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Processing of the structural proteins of human immunodeficiency virus type 1 in the presence of monensin and cerulenin

(virus maturation/syncytia/immunoprecipitation)

RANAJIT PAL*, ROBERT C. GALLO†, AND M. G. SARNGADHARAN*

*Department of Cell Biology, Bionetics Research, Inc., 1330-A Piccard Drive, Rockville, MD 20850; and †Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, MD 20892

Contributed by Robert C. Gallo, August 30, 1988

ABSTRACT **The synthesis and processing of structural proteins of human immunodeficiency virus type 1 (HIV-1) were studied in infected cells treated with monensin and cerulenin. In MOLT-3 cells chronically infected with HTLV-III$_B$, monensin inhibited the proteolytic cleavage of the *env*-coded polyprotein gp160 to gp120, leading to the accumulation of the precursor gp160. The formation of syncytia normally observed when CEM cells are cocultivated with HIV-1-infected MOLT-3 cells was significantly inhibited in the presence of monensin. The effect of the ionophore on the culture was reversible, as withdrawal of monensin from the medium restored the ability of the cells to form syncytia with CEM cells and led to the resumption of the processing of gp160 to gp120. Monensin did not affect the synthesis and processing of *gag*-coded proteins and regulatory proteins. Cerulenin, an inhibitor of *de novo* fatty acid biosynthesis, inhibited the myristoylation and the proteolytic cleavage of the *gag*-coded polyprotein Pr53^{gag} to p24 but did not affect the processing of gp160. However, use for monensin and cerulenin as antiviral agents for treatment of HIV-1 infection cannot be foreseen because of the pronounced *in vitro* toxicity observed.**

The *gag* and *env* gene products of the acquired immunodeficiency syndrome (AIDS) retrovirus, human immunodeficiency virus type 1 (HIV-1) (1–3), are synthesized in the form of precursor polyproteins—Pr53^{gag} and gp160, respectively—in the infected cell (4). Pr53^{gag} undergoes posttranslational cleavage to structural proteins of M_r 17,000, 24,000, and 15,000 (5). The envelope precursor protein is cleaved into two structural glycosylated proteins: gp120, the amino-terminal exterior component, and gp41, the carboxyl-terminal transmembrane portion (6, 7). A number of compounds are known to affect the processing and maturation of viral proteins in the infected cells. The monovalent carboxylic ionophore monensin inhibits the transport and expression of membrane glycoproteins and several secretory proteins (8–11). This ionophore has been demonstrated to affect the maturation of certain enveloped viruses by inhibiting the migration of envelope proteins to the plasma membrane (12–18). The antibiotic cerulenin, an inhibitor of *de novo* fatty acid and sterol synthesis, inhibits the fatty acylation of the glycoproteins of vesicular stomatitis virus and Sindbis virus and the maturation of complete virions (19). In cells infected with Moloney murine leukemia virus, cerulenin appeared to significantly decrease the maturation and release of the virus into the culture medium by inhibiting cleavage of the *gag*- and *env*-coded precursor polyproteins (20). In this communication we report the effect of monensin and cerulenin on the processing of *env* and *gag* proteins of HIV-1.

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MATERIALS AND METHODS

Virus and Cells. Chronically infected MOLT-3/HTLV-III$_B$ cells and uninfected CEM cells were maintained in RPMI-1640 medium (Advanced Biotechnologies, Columbia, MD) supplemented with 10% fetal bovine serum, 1 mM glutamine, and 100 units of penicillin and 100 μg of streptomycin per ml.

Radiolabeling of Cells with [^{35}S]Methionine and Immunoprecipitation Assays. MOLT-3/HTLV-III$_B$ cells were radiolabeled by incubation at 37°C for 7 hr in methionine-free medium supplemented with [^{35}S]methionine (100 μCi/ml; 1 μCi = 37 kBq) (6). Labeled cells were washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and disrupted at 4°C in PBS containing 0.1% $NaDodSO_4$, 1% Triton X-100, and 0.5% sodium deoxycholate. Immunoprecipitation of lysates was as described (6).

