JOURNAL OF VIROLOGY, Sept. 1977, p. 787-798
Vol. 23, No. 3

Printed in U.S.A.

Common Precursor for Rauscher Leukemia Virus gp69/71, p15(E), and p12(E)

W. L. KARSHIN, L. J. ARCEMENT, R. B. NASO, AND R. B. ARLINGHAUS*

Department of Biology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

Received for publication 24 May 1977

Rauscher murine leukemia virus glycoprotein gp69/71 and non-glycosylated p15(E) are synthesized by way of a 90,000-dalton precursor glycoprotein, termed Pr2*a*+*b*. Peptide mapping experiments showed that Pr2*a*+*b* contains all the tyrosine-containing tryptic peptides of gp69/71. Two additional tyrosine-containing tryptic peptides in Pr2*a*+*b* that are not detected in gp69/71 are found in p15(E). Thus, gp69/71 and p15(E) peptide sequences account for all the tyrosine tryptic peptides of Pr2*a*+*b*. The gene order of the two proteins was determined by pulse-labeling infected cells in the presence and absence of pactamycin at concentrations of the inhibitor that prevent initiation of translation, but not elongation. The gene order was found to be: $_2$HN-gp69/71-p15(E)-COOH. A newly identified major viral protein, termed p12(E), migrates in sodium dodecyl sulfate-polyacrylamide gels in the "p12" region. It is related to p15(E) as determined by tryptic mapping experiments. p15(E) and p12(E) are not phosphorylated, and both can be separated from phosphoprotein p12 by guanidine hydrochloride-agarose chromatography. p12(E) and p15(E) elute in the void volume fraction, whereas phosphoprotein p12 elutes between p15 and p10. The two p12 proteins can also be separated from each other by two-dimensional gel electrophoresis involving isoelectric focusing in the first dimension and sodium dodecyl sulfate-gel electrophoresis in the second dimension.

RNA tumor virus (type C) genomes code for several viral proteins, including the reverse transcriptase, the envelope proteins, and four lower-molecular-weight structural proteins termed p30, p15, p12, and p10. The regions of the viral genomic RNA coding for these classes of proteins have been termed "*pol*," "*env*," and "*gag*," respectively (3). Previous work from this laboratory has provided evidence (1a, 2, 21) that the envelope protein gp69/71 and a non-glycosylated envelope protein termed p15(E) are contained in a fucose-deficient glycoprotein precursor termed Pr2*a*+*b* (90,000 daltons). A similar precursor has been observed by Shapiro et al. (25) and Famulari et al. (7). In this report, we provide definitive evidence that shows that gp69/71 and p15(E) are formed by synthesis and cleavage of Pr2*a*+*b* and that p15(E) is apparently C terminal to gp69/71 in Pr2*a*+*b*. A newly identified viral protein, p12(E), is derived from p15(E), probably by proteolytic cleavage.

MATERIALS AND METHODS

Cells and virus. Rauscher murine leukemia virus (RLV)-infected NIH Swiss mouse embryo cells (JLS-V16) and BALB/c spleen-thymus RLV-infected cells (JLS-V5) were used in these studies (20). Intracellular precursors were isolated from infected JLS-V16 cells, whereas mature viral proteins were obtained from virus produced in JLS-V5 cells. The culture medium was a modified Eagle formula containing 10% fetal calf serum (29). Cells were grown in 2-quart (ca. 1.89 liters) roller bottles and were 80% confluent before use. Virus was purified as described previously (29).

Labeling of cells and virus. Cells were rinsed in warm Hanks solution and pulse-labeled at 37°C as indicated. For chase incubations, the radioactive medium was removed, the cell sheet was rinsed with Hanks solution, and incubation was continued in complete growth medium. For tryptic mapping experiments, precursors were isolated from a roller-bottle culture, which was pulse-labeled for 20 min in 25 ml of Hanks solution. Virus was labeled for 48 h in a roller-bottle culture in growth medium containing 1× Eagle amino acids. Under these conditions, lower concentrations of amino acids severely depress virus production. To obtain sufficient quantities of labeled polypeptides for tryptic mapping, infected cells and virus were labeled as follows: 2.5 mCi of ^{3}H-labeled amino acids (reconstituted protein hydrolysate) supplemented with 0.625 mCi each of [^{3}H]lysine (10 Ci/mmol) and [^{3}H]arginine (10 Ci/mmol) or 1.0 mCi of ^{14}C-labeled amino acids (reconstituted protein hydrolysate) supplemented with 0.25 mCi each of [^{14}C]lysine (300 Ci/mol) and [^{14}C]arginine (312 Ci/mol). To obtain arginine- and

lysine-labeled precursors and mature proteins, 0.5 to 1.0 mCi each of [^{14}C]arginine and [^{14}C]lysine or 2.5 mCi each of [^{3}H]arginine or [^{3}H]lysine were used. For tyrosine labeling, 3.0 mCi of [^{14}C]tyrosine and 18 mCi of [^{3}H]tyrosine were used. For methionine labeling, 3.0 mCi of [^{35}S]methionine and 20 mCi of [^{3}H]methionine were used. ^{32}P labeling of virus was performed at one-half Eagle phosphate concentration in a 2-quart (ca. 1.89 liters) roller bottle for 48 h with 25 mCi of [^{32}P]PO_4.

Immune precipitation. The preparation of cytoplasmic extracts and rabbit anti-RLV serum were as described previously (1a). Cell lysis was performed in lysis buffer containing 0.5% Nonidet P-40 (NP-40; Particle Data Laboratories, Ltd.) and 0.5% sodium deoxycholate (1a). For preparative purposes, pulse-labeled cells in 2-quart (ca. 1.89 liters) roller bottles were lysed in 10 ml of lysis buffer. For analytical experiments, 2-ounce (ca. 60 ml) prescription bottles were used, and lysis was done in 2 ml of lysis buffer. The intracellular precursors, including Pr2*a*+*b*, were isolated by direct immune precipitation with anti-RLV serum. A 2-ml portion of cytoplasmic extract was mixed with 0.4 ml of antiserum. The immune precipitates were collected by centrifugation at 10,000 × *g* for 10 min and washed three times with immune buffer containing 0.5% NP-40, 0.5% sodium deoxycholate, 0.02 M Tris-hydrochloride (pH 7.5), and 0.05 M NaCl (20).

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done on 1.5-mm-thick gel slabs by use of the buffer system described by Laemmli (14). The gels were subjected to fluorography as described (4). To obtain a linear response to radioactivity, the X-ray films were preflashed (15).

Purification of viral proteins and precursors. Viral proteins gp69/71 and p15(E) were purified by SDS-PAGE fractionation of virus labeled for 48 h. In addition, p15(E), p12(E), and p12 were purified from virus by guanidine hydrochloride-agarose column chromatography (9); p15(E) and p12(E) eluted in the void volume. The column fractions were dialyzed against 0.05 M NH_4HCO_3 (pH 8.5), freeze-dried, and further fractionated by SDS-PAGE. Pr2*a*+*b* from infected cells was purified by SDS-PAGE (8%) from immune precipitates obtained by direct immune precipitation with anti-RLV serum from 20-min pulse-labeled infected cells.

