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Structure of the Murine Leukemia Virus Envelope Glycoprotein Precursor

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The glycosylated *env* gene precursor ($Pr80^{env}$) of Moloney murine leukemia virus has been isolated by selective immunoprecipitation. Use of the drug tunicamycin to inhibit nascent glycosylation or specific cleavage with endoglycosidase H demonstrated that the precursor contained an apoprotein with a molecular weight of 60,000. The finished virion glycoprotein (gp70) was largely resistant to the action of endoglycosidase H. Chromatography of the glycopeptides of $Pr80^{env}$ in conjunction with endoglycosidase H digestion studies suggested that the precursor contained two distinct major glycosylation sites. Analysis of partial proteolytic cleavage fragments of $Pr80^{env}$ before and after endoglycosidase H treatment placed the two glycosylation sites within a 30,000-dalton region of the apoprotein sequence. Kinetic experiments showed that carbohydrate processing as well as proteolytic cleavage are late steps in the maturation of $Pr80^{env}$.

Genome mapping correlated with in vitro translation studies has shown that a 20 to 22S mRNA encoded from the 3′ third of the murine leukemia virus (MuLV) genome is translated in vivo into a glycosylated polyprotein with a molecular weight of 80,000 to 90,000 designated Pr^{env} (4, 22, 34). Pr^{env} is synthesized and processed in cellular membranes by proteolysis and carbohydrate alteration to the finished *env* gene products gp70 and p15E (5, 13, 16, 18, 26, 37).

Recent work on other viral and cellular glycoprotein systems has shown that the primary glycosylation event involves the en bloc transfer of a large, branched, preformed, lipid-linked, high-mannose-containing structure to the polypeptide chain. This glycosylation can occur on nascent polypeptide chains. Subsequently, these core or high-mannose groups are processed in the rough and smooth endoplasmic reticulum by initial cleavage followed by sequential additions of sugars including fucose and terminal sialic acid into distinct branched-chain patterns referred to as complex carbohydrate groups (7, 8, 19, 20, 29, 35).

The elucidation of the carbohydrate structures of Pr^{env} and gp70, and of the relationship of carbohydrate processing and proteolytic cleavage to the viral budding event, is a central concern of these studies. We have examined the structure of the MuLV glycoprotein precursor (Pr^{env}), using the drug tunicamycin (30, 35), which inhibits formation of the high-mannose carbohydrate groups, and the enzyme endoglycosidase H (endo H) (31), which cleaves en bloc these high-mannose groups from the polypeptide backbone. Our results indicate that the carbohydrate processing of the MuLV Pr^{env} is similar to that described for other animal viral systems (8, 20). In addition, these carbohydrate groups provide a useful marker in studying the structure of the *env* gene precursor and its products.

MATERIALS AND METHODS

Cells and viruses. Wild-type Moloney MuLV, temperature-sensitive Moloney *ts*3 (38), and Rauscher *ts*24 (27) strains were all cloned and grown in NIH/3T3 cells as previously described (36). Cells were labeled with [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) at 50 μCi/ml in Dulbecco minimal essential medium containing 1/100 the normal methionine concentration for the times noted in the text. Cells were labeled with [2-^{3}H]mannose (New England Nuclear Corp.) at 200 μCi/ml in glucose-free phosphate-buffered saline for 60 min. In one experiment, to prepare labeled virions, cells were labeled with [2-^{3}H]mannose (200 μCi/ml) for 2 h in media containing 1/50 the normal glucose concentration; then the media were supplemented to the normal glucose level, and supernatant fluid was collected over 16 h. The virions were purified by sucrose gradient centrifugation as previously described (37).

Immunoprecipitation. Labeled cells were extracted at 0 to 4°C into 0.01 M NaH_2PO_4-Na_2HPO_4 (pH 7.5)-0.1 M NaCl containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) at 10^6 to 2×10^6 cells/ml. Lysates were clarified at 150,000 × *g* for 2 to 3 h, and samples were immunoprecipitated at 0 to 4°C as previously described, with

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the use of *Staphylococcus aureus* Cowen strain I to collect antigen-antibody complexes (11, 37). Sera used included rabbit anti-Moloney gp70, rabbit anti-Moloney p30, and rabbit anti-Moloney virions previously characterized (36).

Washed precipitates were denatured in 1% SDS–1% 2-mercaptoethanol and subjected to electrophoresis on a discontinuous stack, Tris-buffered polyacrylamide gel system as previously described (12, 37). Gels were fluorographed by the procedure of Bonnar and Laskey (1). Standard proteins used as molecular-weight markers included rabbit immunoglobulin heavy (50,000) and light (25,000) chains, MuLV p30 (30,000) and p12 (12,000), and the structural proteins of vesicular stomatitis virus and adenovirus type 2.

