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Analysis of Intracellular Feline Leukemia Virus Proteins

II. Generation of Feline Leukemia Virus Structural Proteins from Precursor Polypeptides[1]

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The synthesis and processing of feline leukemia virus (FeLV) polypeptides were studied in a chronically infected feline thymus tumor cell line, F-422, which produces the Rickard strain of FeLV. Immune precipitation with antiserum to FeLV p30 and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used to isolate intracellular FeLV p30 and possible precursor polypeptides. SDS-PAGE of immune precipitates from cells pulse-labeled for 2.5 min with [^{35}S]methionine revealed the presence of a 60,000-dalton precursor polypeptide (Pp60) as well as a 30,000-dalton polypeptide. When cells were grown in the presence of the proline analogue L-azetidine-2-carboxylic acid, a 70,000-dalton precursor polypeptide (Pp70) was found in addition to Pp60 after a 2.5-min pulse. The cleavage of Pp60 could be partially inhibited by the general protease inhibitor phenyl methyl sulfonyl fluoride (PMSF). This partial inhibition was found to occur only if PMSF was present during pulse-labeling. Intracellular Pp70 and Pp60 and FeLV virion p70, p30, p15, p11, and p10 were subjected to tryptic peptide analysis. The results of this tryptic peptide analysis demonstrated that intracellular Pp70 and virion p70 were identical and that both contained the tryptic peptides of FeLV p30, p15, p11, and p10. Pp60 contained the tryptic peptides of FeLV p30, p15, and p10, but lacked the tryptic peptides of p11. The results of pactamycin gene ordering experiments indicated that the small structural proteins of FeLV are ordered p11-p15-p10-p30. The data indicate that the small structural proteins of FeLV are synthesized as part of a 70,000-dalton precursor. A cleavage scheme for the generation of FeLV p70, p30, p15, p11, and p10 from precursor polypeptides is proposed.

The generation of stable virion proteins from large precursor polypeptides is a well-documented phenomenon observed in many animal virus-infected cells. Post-translational cleavage of precursor polypeptides was initially described for poliovirus-infected cells (8, 23). This phenomenon has been shown to be widespread, occurring in cells infected by coxsackievirus (11), Sindbis virus (21, 22), Semliki forest virus (4, 12, 15), mengovirus (20), and encephalomyocarditis virus (3, 14). The synthesis of large precursor polypeptides has been proposed as a mechanism by which polycistronic mRNA is translated in eukaryotic cells (2).

During the past 3 years several reports of precursor polypeptides in oncornavirus-infected cells have appeared. Avian myeloblastosis virus-infected primary chicken fibroblasts contain a 76,000-dalton precursor polypeptide (27). Tryptic peptide analysis of this precursor polypeptide reveals the presence of four virion proteins within the 76,000-dalton molecule (27, 28). Immune precipitation of Rauscher leukemia virus (RLV) proteins from infected cells was used to demonstrate 82,000- and 65,000-dalton presumptive precursor polypeptides in JLS-V9 cells (26) and 200,000-, 90,000-, 80,000-, and 65,000-dalton polypeptides in JLS-V16 cells (1, 16). Jamjoom et al. (9) demonstrated that RLV contains a non-glycosylated 70,000-dalton polypeptide that is identical to an intracellular polypeptide, immune precipitable by antiserum to RLV. The virion and intracellular 70,000-dalton polypeptide contain RLV p30 tryptic peptides, and the authors suggest a possible precursor-product relationship.

Recently, we identified a 60,000-dalton precursor polypeptide (Pp60) of feline leukemia virus (FeLV) p30 (18). We employed both immune precipitation of FeLV polypeptides from pulse-labeled cells and tryptic peptide analysis of Pp60 and FeLV p30 to demonstrate a precur-

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sor-product relationship. In this report we present evidence that: (i) there exists a 70,000-dalton precursor polypeptide (Pp70) of FeLV p30, p15, p11, and p10; (ii) the cleavage of Pp70 results in the generation of Pp60 and p11; (iii) the cleavage of Pp60 can be partially inhibited by the general protease inhibitor phenyl methyl sulfonyl fluoride (PMSF); (iv) intracellular Pp70 and FeLV virion p70 contain identical tryptic peptide profiles; and (v) the gene order of the low-molecular-weight FeLV structural proteins is p11-p15-p10-p30.

Our evidence indicates that the small structural proteins of FeLV differ from those of avian viruses in both arrangement within and cleavage from precursor polypeptides. FeLV appears to be similar to RLV in that both contain uncleaved precursors of structural protein (9; present publication). The inhibition of precursor cleavage by a general protease inhibitor reported here seems to be unique for FeLV.

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MATERIALS AND METHODS

Source of cells and virus. The chronically infected feline thymus tumor cell suspension (F-422) was used throughout these experiments. This cell line produces the Rickard strain of FeLV and was propagated as previously described (7).

Radioactive labeling of cells. F-422 cells were pulse-labeled with [^{35}S]methionine (1 μCi/10^6 cells) or ^{3}H-amino acid mixture (2 μCi/10^6 cells). The pulse was terminated and cells were chased in the presence of 10 times the normal concentration of methionine as previously described (18).

Purification of FeLV. ^{14}C- or ^{3}H-amino acid-labeled FeLV and unlabeled FeLV were purified from F-422 culture fluids by discontinuous sucrose gradient centrifugation as previously described (18).

Preparation of subcellular fractions. Particulate fractions (PF) and cytoplasmic extracts (CE) were prepared from Nonidet P-40 (NP-40)-disrupted cells as previously described (18). Briefly, cells were disrupted with lysis buffer (0.5% NP-40–0.15 M NaCl–0.01 M Tris [pH 7.4]) and centrifuged at 2,400 rpm for 5 min in an International PR-6 centrifuge. The supernatant was removed and centrifuged at 100,000 $\times$ g for 1 h. The 100,000 $\times$ g supernatant (cytoplasmic extract) was removed, and the pellet was solubilized with lysis buffer containing 0.2% deoxycholate (PF). Both the CE and PF were sonically treated for 2 to 3 min in a 150-W Branson ultrasonic cleaner (Branson Instruments Co., Stamford, Conn.) and centrifuged again at 100,000 $\times$ g for 1 h.

Immune precipitation. Rabbit antiserum to FeLV p30 was prepared and clarified as previously described (7, 18). Rabbit antiserum to bovine serum albumin (BSA) was obtained from E. Sanders (Michigan State University). Clarified antisera were added to subcellular fractions or disrupted virus and incubated for 30 min at 37°C and then overnight at 4°C. Immune precipitates were collected by sedimentation through 1 ml of 10% sucrose (wt/wt) in lysis buffer at 2,000 rpm for 20 min in an International PR-6 centrifuge. This was repeated one additional time, and the final precipitate was solubilized for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (18).