Tryptic mapping. Tryptic digestion was carried out by incubating a dried slab gel band, which was cut up into small pieces in 2 ml of 0.05 M NH_4HCO_3 (pH 8.5) containing 50 μg of trypsin per ml for 16 h at 37°C. An additional 50 μg of trypsin per ml was added, and the incubation was continued for an additional 4 h. Tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Biochemicals Corp.) was stored at 5 mg/ml in 0.01 M HCl containing 1 mM $CaCl_2$ at −20°C. The gel pieces were removed by filtration through 0.45-μm cellulose nitrate filters, and the supernatant containing the soluble tryptic peptides was lyophilized. The peptides were dissolved in 0.2 to 0.4 ml of buffer A (31), and the sample was clarified by centrifugation. A yield of 60 to 70% was obtained from the dry slab gel through this stage of the procedure. The sample was applied to a jacketed column (1 by 23 cm) of Chromo Bead type P resin (Technicon), and the column was eluted with an exponential pyridine-acetic acid gradient with increasing pH. The fractions of 3.33 ml were collected at 50°C at a flow rate of 20 ml/h. The fractions were taken to dryness at 100°C, dissolved in 0.5 ml of 0.01 N HCl, and then mixed with 5 ml of Triton X-100 containing counting fluid (29). Generally an 80 to 90% yield of radioactivity was recovered from the column.

Two-dimensional gels. Two-dimensional PAGE was carried out as described by O'Farrell (22). The first-dimension isoelectric focusing was performed in 2.5-mm cylindrical diameter gels. The second dimension was performed on an 11.25%, 1.5-mm-thick polyacrylamide slab gel by the Laemmli system (14).

RESULTS

Purification of precursor and mature viral proteins. The glycoprotein precursor Pr2*a*+*b* was purified from infected cells pulse-labeled with [^{14}C]tyrosine for 20 min. A cytoplasmic extract was prepared, and immune precipitation with antiserum to disrupted virus was used to isolate virus-specific precursors. The immune precipitate was fractionated by electrophoresis on a preparative 8% polyacrylamide slab gel (Fig. 1). The anti-RLV serum precipitates viral structural proteins and their polyprotein precursors (1, 1a). It also precipitates several proteins present in uninfected cells identified in Fig. 1 as h1, h2, and h3 (1a). Antibodies to these proteins can be removed from the anti-RLV serum by prior treatment with excess uninfected cell proteins (1a). Normal serum precipitates $^{1}/_{20}$ to $^{1}/_{10}$ the amount of radioactive protein from such a pulse-labeled extract. A few of the radioactive bands seen in normal serum precipitates comigrated with viral precursors, but their amounts were $^{1}/_{20}$ to $^{1}/_{10}$ that observed in anti-RLV immune precipitates.

Viral glycoprotein gp69/71 was obtained from purified virus preparations labeled for 48 h with [^{3}H]tyrosine. The viral proteins were fractionated by preparative electrophoresis in 11.25% polyacrylamide slab gels (Fig. 2). The gp69/71 fraction and the Pr2*a*+*b* fraction were found to be greater than 95% pure as determined by reanalysis on an SDS gel.

Viral proteins P15(E) and p12(E) were purified by a two-step procedure, the first step being guanidine hydrochloride-agarose column chromatography. In such a procedure, p15(E) and p12(E) elute in the void volume of the column eluate. The void-volume peak was dialyzed against 0.05 M NH_4HCO_3 and freeze-dried. The

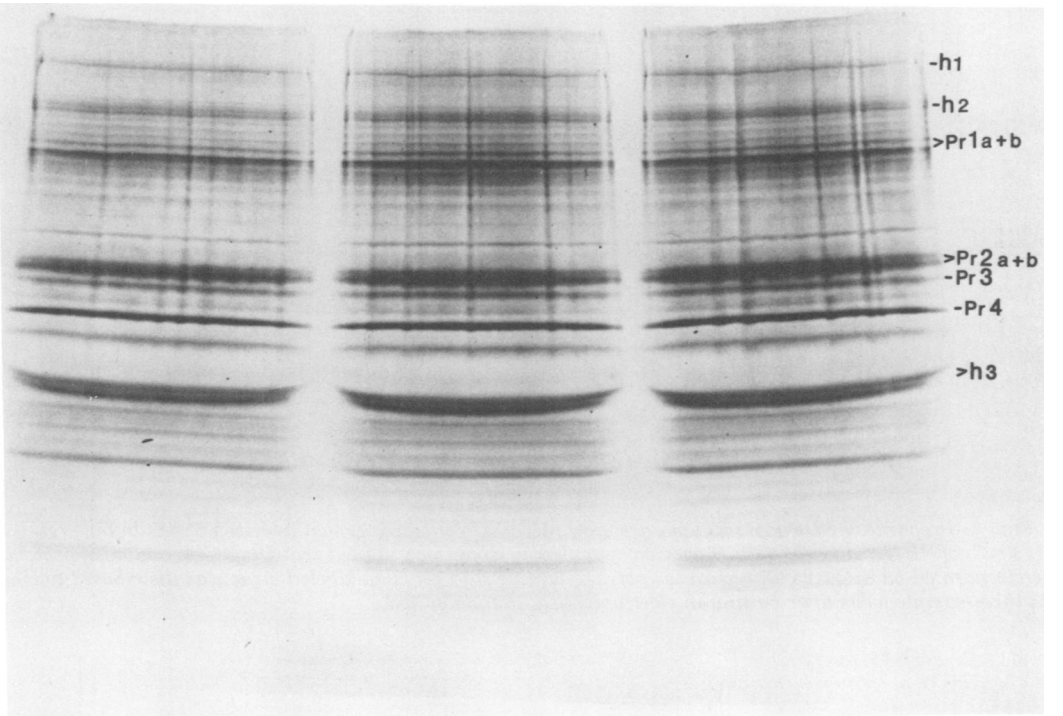

FIG. 1. *Preparative SDS-PAGE of the immune precipitated intracellular glycoprotein precursors. Virus-infected JLS-V16 cells were pulse-labeled for 20 min with 3.0 mCi of [^{14}C]tyrosine in 5 ml of Hanks balanced salt solution. The cytoplasmic extract was precipitated with antiserum to disrupted RLV. The immune precipitate was isolated and fractionated on an 8% preparative polyacrylamide slab gel containing three wells. The immune precipitate from one roller bottle was distributed among the three sample wells after boiling in electrophoresis sample buffer. The protein bands labeled h1, h2, and h3 are normal cellular proteins (1a).*

material was dissolved in 1% SDS and 1% mercaptoethanol and then fractionated by electrophoresis in 11.25% polyacrylamide slab gels. Proteins p15(E) and p12(E) were the only low-molecular-weight components of the guanidine hydrochloride void-volume fraction, although small quantities of unidentifiable high-molecular-weight proteins were also present. This procedure enabled us to effectively separate p12(E) from p12. The latter quantitatively elutes between p15 and p10 on the guanidine hydrochloride-agarose column. Our studies indicated that p12(E) purified in this way was free of the phosphorylated p12 protein as determined by ^{32}P labeling (see Fig. 13 and 14). When virus was applied directly to SDS-polyacrylamide gels, the "p12" region of the gel did contain p12(E), but not phosphorylated p12 (not shown). Trace amounts of the p12 phosphoprotein, seen as two bands, migrated slightly slower than p15(E). It is not known where in the gel fractions the remainder of the p12 phosphoprotein migrated. Thus, phosphoprotein p12 is apparently not solubilized by boiling virus in 1% SDS and 1% 2-mercaptoethanol.

It should be mentioned that p15(E) purified by SDS-PAGE only (as in Fig. 2) was as pure as that purified by the above two-step procedure, as determined by tryptic mapping experiments.

