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Characterization of 40,000- and 25,000-Dalton Intermediate Precursors to Rauscher Murine Leukemia Virus *gag* Gene Products

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Under steady-state labeling conditions, Rauscher murine leukemia virus-infected NIH Swiss mouse cells contain at least three major polyproteins derived from the viral *gag* gene. They have molecular weights of 65,000, 40,000, and 25,000. They have been termed pPr65^{gag}, Pr40^{gag}, and pPr25^{gag}. pPr65^{gag} has been shown by a number of laboratories to be composed of all four core proteins (p15, pp12, p30, and p10). In this paper, Pr40^{gag} was found to contain p30 and p10 antigenic determinants and peptide sequences, whereas pPr25^{gag} was found to contain p15 and pp12. Pr40^{gag} and pPr25^{gag} are rapidly labeled precursor proteins that were detectable early in pulse-chase experiments. Both precursors disappeared during the later stages of the chase period concurrent with the appearance of the mature viral core proteins. pPr65^{gag} and pPr25^{gag} were found to be phosphorylated, pPr25 having a higher specific activity of ^{32}P than pPr65. In spite of this, peptide mapping studies, as well as the identification of the phosphorylated amino acid residues of pPr65, pPr25, and pp12, indicated that the same sites are phosphorylated regardless of whether the precursors or the mature pp12 are examined.

The *gag* gene of mammalian type C retroviruses encodes the genetic information for four proteins that are found in the interior of these viruses. In the murine system these proteins have been identified as p30, p15, pp12, and p10, and they are generally referred to as viral core proteins (3). Previously published results from several laboratories have shown that Rauscher murine leukemia virus (R-MuLV) core proteins are synthesized by way of a high-molecular-weight precursor polyprotein of about 65,000 daltons, termed Pr65^{gag} (1, 2, 4, 20, 23). A similar size precursor has been observed in Moloney murine leukemia virus-infected cells (24). Three size classes of candidate intermediate core protein precursors have also been detected in R-MuLV-infected cells. They have molecular weights of 50,000 to 60,000, 40,000, and 25,000 (1, 4). To shed more light on the nature of these polypeptides, particularly their relationship to the processing of Pr65^{gag} to mature viral proteins, we have examined cytoplasmic extracts from [^{32}P]phosphate-labeled and radioactive amino acid-labeled infected cells by immunoprecipitation with various monospecific antisera raised against viral proteins. Immunoprecipitates were subsequently analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The results, combined with peptide mapping studies, clearly showed that p30 and p10 were found in a 40,000-molecular-weight precursor, termed Pr40^{gag}, whereas viral phosphoproteins p12 and p15 were derived from a 25,000-molecular weight phosphorylated precursor, termed pPr25^{gag}. The results are consistent with a model that involves cleavage of phosphorylated Pr65^{gag} to Pr40^{gag} and pPr25^{gag}. The intermediate precursors are then processed to yield the four viral core proteins.

MATERIALS AND METHODS

Cells, virus, and antisera. NIH Swiss mouse embryo cells (JLS-V16) infected with R-MuLV were used in this study (15). Virus was purified as described by Syrewicz et al. (22). Monospecific antisera were obtained from the Office of Program Resources, Viral Oncology Branch, National Institutes of Health, Bethesda, Md.

Labeling of cells. Pulse-chase experiments were performed by incubating replicate cultures of infected cells for 7.5 min at 37°C in Earle balanced salt solution containing 2% dialyzed fetal calf serum and 100 μCi of [^{3}H]leucine per ml. The chase was performed after rinsing the cells in Earle salt solution (1) followed by addition of complete growth medium (22). In long labeling experiments, virus-infected cells were labeled in T-75 cultures for 20 h with 100 μCi of [^{3}H]leucine

per ml in McCoy's 5A complete growth medium. To pulse-label viral phosphoproteins, 90% confluent T-75 cultures of virus-infected JLS-V16 cells were incubated with 1 mCi of [^{32}P]phosphate (carrier-free) per ml for 30 min in phosphate-free growth medium. In pulse-chase experiments, the cell cultures were rinsed in Hanks balanced salt solution and incubated further in complete growth medium (22). Virus was labeled for 24 h in 2-quart (ca. 1.9-liter) roller bottles at a concentration of 250 μCi of [^{32}P]phosphate (carrier-free) per ml in growth medium containing 25% the usual concentration of phosphate.

For peptide mapping experiments, viral phosphoproteins were labeled in 2-quart roller bottles with [^{32}P]phosphate for 3 h by incubating virus-infected cells with 2 mCi of carrier-free [^{32}P]phosphate per ml in growth medium containing one-tenth the normal concentration of phosphate. Pr65^{gag} and Pr25^{gag} were also labeled with [^{14}C]- and [^{3}H]tyrosine, respectively. Pr65^{gag} was prepared from whole virus which was labeled in a roller culture in the presence of 250 μCi of [^{14}C]tyrosine per ml for 48 h in growth medium containing one-half the usual concentration of amino acids. Pr25^{gag} was prepared by incubating virus-infected cells in a 1-quart (ca. 0.95-liter) prescription bottle for 2 h in Earle solution containing 250 μCi of [^{3}H]tyrosine per ml. Pr40^{gag} was labeled with [^{3}H]leucine by incubating a roller culture of virus-infected cells for 2 h with 100 μCi of [^{3}H]leucine per ml in medium containing one-half the usual concentration of amino acids.

Cell lysis and immunoprecipitation. Cells were pulse-labeled and then lysed in a detergent-containing buffer as described (2). The lysate was centrifuged at either 10,000 × *g* for 10 min or 100,000 × *g* for 60 min as indicated. In general, long pulse-labeling experiments require the higher centrifugation conditions to reduce nonspecific background. Cytoplasmic extracts from ^{32}P-labeled cultures were sometimes treated by incubation at 37°C in the presence or absence of pancreatic RNase (40 μg/ml). The cytoplasmic supernatant fluids were mixed with antisera previously absorbed with uninfected JLS-V16 mouse cells as described (9). The antigen-antibody complexes were obtained either by precipitation with a second antibody (9) or by use of the Cowan strain of Formalin-fixed *Staphylococcus aureus* (11). Generally, 25 μl of a 10% suspension of Formalin-fixed bacteria was used to precipitate 7.5 μl of serum. The immunoprecipitates were collected by centrifugation through a 1 M sucrose cushion as described (9).

Gel electrophoresis. Viral proteins present in virus or immunoprecipitates were fractionated by electrophoresis in SDS-polyacrylamide gels (6 to 12% linear gradient gels or 11.25% gels) as described (9). Radioactive protein bands were detected either by autoradiography (^{32}P-labeled proteins) or fluorography (5) with preflashed film to obtain a linear response (13).

