Inhibitors of Human Immunodeficiency Virus Type 1 Zinc Fingers Prevent Normal Processing of Gag Precursors and Result in the Release of Noninfectious Virus Particles

JIM A. TURPIN,¹ SARA J. TERPENING,¹ CATHERINE A. SCHAEFFER,¹ GANG YU,²
CONSTANCE J. GLOVER,² RONALD L. FELSTED,² EDWARD A. SAUSVILLE,³
AND WILLIAM G. RICE^{1*}

Laboratory of Antiviral Drug Mechanisms, Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis and Centers, National Cancer Institute-Frederick Cancer Research and Development Center, SAIC Frederick, Frederick, Maryland 21702,¹ and Laboratory of Biological Chemistry, Basic Science Division,² and Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis and Centers,³ National Cancer Institute, Bethesda, Maryland 20892

Received 23 February 1996/Accepted 15 May 1996

The Cys-Xaa₂-Cys-Xaa₄-His-Xaa₄-Cys zinc fingers of retroviral nucleocapsid proteins are prime antiviral targets because of conservation of the Cys and His chelating residues and the absolute requirement of these fingers in both early and late phases of retroviral replication. We previously reported that certain disulfide-substituted benzamides (DIBAs) chemically modify the Cys residues of the fingers, resulting in inhibition of human immunodeficiency virus type 1 (HIV-1) replication (W. G. Rice, J. G. Supko, L. Malspeis, R. W. Buckheit, Jr., D. Clanton, M. Bu, L. Graham, C. A. Schaeffer, J. A. Turpin, J. Domagala, R. Gogliotti, J. P. Bader, S. M. Halliday, L. Coren, R. C. Sowder II, L. O. Arthur, and L. E. Henderson, *Science* 270:1194–1197, 1995). We now examine the consequences of the interaction of DIBAs with the zinc fingers of the HIV-1 p7 nucleocapsid protein and its Pr55^{gag} precursor. In HIV-1-infected U1 cells, DIBAs inhibited the release of infectious virions, and even under conditions in which virion particles were produced, the particles were noninfectious. DIBAs caused abnormal processing of Gag precursors, and the inhibitory effect on processing was not due to inhibition of the HIV-1 protease enzyme or Pr55^{gag} myristoylation. Rather, the defect in processing was due to the formation of intermolecular cross-linkages among the zinc fingers of adjacent Gag molecules, rendering the precursors no longer recognizable by HIV-1 protease. Likewise, DIBAs caused intermolecular cross-linkage among recombinant Pr55^{gag} packaged into pseudovirions, thereby generating modified precursors that were resistant to the action of protease. Thus, DIBAs chemically modified the mutationally intolerant retroviral zinc fingers in infected cells, interrupting protease-mediated maturation of virions and leading ultimately to the production of compromised virions.

The human immunodeficiency virus type 1 (HIV-1) p7 nucleocapsid protein (p7NC), which contains two highly conserved zinc fingers with a nonclassical Cys-Xaa₂-Cys-Xaa₄-His-Xaa₄-Cys (CCHC) sequence, is a maturational proteolytic product of the Pr55^{gag} precursor polyprotein (3, 4, 27, 34, 51). The CCHC motif, also known as the retroviral zinc finger, is conserved in all onco- and lentiviruses but is not found in the nucleocapsid proteins of spumaretroviruses (9, 25, 36). These zinc fingers play pivotal roles in both the early and late stages of the retrovirus replication cycle (1, 15, 23). The fingers, while still a component of the HIV-1 Pr55^{gag} (Gag) precursor, function in selection and incorporation of viral RNA into budding virions (5, 13, 21, 22, 39). The fingers of the fully processed p7NC are also required for the initial infection of target cells, promoting initiation and elongation of the nascent transcript and increasing the efficiency of template switching during reverse transcription (10, 49, 52, 56). In addition, p7NC participates in HIV-1 genomic RNA dimerization, protection of the viral RNA from nucleases, and integrase cleavage ac-

tivity (33, 38, 52). Thus, the essential roles of the retroviral zinc fingers in the HIV-1 replication cycle make the fingers choice antiviral targets.

Recently, Rice et al. (46, 48) discovered that certain electrophilic reagents, such as 3-nitrosobenzamide (NOBA), can stoichiometrically and covalently modify the cysteine residues of retroviral zinc fingers, resulting in the ejection of zinc from the fingers and rendering the virus noninfectious. Through chemical modification of the cysteine thiolates of the p7NC zinc fingers, low (micromolar) concentrations of NOBA blocked the ability of the virus to synthesize proviral DNA, even though NOBA did not inhibit reverse transcriptase enzymatic activity or virion binding to target cells (45). More recently, we described a novel class of disulfide-substituted benzamides (DIBAs) that exhibited toxicity profiles that were substantially improved over that of NOBA (44, 47). In those reports we demonstrated a direct correlation between the cellular anti-HIV-1 activity of DIBA compounds and the ability of the compounds to chemically modify and eject zinc from the zinc fingers of HIV-1 p7NC in vitro, utilizing both tryptophan fluorescence and high-pressure liquid chromatography separation techniques. Additionally, DIBA compounds inhibited a wide range of retroviruses and were synergistic with other antiviral agents, and drug-resistant mutant viruses did not arise.

We have postulated that DIBA-induced modifications of

* Corresponding author. Mailing address: Laboratory of Antiviral Drug Mechanisms, Developmental Therapeutics Program, National Cancer Institute-Frederick Cancer Research and Development Center, SAIC Frederick, Building 431T-B, P.O. Box B, Frederick, MD 21702-1201. Phone: (301) 846-5060. Fax: (301) 846-6846. Electronic mail address: rice@dtax2.ncifcrf.gov.

FIG. 1. Structures of DIBA-1 and DIBA-3.

retroviral zinc fingers during the HIV-1 replication cycle would disrupt critical processes during virus maturation, resulting in the formation of aberrant virus particles and interruption of the infection cycle. However, the actual intracellular biochemical processes disrupted by the DIBAs, leading to these antiviral consequences, were unknown. To address these issues, we studied the effects of DIBA treatment on cells actively producing virus, on the virus particles released from those infected cells, and on Gag-laden pseudovirions. Here we report that the action of two selected DIBA compounds (DIBA-1 and DIBA-3) (Fig. 1) on the CCHC zinc fingers of Gag precursors resulted in the formation of noninfectious virus or in the complete inhibition of virus production. Modification of the zinc fingers of Gag precursors altered the precursors in such a manner that they no longer served as a substrate for HIV-1 protease, yet the DIBAs did not inhibit HIV-1 protease activity or prevent precursor myristoylation. These findings demonstrate that zinc finger inhibitors affect virion production and maturation in a fashion that is reminiscent of HIV-1 protease inhibitors.

MATERIALS AND METHODS

Materials. DIBA-1 (NSC 654077) and DIBA-3 (NSC 659881), as well as all other antiviral compounds, were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis and Centers, National Cancer Institute. DIBAs were synthesized by Parke-Davis Pharmaceuticals (Ann Arbor, Mich.). U1, CEM-SS, COS, 174xCEM, pSVrev, and HeLa-CD4-LTR- β gal cells were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health (Bethesda, Md.). All cell lines were maintained in RPMI 1640–10% fetal calf serum–20 μ g of gentamicin per ml–200

mM L-glutamate, except for the HeLa-CD4-LTR- β gal cells, which were maintained in Dulbecco's modified Eagle's medium/F12–10% fetal calf serum–20 μ g of gentamicin per ml–200 mM L-glutamate, and COS cells in transfection experiments, which were maintained without antibiotics or added L-glutamate.

