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N Myristoylation of the Spleen Necrosis Virus Matrix Protein Is Required for Correct Association of the Gag Polyprotein with Intracellular Membranes and for Particle Formation

THOMAS A. WEAVER† AND ANTONITO T. PANGANIBAN*

McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, Wisconsin 53706

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To determine whether myristoylation is required for spleen necrosis virus replication, we constructed a substitution mutation in the *gag* gene that alters the putative myristate acceptor glycine residue. This single amino acid change was lethal for virus replication, resulted in aberrant proteolytic processing, and interrupted virion assembly and the release of virus from cells. Immunofluorescence analysis indicated that the amount of Gag polyprotein at the cell periphery and in Golgi-associated vesicles is severely reduced in the myristoylation mutant, indicating that correct intracellular targeting is affected by a lack of myristoylation. Coexpression of wild-type Gag polyprotein did not complement and rescue the replication-defective phenotype of the myristoylation mutant. Thus, it appears that the nonmyristoylated polyproteins are incapable of interacting with their myristoylated counterparts to form biologically active particles.

The Gag polyprotein precursors of mammalian type C (44), avian type C (49, 52), and human lentiviruses (15) serve as the organizing structural subunits of core particle assembly. Electron microscopic observation indicates that assembly begins as the polyproteins concentrate along the inner surface of the plasma membrane; this process seems to be manifested in the formation of electron-dense crescents beneath semicircular membrane protrusions (9, 12, 14). The subsequent events that result in complete virion assembly are not characterized, but they are thought to occur concomitantly with virion budding through the membrane (4, 14). These newly budded virion particles typically display an electron-dense outer ring encircling an electron-translucent nucleoid core concentrically located within the enveloped particle (9, 12, 21). These immature particles are noninfectious and are composed mainly of unprocessed polyproteins (54). The shape of the immature core changes with time as the polyproteins undergo proteolytic cleavage that results in condensation of the nucleoid into a smaller, electron-dense core particle. This maturation step is absolutely required for viral infectivity (22).

According to this model, the targeting of structural subunits to the membrane prior to particle assembly represents a critical step, initiating virus budding. Although the exact mechanism that results in anchoring of the viral polyproteins at the membrane is still not understood, early observations describing the extremely hydrophobic nature of the N-terminal matrix (MA) protein (3, 27) indicated that this protein may play a key role in this interaction (4). Chemical cross-linking (13, 36) and coimmunoprecipitation (40) studies with avian and mammalian type C virus particles provided direct evidence that the host cell membrane, the transmembrane subunit of the viral envelope glycoprotein, and the MA protein are in close proximity to one another. Moreover, attempts to determine the amino acid sequence of purified murine leukemia virus (MLV) MA protein revealed that the N terminus was blocked to Edman degradation. The nature of this blocking group was as determined by mass spectrometry to be the 14-carbon saturated fatty acid, myristate ($C_{14:0}$) (18). Labeling studies with [^{3}H]myristate have since established that the mature MA protein and the unprocessed Gag precursor polyprotein are myristoylated in the majority of retroviruses tested. The only retroviruses that are known not to be modified in this manner are avian leukosis virus, visna virus, and equine infectious anemia virus (reviewed in reference 41).

Direct evidence for the involvement of myristoylation in the biological activity of retroviruses has come from mutational analysis. Site-directed changes either creating or destroying the myristate acceptor, a penultimate glycine residue, have been engineered into the following viruses: MLV (38), Mason-Pfizer monkey virus (39), Rous sarcoma virus (52), and human immunodeficiency virus (HIV) (15, 16). For each of these viruses, successful myristoylation appeared to be required for viral infectivity. The nature of the block to viral replication in the absence of myristoylation seems to depend on both the virus type and the host cell in which infection is tested. For example, for MLV expressed in mouse fibroblasts or HIV expressed in Cos-7 cells, the loss of infectivity resulted from an apparent inability of the polyproteins to aggregate at the plasma membrane and participate in particle assembly (16, 38, 43). In contrast, close examination of mutant Mason-Pfizer monkey virus expressed in HeLa cells or HIV expressed in insect cells revealed the presence of assembled immature virion particles in the cytoplasm and nuclei of cells (15, 39). In the latter case, particles presumably cannot associate with the plasma membrane in the absence of the myristate and so are blocked in particle release. By using a complementary approach, when a myristate acceptor was created in Rous sarcoma virus, efficient particle assembly and release was observed in mammalian cells; these processes are normally very inefficient in this host (52). Similarly, the creation of a myristate acceptor in the endogenous MLV provirus (Env-3) present in DBA/2 mice rescues the replication-defective phenotype and allows efficient spread of the virus (20).

Finally, detailed biochemical analysis of myristoylation has recently provided further evidence for the necessity of

* Corresponding author.
† Present address: Laboratory for Molecular Biology, Medical Research Council, Cambridge, United Kingdom.

