Obstruction of HIV-1 Particle Release by Interferon-α Occurs Before Viral Protease Processing and Is Independent of Envelope Glycoprotein

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ABSTRACT

The effect of human interferon-α (Hu-IFN-α) on the maturation process of the human immunodeficiency virus type 1 (HIV-1) has been studied using stable cell lines that produce nonenveloped particles. These cell lines secrete particles devoid of the viral envelope proteins gp120 and gp41. The CH-1 cells produce active viral protease that correctly processes its natural substrates, whereas the CH-1Kw cell line expresses an enzymatically inactive viral protease, thus producing immature viral capsids. A block in the secretion of particles was observed in both cell lines when treated with 100–1000 U/ml Hu-IFN-α, as judged by measurements of encapsidated gag proteins. Electron microscopy shows that Hu-IFN-α-treated CH-1 cells are decorated with assembled immature particles at the cell surface. These results suggest that the observed block in particle release on Hu-IFN-α treatment is independent of viral envelope expression and occurs before capsid polyprotein processing. In addition, particles remaining attached to the cell fail to mature into structures with condensed cores. Viral gag proteins from IFN-treated and untreated CH-1 cells were analyzed by 2-D gel electrophoresis. Results suggest a change in posttranslational modifications of gag proteins, as IFN treatment allowed the detection of more basic forms of p55, p39, and p24. Further analysis of cellular or viral protein alterations induced by Hu-IFN-α treatment may identify the mechanism of action by which particle maturation is obstructed.

INTRODUCTION

INTERFERENCE WITH DIFFERENT STEPS OF THE RETROVIRAL LIFE CYCLE LEADS TO THE ANTI-VIRAL PROPERTIES OF INTERFERONS (IFNS). IFNS HAVE BEEN SHOWN TO INHIBIT TRANSCRIPTION AND TRANSLATION PROCESSES, AS WELL AS VIRUS REPLICATION.1–4 THE SPECIFIC PATHWAY INVOLVED IN THEIR ACTION DEPENDS ON THE VIRUS, CELL TYPE, AND SUBTYPE OF IFN USED. THE PRECISE MECHANISMS BY WHICH IFN-α AFFECTS EARLY AND LATER STAGES OF THE REPLICATION CYCLE OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) HAVE NOT BEEN ELUCIDATED, BUT THESE EFFECTS FALL INTO THREE MAJOR CATEGORIES. A LARGE BODY OF EVIDENCE HAS ACCUMULATED SHOWING THAT IFN-α CAN BLOCK BOTH ASSEMBLY AND RELEASE OF VIRIONS IN HIV-1-INFECTED CELLS.5–9 THE EFFECT ON VIRUS RELEASE HAS BEEN LINKED TO (1) A HALT IN THE BUNDELING PROCESS,6,8 (2) A BLOCK IN THE ABILITY TO ASSEMBLE p24 CAPSID PROTEIN,7,10 (3) AND A DEFECT IN THE INCORPORATION OF THE gp120 ENVELOPE GYCOPROTEIN.11,12 SECOND, THE EFFECTS OF IFN-α ON REVERSE TRANSCRIPTION AND POSSIBLY OTHER EARLY HIV-1 REPLICATION EVENTS HAVE BEEN REPORTED.11,12 Finally, a more recently noted effect of IFN-α on HIV-1 replication seems to be mediated by a block of viral-specific miRNA translation.13 IFN-α may also affect yet unidentified cellular proteins that influence the migration of the capsid polypeptide to the plasma membrane and the subsequent particle assembly. A novel cell-free system, designed to monitor de novo assembly of HIV-1 capsids,14 suggests multiple discrete steps in the assembly process, including three independent posttranslational events requiring (1) ATP, (2) a detergent-sensitive host factor, and (3) a detergent-insensitive subcellular fraction. It could be envisioned that IFNs act to modulate one or more of such host factors. Furthermore, it has been proposed that the antiviral effects of IFN-α and IFN-β are mediated by modifications in plasma membrane mechanisms.15 Pitha has suggested that alterations in retroviral (Mu-LV, HIV) assembly in IFN-α-treated cells may be the consequence of a direct IFN-α effect on the fluidity of cellular plasma membranes.3