Enzyme digestions: endo H digestion. Endo β-*N*-acetylglucosaminidase H was prepared by the method described by Tarentino and Maley (31). The final protein concentration in the enzyme stock was 30 μg/ml. The enzyme activity was assayed with dansylated ovalbumin glycopeptides as substrate. Enzyme incubations were carried out as follows. Cell lysates were precipitated as described above with anti-gp70. The *S. aureus* pellets were resuspended in 0.1 M Tris-hydrochloride, pH 6.8, and incubated for 60 min at 30°C with 3 μg of endo H/ml. The enzyme incubation was stopped by the addition of SDS to a final concentration of 2% followed by heating for 2 min at 100°C. In other experiments, the immune precipitates were resuspended in 1% SDS–0.05 M Tris, pH 6.8, heated for 2 min at 100°C, and centrifuged to remove the *S. aureus*. The supernatant fluid was diluted with an equal volume of 0.06 M citrate, pH 5.5, or Tris (0.05 M), pH 6.8, and was incubated for various times with 0.3 μg of endo H/ml. The samples were precipitated by the addition of 2 volumes of ice-cold acetone.

Pronase digestion. Pronase digestions were performed as described by Sefton and Keegstra (25). Briefly, the samples were digested with 1% (wt/vol) Pronase (Calbiochem, grade B) in 0.1 M Tris-hydrochloride (pH 8.0)–0.01 M $CaCl_2$ for 72 h at 50°C. Additional Pronase in amounts of 1% (wt/vol) each were added at 24 and 48 h. The final Pronase concentration was 3% (wt/vol). Virions were prepared as described above. Cell lysates were immunoprecipitated with anti-gp70 before digestion. Samples which were digested with endo H were first boiled for 5 min to inactivate the Pronase, diluted with one-third volume of 0.3 M citrate, pH 5.5, and digested with 0.3 μg of endo H/ml for 12 h. The Pronase-derived glycopeptides were resolved on a Bio-Gel P6 column (1 cm by 100 cm) in 0.01 M Tris, pH 8.0. The fractions (1 ml) were counted in Handifluor (Beckman Instruments, Inc.) in a Beckman LS-230 scintillation counter. Each column run contained [^{14}C]mannose-labeled, in vitro-synthesized, lipid-linked oligosaccharide containing 6 to 9 mannose units (21) as an internal standard, a generous gift of P. Robbins.

Tunicamycin inhibitor. Tunicamycin was a gift from W. Lennarz (Johns Hopkins University). It was dissolved in 0.01 M NaOH and subsequently diluted into tissue culture medium to final concentrations of 10 and 100 μg/ml. Cells were preincubated with tunicamycin (14) for 3 h prior to metabolic labeling.

Peptide cleavage maps. The basic approach of Cleveland et al. (3) was followed. Immunoprecipitated samples released from *S. aureus* were digested in 0.05 M Tris, pH 6.8, 0.5% SDS, and 10% glycerol with a specific protease for the time and temperature noted in the text. Preliminary dose response and kinetics of cleavage with each protease were evaluated (data not shown) on preparations of Moloney Pr80^{env}. Samples were reconstituted into gel sample buffer, boiled for 2 to 3 min, and analyzed by SDS-gel electrophoresis and fluorography. Proteases used included *S. aureus* V8 (Miles Laboratories), trypsin-tolylsulfonyl phenalanyl chloromethyl ketone and α-chymotrypsin (Worthington Biochemical Corp.), and thermolysin (Sigma Chemical Co.). In some experiments samples were digested with endo H prior to or after protease digestion as described above.

Each digestion contained unlabeled rabbit immunoglobulin at approximately 2 mg/ml.

RESULTS

Size of the Pr^{env} apoprotein. Previous studies using 2-deoxyglucose, glucosamine, or cytochalasin to inhibit glycosylation in Rauscher leukemia virus-infected cells demonstrated an apparent molecular weight shift from 90,000 to 70,000 for the glycosylated and nonglycosylated forms of Pr^{env}, respectively (26, 32). To confirm and extend this observation, we used the drug tunicamycin, an inhibitor of glycosylation known to act at the first step of glycoprotein carbohydrate addition. Tunicamycin inhibits the formation of a lipid-linked, high-mannose core structure added en bloc to polypeptide chains (14, 19, 28, 30, 35).

When cells infected with Moloney MuLV were treated with tunicamycin, there was a shift in the apparent molecular weight of Pr^{env} (Fig. 1). Control cells pulse-labeled for 45 min with [^{35}S]methionine showed Pr80^{env} with the normal molecular weight of 80,000 when immunoprecipitated with antisera specific for gp70 (Fig. 1A, lane 1). Cells pretreated for 3 h with 10 μg (Fig. 1B) or 100 μg (Fig. 1C) of tunicamycin/ml and then pulse-labeled and immunoprecipitated showed a shift in the size of the glycoprotein precursor from a molecular weight of 80,000 (Pr80^{env}) to 60,000 (P60^{env}). This result indicated that a significant fraction of the primary glycosylation of Pr80^{env} occurred by transfer of tunicamycin-sensitive, lipid-linked, high-mannose structures similar to that described for several other animal viruses (7, 8, 20). The size of the core precursor (Pr65^{gag}) and its cleavage products was unaffected (Fig. 1A, B and C, lane 2). A significant decrease in the rate of protein synthesis was observed (Fig. 1B and C) with high doses of this tunicamycin preparation.