Double detergent disruption of FeLV. Labeled or unlabeled purified FeLV was disrupted by suspension of viral pellets in lysis buffer containing 0.5% deoxycholate followed by 5 min of sonic treatment in a 150-W Branson ultrasonic cleaner. The disrupted virus was centrifuged at 100,000 $\times$ g for 1 h in an SW 50.1 rotor (Beckman), and the supernatant was used as a source of labeled or unlabeled soluble FeLV polypeptides.

SDS-PAGE. Electrophoresis in the presence of SDS was done as previously described (18), using a 9% polyacrylamide gel described by Fairbanks et al. (5). The gels were fractionated and assayed for radioactivity, using 3a70b scintillation cocktail (RPI Corp., Elk Grove Village, Ill.) as previously described (7).

Tryptic peptide analysis. Tryptic peptides of immune precipitated polypeptides and labeled FeLV polypeptides, eluted from SDS-polyacrylamide gels, were prepared as previously described (18). A 1-mg amount of BSA was added to FeLV proteins separated by gel filtration in the presence of 6 M guanidine hydrochloride (GuHCl), and the proteins were precipitated by dilution of GuHCl to 0.6 M followed by the addition of trichloroacetic acid to a final concentration of 25%. Tryptic peptides were then prepared as previously described (18). Cation-exchange chromatography of tryptic peptides was done by using a high-pressure column of type P chromobeads (Technicon) as previously described (18). Tryptic peptides were lyophilized and stored at −76°C until chromatography.

Pulse-labeling in the presence of pactamycin. Pactamycin was obtained from George B. Whitfield of the Upjohn Co. The drug was stored as a 5 $\times$ 10^{-5} M solution in 1 mM acetic acid at −20°C.

A total of 250 $\times$ 10^6 cells were incubated for 30 s with pactamycin at a final concentration of 5 $\times$ 10^{-7} M and pulse-labeled with ^{3}H-amino acid mixture (2 μCi/10^6 cells) or ^{14}C-amino acid mixture (0.2 μCi/10^6 cells) in a final volume of 5 ml.

RESULTS

Effect of PMSF on the cleavage of Pp60. To determine the effect of PMSF on cell viability and protein synthesis during the short period of exposure to be employed in these experiments, cells were incubated with PMSF for 2 min and then pulse-labeled for 2.5 min in the presence of

PMSF. Both hot trichloroacetic acid-precipitable radioactivity and trypan blue dye exclusion were monitored. The results of this experiment (Fig. 1) indicated that PMSF reduced trichloroacetic acid-precipitable radioactivity by 40% (at 50 μg/ml) during the short period of exposure used in these experiments and reduced cell viability by 10 to 15%.

Pulse-chase-labeling experiments were done in the presence of PMSF to determine whether PMSF, a general protease inhibitor, could inhibit the cleavage of Pp60. In experiment 1 (Fig. 2) cells were pulse-labeled for 2.5 min and then chased for 30 min in the presence or absence of PMSF. SDS-PAGE of immune precipitates from subcellular fractions of pulse-labeled cells demonstrated the presence of Pp60 in the PF and p30 in the CE (Fig. 2A and E) as previously reported (18). When cells were chased for 0.5 h in the presence or absence of PMSF, Pp60 was absent from both subcellular fractions and p30 was found mainly in the CE (Fig. 2B, F, C, and G), indicating no inhibition of Pp60 cleavage.

The effect of PMSF on Pp60 cleavage was also examined with the protease inhibitor present during pulse-labeling. In this experiment (Fig. 3) cells were incubated with PMSF for 2 min and then pulse-labeled for 2.5 min in the presence of PMSF. SDS-PAGE of immune precipitates from subcellular fractions of cells pulse-labeled in the presence of PMSF demonstrated the presence of Pp60 in both the PF and CE and little, if any, p30 (Fig. 3A and D). When cells pulse-labeled in the presence of PMSF were chased in the presence or absence of PMSF for 0.5 h, Pp60 was present in both subcellular fractions as was a small amount of p30, indicating partial inhibition of Pp60 cleavage.

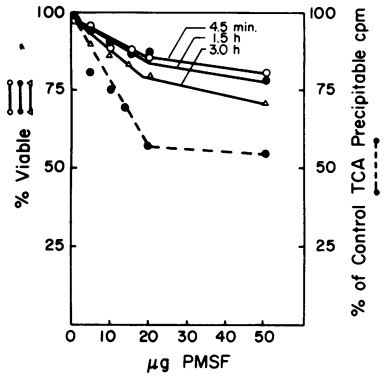

FIG. 1. *Effect of PMSF on cell viability and protein synthesis. Six aliquots of cells (50 × 10⁶ cells per aliquot) were incubated with 0, 5, 10, 15, 20, or 50 μg of PMSF per ml for 2 min and then pulse-labeled for 2.5 min with 10 μCi of [³⁵S]methionine per aliquot in the presence of PMSF. The pulse-labeling was terminated, and the cells were chased for 3.0 h in the presence of PMSF as described in the text. Cell viability was determined by trypan blue dye exclusion immediately after the pulse-label (4.5-min exposure to PMSF) and at 1.5 and 3.0 h. The effect of PMSF on protein synthesis was determined by assaying hot trichloroacetic acid-precipitable radioactivity from NP-40-disrupted cells.*

Nonspecific immune precipitation was monitored by incubating labeled subcellular fractions with 5 μg of BSA and 200 μl of anti-BSA. Figures 2D and H depict typical results obtained from subcellular fractions of pulse-labeled cells. We routinely detect only two polypeptides (labeled a and b) in all control experiments. Identical results were obtained from subcellular fractions of cells incubated with PMSF (data not shown).

Effect of L-azetidine-2-carboxylic acid on the synthesis of FeLV precursor polypeptides. We have previously demonstrated a precursor-product relationship between Pp60 and p30 (18). Several amino acid analogues have been tested for their effect on the appearance and processing of FeLV precursor proteins. Of the analogues tested (fluorophenylalanine, canavanine, and L-azetidine-2-carboxylic acid), only L-azetidine-2-carboxylic acid has yielded any detectable effect. SDS-PAGE of immune precipitates from subcellular fractions of cells pulse-labeled in the presence of L-azetidine-2-carboxylic acid demonstrated the presence of large amounts of 70,000-dalton polypeptide (Pp70) as well as reduced levels of Pp60 and p30 (Fig. 4). A comparison of these results (Fig. 4) with those seen in Fig. 2A and E indicated that the 70,000-dalton polypeptide seen in relatively low amounts in the absence of analogues was now the principal immune precipitable polypeptide when pulse-labeling was done in the presence of L-azetidine-2-carboxylic acid. This suggests that the proline analogue may be inserted into the initial translation product and subsequently inhibit the cleavage step, resulting in the formation of Pp60. Control experiments employing immune precipitation of BSA with anti-BSA yielded results similar to those shown in Fig. 2D and H (data not shown).