TNF- α induction of U1 cells for HIV-1 expression. Fifty thousand U1 cells per milliliter were induced with 5 ng of recombinant tumor necrosis factor alpha (TNF- α) (Sigma Chemical Co., St. Louis, Mo.) per ml for 24 h. At 24 h, 1 ml of medium supplemented with 5 ng of TNF- α per ml and with the appropriate ($2\times$ final) concentration of either DIBA-1 or DIBA-3 was added to the cells. Cell-free supernatants for p24 measurements were collected 48 h later, and the virus particles contained within it were lysed in 1% Triton X-100–0.5% bovine serum albumin (BSA). Samples for cell-associated p24 were obtained by lysis of the cells in 10 μ l of 10% Triton X-100–5.0% BSA. After lysis, 90 μ l of phosphate-buffered saline was added to make a final concentration of 1% Triton X-100–0.5% BSA. All samples underwent a single round of freezing and thawing prior to assay. Viability was determined by trypan blue dye exclusion and XTT dye reduction (7, 54). The p24 assays were performed with p24 enzyme-linked immunosorbent assay (ELISA) kits obtained through the common services of the AIDS Vaccine Program (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Md.).

Virus titration assay. Supernatants derived from TNF- α -induced U1 cells were serially diluted onto 174xCEM cells (5×10^3). Cultures were incubated for 6 days and scored for cytopathic effects, XTT reduction (cell viability), and p24 production upon termination. Viral titrations were initiated with a starting dilution that decreased the initial drug concentration to less than 1 μ M. The virus pellet (centrifugation for 1 h at $18,000 \times g$ and 4°C) obtained from 1.5 ml of culture supernatant was resuspended to 150 μ l in culture medium and then diluted 1:10 as the initial seeding sample. Infectious units were defined by using end-point dilutions and defining positively infected cultures as having absorbances of two times the background (cells, no virus) p24 value. Data were then calculated to determine the infectious units per milliliter of cell-free supernatant.

Western blotting (immunoblotting) of HIV-1 proteins. U1 cells (seeded at 5×10^5 /ml with 4 ml [total volume] per well in six-well plates) were treated as described above for the induction of HIV-1. Twenty-four hours later, 4 ml of medium (total volume, 8 ml) was added with the appropriate concentration of either DIBA-1 or DIBA-3. At 48 h after DIBA addition, the cells were collected by centrifugation and counted, and viability was determined by trypan blue dye exclusion and XTT dye reduction. One million cells were lysed in 20 μ l of sample buffer containing 0.5 M Tris-HCl (pH 6.8), 50% glycerol, 8% sodium dodecyl sulfate (SDS), and 0.4% bromophenol blue. The whole-cell lysates were boiled for 5 min and electrophoresed as described below. Alternatively, cells were lysed with 0.5% Triton X-100–300 mM NaCl–50 mM Tris-HCl (pH 7.6)–10 μ g of leupeptin (Sigma Chemical Co.) per ml–10 μ g of aprotinin (Boehringer Mannheim, Indianapolis, Ind.) per ml–1.8 mg of iodoacetamide (Sigma Chemical Co.) per ml–4 mM Pefabloc (Boehringer Mannheim) per ml, and nuclei and cellular debris were cleared by centrifugation. Protein was determined by the Bradford method (5a). Virus pellets were isolated by centrifugation of supernatants for 1 h at $18,000 \times g$ and 4°C after clarification of supernatants for 10 min at $250 \times g$ and 4°C . The supernatants were removed, the virus pellet was lysed by using the protease inhibitor-containing lysis buffer described above, and protein was determined by the Bradford method (5a). Fifty micrograms of total protein was added per lane for virion protein analyses. For both total-cell and virion preparations, proteins were resolved on 4 to 20% polyacrylamide gels with SDS in Tris-glycine (Novex, San Diego, Calif.). Samples for reduced gels contained 5% β -mercaptoethanol (β -ME) and were boiled for 5 min prior to being loaded. Resolved HIV-1 proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes, and HIV-specific proteins were detected by using a mixture of goat anti-HIV-1 p7NC and anti-p24 antisera (kind gifts of L. E. Henderson, AIDS Vaccine Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Md.). It is important to note that the p7NC and p24 antisera used here are directed toward structural motifs within these proteins and as such detect their polyprotein precursors (Pr55^{gag} and Pr160^{gag-pol}) and other aberrantly processed proteins derived from the Gag precursor. Western blots were developed with standard methodology by chemiluminescence (Dupont-NEN, Wilmington, Del.) with a rabbit anti-goat horseradish peroxidase-conjugated antibody (Bio-Rad, Hercules, Calif.). Reduced molecular weight markers, recombinant p7NC, and recombinant Pr55^{gag} were included on both reduced and nonreduced SDS-polyacrylamide gels.

Cross-linkage of purified Pr55^{gag}. Pr55^{gag} was obtained from the Laboratory of Cell and Molecular Structure, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Md. Pr55^{gag} was prepared in Sf-9 insect cells by using a recombinant baculovirus containing the entire Gag-coding sequence from HIV-1_{HXB2} under the transcriptional control of the polyhedron gene promoter. Pseudovirions were purified by precipitation with polyethylene glycol, banded at 1.16 g/ml on sucrose gradients, and concentrated by ultracentrifugation. When appropriate, Pr55^{gag} monomers were produced from pseudovirions by treatment with 50 mM MES (morpholineethanesulfonic acid) (pH 6.5)–1 mM EDTA–0.05 mM NaCl–1 mM dithiothreitol–0.1% Nonidet P-40 for 1 h at 4°C , vortexed, and stored as aliquots at -80°C . Cross-linkage experiments were performed with either intact Pr55^{gag}-laden pseudovirions or monomers by incubation of 10- μ g equivalents of Pr55^{gag} for 1 h at 37°C with various concentrations of DIBAs. Samples were then resolved by nonreducing or reduc-

ing (5% β -ME) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 20% polyacrylamide). Reaction products were then visualized by Coomassie blue staining or Western blotting, as described above.

Processing of Pr55^{gag} by HIV-1 protease. Recombinant HIV-1 protease (0.25 ng) was treated with various concentrations of DIBAs for 1 h at 37°C in 200 mM MES buffer (pH 6.5)–4 mM EDTA–2 mM NaCl–4 mM dithiothreitol–0.01% Nonidet P-40. After 1 h, 1 μ g of Pr55^{gag} monomers was added, and the incubation was continued for 1 h longer at 37°C. Proteins were resolved by reducing SDS-PAGE (4 to 20% polyacrylamide), and protein expression was detected as described for Western blotting. Alternatively, intact pseudovirions were pre-treated with DIBAs for 1 h at 37°C and then treated with HIV-1 protease for 1 h at 37°C, and then products were analyzed by SDS-PAGE and immunoblotting.

