Articles

Sequence-Independent Acylation of the Vaccinia Virus A-Type Inclusion Protein†

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Received November 11, 2003; Revised Manuscript Received April 19, 2004

ABSTRACT: N-Terminal myristoylation of proteins typically occurs cotranslationally via an amide bond to the penultimate glycine residue within the canonical motif (M)GXXX(S/T/A) in a reaction catalyzed by *N*-myristoyltransferase. A second, less common myristoylation reaction occurs internally at dibasic amino acid doublets of proteins such as α-TNF. In this case, myristoylation occurs within a portion of the preprotein, which is subsequently removed by N-terminal proteolysis. The identity of the enzyme catalyzing internal myristoylation is unknown. Considering this information, the vaccinia virus (VV) A-type inclusion protein (ATI) presents a conundrum. Although this cytosolic protein is clearly myristoylated, the protein does not have the N-terminal myristoylation motif nor is it subject to proteolytic maturation. In the experiments reported here, we cleaved VV ATI with cyanogen bromide and determined that the myristoyl moiety was present in the C-terminal half of the protein. We also subjected a tryptic digest of VV ATI to liquid chromatography electrospray ionization quadrupole ion trap mass spectrometry analyses, which indicated that ATI is randomly myristoylated at six different lysines or arginines. Analysis of the modification sites reveals no obvious conserved acceptor motifs or dibasic doublets. Mutation of these residues alone or in combination does not abrogate myristoylation of the protein, suggesting utilization of alternative modification sites. This information implies that the VV ATI protein is myristoylated in a sequence-independent manner. Because viral acylproteins typically utilize the host cell modification apparatus, this result suggests there may be an alternative type of myristoylation pathway in mammalian cells.

Poxviruses, such as vaccinia virus (VV),[1] are among the largest and most complex of the eukaryotic DNA viruses and are distinguished by replicating exclusively within the cytoplasmic compartment of infected cells (*1*). VV regulates the expression of more than 250 viral gene products in a temporal fashion during the viral replicative cycle, which results in at least four infectious forms. Given the large number of viral encoded proteins, the multiplicity of virion forms, and the number of distinct intracellular sites used during the viral assembly and morphogenesis process, VV must employ a number of targeting mechanisms, including protein acylation, to direct viral proteins to the proper intracellular location.

The two major types of acylprotein modifications are myristoylation and palmitylation. Palmitylation is the post-translational addition of a 16-carbon saturated fatty acid (palmitic acid) via a hydroxylamine-sensitive thioester or ester bond (*2*, *3*). There are six VV palmitylproteins, including A33R, B5R, F13L, A22R, A36R, and A56R. Myristoylation is the addition of a 14-carbon acyl moiety ($C_{14}H_{26}O$) to a protein, and this process can occur via two routes. The most common is the cotranslational addition of myristic acid to the penultimate glycine of the N terminus. This reaction is mediated by *N*-myristoyltransferase (NMT) and contains a conserved motif on the N terminus, G-X-X-X-S/T/A. VV has four *N*-myristoylproteins (L1R, A16L, G9R, and E7R) (*4*). A less frequent occurrence is the posttranslational addition of myrsitic acid to the free amine group of either lysine or arginine. VV has one protein that rapidly incorporates ^{3}H-myristic acid (^{3}H-MA) but does not contain the canonical N-terminal motif (*5*). This protein is known as the A-type inclusion protein (ATI) and is the product of the A25L vaccinia open-reading frame.

Although there are a growing number of cellular proteins that are internally myristoylated (interluekin 1 α and β precursors (*6*), α-tumor necrosis factor (*7*), immunoglobulin M μ chain (*8*), and subunit 1 of cytochrome *c* in *Neurospora crassa* (*9*), there are no viral proteins known to be modified in this manner, other than perhaps the PBCV-1 capsid protein (*10*). Of the known internal myristoylproteins, the specific site of modification has only been mapped in the cytochrome

† This work was supported by NIH Grant AI21335. This publication was made possible in part by Grant P30 ES00210 from the National Institute of Environmental Health Sciences, NIH.

