JOURNAL OF VIROLOGY, Mar. 1983, p. 1200–1206
0022-538X/83/031200-07$02.00/0
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Vol. 45, No. 3

Further Studies on the Glycosylated *gag* Gene Products of Rauscher Murine Leukemia Virus: Identification of an N-Terminal 45,000-Dalton Cleavage Product

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Received 16 July 1982/Accepted 1 December 1982

A glycosylated 45,000-M_r protein containing Rauscher murine leukemia virus p15 and p12 antigenic sites and tryptic peptides was identified in Rauscher murine leukemia virus-infected cells. This glycoprotein, termed gP45^{gag}, was also shown to contain a single tryptic peptide also present in gPr80^{gag} and its unglycosylated apoprotein precursor Pr75^{gag}, but lacking in Pr65^{gag} or Pr40^{gag}. The presence of this peptide only in viral precursor proteins containing the so-called leader (L) sequence strongly suggests that gPr45^{gag} is an N-terminal fragment of larger glycosylated *gag* polyproteins, composed of L sequences in addition to p15 and p12. The kinetics of appearance of radiolabeled gPr45^{gag} and its disappearance during chase-incubation is suggestive of a precursor-like role for this intermediate gene product. An observed 27,000-M_r glycosylated polypeptide, termed gP27^{gag} and containing p15 but not p12, p30, or p10 antigenic determinants, is a candidate cleavage product derived from gPr45^{gag}. These observations suggest that gPr45^{gag} and its putative cleavage product gP27^{gag} represent an authentic pathway for intracellular processing of glycosylated core proteins.

The *gag* gene of murine leukemia virus (MuLV) codes for three primary gene products that have diverse fates. The well-known primary *gag* gene product, Pr65^{gag}, is the principal precursor polyprotein to the four viral core proteins p30, p15, pp12, and p10 (1, 2, 6, 14). Our evidence indicates that the cleavage of Pr65^{gag} is facilitated by phosphorylation (15) and by interaction with viral genomic RNA (7). A second primary *gag* gene proudct (Pr200$^{gag\text{-}pol}$) is synthesized as a joint product of the *gag* and *pol* regions. This *gag-pol* gene product is the precursor to the reverse transcriptase (7, 10, 11). Stoichiometric considerations and pulse-chase studies conducted in the presence and absence of inhibitors of proteolytic cleavage rule out the possibility that Pr200$^{gag\text{-}pol}$ is also a major precursor of *gag* gene products (8). A third primary *gag* gene product is a non-glycosylated polypeptide with an apparent molecular weight (M_r) of about 75,000 daltons (Pr75^{gag}). A Pr75^{gag} is believed to be glycosylated during its synthesis to yield a glycoprotein of approximately 80,000 daltons (gPr80^{gag}) which is glycosylated further, yielding two glycoproteins of 93,000 and 95,000 daltons (3, 19). These latter proteins were first observed by Tung et al. (21) on the surface of AKR mouse leukemic thymocytes and by Evans et al. in Friend MuLV-infected cells (5). Ledbetter et al. made similar observations and provided solid evidence that both gP93^{gag} and gP95^{gag} contained antigenic determinants and peptide sequences found in each of the four core proteins (12). Edwards and Fan (3, 4) and Schultz et al. (18, 19) have further characterized the glycosylated *gag* gene products of MuLV. gPr80^{gag} incorporates mannose and contains additional sequences, termed leader (L) sequences, located N-terminal to core protein sequences. The role of these *gag* gene-derived glycoproteins in virus replication is unclear, but it appears that they are not major constituents of viral particles. In fact, gP93^{gag} and gP95^{gag} appear to be released from the cell surface into the culture medium (3). In this paper, we have investigated the glycosylated *gag* gene products and describe a new glycosylated 45,000-M_r polypeptide (gPr45^{gag}) that was found to contain the proposed L se-

