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O-Linked Glycosylation of Retroviral Envelope Gene Products

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Treatment of [^{3}H]glucosamine-labeled Friend mink cell focus-forming virus (FrMCF) gp70 with excess peptide:*N*-glycanase F (PNGase F) resulted in removal of the expected seven N-linked oligosaccharide chains; however, approximately 10% of the glucosamine label was retained in the resulting 49,000-M_r (49K) product. For [^{3}H]mannose-labeled gp70, similar treatment led to removal of all the carbohydrate label from the protein. Prior digestion of the PNGase F-treated gp70 with neuraminidase resulted in an additional size shift, and treatment with *O*-glycanase led to the removal of almost all of the PNGase F-resistant sugars. These results indicate that gp70 possesses sialic acid-containing O-linked oligosaccharides. Analysis of intracellular *env* precursors demonstrated that O-linked sugars were present in gPr90^{env}, the polyprotein intermediate which contains complex sugars, but not in the primary translation product, gPr80^{env}, and proteolytic digestion studies allowed localization of the O-linked carbohydrates to a 10K region near the center of the gp70 molecule. Similar substituents were detected on the gp70s of ecotropic and xenotropic murine leukemia viruses and two subgroups of feline leukemia virus, indicating that O-linked glycosylation is a conserved feature of retroviral *env* proteins.

Many cellular and viral membrane proteins are modified posttranslationally by glycosylation. A major class of glycoproteins contain N-linked oligosaccharides; these are assembled by transfer of preformed mannose-rich, dolichol-linked oligosaccharides to specific asparagine residues which are part of the recognition sequence Asn-X-Ser(Thr) (18). This transfer occurs cotranslationally on nascent polypeptide chains in the rough endoplasmic reticulum and is generally followed by a maturation process mediated by a series of glycosidases and glycosyl transferases, which occurs during transport of the proteins through the Golgi apparatus (11). A second type of glycosylation has been observed, in which the sugars are attached via the hydroxy group of serine or threonine residues (15). Such O-linked oligosaccharides are present in a number of viral membrane proteins, including herpes simplex virus type 1 glycoproteins (12, 22, 39), vaccinia virus HA protein (32), respiratory syncytial virus GP1 protein (8), mouse hepatitis virus (21), and coronavirus E1 protein (10, 20). The O-linked substituents differ from N-linked oligosaccharides in that they are formed via stepwise addition of carbohydrates posttranslationally by glycosyltransferases located in the Golgi apparatus (30). For several characterized proteins, O-glycosylation occurs on regions containing clusters of serine and threonine residues (36, 21, 40); however, the specific recognition sequences which determine which serine or threonine residues are modified in this manner are not known.

The murine leukemia virus *env* gene product contains six to eight N-linked oligosaccharides, located on the external gp70 subunit (3, 13, 14, 17, 26, 29, 33). In the present article we report the presence of an additional carbohydrate constituent on gp70, which is a conserved feature of a number of murine and feline retroviruses. Evidence is presented that this represents O-glycosylation, and detailed information on the kinetics and site of attachment of the O-linked substituents is presented for one particular case, the Friend mink cell focus-forming virus (FrMCF).

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MATERIALS AND METHODS

Viruses and antisera. The viruses used for this study were dualtropic FrMCF clone 1 grown in NIH 3T3 cells (38), ecotropic Moloney murine leukemia virus (MuLV) clone 1 grown in NIH 3T3 cells (6), xenotropic NZB-6 MuLV grown in mink CCL64 cells (obtained from J. Hartley), and a mixture of feline leukemia virus A and B (FeLV-AB), grown in FLF cells (obtained from W. Hardy). MuLV gp70s were immunoprecipitated with goat anti-Rauscher gp70 serum (lot 79S-603), and FeLV gp70s were immunoprecipitated with goat anti-FeLV gp70 serum (lot 7341), obtained from the Biological Carcinogenesis Branch of the National Cancer Institute. Rat antisera specific for the N-terminal domain of MCF gp70 were prepared in our laboratory by immunization of rats with syngeneic NRK cells infected with the polycythemia-inducing strain of Friend spleen focus-forming virus (F-SFFV) (37).

