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TUNICAMYCIN INHIBITS GLYCOSYLATION OF PRECURSOR POLYPROTEIN ENCODED BY *env* GENE OF RAUSCHER MURINE LEUKEMIA VIRUS

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SUMMARY: The molecular weight of the precursor polyprotein to the envelope proteins of Rauscher murine leukemia virus is reduced from 85,000 to 68,000 daltons when synthesized in the presence of tunicamycin, a specific inhibitor of the synthesis of oligosaccharides that attach to glycoproteins via asparagine residues. The unglycosylated precursor protein ($Pr68^{env}$) is synthesized at a rate comparable to that of the normal carbohydrate-containing envelope precursor ($gPr85^{env}$). $Pr68^{env}$ is not proteolytically processed and remains undegraded in the cell. Thus, most if not all of the carbohydrate content of $gPr85^{env}$ is N-linked, and glycosylation appears to be necessary for normal processing of precursor proteins into viral particles.

INTRODUCTION:

Type C RNA viruses (family Retroviridae) are spherical enveloped particles that bud from the plasma membranes of infected cells. The internal structural proteins that occur in the core-like structure containing the genomic RNA are not glycosylated. A 70,000 dalton viral envelope glycoprotein (gp70) found at the surface of the particle protrudes from the lipid layer and is thought to comprise the surface projections visualized in electron micrographs. The hydrophobic unglycosylated protein p15E (1-3) is also located at the surface of the virion since complement-dependent immune lysis of the virus can be demonstrated with anti-p15E sera (4).

The envelope proteins p15E and gp70 are derived by proteolytic cleavage from an 85,000 dalton glycosylated precursor protein, the *env* gene product designated $gPr85^{env}$ (5, 6). Neither gp70 nor $gPr85^{env}$ is found in unglycosylated form in the absence of inhibitors of glycosylation. Presumably,

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Abbreviations: SDS, sodium dodecyl sulfate; R-MuLV, Rauscher murine leukemia virus.

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carbohydrate attachment occurs on the nascent protein molecule, as is the case for Sindbis virus (7). It has been previously shown that, in the presence of 2-deoxyglucose and cytochalasin B, glycosylation of *env* gene products is inhibited and smaller precursors are produced instead of $gPr85^{env}$ (8, 9). Tunicamycin is an antibiotic that causes specific inhibition of glycosylation reactions mediated by a dolichol-phosphate lipid carrier (10, 11). In this report we describe the effect of tunicamycin on the synthesis and processing of $gPr85^{env}$ in cells infected with Rauscher leukemia virus (R-MuLV).

MATERIALS AND METHODS:

The synthesis and processing of viral precursor proteins were examined in mouse (BALB/c) bone marrow, JLS-V9 cells (12), chronically infected with R-MuLV. Newly synthesized [^{35}S]methionine-labeled proteins were immunoprecipitated with monospecific antisera to envelope proteins and then analyzed by SDS-polyacrylamide gel electrophoresis in 7.5%-17% gradient gels as previously described (13). Briefly, monolayer cultures were incubated for 10 min in methionine-free Eagle's minimal essential medium and then labeled with [^{35}S]methionine (Amersham, 5 μCi/ml) for 15 min. Monolayers were rinsed, lysed in a buffer containing NP40 and deoxycholate, clarified by centrifugation, and treated overnight with antiserum. Antiserum to purified R-MuLV gp70 was prepared in goats as previously described (14). Monoclonal antibody to p15E (15), obtained from Dr. Robert Nowinski (Fred Hutchinson Cancer Research Center, Seattle, Washington), was prepared by the method of Köhler and Milstein (16). Immunoprecipitates were recovered with protein A Sepharose (Pharmacia), and radioactive bands were visualized after electrophoresis by fluorography (17). Tunicamycin was obtained from the Lilly Research Laboratories (Indianapolis, Indiana) through the courtesy of Drs. Irving Johnson and Robert Hamill. Stock solutions of the antibiotic were prepared in dimethyl sulfoxide at 1 mg/ml, kept refrigerated, and used within 2 weeks.

