A. These results suggest that the VV recombinants generated by use of pGNR can be effective tools for protein expression and a means for the rapid generation of recombinant viral vaccines.

5.2 Materials and Methods

5.2.1 Construction of Vectors

The green fluorescent protein gene (*gfp*) was amplified from the vector pGFP-C2 (Cormack, Valdivia, and Falkow, 1996) with primers GFP1: 5' GGA ATTCCATATGGTGAGCAAGGGCGAG 3' and GFP2: 5'CCGGAATTCCTTG TACAGCTCGTC3' which introduced the restriction sites *Nde*I and *Eco*RI respectively. The *neo* gene was also amplified separately from pGFP-C2 with the primers NEO1: 5' CCGGAATTCATGATTGAACAAG 3' introducing an *Eco*RI site and NEO2: 5' CGCGGATCCTCAGAAGAACTCGTC 3' including a *Bam*HI site. The expression vector pSH2.0 (Hansen, 2000) containing the VV synthetic early/late promoter (Chakrabarti, Sisler, and Moss, 1997) was then used as the

cloning vector. First *gfp* was cloned into *Nde*I and *Eco*RI sites of pSH2.0, followed by cloning of *neo* between the *Eco*RI and *Bam*HI sites. The structure and sequence of the vector, pNeoGreen, was confirmed by sequence analysis.

The GFP-NEO recombination vector pGNR was derived from the pUC118 cloning vector with the *Hin*dIII restriction site deleted. pUC118 was digested with *Hin*dIII and the sticky ends were filled in by T4 DNA polymerase (1 µl T4 DNA polymerase, 2µl T4 DNA polymerase buffer, 2µl 10 mM dNTPs, 14° C for 20 min; MBI Fermentas). The two blunt ends were ligated back together, generating an *Nhe*I restriction site. An approximately 600 base pair region flanking the upstream portion of the J2R ORF (encoding for thymidine kinase) of VV WR was PCR amplified using the primer TKUp1 to introduce a 5' *Sac*I restriction site: 5' TCCGAGCTCATACCCGATCCTATT G 3' and TKUp2 to introduce 3' sites *Bam*HI and *Eco*47III: 5' CGCGGATCCAGCGCT GATGACAATAAAGAAT 3'. The J2R upstream fragment was ligated into pUC118-*Hin*dIII between the *Sac*I and *Bam*HI restriction sites, generating pTKUp. The downstream flanking portion of the J2R ORF (TKDown) was then amplified with primers TKDown1 and TKDown2 which introduced 5' *Bam*HI, *Stu*I, and *Cla*I sites and a 3' *Nhe*I site: 5'CGCGGATCCAGGCCTTATTATTATTTTTTATCTAA 3' and 5'CTAGCTAG CGATCCATTGATCTGGAAAC 3', respectively. This segment was inserted into the *Bam*HI and *Nhe*I sites of pTKUp to generate pTKUp/Down. The GFP-NEO fusion was then isolated from pNeoGreen by restriction with *Bam*HI and *Hin*dIII. Sticky ends from the digest were then filled in by T4 DNA polymerase as described

above to generate blunt ends for insertion into the *Eco*47III site of pTKUp/Down. The resulting pGFP-NEO clones were screened for GFP-NEO orientation complementary to the thymidine kinase flanking sequences. The 7.5 synthetic early/late VV promoter was then isolated from the expression vector pDG4.0 (Grosenbach *et al.,* 1997) with oligonucleotide primers 7.5/1 and 7.5/2, introducing an upstream *Bam*HI site and a downstream *Stu*I sites: 5' CGCGGATCCGGTCGACCTGCAGATATAC 3' and 5'AAAAGGC CTGTCACTGTTC TTTATGATTC 3', respectively. The segment was then inserted into the *Bam*HI and *Stu*I sites of pGFP-NEO, generating pGFP-NeoPro. A multiple cloning site (MCS) was designed and synthesized as two complementary primers: 5' AAAAGGCCTACTAGTGTTAACCTTAAGCCCGGGATCGAT GGG 3' and 5'CCCATCGATCCCGGGCTTAAGGTTAACACTAGTAGGCC TTT 3', respectively. The primers were annealed by boiling 80 µmol of each primer with 1 µl annealing buffer (500 mM NaCl, 200 mM TrisCl, and 20 mM $MgCl_2$) and 6 µl water for 3 min and allowing the oligonucleotides to cool to 25°C. The double-stranded MCS was then inserted into the *Stu*I and *Cla*I sites of pGFP-NeoPro, producing a polylinker of 6 unique restriction sites: *Stu*I, *Spe*I, *Hpa*I, *Afl*II, *Sma*I/*Xma*I, and *Cla*I in the completed plasmid, pGNR. All intermediate plasmids were initially confirmed by restriction analysis and agarose DNA gel electrophoresis. Final confirmation was obtained by dideoxy DNA sequencing.

Two recombination vectors containing foreign genes for insertion into the TK locus of VV were derived from pGNR. The chloramphenicol acetyltransferase

gene was amplified (*cat*) by PCR from pVV5.1:CAT (Hruby *et al.*, 1990) using the primers cat1 and cat2, which introduced *Spe*I and *Sma*I restriction sites, respectively: 5' GACTAGTATGGAGAAA AAAATC 3' and 5'TCCCCCGGGTT ACGCCCCGCCCTG 3'. Additionally, chlamydial *Inc*A was amplified from pRB21-*Inc*A (Rockey *et al.*, 1997) using primers incA1, introducing a *Spe*I site and incA2 introducing a *Sma*I site: 5' GGACTAGTATGA CAGTATCCACA 3' and 5' TCCCCCGGGTTAACTATCTTTATG 3'. The PCR products *cat* and *Inc*A were then inserted into separate pGNR plasmids between the *Spe*I and *Sma*I sites of the multiple cloning site, generating the two recombination/expression plasmids pGNR.CAT and pGNR.IncA. The correct plasmid sequence was confirmed by restriction analysis and DNA sequencing.

5.2.2 Cells and Virus

African green monkey kidney cells (BSC_{40}) were cultured in modified Eagle's minimal essential medium (MEM-E, Mediatech) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Biowhittaker), 2mM L-glutamine (LG), 10 μg/ml gentamicin sulfate (GS). Incubation of the cells was at 37°C, 95% humidity, and 5% CO_2. VV western reserve (WR) strain was grown and titered as described previously (Madalinski *et al.*, 1977).

5.2.3 Fluorescent Microscopy

BSC_{40} monolayers were seeded on glass cover slips (VWR Scientific) in 35 mm wells at a density of 1.2 x 10^6 cells/well and cultured at 37° C to 80% confluency. After pre-treating with G418 24 hrs prior to infection at a concentration of 200 mg/ml, cells were infected with VV at a multiplicity of infection (MOI) of 1 and transfected with pGNR as described above. At 12 hpi, infection medium was aspirated and virus-infected cells were washed with 2 ml PBS-M. Cover slips were recovered and placed on glass slides (Fisher Brand). Virus-infected and control cells were visualized using a Leica DMLB fluorescent microscope equipped with a UV filter for green fluorescence. Images were obtained through a 10X eyepiece and 100X oil immersion lens with a Spot digital camera (Diagnostic Instruments).

5.2.4 Engineering of Recombinant Virus

The plasmids described above, pGNR, pGNR.CAT, and pGNR.IncA, were transfected into BSC_{40} cells simultaneously infected with VV. Ten micrograms of each plasmid were added to 1 ml infection media in separate polystyrene conical tubes. Twenty-five microliters of liposome transfection reagent DMRIE-C (Gibco Life Technologies) were added to each tube. The tubes were vortexed and incubated at room temperature for 15 min. Sufficient virus was then added to each tube to achieve an MOI of 1. MEM-E growth medium was aspirated from cell

monolayers prepared in 35 mm wells and replaced by plasmid/liposome/virus inocula.

Recombinant virus was selected for by adding 200 mg/ml (Franke *et al.*, 1985) G418 to BSC_{40} monolayers 24 hpi. Infections were allowed to continue for another 24 h before cells were harvested into a 1.5 ml microcentrifuge tube by washing with culture medium. Virus and cells were pelleted by microcentrifugation at 15000 × G and 4° C for 10 min. Following aspiration of the supernatant, 80 µl of ice-cold PBS-M (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 1mM $MgCl_2$) was used to resuspended the pellet. Virus was released from the cells by a freeze-thaw method in which the resuspended pellet was frozen at -80° C, partially thawed, and vortexed. This process was repeated three times and the solution was subjected to centrifugation at 2000 × G, 4 °C to pellet cellular debris. A dilution of the isolated supernatant was used to infect new cell monolayers for to continue selection of recombinants by plaque purification.

For plaque purification experiments, 40 µl of the viral suspension was added to 1 ml infection media. The inoculum was diluted to 10^{-3} and then added to confluent BSC_{40} monolayers in 35 mm well plates in serial dilutions to 10^{-8}. A 1.2 % agarose overlay supplemented with MEM-E was prepared by combining 1.2 g Sea Plaque agarose (FMC BioProducts) and 50 mL dd H_2O, autoclaving the solution, and adding 50 ml MEM-E after the solution cooled to 37° C. The infection inoculum was removed from the cells at 4 hpi and replaced with 1 ml of

the prepared MEM-E agar overlay. Infections were allowed to continue for 48 hrs and viewed with an inverted fluorescent microscope (Leica DMIL) to identify fluorescent plaques.

Agarose plugs containing recombinant virus were isolated by stabbing fluorescent plaques with Pasteur pipettes. Following resuspension in 80 µl PBS-M, the recombinant virus was added to new monolayers for further amplification, large-scale preparation, and analysis.

5.2.5 SDS-PAGE and Immunoblotting

BSC_{40} monolayers in 35 mm wells were infected with positive control vCAT, described previously (Hruby *et al.*, 1990), vGNR, and vGNR.IncA at a MOI of 10, or transfected with pNeoGreen, pGNR, and pRB21:IncA. Cell lysates were obtained at 12 hpi by harvesting and freezing and then thawing cells as described above. Equal volume (80 µl) 3x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample reducing buffer (50 mM Tris pH 6.8, 1 % (wt/vol) SDS, 0.1 5 (vol/vol) 2-mercaptoethanol, 1 % (vol/vol) glycerol) was added and samples were boiled for 3 min. Proteins were then resolved by discontinuous gel electrophoresis as described previously using 12.5 % polyacrylamide, and transferred from gels to nitrocellulose (Studier, 1973). The proteins were detected by probing blots with primary antibody directed against VV antigen TK and foreign antigens *Inc*A and GFP. TK and *Inc*A antibodies obtained from polyclonal

antisera produced in rabbits were used as described previously (Franke *et al.*, 1985; Rockey *et al.*, 1997). Following primary antibody incubation, secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) was used to facilitate detection. Polyclonal GFP antiserum was obtained from Invitrogen and used according to manufacturer's suggestions. Immunoblots were developed by incubation with Super Signal chemiluminescent substrate solutions (Pierce) followed by exposure to BioMax MR-2 film (Kodak).

5.2.6 Chloramphenicol Acetyltransferase Assays

Monolayers of BSC_{40} cells were grown to confluency on 35 mm plates and infected with vCAT, transfected with pGNR.CAT, or infected with vGNR. At 24 hpi, cells were lysed by the freeze-thaw method described above. Pelleted total cell extracts containing cells and virus was resuspended 80 µl PBS-M. Sixty microliters of the cell extract was incubated with 1 µl 1.0 mM bodipy FL-1-deoxychloramphenicol (Sigma) in 0.1 M Tris-HCl pH 7.8 and methanol (9:1) at 37° C for 5 min. Ten microlitersl of 4 mM acetyl-CoA (Sigma) was added and the incubation was continued for 30 min. The reaction was stopped by adding 1 ml ice-cold ethyl acetate and vortexing to extract the substrate. The phases were separated by brief centrifugation and the top 900 µl of ethyl acetate were transferred to a new tube. The ethyl acetate was then evaporated and the residue was resuspended in 20 µl ethyl acetate.

Aliquots (5 µl) of each sample were then subjected to thin-layer chromatography (TLC). Acetylated and unreacted bands for each sample were scraped from the TLC plate into separate tubes and extracted in 1 ml spectrophotometric-grade methanol. Samples were centrifuged to pellet the silica gel and the supernatant was transferred to a fresh tube. Samples were diluted 1:100 and fluorescence was measured on a flourimeter set at 490 nm for excitation and 512 nm for measurement (Hruby *et al.*, 1990). Results were quantified by relative fluorescence according to the equation:

$$\% \text{ conversion} = \frac{FI_{product} \times 100\ \%}{FI_{substrate} + FI_{product}}$$

where FI is the fluorescence intensity.

5.3 Results

5.3.1 Construction of Recombination Vectors

Insertional inactivation of the vaccinia thymidine kinase ORF (J2R) provides a site within the VV genome for the introduction of foreign genes. Previous studies have shown that inactivation or deletion of this locus does not affect virus growth *in vitro* and has an attenuating effect *in vivo* (Buller *et al.*, 1985), which can be considered advantageous when constructing a candidate vaccine. These considerations led to the design and construction of a

recombination vector using TK gene flanks and the bifunctional enzyme GFP-NEO, hereafter referred to as BiZyme.

BiZyme was constructed by abutting the gene sequences encoding the green fluorescent protein (*gfp*) and neomycin phosphotransferase activity (*neo*) within the context of a standard VV expression vector. The stop codon of *gfp* was removed during PCR-amplification of the gene, allowing direct translation of *gfp* and *neo* as one continuous ORF with termination at the stop codon of *neo*. *Gfp* and *neo* have open reading frames of 797 and 794 base pairs and are predicted to code for proteins of 30 and 29 kDa, respectively. Translation of the complete fusion cassette should result in a single protein of 530 amino acids with a predicted molecular weight of 60 kDa. The fusion gene was cloned downstream of a VV synthetic early/late promoter to produce a complete cassette capable of expression during all times of infection.

A recombination vector was constructed by PCR amplification of the upstream and downstream flanks of the TK gene and inserting them into a pUC-derived expression vector. In order to facilitate selection of recombinants by fluorescence and antibiotic selection, the BiZyme construct was then subcloned into the recombination vector. The pGNR vector was completed with further addition of the native 7.5 VV promoter and 6 unique restriction endonuclease sites for insertion of a foreign gene (Figure 5.1). Care was taken to place the synthetic early late promoter that drives BiZyme expression and the 7.5 promoter in opposite orientation to ensure optimal expression of BiZyme and the putative gene of

interest. It is also of note that different promoters were used for the two cassettes to prevent possible deletion, or "looping out," of intervening sequences during DNA replication in viral-infected cells.

5.3.2 Fluorescent Microscopy to Test BiZyme Activity and Recombination

Following construction of the recombination vector, it was necessary to determine the functionality of BiZyme *in vitro*. BSC_{40} cells pre-treated with G418 were infected with WR strain VV and transfected with pGNR. Following sufficient time for protein expression, monolayers of uninfected, WT VV-infected, and the pGNR transfected cells were visualized by microscopy. Fluorescent and phase-contrast images were overlaid to produce the combined images of Figure 5.2. As seen in Figure 5.2A and Figure 5.2B, uninfected and VV-infected cells showed minimal background fluorescence.

Virus-infected cells also showed the characteristic cell-rounding cytopathic effect (CPE) of vaccinia infection. pGNR-transfected cells also display CPE and had diffuse cellular fluorescence as seen in Figure 5.2C, indicative of intracellular *gfp*. This result also is consistent with the cytoplasmic localization of neomycin phosphotransferase (Franke *et al.*, 1985), which allowed protein expression under these conditions.