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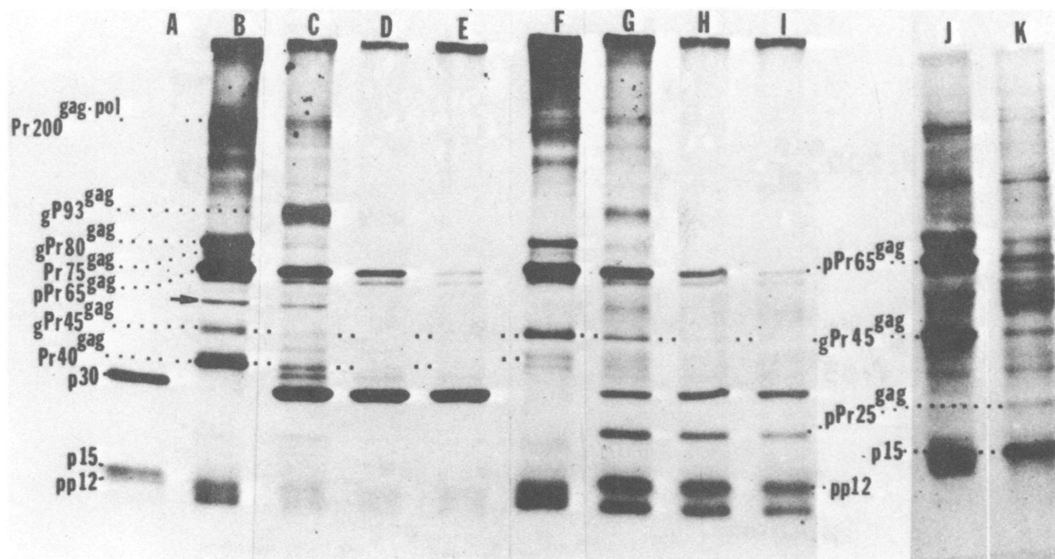

FIG. 1. Pulse-chase kinetics of R-MuLV *gag* gene products. R-MuLV-infected NIH Swiss mouse embryo cells were pulse-labeled in Earle's balanced salts for 7.5 min with [^{3}H]leucine (100 μCi/ml) and chase-incubated in label-free growth media (McCoy's 5A medium; 10% fetal calf serum-50 μg of gentamicin per ml) for 0 min (lanes B, F, and J), 30 min (lanes C and G), 60 min (lanes D, H, and K), or 90 min (lanes E and I) before cell lysis in a Nonidet P-40 detergent-containing buffer (2). The lysate was centrifuged at 10,000 × *g* for 10 min to remove nuclei and debris. The supernatant was treated with antisera to R-MuLV p30 (lanes B through E) or antisera to R-MuLV p15 (lanes J and K). The antigen-antibody complexes were quantitatively precipitated with *Staphylococcus aureus* (15). The supernatant from the anti-p30 precipitations were subsequently treated with antibody to R-MuLV p12, and the immune complex was again collected by centrifugation (lanes F through I). The immunoprecipitates were denatured by boiling in SDS buffer and fractionated by electrophoresis on a 6 to 12% linear gradient of polyacrylamide in the presence of SDS and mercaptoethanol. Bands were detected in dried gels by autofluorography. Lane A, [^{3}H]leucine marker R-MuLV (20).

quences as well as p15 and p12 determinants. A 27,000-M_r glycoprotein containing p15 determinants was also detected.

The *gag* gene products of Rauscher MuLV (R-MuLV) were isolated from cytoplasmic extracts of pulse-labeled and chase-incubated infected cell cultures by immunoprecipitation with anti-p30 and anti-p12 sera (Fig. 1). Anti-p30 serum precipitated the usual precursor polyproteins from extracts of pulse-labeled cells (Fig. 1, lane B). These include Pr200$^{gag\text{-}pol}$, gPr80^{gag}, Pr65^{gag}, and Pr40^{gag}. Pr40^{gag} has previously been shown to be an intermediate-sized precursor of p10 and p30 derived from Pr65^{gag} by intracellular proteolytic cleavage (15). Although clearing the cell extract with anti-p30 removed most of the p30-related precursors, significant amounts of Pr200$^{gag\text{-}pol}$, Pr65^{gag}, and Pr80^{gag} remained in the extract and were precipitable by subsequent treatment with anti-p12 serum. Anti-pp12 serum precipitated each of these precursors from pulse-labeled cells (Fig. 1, lane F) as well as significant amounts of a protein termed gPr45^{gag}