Syncytium Assay. Syncytium assays were performed in 96-well microtiter plates by mixing, in 200 μl of medium, 10^5 CEM cells with 10^4 chronically infected MOLT-3/HTLV-III$_B$ cells. The plates were incubated in a CO_2 incubator at 37°C for 40 hr and the number of giant cells in each well was determined by microscopic examination.

RESULTS

Synthesis of HIV-1 Proteins of MOLT-3/HTLV-III$_B$ Cells Treated with Monensin. To determine the effect of monensin on the synthesis of HIV-1 proteins in infected cells, MOLT-3/HTLV-III$_B$ cells were radiolabeled with [^{35}S]methionine in the presence of various concentrations of monensin (Sigma). It was observed that these chronically infected cells (10^6 cells per ml) could be maintained without any loss of cell viability for 7 hr when treated with 10 μM monensin. After 7 hr of treatment with monensin, the cells were harvested and the immunoreactive proteins were precipitated with human serum containing antibodies to all the major HIV-1 proteins. Monensin markedly inhibited the proteolytic processing of gp160 to gp120 (Fig. 1*A*). At 10 μM concentration there was a significant amount of gp160 accumulated in the infected cells. At all concentrations of monensin, a protein of M_r $\approx$100,000 appeared in the immunoprecipitates (lanes 2–6). The proportion of the *gag* proteins Pr53^{gag} (indicated as p53 in figures) and p24 remained substantially unchanged after monensin treatment. Monensin treatment also had no effect on the expression of *tat*, *vif*, and *nef* (4) gene products (Fig. 1*B*). These studies thus demonstrate that the effect of monensin was specific for the processing of the *env* proteins.

To study the basis for the accumulation of gp160 in monensin-treated cells, a pulse–chase analysis of the stability of the glycoproteins was performed both in the absence and in the presence of monensin. MOLT-3/HTLV-III$_B$ cells treated with 7.5 μM monensin were radiolabeled with [^{35}S]methionine for 1 hr and the label was subsequently

Abbreviation: HIV-1, human immunodeficiency virus type 1.

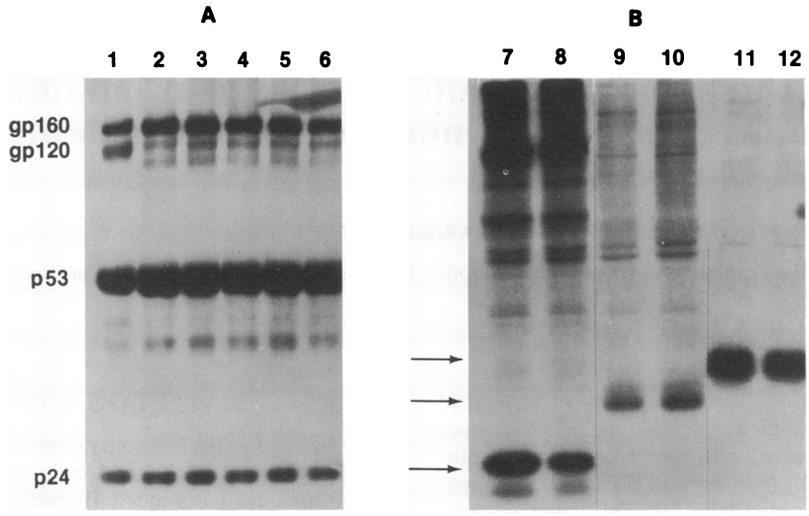

FIG. 1. HIV-1 protein profile of MOLT-3/HTLV-III$_B$ cells treated with monensin. (*A*) Lysates of [^{35}S]methionine-labeled MOLT-3/HTLV-III$_B$ cells were analyzed by immunoprecipitation with HIV-1 antibody-positive human serum. Monensin concentrations were 0 (lane 1), 1.25 (lane 2), 2.5 (lane 3), 5 (lane 4), 7.5 (lane 5), and 10 (lane 6) μM. (*B*) Lysates of cells treated with 10 μM monensin (lanes 8, 10, and 12) and untreated control cells (lanes 7, 9, and 11) were analyzed by immunoprecipitation with rabbit antibodies to HIV-1 *tat* (lanes 7 and 8), *vif* (lanes 9 and 10), and *nef* (lanes 11 and 12) gene products. Arrows mark the respective positions of the gene products.