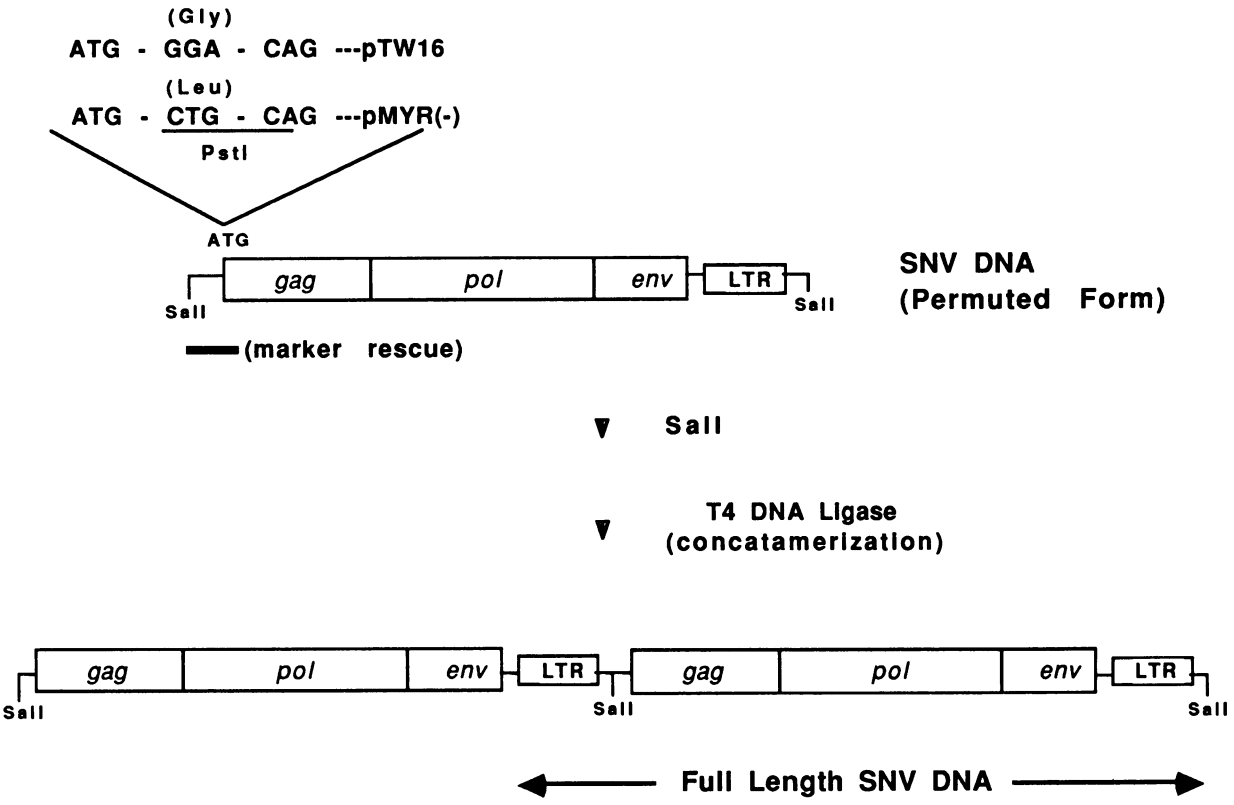

FIG. 1. Schematic representation of permuted wild-type and MYR(−) SNV DNA and strategy for regenerating full-length viral DNA. The permuted 8.3-kilobase *SalI* restriction fragment containing the *gag*, *pol*, and *env* genes and long terminal repeat (LTR) is diagramed. pTW16 was derived from pPB101 (2) by subcloning an 8.3-kilobase *SalI* restriction fragment into the *SalI* site of the phagemid pTZ18R (Pharmacia LKB). Plasmid pMYR(−) was derived from pTW16 and contained a GGA (Gly)-to-CTG (Leu) substitution at the penultimate glycine codon of the *gag* gene, as well as a novel *PstI* restriction site. The mutagenesis reaction was essentially the same as that described by Kunkel (24), except that 100 μg of gene 32 protein per ml was added to the reaction cocktail. The sequence of pMYR(−) was confirmed by dideoxy-sequence analysis of 300 base pairs surrounding the mutation. The location of the 150-base-pair *SalI-XbaI* restriction fragment used for marker rescue experiments is also shown. Full-length viral DNA was generated by concatamerizing permuted *SalI* restriction fragments by using T4 DNA ligase.

fatty acylation during retroviral infection. Myristoylation is a cotranslational process (29, 51), catalyzed by *N*-myristoyltransferase with myristoyl-coenzyme A (48), that results in the formation of a characteristic hydroxylamine-resistant amide linkage with the α-carbon of the N-terminal glycine acceptor (30).

Unlike oxy- and thiolester acylations formed at internal serine, threonine, or cysteine side chains, the N-myristoyltransferase reaction is highly selective for the acyl-chain length of myristoyl-coenzyme A (19). However, myristate analogs containing oxygen or sulfur substitutions at various positions along the hydrocarbon backbone were found to display reduced hydrophobicity without significantly altering N-myristoyltransferase specificity (19). In vivo treatment of cells with these analogs resulted in dose-dependent inhibition of both HIV and MLV replication (5). Similar assays using the drug cerulenin, an inhibitor of de novo fatty acid synthesis, had an inhibitory effect on HIV replication (32). Taken together, these results strongly implicate myristoylation as a key component involved in membrane targeting of viral polyproteins. However, it should be noted that other structural motifs may be involved in this process, since not all retrovirus Gag proteins are myristoylated and other myristoylated proteins are not membrane bound (1, 6, 7, 35, 45).

Lack of Gag myristoylation results in aberrant proteolytic processing and abrogates virus assembly and release. It has been reported that reticuloendotheliosis virus strain A (37) is myristoylated (42). We carried out site-directed mutagenesis (24), thereby changing the N-terminal Gly codon of the *gag* gene into a Leu codon in order to determine whether myristoylation is required for virus production [designated pMYR(−)] (Fig. 1). Concatamerization of *SalI*-cleaved pMYR(−) DNA yielded a subpopulation of molecules in head-to-tail orientation such that continuous viral sequences are generated (Fig. 1; 33). JD214Hyg^r is a spleen necrosis virus (SNV) vector that lacks all of the *trans*-acting sequences required for replication and expresses the dominant hygromycin B resistance gene (Hyg^r) (11). To test the effect of the mutation on viral infectivity, we cotransfected (23) D17 cells with 5 μg of concatameric permuted wild-type (pTW16) or mutant DNA along with 0.1 μg of JD214hygro. Selection with hygromycin B (400 μg/ml) was initiated 24 h posttransfection, and the release and spread of infectious virus were measured 6 days later by directly assaying for the presence of viral antigen through immunofluorescence or by determining the titer of the level of Hyg^r transforming virus particles in culture supernatants. In the case of the wild-type virus (TW16), there was efficient passage of both the replication-competent virus and the vector (JD214hygro), as

evidenced by the presence of viral antigen in 100% of the cells and by the high titer of JD214hygro (>10^5 Hyg^r transforming units per ml). In contrast, we were unable to detect passagable virus in cells transfected with pMYR(−) DNA. Less than 0.1% of these cells expressed viral antigen, which was consistent only with the frequency of successful transfection (data not shown), and there was less than 1 Hyg^r transforming unit per ml.