Immune precipitation of p30 and p70 from disrupted FeLV. The presence of an intracellular 70,000-dalton polypeptide that is both immune precipitable by anti-p30 and electrophoretically identical to virion p70 led us to examine virion p70 for p30 antigenic determinants. In a previous report (18) we demonstrated that the anti-p30 serum could only immune precipitate viral p30 from 0.5% NP-40-disrupted virus. Subsequent experiments have shown that 0.5%

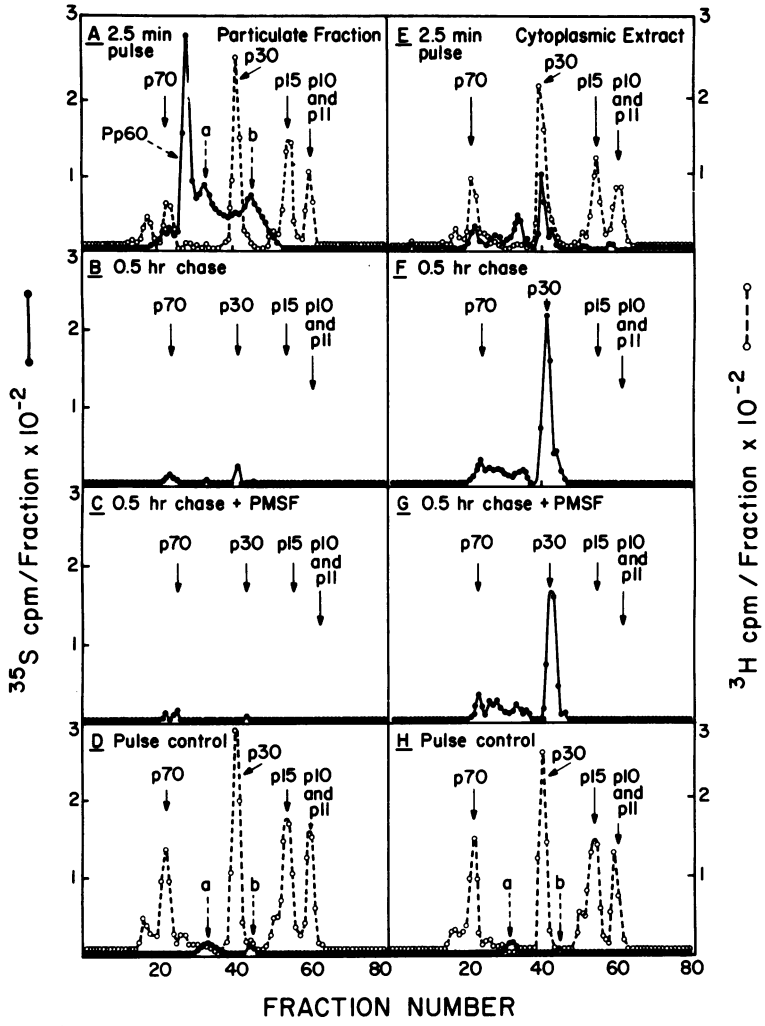

FIG. 2. *SDS-PAGE of immune precipitates from PF and CE of cells incubated with and without PMSF after pulse-labeling. A total of 200 × 10⁶ cells were pulse-labeled with 200 μCi of [³⁵S]methionine in 4 ml of growth medium. The pulse-labeled cells were divided into four aliquots, two of which were chased for 0.5 h either with or without PMSF (50 μg/ml) and the remaining aliquots which were used as a source of pulse-labeled polypeptides. PF and CE were prepared from both pulse-labeled and pulse-chase-labeled cells. Polypeptides were immune precipitated from each fraction with anti-p30 and co-electrophoresed with ³H-amino acid-labeled FeLV as described in the text. Solid arrows indicate the positions of non-glycosylated FeLV polypeptides. (A) and (E) PF and CE, respectively, from pulse-labeled cells; (B) and (F) a PF and CE, respectively, from cells chased in the absence of PMSF; (C) and (G) a PF and CE, respectively, from cells chased in the presence of 50 μg PMSF per ml; (D) and (H) SDS-PAGE of immune precipitates from a PF and CE, respectively, of pulse-labeled cells incubated with 5 μg of BSA and 200 μl of anti-BSA.*

NP-40 alone does not solubilize viral p70 to any considerable degree (data not shown).

^{14}C-amino acid-labeled FeLV was disrupted by treatment with 0.5% NP-40 and sodium deoxycholate and then immune precipitated with anti-p30. The detergent disruption procedure employed in these experiments solubilizes the major FeLV polypeptides (Fig. 5A), although only 60 to 70% of the FeLV p70 is solubilized (data not shown). SDS-PAGE of the resulting immune precipitate demonstrated two labeled FeLV polypeptides. Both FeLV p30 and p70 were immune precipitated (Fig. 5B). FeLV p70 was only partially immune precipitated (30% of the total solubilized p70 counts per minute) in this experiment. The data indicated that FeLV p70 contained some p30 antigenic determinants. Control experiments using 5 μg of BSA and 200 μl of anti-BSA showed virtually no precipitation of labeled viral proteins, indicat-