RESULTS:

Cells chronically infected with R-MuLV were labeled with [^{35}S]methionine for 15 min. Virus-specific envelope proteins synthesized in the presence and absence of tunicamycin were identified by precipitation with anti-p15E and anti-gp70 sera followed by SDS-gel electrophoresis (Fig. 1). In the absence of tunicamycin the major band precipitated by both anti-gp70 serum (lane 1) and anti-p15E serum (lane 2) had a molecular weight of 85,000 daltons. This protein was shown in separate experiments to incorporate [6-^{3}H]glucosamine. The criteria of molecular weight, antigenicity, and glycosylation identify the glycoprotein in the major band as $gPr85^{env}$. Small amounts of radio-labeled gp70 and p15E were also precipitated with the respective antisera, indicating proteolytic

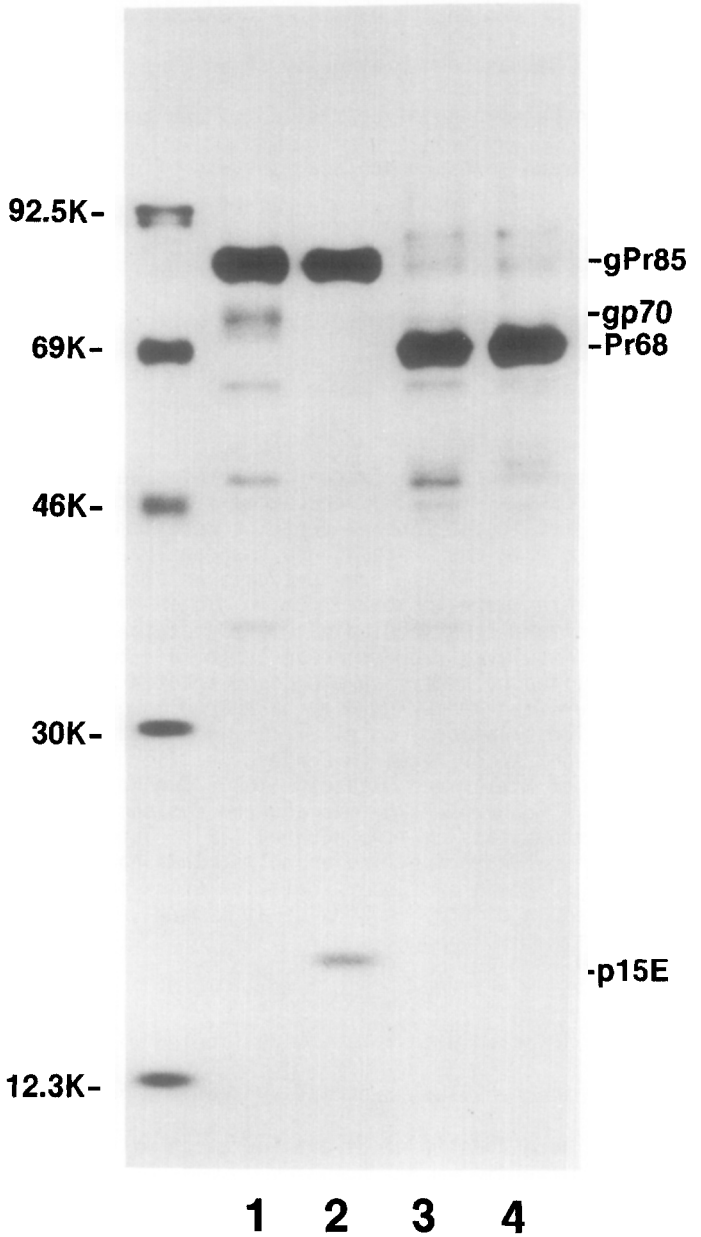

Fig. 1. SDS-gel electrophoresis analysis of immunoprecipitates from extracts of cells labeled for 15 min with [^{35}S]methionine in the presence and absence of tunicamycin. Cells were labeled, extracted, and immunoprecipitated as described in Materials and Methods. Lane 1, no tunicamycin, anti-gp70 serum; lane 2, no tunicamycin, anti-p15E serum; lane 3, 1-h pretreatment with tunicamycin (1 μg/ml) before labeling, anti-gp70 serum; lane 4, 1-h pretreatment with tunicamycin (1 μg/ml) before labeling, anti-p15E serum. Molecular weight markers are [^{14}C]methylated proteins (New England Nuclear): 92.5K, phosphorylase b; 69K, bovine serum albumin; 46K, ovalbumin; 30K, carbonic anhydrase; 12.3K, cytochrome C.

processing of $gPr85^{env}$. The pattern of radioactive bands precipitated by the antisera was altered in the presence of tunicamycin. Instead of $gPr85^{env}$, a 68,000 dalton species ($Pr68^{env}$) was the major product precipitated with both anti-gp70 (lane 3) and anti-p15E (lane 4) sera. Neither gp70 nor p15E appeared in the cell during the pulse period.

