Myristoylation of human immunodeficiency virus (HIV) Gag protein is essential for virus particle budding. Two reactions are involved; activation of free myristate to myristoyl-CoA and transfer of the myristoyl residue to the Gag N-terminal glycine. We have investigated the effects of triacsin C, an inhibitor of long chain acyl-CoA synthetase, on release of HIV Gag virus-like particle (VLP) produced using the recombinant baculovirus system. First, inhibition of acyl-CoA formation by triacsin C, an inhibitor of long chain acyl-CoA synthetase, on release of HIV Gag virus-like particle effects of triacsin C, an inhibitor of long chain acyl-CoA synthetase blocks the production of myristoylated Gag protein, only complete inhibition of Gag myristoylation prevents VLP budding. Thus, relatively few myristoylated Gag molecules are sufficient for plasma membrane targeting and VLP budding.

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome in humans, is classified into the lentiviruses family of retroviridae (1). The life cycle of HIV has been studied extensively, and evidence has accumulated to suggest a mechanism of viral assembly and particle formation (see Ref. 2 for review). The main structure components of HIV particles are encoded by the gag gene, and expression of Gag protein alone in a number of expression systems produces HIV-like particles analogous to the immature type of authentic HIV (2–4). Gag is synthesized initially in the cytosol as a precursor protein, Pr55, and is targeted to the plasma membrane where particle assembly and packaging of viral genomic RNA occur (5). During synthesis, Pr55 Gag is acylated at the N-terminal glycine residue exclusively with myristic acid (6, 7), a modification which, when combined with an N-terminal basic region (8, 9), is essential for Pr55 targeting to the plasma membrane, since non-myristoylated Pr55 obtained by amino acid substitution at the N-terminal glycine fails to bud from the cell surface (4, 10–13). However, this technology is inappropriate for a study of the level of myristoylation required for efficient virus-like particle (VLP) budding. Such studies are necessary as myristoylation has been suggested to be a suitable target for therapeutic drug development against HIV (14), and yet the consequence of partial myristoylation has not been addressed.

Myristoylation consists of two reactions; activation of myristic acid to myristoyl-CoA by acyl-CoA synthetase and transfer of the myristoyl group from myristoyl-CoA to the N-terminal glycine of Pr55 by N-myristoyltransferase. Heteroatom-substituted analogues of myristic acid (15), phospholipid containing such a myristic acid analogue (16), and analogues of N-myristoyl glycine (17–19) have been reported to inhibit HIV replication, and most of these inhibitors are expected to inhibit N-myristoyltransferase activity. However, the biochemical characterization of these compounds in relation to their effect on HIV remains poorly understood, and precise correlation between Gag myristoylation and HIV particle formation remains unclear.

We have previously reported a series of compounds, termed triacsin A to D, isolated from the culture filtrate of Streptomyces sp. SK-1894, which contain 11 carbon alkenyl chains with an N-hydroxytetrazene moiety at the termini (20). Although all triacsin inhibit acyl-CoA synthetase from a wide variety of sources, triacsin C was shown to be the potent inhibitor in a large number of studies in which triacsin were utilized to investigate the function of acyl-CoA synthetase in lipid and related metabolisms (21–26). Here, to investigate the function of HIV Gag myristoyl moiety in the process of HIV assembly and particle budding, we employed to inhibit the expression of myristoylated Pr55 in the recombinant baculovirus system. We show that the inhibition of Gag myristoylation by triacsin C follows dose-dependent kinetics but that the inhibition of VLP budding exhibits sudden shutoff kinetics. These data suggest that only a relatively small proportion of total Gag molecules need to be myristoylated for efficient VLP budding and indicate that total inhibition of myristoylation will be required for effective anti-viral therapy.