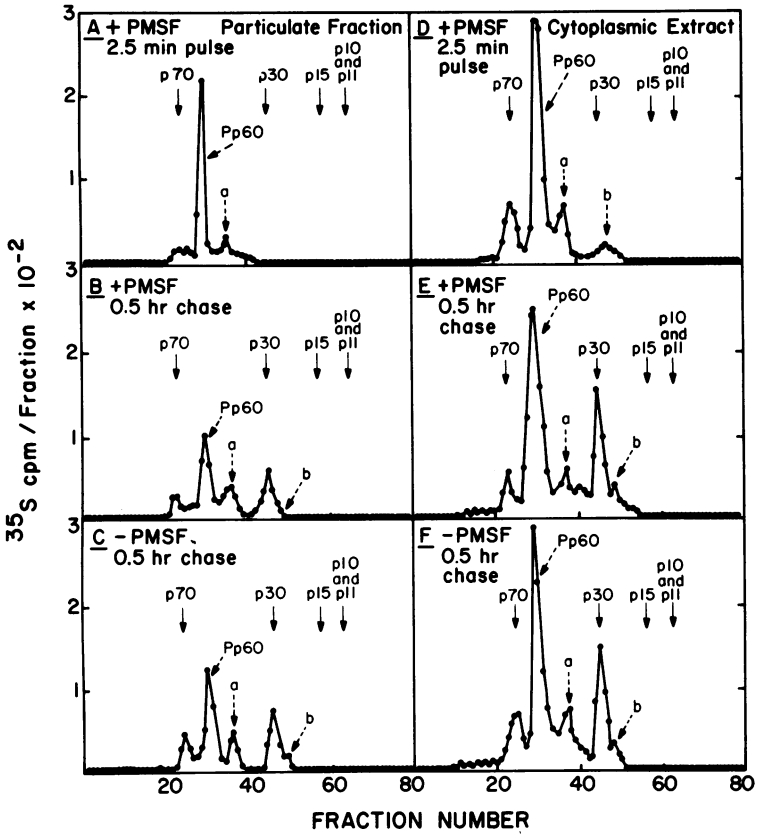

FIG. 3. *SDS-PAGE of immune precipitates from PF and CE of cells pulse-labeled with [35S]methionine in the presence and absence of PMSF. A total of 300 × 10⁶ cells were suspended in 6 ml of growth medium deficient in methionine but containing 50 mg of PMSF per ml for 2 min. The cells were then pulse-labeled for 2.5 min and divided into three aliquots, and two aliquots were chased in the presence or absence of PMSF. PF and CE were prepared and then immune precipitated with anti-p30 and electrophoresed as described in the text. (A) and (D) a PF and CE, respectively, from cells pulse-labeled in the presence of PMSF; (B) and (G) a PF and CE, respectively, from cells pulse-labeled and chased in the presence of PMSF (50 μg/ml); (C) and (F) a PF and CE, respectively, from cells pulsed in the presence of PMSF and chased in the absence of PMSF.*

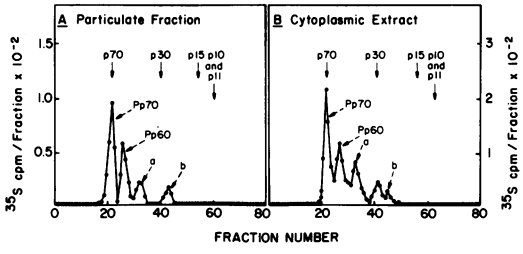

FIG. 4. *SDS-PAGE of immune precipitates from the CE and PF of cells pulse-labeled with [35S]methionine in the presence of L-azetidine-2-carboxylic acid. Prior to pulse-labeling, 100 × 10⁶ cells were incubated for 45 min in growth medium deficient in methionine and proline but containing L-azetidine-2-carboxylic acid (0.21 mg/ml). The cells were pulse-labeled for 2.5 min with 100 μCi of [35S]methionine in the presence of L-azetidine-2-carboxylic acid (0.21 mg/ml), and then a CE and a PF were prepared. The fractions were incubated with 300 μl of anti-p30 and 5 μg of disrupted FeLV. The immune precipitates were collected and co-electrophoresed with 3H-amino acid-labeled FeLV as described in the text. Solid arrows indicate the positions of labeled FeLV polypeptides.*

ing little or no nonspecific trapping (data not shown).

Tryptic peptide analysis of intracellular Pp70, Pp60, and FeLV virion p70, p30, p15, p11, and p10. Intracelular 3H-amino acid-labeled Pp70 and Pp60 tryptic peptides were subjected to high-pressure cation-exchange chromatography on a column of type P chromobeads (Fig. 6). The tryptic peptide fractions are identified by the elution pH of the peak fraction (Fig. 6). Pp70 contained 29 detectable peptide fractions, whereas Pp60 was resolved into 25

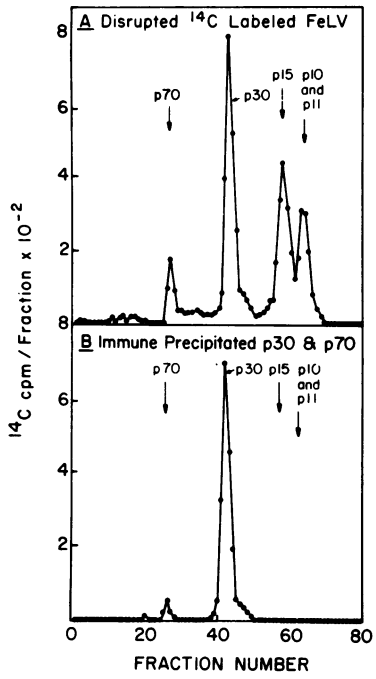

FIG. 5. *SDS-PAGE of immune precipitated p30 and p70 from detergent-disrupted FeLV. ^{14}C-labeled FeLV was prepared from 25 × 10^6 cells incubated with 25 μCi of ^{14}C-labeled amino acid mixture for 24 h in 12.5 ml of growth medium. The virus was purified as described in the text. The viral pellet was suspended in 0.6 ml of lysis buffer containing 0.5% NP-40 and 0.5% deoxycholate by 3 min of sonic oscillation in a 150-W Branson Ultrasonic Cleaner (Branson Instruments Co., Stamford, Conn.). The disrupted virus was then centrifuged for 1 h at 100,000 × g. (A) FeLV polypeptides (20,000 cpm) were precipitated with 9 volumes of acetone and 50 μg of BSA as carrier and then electrophoresed; (B) FeLV polypeptides (20,000 cpm) were immune precipitated with 100 μl of anti-p30 and electrophoresed as described in the text.*

peptide fractions. Twenty-three of the 25 peptide fractions present in Pp60 were also found in Pp70. Two Pp60 tryptic peptide fractions, with elution pH values of 3.85 and 3.94, were not detectable in the tryptic peptide profile of Pp70. Pp70 contained, in addition to the 23 Pp60 tryptic peptide fractions, 6 peptide fractions, with elution pH values of 3.28, 3.62, 3.73, 3.81, 4.12, and 4.14. Because Pp70 and FeLV virion p70 were both immune precipitable with anti-p30 (Fig. 4 and 5) and because Pp70 contained the tryptic peptide fractions of Pp60 (Fig. 5), tryptic peptide analysis of the structural proteins of FeLV was done to determine: (i) whether intracellular Pp70 and FeLV virion p70 were similar, and (ii) whether the additional tryptic peptide fractions seen in Pp70, but not in Pp60, could be assigned to any of the FeLV structural proteins.