Presence of gp69/71 and p15(E) peptide sequences in Pr2*a*+*b*. We have previously shown that Pr2*a*+*b* and gp69/71 contain many tryptic peptides that comigrate on ion-exchange columns (21). However, the elution profiles of each tryptic digest were complex. To simplify the complexity of the tryptic digests and the subsequent comparisons of the peptide sequences, we chose to label proteins with radioactive tyrosine. Figure 3 shows ion-exchange profiles in which [^{14}C]tyrosine-labeled Pr2*a*+*b* tryptic peptides were mixed with tryptic peptides of [^{3}H]tyrosine-labeled gp69/71. The results (Fig. 3) clearly showed that Pr2*a*+*b* shares many tyrosine-containing tryptic peptides with gp69/71. Figure 3 also shows that there are at least two additional tyrosine-containing tryptic peptides in Pr2*a*+*b* that are not found in gp69/71 (see arrows in Fig. 3). An analysis of a [^{14}C]tyrosine-labeled p15(E) tryptic digest mixed with a [^{3}H]tyrosine-labeled Pr2*a*+*b* di-

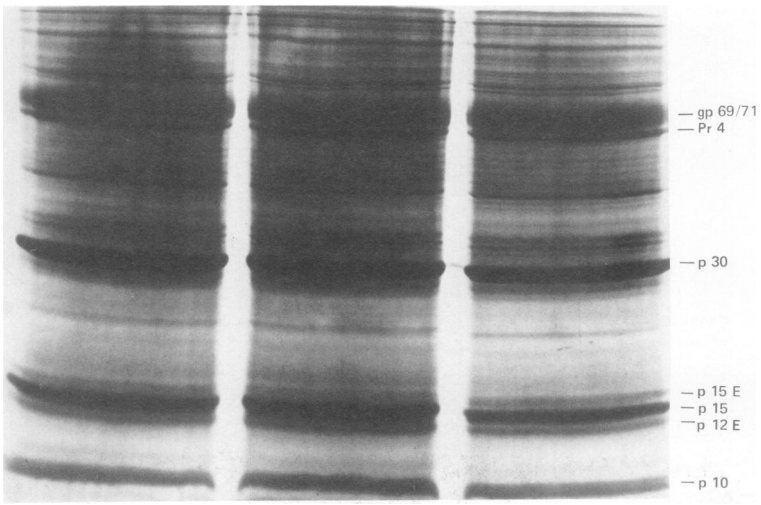

FIG. 2. *Preparative SDS-PAGE of mature viral proteins. Virus was grown in JLS-V5 cells in the presence of 18 mCi of [^{3}H]tyrosine for 48 h. Virus was purified by isopycnic gradient centrifugation, and viral proteins were separated on an 11.25% preparative polyacrylamide slab gel. The labeled virus was distributed among the three sample wells after boiling in electrophoresis sample buffer.*

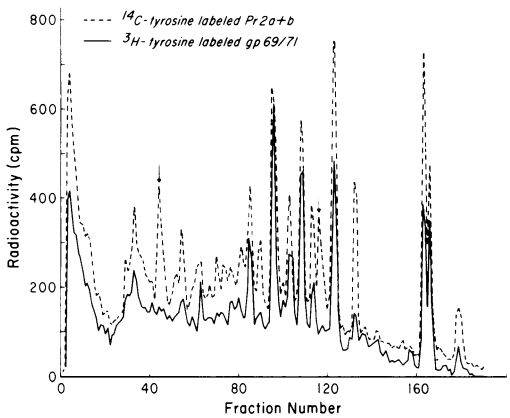

FIG. 3. *Ion-exchange chromatography of tryptic digests of tyrosine-labeled Pr2a+b and gp69/71. The arrows show the two additional tyrosine-containing tryptic peptides in Pr2a+b that are not found in gp69/71.*

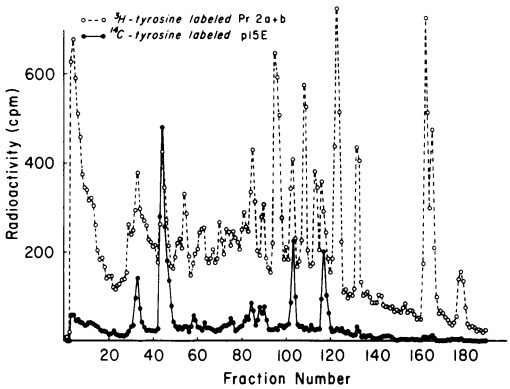

FIG. 4. *Ion-exchange chromatography of tryptic digests of tyrosine-labeled Pr2a+b and p15(E). The p15(E) used in this experiment was purified from virus by SDS-PAGE as described in the legend to Fig. 2.*

gest (Fig. 4) clearly showed that these two additional tryptic peptide fractions are derived from p15(E). We conclude from these results that p15(E) and gp69/71 are cleavage products of Pr2*a*+*b*.

We have previously reported that gp69/71 is deficient in methionine relative to the *env* precursor Pr2*a*+*b* (21). Further studies have clarified this. The results show that Pr2*a*+*b* contains an acidic methionine-containing tryptic peptide fraction characteristic of gp69/71 (data not shown) and a neutral methionine-tryptic peptide fraction characteristic of p15(E) and p12(E) (1a, 21). We note, however, that gp69/71 did contain small amounts of the methionine-tryptic peptide fraction that is characteristic of p15(E). Also, the labeling of gp69/71 with methionine does vary from experiment to experiment. In addition, preparations of anti-gp69/71 also vary in their ability to recognize p15(E). Some anti-gp69/71 sera, supplied by the Virus Cancer Program, do not recognize p15(E) (21), whereas others do precipitate small amounts of p15(E) (R. B. Naso, unpublished data). We conclude from these studies that gp69/71 does contain methionine, but that p15(E) [or p12(E)] sequences are still associated with gp69/71 in

small but variable amounts.

It was also determined that glucosamine-labeled tryptic peptides of gp69/71 eluted from the Chromo Bead cation-exchange column before fraction 30, the majority of the radioactivity eluting in the flow-through material (data not shown). Thus, none of the glucosamine-containing tryptic peptides contributed to any similarities between Pr2*a*+*b* and gp69/71. This is also important to consider since p15(E) is not labeled with either glucosamine or fucose.

The order of the proteins in Pr2*a*+*b* was examined by labeling cells in the presence and absence of pactamycin, a drug that selectively inhibits the initiation of translation at 5×10^{-7} M, but not elongation (5, 28, 30). By incubating cells with radioactive amino acids in the presence of 5×10^{-7} M pactamycin, one preferentially labels the C-terminal ends of nascent polypeptide chains. Thus, it is possible to determine whether p15(E) is N or C terminal in Pr2*a*+*b*. Figure 5 shows that pactamycin treatment for 30 to 60 s before and during the pulse-labeling reduced gp69/71 formation in the chase, whereas p15(E) labeling was increased relative to that in the absence of drug (Fig. 5E to G). The drug also inhibited protein synthesis about 50%, which was compensated for in the gel analyses by applying equal amounts of radioactivity in the pulse (17,000 cpm) and chase (10,000 cpm).

Pulse-labeling experiments showed that pactamycin treatment significantly affected the pattern of synthesis of precursor proteins (Fig. 5B to D), as indicated by the increase in Pr3 relative to Pr4 and Pr1*a*+*b* relative to Pr3 and Pr4. Since Pr3 (≈80,000 daltons) and Pr4 (≈65,000 daltons) have been shown to share tryptic peptide sequences and antigenic determinants with all of the *gag* proteins (1) and that *gag* and *pol* antigenic determinants and p30 tryptic peptides are present in Pr1*a*+*b* (≈200,000 daltons) (1a, 12), these results could be interpreted as follows: (i) that the additional peptide sequences in Pr3 are C terminal to those of Pr4 and (ii) that Pr3 is N terminal to *pol* in Pr1*a*+*b*. However, no firm conclusions can be drawn from these results since pactamycin may have some effect on the processing of precursors during short pulse-labeling experiments.

