0022-538X/78/0025-0408$02.00/0
JOURNAL OF VIROLOGY, Jan. 1978, p. 408-412

Vol. 25, No. 1
Printed in U.S.A.

NOTES

Inhibition of Maturation of Rauscher Leukemia Virus by Amino Acid Analogs

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Received for publication 18 July 1977

Synthesis of primary precursor polyproteins of Rauscher leukemia virus (RLV) core and envelope proteins occurs in the presence of amino acid analogs canavanine and *p*-fluorophenylalanine, but cleavage of these precursors is severely inhibited or slowed down. After treatment with these agents, the release of characteristic virus or stable virus-like particles is greatly depressed.

The synthesis of Rauscher leukemia virus (RLV) core and envelope proteins proceeds by cleavage of high-molecular-weight precursor polyproteins (1a, 8, 14, 20). (We are now using a retrovirus protein nomenclature agreed upon at the meeting held 8-9 March 1977 in Arlington, Va., at a Tumor Viral Immunology Workshop sponsored by the Collaborative Research Branch, RNA Tumor Virus Studies Section of the National Cancer Institute. The Committee was composed of R. B. Arlinghaus, D. Bolognesi (Chairman), R. Eisenman, E. Fleissner, R. Nowinski, S. Orozslan, P. Roy-Burman, and G. VandeWoude. This new nomenclature is related to our former nomenclature as follows: Pr1*a*+*b* (≃200,000 daltons) = $Pr200^{gag\text{-}pol}$; Pr2*a*+*b* (≃90,000 daltons) = $gPr90^{env}$; Pr3 (≃80,000 daltons) = $Pr80^{gag}$; Pr4 (≃65,000 daltons) = $Pr65^{gag}$. Pr is for precursor; gPr is for glycosylated precursor; the number is approximate molecular weight in kilodaltons. The superscripts stand for: *gag*—group antigens, p30, p15, p12 and p10; *pol*—the RNA-directed DNA polymerase or reverse transcriptase (RT); *env*—the envelope proteins, gp69/71, p15E, and p12E.) Synthesis of the viral core proteins, which are coded for by the *gag* gene, has been found to proceed in the following manner: $Pr80^{gag} \rightarrow Pr65^{gag} \rightarrow \rightarrow$ p30 + p15 + p12 + p10 (1, 10). $Pr80^{gag}$ and $Pr65^{gag}$ are intracellular precursor polypeptides that contain tryptic peptides of all the mature core proteins (1). $Pr65^{gag}$ has been observed in variable amounts in mature viral particles (8). Synthesis of the viral envelope proteins gp69/71, p15E, and p12E proceeds by synthesis and cleavage of the glycoprotein precursor $gPr90^{env}$ (15).

We have previously examined the effect of amino acid analogs on processing of viral precursor polypeptides (10). When canavanine is added to virus-producing cells, $Pr80^{gag}$ is synthesized, but cleavage of $Pr80^{gag}$ to $Pr65^{gag}$ and formation of p30 are completely inhibited. In the presence of *p*-fluorophenylalanine, formation of $Pr65^{gag}$ from $Pr80^{gag}$ is not affected, but the cleavage rate of $Pr65^{gag}$ is considerably reduced. Van Zaane et al. (19) have reported similar inhibition of viral precursor polypeptide cleavage in the presence of canavanine.

The purpose of the present study was to examine the effect of inhibiting cleavage of viral precursor polypeptides by canavanine and *p*-fluorophenylalanine on the release of virus particles. This may shed more light on the role of cleavage of precursor polypeptides in viral maturation.

NIH Swiss mouse embryo fibroblasts (JLS-V16) chronically infected with RLV were labeled for 7 h with [^{35}S]methionine either in the absence or in the presence of 4 mM canavanine or 4 mM *p*-fluorophenylalanine. In these experiments, the analogous amino acids, i.e., arginine or tyrosine and phenylalanine, respectively, were removed from the culture medium. Figure 1 indicates that release into the culture fluid of labeled material banding in sucrose gradients at the characteristic viral density (Fig. 1A, fraction 9 through 12) is severely inhibited after treatment with canavanine (Fig. 1B) or *p*-fluorophenylalanine (Fig. 1C). In the presence of these amino acid analogs, instead, reduced amounts of radioactive material appear at a slightly lower density (fractions 7 through 10). The nature of this lower, denser material is unknown, but it clearly did not contain the usual viral proteins (cf. Fig. 2B, D, and F).