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[1] Abbreviations: VV, vaccinia virus; ATI, A-type inclusion; ^{3}H-MA, [9,10 ^{3}H(N)]-myristic acid; CNBr, cyanogen bromide; COP, Copenhagen; WR, Western Reserve; CPV, Cowpox virus; LC−ESI−QIT, liquid chromatography electrospray ionization quadrupole ion trap; MS, mass spectrometry; NMT, *N*-myristoyltransferase; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

10.1021/bi0360197 CCC: $27.50
Published on Web 06/11/2004

Table 1: 5′ and 3′ Primer Sequences of the Six Mutation Sites

site	5′ primer	3′ primer
K-467	ATCGACCGACTTACAGCAGAGATCAAAGA	TCTTTGATCTCTGCTGTAAGTCGGTCGAT
K-470	CTTACAAAAGAGFATCGCAGAACACAGGGA	TCCCTGTGTTCTGCGATCTCTTTTGTAAG
R-502	AGAGAATCGCTTGATGCGGAACGAGAAAT	ATTTCTCGTTCCGCATCAAGCGATTCTCT
R-518	GAACTGGATACTATTGCGAATGGAAAAGT	ACTTTTCCATTCGCAATAGTATCCAGTTC
K-512	CGTTCAGAACTAGAAGCGGAACTGGATAC	GTATCCAGTTCCGCTTCTAGTTCTGAACG
K-538	GTCGTATGTGGCTAGCACAACGCGAT	ATCGCGTTGTGCTAGCCACATACGAC

c subunit of *N. crassa*. This 42 kDa protein (557 amino acids) contains only 8 lysine residues, and the modified lysine was identified by proteolytic and chemical cleavage of the radiolabeled protein followed by gel electrophoresis and liquid scintillation analysis. In contrast, the internally myristoylated VV ATI protein is composed of 725 amino acids (92 kDa) and contains 56 lysine and 59 arginine residues. Because roughly 16% of the ATI protein contains potential myristoyl modification sites, a more precise procedure is needed to map the site of modification.

In this paper, the data obtained demonstrates that the VV ATI protein contains at least six sites that can be modified with myristic acid. ATI labeled with ^{3}H-MA was cleaved with cyanogen bromide (CNBr), and the labeled peptides were all identified. The sites of modification were all within the C-terminal half of the protein. Identification of these sites was obtained through liquid chromatography electrospray ionization quadrupole ion trap (LC−ESI−QIT) mass spectrometry (MS) analysis. Surprisingly, site-directed mutagenesis of the six myristoylation sites produces a mutant protein that can still incorporate ^{3}H-MA, suggesting that the modification can "jump" to a different site if the original site is altered. This also suggests that it is the overall protein hydrophobicity and not the precise acceptor motifs, which dictates its modification.

MATERIALS AND METHODS

Chemicals and Enzymes. CNBr, high-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, and trifluroacetic acid were purchased from Sigma (St. Louis, MO). Media and supplements were purchased from Invitrogen (Calsbad, CA). Sequencing-grade trypsin was purchased from Promega (Madison, WI). Gel buffers and components were purchased from BioRad (Hercules, CA). Acetic acid and dimethyl sulfoxide (DMSO) was purchased from Fisher (Pittsburgh, PA).

Cells and Virus. BSC-40 (African green monkey kidney) cells were maintained in modified Eagle's medium (E-MEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 10 μg of gentamicin/mL, at 37 °C in 5% CO_2 in a humidified incubator. A recombinant VV, vATI, expressing a second copy of ATI containing a 6-histidine tag at the N terminus was produced according to Franke et al. (*11*). The Copenhagen (COP), Western Reserve (WR), and vATI strains of VV were routinely propagated and titered in BSC-40 cells as previously described (*12*).