After a chase-incubation of 30 min, each of the radioactive precursors, including Pr40^{gag} and gPr45^{gag}, could be observed to decrease in amounts of cell extracts (Fig. 1, lanes C and G). There was also the appearance during this chase of two other new proteins in the cells. A protein termed gP93/95^{gag} was present and was precipitable by both antisera (lanes C and G). This polypeptide, which was not present in the pulse-labeled cells, appears to be the cell surface *gag* gene-related glycoprotein (5, 21) which has been found to contain peptides characteristic of p15, pp12, p30, and p10 (12) and which is derived by further glycosylation of gPr80^{gag}. Another protein, termed pPr25^{gag}, was shown to be present but was precipitable in this experiment most efficiently by anti-pp12 serum (Fig. 1, lane G). pPr25^{gag}, although a minor protein in these cells, has been shown to be an intermediate-sized precursor of pp12 and p15 derived by intracellular cleavage of phosphorylated Pr65^{gag} (15). The presence of pPr25^{gag} in short chases is indicative of its origin by the processing of large precursors.

In longer chases (Fig. 1, lanes D, E, H, and I), each of the precursor polyproteins continued to decrease in intensity, with a corresponding increase in intracellular levels of p30 and pp12

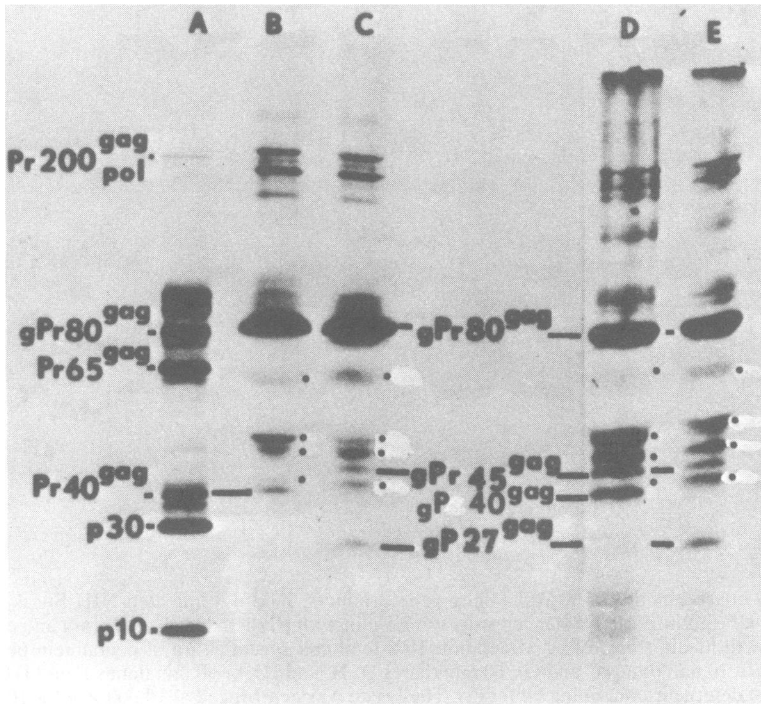

FIG. 2. Incorporation of [^{3}H]mannose into *gag* gene-coded viral proteins. A T75 flask of confluent cells was incubated for 4 h in glucose-free medium, followed by incubation for 30 min with [^{3}H]mannose (200 μCi/ml) as described previously (3). Cells were lysed, and equal aliquots of the cytoplasmic extract were challenged with anti-p10 serum (lane B) or anti-p15 serum (lane C). Lane A. Anti-Rauscher murine leukemia virus serum-precipitated extract of cells pulse-labeled with [^{3}H]leucine mixed with a similarly precipitated extract of pulse-labeled, chase-incubated cells; lanes D and E, cell extracts labeled with [^{3}H]mannose as described above and precipitated with antisera to p30 and pp12 (lane D) and p15 (lane E). The immunoprecipitates were analyzed as described in the legend to Fig. 1. The dots in this figure indicate other mannose-containing proteins which are presumably degradation products of larger glycosylated *gag* gene products or background host proteins.

(lanes D, E, and H, I, respectively). Levels of labeled gP93/95^{gag} also decreased drastically in intensity in cells during the 60-min chase (lanes D and H). It is apparent in this experiment that very low levels of gPr45^{gag} can be precipitated by anti-p30 serum (lane B), owing to contaminating antibody to pp12 in this serum. This interpretation is supported by the ability of the anti-p30 serum to precipitate very low levels of pPr25^{gag} from extracts of pulse-labeled cells (lanes C through E). Likewise, the anti-pp12 serum is contaminated with significant levels of antibody to p30. The preclearing of cell extracts with anti-p30 serum before precipitation with anti-pp12, however, increases the ratio of gPr45^{gag} to Pr40^{gag} precipitated by anti-pp12 serum (cf. lanes F and B). Immunoprecipitation of uncleared extracts with an anti-p15 serum which is free from contaminating anti-p30 activity clearly substantiates the p15 content of gPr45^{gag} (lanes J and K) and pPr25^{gag}

The protein band migrating at about 50,000 daltons in this figure (arrow, lane B) was nonspecifically precipitated by various antisera, including antiserum to gp70 and normal rabbit serum (not shown), and is presumed to be actin.