5.3.3 Recombination Vectors Containing Foreign Genes

Results of fluorescent microscopy indicated that BiZyme retained both the fluorescence and antibiotic resistance functions of its constituent proteins. The possibility that this enzyme could be a potent selectable marker for recombinants suggested that the vector should be further analyzed for use in selection of recombinant virus. To this end, the recombination vectors pGNR.CAT and pGNR.IncA were constructed (data not shown). Chloramphenicol acetyl transferase (CAT), a common antibiotic resistance gene found in bacterial species such as *Escherichia coli*, was chosen for its readily detectable and assayable activity. A vector expressing the foreign antigen *Inc*A, a protein isolated from chlamydia species was also constructed to demonstrate the versatility of the recombination system in generating potential recombinant viral vaccines.

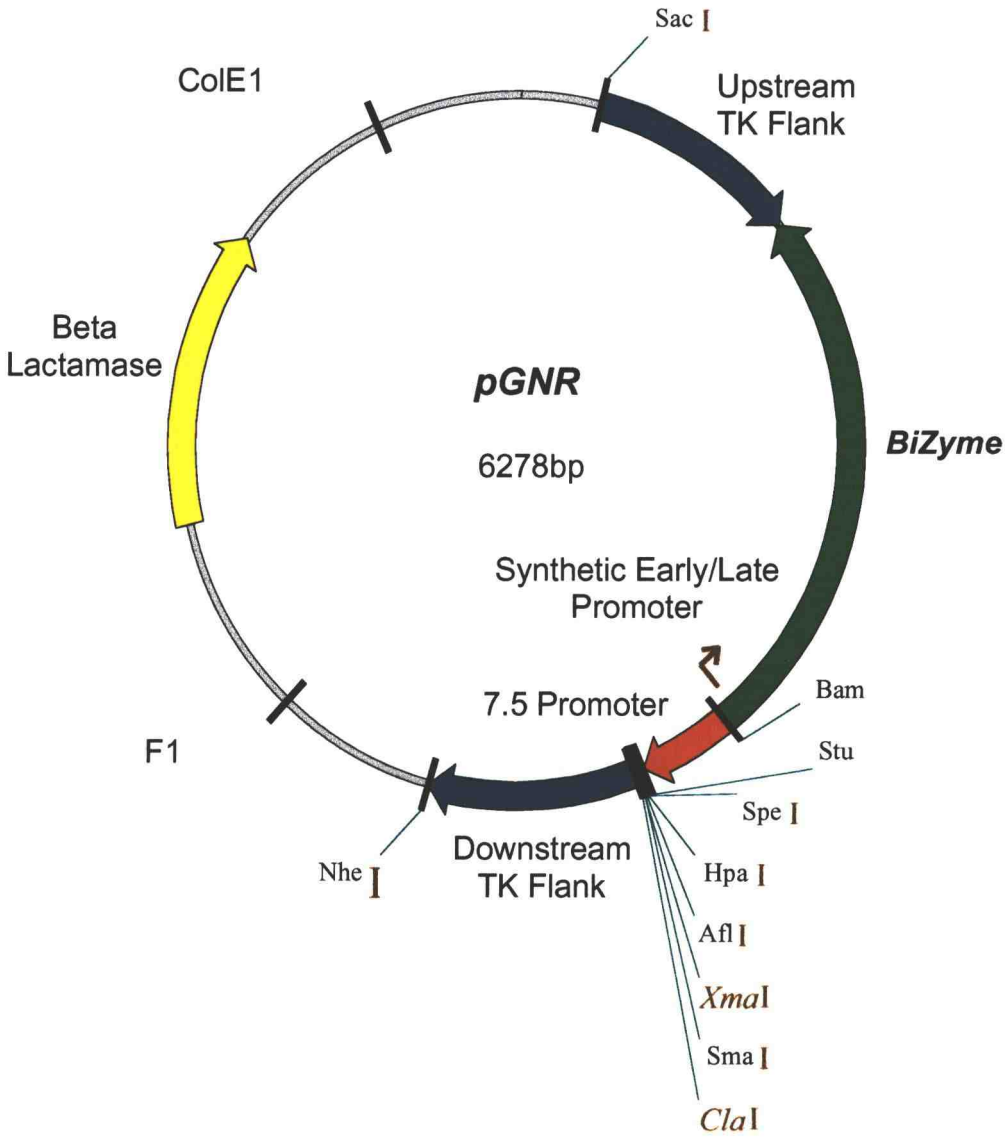

Figure 5.1. Plasmid map of pGNR. The recombination vector pGNR was designed to facilitate homologous recombination directed at the VV thymidine kinase (TK) locus. As a derivative of the pUC family of expression vectors, pGNR contains both bacterial and phage origins of replication and an ampicillin resistance marker for ease in selection during cloning. In addition, the vector contains sequences homologous to the upstream and downstream flanks of the VV J2R open reading frame, the gene coding for TK.

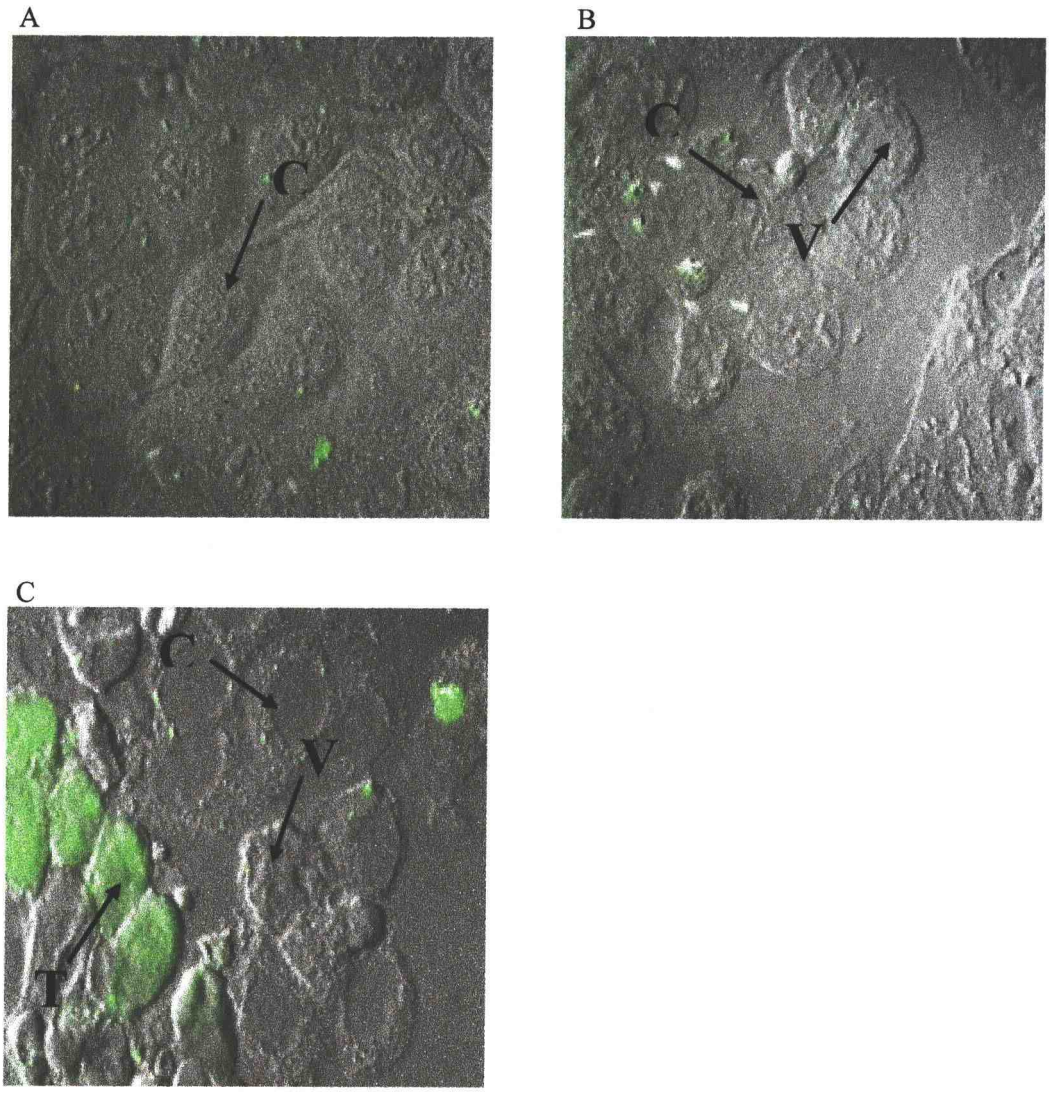

Figure 5.2. Fluorescent microscopy of pGNR transfected cells. Panel A shows a BSC_{40} cell monolayer in G418-containing growth medium. Overlaid phase contract and green fluorescence images show typical uninfected cells (c). The cells in panel B, infected with WT VV under G418, show both uninfected and infected cells (i) that display a rounding cytopathic effect. Infected cells that are also transfected (t) with pGNR are shown in panel C.

5.3.4 Selection of VV Recombinants

The ability of the pGNR vector to mediate homologous recombination and facilitate recombinant selection was next evaluated. The vectors pGNR, pGNR.CAT, and pGNR.IncA were transfected into VV infected BSC_{40} cells. At 24 hpi, the appropriate amount of G418 was added to begin selection for recombinants. After an additional 24 hours, total cell extracts containing both recombinant and non-recombinant virus were used to infect new BSC_{40} monolayers. Following sufficient time for plaque formation to occur, cells were analyzed by inverted light microscopy (Figure 5.3A and C) to identify plaques. To specifically isolate recombinants from the heterogeneous population, the plaques were then viewed by fluorescence microscopy to note those generated by non-recombinant (Figure 5.3B) or recombinant virus (Figure 5.3D). Fluorescent plaques from recombinant viruses vGNR, vGNR.CAT, and vGNR.IncA were recorded and picked for further amplification, purification, and analysis.

5.3.5 Immunoblot Analysis of vGNR Recombinant Gene Expression

Recombination mediated by the pGNR vector is intended to target the TK locus of VV. In order to test for correct insertion of BiZyme into the viral genome and consequent inactivation of the TK locus, immunoblot analysis was used to test

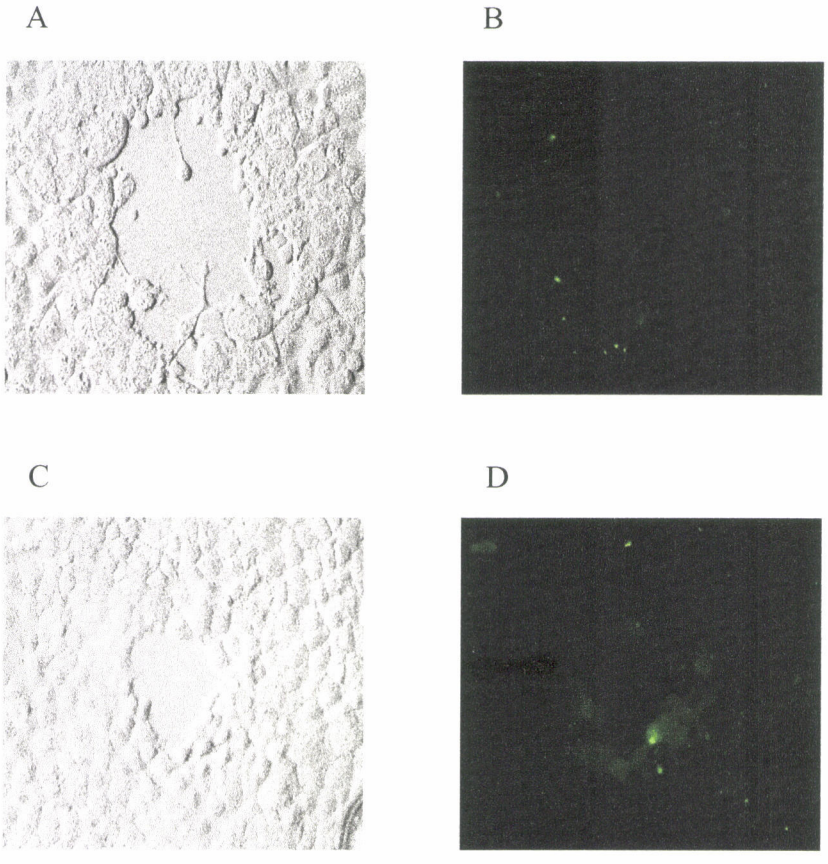

Figure 5.3. Selectable plaques formed by vGNR. Following transfection of BSC_{40} cells with WT VV and pGNR, total cell extracts were used to infect new cell monolayers. At 48 hpi, plaques identified by light microscopy (Panels A and C) were also analyzed for green fluorescence. Plaques formed from WT VV (Panel B) showed no fluorescence while plaques formed by recombinant (vGNR, Panel D) were visibly fluorescent and therefore selectable.

for the presence of BiZyme and the absence of thymidine kinase protein in vGNR-infected cells. Fluorescent plaques containing vGNR recombinants isolated as described above were subjected to two rounds of amplification under G418 antibiotic selection. Total cell extracts harvested from the final infection were

subjected to protein separation by SDS-PAGE and visualized by immunoblotting with anti-GFP and anti-TK polyclonal antibodies separately, as shown in Figure 5.4.

Lanes 1-3 in Figure 5.4A show proteins probed with α-GFP. Controls from cells alone, VV-infected cells, and vCAT showed no protein bands. Lysates from pNEOGreen- and pGNR-transfected and vGNR-infected all show a band of approximately 56 kDa. The predicted molecular weight of the GFP-Neo fusion protein is 60 kDa, as calculated by VectorNTI from the amino acid sequence. In 5.4B, lanes 1 and 3 serve as negative controls, as cells do not express VV TK and vCAT is a known recombinant virus with the thymidine kinase gene replaced by chloramphenicol acetyltransferase. These lanes showed no bands near the 20 kDa molecular weight of VV TK. Positive controls in lanes 2, 4 , and 5 consisting WR-infected cells, pNEOGreen- and pGNR-transfected cells did show reactive bands near 20 kDa as seen in previous experiments (Franke, Roseman, and Hruby, 1985). Finally, lane 6 containing lysates from purified vGNR recombinant virus-infected cells did not contain the 20 kDa TK band. In summary, GFP and TK immunoblots showed expression of BiZyme and no expression of VV TK in vGNR-infected cells, indicating insertional inactivation of TK by the BiZyme gene.

5.3.6 Analysis of vGNR.CAT Recombinants by CAT Assay

Having demonstrated the ability of pGNR to target and insertionally inactivate the TK locus, it was of interest to evaluate the viability and applicability of the recombination system in expressing an enzymatically active protein. To facilitate this, the pGNR.CAT vector was transfected into WR-infected cells and recombinants were selected and purified as described for vGNR. Total cell extracts from recombinant vGNR.CAT-infected and pGNR.CAT-transfected cells were then compared to vCAT (a previously constructed recombinant expressing CAT (Hruby *et al.*, 1990), and CAT derived from *E. coli* for enzyme activity.