Radiolabeling and radioimmunoprecipitation procedures. Cells were radiolabeled for approximately 20 h, except when other time periods are indicated. Cysteine labeling was performed by culturing cells in cysteine-deficient medium in the presence of [^{35}S]cysteine at 50 μCi/ml. Carbohydrate labeling was performed in complete medium supplemented with either [^{3}H]glucosamine or [^{3}H]mannose at 100 μCi/ml. Immunoprecipitations were performed by adjusting labeled cell extracts or supernatant medium to 0.5% Nonidet P-40 (NP-40) and 0.5 M NaCl, followed by treatment with a 1:100 dilution of anti-gp70 serum for 1 h at 37°C. Immune complexes were then collected with fixed *Staphyloccocus aureus* (Pansorbin; Calbiochem), washed with RIP buffer (0.01 M Tris, pH 7.4, 0.5% NP-40, and 0.5 M NaCl), and solubilized by boiling in TN buffer (0.01 M Tris, 0.10 M NaCl, pH 7.4) containing 1% sodium dodecyl sulfate (SDS) and 1% dithiothreitol (DTT). Samples were analyzed by electrophoresis on 8 or 10% SDS–polyacrylamide gels prepared as described by Laemmli (16) and analyzed by fluorography as described by Bonner and Laskey (2).

Enzyme digestions. Endoglycosidase H (endo H), peptide *N*-glycosidase F (PNGase F), and endo-α-*N*-acetyl-D-galac-

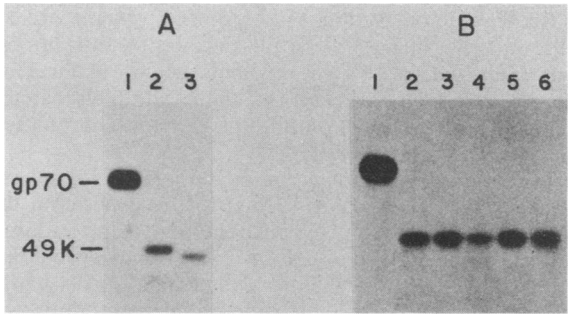

FIG. 1. Presence of PNGase F-resistant carbohydrates on FrMCF gp70. (A) Supernatant of [^{3}H]glucosamine-labeled FrMCF-infected NIH 3T3 cells immunoprecipitated with anti-gp70 serum were analyzed by SDS-PAGE either directly (lane 1), after digestion with PNGase F (lane 2), or after digestion with neuraminidase followed by PNGase F treatment (lane 3). (B) Titration of PNGase F activity. The above sample was treated with the following concentrations of PNGase F: 0, 56, 28, 14, 7, or 3.5 U/ml (lanes 1 to 6, respectively).

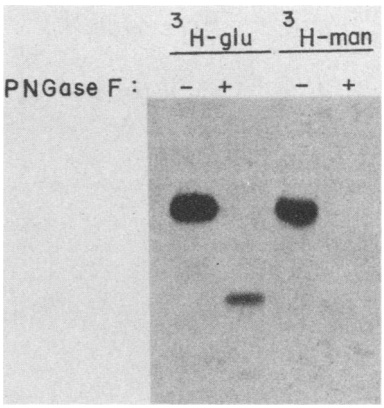

FIG. 2. Lack of mannose in PNGase F-resistant oligosaccharides. gp70 labeled with [^{3}H]glucosamine (^{3}H-glu) and [^{3}H]mannose (^{3}H-man) was analyzed by SDS-PAGE both before (−) and after (+) treatment with PNGase F, as indicated. Equivalent amounts of glucosamine and mannose label were used for each sample.