Construction and description of plasmids. cDNAs of the HIV-1_{HXB2} Responsive element (RRE) (nucleotides 7620 to 8474) sequence and the complete Gag gene (nucleotides 788 to 2291) were prepared by PCR with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) (26, 28, 35, 40). A pSVrrc plasmid containing the RRE was prepared by inserting the RRE cDNA described above into the pSVL vector (Pharmacia Biotech, Piscataway, N.J.) at the *Xho*I site in the proper orientation. The pSVrrc-gag plasmid was then prepared by inserting the Gag cDNA into the *Bam*HI site of pSVrrc just downstream of the RRE.

Expression, radioimmunoprecipitation, and Western blotting of expressed Pr55^{gag}. COS cells were grown to 80% confluence and cotransfected with 10 μ g of pSVrrc-gag and 10 μ g of pSVrrc by Lipofectin (GIBCO-BRL, Gaithersburg, Md.). For radioimmunoprecipitations, the cells were grown for 24 h, split into six-well plates (2 $\times$ 10⁵ cells per well), and incubated overnight. The cells were then washed with Hanks balanced salt solution and incubated with fresh medium containing various concentrations of DIBA plus 100 μ Ci of Trans³⁵S label (ICN, Costa Mesa, Calif.) per ml for 4 h or plus 100 μ Ci of [9,10-³H]tetradecanoic acid (10 to 60 Ci/mmol) (Dupont-NEN) per ml for 6 h. Drug-treated or untreated cells were homogenized in lysis buffer [10 mM Tris-HCl [pH 7.4] containing 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1.5 mM MgCl₂, 1 mM EDTA, 2 mM sodium vanadate, 10 mM NaF, and protease inhibitors [2 μ g of aprotinin per ml, 1 μ g of pepstatin A per ml, 28 μ g of phenylmethanesulfonyl fluoride per ml, and 70 μ g of L-1-chloro-3-(tosylamido)-4-phenyl-2-butanone per ml]] by five passes through a 21-gauge needle and centrifuged in a microcentrifuge for 15 min. Radioimmunoprecipitations were carried out with cell lysate supernatant fractions by using a monoclonal antibody against HIV-1 p24 (NEA-9283; Dupont-NEN) as previously described (8), and the products were subjected to 10 to 20% gradient SDS-PAGE (Integrated Separation Systems, Inc., Hyde Park, Mass.). Gels were either treated with En³Hance (Dupont-NEN) for detection of radiolabeled proteins by fluorography or subjected to Western blotting with a monoclonal antibody against HIV-1 p17 (NEA-9282; Dupont-NEN) and visualized by chemiluminescence. For analysis of pseudovirion particles released into the cell culture medium, the transfected cells were grown for 24 h, split into 12-well plates (10⁵ cells per well), and incubated overnight. The cells were then washed, and new medium containing various concentrations of DIBAs and the cells was incubated for another 12 h. Finally, the cell culture medium was collected, cell debris was removed by centrifugation at 400 $\times$ g for 10 min, and the supernatant fraction was adjusted to 1 M NaCl and 7.5% polyethylene glycol 6000 and incubated at 4°C for 1 h. Pseudovirion particles were collected by centrifugation at 12,000 $\times$ g for 30 min, and the pellet was prepared for immunoblotting with the p17 monoclonal antibody by being dissolved in lysis buffer.

Bovine brain N-myristoyltransferase assay. The effect of 40 μ M DIBA on brain N-myristoyltransferase (EC 2.3.1.97) (500-fold purified) was tested as previously described (19, 20).

RESULTS

DIBAs prevent the release of infectious HIV-1 virions from infected cells. We previously demonstrated that the reaction of the p7NC zinc fingers with DIBA-type compounds results in covalent modification of the metal-chelating cysteine residues of the CCHC motif and the concomitant ejection of zinc (44, 47). We hypothesized that because of the central role of the zinc fingers in virus maturation and infectivity, the disruption of zinc finger organization by DIBA compounds in virus-producing cells would result in the inhibition of virus release. To address this possibility, we utilized U1 cells that carry two TNF- α -inducible copies of the integrated HIV-1 provirus as a model for late events in the HIV-1 replication cycle (17). As shown in Fig. 2A, both DIBAs inhibited HIV-1 expression in TNF- α -stimulated U1 cells in a concentration-dependent manner. In three independent experiments, the concentrations of DIBA-1 and DIBA-3 required for the reduction of virion-associated p24 expression by 50% were 7.5 $\pm$ 1.7 μ M (mean $\pm$ standard deviation) and 17 $\pm$ 1.4 μ M, respectively. No signif-

icant toxicity was observed, by XTT dye reduction, at up to 100 μ M concentrations of either compound; verification of viability by trypan blue dye exclusion also showed no significant decrease in viability at 100 μ M (data not shown).

Since zinc finger inhibitors react stoichiometrically with the fingers (46, 57), we wanted to evaluate the consequences of altering the U1 cell number, which would effectively alter the effector (DIBA)-to-target (zinc finger) ratio. In these studies, either 0.5 $\times$ 10⁵, 1 $\times$ 10⁵, or 4 $\times$ 10⁵ U1 cells were treated with DIBA-1, and the levels of virion-associated p24 and cell-associated p24 and the infectious titer of the released virions were determined (Fig. 2B). The reduction in virion-associated p24 caused by the DIBAs was reversible as the number of cells initially seeded was increased. Nevertheless, the infectivity of the released virions continued to be compromised at the higher cell numbers. Thus, DIBAs exerted an antiviral effect even when there was no apparent decrease in the release of virion-associated p24. Similar results were obtained with DIBA-3 (data not shown).

DIBAs cause Gag cross-linkage and accumulation of unprocessed Pr55^{gag} in infected cells and in the virions released from those cells. Decreases in viral infectivity in the absence of changes in the extracellular levels of virion-associated p24 led us to examine the effects of DIBAs on Gag polypeptide expression within cells and released virions. TNF- α -induced U1 cells were treated with DIBA-1, and cell-associated viral proteins in whole-cell lysates (10⁶ cell equivalents) were analyzed by SDS-PAGE and Western blotting with antiserum to p7NC. Although no major differences were observed when samples were analyzed under reducing conditions (Fig. 3A), a number of DIBA-induced alterations in the protein profiles were observed under nonreducing conditions (Fig. 3B). First, there was an accumulation of unprocessed Pr55^{gag} and a concomitant loss of processed p7NC. In addition, DIBAs caused the formation of high-molecular-weight cross-linked Gag species. The cross-linkage was due to disulfide bond formation, as indicated by the absence of cross-linkage when the lysates were analyzed under reducing conditions (Fig. 3A). The implication that the disulfide cross-linkage between Gag molecules occurs only between the zinc finger cysteine residues, and not among other residues in the polypeptide, is made by our findings that treatment of purified HIV-1 virions with DIBAs results in intermolecular disulfide cross-linkage between the zinc finger cysteine residues of fully processed p7NC proteins and that the only cysteine residues of p7NC are in the finger domains (44, 47, 53). Utilizing an alternate technique for cell lysis, in which the nuclei and cellular debris were removed by centrifugation, we observed that the cross-linked material and a portion of the Gag precursors were removed by the centrifugation step, suggesting that the material may have been associated with the Triton X-100-insoluble cellular architecture removed during centrifugation (53). Together, these findings indicated that DIBAs result in intracellular cross-linkage of Gag precursors and interrupt normal processing of the precursor.