ATI Purification and Preparation for MS Analysis. ATI was isolated and purified according to Chen et al. (*13*). Purified ATI was cleaved either with CNBr (*14*) or trypsin (*13*). The tryptic peptides were analyzed by a conjunction Waters (Milford, MA) 515 HPLC system with a C_{18} column (0.17 × 10 mm, Jupiter 5 μm, 300 Å, packed in house) and an ESI−QIT mass spectrometer (Finnigan LCQ, San Jose, CA). HPLC was performed with a gradient from 70% solvent A (0.1% formic acid and 0.005% trifluoroacetic acid (TFA) in 5% acetonitrile) to 80% solvent B (0.1% formic acid and 0.005% TFA in 95% acetonitrile) over 45 min.

N-Terminal Sequencing. The CNBr fragments of ATI were resolved on a 12.5% SDS−PAGE gel (*15*) with filtered buffers. The gel was blotted using a filtered transfer buffer containing 0.5% SDS and 10% methanol, onto a polyvinylidene fluoride (PVDF, Millipore, Billerica, MA) membrane wetted with HPLC-grade methanol. Protein bands were visualized using 0.1% Comassie R-250, 40% methanol and 1% acetic acid and filtered with a glass filter (Whatman, Clifton, NJ). The membrane was submerged into the stain for 1 min and then destained with 50% methanol until the bands were intensified. Once the blot was dried, the protein bands were excised.

Gels and Fluorography. Virus-infected cells were harvested and pelleted by centrifugation. The supernatant was aspirated, and the pellet was resuspended in phosphate-buffered saline and subjected to 3 cycles of freeze/thaw to lyse the cells. Each sample was then prepared for discontinuous SDS−PAGE by adding equal amounts of a reducing sample buffer and boiled for 10 min. The proteins were resolved on either 12.5 or 10% polyacrylamide gels (*15*). The resulting gels were stained in 0.2% Coomassie R-250, 40% methanol and 10% acetic acid or subjected to fluorography (*16*).

Cloning and Mutagenesis. All primers were purchased from Invitrogen (Carlsbad, CA). The ATI open-reading frame was amplified using polymerase chain reaction (PCR) using the following oligonucleotides: ATI5′ (CATGCCATGGAGGTCACGAAC) and ATI3′ (CGCGGATCCAGACGTCGCATCTCT), which incorporate a *Nco*I site and *Bam*HI site, respectively. The amplified gene was cloned into pTM1/L5R-6His, which when cut with *Nco*1 and *Bam*HI loses the L5R protein but retains the 6His tag at the C terminus. The pTM1/ATI-6His clone was then cut with *Nco*I and *Pst*I (which cuts after the 6-histidine tag) and spliced into pRB21 (*17*), which contains a multiple cloning site upstream of the VV synthetic early/late promoter. This clone, pRB21/ATI, contains a 6-histidine tag at the C terminus and was used in the mutagenesis and transfection experiments. Single mutagenesis of the modified lysine or arginine to alanine was carried out using a QuickChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA) according to the instructions of the manufacturer.

Mutagenesis of the six-site knock out, Δ^6-ATI, was done in two rounds using a QuickChange Multi Site-Directed Mutagenesis kit from Stratagene. Round one used the primers listed in Table 1 and mutated sites K-467, K-512, and K-538. Round two mutated sites K-470, R-502, and R-518 and used

