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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A glycosylated 45,000-Mr 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 gP45gag, was also shown to contain a single tryptic peptide also present in gp80p50 and its unglycosylated apoprotein precursor Pr75gag, but lacking in Pr55gag or Pr40gag. The presence of this peptide only in viral precursor proteins containing the so-called leader (L) sequence strongly suggests that gP45gag 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 gP45gag and its disappearance during chase-incubation is suggestive of a precursor-like role for this intermediate gene product. An observed 27,000-Mr glycosylated polypeptide, termed gP27gag and containing p15 but not p12, p30, or p10 antigenic determinants, is a candidate cleavage product derived from gP45gag. These observations suggest that gP45gag and its putative cleavage product gP27gag 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, Pr65gag, is the principal precursor polyprotein to the four viral core proteins p30, p15, p12, and p10 (1, 2, 6, 14). Our evidence indicates that the cleavage of Pr65gag is facilitated by phosphorylation (15) and by interaction with viral genomic RNA (7). A second primary gag gene product (Pr200gag-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 Pr200gag-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 (Mₐ) of about 75,000 daltons (Pr75gag). A Pr75gag is believed to be glycosylated during its synthesis to yield a glycoprotein of approximately 80,000 daltons (gp80p50) 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 gp93gag and gp95gag 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. gp80gag 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, gp93gag and gp95gag 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-Mr polypeptide (gP45gag) that was found to contain the proposed L se-
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 [³H]leucine (100 μCi/ml) and chase-incubated in label-free growth media (McCoy’s 5 A 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 x 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 autoradiography. Lane A, [³H]leucine marker R-MuLV (20).

quences as well as p15 and p12 determinants. A 27,000-Mr, 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 agreg-pol, gPr80 agreg, Pr65 agreg, and Pr40 agreg. Pr40 agreg has previously been shown to be an intermediate-sized precursor of p10 and p30 derived from Pr65 agreg by intracellular proteolytic cleavage (15). Although clearing the cell extract with anti-p30 removed most of the p30-related precursors, significant amounts of Pr200 agreg-pol, Pr65 agreg, and Pr80 agreg remained in the extract and were precipitable by subsequent treatment with anti-p12 serum. Antip12 serum precipitated each of these precursors from pulse-labeled cells (Fig. 1, lane F) as well as significant amounts of a protein termed gPr45 agreg.

After a chase-incubation of 30 min, each of the radioactive precursors, including Pr40 agreg and gPr45 agreg, 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 agreg 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 agreg. Another protein, termed pPr25 agreg, was shown to be present but was precipitable in this experiment most efficiently by anti-pp12 serum (Fig. 1, lane G). pPr25 agreg, 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 agreg (15). The presence of pPr25 agreg 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 [3H]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 [3H]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-prefractionated extract of cells pulse-labeled with [3H]leucine mixed with a similarly precipitated extract of pulse-labeled, chase-incubated cells; lanes D and E, cell extracts labeled with [3H]mannose as described above and precipitated with antiserum to p30 and p12 (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 gP93/95gag 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 gPr45gag 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 pPr25gag 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 preclaring of cell extracts with anti-p30 serum before precipitation with anti-pp12, however, increases the ratio of gPr45gag to Pr40gag 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 gPr45gag (lanes J and K) and pPr25gag.

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 gPr80gag band, was designated Pr75gag (Fig. 1, lane B). This protein is routinely observed in low amounts in extracts of pulse-labeled cells precipitated by antiserum to core proteins. Pr75gag is similar in size to a 75,000-dalton polypeptide which, like Pr65gag, 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 [3H]mannose (3) and immunoprecipitated the viral proteins with monospecific antiserum prepared against p15 and p10 (Fig. 2). In other experiments, antiserum to p10 did not precipitate p30, p15, or pp12 from extracts of [3H]leucine-labeled cells (15). Antiserum 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 gPr80gag and
two to three proteins which comigrated with Pr200gag-pol (Fig. 2, lane B). Lower-Mr proteins which were also labeled with [3H]mannose included a 40,000-Mr protein, termed gp40gag, which comigrated with Pr40gag (Fig. 2, lanes A and B). Treatment with antiserum to p15 resulted in precipitation of labeled gPr80gag, Pr200gag-pol, a protein which comigrated with gpPr45gag, and a protein termed gp27gag which migrated in the gel only slightly more slowly than did pPr25gag (Fig. 2, lane C). Neither gpPr45gag nor gp27gag were precipitated with antip10 serum (Fig. 2, lane B). Also, [3H]mannose-labeled gp40gag recognized by anti-p10 serum (Fig. 2, lane B) was not precipitated with antip15 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 mannyslated 27,000-Mr protein, gp27gag, 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 gpPr40gag and gpPr45gag. Once again, the previously noted background proteins were observed with either sera. In other studies, nearly identical patterns of [3H]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 Mr, has also been detected in cells infected with Moloney murine leukemia virus (data not shown).

We have previously observed that Pr65gag and one of its intermediate cleavage products, Pr25gag, are phosphorylated (15). The phosphorylation of these precursors presumably occurs at pp12 sites in the polyprotein molecules. Pr75gag, gpR80gag, and gpPr45gag, 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 gpPr45gag is an intermediate glycosylated precursor polyprotein which, like pPr25gag, contains p15 and pp12. Based on the size differences between Pr25gag and gpPr45gag, however, the later polyprotein may also contain additional protein sequences.