As an alternative method for removing carbohydrate from Pr^{env}, we used the enzyme endo H (see Materials and Methods). This endogly-

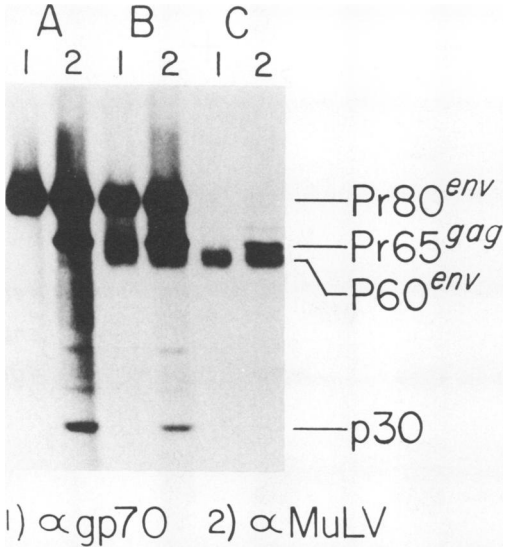

FIG. 1. *Effect of tunicamycin on the size of the env gene precursor. Monolayers of cloned NIH/3T3 cells chronically infected with Moloney MuLV were not treated (control, panel A) or were treated with 10 μg (panel B) or 100 μg (panel C) of tunicamycin/ml for 3 h at 37°C and then were pulse-labeled (30 min) with [^{35}S]methionine (50 μCi/ml) (see Materials and Methods). Cells were extracted, and a sample was immunoprecipitated with either anti-gp70 (lane 1) or anti-Moloney MuLV virions (lane 2). Immune complexes were collected with S. aureus Cowen strain I dissociated with 1% SDS, 1% mercaptoethanol, and heat and then were subjected to electrophoresis on a SDS-10% polyacrylamide gel. The gel was fluorographed and exposed to Kodak XR5 film at −70°C for 1 day.*

cosidase cleaves at the distal side of the first *N*-acetylglucosamine residue attaching the large, branched-chain, high-mannose structure to the protein moiety, leaving only a single *N*-acetylglucosamine residue behind. Endo H is active over a wide pH and temperature range, will function in the presence of moderate concentrations of denaturing detergents such as SDS, and can be extensively purified (31).

When pulse-labeled ([^{35}S]methionine) cell extracts from Moloney MuLV-infected cells were immunoprecipitated with anti-gp70 serum and treated with endo H (Fig. 2A, + lane), the Pr80^{env} had an increased electrophoretic mobility (designated P60^{env}). Determinations of glycoprotein molecular weight by SDS electrophoresis are generally overestimates (24). The shift of molecular weight from Pr80^{env} to P60^{env} does not indicate that 20,000 daltons of carbohydrate was removed, but only that the carbohydrate groups added to an apoprotein with a molecular weight of 60,000 cause it to move at an apparent molecular weight of 80,000 in SDS-gels.

Digestion with endo H had no effect on the size of the Pr65^{gag} precursor or its cleavage products immunoprecipitated with anti-p30 serum. If the Pr80^{env} was labeled with [^{3}H]mannose, however, all of the [^{3}H]mannose label was removed from the protein (data not shown).

When cells infected with other strains of MuLV were treated in the same manner, a similar increase in electrophoretic mobility of Pr^{env} was observed. Moloney *ts*3 Pr80^{env} was shifted to P60^{env} (Fig. 2B), and Rauscher *ts*24 Pr90^{env} was shifted to P70^{env} (Fig. 2C) by endo H treatment. Wild-type Rauscher leukemia virus showed a similarly sized Pr90^{env} (36; P. Traktman and O. Witte, unpublished data). Thus, different common laboratory strains of ecotropic MuLV differed by as much as 10,000 in the molecular weight of their Pr^{env} apoprotein. The position of this additional 10,000 molecular weight of protein within the precursor molecule was not determined. We did not discern any difference in the size of the finished gp70 and p15E gene products when strains of Moloney or Rauscher MuLV were compared (data not shown).

Estimate of the number of carbohydrate side chains in Pr80^{env}. By assuming that each high-mannose core oligosaccharide added at a different site would contribute equally and additively to the established molecular weight in SDS gels (23; J. Rothman, personal communication), one can estimate the number of glycosylation sites from the number of discrete intermediates between the fully glycosylated Pr80^{env} and deglycosylated P60^{env} during a time course of digestion with endo H. Figure 3 demonstrates such a time course for Pr80^{env} immunoprecipitated from cells labeled with [^{35}S]methionine for 30 min. A single intermediate with a molecular weight of 70,000 was found, suggesting that at least two sites per Pr80^{env} are glycosylated.

