Mutational Analysis of N-Linked Glycosylation Sites of Friend Murine Leukemia Virus Envelope Protein

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Received 8 February 1991/Accepted 11 July 1991

The roles played by the N-linked glycans of the Friend murine leukemia virus envelope proteins were investigated by site-specific mutagenesis. The surface protein gp70 has eight potential attachment sites for N-linked glycan; each signal asparagine was converted to aspartate, and mutant viruses were tested for the ability to grow in NIH 3T3 fibroblasts. Seven of the mutations did not affect virus infectivity, whereas mutation of the fourth glycosylation signal from the amino terminus (gs4) resulted in a noninfectious phenotype. Characterization of mutant gene products by radioimmunoprecipitation confirmed that glycosylation occurs at all eight consensus signals in gp70 and that gs2 carries an endoglycosidase H-sensitive glycan. Elimination of gs2 did not cause retention of an endoglycosidase H-sensitive glycan at a different site, demonstrating that this structure does not play an essential role in envelope protein function. The gs3⁻ mutation affected a second posttranslational modification of unknown type, which was manifested as production of gp70 that remained smaller than wild-type gp70 after removal of all N-linked glycans by peptide N-glycosidase F. The gs4⁻ mutation decreased processing of gPr80 to gPr90, completely inhibited proteolytic processing of gPr90 to gp70 and Pr15(E), and prevented incorporation of envelope products into virus particles. Brefeldin A-induced mixing of the endoplasmic reticulum and parts of the Golgi apparatus allowed proteolytic processing of wild-type gPr90 to occur in the absence of protein transport, but it did not overcome the cleavage defect of the gs4⁻ precursor, indicating that gs4⁻ gPr90 is resistant to the processing protease. The work reported here demonstrates that the gs4 region is important for *env* precursor processing and suggests that gs4 may be a critical target in the disruption of murine leukemia virus *env* product processing by inhibitors of N-linked glycosylation.

Retroviral envelope genes (*envs*) encode glycosylated precursor proteins that are converted by oligosaccharide processing and proteolytic cleavage into two mature viral membrane subunits, the surface protein (SU) and the transmembrane protein (TM). These proteins mediate binding of virions to host cell surface receptors and penetration of viral cores into the host cell cytoplasm. In addition to their roles in viral replication, retroviral *env* proteins are important determinants of viral pathogenicity (e.g., see references 34, 55, and 65). The overall structure and processing pathway of murine leukemia virus (MuLV) *env* products have been characterized (reviewed in reference 36). The first identifiable product, gPr80, carries endoglycosidase H (endo H)-sensitive high-mannose glycans. On the next discrete intermediate, gPr90, most of the N-linked glycans have become endo H resistant, and O-linked glycans are present. gPr90 is rapidly cleaved at a dibasic amino acid cleavage site into gp70 (SU) and Pr15(E) (TM), which remain associated via disulfide linkages. After incorporation into virus particles, Pr15(E) is further processed into p15(E) by removal of a small C-terminal peptide.

The work presented here examines the function of the N-linked glycans of the *env* products of Friend MuLV. Inhibitors of N-linked glycosylation (42, 48) or early processing of N-linked oligosaccharides (40) block proteolytic processing and transport of MuLV envelope proteins and production of infectious virus. This suggests that addition and some processing of N-linked glycan is required for

envelope protein function. The earlier studies that yielded these results could not distinguish between a generalized requirement for N-linked glycans and effects due to loss of particular glycans. In this report, site-directed mutagenesis was used to determine which of the eight N-linked glycans of Friend MuLV gp70 are dispensable for protein function. Viral growth was not sensitive to the removal of the glycan at seven of the attachment sites, while mutation of one specific glycan attachment site blocked viral replication by interfering with two steps of *env* protein maturation.

MATERIALS AND METHODS

Construction of mutants. To remove each of the glycans from Friend MuLV *env* proteins, the asparagine codon of each glycosylation signal within the gp70 domain was changed to an aspartic acid codon by oligonucleotide-directed site-specific mutagenesis using the mutagenic oligonucleotides described in Table 1. Mutations that eliminate glycosylation signals, and plasmids or viruses carrying these mutations, are designated gsX⁻, where X is the sequential number of the glycosylation signal, with the numbering beginning at the 5' end of *env* (Table 1). Amino acid numbers begin from the N terminus of mature gp70 (15).