Tryptic peptide analysis of FeLV virion p70 yielded 29 peptide fractions (Fig. 7) that were indistinguishable from the 29 peptides of intracellular Pp70. The results demonstrated that intracellular Pp70 and virion p70 (Fig. 6 and 7) were identical by tryptic peptide analysis. A total of 13 of 15 p30 tryptic peptide fractions were present in virion p70 and intracellular Pp60 and Pp70 (Fig. 6 and 7). Virion p15 contained 9 tryptic peptide fractions, of which 7 were present in virion p70 and intracellular Pp60 and Pp70 (Fig. 6 and 7). The assignment of the p15 tryptic peptide fractions with an elution pH of 3.79 remains uncertain due to the low level of radioactivity found in the p15 tryptic pepide fractions and the high level of radioactivity present in the p70 tryptic peptide fractions, with elution pH values of 3.77 and 3.80. The pH values are accurate to ±0.01 pH unit and thus could allow assignment of this p15 peptide to either the p70 peptide fractions 3.77 or 3.80. We chose to consider this peptide as not

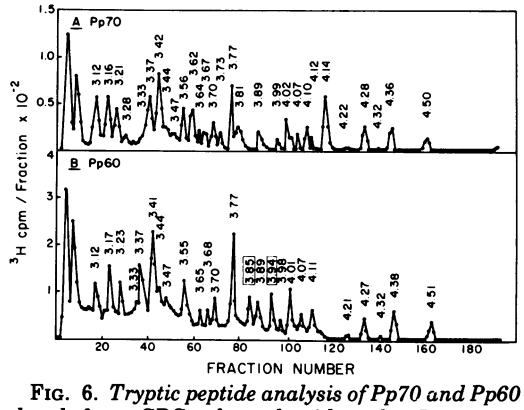

FIG. 6. *Tryptic peptide analysis of Pp70 and Pp60 eluted from SDS-polyacrylamide gels. Pp70 was prepared by SDS-PAGE of immune precipitates from the PF and CE of 500 × 10^6 cells pulse-labeled for 2.5 min with 500 μCi of ^{3}H-amino acid mixture in the presence of L-azetidine-2-carboxylic acid. Pp60 was prepared in a similar manner from the PF of 500 × 10^6 cells pulse-labeled (without L-azetidine-2-carboxylic acid) with 500 μCi of ^{3}H-amino acid mixture. Both polypeptides were eluted from gels, and tryptic peptides were prepared as described in the text. Tryptic peptides were eluted from a column of type P chromobeads, with a linear gradient of pyridine acetate (pH 3.1 to 4.9) at a flow rate of 30 ml/h. Various peptide peaks are identified by the pH of elution determined from reading pH values with a PHM 26 expanded-scale pH meter (Radiometer, Copenhagen, Denmark). Percent recovery ranged from 70 to 80% of the radioactivity eluted from polyacrylamide gels. □, Elution pH values of Pp60 tryptic peptides absent in Pp70.*