Glycopeptides of Pr^{env} and gp70. To characterize further the *env* gene carbohydrate structures, we compared the glycopeptides released from Pr80^{env} and virion gp70 after exhaustive Pronase digestion by chromatography on Bio-Gel P-6. This technique separates on the basis of molecular weight, but terminal charged sugar additions such as sialic acid can have pronounced effects (25). Pulse-labeled ([^{3}H]mannose, 45 min) Pr80^{env} recovered by immunoprecipitation of Moloney MuLV-infected cells or Moloney MuLV virions released from cells labeled during a 2-h interval with [^{3}H]mannose and collected over a 16-h chase period served as substrates. Both were digested with Pronase over 72 h (see Materials and Methods). The glycopeptides of Pr80^{env} (Fig. 4A) resolved into two major peaks. This difference in mobility

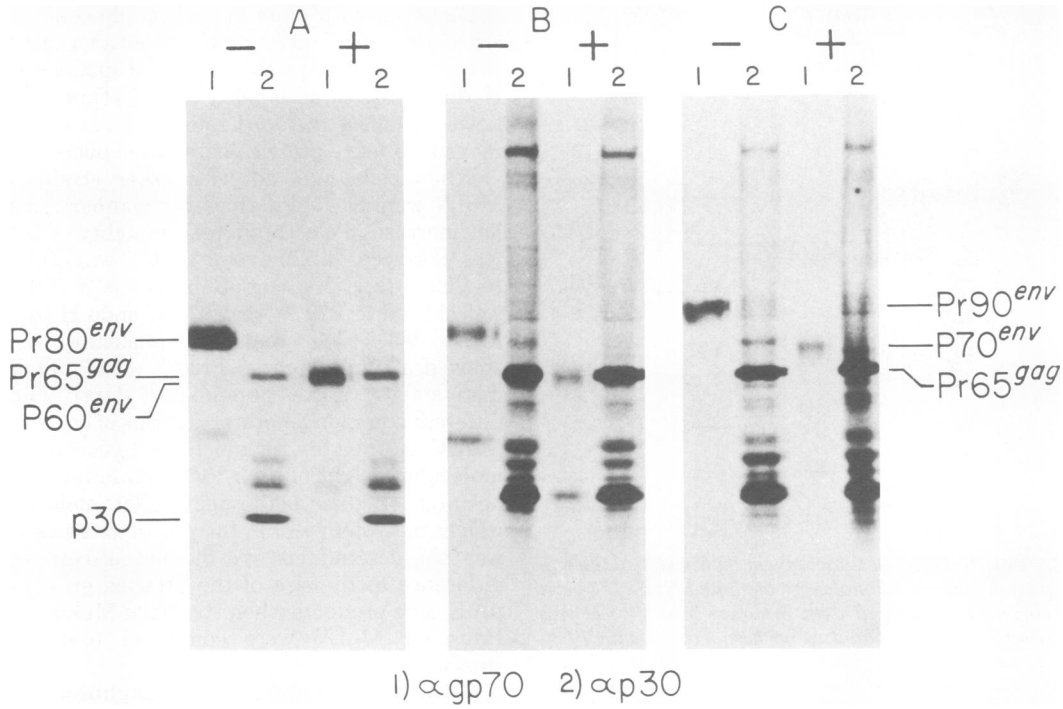

FIG. 2. *Effect of endo H on the size of the env gene precursor. Monolayers of cloned NIH/3T3 cells infected with wild-type Moloney MuLV (panel A), ts3 Moloney MuLV (panel B), or ts24 Rauscher MuLV (panel C) were pulse-labeled with [^{35}S]methionine (50 μCi/ml) at 39°C for 45 min. Cells were extracted and immunoprecipitated with anti-gp70 (lane 1) or anti-p30 (lane 2). Antigen-antibody S. aureus complexes were incubated overnight in citrate buffer (pH 5.5; see Materials and Methods) with endo H, 0.3 μg/ml (+ lanes), or without enzyme (− lanes). Samples were recovered by acetone precipitation and subjected to electrophoresis on SDS–10% polyacrylamide gels developed by fluorography as in Fig. 1.*

could have reflected differences in carbohydrate structure or residual number of amino acids not removed by Pronase digestion. To distinguish between these possibilities, a parallel sample of Pr80^{env} was first Pronase digested and subsequently treated with endo H prior to chromatography (Fig. 4B). The carbohydrate now migrated as a single component of lower molecular weight. This suggested that Pr80^{env} contained at least two identically sized oligosaccharides located at sites which differ in their amino acid composition.