Mutagenesis was performed on single-stranded DNA derived from subclones of Friend MuLV clone 57 (31). Most of the mutations were isolated by the Kunkel method to enrich for mutants (17, 24); for a few mutations the method of Taylor et al. was used (57). In most cases, a new restriction site was introduced along with the glycosylation mutation to provide easy detection and tracking of the mutation. The

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TABLE 1. Glycosylation signal mutations

Signal	Location ^a	Mutant sequence at signal ^b
gs1	Asn-12	His Gln Val Tyr ASP Ile Thr Trp Glu gtc tac Gac att acc
gs2	Asn-169	Thr Val Asp Asn ASP Leu Thr Thr Ser gac aac Gat ctc acc <i>Sau3AI</i>
gs3	Asn-266	Pro Pro Ala Ser ASP Ser Thr Pro Thr c ccc gcc tca Gat tgc act c <i>DdeI</i>
gs4	Asn-302	Tyr Gln Ala Leu ASP Leu Thr Asn Pro ac cag gca cta GaT ctt acc aac c <i>BglII</i>
gs5	Asn-334	Gly Thr Tyr Ser ASP His Thr Ser Ala at tcc Gac cat acc tc
gs6	Asn-341	Ser Ala Pro Ala ASP Cys Ser Val Ala t gcc cca gct Gac tgc <i>PvuII</i>
gs7	Asn-374	Gln Ala Leu Cys ASP Thr Thr Leu Lys c ctg tgc Gac act acc
gs8	Asn-410	Ala Thr Val Leu ASP Arg Thr Thr Asp gtg ctt Gat cgc acc <i>Sau3AI</i>

^a The glycosylation signals of Friend MuLV are numbered sequentially from the N terminus of gp70.

^b The amino acid sequence surrounding each signal is shown, with the mutant Asp residue capitalized. The mutagenic nucleotide sequence is shown as the complement of the oligonucleotide actually used, with non-wild-type bases capitalized. For those mutations for which a new restriction site was introduced to monitor the presence of the mutation, the recognition sequence is shown in italics and the enzyme is indicated below the site.

regions surrounding each mutagenic oligonucleotide were confirmed by DNA sequencing, and mutant *envs* were placed into complete viral genomes for analysis. Some of the mutant *envs* were cloned into viral vectors containing *EcoRI*-permuted genomes of Friend MuLV (50). A vector containing a nonpermuted Friend virus genome with two long terminal repeats, pLRB132, was used for the remainder of the mutants. pLRB132, constructed in this laboratory from p57/19A (50), contains a small amount of *env* duplicated 5' to the complete genome and *gag* and some *pol* sequences 3' to it. To ensure that the phenotypes of the noninfectious *gs4*⁻ mutant and the unexpected mobility alteration of *gs3*⁻ gp70 were due to the Asn→Asp mutations, the complete sequence of a small restriction fragment containing each mutation was determined, and mutant viral genomes that contained only these sequenced fragments from the mutagenized plasmids were constructed. These constructs displayed the same phenotypes as the original *gs3*⁻ and *gs4*⁻ viruses. Sequences were determined by the dideoxy method on single-stranded and double-stranded templates by using Sequenase (U.S. Biochemicals).

Viral culture and immunological and biochemical procedures. Viral culture (40), indirect immunofluorescence (30), and radiolabelling and radioimmunoprecipitation (39) were performed as described previously. The primary antibodies used included a hyperimmune goat anti-Rauscher MuLV gp70 serum (Microbiological Associates), mouse anti-p15(E)

monoclonal antibody 9E8 (21), mouse anti-gp70 monoclonal antibody 307 specific for the Friend and Rauscher MuLV ecotropic *env* proteins (6), hyperimmune rat anti-Gross MuLV p30 provided by W. Hardy, and rat anti-p12^{gag} monoclonal antibody 10CE10 produced in this laboratory. The secondary antibodies were the appropriate rabbit anti-immunoglobulin G and anti-immunoglobulin M mixtures conjugated to fluorescein isothiocyanate (Zymed).

Trypsin digestions using TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin (Sigma) (38) and digestions with endo H and peptide *N*-glycosidase F (PNGase F) (Boehringer Mannheim) (39) were performed as previously described. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the Laemmli method (18).