Another minor band, partly overshadowed by the more intense gPr80^{gag} band, was designated Pr75^{gag} (Fig. 1, lane B). This protein is routinely observed in low amounts in extracts of pulse-labeled cells precipitated by antisera to core proteins. Pr75^{gag} is similar in size to a 75,000-dalton polypeptide which, like Pr65^{gag}, has previously been identified as a primary in vitro translation product of 35S viral RNA (13).

To determine whether any of the proteins shown in Fig. 1 were glycosylated, we pulse-labeled cells with [^{3}H]mannose (3) and immunoprecipitated the viral proteins with monospecific antisera prepared against p15 and p10 (Fig. 2). In other experiments, antiserum to p10 did not precipitate p30, p15, or pp12 from extracts of [^{3}H]leucine-labeled cells (15). Antisera to p10 and p15 were, therefore, considered to be the most specific of the so-called monospecific antisera to R-MuLV core proteins used in this study. Treatment with antiserum to p10 resulted in precipitation of mannosylated gPr80^{gag} and

two to three proteins which comigrated with Pr200$^{gag\text{-}pol}$ (Fig. 2, lane B). Lower-M_r proteins which were also labeled with [^{3}H]mannose included a 40,000-M_r protein, termed gP40^{gag}, which comigrated with Pr40^{gag} (Fig. 2, lanes A and B). Treatment with antiserum to p15 resulted in precipitation of labeled gPr80^{gag}, Pr200$^{gag\text{-}pol}$, a protein which comigrated with gPr45^{gag}, and a protein termed gP27^{gag} which migrated in the gel only slightly more slowly than did pPr25^{gag} (Fig. 2, lane C). Neither gPr45^{gag} nor gP27^{gag} were precipitated with anti-p10 serum (Fig. 2, lane B). Also, [^{3}H]mannose-labeled gP40^{gag} recognized by anti-p10 serum (Fig. 2, lane B) was not precipitated with anti-p15 serum (lane C). Other mannose-containing proteins (Fig. 2, dots) were variably precipitated with both anti-p10 and anti-p15 sera and are presumably either degradation products of larger glycosylated *gag* polyproteins or background host proteins. In another experiment, a similarly labeled extract of cells was precipitated with a mixture of pp12 and p30 antisera (Fig. 2, lane D) or with antiserum to p15 (lane E). It was apparent that the mannosylated 27,000-M_r protein, gP27^{gag}, was precipitated by antiserum to p15 (Fig. 2, lane E) but was not precipitated by the mixed pp12 and p30 antisera (Fig. 2, lane D). This antibody mixture did, however, precipitate both gPr40^{gag} and gPr45^{gag}. Once again, the previously noted background proteins were observed with either sera. In other studies, nearly identical patterns of [^{3}H]mannose-labeled viral proteins have been obtained from cells infected with R-MuLV which had been biologically cloned free of Rauscher spleen focus-forming virus (data not shown). A glycosylated *gag*-related protein of 45,000 M_r has also been detected in cells infected with Moloney murine leukemia virus (data not shown).

We have previously observed that Pr65^{gag} and one of its intermediate cleavage products, Pr25^{gag}, are phosphorylated (15). The phosphorylation of these precursors presumably occurs at pp12 sites in the polyprotein molecules. Pr75^{gag}, gPr80^{gag}, and gPr45^{gag}, however, are apparently not phosphorylated (data not shown), even though each of these polyproteins contains p12 sequences. The results shown in Fig. 1 and 2 suggest that gPr45^{gag} is an intermediate glycosylated precursor polyprotein which, like pPr25^{gag}, contains p15 and pp12. Based on the size differences between Pr25^{gag} and gPr45^{gag}, however, the later polyprotein may also contain additional protein sequences.