The glycopeptides derived from virion gp70 resolved into several components as shown in Fig. 4C. Endo H digestion of the gp70 glycopeptides indicated that most of the glycopeptides were resistant to cleavage by endo H (Fig. 4D). This was in contrast to the glycopeptides derived from the Pr80^{env}, which were sensitive to the action of endo H. A fraction of the gp70 glycopeptides (15 to 20%) were sensitive to cleavage by endo H. This is similar to the Sindbis virus glycoprotein complex, which contains a partially processed high-mannose group susceptible to endo H cleavage in the finished virion (2, 20).

It is interesting to note that treatment of intact virions with endo H did not detectably alter the apparent molecular weight of gp70 in SDS-gel electrophoresis (unpublished data).

Pr^{env} carbohydrate processing and proteolytic cleavage appear temporally linked. It was previously shown that Pr80^{env} was rapidly labeled intracellularly but that the appearance of mature gp70 and p15 was delayed by 45 to 60 min (37).

If Pr80^{env} oligosaccharide was processed before proteolytic cleavage, one might expect to find a population of Pr80^{env} molecules largely resistant to endo H action. Conversely, if proteolytic cleavage of p15E from Pr80^{env} preceded carbohydrate modification, molecules of 65,000 to 70,000 molecular weight sensitive to endo H should be found intracellularly. Pulse-chase analysis coupled with endo H treatment (Fig. 5 and unpublished data) failed to reveal either of these putative intermediate forms accumulating intracellularly, suggesting that carbohydrate modification may be closely timed to the proteolytic processing of Pr80^{env}

Structural map of Pr^{env} by partial prote-

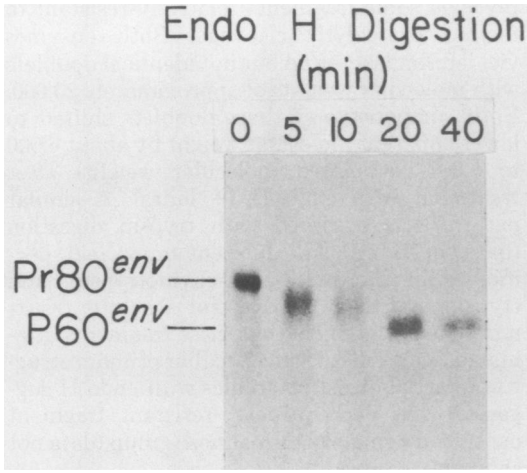

FIG. 3. *Estimation of the number of high-mannose carbohydrate groups on Pr80env by partial endo H digestion. Moloney MuLV-infected NIH/3T3 cells were labeled with [35S]methionine (30 min). Cells were extracted and immunoprecipitated with anti-gp70 serum. The antigen-antibody complexes were resuspended in citrate (pH 5.5) buffer, and 20-μl samples were digested with endo H (3 μg/ml) for 0, 5, 10, 20, or 40 min at 30°C. The reaction was stopped with SDS (final concentration, 2%, wt/vol) and boiling for 2 min. Samples were subjected to electrophoresis on SDS-10% acrylamide gels developed by fluorography.*

olysis in SDS. Cleveland et al. (3) have described a peptide mapping technique based on partial enzymatic proteolysis in SDS and subsequent separation of fragments by SDS-gel electrophoresis. We have applied this technique in combination with differential labeling of carbohydrate and protein and the use of endo H to prepare a preliminary structural map of Prenv. Pulse-labeled Prenv recovered from cellular lysates by immunoprecipitation with anti-gp70 serum was sufficiently pure to serve as substrate for these cleavages. Unlabeled immunoglobulin released from the immune complex served as carrier.

Immunoprecipitated Moloney Pr80env labeled with [35S]methionine was digested for 0, 15, or 30 min (Fig. 6, panel A) with *S. aureus* V8 protease (5.0 μg/ml) in 0.5% SDS and then was analyzed on SDS-10% acrylamide gels. These conditions generated fairly large fragments with molecular weights from 70,000 to 27,000. Pr80env labeled with [2-3H]mannose was similarly treated (Fig. 6, panel B). The fragment with a molecular weight of 27,000 was deficient in carbohydrate label, showing that both sugar side chains are confined to a region less than or equal to one-half of the apoprotein length.

As an alternative to [3H]mannose labeling, we used endo H to identify sugar-containing fragments. Pr80env labeled with [35S]methionine was first digested with a pretitered amount of protease. The proteolysis was stopped by boiling the reaction mixture, and one-half was subsequently digested with endo H.

Pr80env control, not treated with any protease, is shown in Fig. 7A (− lane). Treatment with endo H (+ lane) shifted the apparent molecular weight to P60env. A minor component precipitated with anti-gp70 serum with a molecular weight of approximately 50,000 was also sensitive to treatment with endo H. This may have represented a precursor form for the minor glycoprotein gp45 described for MuLV and shown to overlap with the amino-terminal end of gp70 in its primary structure (6).