tosaminidase (*O*-glycanase) were obtained from Genzyme, *Vibrio cholerae* neuraminidase was from Calbiochem, and TPCK-treated trypsin was from Sigma. Endo H digestions were performed by solubilizing immunoprecipitates of gp70 in TN buffer containing 1% SDS and treating with 0.1 U of endo H per ml for 2 h at 37°C. PNGase F digestions were performed on samples solubilized in TN buffer containing 1% NP-40, 0.25% SDS, 0.25% DTT, and 5 mM EDTA by treating with 7 U of the enzyme per ml at 37°C for 2 h, unless indicated otherwise. Neuraminidase digestions were performed directly on the Staph A pellets suspended in TN buffer containing 0.5% NP-40 and 0.5 M NaCl, by treating with 0.1 U of enzyme per ml for 1 h at 37°C. *O*-Glycanase digestions were performed by suspending immunoprecipitates still absorbed to Staph A in buffer containing 20 mM Tris-maleate and 20 mM sodium citrate, pH 6.0, and treating with 200 mU of enzyme per ml for 24 h at 37°C. In the last two cases, the Staph A complexes were repelleted, washed, and either treated with a second enzyme or dissolved in TN buffer containing 1% SDS and 1% DTT for subsequent analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

RESULTS

Demonstration of O-linked oligosaccharides on FrMCF gp70. Analysis of the sequence of the *env* gene of several FrMCF isolates indicates the existence of seven or eight potential N-linked glycosylation sites in the gp70 coding region (14), and direct biochemical studies of the FrMCF clone 1 gp70 demonstrate the presence of seven N-linked oligosaccharides (26). Digestion of [^{3}H]glucosamine-labeled FrMCF gp70 with PNGase F, an enzyme which completely removes asparagine-linked oligosaccharides from proteins, resulted in a size shift consistent with the loss of all seven predicted N-linked sugars; however, the resulting 49,000-M_r (49K) protein product retained approximately 10% of the original glucosamine label (Fig. 1A). This label was not incorporated into any nonglycosylated viral proteins (data not shown), and a double digestion of gp70 with neuraminidase and PNGase F resulted in an additional size shift (Fig. 1A, lane 3), demonstrating that the PNGase F-resistant glucosamine label represented one or more sialic acid-containing oligosaccharides. A similarly sized product was detected after digestion with a 16-fold concentration range of PNGase F, indicating that the enzyme was present in excess (Fig. 1B). Upon similar digestion of gp70 labeled with [^{3}H]mannose, an invariant constituent of N-linked oligosaccharides, residual radioactivity was not retained in the deglycosylated product (Fig. 2). These results indicate that the PNGase F-resistant glucosamine label does not represent incomplete removal of N-linked sugars and suggest the possible presence of O-linked sugars on gp70.

To confirm the identity of the PNGase F-resistant sugars, the effect of *O*-glycanase, a glycosidase specific for O-linked oligosaccharides, was examined. Digestion of FrMCF gp70 with *O*-glycanase resulted in a small but reproducible reduction of molecular weight (Fig. 3A and B). Subsequent treatment of [^{3}H]glucosamine-labeled gp70 with PNGase F demonstrated that greater than 90% of the residual carbohydrate label in the 49K product was removed (Fig. 3B). This was increased to a greater than 98% when the sample was treated with neuraminidase prior to *O*-glycanase digestion (Fig. 3D), in accord with the reported enhanced sensitivity of desialylated oligosaccharides to this enzyme. Some proteolysis was apparent in the *O*-glycanase-treated samples, but the extent of this reaction was too low to account for the observed loss of radioactivity from the 49K band. This reactivity demonstrates that the PNGase F-resistant carbohydrates on gp70 are in fact O-linked oligosaccharides.