Acetylation of the 3-hydroxyl group of fluorescent bodipy 1-deoxy-chloramphenicol was assayed by *in vitro* catalysis by the above enzymes. Following TLC separation of the reaction products, (Figure 5.5), fluorometry was used to determine relative amounts of the acetylated reaction products (3-acetylated 1-deoxychloramphenicol) and unreacted substrate (Figure 5.6). Comparison of enzymatic activity by percent conversion of substrate to product showed similar activity of vGNR.CAT and vCAT after a 45 minute reaction, 62.2% and 54.9% respectively. Negative controls consisting of whole cell lysates from BSC_{40} cells alone (1.4% conversion), WR-infected cells (0.7%), and a vGNR-infected monolayer (0.1%) showed little to no enzymatic activity. As positive controls, *E. coli* CAT displayed 28.6% conversion while pGNR.CAT-transfected cells had 94.8% conversion.

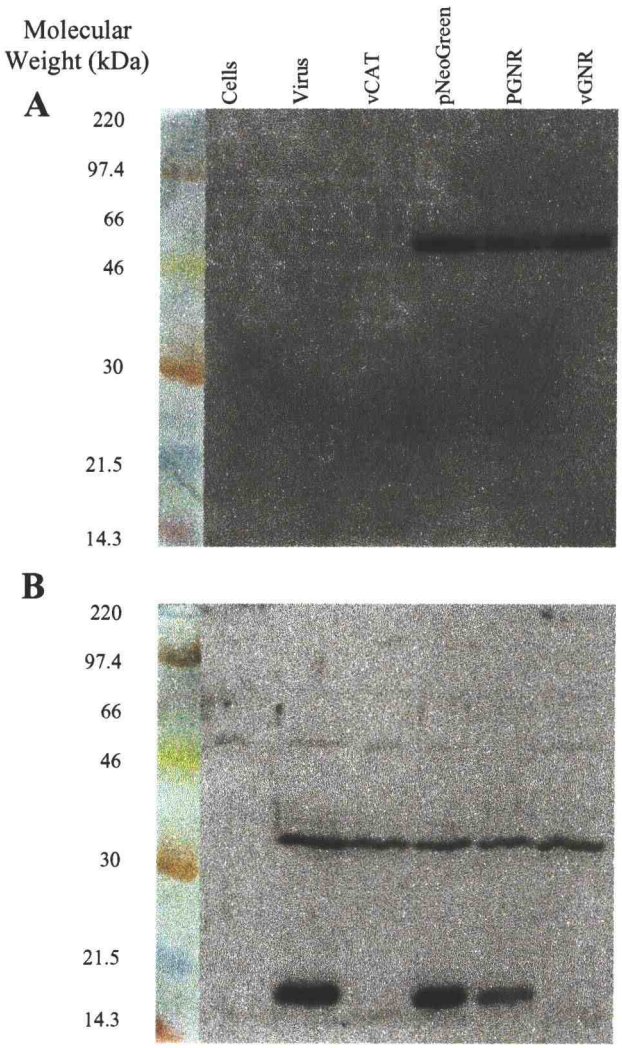

Figure 5.4. BiZyme expression. Total cell extracts were obtained from un-infected and WT VV-infected cells for control lanes 1 and 2. Cells were also infected with a known and titered recombinant, vCAT which has TK insertionally inactivated by the chloramphenicol acetyltransferase gene (lane 3). Lanes 4 and 5 contained extracts from cells transfected with the expression vector pNeoGreen and the pGNR recombination vector, respectively. Lane 7 contains proteins from vGNR-infected cells.

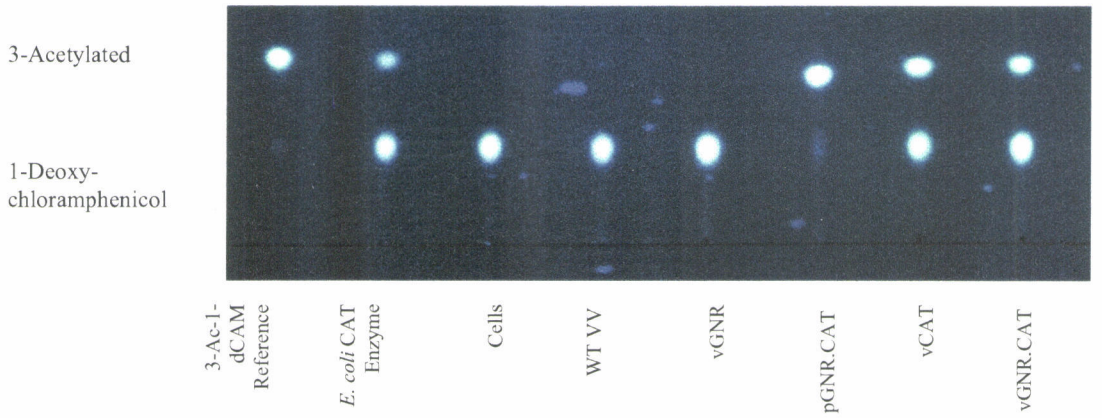

Figure 5.5. Thin-layer chromatography of CAT-activity detection assay. Whole cell extracts from infected BSC40 cells were assayed for chloramphenicol acetyltransferase activity. Fluorescent bodipy 1-deoxychloramphenicol substrate was added to whole cell lysates as well as a CAT enzyme preparations from *Escherichia coli.* After 45 minutes, the reaction was stopped and products were extracted with ethyl acetate. The 3-acetylated products and unreacted substrates where separated by thin-layer chromatography, shown here under ultraviolet illumination.

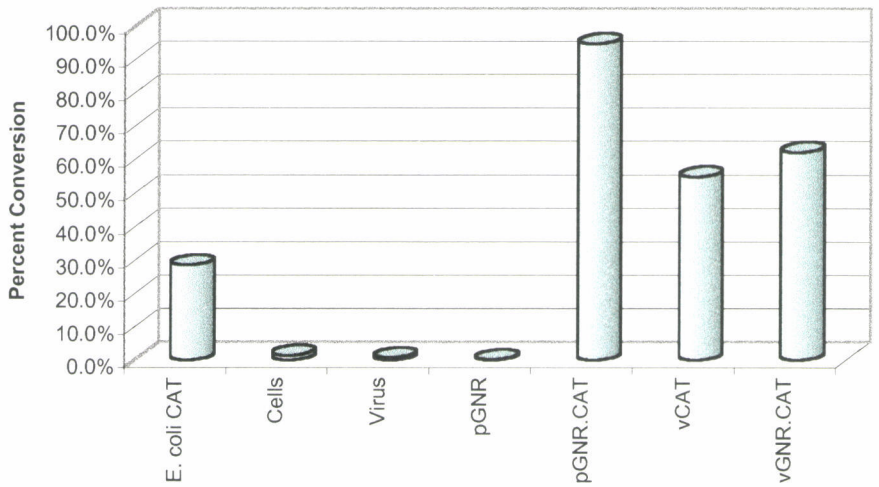

Figure 5.6. Quantitative analysis of CAT assay. Acetylated and unreacted substrates were collected from the TLC plate and resuspended in methanol. Following extraction, samples were diluted 1:100 and analyzed by fluorometry to obtain a fluorescent intensity (FI, data table). CAT activity was measured as percent conversion of the bodipy 1-deoxychloramphenicol to the 3-acelyated enzymatic product.

5.3.7 Immunoblot Analysis of vGNR.IncA Recombinants

Once the ability of this vector to generate recombinant virus capable of producing a biologically active enzyme was confirmed, further applications of the system were explored. Specifically, the ability to create recombinant virus expressing a foreign antigen for potential use as a live vaccine was of interest. The expression of *Inc*A was evaluated in non-infected, VV-infected, vGNR-infected, pGNR-transfected, pGNR.IncA-transfected, and vGNR.IncA-infected cells. Total cell extracts harvested from infected cells were analyzed for endogenous or transiently expressed *Inc*A protein by SDS-PAGE and immunoblotting with anti-*Inc*A antibody (Figure 5.7). *Inc*A typically migrates as multiple bands with an electrophoretic mobility in SDS-PAGE ranging from 39-42 kDa (Rockey *et al.*, 1997). Negative controls in lanes 1-4 consisting of cells alone, WR-infected, vGNR-infected, and pGNR-transfected BSC_{40} cells showed no bands. The *Inc*A expression vector pRB21:IncA (Rockey *et al.*, 1997) was included as a positive control to demonstrate the multiple *Inc*A bands seen in VV-infected cells. Recombinant virus vGNR.IncA seen in lane 6 shows multiple bands identical to the positive control between 39 and 42 kDa. This data indicates that the isolated virus expresses immunoreactive foreign antigen.

5.4 Discussion

The power and utility of recombinant VV as an expression system and potential vaccine would be greatly facilitated by a more rapid and reliable selection system. To address this need, the pGNR vector containing gene flanks to the J2R ORF of VV and a bifunctional protein was constructed. J2R was chosen as the

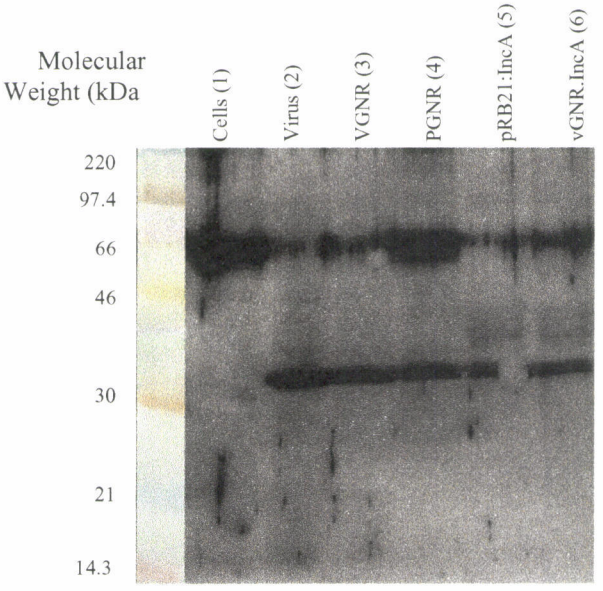

Figure 5.7. Immunoblot analysis of *Inc A* expression from vGNR-IncA. Total cell extracts were obtained from un-infected and WT VV-infected cells for control lanes 1 and 2. Cells were also infected with vGNR and transfected with pGNR for additional controls (lanes 3 and 4). As a positive control, cells were transfected with pRB21:IncA, a vector containing IncA (lane 5). Lane 6 contains cells infected with vGNR-IncA.

target of pGNR due to the attenuating effect insertional inactivation of VV thymidine kinase gene has on the virus. This makes the system suitable for vaccine production. The fusion of the GFP and NEO gene products was chosen to allow for selection of recombinant virus under G418 with the additional advantage of simultaneous detection based on fluorescent phenotype. Acquisition of both phenotypes virtually ensures that each and every plaque selected will be a bonafide recombinant without any background noise due to spontaneous mutants.

Although recombination occurs at a high fidelity, current selection of recombinant virus is a time-intensive process due to the very low frequency of homologous recombination. In one commonly used method, purification is achieved by dilution of WT virus and amplification of recombinants under G418 selection. Recombinants bearing both the foreign gene of interest and the neomycin phosphotransferase selectable marker are preferentially selected. Antibiotic selection, however, is not 100% efficient and non-recombinants are capable of surviving in the presence of G418 due to rescue mediated by recombinants and a naturally high survival capacity of WT virus. Therefore, amplification of the very small (0.1%) recombinant viral population requires multiple passages under antibiotic selection. Sufficient amplification of the recombinant usually requires up to 4 weeks of such serial passages. The use of BiZyme, however, allows a quick and efficient means of circumventing this lengthy process. Visualization of fluorescent plaques can occur very soon after initial transfection and recombination. Amplification of the presumably

homologous virus population under antibiotic selection is the only subsequent requirement for isolation of pure recombinant virus stocks.

To demonstrate the effectiveness of this system, the pGNR vector was transfected into VV infected cells. Fluorescent microscopy showed expression of GFP in the presence of G418, a known inhibitor of protein synthesis (Figure 5.2). This evidence suggested that the two phenotypes could be simultaneously exploited and that BiZyme could be a potent means of selection for VV recombinants. To test the applicability of this selection process, additional recombination vectors were also constructed. One such vector was designed to produce recombinant virus vGNR.CAT, which expresses the bacterial CAT enzyme. Successful generation of these recombinants was intended to demonstrate the ability to generate virus expressing a biologically functional enzyme. The second construct enabled isolation of vGNR.IncA, a potentially immunogenic virus expressing the *Inc*A bacterial antigen from *Chlamydia* species (Rockey *et al.*, 1997).

Examination of plaques formed shortly after transfection and recombination showed that potential recombinants could be quickly and easily identified by fluorescent microscopy (Figure 5.3). By picking specific fluorescent plaques, purified recombinants were obtained in as little as 9 to 11 days and used for analysis or preparation of large scale virus stocks. Immunoblot analysis of recombinant vGNR generated in this fashion showed that BiZyme was expressed from non-transfected cells and migrated in SDS-PAGE analysis at approximately the same molecular weight as predicted by its amino acid sequence (Figure 5.4A).

Insertional inactivation of the J2R locus was also demonstrated by immunoblot, where recombinant vGNR-infected cells lacked expression of thymidine kinase, in contrast to cells transfected with BiZyme containing plasmids or infected with WT VV (Figure 5.4B). This data demonstrates that pGNR specifically targets and inactivates the TK gene by insertion of BiZyme into that locus. Furthermore, the selectable characteristics of G418 resistance and green fluorescence were successfully conferred to the resulting recombinants. Most importantly, the absence of any TK protein demonstrates that purified virus stocks were obtained within 11 days using this method. Inactivation of TK by BiZyme also indicates that other VV genes could be targeted for insertional inactivation, making this an important tool for the study of VV gene function.

This study describes the utility of BiZyme in the construction of recombinant VV. However, using the BiZyme technology in other viral, eukaryotic, and prokaryotic systems will expediate selection of recombinant virus, create stable cell lines expressing foreign and facilitate the isolation of bacterial clones, respectively. The use of the fluorescence activated cell sorter (FACS) could enhance the ease of selection and isolation. FACS would enable individual cells, virally infected cells, and bacteria expressing BiZyme to isolated early during the selection process further reducing the isolation time.

To summarize, the pGNR recombination has provided a significant improvement on the selection mechanisms currently available to isolate recombinant VV. The time and effort required for isolation of pure recombinant

virus stocks has been effectively reduced from 4 weeks with selection by G418 alone, to 1-_ weeks with BiZyme. The pGNR vector has been shown to target the TK locus for homologous recombination, demonstrating that BiZyme could be used in other gene inactivation studies. The production of recombinants expressing biologically active enzymes and foreign antigens also indicates that the pGNR vector system is a highly versatile and effective means of recombinant virus selection.

Chapter 6

Conditional-lethal Expression of the Vaccinia Virus A16L, E7R, G9R Gene Products

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26 pages

6.1 Introduction

Vaccinia virus (VV) belongs to the animal virus, family *Poxviridae.* VV is a large double-stranded DNA virus that replicates entirely with in the cytoplasm of infected cells (Moss, 1996b). VV encodes over 200 proteins, including enzymes such as DNA polymerase, thymidine kinase, topoisomerase, RNA polymerase, numerous replication/transcription factors and many more (Johnson, Goebel, and Paoletti, 1993). With the virus encoding most if not all the proteins that are responsible for its own replication and propagation it is able to replicate in a diverse array of different cell types (Bostock, 1990; Moss *et al.*, 1988; Paoletti, 1990). VV utilizes many of the same post-translation regulatory mechanisms as the host cell such as proteolysis, phosphorylation, glycosylation, and fatty acylation for trafficking proteins to their proper locations within the cell (VanSlyke and Hruby, 1990). Because of this VV has proven to be useful research tool and model system in modern cell biology.

