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Protease Inhibitors Modify Induction of Endogenous Type C Oncornavirus (41019)

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Abstract. The influence of the protease inhibitors, antipain and leupeptin, on type C virus induction from mouse cells by 5-iodo-2-deoxyuridine (IdUrd) and ultraviolet (uv)-irradiated herpes simplex virus (uv-HSV) was studied. Exposure of unaltered intact A1-2 cells, derived from the BALB/c mouse, to these inhibitors decreased the level of endogenous type C virus normally induced by IdUrd or uv-HSV treatment. Dose–response studies showed that antipain was more suppressive of virus induction than leupeptin. Virus induction was suppressed at inhibitor concentrations which did not appreciably affect cellular macromolecular synthesis or capacity of noninduced cells to support helper virus replication upon exogenous infection. These results suggest that the inhibitors affect the virus induction process. Our results are similar to those reported for antipain inhibition of λ-prophage induction in *Escherichia coli*, and corroborate a recent report on suppression of mammalian virus induction by these protease inhibitors.

Cells of many, if not all, animal species possess integrated genetic information for type C RNA tumor viruses or oncornaviruses, which is transmitted from one generation to the next, often in an unexpressed form. Although normally repressed, this information may be expressed after exposure to a variety of environmental agents, such as uv and X radiation (1–3), chemicals (4–7), other viruses (8, 9), or physiological host factors (1, 10–12). Virus induction results in release of infectious virus and/or expression of viral-coded gene products.

Control of virus expression is thought to be under those regulatory processes which affect cellular genes, although the specific mechanism of control is not completely understood. However, since agents which induce prophage λ in lysogenic bacterial strains (13) also induce expression of animal type C viruses, it is possible that similar processes regulate virus expression in both bacterial and mammalian systems. A protein repressor molecule apparently controls λ-prophage expression in *Escherichia coli* (14). It has been postulated that type C virus induction from mouse cells by IdUrd may involve alteration in DNA binding of a control protein (15), whose function may be similar to that of a protein repressor in bacterial lysogenic systems (16).

Antipain (17), a low-molecular-weight protease inhibitor isolated from actinomycetes (18), inhibits λ induction in *E. coli* (19). It has recently been reported that protease inhibitors affect type C oncornavirus induction in chemically treated cells whose membranes have been permeabilized (20). The purpose of this study was to determine whether the protease inhibitors, antipain as well as leupeptin, modify induction of type C oncornavirus from mouse cells by IdUrd treatment or infection with uv-irradiated herpes simplex virus (uv-HSV) in the absence of experimental modification of the cell membrane. The data show that antipain decreases the level of type C virus induction normally observed in A1-2 cells of the BALB/c mouse after IdUrd treatment or uv-HSV infection. Leupeptin, on the other hand, had a minimal effect. Suppression of virus induction occurred at concentrations of protease inhibitors which did not appreciably affect cellular RNA, DNA, and protein synthesis or capacity of noninduced cells to support helper virus replication upon exogenous infection.

Materials and Methods. *Cell cultures.* The A1-2 cell line, a clonal sarcoma-positive, helper-negative (S + H−) derivative of adult BALB/c mouse peritoneal cells

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transformed by Gazdar murine sarcoma virus (Gz-MSV) was used (2). Additional cells included a line of normal rat kidney (NRK) (21) and a cloned line of African green monkey kidney, CV-1 (TC7) (9), cells. Procedures for maintenance of stock and experimental cultures have been reported previously (2, 9).

Viruses. Herpes simplex virus, type 1 (HSV-1), macroplaque strain, was utilized (9). Preparation of virus stocks (9) and determinations of virus titer by plaque-forming assay on CV-1 (TC7) cells have been described (9). Rauscher murine leukemia virus (R-MuLV) was also utilized. Determinations of R-MuLV replication with concomitant MSV rescue, as measured by focus-formation, were made by direct assay on A1-2 cells.

uv-Irradiation. The uv radiation source was a germicidal lamp, emitting principally 254 nm radiation. Calibration procedures (22) and spectral irradiance measurements of the source have been described previously (2). Preparation of uv-HSV has similarly been described (9).

Chemicals. Chemicals used included IdUrd and mitomycin C (Calbiochem) and polybrene (Aldrich Chemical Company). Preparation of solutions has been described (2).