Our studies and those of others have clearly demonstrated that Pr65^{gag} and gPr80^{gag} contain antigenic determinants and peptide sequences found in all four viral core proteins (1, 8, 9). We have also shown that Pr75^{gag} made in vitro appears to contain antigenic determinants of all four core proteins (13). In this study, we compared the peptide maps of gPr80^{gag}, Pr75^{gag}, and Pr65^{gag} with those of gPr45^{gag} and Pr40^{gag} to substantiate the conclusions about the relatedness of these polyproteins reached as a result of immunoprecipitation analyses (Fig. 1 and 2). We prepared [^{3}H]leucine-labeled precursors by means of immunoprecipitation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and digested the purified polypeptides with trypsin. The digests were fractionated on thin-layer plates in two dimensions (Fig. 3). Viral proteins purified by chromatography and SDS-polyacrylamide gel electrophoresis (16) were also digested with trypsin, and the peptides were similarly fractionated. The tryptic peptides of each protein were numbered arbitrarily for identification (Table 1). As expected, gPr80^{gag}, Pr75^{gag}, and Pr65^{gag} were all found to have very similar peptide maps. All three were found to contain [^{3}H]leucine-labeled peptides in the pp12, p30, and p10 core proteins. We also found that Pr65^{gag} contained a peptide (no. 24) that was not detected in p30, pp12, p10, Pr75^{gag}, or gPr80^{gag}.

TABLE 1. Peptide content of R-MuLV proteins

Viral protein	Peptide no.
p30	2, 3, 6, 7, 8A,[a] 8,[b] 10, 11, 12, 14, 16, 17,[c] 18, 19, 20
p15	24[d]
p12	1, 21, 22, 9
p10	4, 5, 15
Pr40^{gag}	2, 3, 4, 5, 6, 7, 8A,[a] 8,[b] 10, 11, 12, 13,[e] 14, 15, 16, 17,[c] 18, 19, 20
gPr45^{gag}	1, 8,[b] 9, 21, 22, 25[f]
Pr65^{gag}	1, 2, 3, 4, 5, 6, 7, 8,[b] 9, 10, 11, 12, 13, 14, 15, 16, 17,[c] 18, 19, 20, 21, 22, 24[d]
Pr75^{gag}	1, 2, 3, 4, 5, 6, 7; 8,[b] 9, 10, 11, 12, 13, 14, 15, 16, 17,[c] 18, 19, 20, 21, 22, 25[f]
gPr80^{gag}	1, 2, 3, 4, 5, 6, 7, 8,[b] 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 23,[g] 25[f]

[a] 8A is a peptide in p30 and Pr40^{gag} that may be altered by association with p12 in Pr65^{gag}, Pr75^{gag}, and gPr80^{gag}.

[b] 8 is a p30 peptide that is also present in p30 precursors, comigrating with a p15-p12 junction peptide present in gPr45^{gag}, but absent in p15 or p12 mature proteins.

[c] 17 is a p30 peptide that may be glycosylated in gPr80^{gag} (no. 23).

[d] 24 is a p15 peptide in p15 and Pr65^{gag} that may be altered by the presence of L peptides in Pr75^{gag} and gPr80^{gag}.

[e] 13 is a presumed p30-p10 junction peptide that is absent in p30 or p10 but present in Pr40^{gag}, Pr65^{gag}, Pr75^{gag}, and gPr80^{gag}.

[f] 25 is a presumed L peptide that is present in gPr45^{gag}, Pr75^{gag}, and gPr80^{gag}.

[g] 23 may be a glycosylated p30 peptide (no. 17).