Peptide mapping. [^{3}H]leucine and [^{3}H]- or [^{14}C]-tyrosine-labeled proteins were digested with TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin, and the digests were fractionated on Chromobead P ion-exchange columns as described (2). ^{32}P-labeled viral phosphoproteins were digested in a similar manner with α-chymotrypsin. The chymotryptic digests were fractionated by two-dimensional fingerprinting on thin-layer cellulose plates (20 by 20 cm) purchased from Eastman Kodak (catalogue no. 13255). In the first dimension, electrophoresis was performed in 27% formic acid at 150 V for 3.5 h. The plates were dried and then subjected to ascending chromatography in the second dimension as described by Erikson et al. (7).

Phosphoamino acid analysis. ^{32}P-labeled α-chymotryptic peptides prepared as described above were dried by lyophilization, dissolved in 35 μl of 2 N HCl, and hydrolyzed in sealed glass capillaries at 110°C for 12 h. The hydrolysates were then lyophilized to dryness, dissolved in 10 μl of water containing 15 μg each of unlabeled phosphoserine and phosphothreonine, and spotted onto Whatman 3 MM paper. Electrophoresis was performed in formic acid, acetic acid, and water according to Brugge et al. (6), but at 300 V for 8 h. Subsequent to electrophoresis, the paper was sprayed with ninhydrin, dried, and subjected to autoradiography.

RESULTS

Characterization of cell-associated R-MuLV proteins by immunoprecipitation with monospecific antisera to the viral core proteins. The antigenic determinants of intracellular viral proteins were analyzed by immunoprecipitation with monospecific sera prepared against viral p30, p15, pp12, and p10. In the experiment shown in Fig. 1, virus-infected cells were incubated for 20 h with [^{3}H]leucine and the postribosomal supernatants were challenged with the various monospecific antisera. Analyses of anti-p30 immunoprecipitates revealed five major polypeptides ranging in size from Pr65^{gag} to p30 (Fig. 1, lane B). The band migrating slower than p30, termed Pr40^{gag}, has a molecular weight estimated at 38,000 to 40,000. The two bands migrating between Pr40^{gag} and Pr65^{gag} appear to be non-specifically precipitated, since they are found in all the immunoprecipitates obtained with the various monospecific sera (Fig. 1, lanes B–G). Figure 1 (lane C) shows that anti-p10 serum precipitated both Pr65^{gag} and Pr40^{gag}, as well as p10. In contrast, neither anti-p15 (lane D) nor anti-p12 (lane E) sera precipitated significant amounts of Pr40^{gag}, although they both recognized Pr65^{gag} and their respective core proteins. The anti-p12 serum was apparently contaminated with low levels of antibody to p10 and, thus, was capable of precipitating small amounts of p10 from cell extracts. Other preparations of anti-p12 sera also contained low levels of antibody to p30 (see Fig. 2). In addition to Pr65^{gag}, both anti-p15 and anti-pp12 sera recognized another polypeptide, termed pPr25^{gag}, which was not recognized by either anti-p30 or anti-p10 sera. (We are using the designation "pp" or "pPr" for phosphoproteins or phosphorylated precursor proteins as agreed upon at a

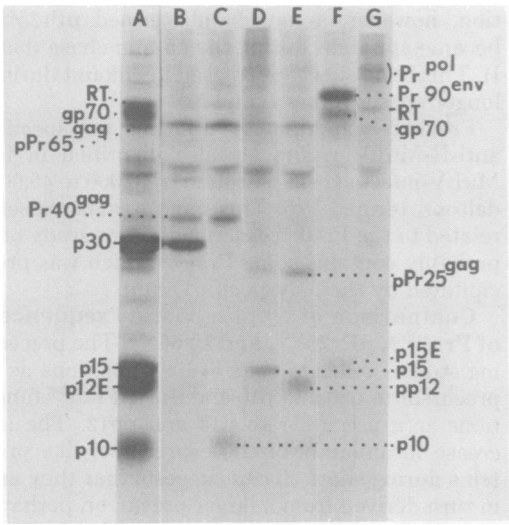


FIG. 1. *Intracellular gag gene products of R-MuLV. R-MuLV-infected cells were labeled for 20 h with [^{3}H]leucine in complete growth medium containing one-half the usual concentration of amino acids. Cytoplasmic extracts were prepared (1), and the extracts were centrifuged at 100,000 × g for 1 h before immunoprecipitation with monospecific sera: anti-p30 (lane B), anti-p10 (lane C), anti-p15 (lane D), anti-pp12 (lane E), anti-gp69/71 (lane F), and anti-RT (lane G). Lane A is [^{3}H]leucine-labeled R-MuLV. Viral proteins were separated on a 11.25% SDS-polyacrylamide gel. In lanes B through G approximately equal amounts of radioactivity were applied to the gel (100,000 dpm). Bands were detected by fluorography, using preflashed film.*

meeting held 8–9 March 1977, in Arlington, Va., at a Tumor Viral Immunology Workshop sponsored by the Collaborative Research Branch, RNA Tumor Virus Studies Section of the National Cancer Institute.) This polypeptide has an estimated molecular weight of 25,000, and, as will be shown below, is phosphorylated. Neither Pr40^{gag} nor pPr25^{gag} was recognized by antisera prepared against gp70 (lane F) or the viral reverse transcriptase (lane G). Thus, Pr40^{gag} and pPr25^{gag} are antigenically related to the viral core proteins but not to viral envelope or reverse transcriptase proteins. We conclude that Pr40^{gag} contains p30 and p10 determinants and that pPr25^{gag} contains p15 and pp12 determinants.

Pulse-chase kinetics of Pr40^{gag} and pPr25^{gag}. The long labeling experiment shown in Fig. 1 identified the antigenic determinants of Pr40^{gag} and pPr25^{gag} as being derived from that region of the *gag* gene coding for p30-p10 and p15-pp12, respectively. To determine whether Pr40^{gag} and pPr25^{gag} are indeed metabolic precursors to the viral core proteins, a pulse-chase experiment was performed (Fig. 2). In this experiment, replicate cultures were pulse-labeled for 7.5 min at 37°C with [^{3}H]leucine. One culture was harvested immediately and an aliquot of the cytoplasmic extract was challenged with either anti-pp12 or anti-p30 serum. The remaining cultures were rinsed and incubated in complete growth medium for periods of 15 min, 30 min, 60 min, 2 h, and 4 h before immunoprecipitation.