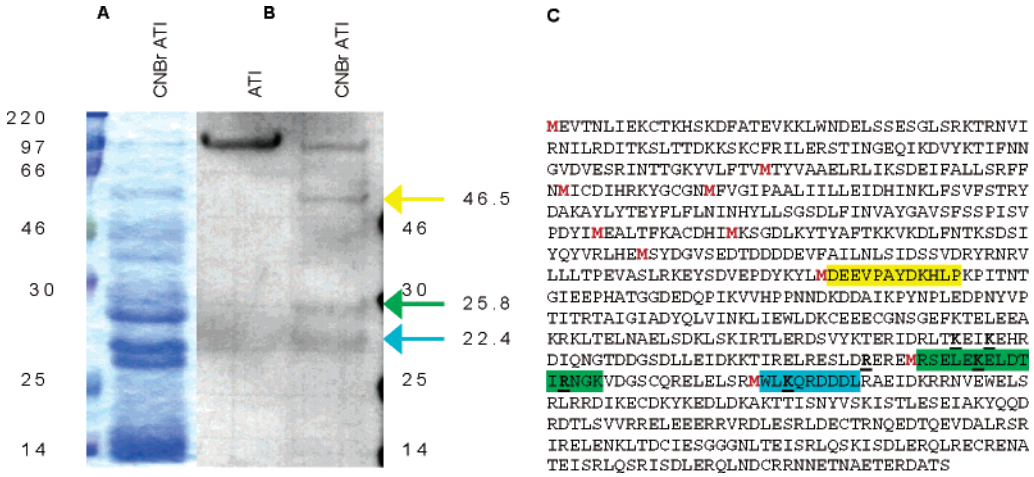

FIGURE 1: CNBr cleavage of ATI. Purified ATI was cleaved with CNBr and resolved by SDS−PAGE. Bands were visualized by either Comassie stain (A) or fluorography (B). Protein peptides that incorporated ^{3}H-MA were sent in for N-terminal sequencing; the predicted molecular masses are shown, and the corresponding sequence information is highlighted (C). Possible cleavage sites (at methionines) are shown in red. Residues that are underlined were found to be modified with a myristoyl moiety.

the primers (Table 1) for R-502 and R-518. A new primer for K-470 was used containing the mutation for K-467: 5′ primer (CTTACAGCAGAGATCGCAGAACACAGGGA) and 3′ primer (TCCCTGTGTTCTGCGATCTCTGCTG-TAAG).

Transfections and Metabolic Labeling. Monolayers (90% confluent) of BSC-40 cells in 35-mm wells were infected with vATI, WR, or COP VV at a multiplicity of infection of 10. Concurrent with infection, cells were transfected with plasmids using the synthetic liposome, DMRIE-C (Invitrogen, Calsbad, CA). As a control, some inoculum lacked DNA (virus alone). The viral-infected cells were place at 37 °C. [9,10 ^{3}H(N)]-Myristic acid was purchased as an ethanolic solution (Perkin−Elmer, Boston, MA) and was dried by nitrogen overflow. The lipids were then dissolved in DMSO at a concentration of 10 μCi/μL. After 4-h postinfection, the transfection medium was replaced with 1 mL of medium containing 10 μCi of [^{3}H]-MA. The cells were then harvested 24-h postinfection and prepared for SDS−PAGE analysis.

RESULTS

Previous studies have shown that the VV 92 kDa protein that rapidly incorporates ^{3}H-MA but does not contain the canonical N-terminal motif (G-X-X-X-S/T/A) is ATI (*5*). This protein is not modified at the N terminus, suggesting that an internal modification occurs on a lysine or arginine residue. The 725 amino acid protein is extremely rich in lysine and arginine (115 potential sites), making site-directed mutagenesis extremely difficult to identify the modification sites. Previous MS studies also showed that there is a discrepancy between the total molecular mass of ATI and its predicted molecular mass. This discrepancy suggests that there are approximately two molecules of the myristoyl moiety ($C_{14}H_{26}O$) associated with each ATI protein (*13*). Considering that there are many potential sites of modification and the fact that there are, on average, two modification sites per protein, we decided to use analytical biochemical approaches to determine if the modifications were in a particular region of the protein.

To determine the regions of modification, ATI was labeled with ^{3}H-MA, purified as described (*13*), and cleaved with CNBr. CNBr was chosen because it should cleave ATI into 10 fragments, most of which are large enough to observe on a polyacrylamide gel (Figure 1). Although CNBr produced a number of bands on a Coomassie-stained gel (Figure 1A), only three fragments were labeled with ^{3}H-MA (Figure 1B). When these three fragments were subjected to N-terminal sequencing, we discovered that they corresponded to sequences in the C-terminal half of the protein (Figure 1C). There was, however, incomplete cleavage using CNBr, not uncommon with this procedure, so that the band with the yellow arrow denotes a peptide from M-326 to the end of ATI (e.g., two missed cleavages). Likewise, this is true for the protein band denoted with the green arrow. Interestingly, the three peptides all resolve at a higher molecular mass on a SDS−PAGE gel than their predicted molecular mass (arrows in Figure 1B), a characteristic observed with the total protein (92 versus 85 kDa, respectively). These results suggest that myristoylation of the ATI protein occurs within the C-terminal region of the protein; however, there are still 73 lysine and arginine residues in this portion of the protein.