When chase experiments were performed following a 15-min pulse-labeling, *env*-precursors synthesized in the presence and absence of tunicamycin exhibited different kinetic behavior. In the absence of tunicamycin (Fig. 2A), radiolabeled $gPr85^{env}$, which was synthesized in 15 min (0 h chase), decreased in amount during the following 15 min to 2 h. Very little gp70, produced by processing of $gPr85^{env}$, was seen at 0 h chase although it appeared at maximal level in the 15 min chase. Since gp70 becomes incorporated into virus particles that bud from the cell membrane, an accumulation of this product as a result of processing was not observed. Both gp70 and $gPr85^{env}$ slowly disappeared during the 2 h chase. No p15E was seen in this experiment because only anti-gp70 serum was employed. When cells were first treated with tunicamycin before labeling, $Pr68^{env}$ was synthesized (Fig. 2B, 0 h chase) as expected from the results shown in Fig. 1. However, $Pr68^{env}$ was stable for at least 2 h (Fig. 2B), in contrast to $gPr85^{env}$ (Fig. 2A). The relative intensities of radioactive bands in the radioautogram suggest that $Pr68^{env}$ is synthesized as efficiently in the presence of tunicamycin as is the glycosylated precursor $gPr85^{env}$ in the absence of the inhibitor.

DISCUSSION:

Tunicamycin, a glucosamine-containing antibiotic obtained from culture filtrates of *Streptomyces lysosuperficus*, possesses potent anti-viral activity (18-20). It acts to prevent glycosylation by specifically inhibiting the synthesis of oligosaccharides that are attached to a dolichol-phosphate lipid carrier. This oligosaccharide unit is normally transferred in a single step to an asparagine residue of a peptide chain (21). Thus, the effect of tunicamycin is to inhibit the formation of N-glycosides in glycoproteins.