Establishment of cell lines expressing noninfectious mutants. The noninfectious *gs4*⁻ mutant was transfected into the ecotropic packaging cell line Ψ_2 (22) or the amphotropic packaging cell line PA317 (25a). Following transfection, Ψ_2 and PA317 cells were cocultured and viral supernatants were collected. The pseudotyped *env*-defective Friend genomes spread through the coculture by a ping-pong effect between the two packaging cell types, generating a high-titer stock of pseudotyped mutant virus particles (2). This viral stock was used to infect NIH 3T3 cells, and then cell lines expressing the mutant Friend virus were cloned and used to characterize the mutant envelope proteins. Five clones from two independent transfections were examined and found to react with Friend virus-specific monoclonal antibody 307, demonstrating that they contain sequences derived from the *gs4*⁻ mutant. The isolates were defective and had similar growth, precursor size, and precursor accumulation phenotypes. These cell lines must therefore express *gs4*⁻ *env*, since genomes containing defective recombinant or revertant *envs* would be present only at levels equal to their formation rates, necessarily far below the level of the parental *gs4*⁻ genome.

Assay for viability. The viability of Friend MuLV mutants was determined by testing their ability to establish a spreading infection in cell culture. NIH 3T3 fibroblasts were transfected by the calcium phosphate method with CsCl-purified or Qiagen Column-purified plasmid DNA at 10 µg/60-mm-diameter dish (or proportionally less for smaller cultures) and Pharmacia CellPect reagents and protocols. For permuted genome vectors, the viral genome was cleaved from the plasmid backbone with *EcoRI* before transfection (31); nonpermuted genome vectors were transfected as supercoiled plasmid. Sixteen hours after exposure to plasmid DNA, the cells were glycerol shocked (day zero). They were passaged 6 h later and every few days thereafter. Slides were prepared at each passage and fixed after 16 to 24 h. The cells on the slides were assayed for viral antigens by immunofluorescence using the anti-p12^{gag} monoclonal antibody. Between 2×10^3 and 1×10^4 cells were assayed at each time point. When the cultures were essentially fully infected, they were used for biochemical characterization of mutant envelope proteins.

RESULTS

Effect of glycosylation signal mutations on infectivity. In order to explore the role(s) of N-linked glycans in the function of retroviral *env* proteins, site-directed mutagenesis was used to destroy the eight glycosylation signals in the SU domain of Friend MuLV *env*. Each signal asparagine was individually changed to aspartic acid (Table 1 contains

FIG. 1. Transfection growth curves of mutant viruses. NIH 3T3 fibroblasts were transfected as described in Materials and Methods. The fraction of cells expressing p12^{gag} was estimated by indirect immunofluorescence at various times, with 0 referring to the time of glycerol shock. Data from two experiments, distinguished by open and filled symbols, are shown. Curves for permuted genome vectors are plotted with solid lines; curves for nonpermuted genome vectors are plotted with dashed lines. Wild-type controls from one of the experiments are shown; those from the second experiment were similar. Symbols: ●—●, wild type; □—□, gs3⁻; ■—■, gs4⁻; ▲—▲, gs5⁻; △—△, gs7⁻; ●—●, wild type; □—□, gs1⁻; ▲—▲, gs2⁻; ■—■, gs6⁻; △—△, gs8⁻.

descriptions of the mutations introduced). Viral genomes carrying each of the Asn→Asp mutations were transfected into NIH 3T3 cells, and the infectivity of each mutant was determined by monitoring the increase in the fraction of cells producing viral antigens. Vectors containing either a permuted or a nonpermuted viral genome were used. Permuted genome vectors carry a single long terminal repeat genome permuted at its *EcoRI* site, the form in which Friend MuLV was originally cloned (31). Transfections with wild-type genomes in such vectors typically resulted in less than 0.05% antigen-positive cells 1 day posttransfection and 50% antigen-positive cells between 8 and 12 days posttransfection. To obtain more efficient transfection, a nonpermuted genome analogous to a provirus was constructed in vitro from the permuted clone of Friend MuLV. Transfections with this vector typically yielded 0.1 to 2% antigen-positive cells 1 day posttransfection and 50% antigen-positive cells between 3 and 5 days posttransfection.