"chased" by incubating the cells with nonradioactive methionine for up to 4 hr. The cells were harvested at various times and the level of intracellular viral proteins was determined by immunoprecipitation with a human serum. In untreated control cells there were significant decreases in labeled gp160 and Pr53^{gag}, with corresponding increases in gp120 and p24, respectively, during the chase (Fig. 2, lanes 1–3). In sharp contrast, in monensin-treated cells only the *gag* proteins underwent normal processing. There was a marked reduction of the proteolytic cleavage of gp160 (lanes 4–6). The labeled gp160 that accumulated in monensin-treated cells could not be processed in the presence of a lysate of untreated infected cells, suggesting that the processing of gp160 took place in intact organelles and could not be accomplished in cell-free extracts (data not shown).

Effect of Monensin on the Syncytium Formation by MOLT-3/HTLV-III$_B$ Cells. One of the consequences of HIV-1 infection is the formation of multinucleated giant cells resulting from cell–cell fusion (1). The envelope glycoprotein gp120 has been shown to interact with the CD4 molecule of the target cells (21–23) to initiate the process of infection, which

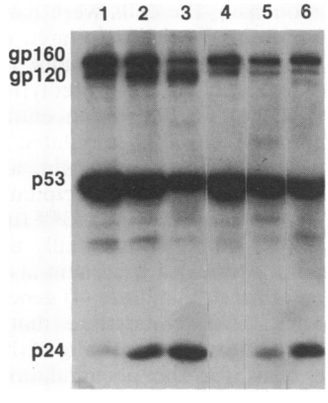

FIG. 2. Analysis of the metabolic fate of HIV-1 proteins in untreated and monensin-treated cells. Cells were pulsed for 60 min with [^{35}S]methionine (100 μCi/ml) in the absence (lanes 1–3) or presence (lanes 4–6) of 7.5 μM monensin. The label was chased for 0 min (lanes 1 and 4), 60 min (lanes 2 and 5), or 240 min (lanes 3 and 6). The labeled proteins were immunoprecipitated with a human antiserum and analyzed by NaDodSO$_4$/PAGE.

in most cases results in the formation of multinucleated giant cells (24). It was of interest to determine whether the inhibition of the processing of gp160 in infected cells would affect the induction of syncytium formation by these cells in uninfected CEM cells. For this, MOLT-3/HTLV-III$_B$ cells were cocultured with uninfected CEM cells in the presence of 1–10 nM monensin. The number of syncytia was markedly reduced for cells treated with >3 nM monensin compared to untreated cells when observed after the normal incubation time of 40 hr (Fig. 3*A*). However, when the incubation was extended for an additional 24 hr an increased number of syncytia was observed in control cells. Under these conditions 3 nM monensin, which gave partial inhibition in the 40-hr experiment, was almost noninhibitory. It is quite likely that some of the ionophore might have degraded in the culture medium during the extended incubation, leading to the apparent reversal of the syncytium blocking.

To understand whether the inhibition of syncytium formation by monensin was reversible, MOLT-3/HTLV-III$_B$ cells were treated with 10 μM monensin for 7 hr, washed free of the ionophore, and cocultured with CEM cells in monensin-free medium. Multiple syncytia resembling those of the untreated cells were observed (Fig. 3*B*). Accompanying this reversal of syncytium blocking was the restoration of the proteolytic processing of gp160 to gp120 after withdrawal of the drug (Fig. 3*C*).

Synthesis of HIV-1 Proteins in MOLT-3/HTLV-III$_B$ Cells Treated with Cerulenin. To determine whether cerulenin affects the processing of HIV-1 proteins, MOLT-3/HTLV-III$_B$ cells were labeled with [^{35}S]methionine in the presence of different concentrations of cerulenin (CalBiochem–Behring). The chronically infected cells could be maintained without any loss of viability when treated with cerulenin (10 μg/ml) for 7 hr. The labeled cells were harvested and their extract was immunoprecipitated with a HIV-1 antibody-positive human serum. There was a general inhibition of viral protein synthesis at higher concentrations of cerulenin (Fig. 4*A*). However, the proteolytic cleavage of Pr53^{gag} to p24 was selectively inhibited in cerulenin-treated cells. Interestingly, at a concentration of cerulenin that virtually stopped the cleavage of Pr53^{gag} to p24 (10 μg/ml), myristoylation of Pr53^{gag} was still detected, albeit at a low level (Fig. 4*B*).