Protease inhibitors. Antipain [(1-carboxy-2-phenylethyl)carbamoyl-L-arginyl-L-valyl-argininal] and leupeptin (acetyl-L-leucyl-L-leucyl-argininal) were provided by Dr. Walter Troll of the New York University Medical Center as part of the U.S.–Japan Cooperative Cancer Research Program. Sterile stock solutions (100 m*M*) were prepared by dissolving the agents in distilled water and passing the solutions through 0.22-μm membrane filters (Millipore Corporation). Working solutions were freshly prepared for each experiment in cell culture growth medium.

Incorporation of [^{3}H]thymidine, and [^{3}H]leucine. Confluent monolayer cultures of A1-2 cells were harvested by trypsinization. The cells were diluted in growth medium and counted in an electronic cell counter (Coulter Electronics, Inc.) with cell viability determined by trypan blue vital dye exclusion. Approximately 2×10^5 cells/ml in aliquots of 0.1 ml were placed in individual wells of microtiter trays (Falcon Plastics). Fifty microliters of antipain or leupeptin at concentrations of 5 or 10 m*M* and 10 or 20 m*M*, respectively, was added to triplicate wells at 0 time, with control cells receiving equivalent amounts of growth medium. Trays were incubated at 37° in a 5% CO_2 atmosphere. Twenty-four hours later, 50 μl of IdUrd (25 μg/ml) was added to appropriate wells, and cultures were reincubated for 23 hr. Cultures were then pulsed for 1 hr with 50 μl of growth medium containing either 20 μCi/ml [^{3}H]thymidine (specific activity 20 Ci/mmole), or [^{3}H]leucine (specific activity 51.6 Ci/mmole) (New England Nuclear). Cultures containing [^{3}H]leucine were additionally supplemented with 200 m*M* glutamine. After incubation, cultures were washed twice with Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium and chased for 30 min with either 100 μg/ml of cold thymidine, or growth medium in appropriate wells. After removal of media, cells were washed with PBS and detached by lidocaine (Astra Pharmaceutical). Cultures were incubated at room temperature for 15 min and adsorbed onto glass filter disks by means of a cell harvester (Model 241, Victor Biomedical Research Institute). Disks were dried, washed with trichloroacetic acid, placed in toluene-base scintillation fluid and counted to determine the incorporation of radioactivity into trichloroacetic acid-insoluble material.

Incorporation of [^{125}I]IdUrd. Log-phase A1-2 cells were incubated at 37° for 4 hr with [^{125}I]IdUrd (specific activity 2000 Ci/mmole) in the presence of antipain (10 m*M*) or leupeptin (20 m*M*). After [^{125}I]IdUrd removal, cells were washed three times with growth medium and lysed by the addition of sodium dodecyl sulfate in ammonium hydroxide for 10 min at room temperature. Cell lysates were adsorbed onto cotton plugs, by means of the Titertek-Supernatant Collection System (Flow Laboratories) and counted in a liquid scintillation counter.

Infectious center assay for endogenous virus induction. Mouse cell lines transformed by murine sarcoma virus (MSV), such as the A1-2, are sensitive indicators

for endogenous leukemia helper virus induction, as described previously (2). The fraction of A1-2 cells induced to release endogenous type C virus was quantified by infectious center focus-forming assay on permissive NRK cells (2, 9). A1-2 cells were seeded at 1×10^5 cells/60-mm-diameter petri dish. Twenty-four hours later, the medium was removed, and cultures were rinsed with PBS and either exposed for 24 hr to fresh medium containing IdUrd or infected with uv-HSV at a multiplicity of infection (moi) of approximately 15, as previously described (9). Cultures were shielded from ambient light following treatment. The effect of antipain or leupeptin on the level of virus induction by IdUrd or uv-HSV was determined by their addition at different concentrations to A1-2 cells treated with the respective inducing agents. After 24 hr, A1-2 cultures were rinsed twice with PBS containing calcium and magnesium and exposed to 5 ml of medium containing 25 μg/ml of mitomycin C for 1 hr at 37° (2). After removal of mitomycin C-containing medium and three rinses with PBS, cultures were harvested by trypsin–EDTA and counted in an electronic cell counter with cell viability determined by trypan blue vital dye exclusion. An appropriate concentration of cells was added to NRK cultures, seeded 24 hr previously at 3×10^4 cells per dish in complete medium with 5% heat-inactivated fetal calf serum and 2 μg/ml polybrene (2). The level of virus induction was determined 6 days later by the enumeration of infectious centers (foci of MSV transformation) in unstained NRK monolayers. The percentage of cells induced to release virus was calculated from the number of infectious centers in the NRK monolayer divided by the total viable cells added to NRK cultures.