The DNA sequence analysis determined for pMYR(−) indicated that the only deviation from the wild-type nucleic acid sequence was the desired Gly-to-Leu substitution mutation introduced at the *gag* gene N-terminal Gly codon. In order to determine whether this single mutation was responsible for the replication-defective phenotype exhibited by the mutant, a marker rescue experiment was performed. A 150-base-pair DNA fragment (a *Sal*I-to-*Xba*I restriction fragment isolated from pTW16 DNA; Fig. 1) encompassing the wild-type N-terminal glycine codon was cotransfected along with *Eco*RI-linearized pMYR(−) DNA into D17 cells under conditions favorable for genetic recombination (28). At 7 days posttransfection, the spread of replication-competent virus was assessed by immunofluorescence with Gag antiserum made in *Escherichia coli* (49). As expected, recombination between the wild-type and pMYR(−) mutation resulted in the generation of replication-competent virus, indicating that the replication-defective phenotype of the pMYR(−) DNA was the result of the site-directed mutation rather than some untoward mutation generated during the process of mutagenesis (data not shown).

To facilitate the characterization of the unmyristoylated Gag protein, we isolated a clonal cell line expressing the DNA harboring and expressing pMYR(−). This was accomplished by cotransfecting *Sal*I-concatamerized pMYR(−) DNA along with pJD214hygro into D17 cells and screening individual Hyg^r colonies for the presence of viral Gag and Env antigens by immunofluorescence. A clonal cell line, designated MYR(−), was isolated and used for subsequent characterization of the mutant phenotype.

MYR(−) or chronically infected cells, plated at a density of 10^6, were labeled for 6 h with 500 μCi of [^{14}C]myristate, and viral antigens from total cell lysates were analyzed by immunoprecipitation with Gag antiserum (data not shown). In addition, total-cell lysates from identical cultures of unlabeled cells were separated in parallel, and the viral antigens were visualized by Western immunoblot analysis with Gag antiserum (data not shown). Several conclusions can be made from this comparative analysis. First, in support of a previous report demonstrating that the reticuloendotheliosis virus strain A MA protein is myristoylated (42), we observed that the intracellular 55-kilodalton (kDa) Gag polyprotein of SNV was also myristoylated. Second, the SNV MA protein, predicted from the nucleotide sequence to be approximately 12 kDa (31), was not detected in cells infected by wild-type virus. This observation may be initially perplexing, but previous characterization of the Gag antiserum indicates that it does not cross-react with the MA domain of the polyprotein (T. A. Weaver and A. T. Panganiban, submitted for publication). Third, the N-terminal Gly-to-Leu mutation present in MYR(−) Gag protein blocks myristoylation of the 55-kDa Gag polyprotein. Fourth, the proteolytic cleavage pattern of the mutant Gag polyprotein is abnormal and results in the accumulation of a Gag derivative of approximately 37 kDa which accumulates at the expense of the 27-kDa mature capsid protein detected in virus-infected cells.

In order to determine whether virion assembly and release were taking place in the MYR(−) cell line, the level of reverse transcriptase activity present in the culture supernatant was determined (34). The observed activity from the mutant cell line (6×10^4 cpm/ml) was only 6% of that from chronically infected cells (1×10^6 cpm/ml) and was approximately equivalent to the level in uninfected D17 cells (3×10^4 cpm/ml). In addition, we were unable to detect virus by immunoprecipitation analysis of pelleted culture supernatant (46) prepared from radiolabeled MYR(−) cells (data not shown). Finally, electron micrographs taken of sectioned MYR(−) cells (53) also failed to detect any intra- or extracellular virus particles (data not shown).

Lack of myristoylation results in an altered intracellular distribution of the Gag polyprotein. In order to determine whether the intracellular distribution of the Gag polyprotein was grossly affected by the absence of myristoylation, we characterized the MYR(−) cell line by immunofluorescence analysis using anti-Gag antiserum. The results of this analysis (Fig. 2) reveal a strikingly different pattern for the nonmyristoylated protein compared with that observed in cells chronically infected with wild-type virus. In cells expressing the wild-type *gag* gene, the cell periphery was distinctly highlighted, presumably because of a concentration of the Gag polyprotein at the plasma membrane. In addition, a strong perinuclear accumulation of Gag antigen was apparent in a large proportion of the cells (Fig. 2A). Double labeling with Texas red-conjugated wheat germ agglutinin, a lectin commonly used to identify the Golgi apparatus (10, 25), demonstrated that the perinuclear Gag antigen of chronically infected cells coincides with the location of the Golgi apparatus (data not shown). In contrast to the wild-type pattern, the intracellular distribution of Gag antigen in the MYR(−) cell line appears to be diffuse throughout the cytoplasm, with no distinct accumulation at the cell surface (Fig. 2B). As expected, uninfected cells did not exhibit detectable staining (data not shown). Additionally, the usual perinuclear and surface distribution of Env protein in MYR(−) cells, as determined by immunofluorescence with anti-Env antiserum, was the same as that observed for chronically infected cells (data not shown).