The stability of the Pr53^{gag} accumulated following cerulenin treatment was determined by pulse–chase experiments.

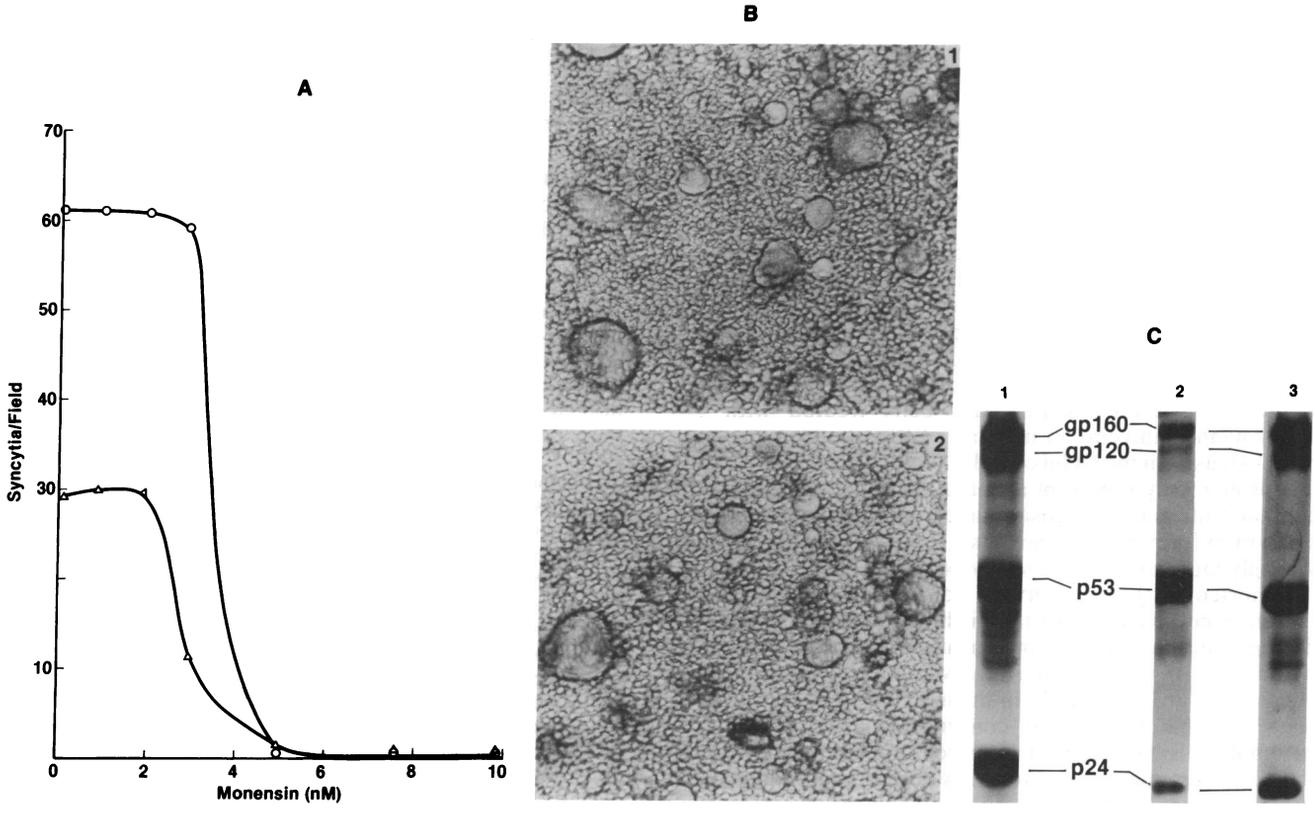

FIG. 3. Reversibility of the monensin effect on glycoprotein processing and on syncytium formation between CEM cells and MOLT-3/HTLV-III$_B$ cells. (*A*) CEM cells cocultured with MOLT-3/HTLV-III$_B$ cells were scored for syncytia after 40 hr (lower curve) or 72 hr (upper curve) in the absence or presence of monensin as indicated. (*B*) Micrograph 1, untreated control culture described in *A* at 40 hr. Micrograph 2, MOLT-3/HTLV-III$_B$ cells (10^6 cells per ml) treated with 10 μM monensin for 7 hr, harvested, washed free of monensin, and cocultured with CEM cells in the absence of monensin for 40 hr. (*C*) [^{35}S]Methionine-labeled MOLT-3/HTLV-III$_B$ cells were lysed and analyzed by immunoprecipitation using a human antiserum. Lane 1, cells not treated with monensin; lane 2, cells labeled in the presence of 10 μM monensin (same as Fig. 1, lane 6); lane 3, cells treated with 10 μM monensin for 7 hr, washed, and cultured overnight in monensin-free medium before labeling.