Transfection of the eight glycosylation signal mutants (Fig. 1) showed that only the gs4⁻ mutation at Asn-302 affected the infectivity of Friend MuLV. Spreading infection was not detected in multiple transfections with this mutant, despite normal levels of antigen-positive cells 1 day posttransfection. This result suggests either that a glycan is necessary at gs4 for envelope protein function or that an aspartic acid residue is not tolerated at position 302. The other seven mutants established infected cultures as rapidly as did wild-type virus, and thus these cultures cannot represent the outgrowth of recombinant or revertant viruses. Therefore, growth in cell culture does not require an N-linked glycan at any one of these seven glycan attachment sites of gp70.

N-linked glycans are added at all eight SU glycosylation signals. The early translation product gPr80 of each mutant was examined to confirm that the glycosylation signal mutations caused loss of N-linked glycan. (For convenience, mutant products and tryptic fragments, as well as their deglycosylated forms, are referred to by standard names that are based on the apparent molecular mass of the wild-type polypeptide.) Infected cell lines expressing each of the

FIG. 2. Examination of cell-associated *env* products. Virus-producing cell lines were labelled for 1 h with [³⁵S]cysteine. The cell lysates were immunoprecipitated with hyperimmune anti-gp70 serum, with or without PNGase F treatment, and analyzed by SDS-PAGE and autoradiography. The genotypes of the samples are indicated above the lanes. (A) No PNGase F treatment. The migration positions of wild-type (wt) gPr80 and gp70 are indicated. (B) PNGase F treatment before separation. The migration positions of untreated and deglycosylated (de-) wild-type gPr80 and gp70 are indicated.

mutants were labelled for 1 h with [³⁵S]cysteine, and cell lysates were immunoprecipitated with hyperimmune anti-gp70 serum. Cell lines for the infectious mutants were derived from transfections like those described above; the cell line for the noninfectious gs4⁻ mutant was generated with pseudotyped virus stocks prepared in retroviral vector packaging cell lines. All eight glycosylation signal mutants produced gPr80 that was slightly smaller than that of the wild type (Fig. 2A). This size shift is due to the loss of N-linked glycan from each mutant product, since each of the mutant gPr80s comigrated with wild-type gPr80 after removal of all N-linked glycans with PNGase F (Fig. 2B). These data demonstrate that all eight glycosylation signal sequences in wild-type Friend MuLV gp70 are utilized, confirming previous reports based on protein sequencing (5, 12).

Characterization of mutant gp70s. Mature gp70s were found in infected cells for the seven infectious mutants but not in cells infected with the defective gs4⁻ mutant (Fig. 2A). The mutant gp70s were all smaller than wild-type gp70, consistent with the absence of an N-linked glycan. Cells expressing the gs5⁻ mutant reproducibly contained a lower ratio of gp70 to gPr80, and gs5⁻ virions contained less *env* protein than wild-type particles (data not shown), but this did not have a significant effect on viral growth. After deglycosylation with PNGase F, six of the seven mutant gp70s comigrated with wild-type gp70, as expected (Fig. 2B). Surprisingly, the gs3⁻ gp70 was smaller than the wild-type gp70 even after removal of all N-linked glycans. This observation, together with the fact that the deglycosylated gs3⁻ gPr80 was the same size as the wild-type gPr80, implies that the gs3⁻ mutation has a second effect on a posttranslational modification that occurs after addition of N-linked glycans and does not directly involve these moieties.

Virion-associated gp70s made by the infectious mutants

FIG. 3. Examination of virion-associated *env* products. [35 S]methionine-labelled gp70s from sucrose gradient purified virions were immunoprecipitated, treated with trypsin (2 μ g/ml) to generate partial digests, and analyzed by SDS-PAGE and autoradiography. The genotype of the gp70 is indicated above each lane. The migration positions of intact wild-type (wt) gp70 and its three largest N-terminal fragments are indicated.