We next used confocal imaging (50) to examine the subcellular distribution of Gag antigen in several focal planes bisecting cells at successive depths (Fig. 2C and D). Beginning at the basal cell surface, strong antigen staining was apparent in cells infected by wild-type virus but not in MYR(−) cells (panels 1 of Fig. 2C and D). Successive 4-μm sections (Fig. 2C and D, panels 2 to 4) traced the location of Gag protein upward through the fried egg-shaped D17 cell and confirmed the lack of perinuclear or plasma membrane staining in the MYR(−) mutant. In addition, no nuclear staining was observed in either cell type, indicating that the apparent nuclear staining seen in chronically infected cells by conventional immunofluorescence (Fig. 2A) is the result of plasma membrane-associated Gag antigen over- and underlying the nucleus, thereby occluding the nucleus.

Lack of myristoylation results in altered association of the Gag protein with membranes. The immunofluorescence data revealed a gross alteration in the intracellular distribution of mutant Gag antigens, consistent with a reduced affinity between viral polyprotein and cellular membranes. In order to determine whether the nature of the local interaction of the Gag proteins with membranes is affected by the lack of myristoylation, we performed a trypsin protection experiment. A cellular fraction enriched for intracellular membranes was isolated from chronically infected or MYR(−) cells and divided, and some samples were detergent solubi-