Palmitylation and N-myristylation are the two predominant forms of fatty acylation known to occur on viral proteins (Magee and Courtneidge, 1985). In the case of palmitylation a 16-carbon saturated fatty acid is added post-translationally to a cysteine, serine, or threonine via a thiolester or ester linkage (Magee and Schlesinger, 1982; Schlesinger *et al.*, 1980; Schmidt, 1983). Palmitylation is a dynamic process meaning that the event can be reversed as a means of cellular regulation (Berger and Schmidt, 1984; Bharadwaj and Bizzozero, 1995).

N-myristylation is the cotranslational addition of a 14-carbon saturated fatty acid to a glycine residue via an amide bond (Hruby and Franke, 1993; Johnson *et al.*, 1994). N-myristylation is catalyzed by N-myristyltransferase, which recognizes the amino terminal motif, MGXXX(S/T/A/C/N) (where X is any amino acid and the M is cleaved prior to modification) (Towler *et al.*, 1987a). In addition to N-myristylation, a second class of myristylation has recently been recognized and termed internal-myristylation (I-myristylation) due to the myristate modification occurring internally on arginines or lysines (Bursten *et al.*, 1988). Two examples of this latter modification include the tumor necrosis factor alpha and interleukin-1 alpha precursors (Stevenson *et al.*, 1993; Stevenson *et al.*, 1992). The enzyme that catalyzes I-myristylation has not been identified.

Many viruses express proteins that are modified by myristic acid. Of these viral myristylproteins, most are associated with the viral capsid or envelope (Chen *et al.*, 1997; Prange *et al.*, 1991). Likewise in those viral myristylproteins that have been carefully examined, the function of both the myristate moiety and the protein are typically essential for the virus life cycle. For example the myristylation of the human immunodeficiency virus' Gag protein is required for proper translocation of the protein to the plasma membrane where virion assembly and budding occur (Bryant and Ratner, 1990; Gheysen *et al.*, 1989; Gottlinger *et al.*, 1989) Myristylation of the picornavirus' VP4 protein is also essential for assembly of infectious virions (Chow *et al.*, 1987). In addition the myristylation of the Rous

sarcoma virus' $pp60^{v\text{-}src}$ protein is essential for the virus to be able to transform cells (Buss and Sefton, 1985; Resh, 1990).

Vaccinia virus encodes numerous myristylproteins of which the best characterized is encoded by the L1R ORF (Ravanello *et al.*, 1993; Ravanello and Hruby, 1994a; Ravanello and Hruby, 1994b). The protein is 29 kDa in size and is myristylated at the amino terminus within the canonical N-myristylation motif. L1R has been shown to be associated with both the IMV and EEV forms of the virus by spanning the IMV membrane at two locations and an extracellular loop that associates with the EEV (Hansen and Hruby, submitted). Conditional lethal expression of L1R has shown that L1R is essential in viral morphogenesis (Ravanello and Hruby, 1994b).

In contrast to L1R, the myristylproteins encoded by the A16L, E7R, and G9R ORFs are far less well characterized (Martin *et al.*, 1997). These viral myristylproteins are unusual in that they all appear to be soluble within the infected cell and that they are found in the virion at low levels (Martin *et al.*, 1997). It has been suggested that these proteins are not associated with any membrane but their presence in the virion is do to the "hitchhiker" process described previously (Hruby, Guarino, and Kates, 1979).

Determining the role of these proteins in the VV life cycle is the focus of experiments reported in this chapter. Previous attempts at creating deletion mutants for each of the ORFs have failed suggesting that these proteins are essential to the virus's maturation. To determine what function these proteins play

we have constructed conditional lethal mutants of the A16L, E7R, and G9R ORFs. We report here their phenotypes and an analysis of the essentiality of the myristylation modification.

6.2 Materials and Methods

6.2.1 Cells and Virus

BSC_{40} (African green monkey kidney) cells were maintained in modified Eagle's medium (MEM-E, Sigma, St. Louis, Mo.), supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Pierce), 2 mM L-glutamine (LG), and 10 μg of gentamicin sulfate (GS) per ml at 37°C, 5% CO_2, and 95% humidity. The recombinant viruses vlacIlac0-A16L, E7R, and G9R were grown in the continuous presence of 5 mM isopropylthiogalactopyranoside (IPTG). VV recombinant vLacI and the viruses vlacO-A16L, E7R, and G9R were purified from infected BSC_{40} cells by methods described previously (Hruby, Guarino, and Kates, 1979).

6.2.2 Vector Construction

Recombination vectors were constructed in the following manner. The primers 5' CTAGCTAGCGATCGTTTGTCAGATGT 3' and 5'TCCCCGCG

GTCTTTAATAATCGTCAG 3' were used for PCR amplification of 1200 base pairs upstream of the A16L open reading frame. The primers inserted a *Nhe*I on the 5' end of the product and a *Sac*II restriction site on the 3' end. The primers 5' CCCCCCGGGATGGGGGCAGCTGTTACTC 3' and 5'AAAAGGCCTT TATCGTCTACGAAC 3' were used to PCR amplify from VV genomic DNA the A16L gene. The primers were designed to incorporate a *Xma*I site on the 5' end and a *Stu*I site on the 3' end of the gene. The primers 5'AAAAGGCCTTTCG TTGATAATTTATTCTAC 3' and 5'ACATGCATGCGAATTAAGATTATT GTTG 3' and 5' ACATGCATGCGAGAATTAAGATTATTGTTG 3' were used to PCR amplify 1200 base pairs downstream of the A16L open reading frame. These primers allowed for the addition of a *Stu*I site and a *Sph*I restriction sites on the 5' and 3' end of the PCR product, respectively.

The primers 5' CTAGCTAGCTGATAGACAAGATATATATAC 3' and 5' TCCCCGCGGTATTTATCAAAAATGTGTGAATTTTAATATG 3', were used to PCR amplify 1200 base pairs upstream of the E7R open reading frame and incorporate a *Nhe*I site and *Sac*II restriction site on the 5' and 3' ends of the product, respectively. The primers 5'CCCCCCGGGATGGGAACTGCTGCAA CAATT 3' and 5' AAAAGGCCTTTATATATTTAGTTTATCTTT 3' , were used to PCR amplify the E7R gene while placing a *Xma*I restriction site and a *Stu*I restriction site on the 5' and 3' ends of the gene, respectively. The primers 5' AAAAGGCCTGTATAATCCCATTCTAATAC 3' and 5' ACATGCATGC

GAGAACGATATTATTTGC 3' were used to PCR amplify 1200 base pairs downstream of the E7R open reading frame. The primers also introduced on the 5' end of the product a *Stu*I restriction site and a *Sph*I restriction site on the 3' end.

The primers 5' CTAGCTAGCTCAAAGTGTTTAGCGTGGC 3' and 5' TCCCCGCGGTATTTATCTCATTGAAAGTATTAATC 3' were used to PCR amplify 1200 base pairs upstream of the G9R open reading frame and incorporate a *Nhe*I site and *Sac*II restriction site on the 5' and 3' ends of the product, respectively. The primers 5' CCCCCCGGGATGGGTGCCGGAGTAAGTG 3' and 5' AAAAGGCCTTTAAATAGCTACAATTAGTAT 3' were used to PCR amplify the G9R gene from VV genomic DNA while introducing a *Xma*I site and a *Stu*I site on the 5' and 3' ends of gene, respectively. The primes 5'AAAAGGCC TATGGGTGCCGCAGCAAGC 3' and 5'ACATGCATGCCTTTATAGGCATA TCACTCA 3' were used to PCR amplify 1200 base pairs downstream of the G9R open reading frame while introducing a StuI site on the 5' end of the product and a *Sph*I restriction site on the 3' end of the product.

The upstream PCR products from A16L, E7R and G9R were cloned into pLacO which contains the Lac Operator sequence using the restriction sites *Nhe*I and *Sac*II contained in the plasmid and on the PCR products, producing pLacO-UpA, pLacO-UpE, and pLacO-UpG. Next the gene products for A16L, E7R, and G9R were cloned into the pLacO-Up plasmids using the restriction sites *Xma*I and *Stu*I located immediately downstream of the LacO sequence and producing the new plasmids pLacO-UpA16L, pLacO-UpE7R and pLacO-UpG9R. After obtaining

these plasmids the downstream PCR products were cloned into the plasmids containing the genes using the restriction sites *Stu*I and *Sph*I, producing pLacO-UpA16LDn, pLacO-UpE7RDn, and pLacOG9RDn. For selection the GNR cassette was subcloned from pGNR into the *Stu*I site and the plasmids were screened for orientation. The plasmid pGNR contains the gene encoding the green fluorescent protein fused in frame with the neomycin resistance gene downstream of the VV synthetic early/late promoter. The recombination plasmids were named pRecOA16L, pRecoE7R, and pRecoG9R. Plasmids were confirmed by restriction analysis and by DNA sequencing.

Transient expression vectors were constructed in the following manner. The primers 5' CCCCCCGGGATGGCGGCAGCTGTTACTC 3' and 5' AAA AGGCCTTTATCGTCTACGAAC 3' were used to PCR amplify from VV genomic DNA a non-myristylating mutant of A16L in which the codon encoding for the myristylated glycine was changed to one that encodes a alanine. The primers 5' CCCCCCGGGATGCAACTGCTGCAACAATTCAG 3' and 5' AAA AGGCCTTTATAATTAGTTTATCTT 3' were used to PCR amplify from the VV genome the E7R open reading while also mutating the codon encoding for glycine to alanine. The primers 5' CCCCCCGGGATGGCTGGCGCAGTAAGTGTT 3' and 5' AAAAGGCCTTTAAATAGCTACAATTAGTAT 3' were used to PCR amplify and mutate the G9R gene. The mutated PCR products were cloned into the vector pRB21 containing the VV synthetic early/late promoter using *Xma*I and *Stu*I restriction sites. The plasmids generated from this cloning were named

pA16LG2A, pE7RG2A, and pG9RG2A. All plasmids were confirmed by restriction analysis and DNA sequencing.

6.2.3 Construction of the Lac-Inducible Recombinant

To generate the recombinant virus vlacIlacOA16L, vlacIlacOE7R, and vlacIlacOG9R, a confluent monolayer of BSC_{40} cells (1.4×10^6) were infected with 0.05 plaque-forming units (PFU) of vlacI per cell. The infected cells were then transfected individually with 30 µl of the synthetic liposome DMRIE-C and 10 µg of pRecOA16L, pRecOE7R, or pRecOG9R (Rose, Buonocore, and Whitt, 1991). The infections were allowed to continue for 24 hours, after which time 500 µg/µl of the antibiotic G418 was added to each well. At 48 post infection the cells and virus were harvested by collecting the cells and virus in a 1.5 ml microcentrifuge tube and spinning at 15,000 x *g* for 20 minutes in a 4°C microcentrifuge. The supernantant was discarded and the pellet was resuspended in 100 µls of PBS-M. The resuspended pellet was frozen, partially thawed and vortexed 3 times to lyse the infected cells to release the virus releasing the virus. The lysed total cell extracts (TCE) were centrifuged at 4°C for 2 minutes at 2000 x g, after which the post nuclear supernatant (PNS) was transferred to a new 1.5 ml microcentrifuge tube. Ten micro liters of each PNS was used to infected new confluent monolayers of BSC_{40} cells (1.2×10^6) that had been pretreated with G418. Forty eight hours post infection cells were harvested and processed as described above. One micro

liter of the PNS from the second infection was diluted 10^{-3} to 10^{-8} in infection media (MEME supplemented with 5% FCS, 2mM LG, 10 μg/ml GS, 500mg/ml) and plated in individual wells of a confluent 6-well plate containing BSC_{40} cells pretreated with G418. Twenty four hours post infection the infection inoculum was aspirated away and replaced with a 1 ml MEME agarose overlay containing G418. At 48 hours post infection plaques were visualized through an inverted fluorescent microscope looking for plaques emitting a green glow. Plaques scoring positive for green fluorescence were picked using a Pasteur pipette, and placed in 1.5 ml microcentrifuge tube containing 100 μl of PBS-M. The resuspended pellets were frozen and then thawed 3 times and the entire 100 μl was then added to 1 ml of infection media and placed on a confluent monolayer of BSC_{40} cells in a 35-millimeter dish. Forty eight hours post infection the virus-infected cells were harvested and resuspended in 100 μl of PBS-M. Ten microliters of the total cell extract was combined with 10 μl of 6 x sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) reducing sample buffer. In addition to immunoblotting, DNA was isolated from the positive plaques and it was screened for the *lac* operator sequence by PCR. Viruses containing the *lac* operator sequence were plaque purified once more and then amplified to produce a viral stock.

6.2.4 Plaque Assay

Confluent monolayers of BSC40 cells in a 35 mm dish were infected with the recombinant vlacIlacOA16l, vlacIlacO-E7R, and vlacIlacO-G9R (~ 100 PFU per well) in the presence and absence of IPTG. At 48 hours post infection, cells were stained with crystal violet and plaques were enumerated.

6.2.5 Transient Expression

The conditionally repressed mutants of A16L, E7R, and G9R were used to infect monolayers of cells (2.5×10^6 cells) contained within a 35 mm dish a multiplicity of infection of 10. Infected cells were transfected with 10 μg of plasmid DNA using DMRIE-C reagent to enable the transient expression of both wild-type and nonmyristylating forms of A16L, E7R, and G9R. The inoculum was prepared by adding 1 ml of infection media to a polystyrene tube. Ten micro grams of plasmid DNA was added to each tube a long with 30 μl of liposomes (DMRIE-C, supplied by Gibco). After 15 minutes at room temperature the appropriate amount of virus was added to each tube and then added to the monolayer of cells from which the culture media had been removed. The cells were then incubated at 37°C for 12 hours, after which cells and virus were harvested in the culture supernatant. Cells were pelleted by centrifugation at 15,000 *g* for 30 minutes at 4°C and then resuspended in 100 μl of 1 x phosphate-buffered saline (PBS). The

resuspended pellet was frozen and then thawed three times after which 50 ml of 6 x reducing sample buffer was added. The sample was then boiled for 3 minutes and then centrifuged at 15,000 *g* for 1 minute to pellet the insoluble material. Fifteen milliliter was loaded on a 12% polyacrylamide gel and resolved by discontinuous gel electrophoresis (SDS-PAGE) as described previously. After electrophoresis, the gels were subjected to immunoblot analysis using anti-A16L, E7R, or G9R antibody. The blots were then developed by chemiluminescent substrate and exposed to Kodak BIOMAX MR film.

6.2.6 Single Cycle Growth Curves

BSC40 cells were infected with VV WR, vlacIlacO-A16L, vlacIlacO-E7R, or vlacIlacO-G9R in the presences or absence of IPTG, at a multiplicity of infection of 10 PFU per cell. Virus yields from cells harvested at the indicated times post-infection were determined by plaque assay on monolayers of BSC_{40} cells in the presence of 5 mM IPTG.