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We have tested the effect of Hu-IFN-α on the production and secretion of capsid particles from two cell lines that constitutively produce nonenveloped HIV-1 particles. These cell lines differ in their ability to express enzymatically active viral protease and thus produce either mature or immature viral particles. Both cell lines encode the entire HXB-2 genome, with the exception of the env gene. The absence of the gp160 protein from these systems can serve to clarify the role of the viral glycoprotein as one of the proposed mechanisms of Hu-IFN-α action. In the absence of gp120, we can then monitor the effect of IFN-α on the assembly and release of nonenveloped particles and detect alterations caused by direct effects on the capsid. Assays in the viral protease-deficient cell line can serve to determine whether Hu-IFN-α affects both p24 assembly and p55 capsid precursor assembly to the same extent, as previous work addressed only the effect on p25-containing capsids. We performed 2-D gel analysis of the capsid proteins in cell extracts from Hu-IFN-α and untreated cells to look for differences in protein migration that might suggest alterations in post-translational modifications.

CH-1 cells display properties of both acute and chronically infected cells by virtue of continuously secreting mature HIV-1 particles, yet they can be handled without the precautions required when dealing with infectious HIV, making them a useful tool for the study of HIV particle assembly and potential inhibitors, such as IFNs.

MATERIALS AND METHODS

Cells and plasmids

The CH-1 cell line was established by stable transfection with vector HIV-gpt as described previously. The CH-1kww cell line was similarly established by stable transfection of the vector HIVkww-gpt. HIVkww-gpt plasmid was generated by introducing mutations D25K, G49W, and 150W into the HIV-gpt genome. Briefly, approximately 1 x 10^6 COS-7 cells were transfected by the calcium phosphate protocol with vector HIVkww-gpt and subsequently placed under selection for gpt expression. Fifteen drug-resistant colonies were isolated after 14 days in culture and gradually expanded. Culture supernatant from these cells was treated with exogenous recombiant HIV-1 protease to convert all p55 to p24, as described previously, and the samples were tested by the p24 Dupont/NEB ELISA kit (following manufacturer’s instructions). The cell line designated CH-1kww was chosen for its ability to secrete >50 ng of p55 per milliliter of medium within 24 h of culture. CH-1 and CH-1kww cells were maintained in Dulbecco’s modified Eagle medium H21 (DMEM) supplemented with 10% dialyzed fetal bovine serum (FBS), and selection for gpt expression was maintained by supplementing culture medium with 250 µg/ml xanthine (Sigma), 14 µg/ml hypoxanthine (Sigma), and 75 µg/ml mycophenolic acid (Calbiochem).

Metabolic labeling of cultures

Cell cultures in 100-mm dishes were labeled for 5 h using 0.1 mCi/ml of [35S]-Translabel (ICN, 70% methionine/30% cysteine, 1000 Ci/mmol specific activity) in 5 ml of DMEM deficient in methionine and supplemented with 3% dialyzed FBS. A 1 h cold chase using DMEM with 10% dialyzed FBS followed. The culture supernatant was then harvested, and the cells were collected by scraping as described below.

Preparation of cell extracts and immunoprecipitations

To obtain whole cell extracts, monolayers were scraped in PBS containing 5 mM EDTA and 1% Triton X-100, followed by centrifugation at 30,000g for 10 min. Whole cell lysates were immunoprecipitated with anti-p24 antibodies to recover the intracellular p24 and p55 proteins. Protein-A Sepharose beads were incubated with rabbit polyclonal anti-p24 antibody (American Biotechnologies) for 1 h at 4°C. Aliquots of cellular extracts from metabolically labeled cells were incubated 2 h at 4°C, and unlabeled cellular extract from COS-7 cells was added to reduce nonspecific binding. Capsid proteins and immunoprecipitated intracellular proteins were separated on 4%-20% polyacrylamide/SDS gels, followed by autoradiography.

Isolation of capsid particles

Secreted virions were isolated from culture supernatants by passing over sucrose cushions, as described previously.

Western blotting

Protein samples were separated by electrophoresis, either one-dimensional SDS-PAGE in 4%-20% polyacrylamide gels or two-dimensional electrophoresis, as described below. Proteins were transferred to nitrocellulose and reacted with polyclonal antibody against HIV-1 p24 (Intracel). Immunoreactive bands were visualized using a secondary antibody conjugated to horseradish peroxidase, followed by the chemiluminescent substrate ECL (Pierce).

p24 ELISA

The intracellular and extracellular p24 protein content was detected by p24 ELISA (NEK-060 kit, NEN/Dupont). This assay permits detection of mature p24 with virtually no reaction with the unprocessed precursor proteins. To detect p55 protein and intermediates, exogenous purified HIV-1 protease was added to digest precursor molecules, as described previously.