Inspection of proteins in the virions released from DIBA-treated U1 cells also revealed alterations in the Gag precursor polypeptides (Fig. 4). DIBA treatment resulted in the formation of high-molecular-weight Gag-containing species and an almost complete loss of identifiable p7NC and p24 from the nonreduced virion preparations (Fig. 4A). Reduction of the virion lysates with β -ME resulted in the recovery of multiple bands between 40 and 60 kDa (Fig. 4B). Reduction also resulted in only a partial recovery of p7NC, suggesting that the large amount of protein between 40 and 60 kDa in reduced samples represented unprocessed and partially processed Pr55^{gag}. DIBA treatment of U1 cells not only resulted in cross-

FIG. 2. DIBAs inhibit the release of HIV-1 from and inactivate HIV-1 released from latently infected TNF- α -induced U1 cells. (A) Fifty thousand U1 cells in 1.0 ml were induced with 5 ng of recombinant TNF- α per ml. Twenty-four hours later, DIBA-1 or DIBA-3 was added at various concentrations in 1 ml of TNF- α -containing medium. Cultures were incubated for 48 h, and cell-free supernatants were assayed for p24 by the ELISA method. Greater than 95% of the supernatant p24 was found to be virion associated as determined by centrifugation (1 h at $18,000 \times g$ and 4°C). Cellular viability was determined by XTT dye reduction. Data are expressed as the percent viability or percent p24 expression with respect to that of the untreated TNF- α -induced control. $\circ$, XTT; $\blacksquare$, virion-associated p24. (B) Either 0.5×10^5 , 1×10^5 , or 4×10^5 U1 cells were placed in wells with 1 ml of tissue culture medium supplemented with 5 ng of recombinant TNF- α per ml for 24 h. After 24 h, DIBA-1 was added in 1 ml of TNF- α -containing medium, and incubation was continued for another 48 h. At 48 h supernatants and cells were harvested for virion-associated p24 and cell-associated p24, respectively. Viability was determined by XTT dye reduction. Infectious units were determined by serial dilution of fresh supernatants on 10^4 174xCEM cells. Infectious units were calculated as the lowest dilution that gave p24 absorbance values of twofold the background absorbance of cells alone 7 days after initiation of culture. Similar results were obtained with DIBA-3. $\times$, XTT; $\bullet$, virion-associated p24; $\blacksquare$, cell-associated p24; $\blacktriangle$, infectious units per milliliter.

linkage of intracellular and virion-associated Pr55^{gag} but also altered processing of the precursor. Likewise, overexposure of Western blots to visualize the Pr160^{gag-pol} revealed the presence of equivalent or enhanced levels of the precursor in viri-

FIG. 3. DIBAs induce cross-linkage of p7NC and accumulation of Pr55^{gag} in U1 cells. Fifty thousand U1 cells per milliliter in six-well plates with 4 ml per well were stimulated with 5 ng of recombinant TNF- α per ml for 24 h. At 24 h various doses of DIBA-1 were added in 4 ml of TNF- α -containing culture medium (total culture volume, 8 ml), and the incubation was continued for 48 h. Cells were recovered by centrifugation and counted, and viability was determined by both XTT reduction and trypan blue dye exclusion. Whole-cell lysates (10^6 cells) were prepared as described in Materials and Methods. Viability of treated cells was found to approximate that of untreated U1 cells. Total cell proteins were resolved under either reducing (A) or nonreducing (5% β -ME) (B) conditions by SDS-PAGE (4 to 20% polyacrylamide) and electroblotted onto PVDF membranes. Reduced molecular weight standards and 10 ng each of recombinant p7NC and Pr55^{gag} were included as controls. Virus-specific proteins were detected by chemiluminescence with p7NC antiserum. Equivalent results were observed with DIBA-3 (data not shown).

FIG. 4. Cross-linkage and accumulation of Pr55^{gag} in virions released from DIBA-treated U1 cells. Fifty thousand U1 cells per milliliter in six-well plates with 4 ml per well were stimulated with 5 ng of recombinant TNF- α per ml for 24 h. At 24 h various doses of DIBA-1 and DIBA-3 were added in 4 ml of TNF- α -containing culture medium (total culture volume, 8 ml), and the incubation was continued for 48 h. At 48 h supernatants were clarified by centrifugation at $250 \times g$ for 10 min, and then virions were isolated by centrifugation for 1 h at $18,000 \times g$ and 4°C . ELISA analysis of p24 in postcentrifugation supernatants showed that greater than 95% of the total p24 was recoverable in the virion pellet. Viral pellets were lysed as described in Materials and Methods, and protein was quantitated by the Bradford method (5a). Fifty-microgram aliquots of total protein were then resolved under either nonreducing (A) or reducing (5% β -ME) (B) conditions by SDS-PAGE (4 to 20% polyacrylamide) and electroblotted onto PVDF membranes. Reduced molecular weight standards (M) and 10 ng each of recombinant p7NC and Pr55^{gag} were included as controls. Virus-specific proteins were detected by chemiluminescence with a mixture of p7 and p24 antisera and a horseradish peroxidase-conjugated detecting antibody.

ons from DIBA-treated U1 cells (data not shown). Thus, the high efficiency of DIBAs in reducing the infectivity of HIV-1 is at least in part due to the production of virions that have internal cross-linkage and altered processing of Gag precursors.

Prevention of Pr55^{gag} processing by DIBAs is not due to inhibition of HIV-1 protease activity or inhibition of myristoylation of the precursor. Since accumulation of unprocessed

FIG. 5. DIBAs do not inhibit HIV-1 protease. Recombinant HIV-1 protease (0.25 ng/ml) was treated with various concentrations of the zinc finger-reactive compounds DIBA-1 and DIBA-3 (A) or the protease inhibitor KNI-272 (B) for 1 h as described in Materials and Methods. After 1 h, 1.0 μ g of Pr55^{gag} monomers was added, and the incubation was continued for another hour at 37°C . Proteins were resolved by reducing SDS-PAGE (4 to 20% polyacrylamide), and protein expression was detected by Western blotting as described in Materials and Methods.

Pr55^{gag} in cells and released virions suggested that one possible mechanism of DIBA action was inhibition of HIV-1 protease activity, we determined the ability of DIBAs to inhibit the *in vitro* processing of Pr55^{gag} by purified HIV-1 protease. Preincubation of DIBA-1 with HIV-1 protease resulted in a slight inhibition of Pr55^{gag} processing at the high test concentration of 100 μ M, while DIBA-3 did not alter processing (Fig. 5A). In contrast, KNI-272, a protease inhibitor, reduced processing of Pr55^{gag} at concentrations as low as 0.001 μ M (Fig. 5B). Thus, the observed effects on Gag processing by DIBA-1 and DIBA-3 were not due to a direct inhibitory action on HIV-1 protease.