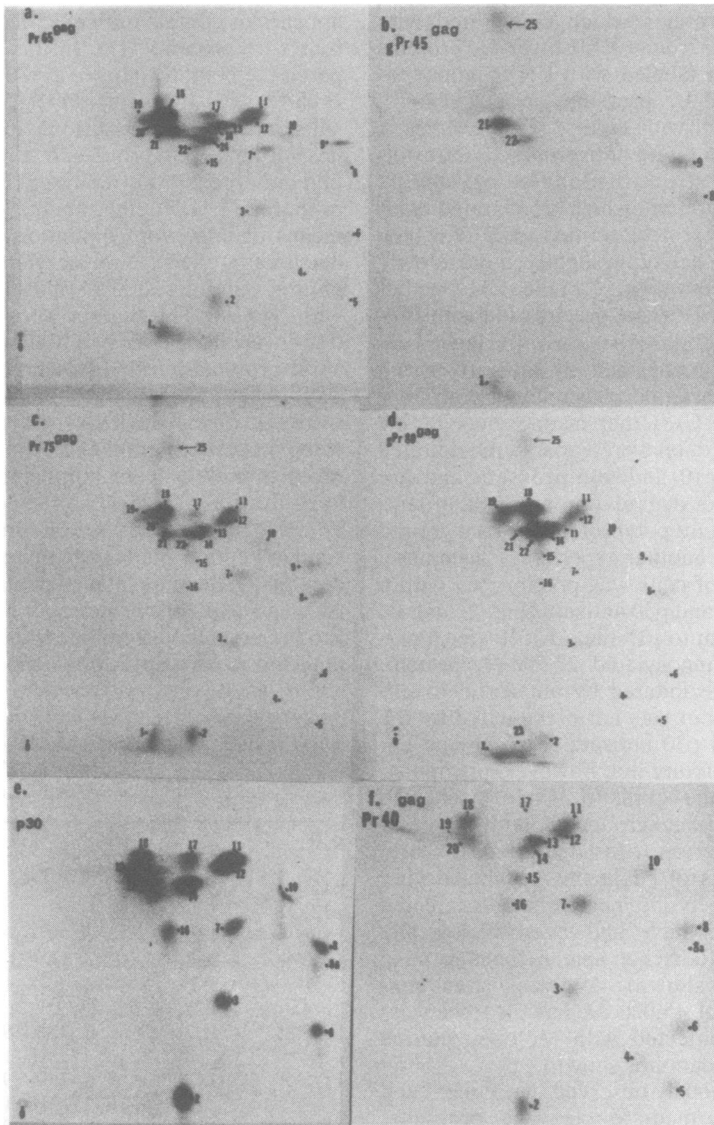

FIG. 3. Peptide maps of R-MuLV precursor polyproteins. Cells were labeled with [^{3}H]leucine (250 μCi/ml) for 20 min, and the cell extracts were precipitated with a mixture of anti-p30 and anti-p10 sera. gPr45^{gag} was precipitated with anti-p12 serum from the supernatants of the extract which had been previously cleared with the anti-p10-anti-p30 sera mixture. The proteins were purified by SDS-polyacrylamide gel electrophoresis as previously described. The [^{3}H]leucine-labeled viral proteins were digested with trypsin as previously described, and the digests were fractionated on thin-layer cellulose plates (11). The peptide maps of viral p30, as well as of p15, p12 and p10 (not shown; see Table 1) were also analyzed.

The single p15 leucine-containing peptide characteristic of p15 appears to comigrate with peptide 24 (not shown). It is possible that the p15 peptide is N-terminal in p15 and present in Pr65^{gag} but altered in Pr75^{gag} and gPr80^{gag} owing to the presence of L sequence peptides more N-terminal in these precursors (see Table 1). It is clear, however, that anti-p15 serum recognizes all three precursors (Fig. 1, lane J). Also of interest was the finding that Pr75^{gag} and gPr80^{gag} reproducibly contained an additional leucine-labeled tryptic peptide, termed spot 25 (Fig. 3, arrow), which was not found in Pr65^{gag}, Pr40^{gag}, or in any of the viral core proteins. This tryptic peptide was also detected in tyrosine-labeled digests of gPr80^{gag} but not those of tyrosine-labeled Pr65^{gag} (11). From immunoprecipitation analyses, we concluded that gPr45^{gag}

contains p15 and p12 sequences, sugar residues, and, on the basis of size, perhaps other protein sequences. Analysis of the tryptic peptide map of gPr45^{gag} substantiated these conclusions. Furthermore, gPr45^{gag} was shown to contain peptide 25, the single peptide characteristic of both gPr80^{gag} and its unglycosylated apoprotein, Pr75^{gag}. Thus, gPr45^{gag} appears to be related to gPr80^{gag} and Pr75^{gag}. In contrast to this, Pr40^{gag} contained p30 and p10 peptides (see Table 1) but lacked peptide 25. Pr40^{gag} also contained the p30-specific peptide 17 which was previously noted to be present in Pr65^{gag} and Pr75^{gag} but absent in gPr80^{gag}.