**EXPERIMENTAL PROCEDURES**

**Materials**—Triacsin C was purified from a culture broth of Streptomyces sp. SK-1894 as reported previously (20). Triacsin C was usually added to every assay described below as an ethanol solution, and the final volume of ethanol never exceeded 2.5%. Equivalent concentrations of ethanol alone showed no effects on the assays. Myristic, palmitic, and oleic acids were purchased from Funakoshi, Japen. [1-14C]Myristic acid (58.0 Ci/mmol), [1-14C]palmitic acid (56.0 Ci/mmol), and [1-14C]oleic acid (56.0 Ci/mmol) were purchased from DuPont NEN, and [1-14C]rainbow protein molecular weight markers and [9,10]-14Imyristic acid were from

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Acmvirus—Sf9 cells were propagated at 27°C in Grace’s insect cell culture medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 50 μg/ml gentamycin (Life Technologies, Inc.).

**Measurement of Cell Viability**—Sf9 cell viability in the presence of triacsin C was measured by trypan blue exclusion method (29).

**Preparation of a Membrane Fraction**—Sf9 Cells—1.5 × 10⁷ Sf9 cells were collected and suspended on ice in 20 ml of 0.1 M potassium phosphate buffer containing 1 mM DTT. The reaction was stopped by adding an isopropanol:heptane:1 M sulfuric acid mixture (40:10:1, v/v), and the aqueous layer containing synthesized [¹⁴C]acyl-CoA was counted by a liquid scintillation spectrometer.

**Activity (dpm/0.5 ml aqueous layer)**

| Activity (dpm/0.5 ml aqueous layer) | Myristic acid | Oleic acid |
|------------------------------------|--------------|------------|
| Complete system                     | 0 (0%)       | 1806.0 (100%) |
| -ATP                               | 23.8 (1.3%)  | 75.5 (4.2%)  |
| -CO₆                               | 43.3 (2.4%)  |            |
| -Mg²⁺                              | 0 (0%)       |            |

**TABLE I**

**Substrate requirements for long chain acyl-CoA synthetase activity in an Sf9 cell membrane fraction**

The assay mixture of the complete system contained 100 mM Tris-HCl (pH 8.0), 15 mM MgCl₂, 150 mM KCl, 5 mM DTT, 10 mM ATP, 1 mM CoA, 1 mM fatty acid (myristic or oleic acid dissolved in 1% Triton X-100 to a final concentration of 0.1%), ¹³C-fatty acid (0.02 μCi, 56 Ci/mmol), and an Sf9 membrane fraction in a total volume of 100 μl.

**Fig. 1. Inhibition of acyl-CoA synthetase activity in the Sf9 cell membrane fraction by triacsin C.** Sf9 cells (1.5 × 10⁶ cells) in 20 μl of 0.1 M potassium phosphate buffer containing 1 mM DTT were sonicated (at 100 watts for 10 s six times) on ice. A membrane fraction precipitated by centrifugation (at 100,000 × g for 1 h) was used as an enzyme source. Acyl-CoA synthetase activity was measured in a 100-μl mixture containing 1 mM Tris-HCl (pH 8.0), 5 mM DTT, 150 mM KCl, 15 mM MgCl₂, 10 mM ATP, 1 mM CoA, 1 mM ¹³C-fatty acid (0.02 μCi), myristic acid; O, palmitic acid; ○, oleic acid; triacsin C (0–48 μM), and the membrane fraction (15 μg of protein). After a 20-min incubation at 27°C, produced [¹⁴C]acyl-CoA and ¹³C-fatty acids were separated, and the radioactivity of [¹⁴C]acyl-CoA and ¹³C-fatty acids was counted by a liquid scintillation spectrometer.

**Fig. 2. Effect of triacsin C on release of HIV Gag protein.** Monolayers of 2 × 10⁶ Sf9 cells in 35-mm tissue culture dishes were infected with the recombinant baculovirus-containing HIV-1 gag gene. After adsorption, the cells were washed and cultured in serum-free Grace’s medium in the presence of various concentrations of triacsin C (0–48 μM) as indicated. At 48 h postinfection, the cells and culture media were harvested separately and analyzed by Western blotting using anti-HIV-1 p24 polyclonal antibodies. Pr55 and its proteolytic products, p47 (p17+p24+p9), p39 (p24+p37), and p24, were detected. lane 1, a prestained molecular weight marker; lanes 2–6, cell lysates; lanes 7–11, culture media.