Two-dimensional electrophoresis

The ISO-DALT system was used as described by Anderson. The isoelectric focusing step is performed first in a pH 3–10 gradient. This is followed by molecular size separation in 10%-20% acrylamide gradient vertical gels, under denaturing and reducing conditions.

Electron microscopy

Cells were gently scraped while in growth medium and collected by low-speed centrifugation. They were fixed with a solution containing 3% glutaraldehyde, 1% paraformaldehyde, and 0.067 M sodium cacodylate (pH 7.5) for 1 h at 25°C. Samples were postfixed in Veronal-buffered 1% osmic acid containing 7% sucrose for 1 h at 25°C, dehydrated in ethanol, and embedded in Epon 812. Thin sections (60–80 nm) were stained in uranyl acetate, contrasted with Reynolds lead stain,
RESULTS

The effect of Hu-IFN-α on the production and release of capsid protein p24 was tested in the stable cell line CH-1 (Fig. 1). The cells were grown in the presence of 0–1000 U/ml of Hu-IFN-α (Schering-Plough Research Laboratories) for 48 h and labeled with [35S]methionine for the last 5 h of treatment. Culture supernatants were collected and subjected to sucrose sedimentation to collect viral capsids. Whole cell extracts were prepared and immunoprecipitated with polyclonal antibodies to the p24 capsid protein. The proteins were separated by SDS-PAGE and detected by autoradiography (Fig. 1A). No decrease was observed in the amount of intracellular p25 or p24 proteins, suggesting that viral protein synthesis and polyprotein processing were unaffected by the IFN treatment. On the other hand, a dose-dependent decrease of p24 protein was observed in the viral capsid samples, indicating a specific effect on particle secretion. Secretion of encapsidated p24 was reduced by

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**FIG. 1.** Detection of capsid protein p24 in cells and particles following Hu-IFN-α treatment of CH-1 cells. (A) CH-1 cells were metabolically labeled with [35S]-Translabel. Whole cell extracts were immunoprecipitated using a polyclonal antibody against p24, and capsids were isolated from culture supernatants. Lanes 1–6 correspond to cells treated for 48 h with 0, 100, 250, 500, 750, and 1000 U/ml Hu-IFN-α, respectively. (B) Samples from CH-1 cells grown for 48 h in the presence of 0, 100, or 1000 U/ml Hu-IFN-α were analyzed by p24 ELISA.

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**FIG. 2.** Effect of Hu-IFN-α on secretion of immature HIV particles from CH-1kww cells. (A) CH-1kww cells were grown in the presence of Hu-IFN-α for 48 h. Capsid protein precursor p55 was detected by immunoblot of whole cell extracts and isolated capsids using a polyclonal antibody against p24. Lanes 1–5 correspond to samples treated with 0, 100, 500, 750, and 1000 U/ml Hu-IFN-α, respectively. (B) Samples from similarly treated CH-1kww cells were analyzed by ELISA to determine the total amount of p24 and p55 proteins present in immature particles. Exogenous viral protease was added to samples to convert all p55 and intermediates into p24 for detection by assay. A value of 100% was assigned to the amount of p24 protein observed for the untreated CH-1 cells.
at least 50% at 100 U/ml of Hu-IFN-α and approximately 98% at 1000 U/ml.

In another experiment, CH-1 cells were treated with 0, 100, or 1000 U/ml Hu-IFN-α for 48 h, and samples were analyzed by p24 ELISA (Fig. 1B). This assay would measure the effect of Hu-IFN-α on whole cell extracts, total culture supernatant, and isolated viral capsids over the full time course of treatment. Only the level of p24 secreted in viral capsids is dramatically affected by the IFN treatment. The amount of soluble p24 protein secreted did not appear affected, suggesting that shedding of p24 is independent of the pathway of particle assembly and, thus, unaffected by Hu-IFN-α. These results are in agreement with previous experiments using HIV-1-infected T cells, where soluble p24 secretion was unaffected by IFN treatment. The 95% reduction in p24 secreted as particles is in agreement with the observation made in Figure 1A.