Immunoprecipitation of cell extracts with

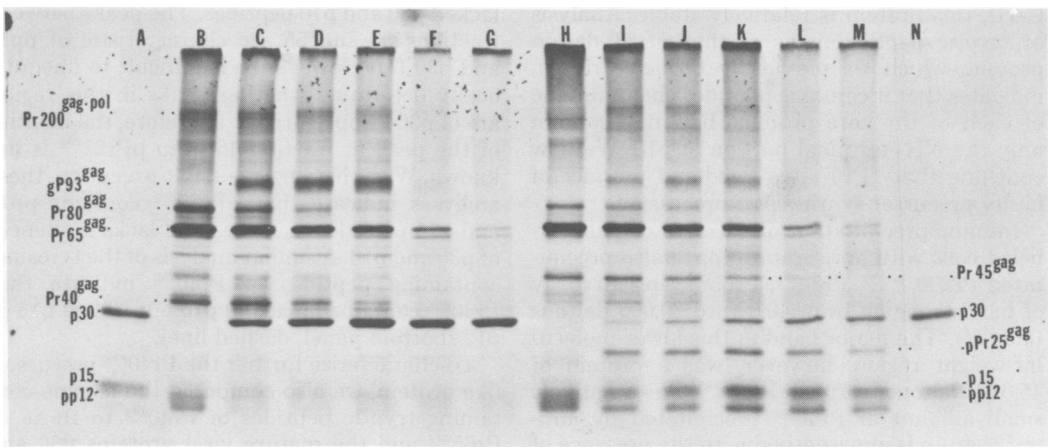


FIG. 2. *Pulse-chase studies on intracellular R-MuLV gag gene proteins. Replicate cultures of R-MuLV-infected cells were pulse-labeled for 7.5 min with [^{3}H]leucine in Earle balanced salt solution supplemented with 2% dialyzed fetal calf serum. One culture was harvested and lysed immediately (lanes B and H). The others were rinsed and incubated in complete growth medium (22) for 15 min (lanes C and I), 30 min (lanes D and J), 60 min (lanes E and K), 2 h (lanes F and L), or 4 h (lanes G and M). After lysis, the cytoplasmic extracts were challenged with anti-p30 (lanes B–G) and anti-pp12 (lanes H–M). The immunoprecipitates were collected by the Staphylococcus A method (11), and the proteins were separated on a 6 to 12% linear gradient polyacrylamide gel. Bands were detected by fluorography, using preflashed film. Lanes A and N are preparations of [^{3}H]leucine-labeled R-MuLV.*

anti-p30 serum resulted in precipitation of the usual p30-related polyproteins (Fig. 2, lane B) including $Pr200^{gag\text{-}pol}$, $Pr80^{gag}$, $Pr65^{gag}$, and an array of bands in the 40,000- to 60,000-dalton region of the gel. The major band in this region, however, was one of 40,000 daltons, termed $Pr40^{gag}$. In the pulse-labeling, $Pr40^{gag}$ and the other p30-precursor polyproteins were detected, whereas p30 was absent. $Pr40^{gag}$ increased slightly in amount during the 15-min chase (lane C) but decreased during chases of 30 min, 60 min, 2 h, and 4 h (lanes D, E, F, and G). Viral p30 increased in amount as $Pr40^{gag}$ and the other p30-precursors decreased. It is also obvious that anti-p30 serum was capable of precipitating small amounts of a 25,000-dalton protein. As discussed below, this is apparently due to a minor amount of contaminating antibody to pp12 or p15, or both, in the anti-p30 serum. The presence of this contaminating antibody was most apparent when *S. aureus* protein was used as an adsorbent to aid in the complete precipitation of the antibody-antigen complex. The minor band migrating at approximately 52,000 daltons in this figure (lane B) is variably precipitated by antisera to viral gp70 and by normal rabbit sera (not shown) and is considered to be a nonspecific background band migrating in the region of the gel associated with the large immunoglobulin chain. Another very minor band migrating slightly faster than $Pr65^{gag}$ is precipitable by antisera to p30, pp12, and p15 but not by anti-p10 serum (not shown). As is evident in the lanes of this figure representing chases (lanes C-G), this protein is relatively stable. Analysis of tryptic peptide maps of this 60,000-dalton protein, which we previously termed Pr5 (1), indicates that it contains peptides characteristic of each of the core proteins but may contain only the NH_2-terminal portion of p10. We now conclude that "Pr5" is a dead-end product of faulty precursor synthesis or processing.

Immunoprecipitation of extracts of pulse-labeled cells with antiserum to pp12 also precipitated $Pr200^{gag\text{-}pol}$, $Pr80^{gag}$, $Pr65^{gag}$, and an array of bands ranging from 40,000 to 60,000 daltons (lane H). The major band in this lower-molecular-weight region, however, was a protein of 45,000 daltons, termed $Pr45^{gag}$. The relatively small amount of $Pr40^{gag}$ precipitated by anti-pp12 serum is apparently due to the presence of contaminating antibody to p30 in the serum. The small amount of mature p30 in the anti-pp12 precipitates shown in lanes J through M confirms this interpretation.

In chase-incubated cells, pp12 appeared at the apparent expense of the larger precursor polyproteins, including $Pr45^{gag}$ (lanes I-M). In addition, however, a new band, termed $pPr25^{gag}$, became apparent during the 15-min chase (lane I). This band slowly decreased in amount during longer chases (lanes L and M).

Previous experiments utilizing polyspecific anti-R-MuLV serum (1) also identified in R-MuLV-infected cells a protein of 40,000 to 45,000 daltons, termed Pr6. This band is most closely related to the $Pr40^{gag}$ described in this study but probably contains some $Pr45^{gag}$ which was precipitated by the polyspecific serum.

Comparison of tryptic peptide sequences of $Pr40^{gag}$, $pPr25^{gag}$, and $Pr65^{gag}$. The preceding studies indicate that $Pr40^{gag}$ functions as a precursor to p30 and p10 and that $pPr25^{gag}$ functions as a precursor to p15 and pp12. The increase in amounts of these precursor-like proteins during short chases suggests that they are in turn derived from a larger precursor, perhaps $Pr65^{gag}$. To characterize these proteins further, we compared the tyrosine-containing tryptic peptides of $Pr40^{gag}$ and $pPr25^{gag}$ to those of $Pr65^{gag}$ and the mature viral proteins (Fig. 3). We have previously identified the core protein tryptic peptides of tyrosine-labeled $Pr65^{gag}$ (1). Viral p15 contains two tyrosine tryptic peptides, only one of which is in $Pr65^{gag}$ (top panel, fraction 115). Viral pp12 contains several tyrosine-containing tryptic peptides, one of which is clearly characteristic of pp12 (top panel, fraction 130). The ion-exchange profile of tryptic peptides of [^{3}H]tyrosine-labeled $pPr25^{gag}$ (top panel, dashed line) indicated that $pPr25^{gag}$ contained p15 and pp12 tyrosine tryptic peptides and lacked p30 and p10 peptides. The peaks between fractions 30 and 55 are characteristic of pp12 and p30 (1). However, it is difficult to unequivocally determine whether peaks in this region are of p30 or pp12 origin. Therefore, the identity of the peak at fraction 45 from $pPr25^{gag}$ is unknown. With this single possible exception, these analyses indicate that $pPr25^{gag}$ contains pp12 and p15 peptide sequences and lacks sequences of p30 and p10. A similar analysis of the tyrosine-containing peptides of $Pr40^{gag}$ indicate that $Pr40^{gag}$ contained p30 and p10 but lacked p15 or p12 (bottom panel, dashed line).