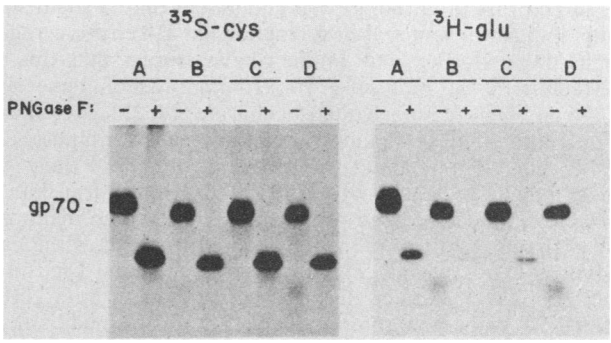

FIG. 3. Sensitivity of PNGase F-resistant oligosaccharides to *O*-glycanase. Immunoprecipitates containing gp70 labeled with [^{3}H]glucosamine or [^{35}S]cysteine (^{35}S-cys) gp70 were analyzed by SDS-PAGE either before treatment (A) or after digestion with *O*-glycanase (B), neuraminidase (C), or neuraminidase plus *O*-glycanase (D).

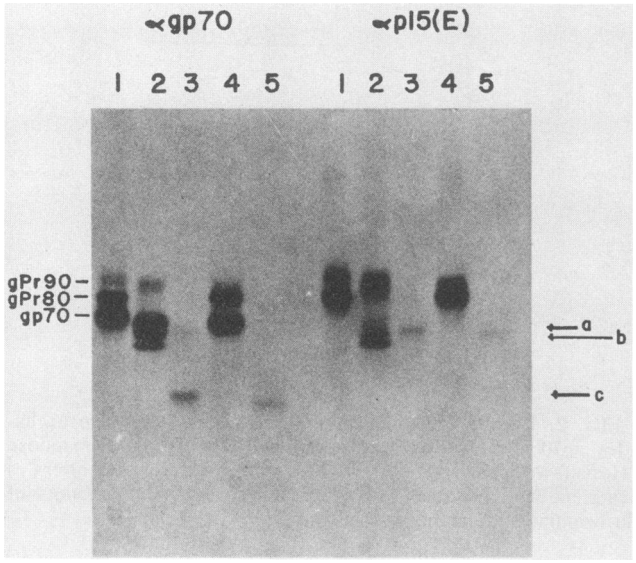

FIG. 4. Analysis of PNGase F-resistant oligosaccharides on intracellular *env* precursor proteins. FrMCF-infected NIH 3T3 cells labeled for 1 h with [^{3}H]glucosamine were lysed and immunoprecipitated with antiserum to either gp70 or p15(E). Samples were then analyzed by SDS-PAGE either directly (lanes 1) or after digestion with endo H (lanes 2), PNGase F (lanes 3), neuraminidase (lanes 4), or neuraminidase plus PNGase F (lanes 5). Arrows denote PNGase F digestion products of gPr90 (a and b) and gp70 (c).