**In Vivo Labeling**—Titers of recombinant baculoviruses were determined by the plaque assay as described elsewhere (32).

**Western Blotting**—At 48 h postinfection, cells and culture media were harvested separately and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 14% acrylamide gels (33). Western blotting was carried out as described elsewhere (34). After transfer, nitrocellulose membrane (Amersham) was incubated with anti-HIV-1 p24 peptide serum or anti-HIV-1 p17 peptide serum (see text and legends for figures) and anti-sheep IgG alkaline phosphatase conjugate. The immunocomplexes were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Promega).

**In Vivo Labeling**—At 36 h postinfection, monolayer cells in 35-mm tissue culture dishes were metabolically labeled with 250 μCi of [9,10(³⁵)S]myristic acid in 0.5 ml of serum-free Grace’s medium with triacin C either for 3 h in the case of immunoprecipitation or overnight.
through 20% (w/v) sucrose cushions in SW55 tubes at 4°C at 147,000 × g for 2 h. The virus pellets were resuspended in 100 ml of phosphate-buffered saline and layered onto 20–60% (w/v) sucrose gradients in SW55 tubes. After centrifugation at 4°C at 147,000 × g overnight, the gradient was fractionated by 300 ml, and 40–50% sucrose fractions were pooled and subjected to SDS-PAGE. Fluorography and autoradiography were carried out as described above.

Electron Microscopy—The procedure for electron microscopic examination was described previously (36). Cells were collected at 36 h postinfection before appreciable cell lysis and washed with 0.05M cacodylate buffer (pH 7.2). The cells were fixed with 2% glutaraldehyde for 2 h at 4°C prior to treatment with 1% osmium tetroxide for 2 h at 4°C. After dehydration in ethanol, the cells were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H-800 electron microscopy.

RESULTS

Inhibition of Myristoyl-CoA Synthesis by Triacsin C—Since acyl-CoA synthetase activity from insect cell had not been previously reported, the characteristics of the enzyme present in the membrane fraction of Sf9 cells was determined. ATP, CoA, and Mg2+ were required to elicit full activity (Table I), similar to the characteristics of the enzyme activity present in rat liver and other animal cells (21, 23, 24). Furthermore, myristic acid appeared to be activated by the same synthetase as oleic acid (Table I). Triacsin C inhibited the activation of both myristic and palmitic acids in a dose-dependent fashion with the IC50 values calculated to be 27 and 21 μM, respectively (Fig. 1). In contrast, inhibition of oleic acid activation required a higher IC50 value of about 50 μM. These studies established that triacsin C inhibited myristoyl-CoA formation by acyl-CoA synthetase in Sf9 cell membrane fractions.

Effect of Triacsin C on Sf9 Cells—To investigate the effect of triacsin C on Sf9 cell viability, confluent monolayers of Sf9 cells were cultured in serum-free Grace’s medium with increasing levels of triacsin C for 48 h at 27°C, and the cell viabilities were determined by the trypan blue staining. No significant difference was observed compared to untreated control with the drug levels of up to 48 μM, although Sf9 cell viability was severely affected at 96 μM (data not shown). Accordingly, levels of 0–48 μM triacsin C were used for further experiments.