Material present in these sucrose gradient peaks was analyzed by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fig. 2D and F) and compared to the pattern of

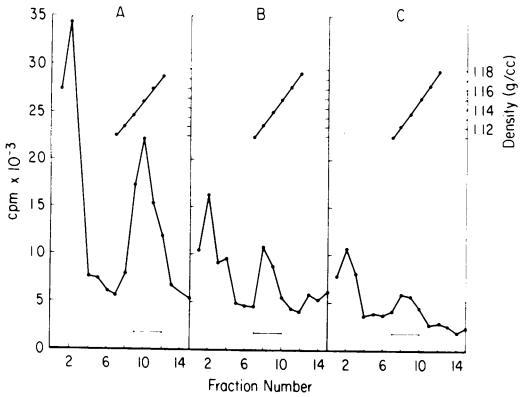

FIG. 1. *Inhibition of virus production by canavanine and p-fluorophenylalanine. RLV-infected cells in quart bottles (ca. 0.946 liter) were labeled for 7 h with [^{35}S]methionine in medium containing the normal amount of amino acids (except the ones indicated below), but one-tenth the concentration of unlabeled methionine, 5% dialyzed fetal calf serum, and no tryptose phosphate. After labeling, the medium was collected and clarified, and the virus was pelleted and purified by sedimentation on a 15 to 60% sucrose gradient. Trichloroacetic acid-precipitable radioactivity and density were measured. Conditions of labeling and virus purification were as previously described (1, 1a, 8). (A) Control gradient; (B) gradient of 4 mM canavanine-treated cells; and (C) gradient of 4 mM p-fluorophenylalanine-treated cells. The growth medium in (B) lacked arginine, whereas that in (C) lacked phenylalanine and tyrosine. Treatment with the analogs was started 20 min before labeling. Brackets under the peaks indicate the fractions that were pooled for analysis on SDS-polyacrylamide gels, as shown in Fig. 2.*

normal viral polypeptides (Fig. 2B). In addition, virus-specific polypeptides present in the cells after labeling in the absence (Fig. 2A) or in the presence of 4 mM canavanine (Fig. 2C) or 4 mM *p*-fluorophenylalanine (Fig. 2E) were analyzed on the same gel.

It is apparent that in the control culture cleavage of intracellular viral precursor polypeptides Pr80^{gag} and Pr65^{gag} and synthesis of mature proteins proceeded normally (Fig. 2A). Pr80^{gag}, which is usually only detected in short pulses, is not seen under conditions of long labeling, whereas Pr65^{gag} is present to a small extent in the cell. Viral p30 can also be seen in the cell extract.

In Fig. 2B the normal pattern of viral polypeptides is seen. The viral envelope glycoprotein gp69/71^{env} is not seen, probably because it is easily lost during virus purification (2), and because it is deficient in methionine relative to gPr90^{env} (15).

Figure 2C shows the pattern of intracellular virus-specific polypeptides synthesized in the presence of 4 mM canavanine. gPr90^{env} and Pr80^{gag} were the major protein precursors synthesized under this condition, whereas Pr65^{gag} and the mature core and envelope proteins were nearly absent. Also observed was the precursor Pr200$^{gag\text{-}pol}$. Pr200$^{gag\text{-}pol}$ was shown to contain both core protein-specific and reverse transcriptase (*pol*)-specific antigenic determinants (10). The cleavage of Pr200$^{gag\text{-}pol}$ is inhibited by canavanine (10).