Digestion with *S. aureus* V8 protease (5.0 μg/ml) (Fig. 7B) for 10 or 40 min generated fragments from full length (80,000) to material ≤10,000 migrating at the buffer front. Subsequent treatment with endo H (+ lanes) shifted many, but not all, fragments to lower molecular weight. These fragments presumably contained at least one high-mannose group sensitive to

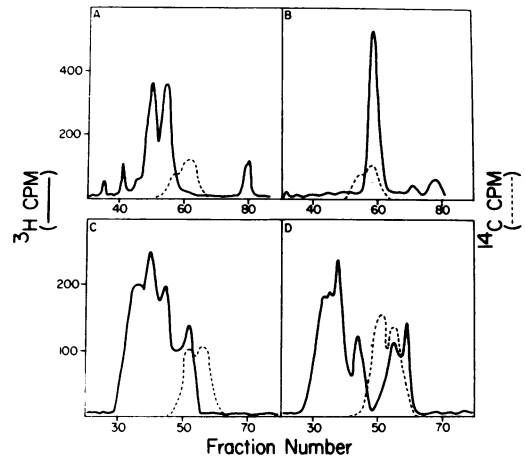

FIG. 4. *Analysis of the glycopeptides of Pr80env and gp70. (A) NIH/3T3 cells infected with Moloney MuLV were labeled with [2-3H]mannose (200 μCi/ml) for 60 min at 37°C. Cells were extracted, and Pr80env was isolated by immunoprecipitation with anti-gp70 serum. Glycopeptides were prepared by exhaustive Pronase digestion and half chromatographed on Bio-Gel P6 as described by Sefton and Keegstra (25). [14C]-mannose-labeled, in vitro-synthesized oligosaccharides (21) were used as an internal standard. (B) After Pronase digestion, one-half of the Pr80env sample was boiled for 5 min and subsequently digested with endo H (0.3 μg/μl) over a 12-h period; it was then analyzed on Bio-Gel P6 with the same internal standards. (C) [2-3H]mannose-labeled gp70 harvested from virions was Pronase digested and analyzed as in A. (D) After Pronase digestion, gp70 was subsequently digested with endo H and analyzed as in B.*

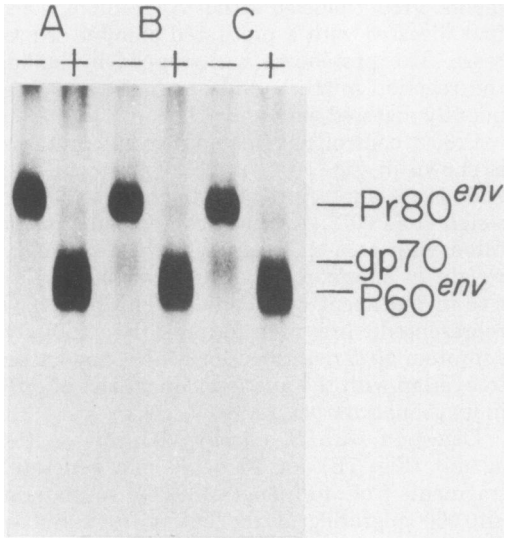

FIG. 5. *Pulse-chase analysis of Pr80^{env}. Plates of NIH/3T3 cells infected with Moloney MuLV were pulse-labeled with [^{35}S]methionine for 30 min (panel A), labeled (30 min) and chased in complete media for 30 min (panel B), or labeled (30 min) and chased for 60 min (panel C), and then were extracted and immunoprecipitated with anti-gp70 serum. One-half of each precipitate was digested with endo H (3 μg/ml) at 30°C for 1 h prior to analysis on SDS-10% polyacrylamide gel developed by fluorography.*

endo H cleavage. This technique is limited by the fact that some fragments after treatment with endo H could shift to a migration position overlapping a fragment not sensitive to endo H. Assignment of a particular fragment as sensitive to endo H was aided by the characteristic intensity and change in migration of each fragment within an experiment. Also, prior titration of the endo H insured a limit digestion in each case and hence a reproducible pattern. Comparison of the size of the protein portion of the smallest fragment retaining endo H-sensitive groups (30,000 to 40,000) and the largest fragment without shift after endo H treatment (25,000 to 30,000) supported the placement of both glycosylation sites in one-half of the apoprotein sequence. Although both gp70 and p15E portions of Pr80^{env} (10, 33) contain methionine residues, some smaller fragments lacking methionine could have been missed by this analysis. Continued digestion with *S. aureus* V8 protease or several other very active proteases, including Pronase, eventually degraded all of Pr80^{env} to material less than 10,000 in molecular weight (data not shown).