Replication of exogenous murine leukemia virus. Infection of A1-2 cells with MuLV of FMR antigenicity rescues the MSV resulting in foci of morphologically altered cells. This permits A1-2 cells to be used as a direct assay for R-MuLV replication with concomitant MSV rescue, as measured by focus formation (2), in the presence of protease inhibitors. A1-2 cells were seeded at 1×10^5/60-mm-diameter petri dish. The medium was removed after 24 hr, and cultures were rinsed with PBS. Appropriate virus dilutions contained in a total volume of 0.4 ml complete medium containing 2 μg/ml of polybrene were added to cell cultures. Virus was adsorbed for 30 min at 37° followed by addition of 4 ml of complete medium with or without different concentrations of antipain or leupeptin. Infected cultures were incubated at 37° for 5 days, at which time foci were counted.

Results. *Effect of protease inhibitors on type C virus induction.* Halogenated pyrimidines are efficient inducers of murine type C virus, with IdUrd relatively more efficient than 5-bromo-2-deoxyuridine (BrdUrd) (2). Under optimal exposure conditions, IdUrd induces approximately 1.0% of treated A1-2 cells to release endogenous type C virus after 24 hr (2). The effect of the protease inhibitors, antipain and leupeptin, on virus induction by IdUrd was determined by simultaneously adding different concentrations of the inhibitors and IdUrd to log-phase A1-2 cells until the time of virus assay 24 hr later. The dose–response curves (Fig. 1) show that both inhibitors decreased the level of virus induction normally observed by IdUrd treatment, with the level of decreased induction related to protease inhibitor concentration. Antipain was more inhibitory for IdUrd induction than leupeptin. Antipain concentrations of 2.5 and 5.0 m*M* decreased induction 65 and 78%, respectively, while 5.0 m*M* of leupeptin decreased induction only 34%. Treatment of cells with IdUrd for 24 hr prior to the addition of protease inhibitors did not lead to a decrease in virus induction. Also, a 24-hr exposure of A1-2 cells to either antipain or leupeptin alone had no observable effect on virus induction.

In order to determine whether the effects of antipain and leupeptin were limited to virus induction by IdUrd, the influence of these agents on oncornavirus induction by uv-HSV [2.3×10^3 J/m^2 of uv, the optimal inducing exposure (9)] was studied. Exposure of log-phase A1-2 cells to uv-HSV induces approximately 0.05% of the cell population to release endogenous type C virus after 24 hr (9). Both antipain and leupeptin decreased virus induction by

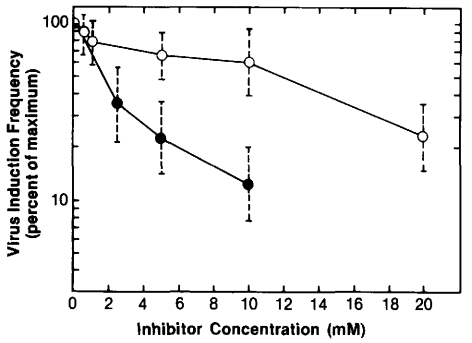

FIG. 1. Effect of protease inhibitors on IdUrd induction of endogenous murine type C virus from A1-2 cells. Log-phase A1-2 cultures were treated for 24 hr with IdUrd (25 μg/ml) in the presence of different concentrations of antipain or leupeptin. The fraction of cells induced to release virus was assayed at 24 hr by adding 1×10^4 viable A1-2 cells to permissive NRK indicator cells and scoring for infectious center focus formation, as described under Materials and Methods. Data are expressed as percentage of virus induction frequency in response to IdUrd. The induction frequency with exposure to IdUrd alone was 1.0%. Each datum point represents the mean for four experiments with 95% confidence limits indicated about the mean for each point. Linear regression analysis, showed that the slopes were significantly different from zero with $P = 0.004$ and 0.000045 for antipain and leupeptin, respectively. Covariance analysis showed that the antipain slope (−0.0593) was significantly steeper than the leupeptin slope (−0.0273) with $P = 0.0387$. ●, antipain; ○, leupeptin.