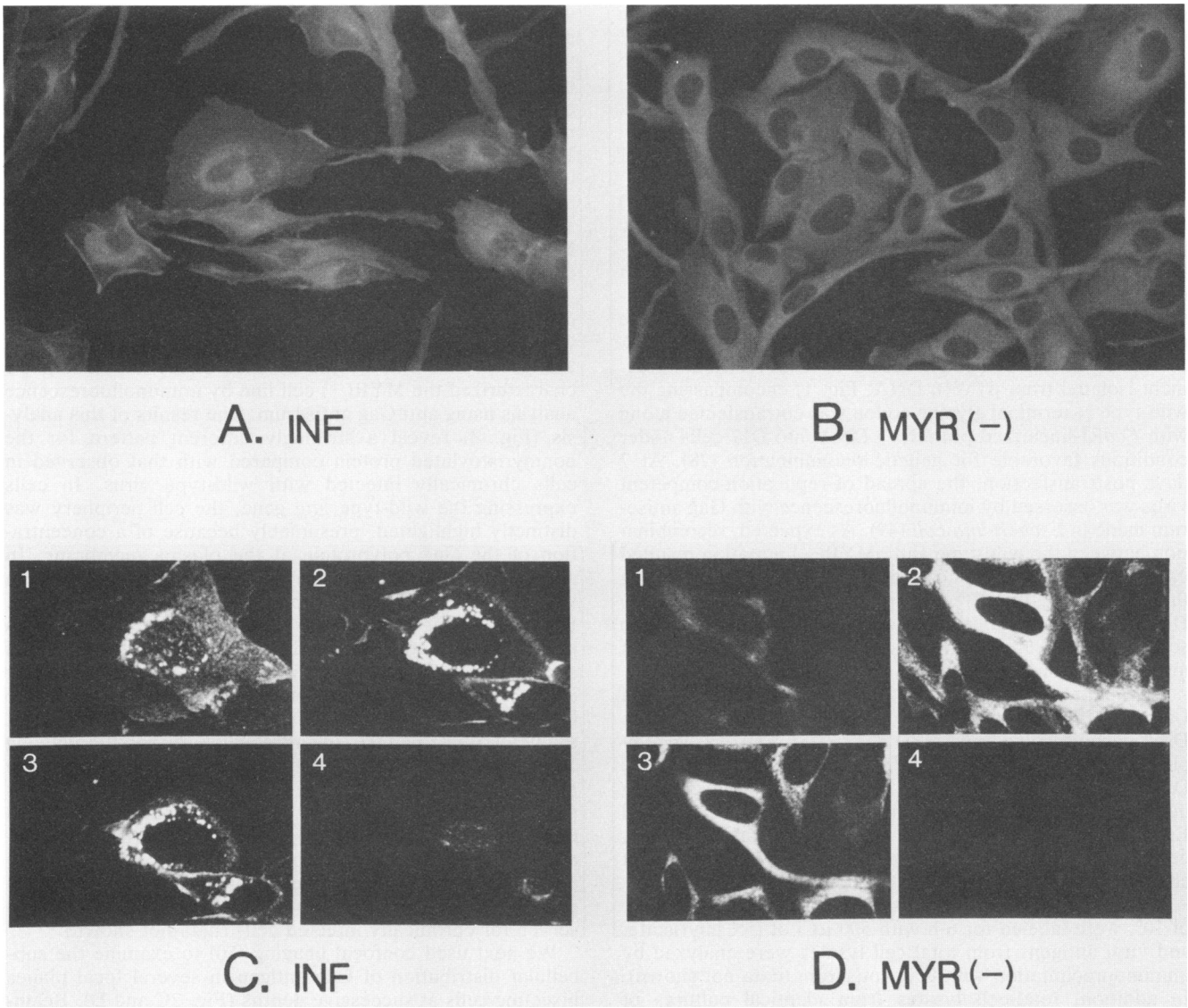

FIG. 2. Comparison of intracellular distribution of viral Gag antigens in chronically infected and MYR(−) cells by conventional and confocal imaging. Chronically infected (INF) and MYR(−) cells were stained with Gag antibody and a fluorescein isothiocyanate-conjugated secondary antibody. Samples were analyzed by conventional imaging (A and B) or by confocal imaging (C and D). Sections: 1, basal cell surface; 2, 4 μm from base; 3, 8 μm from base; 4, apical cell surface, 12 μm from base. Cells were grown overnight on glass cover slips and fixed with ice-cold methanol for 20 min. Rabbit Env or Gag antiserum (1:100 dilution) was incubated with the fixed cells for 60 min at 37°C. The cells were washed three times (3% calf serum in phosphate-buffered saline) for 10 min each wash at room temperature. The bound primary antibody was then detected with a secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody (Sigma Chemical Co.; 1 mg/ml diluted 1:40), and three final washes were performed in the dark. The Golgi apparatus was identified in double-labeling experiments using wheat germ agglutinin conjugated to Texas red-sulfonyl chloride (Molecular Probes; 1 mg/ml diluted 1:100) (data not shown). Cover slips were mounted on glass microscope slides with 30 μl of 10% glycerol–1× phosphate-buffered saline–0.1% *p*-phenylenediamine (Sigma). Viral antigens were visualized by indirect fluorescent microscopy with an Olympus BHTU microscope, fitted with reflected light fluorescence and top mount 35-mm photomicrographic attachments. Dark-field exposures were made with Kodak Ektachrome slide film, ASA 400. Confocal imaging was performed at the University of Wisconsin integrated microscopy resource center with the MRC-500 confocal imaging system (50). Specimens were prepared in the same manner as for direct immunofluorescence microscopy.

lized in 0.1% Nonidet P-40, whereas others were untreated. Trypsin was then added to each sample, and digestion was carried out for increasing periods of time. The digestion reaction was stopped by the addition of trypsin inhibitor, and samples were subsequently analyzed by Western blot analysis using the Gag antiserum (Fig. 3).

The results from this experiment revealed that the membrane-associated Gag proteins present in chronically infected cells are highly resistant to trypsin treatment in the absence of Nonidet P-40 treatment, even for incubation times of up to 15 min (Fig. 3, lanes 2 to 7). These proteins are likely to be derived either from unassembled proteins sequestered within membrane vesicles or possibly from assembled and enveloped intracellular virion particles (21). While the membrane-associated Gag protein in the chronically infected cells was relatively refractory to digestion with