The results of another pulse-chase experiment performed in the presence and absence of pactamycin are shown in Fig. 6. The results were quantitated by preparing densitometer tracings of the autoradiographs. The results clearly showed that p15(E) is increased relative to gp69/71 in the presence of pactamycin compared with that obtained in the absence of pactamycin. The ratio of p15(E) to gp69/71 was 0.39 in the control and 0.76 in the presence of pactamycin.

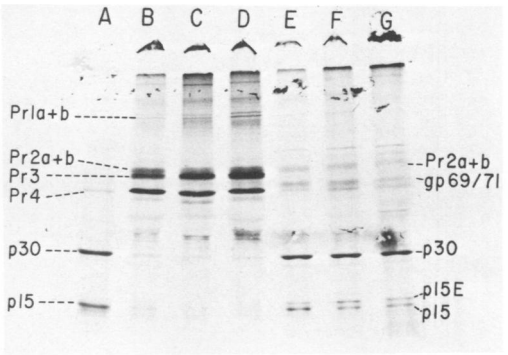

FIG. 5. *Synthesis of RLV proteins in the presence of pactamycin. Virus-infected JLS-V16 cells in 2-ounce (ca. 60 ml) glass bottles were pulse-labeled with 200 μCi of ^{14}C-amino acid hydrolysate in 4 ml of Hanks balanced salts for 15 min at 37°C. Pactamycin, when present, was added at 30 or 60 s prior to the label at a concentration of 5×10^{-7} M. Chases were for 3 h in 8 ml of complete growth medium. (A) Marker virus; (B) 15-min pulse; (C) 15-min pulse with 30-s preincubation with pactamycin; (D) 15-min pulse with 60-s preincubation with pactamycin; (E) 15-min pulse followed by 3-h chase; (F) 30-s preincubation with pactamycin followed by a 15-min pulse and a 3-h chase; (G) 60-s preincubation with pactamycin followed by a 15-min pulse and a 3-h chase. Cytoplasmic extracts were prepared, and immunoprecipitation was performed with anti-RLV serum. The immune precipitates were analyzed by SDS-PAGE on a 6 to 12% gradient slab gel. The dried slab gel was exposed to preflashed X-ray film (DuPont Cronex 2DC). Equal amounts of radioactivity as immune precipitates were applied to each sample well: (B, C, and D) about 20,000 cpm; (E, F, and G) about 10,000 cpm.*

Relationship of p15(E) and p12(E). As discussed above, p15(E) and p12(E) are both present in the void volume of the guanidine hydrochloride-agarose column. Previous studies have shown that they share a methionine-containing tryptic peptide fraction that is also present in Pr2*a*+*b* (1a, 21). To examine further the relationship between p15(E) and p12(E), the proteins were labeled with arginine and lysine, and their tryptic digest chromatograms were compared (Fig. 7). The results indicate that p15(E) and p12(E) share many tryptic peptides. p15(E) is slightly more complex than p12(E) and contains at least two additional tryptic peptides (Fig. 7, fractions ≈105 and ≈125). These observations are consistent with the derivation of p12(E) from p15(E) by proteolytic cleavage.

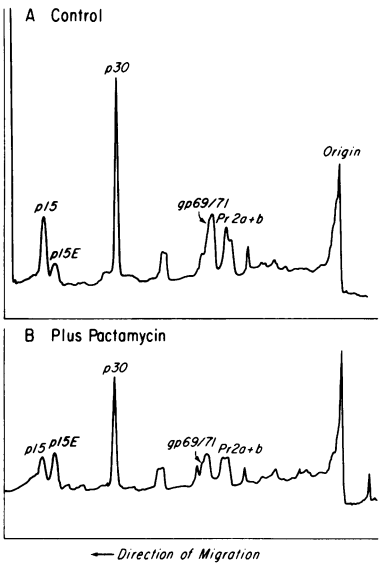

FIG. 6. *Densitometer tracings of autoradiographs of viral proteins isolated from cells treated with and without pactamcyin. Virus-infected JLS-V16 cells were pulse-labeled for 15 min with a ^{14}C-amino acid protein hydrolysate (0.5 mCi) in 5 ml of Hanks balanced salt solution. In (B) the cells were preincubated for 30 s in 5 × 10^{-7} M pactamycin prior to pulsing with ^{14}C-labeled amino acids in the same concentration of pactamycin. In (A) the pulse was done in the absence of pactamycin. Both pulses were then chased for 1 h in complete growth medium in the absence of pactamycin. Cytoplasmic extracts were prepared, and immune precipitation was performed with anti-RLV serum. The immune precipitates were fractionated on a 6 to 12% gradient polyacrylamide gel slab. The dried gel was processed by fluorography, and the appropriate X-ray film strips were analyzed at 590 nm on a spectrophotometer equipped with a gel scanner.*

This conclusion is supported by pulse-chase studies of virus-infected cells. A protein that comigrates with virion p15(E) is present in relatively large amounts in pulse-labeled cells compared with intracellular p12(E). The relative amounts of p12(E) increase during chases at the apparent expense of p15(E) (21; Fig. 8 of reference 11). Furthermore, p12(E) is found in larger amounts than p15(E) in virions released from infected JLS-V16 or JLS-V5 cells.

p12(E), a protein distinct from phosphoprotein p12. Some confusion arose when we reported that a protein in the p12 region of SDS gels shared peptide sequences with the glycoprotein precursor Pr2*a*+*b* (1a). The confusion was amplified when Aaronson and colleagues reported that the p12 protein eluting from the guanidine hydrochloride-agarose column between p15 and p10 was present in a 65,000-dalton polyprotein precursor to the *gag* proteins (26). We examined the possibility that there may be, in fact, two proteins with the molecular weight of p12. We have found this to be the case and have identified them as p12 and p12(E). The first indication that this was the case came from studies with antisera raised against the guanidine hydrochloride-purified

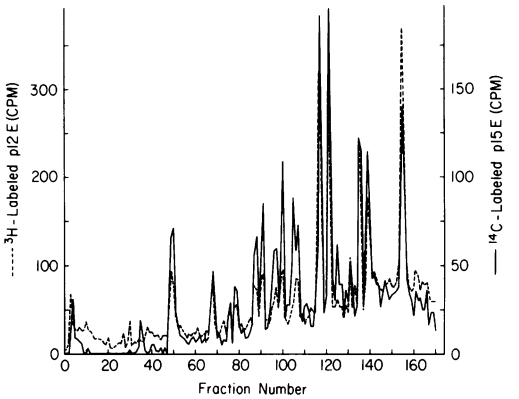

FIG. 7. *Ion-exchange chromatography of tryptic digests of arginine- and lysine-labeled p15(E) and p12(E).*