An examination of the lower-density material accumulating in the culture fluid in the presence of 4 mM canavanine was hampered because it could not be pelleted by centrifugation at 78,000 $\times$ *g* for 2 h. The total sample recovered was applied to the slab gel (Fig. 2D). Only trace levels of polypeptides were seen, and the pattern did not resemble that of virus. In contrast to this, the control virus peak and the *p*-fluorophenylalanine-derived lower-density peak were recoverable in good yields by this procedure and gave characteristic patterns (Fig. 2B and F).

Studies with glucosamine labeling of infected cells treated with 4 mM canavanine (in arginine-free medium) for 7 h showed 80 to 90% inhibition of viral envelope protein glycosylation compared to the control (data not shown). In these experiments, the major component precipitated from the cytoplasmic extracts with anti-gp69/71 serum was gPr90^{env}, whereas only trace amounts of gp69/71^{env} were labeled with glucosamine.

The effect of canavanine on core and envelope precursor cleavage was examined further by treatment of infected cells with growth medium containing [^{3}H]leucine in 0.1 Eagle amino acids ($\simeq$0.06 mM arginine) for 7 h with concentrations of canavanine ranging from 1 to 8 mM. The inclusion of low levels of arginine in the culture medium relieved the protein synthesis inhibition normally observed in the presence of canavanine. Cytoplasmic extracts were prepared and immunoprecipitated with anti-p30 and anti-gp69/71 sera. The results (Table 1) indicated that anti-p30-precipitable material increased twofold compared to the control as canavanine was increased from 1 to 8 mM. This result can be explained by a build-up of core precursors in cells because of lack of cleavage and virus export. In contrast to this build-up, the amount of anti-gp69/71-precipitable material decreased, especially when the concentration of the analog reached 8 mM. The reason for this latter result is unknown.

Formation of p30 and gp69/71 was measured in this experiment by analysis of immunoprecipitates by SDS-polyacrylamide gel electrophoresis. The results (Table 1) showed that p30 formation was completely blocked at 1 to 2 mM canavanine, whereas gp69/71 formation contin-