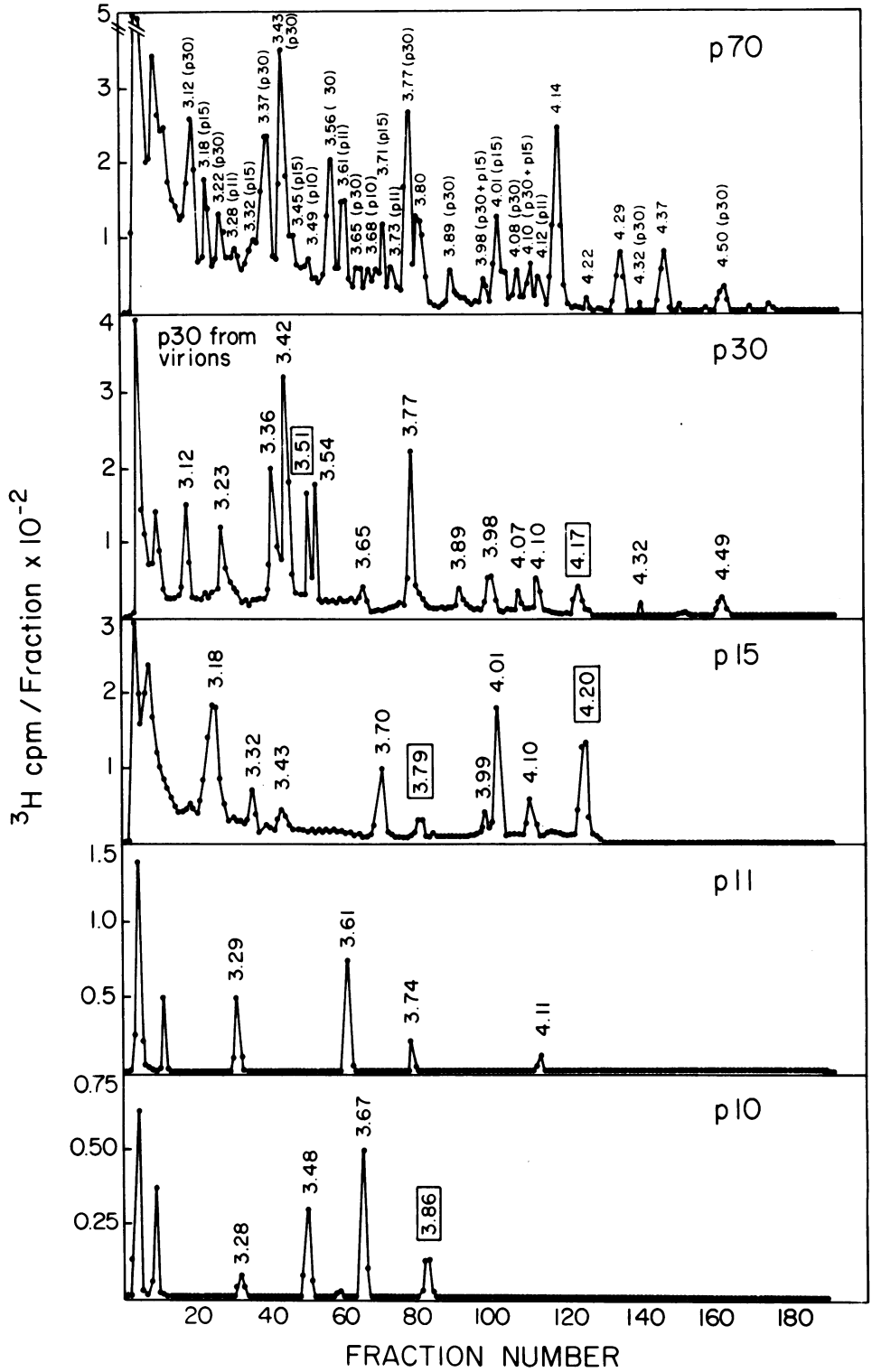

FIG. 7. *Tryptic peptide analysis of FeLV virion p70, p30, p15, p11, and p10. FeLV p70, p30, and p15 were prepared by SDS-PAGE of ^{3}H-amino acid-labeled FeLV. FeLV p11 and p10 were purified by gel filtration of labeled FeLV in GuHCl. Tryptic peptides of the five virion proteins were prepared as described in the text. Tryptic peptides were eluted and pH values were determined as described in the legend to Fig. 6. Percent recovery ranged from 60 to 80% of the radioactivity eluted from polyacrylamide gels or gel filtration columns. Symbols: □, elution pH values of tryptic peptides absent in virion p70 and Pp70 of Fig. 6; ( ), p70 tryptic peptides present in both p70 and virion proteins p30, p15, p11, and p10.*

being present within p70 because of the relatively low level of radioactivity. The tryptic peptide fractions of p11 had elution pH values of 3.29, 3.61, 3.74, and 4.11 (Fig. 7). Three of the p11 tryptic peptide fractions were present in both virion p70 and intracellular Pp70, but were absent in the Pp60 tryptic peptide profile (Fig. 6 and 7). Although the p11 peptide fraction eluting at pH 4.11 could not be assigned unequivocally due to lower radioactivity and a variation of ±0.01 pH units, the presence of a pH 4.12 peptide fraction in Pp70 and p70 that is absent in Pp60 is consistent with the other three p11 fractions. These data show that both virion p70 and intracellular Pp70 contain p11, whereas Pp60 lacks p11. Tryptic peptide analysis of virion p10 revealed four tryptic peptide fractions, with elution pH values of 3.28, 3.48, 3.67, and 3.86. Two of the p10 tryptic peptide fractions (pH values 3.48 and 3.67) were present in p70, Pp70, and Pp60, indicating the presence of p10 in all three molecules. The p10 tryptic peptide fraction with an elution pH value of 3.86 was not present in p70 or Pp70, but was present in Pp60, whereas the p10 peptide designated 3.28 is also seen in p70 and Pp70, but is absent in Pp60. We judge the presence of the p10 peptides 3.86 and 3.28 and the Pp60 peptide 3.85 (Fig. 6) to be expected anomalies of tryptic digestion of precursor and product proteins.

The tryptic peptide analysis of intracellular and virion polypeptides was done in parallel rather than by a double-label procedure, because of the relatively small amounts of p10 and p11 available from FeLV and the expense of generating large quantities of precursor polypeptides of FeLV. To help insure uniformity in this parallel analysis, all of the tryptic peptides of the proteins examined (Fig. 6 and 7) were prepared in parallel and stored as lyophilized tryptic peptides until chromatography (no longer than 3 weeks). Tryptic peptides were eluted with buffers prepared in large quantities, and the column of type P chromobeads was washed with sodium hydroxide, pyridine, and starting buffer before each run. FeLV p10 and p11 were purified by gel filtration in GuHCl (7). To determine the effect of GuHCl-treated proteins on the tryptic peptide profiles, tryptic peptides were prepared and analyzed from FeLV p30 and p15 prepared from both SDS-PAGE and gel filtrations in GuHCl. Polypeptides prepared by both procedures yielded qualitatively identical results when their tryptic peptides were analyzed by high-pressure cation-exchange chromatography (data not shown). The reproducibility of the pH values assigned to the tryptic peptides was examined from several tryptic peptide profiles of FeLV p30 and was found to vary by ±0.01 pH unit (data not shown).

Pactamycin gene ordering. The order of FeLV p30, p15, p11, and p10 within Pp70 was determined by preferential labeling of the carboxy terminal end of newly synthesized polypeptides. Our experimental approach was similar to that employed by other workers to order the proteins of poliovirus (24, 25), encephalomyocarditis virus (3), and recently avian myeloblastosis virus (AMV) (28). In our experiments pactamycin was used to selectively inhibit initiation of polypeptide synthesis, while permitting chain elongation. Cells were incubated for 30 s with 5×10^{-7} M pactamycin, a concentration requiring 10 to 15 min to completely inhibit incorporation of labeled amino acids, and then pulsed-labeled in the presence of pactamycin with ^{3}H- or ^{14}C-amino acid mixtures for 5 min. A parallel culture was pulse-labeled in the absence of pactamycin with the opposite (i.e., either ^{3}H- or ^{14}C-amino acid mixture) labeled amino acid mixture. After pulse-labeling, both cultures were chased for 20 h and released virus was purified from the growth medium. Labeled FeLV proteins were separated by gel filtration in the presence of 6 M GuHCl to allow resolution of FeLV p11 and p10, which are not resolved by SDS-PAGE (7). The relative labeling ratios were determined from the percentage of label in each protein synthesized in the presence of pactamycin over the percentage of label in each protein synthesized in the absence of pactamycin.

This experimental approach, based on an analysis of polypeptides in extracellular virions after a 20-h chase, was necessitated by the lack of high-titer monospecific antisera to FeLV p10 and p11, which is needed to effectively quantitate intracellular polypeptides. Control experiments with immune precipitation by antisera to whole virus demonstrated that the intracellular p30, p15, and p11-p10 peaks detected on SDS-PAGE shortly after a pulse were not detectable 20 h later (G. Okasinski, unpublished data). These results were consistent with our published data (18) concerning incorporation of p30 into extracellular virus in the absence of pactamycin. Immune precipitation, with antiserum to p30 and p15, of subcellular fractions of cells pulse-labeled in the presence of pactamycin and chased for 1 h resulted in no detectable Pp60 (data not shown). The results of these control experiments indicated that pactamycin did not detectably effect either the cleavage of Pp60 or the release of viral polypeptides from these cells.

Table 1 depicts the results of a pactamycin

TABLE 1. *Effect of pactamycin on the relative labeling of FeLV virion proteins*[a]

Viral protein	Relative labeling ratio[b]
p11	0.44
p15	0.65
p10	1.06
p30	1.21

[a] Cells were pulse-labeled in the presence or absence of pactamycin and then chased for 20 h. Purified virus was disrupted, and proteins were chromatographed by gel filtration in the presence of 6 M GuHCl.