6.3 Results

6.3.1 Conditionally Repressed A16L, E7R, and G9R VV Mutants

As shown in Figure 6.1, VV expresses a number of myristylproteins including those encoded by the L1R, A16L, E7R, and G9R open reading frames. The goal of this study was to gain a understanding of the potential function of the A16L, E7R, and G9R gene products in the VV life cycle. We have previously reported that these gene products are myristylated on their N-terminal glycine residue and that the modified proteins are found within the soluble fractions of a subcellular fractionation. Since no temperature-sensitive mutants have been described for these loci we elected to construct recombinant viruses in which the expression of the A16L, E7R and G9R genes are under the control of the lac operator-repressor system. DNA fragments containing the entire A16L, E7R, and G9R open reading frames together with flanking sequences were ligated into the plasmid pLacO. The bifunctional-GNR gene was inserted immediately downstream of the transcriptional stop site for use in selection and detection of the recombinant viruses. The recombination plasmids were transfected into cells infected with a recombinant virus, which expresses the lac repressor protein, vlacI, in the presence of 5 mM IPTG. By using the synthetic antibiotic G418 for positive selection, recombination events between the plasmid vector and the viral genome were amplified. These recombination events resulted in the WT genes being

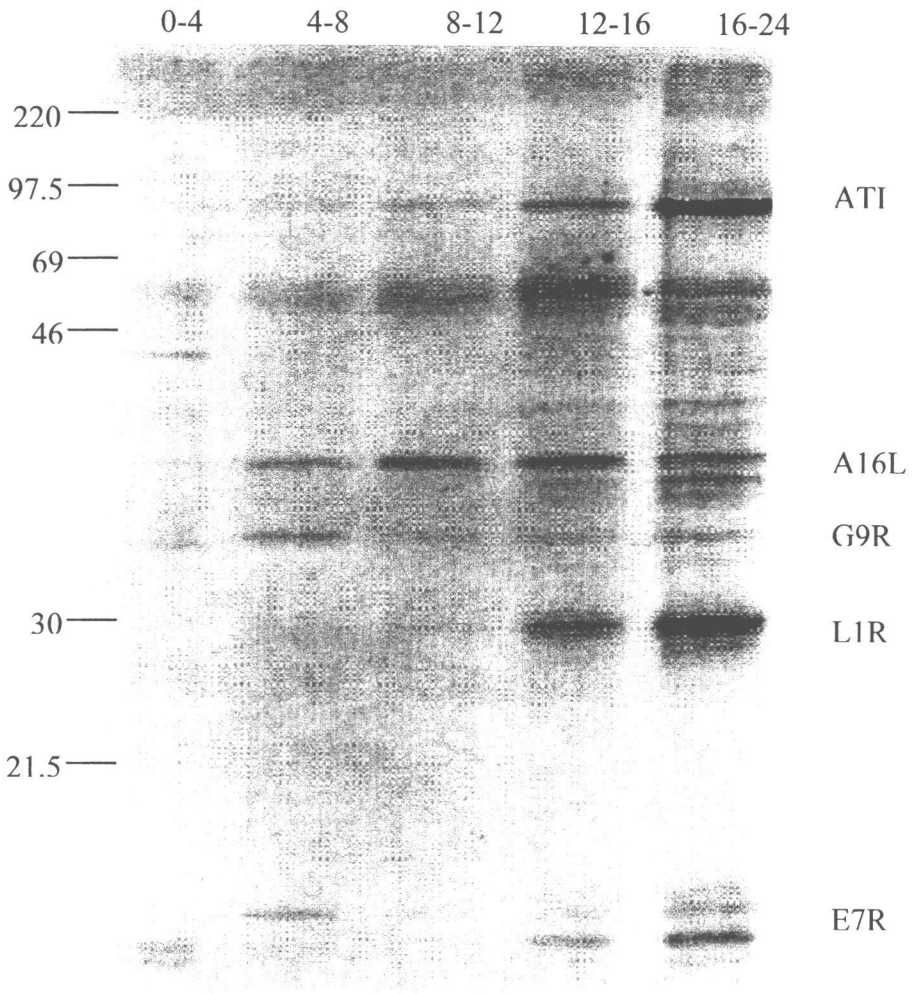


Figure 6.1. Time course labeling experiment with [^{3}H]myristic acid. BSC40 cells were infected with the WR stain of VV in the presence of [^{3}H]myristic. At 4 hours post-infection, virus-infected cells were harvested and fluorgraphed by using 22.2% PPO in DMSO, dried and exposed to Kodak Biomax MR film. The molecular weight marker is indicated to the left and the major myristylproteins are labeled at the right.

replaced by cassettes containing the gene of interest under the control of the lac operator sequence and the GNR gene. After selection with G418 and green fluorescence, we isolated 12 plaques for each putative recombinant. DNA from each virus was isolated and analyzed by PCR for the presence of the lac operator

sequence (data not shown). The recombinants identified by PCR were plaque purified and a virus stock culture was produced in the presence of 5 mM IPTG.

To demonstrate that the expression of the A16L, E7R, and G9R genes in the recombinant viruses were dependent on IPTG, cells were infected with the WR strain of VV or with the transcriptionally controlled mutants in the presence or in the absence of the inducer IPTG. At 12 hours post infection, cells were harvested and the extracts were prepared and analyzed by immunoblot using antibodies specific for the A16L, E7R, or G9R protein. Similar levels of protein were present for VV WR and vlacIlacO-A16L, vlacIlacO-E7R, and vlacIlacO-G9R infected cells in the presence of IPTG (Figure 6.2A, B, and C; lanes 1 and 2). However in the absence of inducer no A16L, E7R, and G9R protein was detected (Figure 6.2A, B, and C; lane 3).

Since the expression of the three genes, A16L, E7R and G9R appeared to be dependent on the addition of 5mM IPTG, a plaque assay was carried out with cells infected with vlacIlacO-A16L, vlacIlacO-E7R, and vlacIlacO-G9R in the presence of 5 mM IPTG to test whether expression of these gene products was essential for viral plaque formation. As shown in Figure 6.3B, C, and D, the size and number of plaques was reduced when the inducer was not present in the medium (Figure 6.3A).

To more precisely investigate the effects the A16L, E7R, and G9R proteins have on virus yield, a single-cycle growth experiment on the recombinants was performed,

and the results were determined by plaque titration assay. Cells infected with vlacIlacO-A16L, vlacIlacO-E7R, and vlacIlacO-G9R produced virus titers comparable to those of wild-type strain of VV WR. However, in the absence of the inducer the A16L, and E7R mutants' ability to replicate was reduced by 4 log units. This is in striking contrast to the G9R mutant, which behaved much like WT VV in the presence or absence of inducer Figure 6.4.

In order to demonstrate that the phenotype of the mutants was due to the repressed gene and not a second-site mutation a trans-complementation assay was used in an attempt to complement the inhibition of L1R protein synthesis in cells infected with vlacOlacI-A16L, vlacOlacI-E7R, and vlacOlacI-G9R in the absence of inducer. Plasmid vectors pA16L, pE7R, and pG9R, which contain the authentic promoter and open reading frame from WR, were transfected in BSC_{40} cells that were infected with the corresponding conditional mutant virus minus inducer. In this system, it was shown that A16L, E7R, and G9R could be expressed from a plasmid. Biological rescue of the recombinants was measured by plaque formation and immunoblot analysis (data not shown). The results from these experiments demonstrate that protein expression can be accomplished and rescue the mutant virus when supplied in *trans* when inducer is absent.

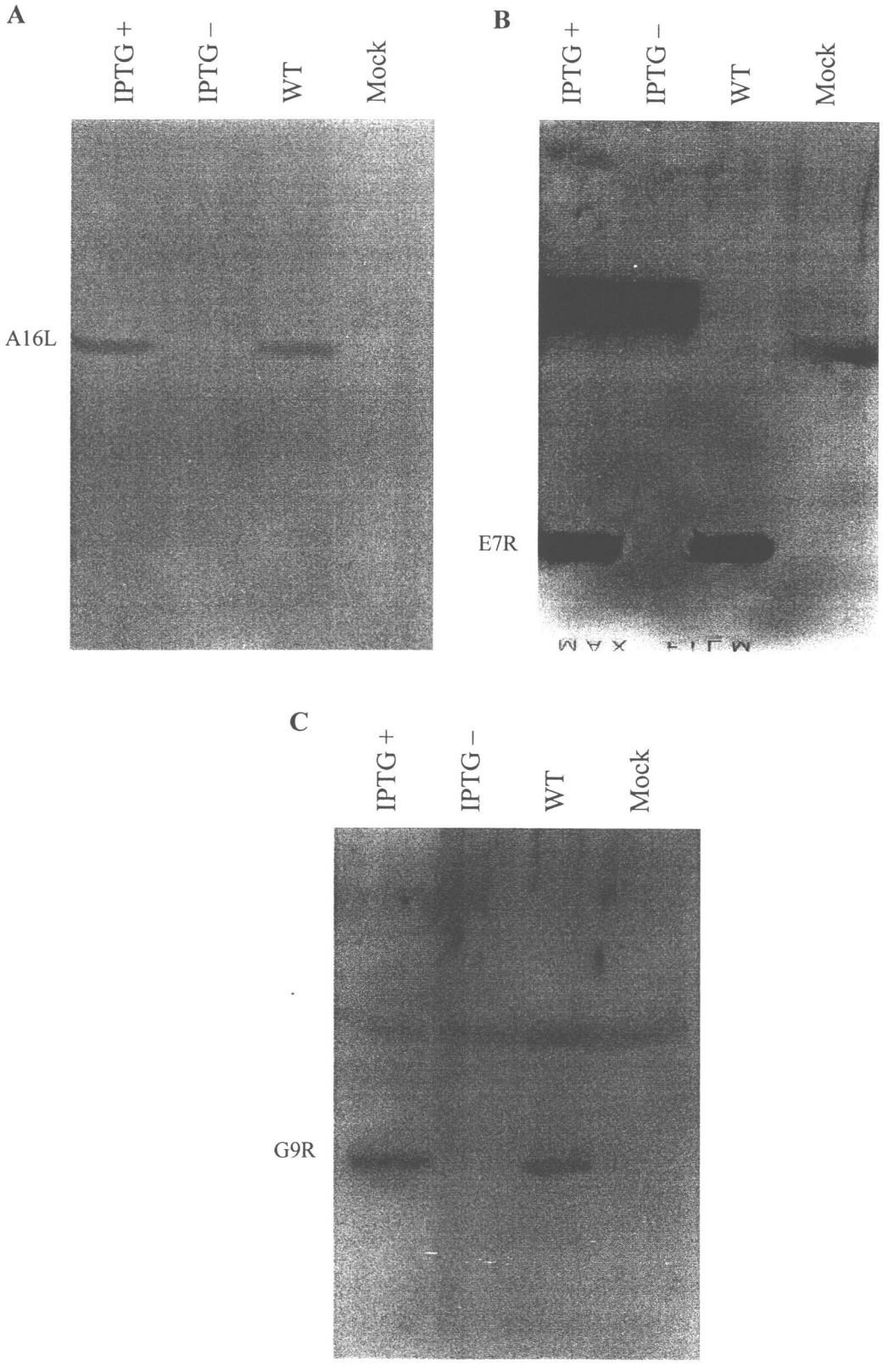
Using the trans-complementation approach, we also addressed the question of whether the myristylation of A16L, E7R and G9R proteins was essential for biological activity. The plasmid constructs used in these experiments have the glycine codon in position two of the three genes mutated to alanine. Therefore, if

the expressed genes produce a form of the proteins, which cannot be myristylated. The pA16lG2A, pE7RG2A, pG9RG2A plasmids were used in single-cycle growth experiments to examine if myristylation was necessary for protein function. Experiments were performed as described previously, however, plasmids were transfected initially and then titered in the presence of 5 mM IPTG. When the nonmyristylating proteins are expressed in this context, replication of the A16L and E7R mutant virus is again reduced considerably as seen previously minus inducer (Figure 6.5). As expected the G9R mutant virus was relatively unaffected with or without the myristate group. These experiments demonstrated that myristylation is necessary for A16L, and E7R protein function.

6.4 Discussion

Both co-translational and post-translational processing of VV proteins has proven to be a common mechanism for exerting regulatory control of function or targeting to subcellular structures. Fatty acylation, most commonly observed as the addition of myristate or palmitate, occurs on numerous VV proteins. Labeling experiments with tritiated myristic or palmitic acid have demonstrated that VV encodes at least six myristylproteins and six palmitylproteins. Almost all of the VV acylproteins that have been characterized play important roles in the viral morphogenesis and that the modification of the protein is either essential or greatly

Figure 6.2. Immunoblot analysis of the lac-inducible recombinants +/- IPTG. Individual wells of BSC40 cells were infected with the recombinant viruses vlacIlacO-A16L, vlacIlacO-E7R, and vlacIlacO-G9R in the presence or absence of the inducer IPTG. Twenty four hours post-infection virus infected cells were harvested and subjected to immunoblotting with α-A16L, α-E7R, or α-G9R polyclonal antibodies.


A
IPTG +
IPTG –
WT
Mock
A16L
B
IPTG +
IPTG –
WT
Mock
E7R
C
IPTG +
IPTG –
WT
Mock
G9R

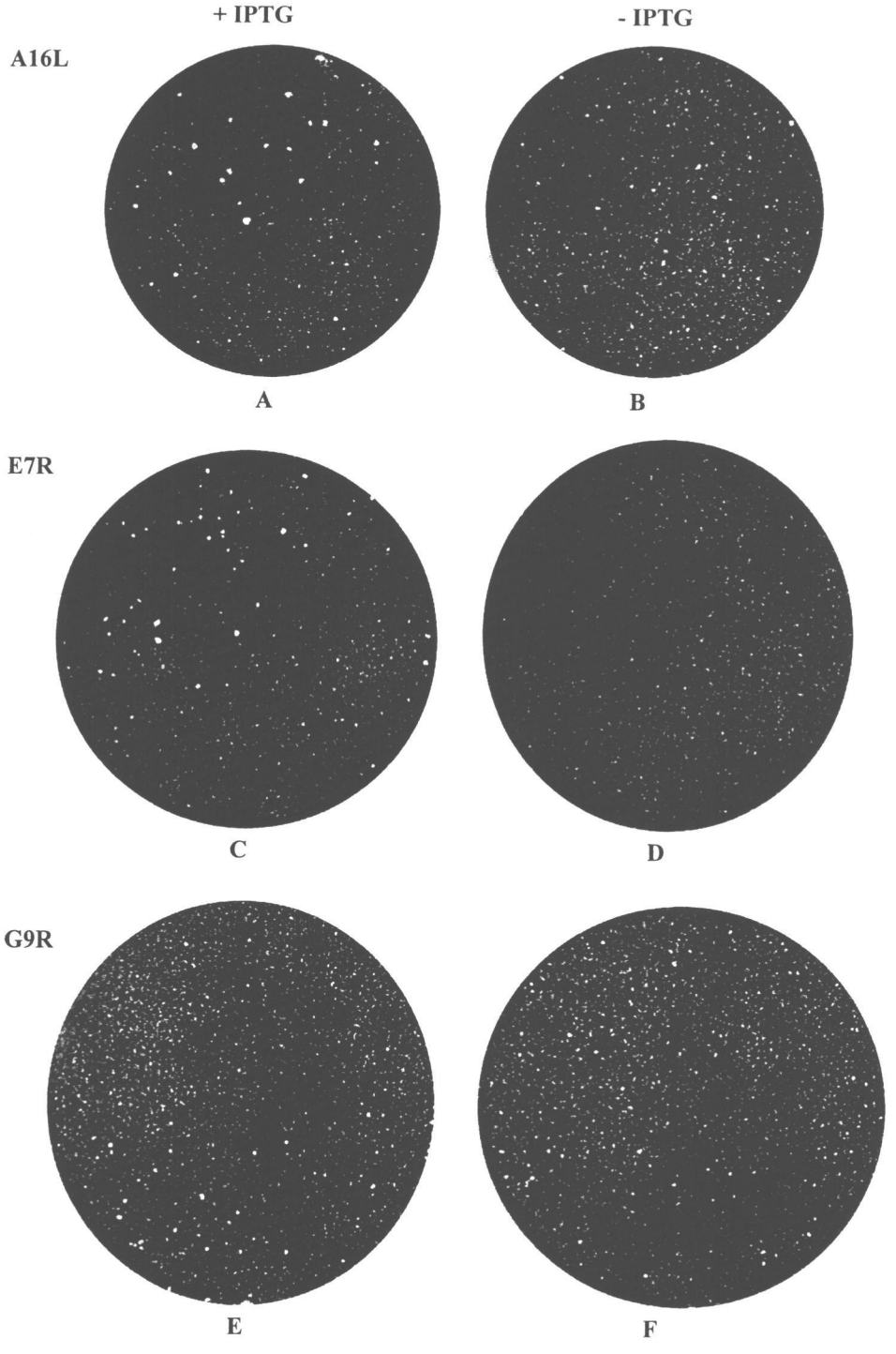
influences the function and biological activity.