In contrast to this pattern, digestion with high concentrations of α-chymotrypsin (10 μg/ml) (Fig. 7C) or thermolysin (15 μg/ml) (Fig. 7D) revealed some fragments relatively resistant to further proteolytic cleavage. Both enzymes yielded similarly sized but not identical doublets with molecular weights of approximately 20,000. Both components of these doublets shifted to lower apparent molecular weight by about 6,000 to 8,000 (estimated molecular weight) after treatment with endo H (+ lanes). A similar pattern was observed with trypsin digestion (data not shown). The different amino acid specificities of thermolysin, α-chymotrypsin, and trypsin, and their relative, not absolute, resistance make it unlikely that these fragments originated as a result of some peculiar primary structure. Partial digestion studies with endo H suggested that each protease-resistant fragment contained a single high-mannose group (data not shown).

Bulky carbohydrate side chains appear to provide specific protection against proteolysis in vivo for many glycoproteins (17). Removal of carbohydrate could reveal new sites for proteolytic cleavage in vitro as well. Pr80^{env} was first digested with endo H, and the resultant P60^{env} was used as substrate for partial proteolytic cleavage with *S. aureus* V8 protease (Fig. 8). A

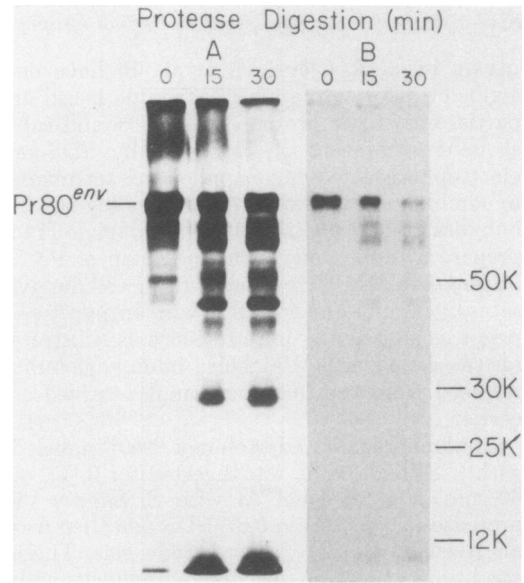

FIG. 6. *Partial proteolytic cleavage of Pr80^{env}. Pr80^{env} labeled with [^{35}S]methionine (panel A) or [2-^{3}H]mannose (panel B) isolated by immunoprecipitation from Moloney MuLV-infected cells was analyzed by the method of Cleveland et al. (3). Samples were digested for 0, 15, or 30 min with S. aureus V8 protease (5.0 μg/ml) in 0.5% SDS and then boiled for 2 min; the fragments were analyzed on a SDS-10% polyacrylamide gel developed by fluorography. Panel A was exposed for 1 day; panel B, for 8 days.*

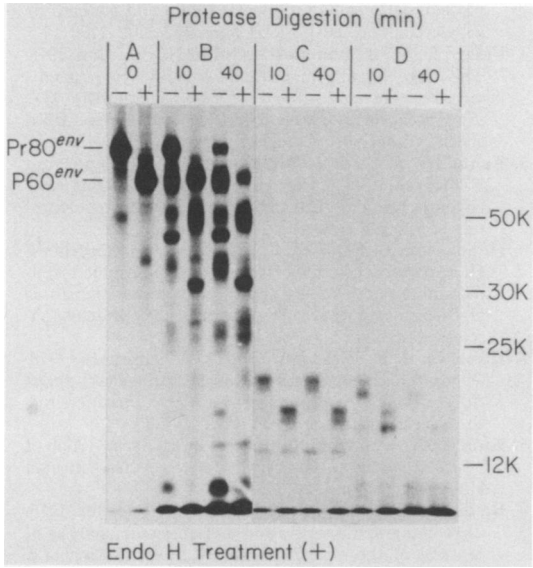

FIG. 7. *Use of endo H to identify carbohydrate-containing fragments of Pr80^{env}. [^{35}S]methionine-labeled Pr80^{env} isolated by immunoprecipitation was used as substrate for partial proteolytic cleavage by the method of Cleveland et al. (3). (A) Control, no protease treatment; (B) S. aureus V8 protease (5.0 μg/ml) for 10 or 40 min at 37°C in 0.5% SDS; (C) α-chymotrypsin (10 μg/ml); (D) thermolysin (15 μg/ml). After protease digestion, samples were boiled and one-half was subsequently digested (+) with endo H (3 μg/ml) for 1 h at 30°C. Fragments were analyzed on a SDS-10% polyacrylamide gel developed by fluorography. Panels A and B were exposed for 3 days; panels C and D, for 1 day.*

shift to lower molecular weight for the deglycosylated fragments of P60^{env} was seen (Fig. 8D) when compared to those generated from Pr80^{env} (Fig. 8B). In addition, several new fragments of lower molecular weight (15,000 to 20,000) (Fig. 8D) appeared in the fragments cleaved from P60^{env}, suggesting that some cleavage sites become more readily available after removal of carbohydrate. The particular region from which these fragments arose has not been mapped.