Inhibition of HIV Gag VLP Budding by Triacsin C—The effect of triacsin C on HIV Gag protein synthesis and subsequent VLP budding was examined using Sf9 cells infected with a recombinant baculovirus-expressing Gag protein (12, 13, 27, 28). Infected cells were cultured in the presence of a range of concentrations of triacsin C, and the culture media and cells were harvested for Western blotting and electron microscopic examination. Western blotting was carried out using an anti-
HIV-1 p24 peptide serum, which recognizes a major antigen located in the central region of Gag protein. Near identical levels of Pr55 were detected in all the cytoplasmic fractions, indicating minimum effect of triacsin C on Gag protein synthesis (Fig. 2). The previously reported Gag proteolytic products, p47 (p17+p24+p9), p39 (p24+p9+p6), and p24, which occur to some degree by cell-directed proteolysis (discussed in Ref. 12), were also visible, and, as these were unaffected by drug treatment, Gag protein processing also appears largely unaffected by triacsin C treatment, although there was a lower level of proteolytic conversion to p24 in the 48 μM sample. In the culture supernatants, however, in contrast to almost identical levels of Gag antigens in 0–24 μM treatments, no detectable antigen was present in the 48 μM sample (Fig. 2). Electron microscopic examination confirmed these findings. Infected cells treated with 24 μM triacsin C showed abundant HIV Gag VLPs budding from the cell surface indistinguishable from non-treated cells (Fig. 3, A and B). In contrast, in the cells treated with 48 μM of the drug, there were no detectable HIV Gag VLPs produced from the cell surface nor any electron-dense structure underneath the plasma membrane (Fig. 3C). However, electron-dense ring structures were frequently observed in the perinuclear area of the cell (Fig. 3D) and resembled those reported in the case of non-myristoylated Gag protein obtained by site-directed mutagenesis (12). We conclude, therefore, 48 μM triacsin C inhibits budding of HIV Gag VLP with little effect on Gag protein synthesis.

The expression system used here, like the gag expression system using vaccinia virus vectors (37, 38), produces baculoviruses as well as HIV Gag VLPs. Since HIV Gag protein is myristoylated and, in contrast, there are no acylated proteins reported in baculovirus (39), the titer of baculoviruses grown in triacsin C-treated cells was used as a general measure of the side effects of triacsin C on non-myristoylated proteins. Equal levels of infectious baculovirus were present in the supernatants from the cells treated with all the dosages of triacsin C used in this study (Fig. 4), suggesting that triacsin C treatment had little effect on the synthesis of non-myristoylated proteins or their folding and incorporation into baculovirus particle.

Inhibition of Myristoylation of HIV Gag Protein by Triacsin C—To examine inhibition of myristoylation of HIV Gag protein in vivo, SF9 cells infected with the recombinant baculovirus-expressing Gag protein were metabolically labeled with [3H]myristic acid for 3 h at 36 h postinfection in the presence of triacsin C. The cell lysates were immunoprecipitated with anti-HIV-1 p17 peptide serum, which recognizes the N-terminal third of the Gag polyprotein, and subjected to SDS-PAGE. From the Western blot analysis of the resulting supernatant fractions and the electron microscopic observation of the infected cells described above, an all-or-none effect of triacsin C on the Gag myristoylation was expected. Surprisingly, however, triacsin C inhibited the Gag myristoylation in a dose-dependent manner (Fig. 5A). Quantitation of [3H] label associated with the immunoprecipitates by liquid scintillation counting confirmed the dose-dependent inhibition of Gag myristoylation by triacsin C with an IC50 value of 6.7 μM (Fig. 5B).

Incorporation of Non-myristoylated Gag Protein with Myristoylated Gag Protein into HIV Gag VLP—Our data showed an obvious discrepancy between the kinetics of inhibition of Gag VLP budding and that of Gag myristoylation. To further understand this phenomenon, we metabolically labeled infected cells overnight to allow labeled Gag proteins to be incorporated into VLPs, fractionated VLPs from the culture media by sucrose density gradient centrifugation, and analyzed them on SDS-PAGE. Western blotting revealed equivalent levels of Pr55 antigens in the VLP fractions from the cells treated with 0–24 μM triacsin C but no antigens of an equal density fraction from the cells with 48 μM (Fig. 6A). Levels of [3H]-myristoylated Pr55 molecules in the VLPs, however, decreased linearly with an increasing dose of triacsin C (Fig. 6B). These results demonstrate that inhibition of myristoylation of almost all Gag molecules is required for the prevention of budding of Gag VLP. Partial inhibition leads to a reduction in the relative level of...
myristoylated Gag but not the level of assembled VLP. This suggests that non-myristoylated and myristoylated Gag molecules co-assemble in various ratios to form complexes that are efficiently targeted to the plasma membrane to produce Gag VLP.