[b] Ratios are the percentage of label in each protein synthesized in the presence of pactamycin over the percentage of label in each protein synthesized in the absence of pactamycin.

experiment. An examination of this data indicates that pactamycin treatment led to a preferential loss of label in p11 and p15 relative to p10 and p30. The relative labeling ratios obtained in this experiment indicate the following order for FeLV proteins: N-p11-p15-p10-p30-C.

DISCUSSION

In this communication we have presented evidence that the major non-glycosylated structural proteins of FeLV are synthesized as part of a large precursor polypeptide. The largest precursor detected in these experiments was a 70,000-dalton polypeptide, present both intracellularly (Fig. 4) and as a component of FeLV (Fig. 5, 6, and 7). Tryptic peptide analysis demonstrated that Pp70 and virion p70 were identical and that both contained the tryptic peptides of FeLV p30, p15, p11, and p10 (Fig. 6 and 7). The presence of p11 tryptic peptides in intracellular Pp70 and the absence of these tryptic peptides in Pp60 indicate that the conversion of Pp70 → Pp60 involves the release of p11 from Pp70. We have also presented evidence suggesting that the conversion of Pp70 → Pp60 can be inhibited by L-azetidine-2-carboxylic acid (Fig. 4), whereas the conversion of Pp60 → FeLV structural proteins was partially inhibited by a general protease inhibitor (Fig. 2 and 3). Pulse-labeling experiments in the presence of pactamycin indicated that the order of small FeLV structural proteins is p11-p15-p10-p30 (Table 1).

The presence of a virion-associated protein (p70) with a tryptic peptide profile identical to intracellular Pp70 indicates the incorporation of an uncleaved precursor into mature virions. A similar finding has recently been demonstrated for Rauscher leukemia virus (RLV) and RLV-infected cells (9) as well as evidence for a possible uncleaved precursor as a component of the feline leukemia virus pseudotype of Moloney sarcoma virus (19). Subviral particles prepared from FeLV contain p70 within the core of FeLV rather than as an envelope component (Behnke and Velicer, manuscript in preparation).

Although immune precipitates (using anti-p30) from pulse-labeled cells routinely contain low levels of a labeled 70,000-dalton moiety (Fig. 2 and 3) (18), this polypeptide becomes the principal immune precipitable polypeptide when cells are grown in the presence of L-azetidine-2-carboxylic acid. We attribute the routine presence of Pp70 at low levels in immune precipitates during normal pulse-labeling conditions (Fig. 2A and B) and with long-term labeling conditions (18) to reflect an intracellular level of uncleaved Pp70 that eventually becomes incorporated into FeLV as virion p70. However, a portion of this Pp70 may also be destined for further cleavage. The proline analogue L-azetidine-2-carboxylic acid could affect cleavage of Pp70 in two ways. The analogue could be incorporated directly into the cleavage enzyme and alter the activity of the enzyme, or the analogue could be incorporated into the substrate (Pp70). In our experiment the cells were preincubated with L-azetidine-2-carboxylic acid for 45 min prior to pulse-labeling (Fig. 4). Similar results are obtained when the cells are preincubated for 0, 5, and 10 min with the analogue (data not shown). If the proline analogue were altering the cleavage enzyme directly, the enzyme would have to have an extremely short half-life. The most likely alternative is that L-azetidine-2-carboxylic acid is incorporated directly into Pp70 and thus prevents the conversion of Pp70 → Pp60.

The effect of the general protease inhibitor upon the cleavage of Pp60 does not provide an unequivocal interpretation. We cannot attribute the rather dramatic changes in the levels and distribution of Pp60 (Fig. 3) to a general effect of PMSF on cell viability because the decrease in cell viability is rather small. Although PMSF does reduce the level of protein synthesis, this reduction appears to be quantitative rather than selective for viral protein synthesis. Furthermore, Fig. 3 shows that a change in the total length of exposure from 4.5 min (Fig. 3A and D plus C and F) to 34.5 min (Fig. 3B and E) did not cause any significant changes in the electrophoretic profiles. Figures 2 and 3 show that a partial inhibition of Pp60 by PMSF occurs only if PMSF is present during and shortly after the synthesis of Pp60. It is difficult to attribute the inhibition of Pp60 cleavage to a simple inactivation of a protease due to the short time frame of activity seen in

these experiments. Figure 2 shows that Pp60 becomes committed to cleavage within the first few minutes after synthesis, whereas Fig. 3 indicates that PMSF inhibition requires only a short exposure to PMSF during pulse-labeling. Because PMSF inhibition requires the presence of the protease inhibitor only during pulse-labeling, we interpret its activity to an aberration of the normal juxtaposition of a substrate Pp60 and enzyme, possibly induced by the interaction of PMSF with either Pp60 or a protease. In support of our interpretation is the change in distribution of Pp60 within the PF and CE. Under normal conditions Pp60 is limited to the PF, whereas, in the presence of PMSF, Pp60 is found in both the PF and CE immediately after pulse-labeling. Lindell has recently reported (13) that the addition of PMSF to isolated nuclei inhibits the release of RNA polymerase, which also indicates that PMSF may have additional activities aside from the protease inactivation activity. Further experimentation involving isolation of intracellular membranes and precursor polypeptides will be required to more clearly elucidate the activity of PMSF on Pp60 cleavage. Previous work with both AMV-infected cells (27, 28) and RLV-infected cells (26) has not provided evidence for any inhibition of precursor cleavage by general protease inhibitors. However, RLV precursor cleavage may be inhibited by tolylsulfonyl phenylalanyl chloromethyl ketone and carbobenzyloxy-phenylalanyl chloromethyl ketone (R. Arlinghaus, personal communication). Our results with FeLV-infected cells may reflect differences in experimental design (i.e., concentrations of protease inhibitor) or in differing susceptibilities of avian, murine, and feline oncornavirus precursor polypeptides.

Tryptic peptide analysis of intracellular precursor polypeptides and virion structural proteins (Fig. 6 and 7) demonstrates that the major structural non-glycosylated polypeptides of FeLV are synthesized as part of a 70,000-dalton precursor. The presence of p30, p15, p11, and p10 tryptic peptides within the Pp70 molecule and the absence of the p11 tryptic peptides in Pp60 indicate that the conversion of Pp70 → Pp60 results in the formation of p11. These data would suggest that Pp70 contains the p11 moiety at either its NH_2-terminal or COOH-terminal end and that removal of p11 results in the formation of Pp60.