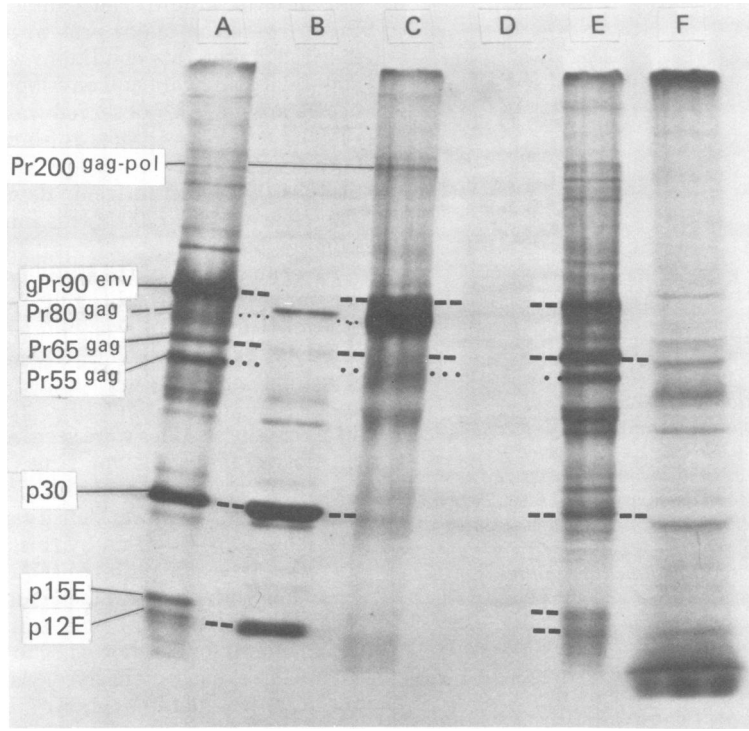

FIG. 2. *Analysis of cytoplasmic extracts of cells and growth media after treatment with canavanine and p-fluorophenylalanine. Cytoplasmic extracts of cells labeled as described in the legend of Fig. 1 were prepared after 7 h of labeling, and direct immunoprecipitation with anti-RLV serum was performed as described previously (1, 8). In addition, the gradient peak fractions indicated in Fig. 1 were pooled, diluted with TNE (0.01 M Tris-hydrochloride, pH 7.5–0.1 M NaCl–0.001 M EDTA), and pelleted at 78,000 × g for 2 h after addition of carrier RLV. The immune pellet of the cytoplasmic extract and the corresponding gradient peak were analyzed on 6 to 12% gradient SDS-polyacrylamide gels. (A) Control cells; (B) control gradient peak; (C) 4 mM canavanine-treated cells; (D) canavanine gradient peak; (E) 4 mM p-fluorophenylalanine-treated cells; and (F) p-fluorophenylalanine gradient peak. Cytoplasmic immunoprecipitates from similar numbers of cells were analyzed on the gels. The entire sample of gradient material from analog-treated cells was applied to the gels; only one-half of the control gradient material was applied.*

ued at a low rate up to 8 mM canavanine, although its formation was inhibited by more than 80% at the 8 mM concentration (Table 1). Thus, cleavage of the envelope precursor $gPr90^{env}$ seems less sensitive to treatment with canavanine than cleavage of the core precursors. It should be noted that at lower canavanine concentrations (1 to 2 mM), cleavage of $Pr80^{gag}$ still occurred but at a reduced rate.

Virus production was also monitored in this experiment (Table 1). As little as 1 mM canavanine inhibited virus release about 85%. The virus produced under these conditions had normal density and contained nearly normal amounts of p30 but was slightly enriched in $Pr65^{gag}$. Concentrations of 2 mM or above inhibited virus production more than 99% (Table 1). No peak of lower-density material was observed in this experiment, in contrast to that observed in Fig. 1B. The presence of 0.06 mM arginine in the medium may or may not account for absence of the low-density material.

Figure 2E shows accumulation in infected cells of $Pr65^{gag}$ when labeling was carried out in the presence of *p*-fluorophenylalanine. Additional virus-specific polypeptides of a lower molecular weight than $Pr65^{gag}$ were also observed. One of these polypeptides comigrated with normal p30. Thus, it is apparent that the inhibition of cleavage with *p*-fluorophenylalanine is not as strict as that with canavanine.

Examination of the material accumulated in the culture fluid of *p*-fluorophenylalanine-treated cells (Fig. 2F) revealed the presence of several discrete bands, one of which migrated in the region of p30. However, most of these bands did not comigrate with the virus-specific polypeptides that accumulated in the cell. It is not known whether or not the polypeptides of the material secreted in the culture medium

TABLE 1. *Effect of canavanine treatment on viral protein synthesis and virus release*[a]

Canavanine concn (mM)	Virus production (%)	Anti-p30-precipitable radioactivity (%)	Anti-gp69/71-precipitable radioactivity (%)	p30[b] (%)	gp69/71[b] (%)
0	100 (2,456 cpm)	100 (5,779 cpm)	100 (10,604 cpm)	100 (1,420 cpm)	100 (2,886 cpm)
1	15	140	85	1	67
2	<1	158	87	0	35
4	<1	197	85	0	42
8	<1	201	38	0	18

[a] Parallel cultures of RLV-infected JLS-V16 cells were treated with various concentrations of canavanine for 7 h in a medium containing 0.1 Eagle amino acids (≈0.06 mM arginine). The cells in T-75 flasks were incubated at 250 μCi of [^{3}H]leucine in 10 ml of medium. Virus production was measured by subjecting the clarified supernatant to centrifugation at 78,000 × *g* for 2 h, followed by isopycnic sucrose gradient centrifugation (10). Cell lysis and immunoprecipitation were performed as described (10).

[b] Amount of radioactivity in the p30 and gp69/71 bands as determined by swelling the gel band in counting fluid containing NCS (Amersham/Searle) (14). A background value, corresponding to an average region of the gel containing no detectable bands, was substracted from each fraction in which radioactivity was determined.

represent virus-specific polypeptides, since they were analyzed directly without being subjected to immunoprecipitation with anti-viral serum. However, it is clear that the major virus-specific precursor polypeptides, such as Pr65^{gag}, accumulated in *p*-fluorophenylalanine-treated cells were not released into the culture medium in proportion to their cellular amounts.