O-glycosylation of intracellular *env* polyprotein. The MuLV *env* gene is expressed initially as a polyprotein consisting of covalently linked gp70 and p15(E) sequences, which is then modified by processing of N-linked sugars and proteolysis (5). To determine at what stage in the biosynthesis of the viral *env* proteins O-linked glycosylation occurs, the presence of PNGase F-resistant oligosaccharides on various intracellular *env* precursors was examined. After FrMCF-infected cells were labeled with [^{3}H]glucosamine for 1 h, two proteins in addition to gp70 were immunoprecipitated with anti-gp70 serum; these had molecular weights of approximately 80,000 (gPr80) and 90,000 (gPr90) (Fig. 4). Both of these components were also recognized by antisera to p15(E), indicating that they represent polyprotein precursors of the mature *env* proteins. After treatment with endo H, gPr80 was converted to a 60K molecule, consistent with removal of all seven N-linked oligosaccharide chains (lane 2). In contrast to gPr80, gPR90 underwent only a small size shift, indicating removal of a single endo H-sensitive sugar chain (lane 2). Previous studies have shown that this is characteristic of mature gp70 proteins, which generally retain one high-mannose oligosaccharide (24, 25). Treatment with neuraminidase resulted in an increased mobility for gPr90, but did not affect the migration of gPr80 (lane 4). These results indicate that gPr80 is the primary translation product of the *env* gene and contains only high-mannose sugar chains, while gPr90 is a more mature form of the *env* polyprotein possessing complex oligosaccharides. Digestion with PNGase F resulted in the disappearance of both gPr80 and gPr90, and a new component of 65K was formed (lane 3, band a). This component was larger than the deglycosylated form of gPr80 and thus must be a product of gPr90. Digestion with neuraminidase in addition to PNGase F resulted in the further reduction in size of this product to 63K (lane 5, band b). A band similar in size to the 60K endo H digestion product of gPr80 was not detected after digestion with PNGase F, demonstrating that O-linked sugars are not present on the primary translation product. It thus appears that O-glycosylation of these proteins occurs in the Golgi apparatus, coincident with processing of the high-mannose N-linked oligosaccharides on gPr80 and prior to proteolytic cleavage to gp70 and Pr15(E).

Localization of O-glycosylation site on gp70. Limited proteolysis of gp70s with trypsin leads to the generation of a number of fragments which correspond to specific structural domains of these molecules (24, 25, 27). We have previously shown that individual domains of dualtropic gp70s can be readily distinguished by their differential reactivities with antisera prepared against ecotropic or xenotropic gp70s (25). A rat serum prepared against F-SFFV-infected syngeneic rat cells reacted preferentially with the amino-terminal domain of MCF gp70s. This antiserum precipitated three amino-terminal fragments, with molecular weights of 41K, 32K, and 29K (Fig. 5A, lane a). Analysis of the sensitivities of these products to endo H and PNGase F indicated that all three components had one complex sugar chain and one endo H-sensitive oligosaccharides (lanes b and c). This endo H-sensitive sugar chain has previously been localized to Asn-11 of the FrMCF gp70 sequence (25) and thus serves as a convenient marker for the amino terminus of gp70. Only the largest of these fragments exhibited changes in mobility after neuraminidase treatment, indicating that this component, but not the smaller products, contains sialic acid residues (lane d). Digestion with PNGase F removed both N-linked oligosaccharides from all three fragments, leading to products which were smaller by approximately 6K, which can be readily visualized in the [^{35}S]cysteine-labeled sample. PNGase F treatment of [^{3}H]glucosamine-labeled gp70 resulted in the loss of all sugar label from the digestion products of the 32K and 29K fragments, but the product of the 41K component (band a) retained [^{3}H]glucosamine (lane c). This component increased in mobility after neuraminidase treatment, consistent with the presence of sialic acid residues (lane e). This indicates that O-linked sugars are present in the 41K product but not in the smaller amino-terminal fragments.

An antiserum prepared against ecotropic Rauscher MuLV gp70 recognized two different heavily glycosylated fragments, with mobilities corresponding to apparent molecular weights of 49,000 and 39,000 (Fig. 5B, lane a). These represent carboxy-terminal components (25), and by virtue of its size, the 49K fragment must contain overlapping sequences with the 41K amino-terminal fragment described above. In contrast to the components recognized by the rat serum, the carboxy-terminal fragments were mostly resistant to endo H (lane b). The larger component but not the smaller one underwent a significant change in mobility upon neuraminidase digestion (lane d). Digestion of the [^{35}S]cysteine-labeled sample with PNGase F (lane c) resulted in conversion of the 49K fragment to a doublet of 28K and 25K (bands b and c) and the 39K component to a band of 14K (band d). The ratio of the 28K and 25K bands was sensitive to the trypsin concentration used, and these components appear to represent related products generated by trypsinization at closely spaced sites. Analysis of the digestion products of the [^{3}H]glucosamine-labeled sample demonstrated that only the 28K and 25K products contained PNGase F-resistant sugars (lane c); these represent sialylated oligosaccharides, as evidenced by the increased mobility of these components, but not the 14K product, upon neuraminidase treatment (lane e). These results indicate that the 49K carboxy-terminal product, but not its 39K fragment,