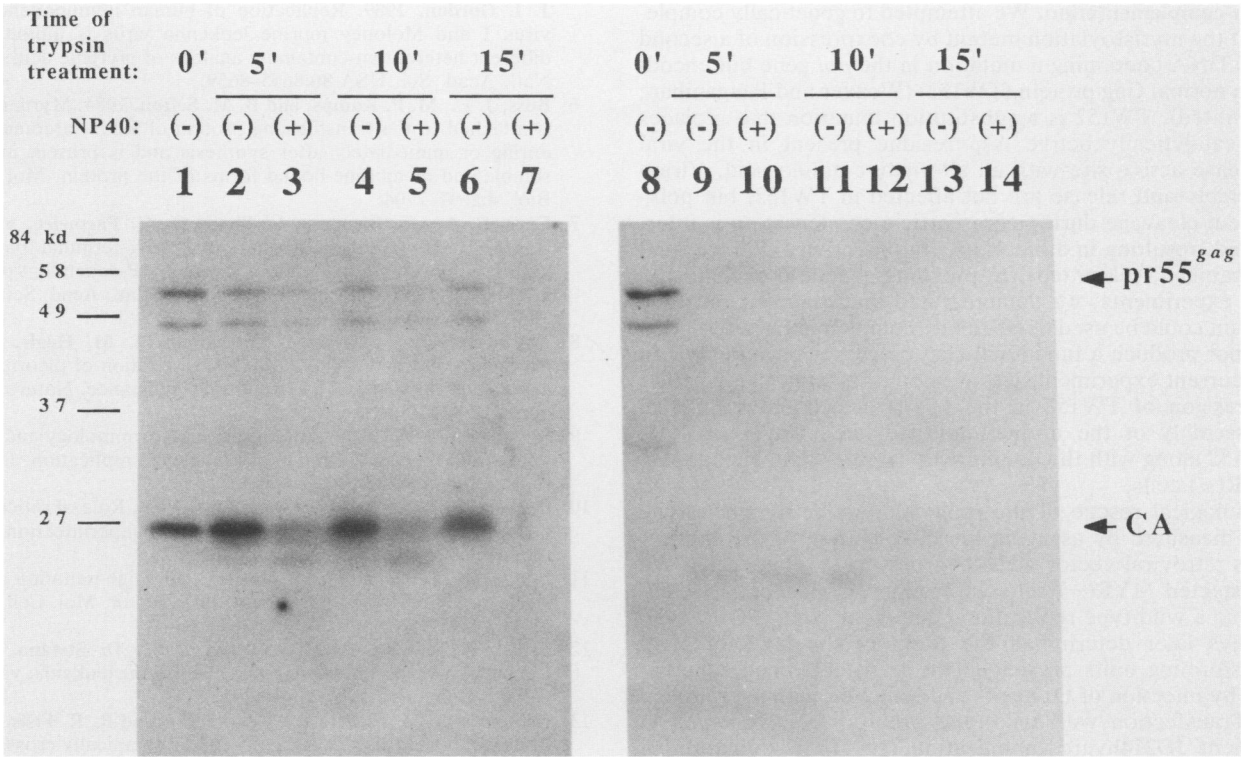

FIG. 3. Trypsin protection of intracellular smooth membrane-associated viral Gag antigens in chronically infected and MYR(−) cells. The smooth membrane fractions obtained from chronically infected cells (lanes 1 to 7) or MYR(−) cells (lanes 8 to 14) were used as the source of viral antigens for trypsin protection analysis. Samples were either detergent solubilized with 0.1% Nonidet P-40 (NP40) (+) or left untreated (−) as indicated. Trypsin (100 μg/ml) was then added to all samples and allowed to incubate at 0°C for 0, 5, 10, or 15 min as indicated. $pr55^{gag}$, 55-kDa (kd) Gag polyprotein; CA, 27-kDa viral capsid protein. Cells (10^6) seeded on a 10-cm plate were rinsed three times in ice-cold phosphate-buffered saline containing 0.1% D-glucose and then incubated for 10 min with 1 ml of ice-cold hypotonic buffer (10 mM Tris hydrochloride, pH 7.9, 10 mM NaCl, 2 mM $MgCl_2$). The cells were collected by scraping with a plastic cell lifter (Costar) and pelleted by centrifuging at 1,000 × *g* for 3 min. The pellet was washed an additional two times before the cells were homogenized by 25 strokes in a Dounce homogenizer. The smooth membrane vesicle fraction of the lysate was separated from the nuclear, plasma membrane, and microsomal fractions by successive centrifugations: 3,000 × *g* for 10 min (pellets, nuclei, and plasma membrane), 10,000 × *g* for 20 min (pellets and microsomes), and 100,000 × *g* for 40 min (pellets and smooth membrane vesicles away from cytoplasmic proteins) (17). The high-speed smooth membrane fraction obtained from cell lysates was suspended in 50 mM Tris hydrochloride (pH 7.9)–50 mM KCl–5 mM $MgCl_2$, and 100-μl samples were separated into Eppendorf tubes. One-half of the samples were detergent solubilized with 0.1% Nonidet P-40. Trypsin (Sigma) was added to both detergent-solubilized and untreated samples at a final concentration of 100 μg/ml. Samples were incubated at 0°C for 5, 10, and 15 min, and each reaction was stopped by the addition of 100 μg of soybean trypsin inhibitor (Sigma) per ml. For Western blot analysis, the sample was separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose filter paper by standard methods (47). After transfer, filters were incubated in blocking buffer (1% dry nonfat milk [Carnation] in 1× phosphate-buffered saline for 1 h, after which primary Gag antisera was added (1:5,000 final dilution) and allowed to incubate for another hour. Filters were washed three times in blocking buffer before ^{125}I-protein A (Dupont, NEN Research Products) was added (2×10^5 dpm/ml in blocking buffer). After 1 h of incubation with label, filters were washed three times (0.3% Tween 20 in 1× phosphate-buffered saline), air dried, and autoradiographed.

trypsin, the Gag protein in the myristoylation mutant was trypsin sensitive even in the absence of detergent (Fig. 3, lanes 9 to 14). This sensitivity to trypsin digestion is intriguing and likely indicates that the nonmyristoylated protein is nonspecifically bound to the exterior of the purified membranes in a form easily accessible to proteolysis. It is also likely that the extremely hydrophobic nature of the SNV MA protein (41% hydrophobic residues) (31) may provide enough membrane affinity to account for some myristoylation-independent association. However, close association with membranes, as assayed by trypsin resistance, appears to require correct fatty acylation.

The added hydrophobicity provided by the myristate moiety not only could affect the ability of the Gag protein to successfully interact with membranes but might also be required for correct overall polyprotein structure. Support for this possibility comes from the recent description of a myristoylation mutation in the VP4 structural protein of poliovirus which interrupts polyprotein processing and possibly particle assembly (26). The fact that picornaviruses are not enveloped and that the myristate moiety is likely buried within the capsid (8) suggests that fatty acylation may serve a non-membrane-dependent structural function such as the stabilization of a particular tertiary fold that in turn allows subunit interaction during capsid assembly. However, it should be noted that assembly is not affected in type D retroviruses bearing a myristoylation mutation or when nonmyristoylated HIV Gag polyproteins are overexpressed in heterologous cells (15, 39).

The myristoylation mutant cannot be genetically rescued by

***trans*-complementation.** We attempted to genetically complement the myristoylation mutant by coexpression of a second viral DNA containing a mutation in the *pol* gene but encoding a normal Gag protein (TW152) (Weaver and Panganiban, submitted). TW152 is a substitution mutation that replaces the catalytically active Asp residue present in the viral protease active site with an alternative amino acid. Virion assembly and release are not affected in TW152, but polyprotein cleavage during core particle condensation is interrupted, resulting in a block to viral infectivity (Weaver and Panganiban, submitted). In previous genetic complementation experiments, we demonstrated that a similar protease mutant could be used to efficiently complement a mutant that did not produce a functional Gag precursor protein (49). In the current experiment, we attempted to determine whether expression of TW152 in the MYR(−) cell line results in coassembly of the normal, myristoylated Gag protein of TW152 along with the nonmyristoylated protein encoded by MYR(−) cells.

Biological rescue of the replication-defective phenotype was measured by assaying the mobilization of the endogenous retroviral vector JD214hygro from MYR(−) cells. We transfected MYR(−) cells with either TW152 or DNA encoding a wild-type replication-competent virus (PB101) and 7 days later determined the titers of the level of Hyg^r transforming units present in the transfected-cell supernatant by infection of D17 cells and selection with hygromycin B. Transfection with wild-type proviral DNA resulted in efficient JD214hygro mobilization (10^5 Hyg^r transforming units per ml). In contrast, no JD214hygro virus was detected after transfection with TW152 DNA. Therefore, there was no apparent complementation between these two mutants. The most likely hypothesis that would explain this observation is that the nonmyristoylated Gag polyprotein is unable to participate in core particle assembly, even in the presence of myristoylated Gag protein, because of an improper intracellular location (Fig. 2) or incorrect interaction with membranes (Fig. 3) or both. This experiment, taken together with the experiments that indicate that the nonmyristoylated protein exhibits improper intracellular distribution and association with membranes, may indicate that the myristoylated and nonmyristoylated proteins are intracellularly segregated.