To characterize further the $Pr40^{gag}$ precursor-like protein, we also compared the leucine-containing tryptic peptides of $Pr40^{gag}$ to those of $Pr65^{gag}$ and the mature viral proteins p30 and p10 (Fig. 4). We have found that p10 and p30 each contained several characteristic leucine-containing tryptic peptides, some of which are easily resolved by ion-exchange chromatography (Fig. 4, bottom panel). Viral p15 contained just two detectable leucine-containing tryptic peptides, whereas pp12 peptides eluted in the flow-

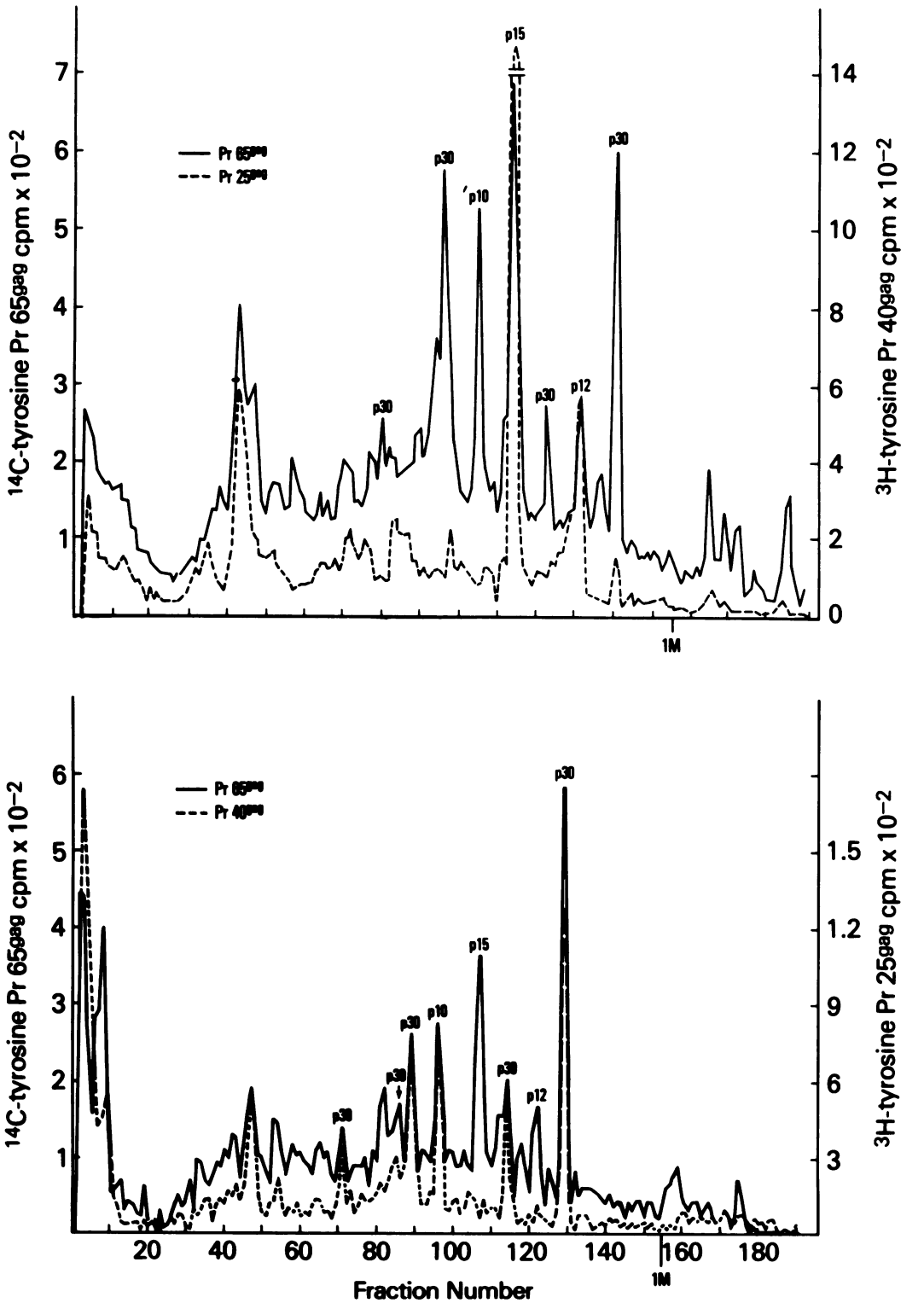


FIG. 3. *Analyses of tryptic digests of [^{3}H]tyrosine-labeled pPr25^{gag} or Pr40^{gag} and [^{14}C]tyrosine-labeled Pr65^{gag} by ion-exchange chromatography. Digests were mixed and then fractionated on a standard Technicon Chromobead type P exchange column. Peak fractions were marked to identify the viral protein from which they originate (1). All [^{3}H]tyrosine-labeled precursors were immunoprecipitated from cells pulse-labeled for 20 min. The [^{14}C]tyrosine-labeled Pr65^{gag} was also from a 20-min pulse of infected cells. Anti-p10 was used to precipitate Pr40^{gag} and anti-p12 was used for Pr25^{gag}, whereas Pr65^{gag} was obtained by use of anti-p30. The yield ranged from 80 to 90%.*

through volume of the column (data not shown). Analysis of the leucine-labeled tryptic peptides of $Pr65^{gag}$ (Fig. 4, top panel) showed that $Pr65^{gag}$ contained the two leucine-containing tryptic peptides characteristic of p15 and all but one of the major tryptic peptides of p30 and p10. These missing peptides characteristic of p30 and p10 are identified as fractions 139 and 92, respectively (upper and lower panels). $Pr65^{gag}$ also contained a leucine tryptic peptide (fraction 80, marked with an asterisk) not found in any of the core proteins. Other such extra peptides may also be eluted in the void fraction of the column. $Pr40^{gag}$ also contained the extra peptide eluting at fraction 80 and all the p30 and p10 peptides found in $Pr65^{gag}$. In addition, $Pr40^{gag}$ contained that p30 peptide (fraction 139, middle panel) that was absent in $Pr65^{gag}$. $Pr40^{gag}$, like $Pr65^{gag}$, also lacked the p10 peptide in fraction 92.