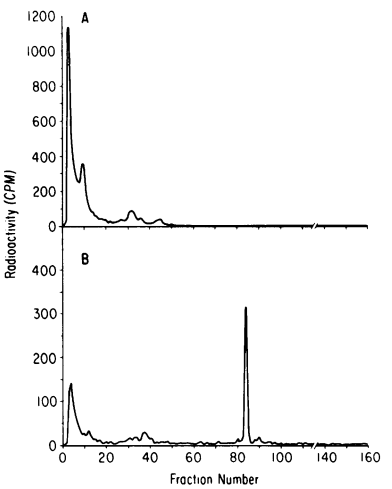

FIG. 8. *Ion-exchange chromatography of tryptic digests of methionine-labeled p12 and p12(E). Both p12 (A) and p12(E) (B) were isolated by guanidine hydrochloride-agarose column chromatography. They were further purified by SDS-PAGE and autoradiographed, and the bands were cut out and minced into small pieces. The proteins were extracted in 0.1% SDS in 0.05 M NH_4HCO_3 (pH 8.5) at 37°C for 18 to 22 h. The proteins were then precipitated, oxidized, and treated with trypsin by the procedure of Vogt et al. (31). The samples were then applied to a Chromo Bead P ion-exchange column (1 by 23 cm) as described in the text. The proteins were labeled with [^{35}S]methionine.*

p12 protein (termed p12), kindly supplied by C. J. Scherr of the National Cancer Institute. Such antisera precipitated *gag* precursors Pr3 (80,000 daltons) and Pr4 (≃65,000 daltons), but not the glycoprotein precursor Pr2*a*+*b* (≃90,000 daltons). This sharply contrasted with our tryptic mapping data (1a), which clearly showed that a p12 protein [p12(E)], isolated by SDS-PAGE, shared a methionine-containing tryptic peptide fraction with Pr2*a*+*b*, but which was not present in *gag* precursors Pr3 and Pr4. As indicated above, the *gag* p12 or phosphoprotein p12 of RLV is not solubilized by denaturation in SDS-PAGE sample buffer at 100°C. The net result is that p12 does not comigrate with p12(E) unless it is first isolated on a guanidine column (see Fig. 13 and 14).

We then set out to compare the p12 proteins that are easily separated by guanidine hydrochloride-agarose column chromatography. We first established by radiolabeling experiments that p12 and p12(E) contained methionine. The two "p12" preparations, each labeled with methionine, were purified as follows. [^{35}S]methionine-labeled virus was denatured in 8 M guanidine at 100°C for 5 min and applied to a guanidine hydrochloride-agarose column (9). The profile was similar to that shown in the experiment described in the legend to Fig. 12A (solid line). The void-volume reaction and the p12 peak were pooled separately, dialyzed extensively versus 0.05 M NH_4HCO_3, and lyophilized. The residue was denatured in SDS-PAGE sample buffer, and the samples were fractionated on 11.25% polyacrylamide gels. The void-volume fraction contained p15(E) and p12(E) (neither one being phosphorylated; see Fig. 14). The p12 region of the column contained a phosphoprotein (see Fig. 13). The methionine-containing tryptic peptides of these two proteins were compared (Fig. 8). The results showed that p12 contained only an acidic methionine-containing tryptic peptide fraction that eluted in the ion-exchange flow-through eluate (Fig. 8A), whereas p12(E) contained a more neutral methionine-containing tryptic peptide (Fig. 8B). Variable amounts of acidic flow-through material were present in p12(E). An analysis of the total amino acid-labeled tryptic maps of the two p12 preparations showed vast differences. The p12 eluting between p15 and p10 from the guanidine hydrochloride-agarose column (see Fig. 12A, dashed line) contained mostly acidic tryptic peptides that eluted in the flow-through fraction, and one basic tryptic peptide fraction was detected (Fig. 9). This basic peptide was also present in the *gag* precursor Pr4 (Fig. 9). Peptide maps of p12(E) (Fig. 7) and p12 (Fig. 9) showed large differences, indicating that the

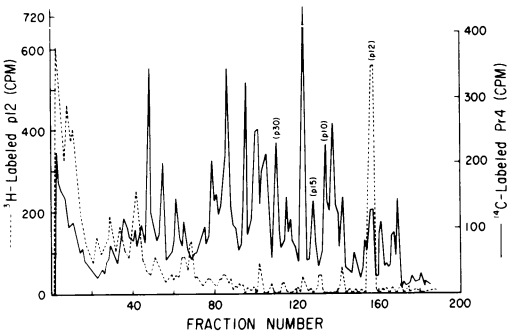

FIG. 9. *Ion-exchange chromatography of tryptic digests of amino acid-labeled p12 and Pr4. The proteins were labeled with a mixture of amino acids supplemented with arginine and lysine, purified, and digested with trypsin as described in the text.*

two proteins are vastly different in chemical structure. We have previously shown, (1) that p12 shares tryptic peptides with *gag* precursors Pr3 through Pr5 (≃80,000 to 55,000 daltons). Thus, the two p12 proteins have different peptide sequences, and they originate from different precursors, one [p12(E)] from the *env* precursor and the other (p12) from the *gag* precursor.

Analysis of viral proteins by two-dimensional gel electrophoresis. In an effort to better separate the two p12 proteins, we fractionated the viral proteins by the two-dimensional gel procedure of O'Farrell (22). This procedure consists of electrophoresis through an isoelectric focusing medium in the first dimension followed by SDS-PAGE in the second dimension. The results yielded a number of interesting findings. Surprisingly, p30 exhibited a large amount of charge heterogeneity, much more so than p15, p12, and p10. Fourteen to fifteen spots, ranging from pH 6 to pH 7+, were observed in the p30 molecular weight range (Fig. 10). Similar charge heterogeneity has been observed with VP-1 protein of simian virus 40 (23). The structural protein VP-1 satellite bands were in part attributed to in vivo alterations (23). The p15(E) region had about four species, three of which have the same molecular size. p15 had one major species migrating in the pH 7 region; p10 had one species in the pH 7+ region. The p12 region contained one major species at pH 7, a few minor ones between pH 6.5 and 7, and a faint p12 species detectable in the pH 5 region. This latter minor species was found to be phosphoprotein p12, whereas those at about pH 7 were attributable to p12(E). This was determined by analyzing the guanidine hydrochloride column void-volume fraction [containing p15(E) and p12(E)]

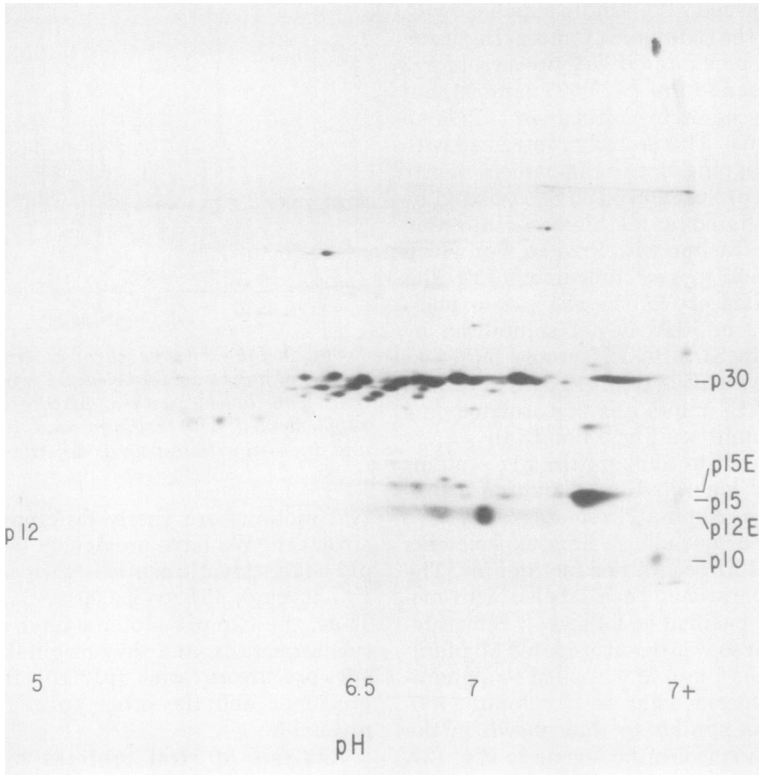

FIG. 10. *Two-dimensional electrophoresis of RLV labeled with ^{14}C-amino acids, supplemented with [^{14}C]arginine and [^{14}C]lysine. The procedure is described in the text and is the method of O'Farrell (22). The mixture of ampholines used was 4 parts of the pH 5 to 7 and 1 part of pH 3 to 10, yielding a pH range from slightly below pH 5 to greater than pH 7. The origin is at the right of the figure.*

and the p12 fraction (Fig. 11A and B). It is clear that the p12 protein species migrating on the guanidine column between p15 and p10 migrates in two-dimensional gels in the p12 region, with an isoelectric point of pH 5 (Fig. 11B), and that the p12 protein [p12(E)] remaining in the void volume of the guanidine column had an isoelectric point above pH 7 (Fig. 11A). Figure 11A shows some material from the void volume in p15(E) and p12(E) molecular weight range that remained at the origin of the first-dimension isoelectric focusing gel. This was not the case when intact virus was analyzed directly (Fig. 10). Thus, the void-volume aggregate apparently was not entirely solubilized during the first-dimension analysis. These results indicate that p12 is an acidic protein, whereas p12(E) is more neutral.