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LITERATURE CITED

1. **Aitken, A., P. Cohen, S. Santikarn, D. H. Williams, A. G. Calder, A. Smith, and C. B. Klee.** 1982. Identification of the NH_2-terminal blocking group of calcineurin B as myristic acid. FEBS Lett. **150**:314–318.
2. **Bandyopadhyay, P. K., and H. M. Temin.** 1984. Expression from an internal AUG codon of herpes simplex thymidine kinase gene inserted in a retrovirus vector. Mol. Cell. Biol. **4**:743–748.
3. **Barbacid, M., and S. A. Aaronson.** 1978. Membrane properties of the *gag* gene-coded p15 protein of mouse type-C RNA tumor viruses. J. Biol. Chem. **253**:1408–1414.
4. **Bolognesi, D. P., R. C. Montelaro, H. Frank, and W. Schafer.** 1978. Assembly of type-C oncornaviruses: a model. Science **199**:183–186.
5. **Bryant, M. L., R. O Heuckeroth, J. T. Kimata, L. Ratner, and J. I. Gordon.** 1989. Replication of human immunodeficiency virus 1 and Moloney murine leukemia virus is inhibited by different heteroatom-containing analogs of myristic acid. Proc. Natl. Acad. Sci. USA **86**:8655–8659.
6. **Buss, J. E., M. P. Kamps, and B. M. Sefton.** 1984. Myristic acid is attached to the transforming protein of Rous sarcoma virus during or immediately after synthesis and is present in both soluble and membrane-bound forms of the protein. Mol. Cell. Biol. **4**:2697–2704.
7. **Carr, S. A., K. Biemann, S. Shoji, D. C. Parmelee, and K. Titani.** 1982. n-Tetradecanoyl is the NH_2-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. Proc. Natl. Acad. Sci. USA **79**:6128–6131.
8. **Chow, M., J. F. Newman, D. Filman, J. M. Hogle, D. J. Rowlands, and F. Brown.** 1987. Myristoylation of picornavirus capsid protein VP4 and its structural significance. Nature (London) **327**:482–486.
9. **Dalton, A. J.** 1972. RNA tumor viruses—terminology and ultrastructural aspects of virion morphology and replication. J. Natl. Cancer Inst. **49**:323–327.
10. **Delwart, E. L., and A. T. Panganiban.** 1989. Role of reticuloendotheliosis virus envelope glycoprotein in superinfection interference. J. Virol. **63**:273–280.
11. **Dougherty, J. P., and H. M. Temin.** 1986. High mutation rate of a spleen necrosis virus-based retrovirus vector. Mol. Cell. Biol. **6**:4387–4395.
12. **Feller, U., R. M. Dougherty, and H. S. Di Stefano.** 1971. Comparative morphology of avian and murine leukemia viruses. J. Natl. Cancer Inst. **47**:1289–1298.
13. **Gebhardt, A., J. V. Bosch, A. Ziemiecki, and R. R. Friis.** 1984. Rous sarcoma virus p19 and gp35 can be chemically crosslinked to high molecular weight complexes: an insight into virus assembly. J. Mol. Biol. **174**:297–317.
14. **Gelderblom, H. R., E. H. S. Hausmann, M. Ozel, G. Pauli, and M. A. Koch.** 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology **156**:171–176.
15. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde.** 1989. Assembly and release of HIV-1 precursor $pr55^{gag}$ virus-like particles from recombinant baculovirus-infected cells. Cell **59**:103–112.
16. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86**:5781–5785.
17. **Graham, J.** 1984. Isolation of subcellular organelles and membranes, p. 161–182. *In* D. Rickwood (ed.), Centrifugation: a practical approach, 2nd ed. IRL Press Ltd., Oxford.
18. **Henderson, L. E., H. C. Krutzsch, and S. Oroszlan.** 1983. Myristyl amino-terminal acylation of murine retrovirus proteins: an unusual post-translational protein modification. Proc. Natl. Acad. Sci. USA **80**:339–343.
19. **Heuckeroth, R. O., L. Glaser, and J. I. Gordon.** 1988. Heteroatom-substituted fatty acid analogs as substrates for N-myristoyltransferase: an approach for studying both the enzymology and function of protein acylation. Proc. Natl. Acad. Sci. USA **85**:8795–8799.
20. **Jørgensen, E. C., N. O. Kjeldgaard, F. S. Pedersen, and P. Jørgensen.** 1988. A nucleotide substitution in the *gag* N terminus of the endogenous ecotropic DBA/2 virus prevents $pr65^{gag}$ myristoylation and virus replication. J. Virol. **62**:3217–3223.
21. **Kang, C.-Y., T. C. Wong, and K. V. Holmes.** 1975. Comparative ultrastructural study of four reticuloendotheliosis viruses. J. Virol. **16**:1027–1038.
22. **Katoh, I., Y. Yoshinaka, A. Rein, M. Shibuya, T. Odaka, and S. Oroszlan.** 1985. Murine leukemia virus maturation: protease region required for conversion from "immature" to "mature" core form and for virus infectivity. Virology **145**:280–292.
23. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4**:1172–1174.
24. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis