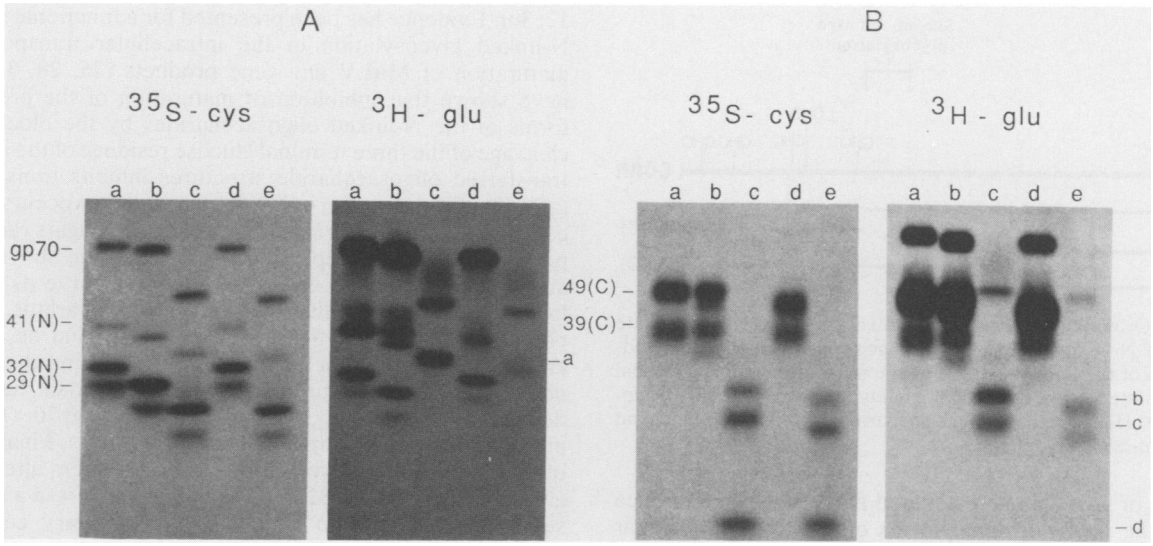

FIG. 5. Localization of O-linked sugars to specific tryptic fragment of FrMCF gp70. Supernatant medium of [^{3}H]glucosamine- and [^{35}S]cysteine-labeled FrMCF-infected NIH 3T3 cells were treated with trypsin at 2 μg/ml and immunoprecipitated with antisera specific for either the amino terminus (A) or carboxy terminus (B) of gp70. Samples were then analyzed by SDS-PAGE either directly (lanes a) or after digestion with endo H (lanes b), PNGase F (lanes c), neuraminidase (lanes d), or neuraminidase plus PNGase F (lanes e). The [^{3}H]glucosamine-labeled band migrating above the 41K component [41(N)] which is visible in panel A, lanes a and b, corresponds to the 49(C) fragment, which was present as a contaminant in this sample. The PNGase F digestion products of 41(N) (band a), 49(C) (band b and c), and 39(C) band d) are indicated.

also contained O-linked sugars. Thus it appears that O-glycosylation is localized to an overlapping region of approximately 10K which is present in both the larger amino- and carboxy-terminal gp70 fragments (see Fig. 7).