Accumulation of Pr55^{gag} in DIBA-treated cells could also have resulted from a decrease in the association of Gag precursor molecules with the cytoplasmic face of the cellular membrane. Since myristoylation of the Pr55^{gag} polyprotein

FIG. 6. DIBAs inhibit release of pseudovirion particles without inhibiting synthesis or *N*-myristoylation of expressed Pr55^{gag}. COS cells were transfected with an expression vector containing the Gag gene open reading frame of HIV-1_{HXB2} and treated with either DIBA-1 or its inactive congener DIBA-5 (47). At 48 h posttransfection the pseudovirion particles released into the culture supernatant medium were collected by polyethylene glycol precipitation and centrifugation and analyzed by SDS-PAGE and Western blotting with an anti-p17 monoclonal antibody (A). The cells were also washed and either incubated for 6 h with 100 μ Ci of [9,10-³H]myristic acid per ml (B) or incubated for 4 h with 100 μ Ci of Trans-³⁵S label ([³⁵S]cysteine-methionine) per ml (C). The radiolabeled Pr55^{gag} in cell lysates was immunoprecipitated with an anti-p24 monoclonal antibody and analyzed by SDS-PAGE and fluorography.

plays an essential role in directing this protein to the inner surface of the cell membrane (6, 24), we examined the effects of DIBAs on Gag myristoylation. COS cells were transfected with an expression vector containing the HIV-1 Gag open reading frame. Gag proteins were metabolically labeled with [³H]myristic acid or [³⁵S]cysteine-methionine, isolated by radioimmunoprecipitation, separated by SDS-PAGE, and visualized by fluorography. DIBA-1 inhibited the release of Pr55^{gag}-laden pseudovirions (Fig. 6A) but did not alter the level of myristoylation or synthesis of the precursor polypeptide (Fig. 6B and C, respectively). An inactive congener (DIBA-5) (47) served as a negative control in these studies. We also observed that as much as 40 μ M DIBA-1 or DIBA-5 had no inhibitory effect on bovine brain *N*-myristoyltransferase in vitro assays (data not shown). These data indicated that the accumulation of Gag precursors caused by zinc finger inhibitors was not due to hypomyristoylation of those precursors.

Prevention of Pr55^{gag} processing by DIBAs results from modification of the precursor. The lack of any direct effect on HIV-1 protease activity or Gag myristoylation suggested that disruption of the zinc fingers and cross-linkage of the Gag polyprotein precursors may prevent efficient processing of the precursor by the HIV-1 protease enzyme (11, 55). To model the conditions of closely packed Gag molecules within infected

cells where cross-linkage occurs, we used Pr55^{gag}-laden pseudovirions. These pseudovirions are composed of tightly packed Pr55^{gag} molecules that form structures that are morphologically similar to viral particles except that they do not contain viral RNA or enzymes (18). Ten-microgram equivalents of Pr55^{gag} representing either intact pseudovirions or solubilized Pr55^{gag} monomers from disrupted pseudovirions were incubated for 1 h at 37°C with DIBAs and resolved by nonreducing or reducing SDS-PAGE (Fig. 7). Pr55^{gag} from intact pseudovirions was effectively cross-linked in intact pseudovirions by DIBA-1 in a dose-dependent manner as evidenced by the disappearance of the Pr55^{gag} and the appearance of high-molecular-weight species that failed to enter the gel (Fig. 7A). Cross-linkage was reversible by β -ME treatment, demonstrating the disulfide nature of the cross-linkages (Fig. 7B). In contrast, solubilized Pr55^{gag} monomers were not cross-linked by DIBA-1 (Fig. 7C). Similar data were obtained with DIBA-3, while 3'-azido-3'-deoxythymidine did not promote cross-linkage (data not shown) (53). These data clearly illustrate that exposure of highly approximated Gag precursors to DIBAs results in extensive intermolecular cross-linkage.

Because the cross-linkage observed with the pseudovirions was analogous to that seen in proteins derived from infected cells and released virions, we utilized this system to next determine the effect of cross-linking on the ability of HIV-1 protease to process Pr55^{gag}. Pr55^{gag} monomers were treated with DIBA-1 or DIBA-3 to determine if disruption of the zinc finger array without cross-linkage of adjacent molecules was sufficient to disrupt HIV-1 protease processing. As shown in Fig. 8A, the solubilized Pr55^{gag} that had been treated with DIBAs was processed normally by HIV-1 protease. However, the findings were quite different when intact Pr55^{gag}-laden pseudovirions were utilized in these studies. In pseudovirions that were DIBA-1 treated and cross-linked prior to addition of HIV-1 protease, processing of Pr55^{gag} failed to occur (Fig. 8B, lanes 5 and 6). In contrast, pseudovirions that had not been treated with DIBA-1 were processed normally by HIV-1 protease, yielding mature p7NC in a pattern similar to that for HIV-1 protease processing of Pr55^{gag} monomers (Fig. 8B, compare lanes 4 and 10). Even though the protease cleavage reaction buffer contains detergent that should allow for adequate penetration of the protease into DIBA-treated pseudovirions, we sought to ensure efficient disruption of the DIBA-treated pseudovirions by two additional cycles of freeze-thawing prior to addition of protease. As shown in lanes 7 and 8 of Fig. 8B, further pseudovirion disruption prior to addition of protease did not promote cleavage of Pr55^{gag}. These data clearly illustrate that the DIBA-induced cross-linkage of closely approximated Gag molecules by disulfide bridging between the zinc finger cysteine thiolates in virus-producing cells and in Pr55^{gag}-laden pseudovirions leads to significant alterations in Gag polyprotein processing and decreased infectivity of progeny virions.

DISCUSSION

In this study we examined the biochemical mechanisms by which DIBA inhibitors of retroviral CCHC zinc fingers disrupt the late-stage production of infectious HIV-1. In U1 cells, DIBAs chemically modified the Gag-associated zinc fingers during the late phases of viral replication, causing intermolecular disulfide cross-linkage between the zinc fingers of adjacent Gag precursor molecules. The Gag cross-linkage resulted in the formation of noninfectious virus particles because of failure of the precursors to undergo normal proteolytic processing by the HIV-1 protease enzyme or resulted in the complete

FIG. 7. Cross-linkage of Pr55^{gag}-laden pseudovirions but not soluble Pr55^{gag} monomers by DIBAs. Recombinant Pr55^{gag} (10 μg) either in pseudovirion particles or as solubilized monomers was treated with DIBA-1 at various concentrations for 1 h at 37°C. Reaction mixtures were resolved by SDS-PAGE (4 to 20% polyacrylamide), and proteins were identified by Coomassie blue staining. Similar results were obtained with DIBA-3. (A) Reaction products of DIBA-1 interaction with Pr55^{gag} pseudovirions separated under nonreducing conditions;

inhibition of virion particle release at higher DIBA concentrations. Consistent with these findings, DIBAs promoted cross-linkage among the zinc fingers of Gag precursors in Gag-laden pseudovirions, and HIV-1 protease was no longer able to process the cross-linked Gag in a normal fashion. Conversely, DIBAs did not promote intermolecular cross-linkage of Pr55^{gag} polyprotein monomers in solution, and these precursors were efficiently processed by HIV-1 protease. Together, our findings indicate that the chemical attack of DIBAs on closely approximated retroviral zinc finger proteins yields disulfide cross-linkage among the zinc finger cysteine residues of adjacent molecules, thereby preventing HIV-1 protease from processing the Gag precursors in newly forming virus particles. The major consequence of this defective processing is the greatly reduced infectivity of new progeny virions and disruption of the HIV-1 replication cycle.