were also smaller than the wild-type product (Fig. 3). The location of the glycan removed by each mutation was mapped to the appropriate domain of gp70 by examination of fragments generated by limited digestion of [35 S]methionine-labelled gp70 with trypsin (Fig. 3). Since there are no trypsin-sensitive sites between the N terminus of Friend MuLV gp70 and the single methionine at amino acid 47, only N-terminal tryptic fragments are detected with this label. For wild-type virus, undigested gp70 and three large fragments of 51, 36, and 34 kDa were detected. For the *gs1*⁻ and *gs2*⁻ mutants, intact gp70 and all three N-terminal fragments were smaller than the wild-type polypeptides, consistent with the location of these glycosylation sites in the N-terminal domain of wild-type gp70. The mutant forms of the 36- and 34-kDa fragments were not resolved from other fragments on the gel shown in Fig. 3, but they were clearly distinguished in other experiments (data not shown). The gp70s from the *gs5*⁻, *gs6*⁻, *gs7*⁻, and *gs8*⁻ mutants were smaller than that from the wild type, while the three N-terminal tryptic fragments from these mutants comigrated with those from the wild type, consistent with the location of these glycosylation signals in the C-terminal domain of gp70.

For the *gs3*⁻ mutant, gp70 and its 51-kDa fragment were smaller than those from the wild type, whereas its 36- and 34-kDa fragments comigrated with the corresponding wild-type fragments. In this case, the shift in the size of the 51-kDa fragment was not due to the loss of the glycan at *gs3*, since removal of N-linked glycans with PNGase F failed to eliminate the size difference (data not shown), and as shown below, the 51-kDa fragment contains only *gs1* and *gs2*. The mobility shift of the *gs3*⁻ 51-kDa fragment therefore resulted from the second processing alteration associated with the *gs3*⁻ mutation, as noted above (Fig. 2B). These data map this modification to the region of gp70 between *gs2* and *gs3*, since *gs2* is present on the 36-kDa fragment that does not carry this modification and the 51-kDa fragment that carries this modification terminates before *gs3*. This is a region in which O-linked carbohydrate is attached to gp70 (reference 39 and unpublished data). Although *gs3*⁻ gp70 does carry O-linked carbohydrate (data not shown), it is possible that *gs3*⁻ gp70 carries less O-linked carbohydrate than wild-type gp70. Alternatively, there may be a different posttranslational modification in this region of gp70 that is affected by the *gs3*⁻ mutation.

An endo H-sensitive glycan is attached at *gs2*. Endo H removes high-mannose and some hybrid N-linked glycans but not complex forms (59). Whereas most of the N-linked glycans on mature gp70 are of the complex form, MuLV gp70s generally retain one endo H-sensitive N-linked glycan

FIG. 4. Identification of the attachment site of the endo H-sensitive glycan of gp70. [35 S]methionine-labelled gp70s from sucrose gradient-purified wild-type and mutant viruses were treated with limiting trypsin as described in the legend to Fig. 3 (lanes A) and then further treated with endo H (lanes B) or PNGase F (lanes C) before analysis by SDS-PAGE and autoradiography. The region of the 51-kDa N-terminal fragment is shown.

on their N-terminal domain (37, 38). Biochemical data suggesting that a significant fraction of Friend MuLV gp70 carries an endo H-sensitive glycan at *gs1* (47) and that essentially all gp70 molecules carry an endo H-sensitive glycan at *gs2* (12) have been reported. The glycosylation mutants provided an opportunity to further explore the question of which Friend MuLV gp70 glycosylation sites carry predominantly endo H-sensitive glycans and to determine whether such a glycan is required for viral replication.

It was previously shown that the endo H-sensitive glycan of Friend MuLV gp70 is present on its 51-kDa N-terminal tryptic fragment (38). After digestion with endo H, the wild-type fragment comigrated with untreated, singly mutant fragments, consistent with removal of a single glycan by endo H (Fig. 4). Endo H digestion of the fragment from *gs1*⁻ virus converted it into a product that comigrated with the untreated fragment from the *gs1*⁻ *gs2*⁻ double mutant; however, endo H had no effect on the mobility of the fragment from *gs2*⁻ virus (or on *gs2*⁻ gp70 [data not shown]), consistent with the lack of an endo H-sensitive glycan in the *gs2*⁻ mutant. These results confirm that the glycan at *gs2* in Friend MuLV is endo H sensitive and indicate that the bulk of the glycan attached at *gs1* is endo H resistant. The reason for this difference from the earlier report of endo H-sensitive glycans at *gs1* (47) is not clear. In the absence of *gs2*, Friend MuLV gp70 did not carry an endo H-sensitive glycan at a different site in the N-terminal domain (Fig. 4) or elsewhere on the protein. Thus, retention of the glycan in an endo H-sensitive form is specific to *gs2*, and an endo H-sensitive glycan is not required for growth in culture. Treatment of 51-kDa fragments from wild-type, *gs1*⁻, and *gs2*⁻ viruses with PNGase F resulted in fragments that comigrated with the untreated *gs1*⁻ *gs2*⁻ fragment, which was itself insensitive to either glycosidase. These data demonstrate that *gs1* and *gs2* are the only signals for N-linked glycosylation present on the 51-kDa N-terminal fragment of gp70.