DISCUSSION

The data presented demonstrate that the Moloney MuLV glycoprotein precursor (Pr80^{env}) is a molecule with a apoprotein portion having a molecular weight of 60,000 and at least two large high-mannose carbohydrate groups. The inability to find pulse-labeled intracellular *env* precursors with molecular weights of less than 80,000 (16, 26, 37) suggests that these primary carbohydrate additions occur during protein elongation in a manner similar to that in other animal virus models (23). The stable nature of Pr80^{env} (37), combined with pulse-chase data of Fig. 5, suggests that carbohydrate modification and proteolytic cleavage are both late events in the intracellular processing.

The precise position of the high-mannose carbohydrate additions along the apoprotein sequence is not known. However, several constraints on the structure of Pr^{env} can be deduced from the peptide fragment data of Fig. 6, 7, and 8. Glycosylated fragments with molecular weights of 45,000 to 50,000 which shift to 30,000 to 35,000 after endo H treatment (Fig. 7 and 8) and a fragment which has a molecular weight of

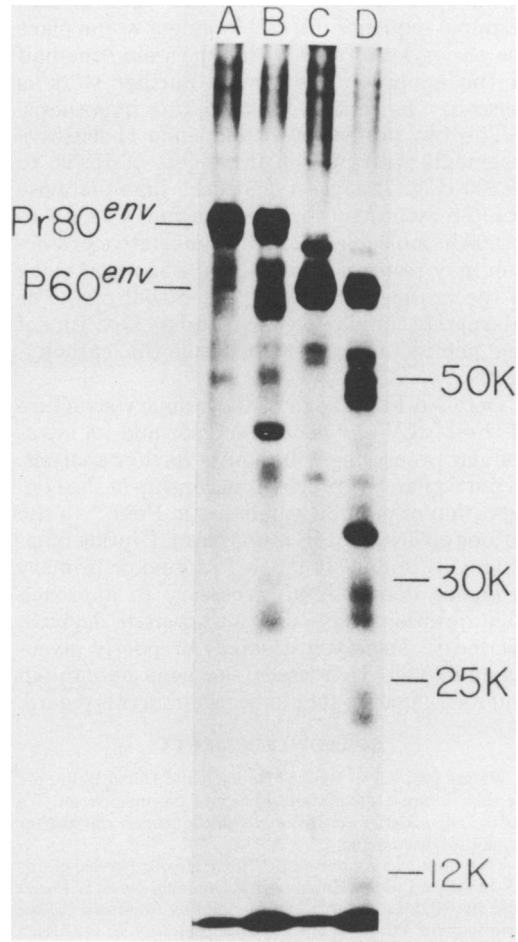

FIG. 8. *Proteolytic cleavage of Pr80^{env} after removal of carbohydrate. Pr80^{env} pulse-labeled with [^{35}S]methionine (30 min) was isolated by immunoprecipitation as in Fig. 5. (A) No treatment; (B) treated with S. aureus V8 protease (5.0 μg/ml), 30 min, 37°C; (C) digested with endo H (3 μg/ml), 1 h, 30°C; (D) digested first with endo H as in C and then boiled for 2 min and treated with S. aureus V8 protease as in B. All samples were analyzed on SDS-10% polyacrylamide gel developed by fluorography.*

27,000 and lacks mannose label (Fig. 6) demonstrate that both sugar groups are confined to a region of one-half the apoprotein length.

The reported orientation of NH2gp70-p15E-COOH for Pr^{env} and lack of carbohydrate on p15E (5, 9, 10, 15, 33) would limit the carbohydrate sites to an N-terminal molecular-weight region of 45,000, within the gp70 sequence. If the naturally occurring fragment with a molecular weight of 50,000 to 55,000 cross-reactive with gp70 which shifts to a molecular weight of 35,000 after endo H treatment (Fig. 7) does represent a precursor form to gp45, then a further constraint on the structure of Pr^{env} can be noted. Since gp45 and gp70 share the same amino-terminal sequence (6), this fragment would place the glycosylation sites within the amino one-half of the apoprotein sequence. Further work is necessary to confirm this working hypothesis.

The two protease-resistant endo H-sensitive fragments with molecular weights of 15,000 to 20,000 (Fig. 7) suggest that each high-mannose group may protect a peptide region of 12,000 to 15,000 in molecular weight. This relative protection may result from an actual structural block of the proteolytic sites by the carbohydrate or alternately could reflect a secondary structure of the peptide region surrounding the carbohydrate.

Detailed knowledge of the primary structure of the MuLV *env* gene precursor and its intracellular processing will require further analysis. In particular, the precise relationship of the conservation of peptide sequences in $Pr80^{env}$ to the virions gp70 and p15E is unknown. Peptide mapping with uniformly labeled protein or primary sequence data will be necessary to approach such questions. The ability to prepare deglycosylated (tunicamycin-treated) or poorly glycosylated (endo H-treated) *env* gene apoprotein and its fragments should be useful in this regard.

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