Phosphoprotein p12 and non-phosphorylated p12(E). Since murine type C p12 proteins have been reported to be phosphorylated (24), we examined the possibility that p12 is phosphorylated, as is suggested by its low isoelectric point. In these studies we took advantage of the fact that RLV p12 contains methionine, whereas p15 and p10 are methionine-free (Fig. 12A). Virus was separately labeled with a [^{3}H]arginine-lysine mixture and with [^{35}S]methionine. Appropriate amounts of each purified virus preparation were mixed together, denatured, and analyzed by chromatography on a 6 M guanidine hydrochloride-agarose column (Fig. 12A). The results clearly showed that p15 and p10 lacked methionine, whereas p12 contained methionine. The presence of methionine in p12 and its absence in p15 and p10 allowed us to determine whether p12 is phosphorylated. In this experiment RLV was labeled separately with [^{3}H]methionine and [^{32}P]PO_4 and then mixed together in suitable proportions. The virus preparation was treated differently than usual prior to chromatography on the guanidine column. In this case virus was incubated for 2 h in 0.5% Triton X-100 containing 25 μg of pancreatic RNase per ml. This procedure hydrolyzes the ^{32}P-labeled viral

RNA, but also results in some proteolysis (not dependent upon the presence of RNase) since virion Pr4 (the *gag* precursor of ≈65,000 daltons) is specifically destroyed by this treatment (11). Analysis of the RNase-treated preparation by guanidine hydrochloride-agarose column chromatography gave the profile shown in Fig. 12B. ^{32}P radioactivity was present in the void-volume fraction and in the p12 region (the latter as two peaks) as well as in the low-molecular-weight fraction (less than 10,000). Since p15 and p10 do not contain methionine and p12 does, [^{3}H]methionine and ^{32}P peaks in the p12 region must be p12 proteins. The smaller peak may result from proteolysis of the higher-molecular-weight *gag* precursor protein Pr4. We note that p30 appears intact and lacks ^{32}P radioactivity, as do p15 and p10.

Fractions from the void-volume peak and the major p12 peak were pooled, respectively, and analyzed on 11.25% SDS-polyacrylamide gels (Fig. 13 and 14). The results showed that the major p12 peak eluting from the guanidine column in fractions 96 to 101 was homogeneous and contained radioactive phosphorous (Fig. 13). p15(E) and p12(E) from the void-volume fraction of the column were resolved by SDS-PAGE, and neither contained radioactive phosphorous (Fig. 14). The minor p12 peak from fractions 102 to 108 of the guanidine hydrochlo-

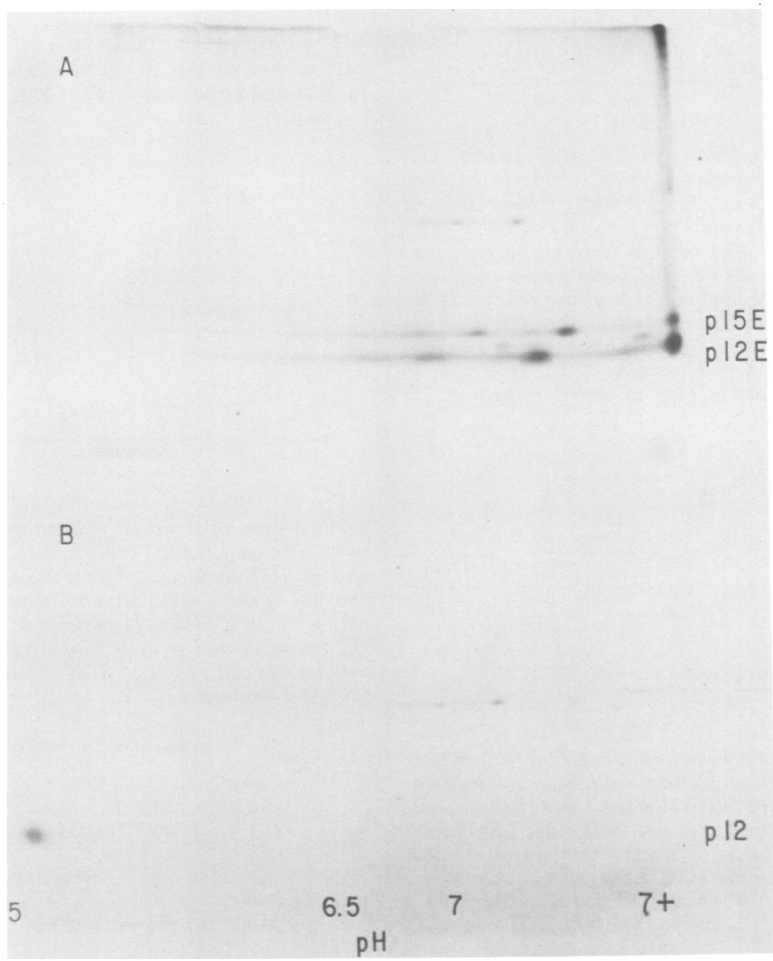

FIG. 11. *Two-dimensional electrophoresis of the guanidine hydrochloride-agarose column chromatography-purified void and p12 regions. The virus was labeled with ^{14}C-amino acids supplemented with [^{14}C]arginine and [^{14}C]lysine. (A) is the void fraction, and (B) is the p12 region that elutes between p15 and p10. The column fractions were dialyzed against 0.05 M NH_4HCO_3 (pH 8.5) and lyophilized. The two-dimensional procedure is described in the text and is the method of O'Farrell (22). The origin is at the right of the figure.*

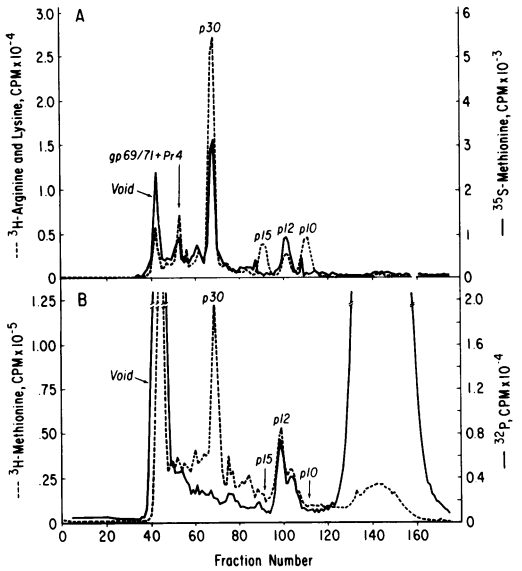

FIG. 12. *Guanidine hydrochloride-agarose column chromatography of labeled virus. In (A) one virus preparation was labeled with [^{3}H]arginine and [^{3}H]lysine and the other was labeled with [^{35}S]methionine, and the two were mixed in appropriate amounts. In (B) one virus preparation was labeled with [^{3}H]methionine and the other was labeled with [^{32}P]PO_4, and the two were mixed in appropriate amounts. The latter preparation was pretreated prior to chromatography with 0.5% Triton X-100 and pancreatic RNase, as stated in the text.*