The effect of Hu-IFN-α was also tested in CH-1kww cells, which lack the ability to process the p55 polyprotein precursor by virtue of expressing an inactive viral protease. CH-1kww cells were treated Hu-IFN-α (0–1000 U/ml) for 48 h (Fig. 2A). The p55 protein was detected by immunoblotting of whole cell extracts or capsids isolated from culture supernatants. No significant changes could be observed in the amount of p55 detectable in whole cell extracts. On the other hand, the p55 detected in secreted capsids decreased by at least 50% in samples treated with 500 U/ml of Hu-IFN-α and 90% in the presence of 1000 U/ml. Samples from a similar experiment were analyzed by p24 ELISA (Fig. 2B). The amounts of processed p24 protein were detected directly, whereas the amount of p55 was determined following digestion with exogenous viral protease to convert all p55 to the p24 form measured by the p24-specific ELISA. Results indicate that only p55 secreted as particles is significantly affected by IFN treatment, with 90% inhibition reached at 1000 U/ml Hu-IFN-α. Thus, Hu-IFN-α exerts a comparable block in particle release regardless of the degree of capsid protein maturation.

The effect of IFN treatment on viral capsid protein biosynthesis was analyzed by 2-D gel electrophoresis of whole cell extracts (Fig. 3). CH-1 cells were grown for 48 h in the presence or absence of 500 U/ml Hu-IFN-α and then lysed. Whole cell extracts were separated by IEF followed by SDS-PAGE, and gels were subjected to immunoblotting using antibodies against p24. Comparison of the immunoblots reveals a significant effect in the charge migration of all the gag proteins detectable by this antibody (p55, p39, and p24). The more acidic forms of the p24 capsid protein (pH 6.7–7.2) and of its precursor p55 (pH 6.1–6.4) are missing in the sample of IFN-treated CH-1 cells (Fig. 3B), and the pattern of the intermediate p39 is shifted to the more basic range of the gel (from pH 6.9–7.2 to pH 7.2–7.5). The observed increase in migration toward the cathode for capsid proteins from Hu-IFN-α-treated cells is consistent with an alteration in a posttranslational modification, such as phosphorylation (phosphorylation would serve to acidify the proteins). The fact that the modifications are seen in the initial p55 precursor form as well as in the intermediate and mature capsid protein suggests that the effect of Hu-IFN-α occurs before the proteolytic processing and is independent of this event.

Thin section electron microscopy was performed on CH-1 cells, untreated and treated with 500 U/ml Hu-IFN-α, to determine the presence and location of capsid particles (Fig. 4). In IFN-treated cells, viral particles were associated with the plasma membrane of the cell, they appeared immature, and they displayed aberrant morphologies, such as C-shaped electron density. No viral particles were detected in intracellular compartments or in the cytoplasm. Although we noted an increase in the number of viral protein crescents protruding from the plasma membrane, the observed number of capsids detectable on the cell surface was reduced by about 50% as compared with untreated CH-1 cells (this report and previous observations[15]). A similar accumulation of immature particles was observed when CH-1 cells were incubated with both Hu-IFN-α and the potent viral protease inhibitor U75879 (data not shown) or with the protease inhibitor alone.[15] These observations suggest that Hu-IFN-α may have an effect in the actual assembly of particles at the plasma membrane, as a reduction in number of fully formed budding structures was detected. Also, the presence of
FIG. 4. Thin section electron microscopy of untreated and Hu-IFN-α-treated CH-1 cells. CH-1 cells were grown for 48 h in the absence (top) or in the presence of 500 U/ml Hu-IFN-α (center and bottom). Arrows (center) indicate viral particles attached to the cell surface. (Bottom) A magnified section of the same cell showing immature core morphology and connections to plasma membrane.
no particles with condensed cores indicated that proteolytic processing did not occur within the particles that remain attached to the IFN-treated cells.

DISCUSSION

The assembly of HIV-1 particles is a multistep process involving the interplay of host and viral proteins. The effect of IFN-α on viral maturation, as studied here, may reflect the involvement of cellular proteins in the late stages of assembly, as the capsids begin the process of release and concomitant proteolytic processing. We designed these experiments to study the effects of IFN on the late stages of particle assembly, and we have focused on modifications in viral capsids by excluding the viral envelope from this test system.