To more precisely map the modification sites of ATI, MS was utilized. Purified ATI was digested with trypsin, and the resulting peptides were analyzed by a LC−ESI−QIT mass spectrometer. A universal loss of 210 Da (the mass of the myristoyl moiety, $C_{14}H_{26}O$) was observed in 6 different peptides (underlined residues in Figure 1C). Figure 2A shows an example of a tandem MS spectrum of the myristoylated lysine at position 512. The ion at *m*/*z* 1824.7 resulted from the loss of water from the parent ion (*m*/*z* 1842.6); this is due to the energy used to fragment the parent ion. Ions present at *m*/*z* 917.0, 1383.5, 1511.6, and 1626.4 (fragmentation from the C terminus), together with ions at *m*/*z* 797.4, 926.5, 1039.6, 1154.9, and 1525.4 (fragmentation from the N terminus), provide the sequence information of the peptide. Two key ions at *m*/*z* 1314.7 and 1614.4 depict the loss of a myristoyl moiety (210 Da) from the b_{11}^+ ion (*m*/*z* 1525.4) and parent-water ion (*m*/*z* 1824.7), respectively. The unambiguous loss of 210 Da has also been observed with peptides synthetically myristoylated at internal lysine and arginine residues (*18*). The difference between the *m*/*z* at ion y_8^+ (917.0) and y_{10}^+ (1383.5) is 466.5 (K−E peptide). The