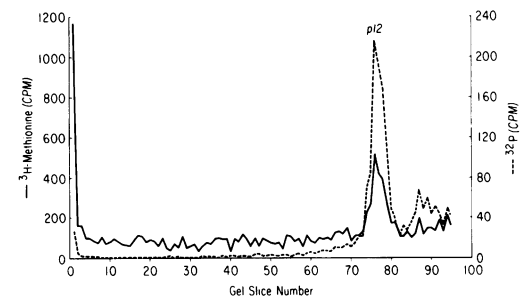

FIG. 13. *SDS-polyacrylamide gel of p12 region from a guanidine hydrochloride-agarose column. p12, labeled with [^{3}H]methionine and [^{32}P]PO_4 and isolated from the guanidine hydrochloride-agarose column above, was electrophoresed on an 11.25% SDS-polyacrylamide slab. After slicing into 1-mm cross-section strips, each gel slice was treated with 5 ml of NCS-based counting fluid (20) and analyzed.*

ride column (Fig. 12B) migrated on SDS gels slightly faster than did the major p12 peak and also contained ^{32}P radioactivity (not shown). These data indicate that p12 is phosphorylated, confirming earlier work of others (24), and that p15(E) and p12(E) are not phosphorylated.

DISCUSSION

The results presented in this paper provide strong evidence that RLV glycoprotein gp69/71 and a non-glycosylated viral protein termed p15(E) are formed by synthesis and cleavage of a 90,000-molecular-weight glycosylated precursor that we have identified as Pr2*a*+*b*. Similar results by others (7, 25) have confirmed our initial findings (1a, 21).

SDS-PAGE patterns of viral proteins labeled with radioactive glucosamine and fucose showed that gp69/71 is readily labeled, but no evidence was found for the incorporation of label into proteins migrating in the p15(E) or p12(E) region of the gel (10, 21). Thus, p15(E) and p12(E) appear to have little or no carbohydrate.

The data presented in this paper and in our earlier reports (1a, 21) have made it possible for us to propose a model (Fig. 15). In this model

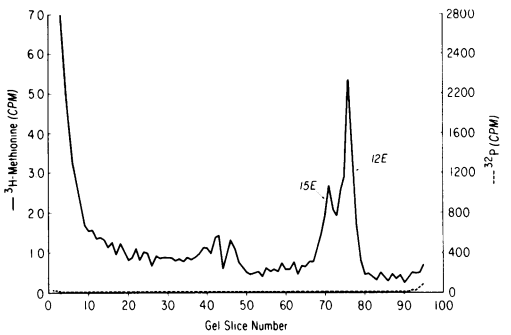

FIG. 14. *SDS-polyacrylamide gel of void region isolated from a guanidine hydrochloride-agarose column. The void region, labeled with [^{3}H]methionine and [^{32}P]PO_4 and isolated from the guanidine hydrochloride-agarose column above, was electrophoresed on an 11.25% SDS-polyacrylamide slab gel. After slicing into 1-mm cross-section strips, each gel slice was treated with 5 ml of NCS-based counting fluid (20) and analyzed.*

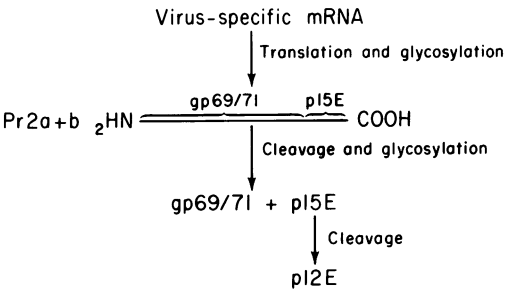

FIG. 15. *Model showing the order of the env proteins within the precursor Pr2a+b and the subsequent cleavage pattern from this precursor to complete env proteins.*

gp69/71 and p15(E) exist in tandem in Pr2*a*+*b*. We have tentatively ordered the two proteins within Pr2*a*+*b* by using the drug pactamycin. Treatment of cells with 5×10^{-7} M pactamycin allows preferential labeling of the C-terminal ends of growing nascent polypeptide chains (5, 28, 30) by inhibiting the initiation of new polypeptide chains while allowing elongation to proceed. The results (Fig. 5 and 6) indicate that p15(E) is C terminal to gp69/71.

Viral protein p15(E) has been reported to be present on the virion surface (8). Thus, the antigenic sites of p15(E) exposed on the virion envelope are sufficient to mediate immunoprecipitation of intact virus (7, 8). High-titer antiserum against this protein, however, does not strongly neutralize virus (8). p15(E) has similarities to a protein termed HA_2 associated with the envelope of influenza virus (16). HA_2 is a subunit of influenza virus hemagglutinin and is poorly glycosylated in comparison to the second subunit HA_1 (16). Furthermore, HA_2, like p15(E) and p12(E), aggregates in guanidine hydrochloride (16), and both HA_1 and HA_2 are derived from a common precursor (13, 17).

It is not at all clear why there are two polypeptides in the precursor fraction Pr2*a*+*b* as well as in the mature viral glycoprotein. Our initial tryptic mapping studies indicate that Pr2*a* is very similar to Pr2*b*. The same can be said for gp69 and gp71 (L. J. Arcement, unpublished data). The observed minor differences in the tryptic digest ion-exchange profiles of Pr2*a* and Pr2*b* may not be attributable solely to glycosylation differences because the glucosamine-labeled tryptic peptides of these glycoproteins elute in the void volume of the cation-exchange column under the conditions employed (Arcement, unpublished data).

Strand and August (27) originally isolated and purified the RLV glycoprotein gp69/71. This material is treated by them as a single protein. Our results suggest that only the gp71 is labeled with fucose (21; Naso, unpublished data), suggesting that the only difference between gp69 and gp71 is the degree and amount of glycosylation. Our tryptic peptide mapping results are also consistent with this interpretation. Our peptide mapping results also show that relatively small but significant amounts of p15(E) can be present in the gp69/71 region of a polyacrylamide gel. We have also observed that the intensity of the gp69/71 region in an autoradiogram varies from experiment to experiment (21) when labeling infected cells with radioactive methionine. These data together with the fact that different preparations of anti-gp69/71 serum vary in their ability to recognize p15(E) suggest that p15(E) can become variably associated with gp69/71 during isolation of proteins.

Viral glycoprotein gp45 (6, 19) was not studied in this report. In our hands this glycoprotein is present in small but variable amounts in virions, and it is labeled to a minor extent with radioactive glucosamine (10, 20, 21). Long-labeled virus (16 h with [^{14}C]glucosamine) had a significant peak of gp45 (10), whereas 5- to 6-h glucosamine-labeled virus contained little or no gp45 (21). McLellan and August (18) could not convincingly detect gp45 on the virus envelope, whereas gp69/71 was readily observed. Molecules of 45,000 molecular weight were observed, but they appear to be minor components and were precipitated by anti-gp69/71 serum (18). Other studies have indicated that gp45 may be a subglycosylated form of gp69/71 (H. Marquardt and S. Oroszlan, Fed. Proc. 35:1610, 1976).