We have shown that treatment of CH-1 cells with IFN-α prevents particle secretion in the absence of gp120, thus eliminating this protein as the mediator of the block in particle secretion. The effect on gp120 loading onto viral particles, as reported by Hansen et al., may be because of the effect of IFN-α on plasma membrane fluidity and not a specific effect on envelope packaging. Previous observations with the other C-type retrovirus, Mu-LV, indicated that IFN-α did not affect an early event in the replication of this virus, but it affected particle release by perturbing the incorporation of the major envelope glycoprotein. One possible mechanism involving Hu-IFN-α blocking retroviral particle secretion is by modulation of the plasma membrane, as alterations of microfilaments that affect the mobility of membrane-bound proteins have been detected in IFN-α-treated cells. Our results using HIV-1 capsids lacking envelope show that IFN-α can prevent release of these particles, suggesting that multiple mechanisms contribute to the antiviral effect of this cytokine on retroviruses.

An alternative or additional mode of action of Hu-IFN-α could involve its effects on host proteins, such as kinases. Changes in the activity and cellular localization of protein kinase C have been detected following treatment of cells with IFN-α. A number of HIV proteins, including matrix p17 and capsid p24, undergo phosphorylation, and these posttranslational modifications may determine the fate of viral protein assembly and particle budding. Differential packaging of phosphorylated and unphosphorylated forms of p24 have been shown, suggesting a selectivity during particle assembly. In addition, mutations in p17 sequences that prevent protein kinase C-mediated phosphorylation have been shown to affect p17 cellular localization and packaging. Posttranslational modifications of gag proteins may serve to regulate particle secretion, and a block in this process by the action of Hu-IFN-α may thus prevent virus budding. The ideal system to study the effect of IFN on the posttranslational modification of viral proteins and their subsequent assembly would be one where the assembly process could be synchronized and the host cell components could be modulated as in a cell-free reconstitution assay. The recent report of cell-free assembly of HIV-1 particles is one such example of a potentially useful system. Identification of IFN-induced changes in viral proteins could point to a new target for antiviral intervention, possibly one common to all viral isolates or drug-resistant strains. We speculate that a modification of the capsid proteins is responsible for the inability of virions to properly assemble and exit a cell under the effect of Hu-IFN-α. Many posttranslational modifications, such as acetylation, phosphorylation, proline, or sulfation, can cause changes of isoelectric properties like the ones we have observed in this study. Further analysis in the subcellular distribution of particular viral protein isoforms should prove insightful in dissecting the assembly process. The identification of other compounds besides IFN-α capable of blocking virus budding by such a mechanism may lead to a new class of antiviral compounds.

The extent of the block in particle release observed on treatment with IFN-α of both CH-1 and CH-Kww cells was comparable and exclusive to the encapsidated proteins, not the shedded soluble forms of p24 and p55. It has been proposed that HIV-1 gag proteins are processed in two cellular compartments. The membrane-associated processing pathway, which gives rise to virions, seems to be the only one affected by IFN-α treatment, suggesting alterations of the plasma membrane or membrane-entrapped host proteins. These host proteins may in turn modify the capsid proteins to alter the virus budding mechanisms. The proteolytic processing that occurs in the cytoplasm does not appear affected in our IFN-α experiments.

The observation that IFN causes viral particles to retain an immature morphology when attached to the plasma membrane is in agreement with previous proposals that protease maturation occurs concomitantly with viral maturation. It confirms that virus budding is a prerequisite to proteolytic processing within assembled virions. Likewise, it was reported recently that p55 polyprotein displays an enhanced stability in IFN-treated HIV-1-infected cells, suggesting that proteolytic processing was also suppressed in those infected T cells. We propose that the effect of Hu-IFN-α is thus twofold, preventing virus budding and proteolytic maturation. This suggests that IFN could provide additional antiviral activity when combined with current viral protease inhibitor therapeutics.

Because Hu-IFN-α has been shown to affect HIV replication at multiple stages of the virus life cycle (see reviews), it is useful to find a cell that responds selectively at the level of particle secretion. The CH-1 cell line may serve as a safe assay system to identify compounds that affect late events in capsid maturation and release. A compound with those characteristics but lacking the undesired toxic effects of IFN could be a very potent antiviral agent.

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