Our previous findings with the zinc finger inhibitor NOBA (45, 46) demonstrated that the zinc-coordinating cysteine thiolates of the retroviral zinc finger motifs are amenable to covalent modification and the resultant ejection of zinc. Those studies with NOBA suggested that such a chemical modification on CCHC zinc finger motifs proceeds in a stepwise process, wherein initially reagents form adducts with the cysteine thiolates and then intra- and interarray disulfide bond formation occurs among the cysteine residues of the two fingers of p7NC (57) (also see Fig. 3 in reference 44). However, if other retroviral zinc finger motifs exist in close spatial proximity, then intermolecular cross-linking can occur (44, 47). When zinc finger-containing proteins were housed in highly ordered structures, such as virions or Pr55^{gag}-laden pseudovirions, DIBAs cross-linked the proteins through disulfide bonds between cysteine residues of zinc fingers in adjacent molecules. Thus, for gross alterations in the Gag precursors to occur, the DIBAs must encounter closely approximated zinc fingers and modify the cysteine thiolates, producing intermolecular cross-linkages. The presence of this cross-linkage is then directly associated with a precipitous loss of infectivity of the newly forming virus particles. We also observed that DIBAs entered intact HIV-1 virions and cross-linked the mature p7NC, whereas monomeric p7NC in solution was not cross-linked by DIBAs (47, 53).

More subtle changes in the zinc finger motif, which would be undetectable by the methods employed here, may also contribute to the loss of virus survivability. DIBA-induced actions on the zinc fingers that result in only localized alterations in Gag (i.e., only in the region of the fingers) or only minimal cross-linkage might also diminish the production of infectious virions. Indeed, the conformation of the retroviral zinc finger has been reported to be crucial for packaging of RNA into virions, as well as for the stability of the virions and virus infectivity (12, 14). In addition, the mature p7NC plays a critical role in the stabilization of the HIV-1 genomic RNA dimer and in its protection from endonuclease attack (33), and site-directed mutagenesis of the four basic amino acids in the 7-amino-acid linker region between the two zinc fingers of the p7NC portion of Gag reversed the association of Gag polyproteins with membrane fractions from transfected cells (16, 42). Thus, maintenance of the structural integrity of the zinc fingers is essential for successful completion of the HIV-1 replication cycle, and antiviral agents such as those described here can have dramatic

(B) reaction products of DIBA-1 interaction with Pr55^{gag} pseudovirions separated under reducing conditions; (C) reaction products of DIBA-1 interaction with Pr55^{gag} monomers electrophoresed under nonreducing conditions.

FIG. 8. DIBA-mediated cross-linkage of pseudovirions prevents subsequent processing of Pr55^{gag} by HIV-1 protease but does not alter processing of solubilized Pr55^{gag}. (A) Pr55^{gag}-laden pseudovirions solubilized with Nonidet P-40, as described in Materials and Methods, to create Pr55^{gag} monomers were incubated with either DIBA-1 or DIBA-3 for 1 h at 37°C. HIV-1 protease was then added, and the incubation was continued for another hour at 37°C. The resulting cleavage products were resolved by reducing SDS-PAGE (4 to 20% polyacrylamide) and electroblotted onto PVDF membranes. Protease cleavage products were detected by Western blotting with a p24 antiserum. Equivalent results were obtained with p7NC antiserum. (B) Pr55^{gag} in intact pseudovirions was incubated with DIBA-1 for 1 h at 37°C. All Pr55^{gag} pseudovirion or monomer preparations were resolved under reducing conditions by SDS-PAGE (4 to 20% polyacrylamide), and proteins were detected by Western blotting with a mixture of p7NC and p24 antisera. Treated pseudovirions were resolved either without prior protease treatment (lanes 1 to 3), after treatment with recombinant HIV-1 protease for 1 h at 37°C (lanes 4 to 6), or after disruption by two cycles of freezing (–80°C) and thawing (37°C) followed by treatment with protease (lanes 7 and 8). Finally, Pr55^{gag} monomers were resolved by electrophoresis with (lane 10) or without (lane 9) HIV-1 protease treatment. Similar results were obtained with DIBA-3.

effects on virus survival without inducing gross structural alterations in the retroviral zinc fingers or the Gag polypeptide precursors.

Significant levels of processed p7NC were identified within the TNF- α -induced U1 cells. Similar results have been found for acutely infected cells, even though p7NC is more often considered to be associated with mature HIV-1 virions as the result of HIV-1 protease action during virion maturation (29). DIBAs caused accumulation of the Pr55^{gag} precursor polypeptide and loss of processed p7NC within treated U1 cells and in

the virions released from those cells. Accumulation of the precursors suggested a mechanism involving inhibition of HIV-1 protease, yet in tests to determine if DIBAs could inhibit recombinant HIV-1 protease, we observed no significant direct inhibition of protease activity (Fig. 5A). These findings indicated that the interaction of DIBA-type drugs with HIV-1-infected cells altered processing of Gag precursors without directly inhibiting protease activity. Likewise, association of Pr55^{gag} with the cell membrane and incorporation into virions requires myristoylation of the precursor; however, neither myristoylation of Pr55^{gag} nor the activity of the myristoyl-transferase was altered by DIBA treatment (Fig. 6 and data not shown). Consistent with these findings, another DIBA congener (DIBA-2) was found to prevent normal protease processing of the Pr65 Gag precursor in cells chronically infected with murine leukemia virus (43).

The body of evidence presented here indicates that the abnormal Gag processing was caused by alterations in tertiary structures of the precursors due to adduct formation and/or intermolecular cross-linking (Fig. 3 and 4). This contention is further strengthened by the inability of HIV-1 protease to process recombinant Pr55^{gag} that had undergone cross-linkage after treatment of pseudovirions with DIBAs, even though soluble Pr55^{gag} monomers are an efficient substrate for the HIV-1 protease (Fig. 8). Modifications in Gag precursors might also block or significantly reduce the expression of other membrane localization or packaging signals within the precursors, as well as prevent the formation of mature protease. Other investigators have shown that dimerization of Pr160^{gag-pol} during condensation of the budding virion is required for the release of the protease dimer through autocatalysis, with the retroviral zinc fingers playing a crucial role in this dimerization (32, 41, 58). Attack on the zinc fingers by the DIBAs may prevent proper dimerization of the precursors, thereby reducing the amount of or preventing the formation of active HIV-1 protease. Ironically, within the cell the close approximations required for condensation of Pr55^{gag} and Pr160^{gag-pol} to form the newly budding virus may allow retroviral zinc finger inhibitors to disrupt protease-driven viral maturation. Finally, modifications of the Gag zinc fingers would likely result in abnormal packaging of viral genomic RNA (1, 23). Hence, the cross-linkage of Gag zinc fingers not only might result in abnormal processing due to modification of the HIV-1 protease cleavage pattern but might also result in other aberrant features of viral packaging and maturation. These aspects are presently under investigation.

It is noteworthy that the consequences of zinc finger inhibitors mimic those of reverse transcriptase inhibitors and protease inhibitors in many ways (2, 30, 31, 37, 50). Studies with both NOBA (45) and DIBAs (47, 53) found that the action of zinc finger inhibitors on cell-free virions blocked the ability of those virions to synthesize proviral DNA after target cell binding, even though the zinc finger inhibitors did not directly inhibit the reverse transcriptase enzyme activity or prevent virus from binding to cells. Similarly, the findings obtained in the current study indicate that zinc finger inhibitors can also prevent Gag precursor processing and maturation in the manner of protease inhibitors, without any evidence for a significant inhibition of the protease enzyme. These paradoxes not only illustrate the utility and multiple consequences of zinc finger inhibitors but also indicate that one must be cautious in defining the mechanisms of action of new antiviral compounds. Toward this end, the findings in the current study clearly define one of the cellular biochemical mechanisms by which zinc finger inhibitors affect virion production and maturation.