The results of pactamycin gene ordering experiments (Table 1) confirm the suggested position of p11 within Pp70. These results combined with the tryptic peptide analysis and immune precipitation experiments lead us to propose

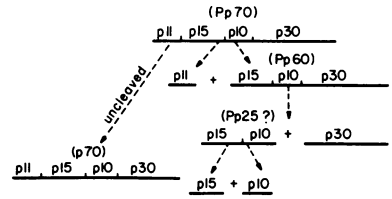

FIG. 8. *Proposed scheme for the generation of the non-glycosylated structural proteins of FeLV. The order of FeLV proteins within Pp70 was that obtained from pactamycin gene ordering experiments. The existence of Pp25 is uncertain.*

the cleavage scheme shown in Fig. 8 for the generation of FeLV non-glycosylated structural proteins. The first step in this scheme involves the generation of p11 and Pp60 from Pp70. Because p11 is the NH_2-terminal moiety, this cleavage may occur on a nascent polypeptide and thus could explain the detection of only low levels of Pp70 under normal labeling conditions (Fig. 2A and B). The absence of any anti-p30 immune precipitable intracellular polypeptides other than Pp60 and p30 (Fig. 2) argues that p30 is generated by direct cleavage of Pp60. The order of FeLV structural proteins also supports this interpretation in that p30 is carboxy-terminal on the Pp60 moiety. The generation of p30 from Pp60 should result in the formation of a precursor polypeptide of about 25,000 daltons (Pp25) containing p15 and p10. Immune precipitation of subcellular fractions from pulse-labeled cells using anti-p15 under the same conditions used for anti-p30 also yields Pp70 and Pp60, but not a 25,000-dalton precursor (G. Okasinski, unpublished data). This observation leads us to believe that p15 and p10 may be rapidly generated directly from a would-be Pp25. The final feature of this proposed generation of structural proteins is the incorporation of Pp70 into assembled virus as p70.

A comparison of the arrangement and proposed processing of FeLV structural proteins with that recently reported for AMV reveals some distinct differences. The order of AMV structural proteins within a 76,000-dalton precursor (Pr76) is N-p17-p28-p14-C, with the position of p10 being uncertain (28). The major group-specific antigen (p28) is located internally within AMV Pr76, whereas the corresponding FeLV protein is COOH-terminal on FeLV Pp70. Vogt et al. (28) have proposed that the first cleavage of Pr76 results in precursors of 66,000 and 12,000 daltons (Pr66 and Pr12), the latter arising from cleavage at the COOH-terminal end and ultimately giving rise to AMV p14. Thus AMV precursor processing may differ from that proposed for FeLV in that the

first cleavage is at the COOH-terminal end in the former and at the NH_2-terminal end in the latter. Avian Pr76, thus, requires complete or almost complete synthesis prior to the first cleavage, whereas feline Pp70 could be cleaved as a nascent polypeptide. These differences could explain the relative ease of avian Pr76 isolation and the absence of abundant feline Pp70 under normal labeling conditions. Pr66 is believed to be further processed to a 60,000-dalton precursor (Pr60). At this point the two proposed schemes become very similar. It is believed that AMV p28, p10, and p17 are generated directly from AMV Pr60. This process may be analogous to the generation of FeLV p30, p15, and p10 from FeLV Pp60. Although Vogt et al. (28) have detected a 32,000-dalton precursor (Pr32), it is not believed to be an essential step in the generation of AMV proteins. Recent published work with RLV precursor proteins has also failed to detect precursors of RLV p30 smaller than 65,000 daltons (1, 16, 26), although this point is still under investigation and there is some unpublished evidence for smaller precursors (Arlinghaus, personal communication). Thus FeLV appears to differ from AMV in both the arrangement of structural proteins in precursor polypeptides and in the initial cleavage from precursors, but may be similar in the processing of 60,000-dalton precursor polypeptides. The incorporation of a precursor polypeptide into assembled virions also appears to be a unique phenomenon of FeLV and RLV (9), while absent from AMV.

The identification of precursor polypeptides in FeLV-infected cells is consistent with similar reports of precursor polypeptides in AMV-infected cells (18, 27) and in RLV-infected cells (9, 16, 26). Precursor polypeptides similar to those identified in AMV- and RLV-infected cells have also been synthesized in cell-free translation systems (6, 10, 17, 29). In addition to the presence of precursor polypeptides in cells productively infected with oncornaviruses, these precursors have in every instance been indicated to have a membrane association (9, 16, 18, 26–28). Although the synthesis of oncornavirus proteins in productively infected cells results in the formation of high-molecular-weight, membrane-associated precursor polypeptides, the size of detectable precursors and their subsequent processing appears to vary. When amino acid analogues have been utilized to inhibit precursor processing, the results have varied from no effect in AMV-infected cells (27) with fluorophenylalanine and canavanine to inhibition of formation of a 65,000-dalton precursor in RLV-infected cells with canavanine and no effect with fluorophenylalanine and L-azetidine-2-carboxylic acid (26). In our hands L-azetidine-2-carboxylic acid results in an accumulation of Pp70 (Fig. 5), whereas canavanine and fluorophenylalanine yield no detectable alterations in electrophoretic profiles of immune precipitable precursor polypeptides (G. Okasinski, unpublished data). Our finding of partial inhibition of Pp60 cleavage by a general protease inhibitor (Fig. 3 and 4) is, to our knowledge, the first such published report and serves to point out what may be fundamental differences in various oncornavirus precursor polypeptides and/or in host cell influences on precursor processing.

The synthesis and processing of RLV precursor polypeptides as discerned by two different groups of workers using different host cells may best exemplify the effects of host cells on precursor processing (16, 26). One group of workers, using JLS-V9 cells infected with RLV, could identify presumptive precursor polypeptides with molecular weights of 82,000 and 65,000. These precursor polypeptides are believed to be precursors of RLV p30, p15, and p12b (26). Results of immune precipitation of intracellular protein with anti-RLV indicated that very little, if any, RLV p30 was present intracellularly and suggested that production of p30 from precursor polypeptides is immediately followed by assembly of RLV p30 into extracellular virus. When JLS-V16 cells were examined for the presence of RLV precursor polypeptides, four large polypeptides were identified, with molecular weights of 200,000, 90,000, 80,000, and 65,000, respectively (1, 16). JLS-V16 cells, however, do contain readily identifiable intracellular RLV p30, suggesting relatively larger pools of intracellular RLV p30. These differences in the number of identifiable precursor polypeptides, their size, and their processing rates (as judged by intracellular RLV p30 levels) all argue that due caution must be exercised in developing a general mode of oncornavirus protein synthesis and processing.

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