without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
25. **Machamer, C. E., R. Z. Florkiewicz, and J. K. Rose.** 1985. A single N-linked oligosaccharide at either of the two normal sites is sufficient for transport of vesicular stomatitis virus G protein to the cell surface. Mol. Cell. Biol. **5**:3074–3083.
26. **Marc, D., G. Drugeon, A.-L. Haenni, M. Girard, and S. van der Werf.** 1989. Role of myristoylation of poliovirus capsid protein VP4 as determined by site-directed mutagenesis of its N-terminal sequence. EMBO J. **8**:2661–2668.
27. **Marcus, S. L., S. W. Smith, J. Racevskis, and N. H. Sarkar.** 1978. The relative hydrophobicity of oncornaviral structural proteins. Virology **86**:398–412.
28. **Miller, C. K., and H. M. Temin.** 1983. High-efficiency ligation and recombination of DNA fragments by vertebrate cells. Science **220**:606–609.
29. **Olson, E. N., and G. Spizz.** 1986. Fatty acylation of cellular proteins. J. Biol. Chem. **261**:2458–2466.
30. **Olson, E. N., D. A. Towler, and L. Glaser.** 1985. Specificity of fatty acid acylation of cellular proteins. J. Biol. Chem. **260**: 3784–3790.
31. **O'Rear, J. J., and H. M. Temin.** 1982. Spontaneous changes in nucleotide sequence in proviruses of spleen necrosis virus, an avian retrovirus. Proc. Natl. Acad. Sci. USA **79**:1230–1234.
32. **Pal, R., R. C. Gallo, and M. G. Sarngaharan.** 1988. Processing of the structural proteins of human immunodeficiency virus type 1 in the presence of monensin and cerulenin. Proc. Natl. Acad. Sci. USA **85**:9283–9286.
33. **Panganiban, A. T., and H. M. Temin.** 1983. The terminal nucleotides of retrovirus DNA are required for integration but not virus production. Nature (London) **306**:155–160.
34. **Paskind, M. P., R. A. Weinberg, and D. Baltimore.** 1975. Dependence of Moloney murine leukemia virus production on cell growth. Virology **67**:242–248.
35. **Paul, A. V., A. Schultz, S. E. Pincus, S. Oroszlan, and E. Wimmer.** 1987. Capsid protein VP4 of poliovirus is N-myristoylated. Proc. Natl. Acad. Sci. USA **84**:7827–7831.
36. **Pepinsky, R. B., and V. M. Vogt.** 1979. Identification of retrovirus matrix proteins by lipid-protein cross-linking. J. Mol. Biol. **131**:819–837.
37. **Purchase, H. G., C. Ludford, K. Nazerian, and H. W. Cox.** 1973. A new group of oncogenic viruses: reticuloendotheliosis, chick syncytial, duck infectious anemia, and spleen necrosis viruses. J. Natl. Cancer Inst. **51**:489–499.
38. **Rein, A., M. R. McClure, N. R. Rice, R. B. Luftig, and A. M. Schultz.** 1986. Myristoylation site in pr65gag is essential for virus particle formation by Moloney murine leukemia virus. Proc. Natl. Acad. Sci. USA **83**:7246–7250.
39. **Rhee, S. S., and E. Hunter.** 1987. Myristoylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. J. Virol. **61**:1045–1053.
40. **Schlesinger, M. J.** 1976. Formation of an infectious virus-antibody complex with Rous sarcoma virus and antibodies directed against the major virus glycoprotein. J. Virol. **17**: 1063–1067.
41. **Schultz, A. M., L. E. Henderson, and S. Oroszlan.** 1988. Fatty acylation of proteins. Annu. Rev. Cell Biol. **4**:611–647.
42. **Schultz, A. M., and S. Oroszlan.** 1983. In vivo modification of retroviral *gag* gene-encoded polyproteins by myristic acid. J. Virol. **46**:355–361.
43. **Schultz, A. M., and A. Rein.** 1989. Unmyristoylated Moloney-murine leukemia virus $pr65^{gag}$ is excluded from virus assembly and maturation events. J. Virol. **63**:2370–2373.
44. **Shields, A., O. N. Witte, E. Rothenberg, and D. Baltimore.** 1978. High frequency of aberrant expression of Moloney murine leukemia virus in clonal infections. Cell **14**:601–609.
45. **Streuli, C. H., and B. E. Griffin.** 1987. Myristic acid is coupled to a structural protein of polyoma virus and SV40. Nature (London) **326**:619–622.
46. **Tanese, N., M. J. Roth, and S. P. Goff.** 1986. Analysis of retroviral *pol* gene products with antisera raised against fusion proteins produced in *Escherichia coli*. J. Virol. **59**:328–340.
47. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
48. **Towler, D., and L. Glaser.** 1986. Protein fatty acid acylation: enzymatic synthesis of an N-myristoylglycyl peptide. Proc. Natl. Acad. Sci. USA **83**:2812–2816.
49. **Weaver, T. A., K. J. Talbot, and A. T. Panganiban.** 1990. Spleen necrosis virus *gag* polyprotein is necessary for particle assembly and release but not for proteolytic processing. J. Virol. **64**:2642–2652.
50. **White, J. G., W. B. Amos, and M. Fordham.** 1987. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. J. Cell Biol. **105**: 41–48.
51. **Wilcox, C., J.-S. Hu, and E. N. Olson.** 1987. Acylation of proteins with myristic acid occurs cotranslationally. Science **238**:1275–1278.
52. **Wills, J. W., R. C. Craven, and J. A. Achacoso.** 1989. Creation and expression of myristoylated forms of Rous sarcoma virus Gag protein in mammalian cells. J. Virol. **63**:4331–4343.
53. **Yeger, H., V. I. Kalnins, and J. R. Stephenson.** 1976. Electron microscopy of mammalian type-C RNA viruses: use of conditional lethal mutants in studies of virion maturation and assembly. Virology **74**:459–469.
54. **Yoshinaka, Y., and R. B. Luftig.** 1977. Murine leukemia virus morphogenesis: cleavage of P70 *in vitro* can be accompanied by a shift from a concentrically coiled internal strand ("immature") to a collapsed ("mature") form of the virus core. Proc. Natl. Acad. Sci. USA **74**:3446–3450.