O-glycosylation of other retroviral *env* proteins. To determine whether O-glycosylation is a common characteristic of retroviral glycoproteins, a number of other murine and feline gp70s were analyzed for the presence of O-linked oligosaccharides. Viruses studied included ecotropic Moloney MuLV (Fig. 6B), xenotropic NZB-6 MuLV (Fig. 6C), and a mixture of A and B subtypes of FeLV (Fig. 6D). In each of these cases, residual carbohydrate label was retained after treatment of [^{3}H]glucosamine-labeled viral glycoproteins with excess PNGase F (lane 2). PNGase F digestion led to a size shift similar to that observed for FrMCF gp70 (Fig. 6A), indicating removal of all N-linked sugars, and neuraminidase treatment induced a further size shift in all of the deglycosylated products (lane 3). Similar results were obtained for the gp70s of several other ecotropic MuLVs, including Friend, Rauscher, and Akv viruses (data not shown). For the FeLV-AB sample, two bands were obtained after deglycosylation (Fig. 6D); the larger band was similar in size to the product obtained for pure FeLV-B (data not shown), and thus the smaller band apparently corresponds to FeLV-A gp70. These results indicate the presence of sialic acid-containing O-linked oligosaccharides in each of the viruses examined.

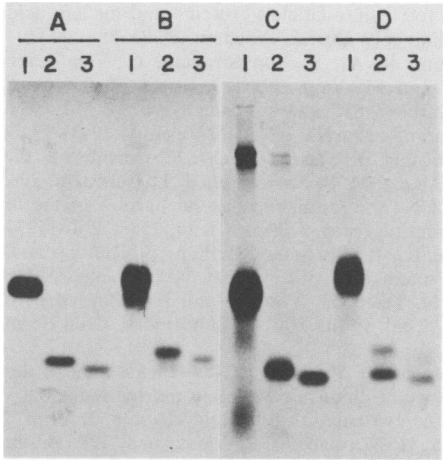

FIG. 6. Demonstration of PNGase F-resistant oligosaccharides on other retroviral glycoproteins. [^{3}H]glucosamine-labeled proteins from FrMCF (A), Moloney MuLV (B), NZB-6 MuLV (C), and FeLV-AB (D) were immunoprecipitated with anti-gp70 serum and analyzed by SDS-PAGE directly (lanes 1), after treatment with PNGase F (lanes 2), or after treatment with neuraminidase plus PNGase F (lanes 3).

DISCUSSION

The data presented above demonstrate that a number of murine and feline retroviral gp70 proteins possess PNGase F-resistant carbohydrate substituents. The PNGase F enzyme preparation used was free of contaminating endoglycosidase F activity, as evidenced by the complete removal of the sugar label from the primary translational product, gPr80 (Fig. 4), as well as from the amino-terminal and 39K carboxy-terminal gp70 fragments (Fig. 5). PNGase F is believed to cleave all asparagine-linked oligosaccharides (35), suggesting that the resistant sugars are not N-linked; this conclusion is supported by the absence of mannose in these structures. The sensitivity of these structures to *O*-glycanase provides direct confirmation that they represent O-linked glycosylation.

Approximately 10% of the total [^{3}H]glucosamine label was present in the PNGase F-resistant fraction, suggesting the presence of a limited number of such substituents. The contribution of the O-linked sugars to the apparent molecu-

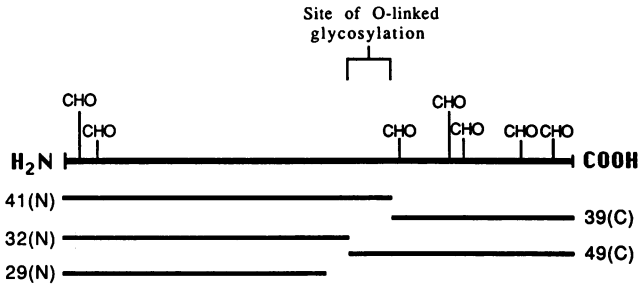

FIG. 7. Orientation of tryptic fragments of gp70. Approximate positions of N-linked and O-linked oligosaccharides are indicated. The lengths of the lines representing the fragments are proportional to the estimated sizes of the completely deglycosylated polypeptides. CHO, High-mannose oligosaccharide. Refer to Fig. 5 legend for fragment designations.