L1R is the most characterized VV myristylprotein. The L1R protein is modified on its amino terminal and this modification is essential for virus maturation. L1R has been shown to be membrane associated by subcellular fractionation and immunoblotting, which is typical of a fatty acylated protein. Likewise, the A16L, E7R and G9R open reading frames all encode amino terminal myristylproteins. However, these proteins are not found associated with membranes, but are found soluble within the cytoplasm of an infected cell.

To study the A16L, E7R, and G9R it was necessary to construct recombinant viruses in which the genes were insertionally inactivated or one that could be transcriptionally repressed. Attempts at constructing an insertionally inactivated virus failed, suggesting that these genes may be essential to VV maturation. Therefore, we decided to utilize the *E. coli lac* operator-repressor system to construct individual recombinant viruses in which the transcriptional start site for A16L, E7R, and G9R is interrupted by the *lac* operator sequence. Immunoblotting of the individual recombinants showed that in the absence of inducer that no A16L, E7R, or G9R but when present protein could be detected. This data indicated to us that we had bonafide recombinants and expression of A16l, E7R and G9R could be transcriptionally repressed.

To see whether the proteins produced by the A16L, E7R, and G9R open reading frames are involved in virus maturation a plaque assay was done.

Figure 6.3. Plaque assay +/- IPTG. A confluent monolayers of BSC_{40} cells were infected with vlacIlacO-A16L, vlacIlacO-E7R, or vlacIlaco-G9R. At 48 hours post-infection infected cell monolayers were stained with crystal violet.


+ IPTG
- IPTG
A16L
A
B
E7R
C
D
G9R
E
F

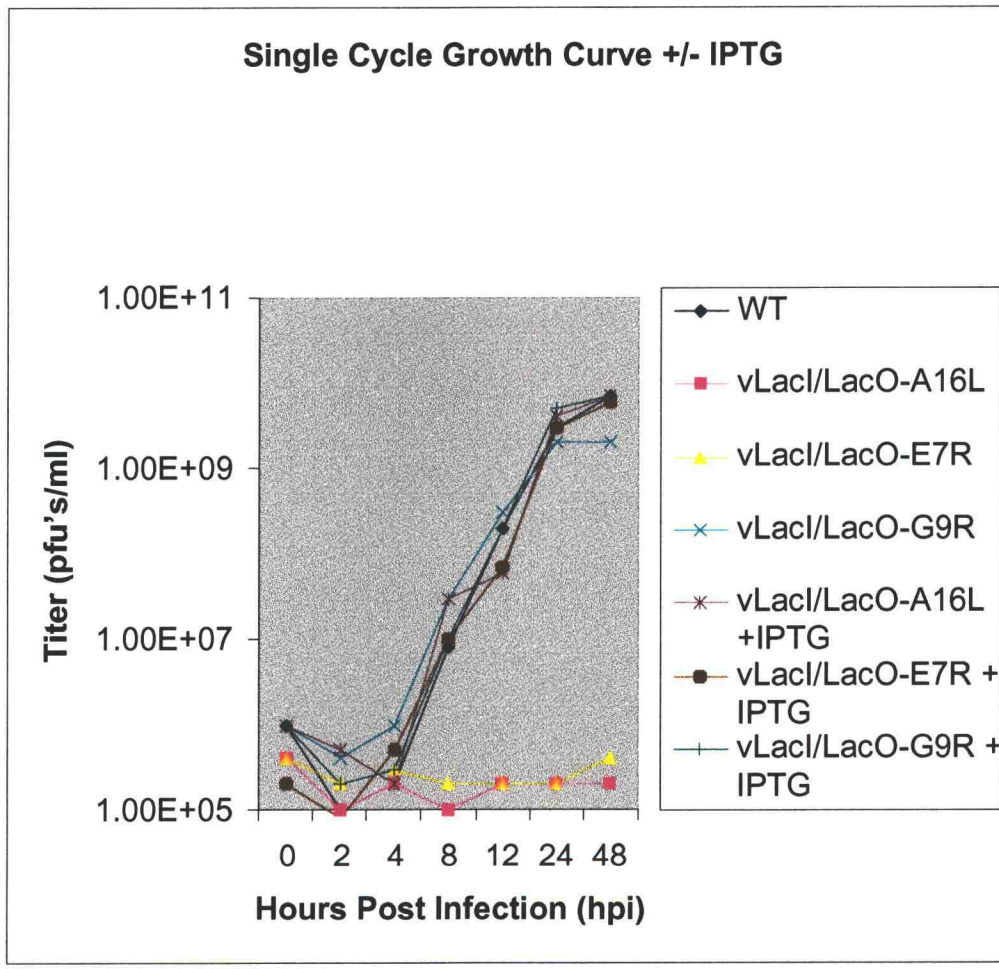


Figure 6.4. Effect of IPTG on the ability to rescue growth of vlacIlacO-A16L, vlacIlacO-E7R, and vlacIlacO-G9R. BSC_{40} cells were infected with VV WR or with the conditional lethal mutants in the presence and absence of IPTG. Infected cells were harvested at the indicated times, and the virus yield was determined by plaque assay of harvested virus in the presence of 5 mM IPTG.

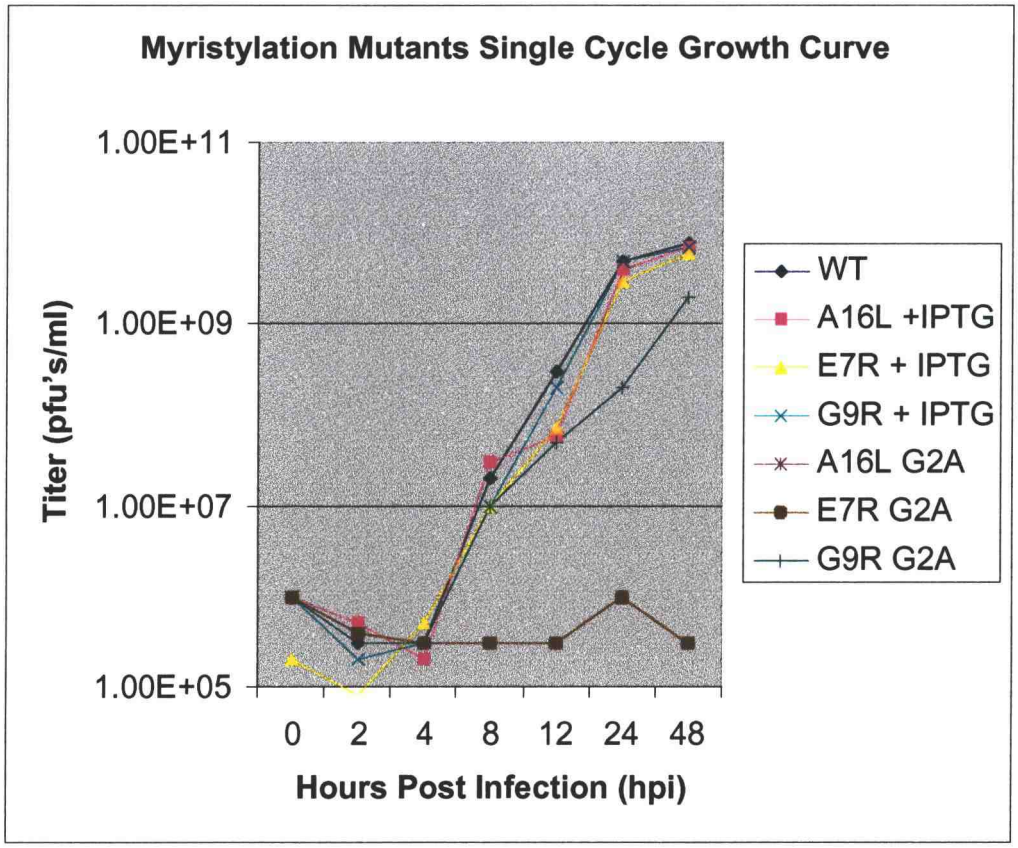


Figure 6.5. Effect of myristylation on the growth of vlacIlacO-A16L, vlacIlacO-E7R, and vlacIlacO-G9R. BSC40 cells were transfected with WT expression plasmids or plasmids that had the myristate acceptor glycine mutated to alanine. Infected cells were harvested at the indicated times, and the virus yield was determined by plaque assay of harvested virus in the presence of 5 mM IPTG.

Monolayers of cells were infected in the presence or absence of IPTG. The plaque assay data clearly shows that when the recombinants are grown in the absence of inducer that there is a reduction in plaque size and number compared to wild-type virus and the recombinants grown in the presence of IPTG.

To determine if the proteins were essential for virus replication single cycle growth experiments were conducted with the recombinants +/- the inducer IPTG

and with wild-type VV WR. Wild-type virus and the recombinants grown and titered in the presence of IPTG behaved in an identical manner. However, when the inducer was not present the A16L and E7R mutants failed to replicate as efficiently with over a 4 fold log unit decrease in the titter. No significant difference was seen with the G9R recombinant virus plus or minus IPTG. Taken together with the plaque assay data and the failure to isolate insertionally inactivated virus in the A16L, and E7R locus it is evident that these genes are essential for VV replication. However, the role G9R plays in the VV life cycle remains unclear.

Next it was of interest to see if myristylation or lack thereof has any affect on the biological function of the proteins. Transient expression plasmids were constructed that have the codon for the myristate acceptor glycine mutated to an alanine. Single-cycle growth experiments were conducted using these vectors minus inducer. When the A16L and E7R proteins are not myristylated, the virus is unable to replicate efficiently and as the same phenotype as all the recombinants minus inducer. This data indicates that the myristate moiety attached to A16L, and E7R is essential for the proteins biological function. The G9R myristylation mutant behaved as WT, and thus myristylation is not necessary for this protein's function.

The use of the *lac* operator-repressor system to construct recombinant conditional viruses has proved to be an invaluable to in determining the phenotype of a virus in the absence of a specific gene product. There remains much to learn

about the functions of A16L, E7R, and G9R gene products. Studies need to be performed to determine the exact functions these proteins have and what role these proteins play in the VV life cycle. It is of interest to determine if myristylation can act as a switch in turning protein function on or off for A16L, E7R, and G9R or see what role the fatty acid moiety plays on a cytoplasmic protein. We are also interested in determining if these proteins have any protein partners and experiments are currently underway to address this question.

Chapter 7

Conclusions

Scott G. Hansen

7.1 Significance and Summary of the Research

VV is proving to be an ideal system in which to study protein processing and modification. This laboratory and others are continually constructing recombinant VV expressing bacterial, eukaryotic, and viral proteins that can be modified and processed in a similar manner to their wild-type environment. VV is again becoming a useful tool for humanity, this time not as a vaccine to cure smallpox, but rather a tool for molecular biologists. Continual study of VV and its proteins may provide insights that can be applied to other viral systems.

Vaccinia virus encodes a 37-kDa envelope protein (p37) that is palmitylated on cysteine residues 185 and 186 of the 372 amino acid protein. We have previously reported on a loosely conserved consensus motif. Further analysis has identified a conserved consensus sequence, Hydro*AAC(C)A (Hydro* represents a hydrophobic portion of a protein determined by any one of the following: a hydrophobic sequence, a transmembrane domain 1 to 12 amino acids away from the modification site, or the prior addition of a hydrophobic molecule; C, palmitate acceptor cysteines; A, aliphatic residue) that is responsible for directing palmitylation of certain classes of palmitylproteins. We have analyzed the amino acid site occupancy upstream and downstream of the palmitate acceptor residues in p37 by site-directed mutagenesis and transient expression of mutated proteins in VV infected cells. The two aliphatic alanines naturally found at positions 183 and 184 of the wild-type p37 allow for efficient palmitylation. In contrast, the

replacement of leucine at position 187 with glycine increases palmitylation efficiency. The 10 amino acids immediately upstream of the palmitate acceptor site are absolutely necessary while the downstream 10 amino acids are dispensable. These results together with previous data suggests that the Hydro*AAC(C)A motif is required for efficient palmitylation of p37 .

Vaccinia virus encodes at least eight proteins that incorporate label from tritiated palmitic acid when it is added to infected cell cultures. Three of these palmitylproteins are encoded by the A33R, B5R, and F13L open reading frames and migrate by gel electrophoresis with relative molecular masses of 23-28 kDa, 42 kDa and 37 kDa respectively. In this report we provide evidence that the A22R and A36R open reading frames also encode palmitylproteins with apparent molecular masses of 22 kDa and 50-55 kDa respectively. Furthermore, the hemagglutinin protein (A56R) from the Copenhagen strain is shown to be palmitylated while the hemagglutinin protein from the WR and IHD-J strains is not. A 94-kDa VV palmitylprotein appears to be a multimeric complex composed of the B5R protein and possibly others. All vaccinia-encoded palmitylproteins are present in the membranous fraction of cells and are specific for the trans-Golgi network membrane-enveloped forms of the virus suggesting that these proteins play a role in the envelopment and egress of virions or the infectivity of released virus.

The vaccinia virus L1R open reading frame encodes a 25-KDa acylprotein expressed during the late stage of infection, which is covalently modified by myristic acid at the penultimate NH_2-terminal glycine. L1R is associated

exclusively with the membranes surrounding the virion core, making it a constituent of both intracellular virus particles and extracellular enveloped virions. Previously, we have demonstrated that myristylation is necessary for appropriate membrane targeting. In view of these facts, it was of interest to determine the membrane topology of L1R as it exists within the envelope surrounding the virion core. The entire amino acid sequence of L1R was analyzed to predict regions of hydrophobicity, and putative transmembrane spanning domains. This analysis suggested two putative transmembrane domains, but gave no insights to how the protein was orientated in the lipid bilayer. To address this question, L1R mutants have been constructed that contain either a reporter epitope (myc) or artificial glycosylation site, preceding or following each of the potential transmembrane domains. These mutants were expressed *in vitro* by translation in reticulocyte lysate containing canine pancreatic microsomes, and *in vivo* by transient expression. The utilization of the glycosylation sites and sensitivity of the reporter epitopes to protease digestion has allowed us to determine the membrane orientation of L1R. The data strongly suggest that L1R spans the membrane of the IMV particle twice with an extra viral loop of 80 amino acids with both termini of the protein oriented towards the interior of the virus particle. This result has implications for probable regions of L1R, which are involved in IMV assembly and/or acquisition of the EEV envelope.