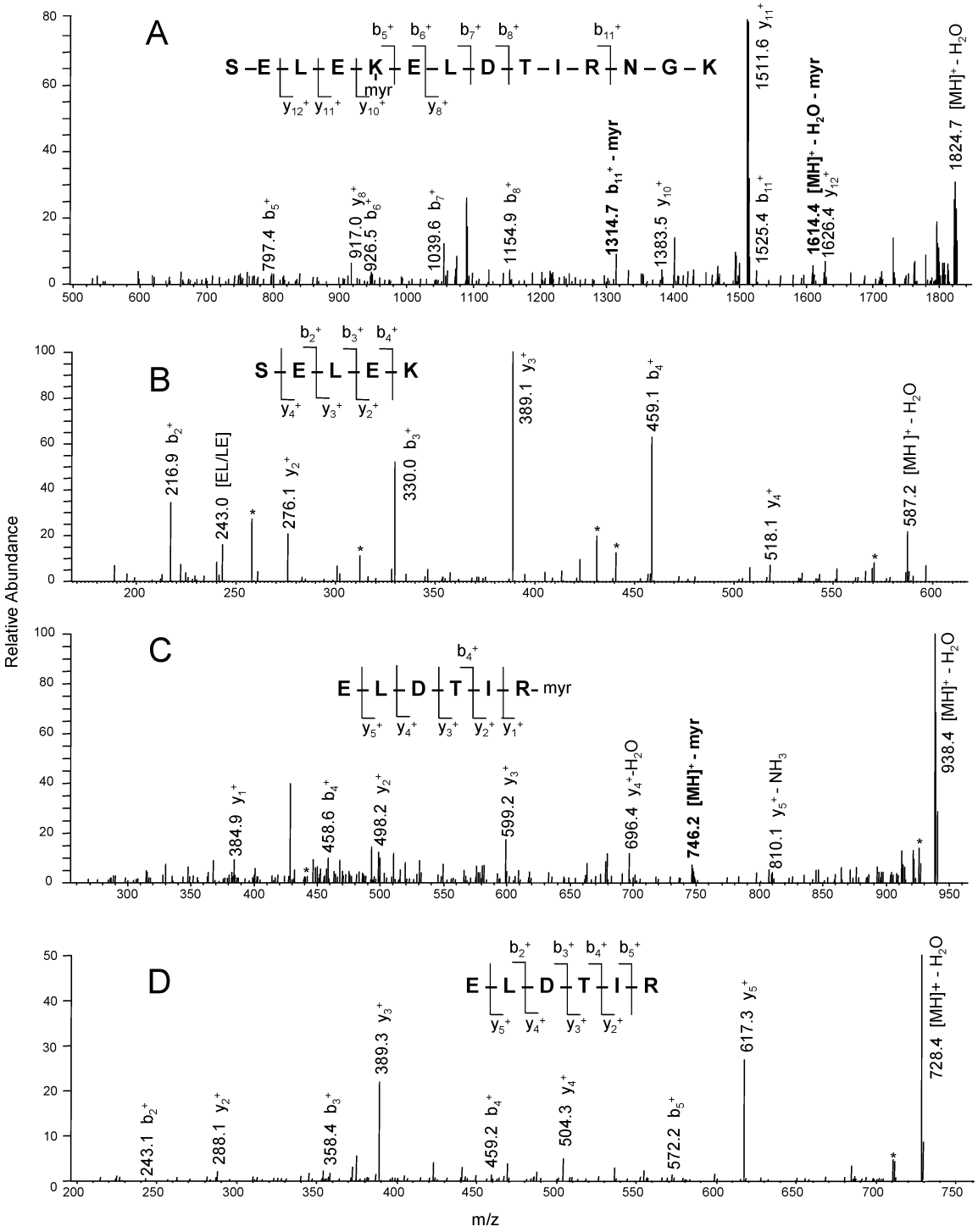

FIGURE 2: MS of the internal myristoylated peptides of ATI and the corresponding unmodified peptides. (A) Tandem mass spectrum of the myristoyl modification at K-512 shows the sequence information for amino acids 508−521, with a parent ion mass of 1842.7. The mass loss of 210 Da is indicated at ions *m*/*z* 1614.4 (parent ion − H_2O − myr) and 1314.7 (b_{11}^+ ion − myr). (B) Tandem mass spectrum of a peptide containing an unmodified K-512 residue. (C) Tandem mass spectrum of the myristoyl modification at R-518 shows the sequence information for amino acids 513−518, with a parent ion mass of 956.4. The mass loss of 210 Da is indicated at ions *m*/*z* 746.2 (parent ion − myr). (D) Tandem mass spectrum of a peptide containing an unmodified R-518 residue.

theoretical mass of a K−E peptide is 256.2 Da. The observed increase in mass (210.3 Da) is related to the myristoyl moiety, and therefore the site of modification is assigned to K-512. Interestingly, in the same sample an unmodified K-512 spectrum is also observed (Figure 2B). The loss of water from the parent ion (*m*/*z* 605.3) resulted in the ion at *m*/*z* 587.2. Ions at *m*/*z* 276.1, 389.1, and 518.1 and ions at *m*/*z* 216.9, 330.0, and 459.1 are the C and N termini fragments, respectively. The ion at *m*/*z* 243.0 is the internal fragment of the dipeptide E−L or L−E. The arginine modification at R-518 is shown in Figure 2C. The ion at *m*/*z* 938.4 is the loss of water from the parent ion (*m*/*z* 956.4). The ions at *m*/*z* 384.9, 498.2, 599.2, and 696.4 are the C-terminal fragments, and the ion at *m*/*z* 458.6 is the N-terminal fragment. The ion at *m*/*z* 746.2 indicates the key fragment loss of the myristoyl moiety (210 Da) from the

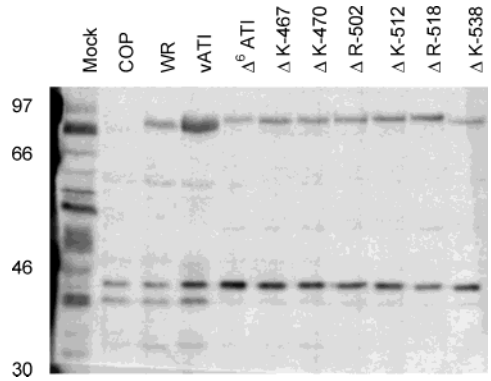

FIGURE 3: Fluorography of ATI. Lanes 1−4 are various controls. Lanes 5−11 are transfections using the COP strain of VV and either the plasmid-born Δ^6 mutant or the plasmid-born mutants of the individual myristoyl sites (amino acids underlined in Figure 1C).