ACKNOWLEDGMENTS

This research was supported by the National Cancer Institute (contract N01-C0-56000).

We thank Louis E. Henderson, Larry O. Arthur, and Alan Rein for many helpful conversations and the sharing of reagents. In addition, we thank Ming Bu and Lisa Graham for technical assistance, Judy Duears for help in the preparation of the manuscript, and Terry Williams for preparation of the figures.

REFERENCES

- Aldovini, A., and R. A. Young. 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. *J. Virol.* **64**:1920–1926.
- Ashorn, P., T. J. McQuade, S. Thaisrivongs, A. G. Tomasselli, W. G. Tarpley, and B. Moss. 1990. An inhibitor of the protease blocks maturation of human and simian immunodeficiency viruses and spread of infection. *Proc. Natl. Acad. Sci. USA* **87**:7472–7476.
- Berg, J. M. 1986. Potential metal-binding domains in nucleic acid binding proteins. *Science* **232**:485–487.
- Bess, J. W., Jr., P. J. Powell, H. J. Issaq, L. J. Schumack, M. K. Grimes, L. E. Henderson, and L. O. Arthur. 1992. Tightly bound zinc in human immunodeficiency virus type 1, human T-cell leukemia virus type 1, and other retroviruses. *J. Virol.* **66**:840–847.
- Bowles, N. E., P. Damay, and P.-F. Spahr. 1993. Effect of rearrangement and duplications of the Cys-His motifs of Rous sarcoma virus nucleocapsid protein. *J. Virol.* **67**:623–631.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Bryant, M., and L. Ratner. 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl. Acad. Sci. USA* **87**:523–527.
- Buckheit, R. W., Jr., M. G. Hollingshead, J. Germany-Decker, E. L. White, J. B. McMahon, L. B. Allen, L. J. Ross, W. D. Decker, L. Westbrook, W. M. Shannon, O. Weislow, J. P. Bader, and M. R. Boyd. 1993. Thiazolobenzimidazole: biological and biochemical anti-retroviral activity of a new non-nucleoside reverse transcriptase inhibitor. *Antivir. Res.* **21**:247–265.
- Burnette, B., G. Yu, and R. L. Felsted. 1993. Phosphorylation of HIV-1 gag proteins by protein kinase C. *J. Biol. Chem.* **268**:8698–8703.
- Covey, S. N. 1986. Amino acid sequence homology in gag region of reverse transcribing elements and the coat protein gene of cauliflower mosaic virus. *Nucleic Acids Res.* **14**:623–633.
- Darlix, J.-L., A. Vincent, C. Gabus, H. De Rocquigny, and B. Roques. 1993. Trans-activation of the 5' to 3' viral DNA strand transfer by nucleocapsid protein during reverse transcription of HIV-1 RNA. *C. R. Acad. Sci. Ser. III* **316**:763–771.
- Debouch, C. 1992. The HIV-1 protease as a therapeutic target for AIDS. *AIDS Res. Hum. Retroviruses* **8**:153–164.
- Déméné, H., C. Z. Dong, M. Ottmann, M. C. Rouyez, N. Jullian, N. Morellet, Y. Mely, J. L. Darlix, M. C. Fournié-Zaluski, S. Saragosti, and B. P. Roques. 1994. ¹H NMR structure and biological studies of the His²³→Cys mutant nucleocapsid protein of HIV-1 indicate that the conformation of the first zinc finger is critical for virus infectivity. *Biochemistry* **33**:11707–11716.
- De Rocquigny, H., C. Gabus, A. Vincent, M.-C. Fournié-Zaluski, B. Roques, and J.-L. Darlix. 1992. Viral RNA annealing activities of human immunodeficiency virus type 1 nucleocapsid protein require only peptide domains outside the zinc fingers. *Proc. Natl. Acad. Sci. USA* **89**:6472–6476.
- Dorfman, T., J. Luban, S. P. Goff, W. A. Haseltine, and H. G. Göttlinger. 1993. Mapping of functionally important residues of a cysteine-histidine box in the human immunodeficiency virus type 1 nucleocapsid protein. *J. Virol.* **67**:6159–6169.
- Dupraz, P., S. Oertle, C. Meric, P. Damay, and P.-F. Spahr. 1990. Point mutations in the proximal Cys-His box of Rous sarcoma virus nucleocapsid protein. *J. Virol.* **64**:4978–4987.
- Felsted, R. L., et al. Unpublished data.
- Folks, T. M., J. Justement, A. Kinter, J. Orenstein, G. Poli, and A. S. Fauci. 1988. Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J. Immunol.* **140**:1117–1122.
- Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde. 1989. Assembly and release of HIV-1 precursors Pr55^{gag} virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**:103–112.
- Glover, C. J., and R. L. Felsted. 1995. Identification and characterization of multiple forms of bovine N-myristoyltransferase. *J. Biol. Chem.* **270**:23226–23233.
- Glover, C. J., M. R. Tellez, F. S. Guziec, Jr., and R. L. Felsted. 1991. Synthesis and characterization of inhibitors of myristoyl-CoA:protein N-myristoyltransferase. *Biochem. Pharmacol.* **41**:1067–1074.
- Gorelick, R. J., D. J. Chabot, A. Rein, L. E. Henderson, and L. O. Arthur. 1993. The two zinc fingers in the human immunodeficiency virus type 1 nucleocapsid protein are not functionally equivalent. *J. Virol.* **67**:4027–4036.
- Gorelick, R. J., L. E. Henderson, J. P. Hanser, and A. Rein. 1988. Point mutations of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a "zinc finger-like" protein sequence. *Proc. Natl. Acad. Sci. USA* **85**:8420–8424.
- Gorelick, R. J., S. M. Nigida, Jr., J. W. Bess, Jr., L. O. Arthur, L. E. Henderson, and A. Rein. 1990. Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA. *J. Virol.* **64**:3207–3211.
- Göttlinger, 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.
- Green, L. M., and J. M. Berg. 1989. A retroviral Cys-Xaa₂-Cys-Xaa₂-His-Xaa₂-Cys peptide binds metal ions: spectroscopic studies and a proposed three-dimensional structure. *Proc. Natl. Acad. Sci. USA* **86**:4047–4051.
- Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Anthanassopoulos, A. Tse, and G. N. Pavlakis. 1989. *rev* (*trsl/art*) protein expression via a *cis*-acting sequence in the *env* region. *J. Virol.* **63**:1265–1274.
- Henderson, L. E., T. D. Copeland, R. C. Sowder, G. W. Smythers, and S. Oroszlan. 1981. Primary structure of the low molecular weight nucleic acid-binding proteins of murine leukemia viruses. *J. Biol. Chem.* **256**:8400–8406.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51–59.
- Kaplan, A. H., and R. Swanstrom. 1991. Human immunodeficiency virus type 1 Gag proteins are processed in two cellular compartments. *Proc. Natl. Acad. Sci. USA* **88**:4528–4532.
- Kaplan, A. H., J. A. Zack, M. Knigge, D. A. Paul, D. J. Kempf, D. W. Norbeck, and R. Swanstrom. 1993. Partial inhibition of the human immunodeficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. *J. Virol.* **67**:4050–4055.
- Kohl, N. E., E. A. Emini, W. A. Schleif, L. J. Davis, J. C. Heimbach, R. A. F. Dixon, E. M. Scolnick, and I. S. Sigal. 1988. Active human immunodeficiency virus protease is required for viral infectivity. *Proc. Natl. Acad. Sci. USA* **85**:4686–4690.
- Kräusslich, H.-G. 1991. Human immunodeficiency virus proteinase dimer as component of the viral polyprotein prevents particle assembly and viral infectivity. *Proc. Natl. Acad. Sci. USA* **88**:3213–3217.
- Lapadat-Tapolsky, M., H. De Rocquigny, D. Van Gent, B. Roques, R. Plasterk, and J.-L. Darlix. 1993. Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle. *Nucleic Acid Res.* **21**:831–839.
- Leis, J., D. Baltimore, J. M. Bishop, J. Coffin, E. Fleissner, S. P. Goff, S. Oroszlan, H. Robinson, A. M. Skalka, H. M. Temin, and V. Vogt. 1988. Standardization and simplified nomenclature for proteins common to all retroviruses. *J. Virol.* **62**:1808–1809.
- Malim, M. H., J. Hauber, S.-Y. Lee, J. V. Maizel, and B. Cullen. 1989. The HIV-1 *rev* trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature (London)* **338**:254–257.
- Maurer, B., H. Bannert, G. Darai, and R. M. Flügel. 1988. Analysis of the primary structure of the long terminal repeat and the *gag* and *pol* genes of human spumaretrovirus. *J. Virol.* **62**:1590–1597.
- McQuade, T. J., A. G. Tomasselli, L. Liu, V. Karacostas, B. Moss, T. K. Sawyer, R. L. Heinrikson, and W. G. Tarpley. 1990. A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation. *Science* **247**:454–456.
- Méric, C., and S. P. Goff. 1989. Characterization of Moloney leukemia virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein. *J. Virol.* **63**:1558–1568.
- Méric, C., E. Gouilloud, and P.-F. Spahr. 1988. Mutations in Rous sarcoma virus nucleocapsid protein p12 (NC): deletions of Cys-His boxes. *J. Virol.* **62**:3328–3333.
- Meyers, G., J. A. Berzofsky, A. B. Rabson, T. F. Smith, and F. Wong-Staal (ed.). 1990. Human retrovirus and AIDS. Los Alamos National Laboratory, Los Alamos, N. Mex.
- Navia, M. A., P. M. D. Fitzgerald, B. M. McKee, C.-T. Leu, J. C. Heimbach, W. K. Herber, I. S. Sigal, P. L. Darke, and J. P. Springer. 1989. Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. *Nature (London)* **337**:615–620.
- Platt, E. J., and O. K. Haft. 1994. Characterization of human immunodeficiency virus type 1 Pr55^{gag} membrane association in a cell free system: requirement for a C-terminal domain. *Proc. Natl. Acad. Sci. USA* **91**:4594–4598.
- Rein, A., D. E. Ott, J. Mirro, L. O. Arthur, W. Rice, and L. E. Henderson. 1996. Inactivation of murine leukemia virus by compounds that react with the zinc finger in the viral nucleocapsid protein. *J. Virol.* **70**:4966–4972.
- Rice, W. G., L. O. Arthur, L. E. Henderson, and J. A. Turpin. 1996. Discovery and development of disulfide-substituted benzamides as candidate anti-HIV drugs. *Int. Antivir. News* **4**:3–6.
- Rice, W. G., C. A. Schaeffer, L. Graham, M. Bu, J. McDougal, S. L. Orloff, F. Villinger, M. Young, S. Oroszlan, M. R. Fesen, Y. Pommier, J. Mende-