Recombinant vaccinia virus is a useful and powerful tool for the expression and study of foreign genes. Methods that are currently available for the selection of

VV recombinants include restoration of viral plaque-forming phenotype, replication of viral DNA in the presence of BUdR or mycophenolic acid, and maturation and propagation of virus under antibiotic selection. Although effective, each of these methods require several weeks of concerted effort to isolate, purify and amplify a potential recombinant virus. We report here the development of a bifunctional enzyme (BiZyme) to simplify and expedite the isolation and purification of VV recombinants. This novel selection marker is composed of an in-frame fusion between the genes encoding the green fluorescent protein (*gfp*) and the neomycin phosphotransferase enzyme (*neo*). Remarkably, expression of the chimeric *gfp-neo* cassette in the presence of G418 confers both viability and fluorescence to transfected or recombinant virus infected cells, indicating both activities are retained within the fusion protein. BiZyme was therefore incorporated into a recombination plasmid (pGNR) to enable the concomitant insertion of a foreign gene of interest. We demonstrate here that this selection/amplification process requires little more than a week to produce the desired VV recombinants. Furthermore, recombinants produced in this fashion have been shown to express both biologically-active enzymes and antigenically authentic foreign antigens. In addition to its use in the VV vector system, the BiZyme bifunctional selection scheme should be applicable to other eukaryotic and prokaryotic expression systems simply by coupling it to appropriate host-specific transcription regulatory signals.

Vaccinia virus encodes three myristylproteins of unknown function: A16L, E7R, and G9R with relative masses of 35-, 17- and 37-kDa, respectively. By subcellular fractionation of VV-infected cells it was determined that, unlike most acylated viral proteins that are membrane-associated, each of these proteins are soluble within the cytosol of the infected cell. Recently, we have attempted to insertionally inactivate these genes, but were unable to isolate recombinant virus in which the genes were not expressed suggesting that the genes are essential to VV replication. To circumvent this problem, three recombinant viruses have been constructed in which the expression of the A16L, E7R, and G9R genes can be inducibly repressed at the transcriptional level by absence of isopropylthiogalactopyranoside (IPTG.) Using this system we have examined the role of both the wild-type and nonmyristylated forms of the proteins in the VV life cycle. Data suggests that both A16L, and E7R are essential for VV replication and that myristylation is an important part of protein function. Whereas surprisingly, G9R expression has little, if any, effect on VV replication in tissue culture, regardless of myristylation state.

7.2 Suggestions for Future Research

Characterization of the virus continues to be paramount due to it's growing popularity in recombinant vaccine technology and the threat of using variola virus as a biological warfare agent. In addition the use of VV is proving to be a model

system for the study of other viral, eukaryotic and prokaryotic proteins. The focus of research in our lab over the past decade has been the identification, analysis, and characterization of VV acylproteins. Viral acylproteins have been shown to be important players in many life cycles, playing roles in enzymatic activities, or structural functions.

Currently, there has been eight palmitylproteins observed during a VV infection and at least six myristylproteins. Our laboratory has either identified or confirmed the identity of five palmitylproteins and five myristylproteins. However, many of these still remain uncharacterized and there functions are unknown. During this thesis period we have identified three new palmitylproteins encoded by the A22R, A36R, and A56R open reading frames. The protein encoded by the A22R open reading frame is the VV recombinase. The protein encoded by the A56R open reading frame is hemagglutinin protein. The protein encoded by the A36R open reading is an EEV specific protein that is essential for virus maturation. These proteins have been or are being characterized by other laboratories. However, the two remaining palmitylproteins are still unidentified and need to be further characterized. Palmitylation of these protein might follow different patterns, or possibly be enzymes in which the modification is turned on and off during the viral infection, causing them to be difficult to identify.

Now that the membrane orientation of the L1R protein has been identified, there are several directions that future studies can proceed in. First, the protein can be used to construct fusion proteins with other foreign antigens to specifically

deliver peptides to the surface of the IMV particles. In preliminary studies I demonstrated that the entire 80 amino acid loop with a foreign antigen can be replaced or a foreign sequence can be inserted, in frame, into the wild-type L1R sequence. Whether it still forms viable virus is a question that has yet to be answered. This also presents the possibility of a more potent vaccine delivery virus.

Since the start of the L1R project it has been determined that some of the cysteines of L1R are involved in disulfide bond formation. It is of interest to determine which cysteines are involved in the formation of disulfide bonds to possibly determine how the IV particle is wrapped with the IMV envelope. It is possible that the formation of the disulfide bonds wraps the envelope around the virus particle, which became possible through knowing how L1R is orientated in the IMV envelope.

Construction of *lac* inducible recombinants in the A16L, E7R, and G9R open reading frames has opened up the doors for the characterization of these proteins. Using these recombinant viruses one could perform both immunoelectron microscopy, and immunofluorescent microscopy to determine intracellular location plus or minus inducer, and determine which part of the virus life cycle these mutants affect. Further characterization of these proteins is made possible by using the viruses and transient expression plasmids of these genes to study different mutated forms of the proteins. Lastly for all the above mentioned proteins it will

be of interest to determine possible protein partners, both cellular and viral, to determine their roles in viral morphogenesis.

Bibliography

Ball, L. A. (1987). High-frequency homologous recombination in vaccinia virus DNA. *J Virol* **61**(6), 1788-1795.

Ball, L. A. (1995). Fidelity of homologous recombination in vaccinia virus DNA. *Virology* **209**(2), 688-691.

Bano, M. C., Jackson, C. S., and Magee, A. I. (1998). Pseudo-enzymatic S-acylation of a myristoylated yes protein tyrosine kinase peptide in vitro may reflect non-enzymatic S-acylation in vivo. *Biochem J* **330**(2), 723-731.

Belyakov, I. M., Moss, B., Strober, W., and Berzofsky, J. A. (1999). Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. *Proc Natl Acad Sci U S A* **96**(8), 4512-4517.

Berger, M., and Schmidt, M. F. (1984). Cell-free fatty acid acylation of Semliki Forest viral polypeptides with microsomal membranes from eukaryotic cells. *J Biol Chem* **259**(11), 7245-52.

Bernards, R., Destree, A., McKenzie, S., Gordon, E., Weinberg, R. A., and Panicali, D. (1987). Effective tumor immunotherapy directed against an oncogene-encoded product using a vaccinia virus vector. *Proc Natl Acad Sci U S A* **84**(19), 6854-6858.

Berthiaume, L., and Resh, M. D. (1995). Biochemical characterization of a palmitoyl acyltransferase activity that palmitoylates myristoylated proteins. *J Biol Chem* **270**(38), 22399-22405.

Bharadwaj, M., and Bizzozero, O. A. (1995). Myelin P0 glycoprotein and a synthetic peptide containing the palmitoylation site are both autoacylated. *J Neurochem* **65**(4), 1805-15.

Bizzozero, O. A., Fridal, K., and Pastuszyn, A. (1994). Identification of the palmitoylation site in rat myelin P0 glycoprotein. *J Neurochem* **62**(3), 1163-71.

Bizzozero, O. A., Tetzloff, S. U., and Bharadwaj, M. (1994). Overview: protein palmitoylation in the nervous system: current views and unsolved problems. *Neurochem Res* **19**(8), 923-33.

Blasco, R., and Moss, B. (1991). Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000- Dalton outer envelope protein. *J Virol* **65**(11), 5910-5920.

Bostock, C. J. (1990). Viruses as vectors. *Vet Microbiol* **23**(1-4), 55-71.

Brown, M. S., and Goldstein, J. L. (1993). Protein prenylation. Mad bet for Rab. *Nature* **366**(6450), 14-15.

Bryant, M., and Ratner, L. (1990). Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc Natl Acad Sci U S A* **87**(2), 523-7.

Buller, R. M., Smith, G. L., Cremer, K., Notkins, A. L., and Moss, B. (1985). Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* **317**(6040), 813-815.

Bursten, S. L., Locksley, R. M., Ryan, J. L., and Lovett, D. H. (1988). Acylation of monocyte and glomerular mesangial cell proteins. Myristyl acylation of the interleukin 1 precursors. *J Clin Invest* **82**(5), 1479-88.

Buss, J. E., and Sefton, B. M. (1985). Myristic acid, a rare fatty acid, is the lipid attached to the transforming protein of Rous sarcoma virus and its cellular homolog. *J Virol* **53**(1), 7-12.

Campbell, M. J. (1995). Lipofection reagents prepared by a simple ethanol injection technique. *Biotechniques* **18**(6), 1027-1032.

Caras, I. W., Weddell, G. N., Davitz, M. A., Nussenzweig, V., and Martin, D. W., Jr. (1987). Signal for attachment of a phospholipid membrane anchor in decay accelerating factor. *Science* **238**(4831), 1280-1283.

Chakrabarti, S., Sisler, J. R., and Moss, B. (1997). Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques* **23**(6), 1094-1097.

Challberg, M. D., and Englund, P. T. (1979). The effect of template secondary structure on vaccinia DNA polymerase. *J Biol Chem* **254**(16), 7820-7826.

Chen, Y. L., Ts'ai, P. W., Yang, C. C., and Wang, C. T. (1997). Generation of infectious virus particles by transient co-expression of human immunodeficiency virus type 1 gag mutants. *J Gen Virol* **78**(Pt 10), 2497-501.

Clegg, C. H., Ran, W., Uhler, M. D., and McKnight, G. S. (1989). A mutation in the catalytic subunit of protein kinase A prevents myristylation but does not inhibit biological activity. *J Biol Chem* **264**(33), 20140-6.

Child, S. J., and Hruby, D. E. (1992). Evidence for multiple species of vaccinia virus-encoded palmitylated proteins. *Virology* **191**(1), 262-271.

Chow, M., Newman, J. F., Filman, D., Hogle, J. M., Rowlands, D. J., and Brown, F. (1987). Myristylation of picornavirus capsid protein VP4 and its structural significance. *Nature* **327**(6122), 482-6.

Clarke, S. (1992). Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu Rev Biochem* **61**, 355-386.

Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**(1), 33-38.

Coupar, B. E., Oke, P. G., and Andrew, M. E. (2000). Insertion sites for recombinant vaccinia virus construction: effects on expression of a foreign protein. *J Gen Virol* **81**, 431-439.

Cox, A. D., and Der, C. J. (1992). Protein prenylation: more than just glue? *Curr Opin Cell Biol* **4**(6), 1008-1016.

Dales, S., and Pogo, B. G. (1981). Biology of poxviruses. *Virol Monogr* **18**, 1-109.

Deichaite, I., Casson, L. P., Ling, H. P., and Resh, M. D. (1988). In vitro synthesis of pp60v-src: myristylation in a cell-free system. *Mol Cell Biol* **8**(10), 4295-4301.

Dietzen, D. J., Hastings, W. R., and Lublin, D. M. (1995). Caveolin is palmitoylated on multiple cysteine residues. Palmitoylation is not necessary for localization of caveolin to caveolae. *J Biol Chem* **270**(12), 6838-6842.

Dohlman, H. G., Goldsmith, P., Spiegel, A. M., and Thorner, J. (1993). Pheromone action regulates G-protein alpha-subunit myristoylation in the yeast Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **90**(20), 9688-9692.

Duncan, S. A., and Smith, G. L. (1992). Identification and characterization of an extracellular envelope glycoprotein affecting vaccinia virus egress. *J Virol* **66**(3), 1610-21.

Dunphy, J. T., Greentree, W. K., Manahan, C. L., and Linder, M. E. (1996). G-protein palmitoyltransferase activity is enriched in plasma membranes. *J Biol Chem* **271**(12), 7154-7159.

Duronio, R. J., Rudnick, D. A., Adams, S. P., Towler, D. A., and Gordon, J. I. (1991). Analyzing the substrate specificity of Saccharomyces cerevisiae myristoyl-CoA:protein N-myristoyltransferase by co-expressing it with mammalian G protein alpha subunits in Escherichia coli. *J Biol Chem* **266**(16), 10498-10504.

Engelstad, M., Howard, S. T., and Smith, G. L. (1992). A constitutively expressed vaccinia gene encodes a 42-kDa glycoprotein related to complement control factors that forms part of the extracellular virus envelope. *Virology* **188**(2), 801-10.

Engelstad, M., and Smith, G. L. (1993). The vaccinia virus 42-kDa envelope protein is required for the envelopment and egress of extracellular virus and for virus virulence. *Virology* **194**(2), 627-637.

Fenner, F. (1989). Risks and benefits of vaccinia vaccine use in the worldwide smallpox eradication campaign. *Res Virol* **140**(5), 465-466.

Ferguson, M. A., and Williams, A. F. (1988). Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Annu Rev Biochem* **57**, 285-320.

Franke, C. A., Reynolds, P. L., and Hruby, D. E. (1989). Fatty acid acylation of vaccinia virus proteins. *J Virol* **63**(10), 4285-4291.

Franke, C. A., Rice, C. M., Strauss, J. H., and Hruby, D. E. (1985). Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. *Mol Cell Biol* **5**(8), 1918-1924.

Franke, C. A., Roseman, N. A., and Hruby, D. E. (1985). Expression and regulation of the vaccinia virus thymidine kinase gene in non-permissive cells. *Virus Res* **3**(1), 13-17.

Franke, C. A., Wilson, E. M., and Hruby, D. E. (1990). Use of a cell-free system to identify the vaccinia virus L1R gene product as the major late myristylated virion protein M25. *J Virol* **64**(12), 5988-96.

Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc Natl Acad Sci U S A* **83**(21), 8122-8126.

Gheysen, D., Jacobs, E., de Foresta, F., Thiriart, C., Francotte, M., Thines, D., and De Wilde, M. (1989). Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**(1), 103-12.

Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. (1990). The complete DNA sequence of vaccinia virus. *Virology* **179**(1), 247-266.

Gordon, J. I., Duronio, R. J., Rudnick, D. A., Adams, S. P., and Gokel, G. W. (1991). Protein N-myristoylation. *J Biol Chem* **266**(14), 8647-8650.

Gottlinger, H. G., Sodroski, J. G., and Haseltine, W. A. (1989). Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **86**(15), 5781-5.

Grosenbach, D. W., and Hruby, D. E. (1998). Analysis of a vaccinia virus mutant expressing a nonpalmitylated form of p37, a mediator of virion envelopment. *J Virol* **72**(6), 5108-20.

Grosenbach, D. W., Ulaeto, D. O., and Hruby, D. E. (1997). Palmitylation of the vaccinia virus 37-kDa major envelope antigen. Identification of a conserved acceptor motif and biological relevance. *J Biol Chem* **272**(3), 1956-1964.

Gutierrez, L., and Magee, A. I. (1991). Characterization of an acyltransferase acting on p21N-ras protein in a cell-free system. *Biochim Biophys Acta* **1078**(2), 147-154.

Hansen, S. G. (2000). Oregon State University, Corvallis.

Hansen, S. G., Grosenbach, D. W., and Hruby, D. E. (1999). Analysis of the site occupancy constraints of primary amino acid sequences in the motif directing palmitylation of the vaccinia virus 37-kDa envelope protein. *Virology* **254**(1), 124-37.

Hiller, G., and Weber, K. (1985). Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *J Virol* **55**(3), 651-659.

Hirt, P., Hiller, G., and Wittek, R. (1986). Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. *J Virol* **58**(3), 757-64.

Hofmann, K., and Stoffel, W. (1993). . *Biol. Chem. Hoppe-Seyler* **347,** 166.