parent ion (*m*/*z* 956.4). Again, the corresponding unmodified peptide was also observed in the same tryptic mixture (Figure 2D). A loss of water from the parent ion (*m*/*z* 728.4), the C-terminal fragment ions (*m*/*z* 288.1, 389.3, 504.3, and 617.3), and the N-terminal fragment ions (*m*/*z* 243.1, 358.4, 459.2, and 572.2) were observed. Similar spectra were observed for both the modified and unmodified residues: K-467, K-470, R-502, and K-538 (data not shown). Because there are six potential sites and an average of two myristic acid molecules associated with each ATI protein, this suggests that each of the six sites is incompletely and randomly modified.

Because six sites were observed by MS to contain a myristoyl moiety, site-directed mutagenesis was used to mutate the lysine or arginine residues (individually and as a group) to an alanine residue, to create a nonmyristoylated version of the ATI protein. The mutant ATI genes were amplified and cloned into a VV expression plasmid (pRB21), which contains a vaccinia synthetic early/late promoter. In total, seven mutants were made, one of each individual site and one containing the mutation at all six sites (Δ^6-ATI). Each of the mutants were transfected into BSC-40 cells in the presence of ^{3}H-MA using the COP strain of VV as the superinfecting virus, because it does not produce the 92 kDa ATI protein. Controls included mock-infected and COP-infected cells (negative controls), WR strain of VV, and vATI VV (positive controls). The cells were harvested, and the proteins were resolved on a SDS−PAGE gel and subjected to fluorography. Figure 3 shows the results of the site-directed mutagenesis experiment. Mock- and COP-infected cells do not have the ^{3}H-MA-labeled protein band at 92 kDa, while WR and vATI do express the 92 kDa band. The recombinant virus, vATI, has a higher level of expression because it actually produces two copies of ATI, the native protein as well as an engineered copy containing a 6-histidine tag. Each of the single mutations still label with ^{3}H-MA. Surprisingly, the Δ^6-ATI mutant (changing at all six sites) still labels with ^{3}H-MA, albeit slightly fainter than the positive controls.

Because the mutation of the six sites produced a protein that still incorporated ^{3}H-MA, Δ^6-ATI was isolated and digested with trypsin. The peptide mixture was then analyzed by LC−QIT−ESI MS to evaluate the modification status of the mutant sequences. A total of five of the six mutation sites were resolved by this analysis (data not shown). During this analysis, two new myristoylation sites, R-497 and K-521, were found (both within the C terminus of the ATI), which were not modified in the native protein (data not shown). This result suggests that, when the preferred myristoylation sites are removed, modification can occur at alternative cryptic acceptor sites. Each of the eight (original six plus two cryptic) modification sites were aligned to see if there were any conserved features (Figure 4). Other than being located within the C-terminal half of the protein, there is neither an obvious homology surrounding the acceptor residues nor any distinguishing protein characteristics.

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K-467 YKTERIDRLT K EIKEHRDIQN
K-470 ERIDRLTKEI K EHRDIQNGTD
R-502 TIRELRESLD R EREMRSELEK
K-512 REREMRSELE K ELDTIRNGKV
R-518 SELEKELDTI R NGKVDGSCQR
K-538 RELELSRMWL K QRDDDLRAEI

R-497 EIDKKTIREL R ESLDREREMR
K-521 EKELDTIRNG K VDGSCORELE
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FIGURE 4: Alignment of ATI myristoylation sites. Amino acids of interest are in bold with the corresponding up- and downstream sequences. The top six amino acids are the original modification sites. The two residues listed at the bottom are the alternative cryptic acceptor sites.