MOLT-3/HTLV-III$_B$ cells were radiolabeled for 1 hr in the presence of cerulenin (7.5 μg/ml) and the label was then chased for up to 4 hr. The cells were harvested at various times and the intracellular protein profile was determined by immunoprecipitation. Pr53^{gag} was processed to p24 in untreated MOLT-3/HTLV-III$_B$ cells (Fig. 4*C*, lanes 10–12).

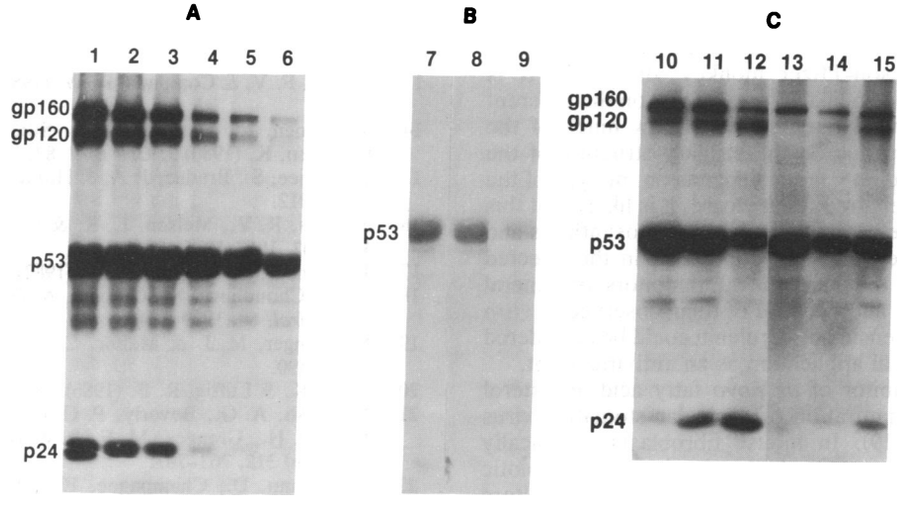

FIG. 4. Intracellular protein profile of MOLT-3/HTLV-III$_B$ cells treated with various concentrations of cerulenin. (*A*) Lysates of [^{35}S]methionine-labeled MOLT-3/HTLV-III$_B$ cells were immunoprecipitated with a HIV antibody-positive human serum. Concentrations of cerulenin for lanes 1–6 were 0, 2.5, 5, 7.5, 10, and 12.5 μg/ml, respectively. (*B*) MOLT-3/HTLV-III$_B$ cells (10^6 cells per ml) were labeled with [^{3}H]myristic acid (100 μCi/ml) for 7 hr. Cerulenin was absent (lane 7) or present at 7.5 (lane 8) or 10 (lane 9) μg/ml. (*C*) Pulse–chase study of HIV-1 proteins in untreated (lanes 10–12) and cerulenin (7.5 μg/ml)-treated cells (lanes 13–15) was performed by metabolic labeling of MOLT-3/HTLV-III$_B$ cells with [^{35}S]methionine as described in the legend to Fig. 2. Chase periods were 0 min (lanes 10 and 13), 60 min (lanes 11 and 14), and 240 min (lanes 12 and 15).