DISCUSSION

It is well established that HIV Gag myristoylation is essential for plasma membrane targeting of Gag protein, as when the myristoylation acceptor glycine located at the N terminus of Gag protein is mutated, the budding of HIV Gag VLP is completely abolished, and their assembly is restricted to intracellular locations (4, 10–13). However, it has also been reported that the non-myristoylated Gag proteins obtained by site-directed mutagenesis can be rescued into Gag VLPs by co-expression with myristoylated Gag (40, 41). Similar findings have been reported for studies with Rous sarcoma virus (42), although not for murine leukemia virus (43). One would assume, therefore, partial reduction in the level of Gag myristoylation might reduce the number of Gag molecules located to plasma membrane and result in a reduction in the level of Gag VLP release proportional to the level of myristoylation inhibition. In this paper, we used triacsin C, a well defined inhibitor of acyl-CoA synthetase, and found that activation of myristic acid to myristoyl-CoA catalyzed by acyl-CoA synthetase was dose dependently inhibited by triacsin C (Fig. 1), and correspondingly, dose-dependent inhibition of Gag myristoylation occurred in the Gag-expressing cells (Fig. 5).

In contrast to the dose-dependent inhibition of Gag myristoylation (Fig. 5), the budding of Gag VLP from the plasma membrane of infected cells exhibited an “all-or-none” phenotype (Fig. 3). The aspect was much more clearly highlighted by analyzing the ratio of total to [3H]-myristoylated Gag protein in the budded VLP in the presence of triacsin C (Fig. 6). This observation suggests that non-myristoylated and myristoylated Gag proteins co-assemble efficiently to form Gag VLP, presumably because the addition of the myristoyl group does not affect the conformation of the putative assembly domains of Gag proteins. It has been shown previously that myristoylated Gag proteins can co-assemble with non-myristoylated Gag-Pol proteins and lead to release as part of an assembled particle (40, 41). However, it is difficult to assess the exact level of Gag myristoylation necessary for Gag VLP budding, as the level of Gag-Pol incorporation found in mature HIV particles was typically 5% of total Gag antigen. Semi-quantitative measures of the relative levels of myristoylated and non-myristoylated Gag in our experiments (Fig. 6) suggest that as little as 25% of the total Gag molecules needs to be derived from myristoylated Gag to ensure VLP release.

The assembly of Gag proteins is believed to occur after Gag proteins reach the plasma membrane, since electron microscopic observation shows that an electron-dense half-ring corresponding to the gathering of Gag proteins is present underneath locally extruded plasma membrane (2, 44). Nonetheless, electron-dense ring structures in cytoplasmic VLP have also been documented in the case of non-myristoylated Gag protein (12, 45), and we found similar structures in a perinuclear area of the cells expressing Gag proteins in the presence of 48 μM triacsin C. Pre-assembly in the cytoplasm has been traditionally thought of as the morphological pathway for D-type retroviruses and plasma membrane assembly the pathway for C-type (as for HIV) retroviruses (1). Our data suggest this distinction is not as clear cut as is commonly thought, especially when non-myristoylated form of Gag protein is concerned.

We consider that myristoylation of retrovirus Gag protein is a potential target for development of new antiviral agents. We showed here that Gag VLP was produced up to and including 24 μM of triacsin C and composed of mixture of non-myristoylated and myristoylated Gag proteins. In this system, we were unable to assess whether or not HIV particles containing predominantly non-myristoylated Gag proteins are as stable or as infectious as authentic HIV particles whose Gag molecules are believed to be fully myristoylated. It is possible that, although budding of VLP is not prevented because of partial myristoylation, there is an effect on processing or uncoating. As non-processed Gag protein always results in an infectious state (46–48), anti-myristoylation therapy may prove effective even if particles continue to be produced.

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