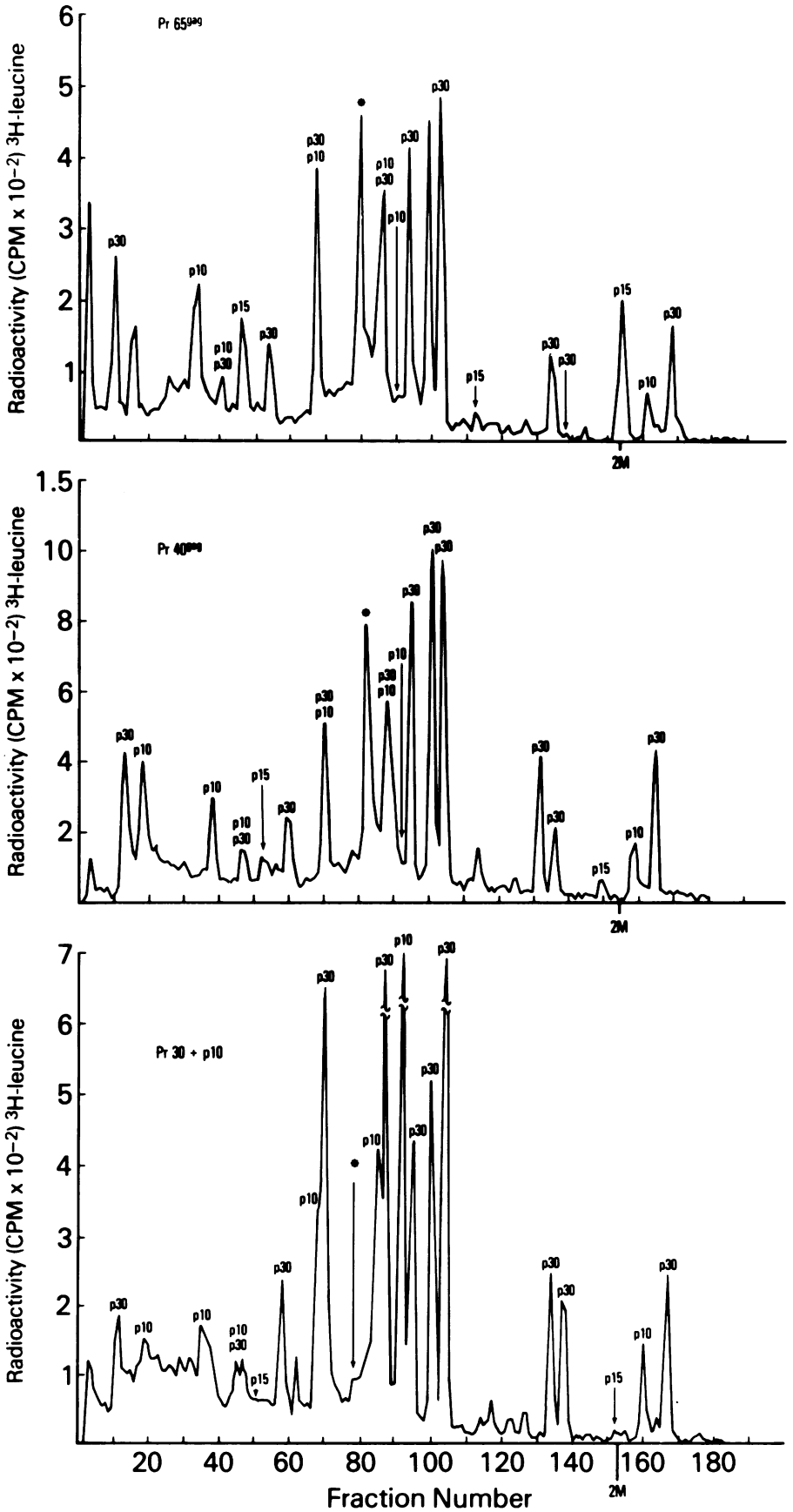
R-MuLV-specified phosphoproteins in virus-infected cells. Because it is well known that pp12 is a phosphoprotein (17), it was of interest to determine whether the precursors to pp12 are phosphorylated. Thus, virus-infected cells were pulse-labeled for 30 min with [^{32}P]-phosphate to examine the virus-specified phosphoproteins. Cytoplasmic extracts were prepared and incubated with pancreatic RNase before immunoprecipitation with antiserum to phosphoprotein p12 (anti-pp12). The immunoprecipitates were denatured in boiling SDS-mercaptoethanol and fractionated by SDS-polyacrylamide gel electrophoresis. The results are shown in Fig. 5 (left). The autoradiogram (left, lane B) showed that two major polypeptides and a number of minor polypeptides became radioactive in the pulse-labeling experiment. One of the major labeled polypeptides, termed $pPr65^{gag}$, comigrated with $Pr65^{gag}$, whereas the other, termed $pPr25^{gag}$, migrated significantly faster than viral p30. Another protein, which labeled poorly in a 30-min pulse, migrated at the position expected for pp12.

The stability of these phosphoproteins was determined by performing a pulse-chase experiment (Fig. 5, left, lane C). In this experiment, virus-infected cells were pulse-labeled with [^{32}P]phosphate for 30 min; the radioactive medium was removed, the cell sheet was rinsed, and nonradioactive complete growth medium was added. After incubation for 2 h, the cells were lysed, the cytoplasmic extracts were treated with pancreatic RNase to hydrolyze RNA, and then immunoprecipitation with anti-pp12 was performed. The results showed that $pPr65^{gag}$ also decreased significantly during the chase compared to the pulse-labeling experiment. We emphasize that the amounts of radioactivity recovered in the anti-pp12 immunoprecipitates from both the pulse-labeling and the pulse-chase experiments were approximately the same, a result which underscores the validity of the pulse-chase experiment.

Characterization of intracellular R-MuLV-specified phosphoproteins by immunoprecipitation with monospecific sera prepared against purified viral proteins. Virus-infected cells were pulse-labeled with [^{32}P]phosphate for 30 min, and cytoplasmic extracts were prepared with RNase treatment. Immunoprecipitation was performed with anti-R-MuLV, anti-p30, anti-p15, anti-pp12, anti-p10, anti-gp70, and anti-reverse transcriptase monospecific sera (Fig. 5, right, lanes A–G). Immunoprecipitation with serum to disrupted virus precipitated $pPr65^{gag}$ and $pPr25^{gag}$ (Fig. 5, right, lane A). Immunoprecipitation of the cytoplasmic extract with various monospecific sera (Fig. 5, right, lanes B–G) clearly showed that $pPr25^{gag}$ contained antigenic determinants of pp12 (lane D) but lacked p30 (lane B), p10 (lane E), gp70 (lane F), and reverse transciptase (lane G) determinants. Anti-p15 (lane C) recognized only trace amounts of $pPr25^{gag}$ as well as $pPr65^{gag}$. This can be explained by the low titer of the p15 serum used in this experiment and the relative ineffeciency of anti-p15 serum to precipitate phosphopolyproteins.