These studies indicate that viral precursors that accumulate in infected cells in the presence of the amino acid analogs (i.e., gPr90^{env} and Pr80^{gag} in the presence of canavanine, and gPr90^{env} and Pr65^{gag} in the presence of *p*-fluorophenylalanine) are not exported as part of virus or virus-like particles in the culture medium. This suggests that virus or virus-like particles are not formed in the presence of these analogs. The accumulation of virus-specific precursors in cells in the analogs' presence indicates that no substantial release of disaggregated viral components occurred.

The effect of canavanine on cleavage of gPr90^{env} and Pr80^{gag} is most likely due to a direct inhibition of the cleavage reaction itself. Inhibition of proteolytic cleavage could be due to the resistance of canavanine-containing molecules to cleavage or to the inhibition by canavanine of the proteolytic enzyme responsible for cleavage (19). In the case of gPr90^{env}, canavanine strongly inhibited [^{14}C]glucosamine incorporation, and this may have contributed to inhibition of this precursor's processing.

We interpret the observed interference of virus release to be mainly due to the inhibition by the analogs of the cleavage of the viral core precursors and, to a lesser extent, envelope proteins. Other mechanisms based on a decreased affinity of analog-containing polyproteins for the cell membrane or the inhibition of glycosylation cannot be excluded by the present study. In addition, the analogs used here have an inhibitory effect on protein synthesis. But, it was observed that the presence of low levels of arginine relieves the inhibitory effect of canavanine on protein synthesis but not on virus release (Table 1).

This interpretation of the effect of the analogs on virus release is consistent with the findings of several other investigators, which suggest that defective maturation of RNA tumor viruses is associated with a lack of cleavage of precursors of the virus core proteins (3, 16, 18). On the other hand, where cleavage of the viral core precursors occurs, release of physical particles is usually observed, although abnormal cleavage of these precursors may result in release of particles with abnormal morphology (4, 7, 17). But a role for cleavage of the precursors of the viral envelope proteins in normal virus maturation cannot be excluded. In RLV, the envelope glycoprotein precursor gPr90^{env} is not observed in viral particles, and its processing to the mature envelope proteins gp69/71 and p12E could be essential for virus release under normal conditions. The existence of envelope protein defective mutants of Rous sarcoma virus which nevertheless can be released from the cell (12) suggests that the processing of the envelope proteins is of lesser importance than that of the core proteins. However, it should be noted that cleavage of an envelope glycoprotein precursor was found a necessary step in the process of release of α-togaviruses (11) and Semliki Forest virus (13).

Similarly, the effect of the processing of precursors of the reverse transcriptase on virus assembly, if any, cannot yet be assessed. As mentioned above, canavanine inhibits the processing of these precursors (10). However, the existence of a reverse transcriptase-deficient mutant of

Rous sarcoma virus, RSVα (5), suggests that this enzyme does not play an important role in virus assembly.

Finally, the results of Yoshinaka and Luftig (21) suggested that cleavage of the viral precursor polypeptide, Pr65^{gag}, may be accompanied by the postbudding morphological changes observed in RNA tumor viruses by electron microscopy. Our interpretation of the present and previous (9) results favors the occurrence of such cleavage during rather than after budding. We have proposed a model of virus assembly, one feature of which is the requirement for a substantial cleavage of Pr65^{gag} for virus release (9). Postrelease morphological changes may still depend on cleavage of the precursor proteins, which we suggest occurs earlier during budding. Further experiments are needed to clarify this point.

This work was supported by Public Health Service contract CP-61017 from the National Cancer Institute and by a grant from The Robert A. Welch Foundation (G-429). G. A. J. was supported by the Riyadh University, Saudi Arabia.

We thank J. Syrewicz for technical assistance. We also thank R. B. Luftig for providing us with manuscripts before publication.

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