DISCUSSION

The VV ATI protein belongs to a family of poxvirus proteins designed to form large intracellular aggregates or inclusions. Because one known function of myristoylation is to increase protein hydrophobicity of the proteins involved in protein−protein interactions, it was not surprising to discover that both VV and Cowpox (CPV) ATI proteins were labeled with myristic acid (*5*). What was unusual was that although these proteins share a high degree of homology at the N terminus (the VV 92 kDa ATI protein is a C-terminal truncated version of the CPV 160 kDa ATI) neither protein contained the traditional myristoylation modification acceptor site (M)GXXX(S/T/A) at their N termini. This suggested that these proteins were myristoylated within the body of the protein, most likely on the amine group of an arginine or lysine residue. Although many viral proteins are subject to N-terminal myristoylation, this would be the first known example of an internally myristoylated viral protein, although previous work with the PBCV-1 capsid protein has suggested it may also be modified internally (*10*). Furthermore, analysis of the few known examples of internally myristoylated proteins has revealed that they are typically modified within the portion of a preprotein that is subsequently removed by proteolysis, such that the mature protein is not myristoylated (*7*). This would suggest that the function of this modification might be to direct the precursor protein to the correct location or compartment within the cell for its subsequent maturation. Neither the VV or CPV ATI proteins appear to be subject to any sort of proteolytic maturation, implying a different function of the myristoylation modification on these proteins. For these reasons, it was of interest to determine the location of the myristoylation modification sites within the VV ATI protein.

Previously, it has been demonstrated that the VV ATI protein is labeled by ^{3}H-MA, the modification is via an amide bond, and there are about two myristoyl moieties linked to

the ATI protein molecule (*5*, *13*). Although the ATI protein lacks a N-terminal (M)GXXX(S/T/A) signal, three internal occurrences of a G-X-X-X-S/T/A with K residues upstream were noted, but mutagenesis of all three did not effect myristoylation of the protein, suggesting that the protein was modified elsewhere (*5*). Because of the large number of K and R residues contained within the ATI coding sequence (115 total), site-directed mutagenesis was not a viable approach; therefore, we used MS and peptide sequencing of ATI protein fragments to identify the modification sites.

When ^{3}H-MA-labeled ATI protein was cleaved with CNBr, only three labeled fragments were detected. N−Terminal peptide sequencing confirmed that the three fragments corresponded to overlapping regions from the C-terminal half of the ATI protein, suggesting that N terminus was free of the label. A complete MS analysis of the tryptic peptides corresponding to this region of the protein revealed six myristoylation sites (K-467, K-470, R-502, K-512, R-518, and K-538). Interestingly, however, in each case, spectra indicated a mixture of modified and unmodified peptides, suggesting that modification at each site was incomplete. When all six of the acceptor residues were mutated, the mutant ATI protein was still myristoylated, albeit at two new sites R-497 and K-521. When all of the ATI myristoylation sites were aligned, there was no obvious protein feature or conserved motif evident to explain the selection of these 8 sites of the 115 available on the protein.

These results were unexpected and most surprising. They suggest that the VV ATI protein is myristoylated in a somewhat random and sequence-independent fashion. The fact that there are some preferred acceptor sites may be related to their surface accessibility on the folded ATI protein. Despite the promiscuous modification, it is of note that the protein ends up with two myristoyl moieties. Perhaps this level of increased hydrophobicity is sufficient to either target or fold the protein properly for its biological activity. In any case, it will be interesting to determine if the VV ATI protein represents a biological anomaly or is representative of a new pathway by which proteins that are destined for protein−protein interactions undergo multiple modifications. Given the fact that viruses such as VV typically utilize host pathways for their development, the latter hypothesis seems most likely.

ACKNOWLEDGMENT

We thank Brian Arborgast of the Department of Chemistry and the Environmental Health Science Center, Oregon State University, for his assistance with the LC−ESI−QIT MS analysis. We also thank Tove Bolken of Siga Research Labs for her assistance with the mutagenesis work. The N-terminal protein sequencing was done by Steve Smith at the University of Texas Medical Branch. The DNA sequencing work was done at the Central Services Lab in the Center for Gene Research and Biotechnology at Oregon State University. We also thank Dr. Claudia Maier for her helpful advice.

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BI0360197