The phosphorylated precursor $pPr65^{gag}$ was recognized by anti-p30, anti-pp12, and anti-p10 sera and to a small extent by anti-p15 serum. We note that in other experiments (not shown) higher concentrations of anti-p15 gave a more prominent band of $pPr25^{gag}$ and $pPr65^{gag}$. Anti-

FIG. 4. *Analyses of tryptic digests of [^{3}H]leucine-labeled $Pr65^{gag}$, $Pr40^{gag}$, and viral proteins p30 and p10 by ion-exchange column chromatography. A standard column containing Technicon Chromobead type P ion-exchange beads was used to fractionate the tryptic digests of $Pr65^{gag}$, $Pr40^{gag}$, and a mixture of p30 and p10. About 20,000 to 30,000 cpm of [^{3}H]leucine-labeled peptides were applied along with 10,000 cpm of [^{14}C]tyrosine-labeled p30 peptides as an internal marker. Only ^{3}H-labeled peptides are shown. The yield ranged from 80 to 90%. Peak fractions were marked to identify the viral protein from which they originate (1). $Pr65^{gag}$ was isolated from infected cells by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE) of 30-min pulse-labeled cells with anti-p30 serum. $Pr40^{gag}$ was isolated by immunoprecipitation with anti-p30 and SDS-PAGE of a 30-min pulse–2-h chase culture. Viral p30 and p10 were obtained from purified virus, and the proteins were purified by guanidine HCl chromatography and subsequent SDS-PAGE.*


Pr 65gag
Pr 40gag
Pr 30 + p10
Radioactivity (CPM x 10−2) 3H-leucine
Fraction Number
2M
p30
p10
p15

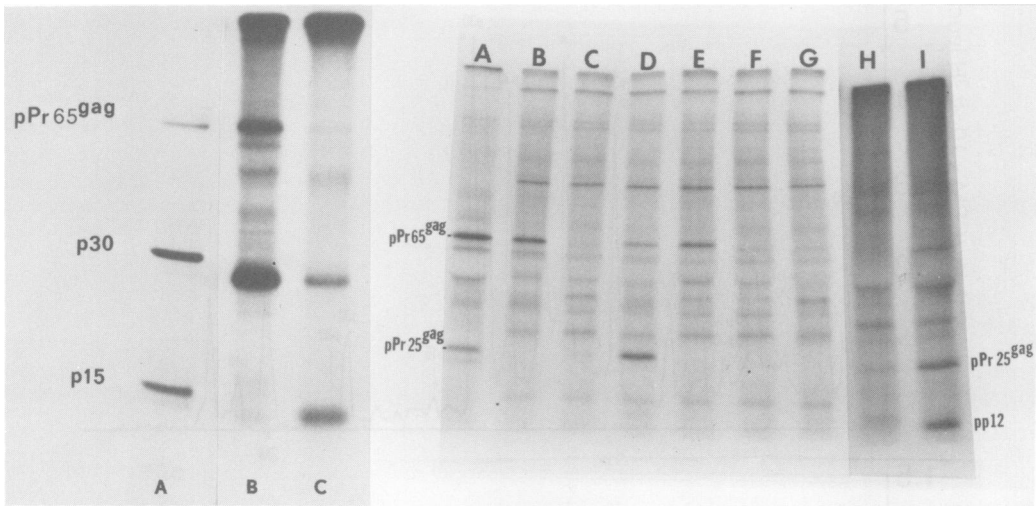


FIG. 5. *Intracellular R-MuLV-specified phosphoproteins. (Left) In a pulse-chase experiment, cultures of R-MuLV-infected cells were pulse-labeled for 30 min with [^{32}P]phosphate (lane B) or pulse-labeled for 30 min and chased with complete growth medium for 2 h (lane C). Cytoplasmic extracts were prepared and treated with RNase (100 μg/ml) for 30 min at 37°C before immunoprecipitation with anti-pp12 serum. Lane A is [^{3}H] leucine-labeled R-MuLV. The samples were fractionated on 11.25% SDS-polyacrylamide gels. Approximately equal volumes of cytoplasmic extracts were applied to each lane. (Right) For antigenic characterization of R-MuLV-specified intracellular proteins, replicate cultures of R-MuLV-infected cells were pulse-labeled for 30 min with [^{32}P]phosphate. The cytoplasmic extracts were treated with RNase and then incubated with: antisera to disrupted R-MuLV (lane A); anti-p30 (lane B); anti-p15 (lane C); anti-pp12 (lane D); anti-p10 (lane E); anti-gp69/71 (lane F); and anti-reverse transcriptase (lane G). Immunoprecipitation was performed by absorption to Staphylococcus A as described in the text. The immunoprecipitate from equivalent volumes of cytoplasmic extracts were applied to the 11.25% SDS-polyacrylamide gel and electrophoresed. In a separate experiment, similarly pulse-labeled cells were chase-incubated for 1 h before precipitation with anti-p15 (lane H) or anti-pp12 (lane I) sera. Cytoplasmic extracts in the chase experiment were not treated with RNase before incubation with antisera.*

gp70 or anti-reverse transcriptase serum did not immunoprecipitate either pPr65^{gag} or pPr25^{gag}. These results show that pPr65^{gag} contains antigenic determinants of the four viral core proteins but that pPr25^{gag} only contains pp12 and p15 determinants. Lanes H and I (Fig. 5) show the profiles of phosphorylated proteins immunoprecipitated with anti-p15 and anti-pp12 sera from cytoplasmic extracts of cells pulse-labeled with [^{32}P]phosphate and chase-incubated for 1 h. Both sera precipitated pPr25^{gag}, whereas only the anti-pp12 serum precipitated pp12. Evident in these studies are numerous background bands which represent nonspecific precipitation during the immunoprecipitation procedure. These bands are most obvious when [^{32}P]phosphate is used as label. Their identity as nonspecific precipitates is determined by their presence in immunoprecipitates using antiserum prepared against core proteins as well as other viral gene products.

Analyses of the chymotryptic peptides of pPr65^{gag}, pPr25^{gag}, and pp12 by two-dimensional fingerprinting. Chymotryptic digestion and two-dimensional peptide mapping of SDS-polyacrylamide gel electrophoresis-purified, ^{32}P-labeled pPr65^{gag}, pPr25^{gag}, and pp12 isolated from infected cells was used to compare the phosphopeptides of the three phosphoproteins (Fig. 6). The results showed that all three polypeptides have similar chymotryptic maps (Fig. 6) consisting of a major group of eight peptides near the center of the chromatogram and a minor spot at the top-middle portion of the two-dimensional map. The pp12 map also had a trace amount of this minor spot that is not readily seen in Fig. 6.

In other analyses in which trypsin was used instead of chymotrypsin, a different overall pattern was observed, but again each tryptic map was similar among the three proteins (not shown).