However, no significant processing of $Pr53^{gag}$ was observed under similar conditions in cerulenin-treated cells (lanes 13–15).

DISCUSSION

We describe here the effects of a carboxylic ionophore, monensin, and an inhibitor of fatty acid biosynthesis, cerulenin, on the processing of HIV-1 proteins. Monensin up to a concentration of 10 μM was found to have little effect on the *de novo* protein synthesis in the HIV-1-infected cells. However, treatment of HTLV-III_B-infected MOLT-3 cells with monensin for 7 hr inhibited the proteolytic conversion of gp160 to gp120 and gp41. In addition, a protein of M_r $\approx$100,000 was immunoprecipitated. A similar protein was formed in MOLT-3/HTLV-III_B cells treated with 1-deoxymannojirimycin, an inhibitor of the trimming enzyme mannosidase I in the Golgi complex (unpublished data). Thus this protein may represent a cleavage product of gp160 and contain untrimmed oligosaccharide chains. Inhibition of syncytium formation by monensin tends to suggest that the viral glycoproteins in the ionophore-treated cells were not transported to the cell surface from Golgi membrane and therefore not available for the binding to the CD4 molecule. The inhibition of syncytium formation was reversible upon withdrawal of the ionophore, as was the inhibition of the cleavage of gp160 to gp120 in the infected cells. It has been suggested that at moderate concentrations (0.1–1 μM) the metabolic effects of monensin on cells are ultimately reversible (11). This might be due to a slow degradation of monensin in the culture medium. Presumably for the same reason syncytium assays performed over long incubation periods led to a restoration of syncytia, provided that the initial concentration of monensin was low.

The effect of monensin on the processing and maturation of membrane glycoproteins has been studied in a number of enveloped viruses. Although monensin blocks the transfer of newly synthesized G glycoprotein of vesicular stomatitis virus from the Golgi complex to the plasma membrane of the infected cells, thereby severely inhibiting the release of virus particles into the medium (12), hemagglutinin of influenza virus was transported in its presence to the plasma membrane in a functional form (13). Among retroviruses, monensin inhibited the processing and transport to the plasma membrane of the glycoprotein precursor in Friend murine leukemia virus (16) but did not affect the intracellular migration of glycoproteins in Mason–Pfizer monkey virus (15). It is interesting that monensin has diverse effects on the different virus systems that have been studied. How much of the differential effect depends on the primary structure of the viral glycoprotein and how much depends on the type of the host cell are not understood. In any case, it is likely that this ionophore will be useful to understand the biosynthesis and transport of HIV-1 envelope glycoproteins in the infected host cells. Because they are potent inhibitors of general metabolic pathways, and because of their observed *in vitro* toxicity, neither monensin nor cerulenin could be considered a candidate for clinical application as an antiviral agent.

Cerulenin, an inhibitor of *de novo* fatty acid and sterol synthesis, inhibits maturation of vesicular stomatitis virus and Sindbis virus (19). In mouse fibroblasts chronically infected with Moloney murine leukemia virus, the antibiotic inhibited the release of the mature virions into the culture medium by inhibiting the proteolytic cleavage of *gag*- and *env*-coded precursor polyproteins (20). In this communication we have demonstrated that the treatment of HTLV-III_B-infected MOLT-3 cells with cerulenin inhibited the cleavage of $Pr53^{gag}$ to p24. Myristoylation of $Pr53^{gag}$ was also inhibited by cerulenin. It is not clear how the cleavage of $Pr53^{gag}$ was inhibited by cerulenin. One possible explanation is that the antibiotic acts as an inhibitor of viral protease, which is responsible for processing the *gag* polyprotein. Another possibility is that the myristoylation of the *gag* protein is essential for its interaction with intracellular membranes where the cleavage of $Pr53^{gag}$ to p24 takes place. Inhibition of myristoylation by cerulenin might affect the interaction of the *gag* polyprotein with the membrane, thereby affecting its accessibility to the protease.

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