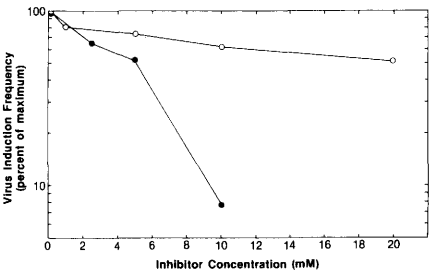

FIG. 2. Effect of protease inhibitors on induction of endogenous murine type C virus from A1-2 cells by infection with uv-HSV. Log-phase A1-2 cultures were infected with uv-HSV (2.3×10^3 J/m^2 of uv) in the presence of different concentrations of antipain or leupeptin. The fraction of cells induced to release virus was assayed at 24 hr by adding 3×10^4 viable A1-2 cells to permissive NRK indicator cells and scoring for infectious center focus formation, as described under Materials and Methods. Results are expressed as percentage of virus induction frequency in response to infection by uv-HSV. The induction frequency with uv-HSV alone was 0.05%. Each datum point represents the average of two experiments. ●, antipain; ○, leupeptin.

uv-HSV, with the level of inhibition dependent on protease inhibitor concentration (Fig. 2). As seen with IdUrd induction, antipain was more suppressive than leupeptin. Antipain at a concentration of 5.0 m*M* decreased induction approximately 48%, while 5.0 m*M* of leupeptin decreased induction by 20% of that observed in its absence.

Protease inhibitors and cellular capacity to support type C viral replication. In order to test the possibility that inhibition of virus induction by the protease inhibitors described above could be due to interference with a step in virus induction occurring subsequent to the inductive event, i.e., inhibition of virus replication, the influence of antipain and leupeptin on the replication of type C virus exogenous to the system was studied. Log-phase cultures of A1-2 cells were infected with R-MuLV. After virus adsorption, growth medium containing different concentrations of antipain or leupeptin was added. Foci of morphologically altered cells, indicative of R-MuLV replication with concomitant rescue of Gz-MSV were counted at 5 days. The results indicate that antipain and leupeptin had no appreciable effect on exogenous type C virus replication at those concentrations (5 m*M* or less) which decreased virus induction by IdUrd and uv-HSV (Fig. 3). However, it was noted that concentrations of 10 m*M* or greater did reduce cellular capacity to support virus replication (Fig. 3). This suggests that suppressed virus induction by the protease inhibitors is most likely due to an effect on the induction process rather than an effect on induced virus replication.

Sensitivity of cellular functions to protease inhibitors. To determine the influence of antipain and leupeptin on A1-2 cellular metabolism, [^{3}H]thymidine and [^{3}H]leucine incorporation were measured in the presence of different protease inhibitor concentrations.

In pulse chase experiments, control cell

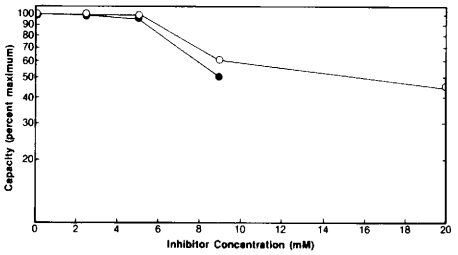

FIG. 3. Influence of protease inhibitors on capacity of noninduced A1-2 cells to support R-MuLV replication. Different concentrations of antipain or leupeptin were added immediately after R-MuLV adsorption. Foci were counted 5 days after infection. Data are expressed as the percentage of capacity in response to R-MuLV infection. The virus titer in the absence of protease inhibitors was 4.7×10^7 focus-forming units (FFU)/0.2 ml of virus suspension. Each datum point represents the average of two experiments. ●, antipain; ○, leupeptin.

cultures incorporated 3.05 pmole of [^{3}H]thymidine into acid-insoluble material (Table I). No substantial difference in [^{3}H]thymidine incorporation was seen in cultures containing 5 m*M* antipain relative to the control. Cultures containing antipain at 10 m*M* and leupeptin at 10 and 20 m*M*, however, demonstrated an average 82% reduction in [^{3}H]thymidine incorporation. In the presence of IdUrd, [^{3}H]thymidine incorporation was reduced 96%. The addition of 5 m*M* antipain did not affect the observed reduction while 10 m*M* antipain decreased incorporation by 82%. Concentrations of 10 and 20 m*M* leupeptin did not substantially influence [^{3}H]thymidine incorporation in IdUrd-treated cells.

The incorporation of [^{3}H]leucine into acid-precipitable material was utilized to measure cellular protein synthesis (Table I). Cultures containing antipain and leupeptin at concentrations of 5 and 10 m*M*, respectively, demonstrated slightly increased [^{3}H]leucine incorporation relative to control cultures. Increasing the concentrations of each protease inhibitor twofold slightly reduced [^{3}H]leucine incorporation by 14%. In IdUrd-treated cultures, [^{3}H]-leucine incorporation was reduced approximately 7% relative to control values. Leupeptin at concentrations of 10 and 20 m*M* reduced this [^{3}H]leucine incorporation by 3 and 23%, respectively, while 5 and 10 m*M* concentrations of antipain reduced [^{3}H]leucine incorporation by 23 and 38%. Similar trends were obtained utilizing the incorporation of [^{3}H]uridine to measure cellular RNA synthesis (data not shown).