To characterize further the nature of the phosphoproteins pPr65^{gag}, pPr25^{gag}, and pp12, we determined whether the phosphorylation occurred through a serine or a threonine residue. Figure 7 shows that in all cases the phosphate was predominantly linked to serine. There was, how-

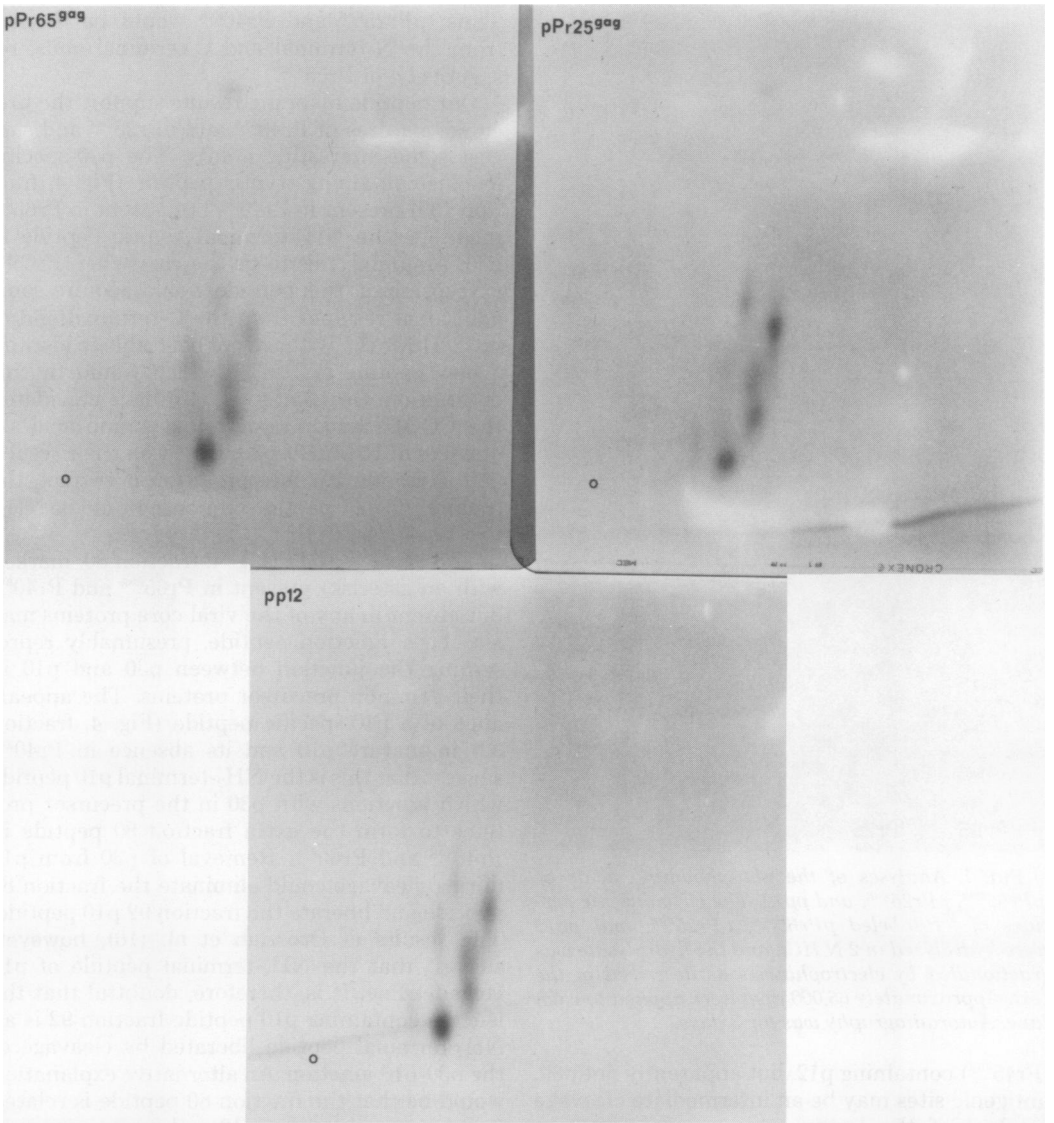


FIG. 6. *Chymotryptic peptide maps of p65gag, pPr25gag, and pp12. A 2-quart roller culture of R-MuLV-infected cells was incubated for 2 h with [32P]phosphate, and the viral phosphoproteins were isolated from cytoplasmic extracts by immunoprecipitation with anti-pp12 serum. The immunoprecipitates were absorbed to Staphylococcus A and fractionated by SDS-polyacrylamide gel electrophoresis on 11 to 25% gels. The bands were digested with 50 μg of α-chymotrypsin per ml as described (1), and the soluble peptides were fractionated by a two-dimensional procedure on thin-layer plates. Approximately 33,000 dpm of radioactivity were applied to each plate. Autoradiography was for 3 days.*

ever, a trace of phosphothreonine in pPr65^{gag} that was not detected in either pPr25^{gag} or pp12.

DISCUSSION

The results presented here indicate that the four core proteins coded by the *gag* gene can be formed in infected cells by cleavage of Pr40^{gag} and pPr25^{gag}. The latter contains p15 and pp12, whereas the former contains p30 and p10. Furthermore, Pr40^{gag} and pPr25^{gag} appear to be derived by cleavage of pPr65^{gag}. These conclusions stem from pulse-chase, immunological, and peptide mapping studies. However, other alternate cleavage pathways involving processing of Pr65^{gag} and other precursors to the viral core proteins cannot be completely eliminated at this point. The 45,000-dalton precursor-like protein

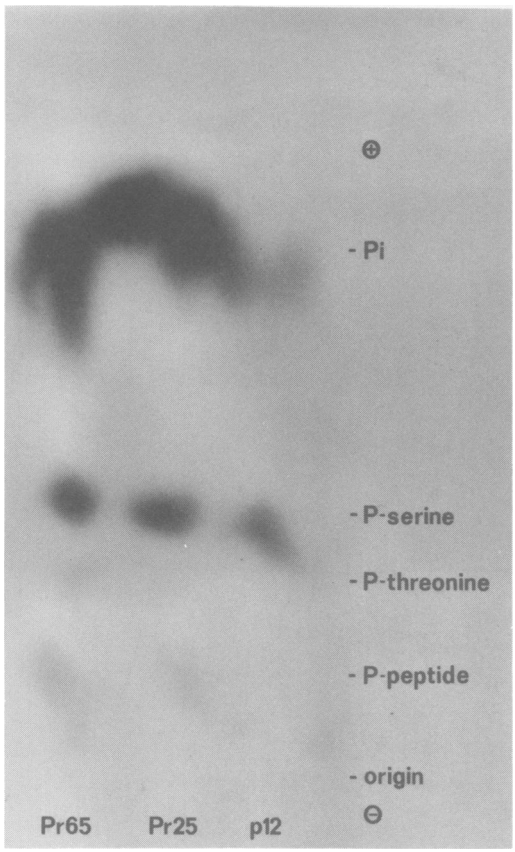


FIG. 7. *Analyses of the phosphoamino acids of pPr65^{gag}, pPr25^{gag}, and pp12. The chymotryptic peptides of ^{32}P-labeled pPr65^{gag}, pPr25^{gag}, and pp12 were hydrolyzed in 2 N HCl, and the hydrolysate was fractionated by electrophoresis as described in the text. Approximately 65,000 dpm were applied to each lane. Autoradiography was for 3 days.*

(Pr45^{gag}) containing p12, but apparently not p30, antigenic sites may be an intermediate cleavage product of the larger core protein precursor Pr80^{gag}.