Our studies and those of others have clearly demonstrated that Pr65gag and Pr80gag contain antigenic determinants and peptide sequences found in all four viral core proteins (1, 8, 9). We have also shown that Pr75gag made in vitro appears to contain antigenic determinants of all four core proteins (13). In this study, we compared the peptide maps of gPr80gag, Pr75gag, and Pr65gag with those of gpPr45gag and Pr40gag to substantiate the conclusions about the relatedness of these polyproteins reached as a result of immunoprecipitation analyses (Fig. 1 and 2). We prepared [3H]leucine-labeled precursors by means of immunoprecipitation and sodium deoxy sulfate (SDS)-polyacrylamide gel electrophoresis and digested the purified polyproteins 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, gPr80gag, Pr75gag, and Pr65gag were all found to have very similar peptide maps. All three were found to contain [3H]leucine-labeled peptides in the pp12, p30, and p10 core proteins. We also found that Pr65gag contained a peptide (no. 24) that was not detected in p30, pp12, p10, Pr75gag, or gPr80gag.

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<th>TABLE 1. Peptide content of R-MuLV proteins</th>
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* 8A is a peptide in p30 and Pr40gag that may be altered by association with p12 in Pr65gag, Pr75gag, and gPr80gag.
* 8 is a p30 peptide that is also present in p30 precursors, comigrating with a p15-p12 junction peptide present in gpPr45gag, but absent in p15 or p12 mature proteins.
* 17 is a p30 peptide that may be glycosylated in gPr80gag (no. 23).
* 24 is a p15 peptide in p15 and Pr65gag that may be altered by the presence of L peptides in Pr75gag and gPr80gag.
* 25 is a presumed p30-p10 junction peptide that is absent in p30 or p10 but present in Pr40gag, Pr65gag, Pr75gag, and gPr80gag.
* 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$^{ag}$ 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$^{ag}$ but altered in Pr75$^{ag}$ and gPr80$^{ag}$ 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$^{ag}$ and gPr80$^{ag}$ reproducibly contained an additional leucine-labeled tryptic peptide, termed spot 25 (Fig. 3, arrow), which was not found in Pr65$^{ag}$, Pr40$^{ag}$, or in any of the viral core proteins. This tryptic peptide was also detected in tyrosine-labeled digests of gPr80$^{ag}$ but not those of tyrosine-labeled Pr65$^{ag}$ (11). From immunoprecipitation analyses, we concluded that gPr45$^{ag}$
contains p15 and p12 sequences, sugar residues, and, on the basis of size, perhaps other protein sequences. Analysis of the tryptic peptide map of gPr45gag substantiated these conclusions. Furthermore, gPr45gag was shown to contain peptide 25, the single peptide characteristic of both gPr80gag and its unglycosylated apoprotein, Pr75gag. Thus, gPr45gag appears to be related to gPr80gag and Pr75gag. In contrast to this, Pr40gag contained p30 and p10 peptides (see Table 1) but lacked peptide 25. Pr40gag also contained the p30-specific peptide 17 which was previously noted to be present in Pr65gag and Pr75gag but absent in gPr80gag.

Several findings indicate that Pr75gag observed in and isolated from whole cells is a distinct protein not contaminated with gPr80gag. First, Pr65gag and Pr75gag contained a p30-specific peptide (spot 17; Fig. 3a, c, and e) not found in gPr80gag (Fig. 3d). Likewise, gPr80gag appeared to contain a peptide (Fig. 3d, spot 23) not present in either Pr75gag or Pr65gag or in any of the mature viral proteins (see Table 1). Furthermore, two minor spots located above spot 18 in Pr65gag (Fig. 3a) also appear in Pr75gag (Fig. 3c) but are absent from gPr80gag (Fig. 3d). Most significantly, the map of Pr75gag shown in Fig. 3 (panel c) is nearly identical to the map obtained from Pr75gag synthesized in a cell-free translation system or from Pr75gag 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 gPr80gag. The new peptide (no. 23 in Fig. 3d) in gPr80gag may be glycosylated p30 peptide 17. Consistent with this possibility are the reports that glycosylation of gPr80gag 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 Pr75gag and glycosylated gPr80gag and the absence of this peptide in Pr65gag strongly suggests that peptide 25 is unique to the L sequence that is also known to be present in both Pr75gag and gPr80gag but absent in Pr65gag. The presence of this same peptide in a newly described intracellular polypeptide, termed gPr45gag, indicates that this polypeptide also contains the L sequence. The fact that gPr45gag is glycosylated and contains both p15 and p12 antigenic sequences and p12 peptide sequences supports the conclusion that gPr45gag is a polypeptide representing the N-terminal region of the glycosylated gag gene product. Given the structure of gPr80gag as NH2-L-gp15-p12-gp30-p10-COOH, the proposed structure of gPr45gag is NH2-L-gp15-p12-COOH. Kinetics of gPr45gag disappearance during chase incubation of cells and the comcomitant appearance of a lower-Mr glycoprotein, termed gP27gag, which contains p15 but not p12 sequences suggest specific processing of glycosylated gag gene products in infected cells.

Our results concerning the synthesis of gPr45gag containing p15, p12 and L sequences is supported by the recent observations of others (E. Pillemer and L. 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 Pr65gag 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 polypeptides. Two glycosylated gag polypeptides, as well as the apoprotein, were detected by this antibody on the cell surface of AKR MuLV-infected cells. One glycoprotein is gP93.95gag 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 gPr45gag described here has been detected at the surface of AKR virus-infected cells, using antibody specific for the L peptide unique to glycosylated gag polypeptides. The role of gPr45gag, gp27gag, or other glycosylated MuLV core proteins, if any, in retrovirus replication remains to be established.

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LITERATURE CITED