Another interesting finding in these studies is the existence of two viral p12 proteins. One viral protein (p12) is acidic, having an isoelectric point of about pH 5, and it is phosphorylated; the other [p12(E)] has a more neutral isoelectric point and is not phosphorylated. We have termed the acidic phosphoprotein p12 and the neutral protein p12(E) because of its relationship to p15(E). We have been able to separate the two p12 proteins by chromatography on 6 M guanidine hydrochloride-agarose columns. p12 elutes between p15 and p10, whereas p12(E) elutes in the void volume of the column as an aggregate. However, p12 is not readily observed in the SDS-PAGE system, except for minor amounts in the 17,000-molecular-weight region. This minor component can readily be observed with ^{32}P-labeled virus. If virus is treated with NP-40 and RNase prior to SDS-PAGE, minor amounts of this phosphoprotein migrate in the p12(E) region of the gel (W. L. Karshin, unpublished data). Also, p12 isolated by chromatography on guanidine hydrochloride-agarose columns migrates in the same region as p12(E) on SDS gels (Fig. 13 and 14). The question remains as to why large amounts of p12 are obtained by guanidine hydrochloride-agarose column chromatography, whereas direct SDS-PAGE of virions yields little p12. Possibly p12 is incompletely solubilized or denatured by treatment with SDS-mercaptoethanol at 100°C, whereas the conditions of the guanidine hydrochloride column are capable of denaturing p12.

Viral protein p12 is present in the *gag* precursor polyproteins Pr3, Pr4, and Pr5 (1), whereas p12(E) shares tryptic peptide sequences with p15(E) and *env* precursor Pr2*a*+*b* (1a, 2, 21). Our results (Fig. 7 and reference 11) indicate that p12(E) is a cleavage product of

p15(E). The importance of this cleavage in virus structure or function is not known.

ACKNOWLEDGMENTS

This work was supported by Public Health Servic contract CP-61017 and grant G-429 from The Robert A. Welch Foundation.

We thank Elena Leroux and James Syrewicz for expert technical assistance.

LITERATURE CITED

1. **Arcement, L. J., W. L. Karshin, R. B. Naso, and R. B. Arlinghaus.** 1977. 'gag' polyprotein precursors of Rauscher murine leukemia virus. Virology 81:284–297.

1a. **Arcement, L. J., W. L. Karshin, R. B. Naso, G. A. Jamjoom, and R. B. Arlinghaus.** 1976. Biosynthesis of Rauscher leukemia viral proteins: presence of p30 and envelope p15 sequences in precursor polypeptides. Virology 69:763–774.

2. **Arlinghaus, R. B., R. B. Naso, G. A. Jamjoom, L. J. Arcement, and W. L. Karshin.** 1976. Animal virology, p. 689–716. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), ICN-UCLA Symposium on Molecular and Cellular Biology, vol. iv. Academic Press Inc., New York.

3. **Baltimore D.** 1974. Tumor viruses. Cold Spring Harbor Symp. Quant. Biol. 39:1187–1200.

4. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83–88.

5. **Butterworth, B. E., and R. R. Rueckert.** 1972. Gene order of encephalomyocarditis virus as determined by studies with pactamycin. J. Virol. 9:823–828.

6. **Duesberg, P., G. Martin, and P. Vogt.** 1970. Glycoprotein components of avian and murine RNA tumor viruses. Virology 41:631–646.

7. **Famulari, N. G., D. L. Buchhagen, H. D. Klenk, E. Fleissner.** 1976. Presence of murine leukemia virus envelope proteins gp70 and p15(E) in a common polyprotein of infected cells. J. Virol. 20:501–508.

8. **Ihle, J., M. Hanna, W. Schafer, G. Hunsmann, D. Bolognesi, and G. Huper.** 1975. Polypeptides of mammalian oncornaviruses. III. Localization of p15 and reactivity with natural antibody. Virology 58:449–456.

9. **Ikeda, H., W. Hardy, Jr., E. Tress, and E. Fleissner.** 1975. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. V. Identification of a new murine viral protein, p15(E). J. Virol. 16:53–61.

10. **Jamjoom, G., W. L. Karshin, R. B. Naso, L. J. Arcement, and R. B. Arlinghaus.** 1975. Proteins of Rauscher murine leukemia virus: resolution of a 70,000-dalton, nonglycosylated polypeptide containing p30 peptide sequences. Virology 68:135–145.

11. **Jamjoom, G. A., R. B. Naso, and R. B. Arlinghaus.** 1976. Selective decrease in the rate of cleavage of an intracellular precursor to Rauscher leukemia virus p30 by treatment of infected cells with actinomycin D. J. Virol. 19:1054–1072.

12. **Jamjoom, G. A., R. B. Naso, and R. B. Arlinghaus.** 1977. Further characterization of intracellular polyprotein precursors of Rauscher leukemia virus. Virology 78:11–34.

13. **Klenk, H. D., C. Scholtissek, and R. Rott.** 1972. Inhibition of glycoprotein biosynthesis of influenza virus by D-glucosamine and 2-deoxy-D-glucose. Virology 49:723–734.

14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

15. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335–341.

16. **Laver, W. G.** 1971. Separation of two polypeptide chains from the hemagglutinin subunit of influenza virus. Virology 42:275–288.

17. **Lazarowitz, S. G., R. W. Compans, and P. W. Choppin.** 1971. Influenza virus structural and nonstructural proteins in infected cells and their plasma membranes. Virology 45:830–843.

18. **McLellan, W. L., and J. T. August.** 1976. Analysis of the envelope of Rauscher murine oncornavirus: in vitro labeling of glycopeptides. J. Virol. 20:627–636.

19. **Moroni, C.** 1972. Structural proteins of Rauscher leukemia virus and Harvey sarcoma virus. Virology 47:1–7.

20. **Naso, R. B., L. J. Arcement, and R. B. Arlinghaus.** 1975. Biosynthesis of Rauscher leukemia viral proteins. Cell 4:31–36.

21. **Naso, R. B., L. J. Arcement, W. L. Karshin, G. A. Jamjoom, and R. B. Arlinghaus.** 1976. A fucose-deficient glycoprotein precursor to Rauscher leukemia virus gp69/71. Proc. Natl. Acad. Sci. U.S.A. 73:2326–2330.

22. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007–4021.

23. **O'Farrell, P. Z., and H. M. Goodman.** 1976. Resolution of Simian virus 40 proteins in whole cell extracts by two-dimensional electrophoresis: heterogeneity of the major capsid protein. Cell 9:289–298.

24. **Pal, B. K., R. M. McAllister, M. B. Gardner, and P. Roy-Burman.** 1975. Comparative studies on the structural phosphoproteins of mammalian type C viruses. J. Virol. 16:123–131.

25. **Shapiro, S. Z., M. Strand, and J. T. August.** 1976. High molecular weight precursor polypeptides to structural proteins of Rauscher murine leukemia virus. J. Mol. Biol. 107:459–477.

26. **Stephenson, J. R., S. R. Tronick, and S. A. Aaronson.** 1975. Murine leukemia virus mutants with temperature-sensitive defects in precursor polypeptide cleavage. Cell 6:543–548.

27. **Strand, M., and J. T. August.** 1976. Structural proteins of ribonucleic acid tumor viruses. J. Biol. Chem. 251:559–564.

28. **Summers, D. F., and J. V. Maizel, Jr.,** 1971. Determination of the gene sequence of poliovirus with pactamycin. Proc. Natl. Acad. Sci. U.S.A. 68:2852–2956.

29. **Syrewicz, J. J., R. B. Naso, C. S. Wang, and R. B. Arlinghaus.** 1972. Purification of large amounts of murine ribonucleic acid tumor viruses in roller bottle cultures. Appl. Microbiol. 24:488–498.

30. **Taber, R., D. Rekosh, and D. Baltimore.** 1971. Effect of pactamycin on synthesis of poliovirus proteins: a method for genetic mapping. J. Virol. 8:395–401.

31. **Vogt, V. M., R. Eisenman, and H. Diggelmann.** 1975. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. J. Mol. Biol. 96:471–493.