Several findings indicate that Pr75^{gag} observed in and isolated from whole cells is a distinct protein not contaminated with gPr80^{gag}. First, Pr65^{gag} and Pr75^{gag} contained a p30-specific peptide (spot 17; Fig. 3a, c, and e) not found in gPr80^{gag} (Fig. 3d). Likewise, gPr80^{gag} appeared to contain a peptide (Fig. 3d, spot 23) not present in either Pr75^{gag} or Pr65^{gag} or in any of the mature viral proteins (see Table 1). Furthermore, two minor spots located above spot 18 in Pr65^{gag} (Fig. 3a) also appear in Pr75^{gag} (Fig. 3c) but are absent from gPr80^{gag} (Fig. 3d). Most significantly, the map of Pr75^{gag} shown in Fig. 3 (panel c) is nearly identical to the map obtained from Pr75^{gag} synthesized in a cell-free translation system or from Pr75^{gag} synthesized in whole cells in the presence of tunicamycin (data not shown). Peptide 17 in p30 and unglycosylated core-related precursors is thought to be modified by glycosylation in gPr80^{gag}. The new peptide (no. 23 in Fig. 3d) in gPr80^{gag} may be glycosylated p30 peptide 17. Consistent with this possibility are the reports that glycosylation of gPr80^{gag} occurs at one location in the p30 region and at a second location in p15 or in the L sequences (4, 17).

The presence of a common peptide (no. 25) in both unglycosylated Pr75^{gag} and glycosylated gPr80^{gag} and the absence of this peptide in Pr65^{gag} strongly suggests that peptide 25 is unique to the L sequence that is also known to be present in both Pr75^{gag} and gPr80^{gag} but absent in Pr65^{gag}. The presence of this same peptide in a newly described intracellular polyprotein, termed gPr45^{gag}, indicates that this polyprotein also contains the L sequence. The fact that gPr45^{gag} is glycosylated and contains both p15 and p12 antigenic sequences and p12 peptide sequences supports the conclusion that gPr45^{gag} is a polyprotein representing the N-terminal region of the glycosylated *gag* gene product. Given the structure of gPr80^{gag} as NH_2-L-gp15-p12-gp30-p10-COOH, the proposed structure of gPr45^{gag} is NH_2-L-gp15-p12-COOH. Kinetics of gPr45^{gag} disappearance during chase incubation of cells and the comcomitant appearance of a lower-M_r glycoprotein, termed gP27^{gag}, which contains p15 but not p12 sequences suggest specific processing of glycosylated *gag* gene products in infected cells.

Our results concerning the synthesis of gPr45^{gag} containing p15, p12 and L sequences is supported by the recent observations of others (E. Pillemer and I. Weisman, Stanford University, Stanford, Calif., personal communication) who have prepared a monoclonal antibody that reacts with glycosylated *gag* polypeptides on the surface of cells infected with and producing AKR MuLV. The antibody does not react with Pr65^{gag} but does react with the apoprotein of glycosylated *gag*. These results suggest that this antibody is specific for a determinant in the N-terminal leader or L peptide of glycosylated *gag* polyproteins. Two glycosylated *gag* polyproteins, as well as the apoprotein, were detected by this antibody on the cell surface of AKR MuLV-infected cells. One glycoprotein is gP93/95^{gag} and the other is a smaller protein containing L, p15, and p12 determinants but lacking p30 and p10 determinants. Thus, a protein similar in size and structure to gPr45^{gag} described here has been detected at the surface of AKR virus-infected cells, using antibody specific for the L peptide unique to glycosylated *gag* polyproteins. The role of gPr45^{gag}, gP27^{gag}, or other glycosylated MuLV core proteins, if any, in retrovirus replication remains to be established.

This research was supported in part by grant G-429 from the Robert A. Welch Foundation, Public Health Service grants CA-25465 and CA-16672 from the National Cancer Institute, grant MV-86 from the American Cancer Society, grant KS-994 from the Kelsey-Seybold Foundation, and a grant from the Mary K. Ash Fund for Cancer Research. R.B.N. is a Leukemia Society of America Scholar. V.L.N. and J.J.K. were predoctoral fellows supported by the Rosalie B. Hite Foundation. L.H.S. was a postdoctoral fellow supported by Public Health Service training grant CA-09299 from the National Cancer Institute.

We thank James Syrewicz, Elena Leroux, and Tania Busch for technical assistance and Becky Bazer for assistance in manuscript preparation.

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