- leyev, and E. Kun. 1993. The site of action of 3-nitrosobenzamide on the infectivity process of human immunodeficiency virus in human lymphocytes. *Proc. Natl. Acad. Sci. USA* **90**:9721–9724.
46. Rice, W. G., C. A. Schaeffer, B. Harten, F. Villinger, T. L. South, M. F. Summers, L. E. Henderson, J. W. Bess, Jr., L. O. Arthur, J. S. McDougal, S. L. Orloff, J. Mendeleyev, and E. Kun. 1993. Inhibition of HIV-1 infectivity by zinc-ejecting aromatic C-nitroso compounds. *Nature (London)* **361**:473–475.
47. Rice, W. G., J. G. Supko, L. Malspeis, R. W. Buckheit, Jr., D. Clanton, M. Bu, L. Graham, C. A. Schaeffer, J. A. Turpin, J. Domagala, R. Gogliotti, J. P. Bader, S. M. Halliday, L. Coren, R. C. Sowder II, L. O. Arthur, and L. E. Henderson. 1995. Inhibitors of HIV nucleocapsid protein zinc fingers as candidates for the treatment of AIDS. *Science* **270**:1194–1197.
48. Rice, W. G., J. A. Turpin, L. O. Arthur, and L. E. Henderson. 1995. Highly conserved retroviral zinc fingers as targets for HIV-1 therapeutic management. *Int. Antivir. News* **3**:87–89.
49. Rodriguez-Rodriguez, L., Z. Tsuchihashi, G. M. Fuentes, R. A. Bambara, and P. J. Fay. 1995. Influence of human immunodeficiency virus nucleocapsid protein on synthesis and strand transfer by the reverse transcriptase *in vitro*. *J. Biol. Chem.* **270**:15005–15011.
50. Rosé, J. R., L. M. Babé, and C. S. Craik. 1995. Defining the level of human immunodeficiency virus type 1 (HIV-1) protease activity required for HIV-1 particle maturation and infectivity. *J. Virol.* **69**:2751–2758.
51. South, T. L., P. R. Blake, R. C. Sowder III, L. O. Arthur, L. E. Henderson, and M. F. Summers. 1990. The nucleocapsid protein isolated from HIV-1 particles binds zinc and forms retroviral-type zinc fingers. *Biochemistry* **29**:7786–7789.
52. Tranchou, V., C. Gabus, V. Rogemond, and J.-L. Darlix. 1995. Formation of stable and functional HIV-1 nucleoprotein complexes *in vitro*. *J. Mol. Biol.* **252**:563–571.
53. Turpin, J. A., and W. G. Rice. Unpublished data.
54. Weislow, O. S., R. Kiser, D. L. Fine, J. P. Bader, R. H. Shoemaker, and M. R. Boyd. 1989. New soluble formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS antiviral activity. *J. Natl. Cancer Inst.* **81**:577–586.
55. Wills, J. W., and R. C. Craven. 1991. Form, function, and use of retroviral Gag proteins. *AIDS* **5**:639–654.
56. You, J. C., and C. S. McHenry. 1994. Human immunodeficiency virus nucleocapsid protein accelerates strand transfer of the terminally redundant sequences involved in reverse transcription. *J. Biol. Chem.* **269**:31491–31495.
57. Yu, X., Y. Hathout, C. Fenselau, R. C. Sowder II, L. E. Henderson, W. G. Rice, J. Mendeleyev, and E. Kun. 1995. Specific disulfide formation in the oxidation of HIV-1 zinc finger protein nucleocapsid p7. *Chem. Res. Toxicol.* **8**:586–590.
58. Zybarth, G., and C. Carter. 1995. Domains upstream of the protease (PR) in human immunodeficiency virus type 1 Gag-Pol influence PR autoprocessing. *J. Virol.* **69**:3878–3884.