Since concentrations of antipain and leupeptin which suppressed virus induction did not significantly inhibit DNA, RNA, or protein synthesis, as assayed by radioactively labeled substrate incorporation, it is unlikely that their effect on virus induc-

TABLE I. EFFECT OF PROTEASE INHIBITORS ON CELLULAR DNA, PROTEIN SYNTHESIS, AND IdUrd INCORPORATION

	No IdUrd		IdUrd		
	[^{3}H]Thymidine (pmole/10^6 cells)	[^{3}H]Leucine (pmole/10^6 cells)	[^{3}H]Thymidine (pmole/10^6 cells)	[^{3}H]Leucine (pmole/10^6 cells)	[^{125}I]IdUrd (cpm)
Control	3.05	0.070	0.110	0.065	18,743
Antipain					
(5 m*M*)	3.25	0.080	0.120	0.050	ND[b]
(10 m*M*)	2.65	0.060	0.090	0.040	19,067
Leupeptin					
(10 m*M*)	2.45	0.075	0.105	0.063	ND[b]
(20 m*M*)	2.35	0.060	0.120	0.050	14,008

[a] A1-2 cells were cultured in microtiter trays in the presence of either antipain (5 and 10 m*M*) or leupeptin (10 and 20 m*M*). After 24 hr of culture, 25 μg/ml of IdUrd was added to appropriate wells which were incubated for an additional 23 hr with 20 μCi/ml of either [^{3}H]thymidine or [^{3}H]leucine. The effect of protease inhibitors on [^{125}I]IdUrd incorporation was tested over a 4-hr incubation period.

[b] Not done.

tion is due to generalized effects on cellular macromolecular synthesis.

Effect of antipain and leupeptin on IdUrd incorporation. It is possible that the observed suppression of IdUrd virus induction by protease inhibitors could be due to inhibition of IdUrd incorporation. To test this possibility, log-phase A1-2 cells were incubated with [^{125}I]IdUrd in the presence of antipain (10 m*M*) and leupeptin (20 m*M*). The results show that 10 m*M* antipain had no inhibitory effect, whereas 20 m*M* leupeptin reduced IdUrd incorporation by 25% (Table I). This argues against the possibility that decreased IdUrd induction in the case of antipain was due to reduced IdUrd incorporation into the cell. However, it does not preclude the possibility of direct competition between the protease inhibitor and IdUrd at the induction site.

Discussion. Our results demonstrate that antipain and, to a lesser degree, leupeptin suppress endogenous type C oncornavirus induction from unaltered intact cells. This observation confirms recently published data (20) reporting that these protease inhibitors decreased virus induction in IdUrd- and cycloheximide-treated cells whose membranes had been altered. In view of the permanent alteration of the cell membrane, these authors were unable to adequately address the question of whether the protease inhibitors affected cellular capacity to support exogenous virus replication.

Certain conclusions may be drawn from our studies. These include the fact that suppression of IdUrd and uv-HSV induction of virus by protease inhibitors is inhibitor dose dependent. Moreover, virus induction is not influenced by protease inhibitors once the induction process has been initiated by treatment of cells with the inducing agent. In addition, antipain, the more suppressive inhibitor, as well as leupeptin, inhibited virus induction at concentrations which had no appreciable influence on cellular macromolecular synthesis or capacity of noninduced cell cultures to support type C virus replication upon exogenous infection. These results suggest that the inhibition is due to an effect on the virus induction process.

Our antipain suppression data are similar to those reported for antipain inhibition of λ-prophage induction in *E. coli* (19). In this bacterial system, such induction seems to be one expression of certain coordinately controlled inducible SOS functions (23). Since antipain does not affect SOS-independent λ-prophage induction (19), it is believed that the antipain-sensitive event most likely involves proteolytic inactivation of the λ-protein repressor molecule (19). A repressor affecting expression of endogenous viral genes in mammalian cells has, as yet, not been demonstrated. However, the observed induction of endogenous type C murine virus by the protein synthesis inhibitor, cycloheximide ((24), Hellman, K. B. and Brewer, P. P., unpublished data), suggests that such agents may act to block synthesis of a labile control protein (24). Since protease inhibitors suppressed endogenous type C virus induction and, since proteases are recognized as being involved in cellular control mechanisms in a variety of organisms including mammals (25), our system may provide the means for answering the question of whether protein repressors function similarly in mammalian cell systems as they do in bacterial systems.

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