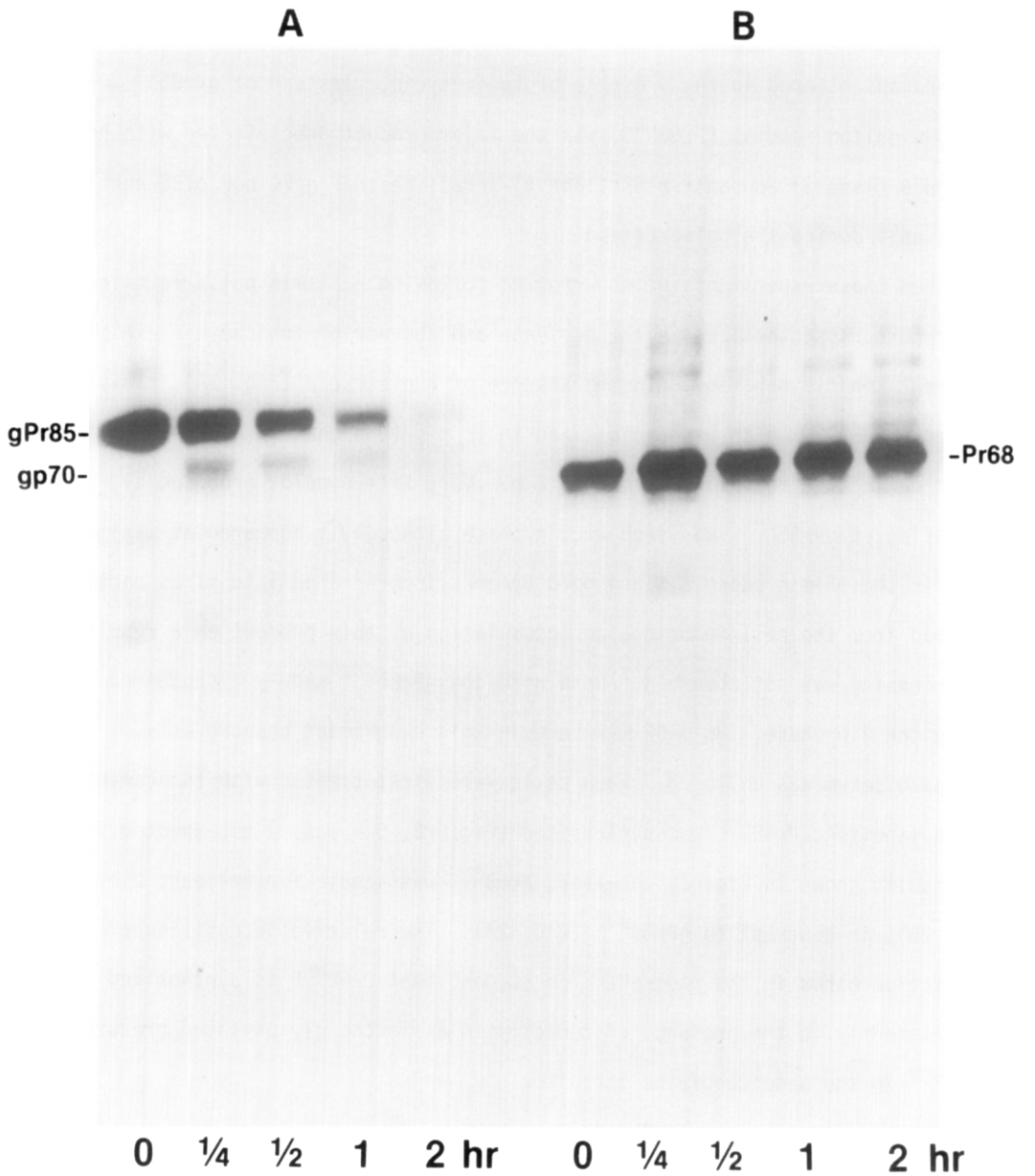

Fig. 2. SDS-gel electrophoresis analysis of a pulse-chase labeling experiment performed in the presence and absence of tunicamycin. A, no tunicamycin; B, 1-h pretreatment with tunicamycin (1 μg/ml) before labeling. Cells were labeled with [^{35}S]methionine for 15 min and chased for indicated times. At the end of each chase period, extracts were prepared as described in Materials and Methods and immunoprecipitated with anti-gp70 serum.

In R-MuLV-infected JLS-V9 cells in the presence of tunicamycin, an unglycosylated *env* precursor with a molecular weight of 68,000 daltons was synthesized in amounts equivalent to the amounts of gPr85^{env} synthesized in

the absence of inhibitor. The size of an unglycosylated *env* precursor in R-MuLV produced by 2-deoxyglucose (8, 9) or cytochalasin B (9) was previously found to be approximately 70,000 daltons. The difference between 68,000 and 70,000 daltons is within the range of inter-laboratory variation for molecular weight determinations by SDS-gel electrophoresis. However, since 2-deoxyglucose and cytochalasin B indirectly block glycosylation, it is possible that glycoproteins synthesized in the presence of these inhibitors possess short oligosaccharide chains and are not entirely free of carbohydrate. Since the molecular weight reduction of $Pr85^{env}$ in the presence of tunicamycin is as great or greater than that obtained in the presence of generalized inhibitors of glycosylation, it may be that most if not all $gPr85^{env}$ oligosaccharides are N-linked. In this respect, the attachment site of one large oligosaccharide in gp70 has been determined to be an asparagine residue near the NH_2-terminus (Henderson and Oroszlan, manuscript in preparation).

The peptide chain of the *env* precursor is synthesized whether tunicamycin is present or not. However, the unglycosylated precursor is not proteolytically cleaved to its constituent proteins, whereas normal $gPr85^{env}$ is cleaved to gp70 and p15E. In contrast, unglycosylated forms of viral glycoproteins in vesicular stomatitis virus (VSV) and Semliki forest virus (SFV) do undergo proteolytic cleavage (22-24). In cells producing Rous sarcoma virus, only small amounts of an unglycosylated *env* precursor were detected when glycosylation was inhibited with glucosamine (25) or tunicamycin (26). This may be similar to the nonspecific degradation of unglycosylated precursor molecules reported for influenza virus (26).

Tunicamycin could affect the cleavage of the *env* precursor by several mechanisms. Direct inhibition of the cleavage enzyme is possible, although we have found that the proteolytic processing of the *gag* gene precursor polyprotein of R-MuLV is not blocked (27). Alternatively, glycosylation may specify the cleavage site and lack of the signal would result in no cleavage, or it is possible that the unglycosylated *env* precursor may remain firmly bound to

intracellular membranes and not be accessible to the processing enzymes. In cells infected with VSV and SFV, unglycosylated viral glycoproteins remain associated with internal membranes and do not migrate to the cell surface (23, 24).

Tunicamycin is an effective inhibitor of VSV and SFV particle production (28). In the case of avian sarcoma virus, noninfectious particles free of envelope proteins are still produced at 60% yield of the normal virions (26). In preliminary experiments, we found that tunicamycin also significantly inhibited R-MuLV production, and that the produced particles did not contain gp70, p15E, $Pr68^{env}$, or an unglycosylated form of gp70. Tunicamycin thus is a useful tool for studying the effect of glycosylation on type C virus maturation.

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