Yoshinaka and Luftig (25) first identified a 40,000-dalton polyprotein, termed P40, which contained p30 and p10 antigenic determinants and which resulted from in vitro cleavage of R-MuLV Pr65^{gag}. A 25,000-dalton polyprotein containing p15 and pp12 antigenic determinants was observed originally by Barbacid et al. (4) in R-MuLV-infected cells and by Stephenson and his colleagues in a variety of type C virus-infected cells (10, 21). Our studies strongly suggest an actual precursor-product relationship of these polyproteins with the viral core proteins.

The composition of Pr40^{gag} and pPr25^{gag} indicated by the present study is also consistent with the *gag* gene order of R-MuLV, which is known to be NH_2---p15-pp12-p30-p10---COOH (4, 14). Thus, pPr25^{gag} and Pr40^{gag} would be derived from the N-terminal and C-terminal ends, respectively, of Pr65^{gag}.

Our peptide mapping results support the precursor natures of Pr40^{gag} and pPr25^{gag} and suggest some interesting points. The p30-specific leucine-containing tryptic peptide (Fig. 4, fraction 139) present in Pr40^{gag} but absent in Pr65^{gag} might be the NH_2-terminal tryptic peptide in p30. Assuming this to be the case when Pr65^{gag} is trypsinized, this peptide would acquire some additional residues from the C-terminal end of pp12. However, we have not been able to identify a new peptide in Pr65^{gag} which would fit this description. Oroszlan et al. (16) have elucidated the COOH- and NH_2-terminal amino acid sequences of R-MuLV p30. Based on their results and assuming our interpretation is correct, the fraction 139 p30 peptide sequence would be NH_2-Pro-Leu-Arg-COOH.

The extra peptide (Fig. 4, fraction 80, marked with an asterisk) present in Pr65^{gag} and Pr40^{gag} but absent in any of the viral core proteins may also be a junction peptide, presumably representing the junction between p30 and p10 in their common precursor proteins. The appearance of a p10-specific peptide (Fig. 4, fraction 92) in mature p10 and its absence in Pr40^{gag} suggest that this is the NH_2-terminal p10 peptide which junctions with p30 in the precursor proteins to form the extra fraction 80 peptide in Pr40^{gag} and Pr65^{gag}. Removal of p30 from p10 during cleavage could eliminate the fraction 80 peptide and liberate the fraction 92 p10 peptide. The results of Oroszlan et al. (16), however, suggest that the NH_2-terminal peptide of p10 lacks leucine. It is, therefore, doubtful that the leucine-containing p10 peptide fraction 92 is an NH_2-terminal peptide liberated by cleavage of the p30-p10 junction. An alternative explanation would be that the fraction 80 peptide is related to a junction between p10 and sequences more COOH-terminal to p10 in Pr65^{gag} and Pr40^{gag}. Removal of the COOH-terminal sequences when Pr40^{gag} is cleaved to p30 and p10 could be expected to liberate the fraction 92 p10 peptide. Since no published results support the existence of peptide sequences C-terminal to p10 in Pr65^{gag}, the identity and structures of the fraction 80 and 92 peptides remain unclear.

The phosphorylation studies show that pp12 becomes phosphorylated while part of the core protein precursor pPr65^{gag}. Our studies indicate that pPr25^{gag} and pPr65^{gag} are the major pp12 precursors that are phosphorylated. Pr200$^{gag\text{-}pol}$, Pr80^{gag}, and Pr45^{gag} did not appear to be phosphorylated. Pal and Roy-Burman (18) have recently characterized intracellular wild mouse type C retrovirus phosphoproteins, using im-

munoprecipitation with anti-p30 and anti-pp12. In contrast to our findings with R-MuLV, they detected a number of phosphoproteins which include a >100,000-dalton phosphoprotein, as well as polypeptides termed Pr100, Pr77, Pr62, and Pr50. These proteins were rapidly labeled and they disappeared in the chase. The major phosphoprotein found in the chase was pp12. The reasons for the differences in the two viral systems are unknown.

Concerning the mechanism of pp12 phosphorylation, the following points are pertinent. Peptide mapping studies revealed that pp12 has eight major chymotryptic phosphopeptides and that these same phosphopeptides are also found in $pPr25^{gag}$ and $pPr65^{gag}$. Furthermore, phosphoserine is the major phosphorylated amino acid found in $pPr65^{gag}$, $pPr25^{gag}$, and pp12. These findings indicate that the same sites that are phosphorylated in pp12 are also phosphorylated in $pPr25^{gag}$ and $pPr65^{gag}$. Inspection of [^{3}H]leucine pulse-labeled extracts immunoprecipitated with monospecific serum to pp12 has shown that $Pr25^{gag}$ is minor in amount compared to $Pr65^{gag}$ (Fig. 2, lane I). Yet in phosphorylation studies, $pPr25^{gag}$ is more highly labeled than $pPr65^{gag}$ in short pulses or chases (Fig. 5). Thus, it appears that most of the $Pr25^{gag}$ molecules are phosphorylated, whereas only a few $Pr65^{gag}$ molecules undergo phosphorylation. However, as determined by our peptide mapping studies, it seems clear that no new sites of phosphorylation are present in the lower-molecular-weight proteins.

Although alternate pathways are possible, our results suggest that during its synthesis or after its release from the ribosome, a portion of the $Pr65^{gag}$ molecules is phosphorylated and cleaved, yielding $pPr25^{gag}$ and $Pr40^{gag}$, which are in turn cleaved to yield the four core proteins. Although it is quite likely that unphosphorylated $Pr65^{gag}$ is also cleaved, we favor the view that the rate of cleavage of phosphorylated $Pr65^{gag}$ is higher than cleavage of unphosphorylated molecules. Such an interpretation receives support from the results indicating rapid processing of $pPr65^{gag}$ to $pPr25^{gag}$ (Fig. 5), from the observation that cleavage of $Pr65^{gag}$ is accelerated by interaction with viral RNA (8), and from the fact that phosphorylated p12 binds to viral RNA, whereas unphosphorylated p12 does not (19). Thus, phosphorylated $Pr65^{gag}$ would readily bind to viral genomic RNA, thereby facilitating $Pr65^{gag}$ processing. We emphasize that $Pr65^{gag}$ cleavage does occur even in the absence of synthesis of viral genomic RNA, but at a slower rate (8).

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ADDENDUM IN PROOF

We have recently shown that $Pr80^{gag}$ is a glycoprotein ($gPr80^{gag}$) because it is labeled with [^{3}H]mannose, whereas neither $Pr65^{gag}$ nor p30 becomes labeled under the same conditions. In addition, $gPr80^{gag}$ binds to a lectin column and is eluted with α-methyl-D-mannoside (V. Ng, J. Kopchick, and R. Arlinghaus, unpublished data). Furthermore, $gPr80^{gag}$ lacks two p30 tyrosine-containing tryptic peptides that are seen in $Pr65^{gag}$ (J. Kopchick, W. L. Karshin, and R. Arlinghaus, J. Virol. **30**:610–623, 1979).

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