lar weight of gp70 can be estimated from the size difference between the deglycosylated forms of the two intracellular *env* precursors, gPr80 and gPr90. Digestion with PNGase F converted gPr90 to a 65K product, whereas the digestion product of gPr80 was approximately 60K, indicating an apparent molecular weight for the O-linked sugars of about 5,000 (Fig. 4). Sialic acid residues account for almost half of this effect, as neuraminidase treatment converted the 65K band to a 63K product. Carbohydrate substituents are known to cause anomalous changes in the electrophoretic mobility of different glycoproteins in SDS-PAGE, and thus care must be taken in interpreting the above data. It is, however, informative to compare these results with those obtained for other systems. The precursor and mature forms of the human low-density lipoprotein (LDL) receptor differ in molecular weight by 40,000, and this size difference is believed to be due to the maturation of O-linked sugars (4). These proteins contain as many as nine O-linked oligosaccharides, indicating a contribution of 4,000 to 5,000 M_r for each of these substituents to the apparent size of the parent molecule. Assuming a similar structure for the O-linked substituents in these two systems, this would imply the presence of only a single O-linked oligosaccharide on gp70.

The fragmentation analysis described in Fig. 5 demonstrates that the O-linked oligosaccharides are present on both the 41K amino-terminal fragment and the 49K carboxy-terminal fragment, but not on smaller fragments derived from the two ends of the gp70 molecule. This localizes the O-linked sugars to an internal 10K peptide shared by the two large terminal fragments (Fig. 7). Approximate assignments of the termini of the tryptic fragments identified in these experiments can be made from the sequence of the FrMCF gp70 gene (14) by taking into consideration the size of the deglycosylated fragments and the distribution of trypsin cleavage sites and N-linked glycosylation sites. Likely cleavage sites for the generation of the two large terminal fragments are Arg-216 and Arg-252 of the pFM1 clone of FrMCF gp70 (14). This brackets a region of 36 amino acids, of which fully one-third are prolines. Such proline-rich regions are common characteristics of gp70s and have been proposed to be potential determinants of receptor specificity (14). This sequence also includes two serine and two threonine residues, one or more of which are presumably the sites of O-glycosylation.

The presence of O-linked sugars on gPr90 but not gPr80 indicates that O-glycosylation is a posttranslational process, linked temporally to the maturation of the N-linked sugars. This is consistent with similar studies of other systems (9, 12, 30). Evidence has been presented for a functional role for N-linked glycosylation in the intracellular transport and maturation of MuLV *env* gene products (26, 28, 31). We have shown that inhibition of maturation of the precursor forms of the N-linked oligosaccharides by the blocking of cleavage of the three terminal glucose residues of the initially transferred oligosaccharide structures inhibits transport of gp70 (26). The discovery that O-glycosylation occurs and is coordinated with the processing of N-linked sugars raises the possibility that this step may play a role in the above effect and that the O-linked carbohydrates may serve as signals facilitating transport through the Golgi apparatus. Other potential functions of these substituents should be considered as well. These include roles in modulating the immunoreactivity of these proteins, a function which has been demonstrated for the N-linked sugars of gp70 (1), and protection of sensitive regions from proteolysis. Finally, it is of interest that a correlation exists between altered O-glycosylation of the LDL receptor which occurs in a monensin-resistant mutant of Chinese hamster ovary cells and reduced LDL binding by these cells (41) and that O-linked oligosaccharides have been reported to be determinants of the sperm receptor activity of the mouse oocyte protein ZP3 (7). Thus, precedents exist for a function for similar oligosaccharides in cell surface binding reactions, and the possibility that O-linked sugars may play some role in the binding of gp70 to its cell surface receptor must be considered. Determination of the sequence of the O-glycosylation site would allow the application of site-specific mutagenesis methods towards elucidation of the functional significance of this substituent.

ACKNOWLEDGMENTS

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