Hruby, D. E., Brinkley, J. M., Kang, H. C., Haugland, R. P., Young, S. L., and Melner, M. H. (1990). Use of a fluorescent chloramphenicol derivative as a substrate for CAT assays. *Biotechniques* **8**(2), 170-171.

Hruby, D. E., and Franke, C. A. (1993). Viral acylproteins: greasing the wheels of assembly. *Trends Microbiol* **1**(1), 20-5.

Hruby, D. E., Guarino, L. A., and Kates, J. R. (1979). Vaccinia virus replication. I. Requirement for the host-cell nucleus. *J Virol* **29**(2), 705-15.

Hruby, D. E., Hodges, W. M., Wilson, E. M., Franke, C. A., and Fischetti, V. A. (1988). Expression of streptococcal M protein in mammalian cells. *Proc Natl Acad Sci U S A* **85**(15), 5714-5717.

Isaacs, S. N., Wolffe, E. J., Payne, L. G., and Moss, B. (1992). Characterization of a vaccinia virus-encoded 42-kilodalton class I membrane glycoprotein component of the extracellular virus envelope. *J Virol* **66**(12), 7217-7224.

Ivanova, L., and Schlesinger, M. J. (1993). Site-directed mutations in the Sindbis virus E2 glycoprotein identify palmitoylation sites and affect virus budding. *J Virol* **67**(5), 2546-51.

Johnson, D. R., Knoll, L. J., Rowley, N., and Gordon, J. I. (1994). Genetic analysis of the role of Saccharomyces cerevisiae acyl-CoA synthetase genes in regulating protein N-myristoylation. *J Biol Chem* **269**(27), 18037-46.

Johnson, G. P., Goebel, S. J., and Paoletti, E. (1993). An update on the vaccinia virus genome. *Virology* **196**(2), 381-401.

Kao, S. Y., Ressner, E., Kates, J., and Bauer, W. R. (1981). Purification and characterization of a superhelix binding protein from vaccinia virus. *Virology* **111**(2), 500-508.

Kasinathan, C., Grzelinska, E., Okazaki, K., Slomiany, B. L., and Slomiany, A. (1990). Purification of protein fatty acyltransferase and determination of its distribution and topology. *J Biol Chem* **265**(9), 5139-5144.

Knoll, L. J., Levy, M. A., Stahl, P. D., and Gordon, J. I. (1992). Analysis of the compartmentalization of myristoyl-CoA:protein N- myristoyltransferase in Saccharomyces cerevisiae. *J Biol Chem* **267**(8), 5366-5373.

Koegl, M., Zlatkine, P., Ley, S. C., Courtneidge, S. A., and Magee, A. I. (1994). Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif. *Biochem J* **303**(Pt 3), 749-53.

Kunkel, T. A., Bebenek, K., and McClary, J. (1991). Efficient site-directed mutagenesis using uracil-containing DNA. *Methods Enzymol* **204**, 125-139.

Larder, B. A., Kemp, S. D., and Darby, G. (1987). Related functional domains in virus DNA polymerases. *Embo J* **6**(1), 169-175.

Madalinski, W., Bankovski, A., and Korbecki, M. (1977). Purification of vaccinia virus by zonal centrifugation and analysis of viral protein composition. *Acta Virol* **21**(2), 104-108.

Magee, A. I., and Courtneidge, S. A. (1985). Two classes of fatty acid acylated proteins exist in eukaryotic cells. *Embo J* **4**(5), 1137-44.

Magee, A. I., and Schlesinger, M. J. (1982). Fatty acid acylation of eucaryotic cell membrane proteins. *Biochim Biophys Acta* **694**(3), 279-89.

Manenti, S., Sorokine, O., Van Dorsselaer, A., and Taniguchi, H. (1994). Demyristoylation of the major substrate of protein kinase C (MARCKS) by the cytoplasmic fraction of brain synaptosomes. *J Biol Chem* **269**(11), 8309-8313.

Martin, K. H. (1997). Oregon State University, Corvallis.

Martin, K. H., Grosenbach, D. W., Franke, C. A., and Hruby, D. E. (1997). Identification and analysis of three myristylated vaccinia virus late proteins. *J Virol* **71**(7), 5218-5226.

Merchlinsky, M., and Moss, B. (1992). Introduction of foreign DNA into the vaccinia virus genome by in vitro ligation: recombination-independent selectable cloning vectors. *Virology* **190**(1), 522-526.

Meyer, H., and Rziha, H. J. (1993). Characterization of the gene encoding the A-type inclusion protein of camelpox virus and sequence comparison with other orthopoxviruses. *J Gen Virol* **74**(Pt 8), 1679-84.

Moores, S. L., Schaber, M. D., Mosser, S. D., Rands, E., MB, O. H., Garsky, V. M., Marshall, M. S., Pompliano, D. L., and Gibbs, J. B. (1991). Sequence dependence of protein isoprenylation. *J Biol Chem* **266**(22), 14603-14610.

Moss, B. (1990). Poxviruses. *In* "Virology" (B. N. Fields, Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J., Monath, T. P., and Roizmain, B., eds, Ed.), Vol. 2, pp. 2079-2111. 2 vols. Raven Publishing, New York.

Moss, B. (1991). Vaccinia virus: a tool for research and vaccine development. *Science* **252**(5013), 1662-1667.

Moss, B. (1996a). Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc Natl Acad Sci U S A* **93**(21), 11341-11348.

Moss, B. (1996b). Poxviridae: The Viruses and Their Replication. 3rd ed. *In* "Fundamental Virology" (B. N. Fields, Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J., Monath, T. P., and Roizmain, B., eds., Ed.), pp. 1163- 1185. Lippencott-Raven Publishers, Philadelphia.

Moss, B., Fuerst, T. R., Flexner, C., and Hugin, A. (1988). Roles of vaccinia virus in the development of new vaccines. *Vaccine* **6**(2), 161-3.

Oie, M., and Ichihashi, Y. (1987). Modification of vaccinia virus penetration proteins analyzed by monoclonal antibodies. *Virology* **157**(2), 449-459.

Paoletti, E. (1990). Poxvirus recombinant vaccines. *Ann N Y Acad Sci* **590**, 309-25.

Parkinson, J. E., and Smith, G. L. (1994). Vaccinia virus gene A36R encodes a M(r) 43-50 K protein on the surface of extracellular enveloped virus. *Virology* **204**(1), 376-390.

Patel, D. D., Pickup, D. J., and Joklik, W. K. (1986). Isolation of cowpox virus A-type inclusions and characterization of their major protein component. *Virology* **149**(2), 174-89.

Payne, L. G. (1980). Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. *J Gen Virol* **50**(1), 89-100.

Payne, L. G. (1992). Characterization of vaccinia virus glycoproteins by monoclonal antibody precipitation. *Virology* **187**(1), 251-60.

Payne, L. G., and Norrby, E. (1976). Presence of haemagglutinin in the envelope of extracellular vaccinia virus particles. *J Gen Virol* **32**(1), 63-72.

Pietrini, G., Aggujaro, D., Carrera, P., Malyszko, J., Vitale, A., and Borgese, N. (1992). A single mRNA, transcribed from an alternative, erythroid-specific, promoter, codes for two non-myristylated forms of NADH-cytochrome b5 reductase. *J Cell Biol* **117**(5), 975-986.

Ponimaskin, E., and Schmidt, M. F. (1995). Acylation of viral glycoproteins: structural requirements for palmitoylation of transmembrane proteins. *Biochem Soc Trans* **23**(3), 565-568.

Prange, R., Clemen, A., and Streeck, R. E. (1991). Myristylation is involved in intracellular retention of hepatitis B virus envelope proteins. *J Virol* **65**(7), 3919-23.

Ravanello, M. P., Franke, C. A., and Hruby, D. E. (1993). An NH2-terminal peptide from the vaccinia virus L1R protein directs the myristylation and virion envelope localization of a heterologous fusion protein. *J Biol Chem* **268**(10), 7585-7593.

Ravanello, M. P., and Hruby, D. E. (1994a). Characterization of the vaccinia virus L1R myristylprotein as a component of the intracellular virion envelope. *J Gen Virol* **75**(Pt 6), 1479-1483.

Ravanello, M. P., and Hruby, D. E. (1994b). Conditional lethal expression of the vaccinia virus L1R myristylated protein reveals a role in virion assembly. *J Virol* **68**(10), 6401-10.

Resh, M. D. (1990). Membrane interactions of pp60v-src: a model for myristylated tyrosine protein kinases. *Oncogene* **5**(10), 1437-44.

Resh, M. D. (1996). Regulation of cellular signalling by fatty acid acylation and prenylation of signal transduction proteins. *Cell Signal* **8**(6), 403-412.

Rockey, D. D., Grosenbach, D., Hruby, D. E., Peacock, M. G., Heinzen, R. A., and Hackstadt, T. (1997). Chlamydia psittaci IncA is phosphorylated by the host cell and is exposed on the cytoplasmic face of the developing inclusion. *Mol Microbiol* **24**(1), 217-228.

Rodriguez, J. F., and Smith, G. L. (1990). IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. *Nucleic Acids Res* **18**(18), 5347-5351.

Rodriguez, J. R., Risco, C., Carrascosa, J. L., Esteban, M., and Rodriguez, D. (1997). Characterization of early stages in vaccinia virus membrane biogenesis: implications of the 21-kilodalton protein and a newly identified 15-kilodalton envelope protein. *J Virol* **71**(3), 1821-33.

Rodriguez, J. R., Risco, C., Carrascosa, J. L., Esteban, M., and Rodriguez, D. (1998). Vaccinia virus 15-kilodalton (A14L) protein is essential for assembly and attachment of viral crescents to virosomes. *J Virol* **72**(2), 1287-1296.

Roper, R. L., Payne, L. G., and Moss, B. (1996). Extracellular vaccinia virus envelope glycoprotein encoded by the A33R gene. *J Virol* **70**(6), 3753-3762.

Rose, J. K., Buonocore, L., and Whitt, M. A. (1991). A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *Biotechniques* **10**(4), 520-525.

Rottger, S., Frischknecht, F., Reckmann, I., Smith, G. L., and Way, M. (1999). Interactions between vaccinia virus IEV membrane proteins and their roles in IEV assembly and actin tail formation. *J Virol* **73**(4), 2863-75.

Rudnick, D. A., McWherter, C. A., Rocque, W. J., Lennon, P. J., Getman, D. P., and Gordon, J. I. (1991). Kinetic and structural evidence for a sequential ordered Bi Bi mechanism of catalysis by Saccharomyces cerevisiae myristoyl- CoA:protein N-myristoyltransferase. *J Biol Chem* **266**(15), 9732-9739.

Schlesinger, M. J., Magee, A. I., and Schmidt, M. F. (1980). Fatty acid acylation of proteins in cultured cells. *J Biol Chem* **255**(21), 10021-4.

Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E. J., Shida, H., Hiller, G., and Griffiths, G. (1994). Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. *J Virol* **68**(1), 130-147.

Schmidt, M. F. (1983). Fatty acid binding: a new kind of posttranslational modification of membrane proteins. *Curr Top Microbiol Immunol* **102**, 101-29.

Schmidt, M. F., and Burns, G. R. (1989). Hydrophobic modifications of membrane proteins by palmitoylation in vitro. *Biochem Soc Trans* **17**(4), 625-626.

Schmidt, M. F., McIlhinney, R. A., and Burns, G. R. (1995). Palmitoylation of endogenous and viral acceptor proteins by fatty acyltransferase (PAT) present in erythrocyte ghosts and in placental membranes. *Biochim Biophys Acta* **1257**(3), 205-13.

Schmutz, C., Rindisbacher, L., Galmiche, M. C., and Wittek, R. (1995). Biochemical analysis of the major vaccinia virus envelope antigen. *Virology* **213**(1), 19-27.

Shahinian, S., and Silvius, J. R. (1995). Doubly-lipid-modified protein sequence motifs exhibit long-lived anchorage to lipid bilayer membranes. *Biochemistry* **34**(11), 3813-3822.

Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1994). Cysteine3 of Src family protein tyrosine kinase determines palmitoylation and localization in caveolae. *J Cell Biol* **126**(2), 353-363.

Silvius, J. R., and l'Heureux, F. (1994). Fluorimetric evaluation of the affinities of isoprenylated peptides for lipid bilayers. *Biochemistry* **33**(10), 3014-3022.

Smith, G. L., Chan, Y. S., and Howard, S. T. (1991). Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat. *J Gen Virol* **72**(Pt 6), 1349-76.

Spencer, E., Shuman, S., and Hurwitz, J. (1980). Purification and properties of vaccinia virus DNA-dependent RNA polymerase. *J Biol Chem* **255**(11), 5388-5395.

Stahl, N., Borchelt, D. R., and Prusiner, S. B. (1990). Differential release of cellular and scrapie prion proteins from cellular membranes by phosphatidylinositol-specific phospholipase C. *Biochemistry* **29**(22), 5405-5412.

Stern, W., and Dales, S. (1976). Biogenesis of vaccinia: isolation and characterization of a surface component that elicits antibody suppressing infectivity and cell-cell fusion. *Virology* **75**(1), 232-241.

Stevenson, F. T., Bursten, S. L., Fanton, C., Locksley, R. M., and Lovett, D. H. (1993). The 31-kDa precursor of interleukin 1 alpha is myristoylated on specific lysines within the 16-kDa N-terminal propiece. *Proc Natl Acad Sci U S A* **90**(15), 7245-7249.

Stevenson, F. T., Bursten, S. L., Locksley, R. M., and Lovett, D. H. (1992). Myristyl acylation of the tumor necrosis factor alpha precursor on specific lysine residues. *J Exp Med* **176**(4), 1053-62.

Studier, F. W. (1973). Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J Mol Biol* **79**(2), 237-248.

Towbin, H., Staehelin, T., and Gordon, J. (1992). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. *Biotechnology* **24**, 145-9.

Towler, D. A., Adams, S. P., Eubanks, S. R., Towery, D. S., Jackson-Machelski, E., Glaser, L., and Gordon, J. I. (1987a). Purification and characterization of yeast myristoyl CoA:protein N- myristoyltransferase. *Proc Natl Acad Sci U S A* **84**(9), 2708-2712.

Towler, D. A., Eubanks, S. R., Towery, D. S., Adams, S. P., and Glaser, L. (1987b). Amino-terminal processing of proteins by N-myristoylation. Substrate specificity of N-myristoyl transferase. *J Biol Chem* **262**(3), 1030-1036.

VanSlyke, J. K., and Hruby, D. E. (1990). Posttranslational modification of vaccinia virus proteins. *Curr Top Microbiol Immunol* **163**, 185-206.

Veit, M., Reverey, H., and Schmidt, M. F. (1996). Cytoplasmic tail length influences fatty acid selection for acylation of viral glycoproteins. *Biochem J* **318**(Pt 1), 163-72.

Wedegaertner, P. B., Wilson, P. T., and Bourne, H. R. (1995). Lipid modifications of trimeric G proteins. *J Biol Chem* **270**(2), 503-506.

Wilcox, C., Hu, J. S., and Olson, E. N. (1987). Acylation of proteins with myristic acid occurs cotranslationally. *Science* **238**(4831), 1275-1278.

Wolffe, E. J., Vijaya, S., and Moss, B. (1995). A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. *Virology* **211**(1), 53-63.

Yang, C., and Compans, R. W. (1996). Palmitoylation of the murine leukemia virus envelope glycoprotein transmembrane subunits. *Virology* **221**(1), 87-97.

Zhou, W., Parent, L. J., Wills, J. W., and Resh, M. D. (1994). Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J Virol* **68**(4), 2556-2569.