The *gs4*⁻ mutation blocks *env* protein processing and incorporation into virions. In order to explore the biochemical basis for the noninfectious phenotype of the *gs4*⁻ mutant, infected cells were labelled with a mixture of [35 S]cysteine and [35 S]methionine for 2 h, and the lysates were immunoprecipitated with hyperimmune anti-gp70 serum or anti-p15(E) monoclonal antibody 9E8. Only uncleaved *env* precursors were detected in *gs4*⁻ virus-infected cells (Fig. 5A). Note that the anti-p15(E) monoclonal antibody precipitated a much larger fraction of *gs4*⁻ gPr80 than of wild-type gPr80, suggesting that its epitope is more accessible in the mutant precursor than in the wild-type precursor. Comparison of the protein composition of *gs4*⁻ virions with that of wild-type virions revealed two reproducible differences (Fig. 5B). First, a band at 15 kDa, shown to be p15(E) by immunoprecipitation, was totally absent from *gs4*⁻ virions. Second,

FIG. 5. *gs4⁻* *env* products. (A) Wild-type and *gs4⁻* virus-infected cells were labelled for 2 h with a mixture of [³⁵S]cysteine and [³⁵S]methionine. Cell lysates were immunoprecipitated with hyperimmune goat anti-gp70 serum (lanes A), anti-p15(E) monoclonal antibody 9E8 (lanes B), hyperimmune goat anti-p30^{gag} serum (lanes C), or normal goat serum (lane D) and analyzed by SDS-PAGE and autoradiography. The migration positions of gp70 and Pr15(E) are indicated. (B) Wild-type and *gs4⁻* virus particles labelled with a mixture of [³⁵S]cysteine and [³⁵S]methionine were purified on sucrose gradients. Complete virions (lane A, wild type; lane B, *gs4⁻*), as well as immunoprecipitates obtained by using hyperimmune anti-gp70 serum (lane C, wild type; lane E, *gs4⁻*) and the anti-p15(E) monoclonal antibody (lane D, wild type; lane F, *gs4⁻*), were examined by SDS-PAGE and autoradiography. The migration positions of gp70 and p15(E) are indicated.

only a small amount of 70-kDa protein was associated with *gs4⁻* virions. This material is not gp70, since it was not immunoprecipitated by the hyperimmune anti-gp70 serum (Fig. 5B) even though this serum did precipitate *gs4⁻* gPr80 (Fig. 5A).

The kinetics of processing of *gs4⁻* *env* products was investigated with a 1-h pulse label followed by chase periods of various times (Fig. 6). Proteolytic processing of gPr90 to gp70 and Pr15(E) normally occurs very rapidly, and gPr90 is therefore best detected by labelling with [³H]glucosamine, a label that is enriched in gPr90 and gp70 relative to gPr80. By the end of the 1-h pulse, most of the label in wild-type *env* products was already associated with gp70, indicating the efficient and rapid processing of the wild-type precursors. The half-life of *gs4⁻* gPr80 was significantly longer than that of wild-type gPr80, with the majority of *env* label found in gPr80 throughout the experiment. gPr90 was also seen in *gs4⁻* virus-infected cells, where it accumulated to abnormally high levels. Consistent with the results described

FIG. 6. Accumulation of *gs4⁻* intermediates. Wild-type and *gs4⁻* virus-infected cells were labelled with [³H]glucosamine for 1 h and then chased for the times indicated above each lane in hours. The cell lysates were immunoprecipitated with hyperimmune anti-gp70 serum and analyzed by SDS-PAGE and autoradiography. The migration positions of wild-type gPr90, gPr80, and gp70 are indicated. The two bands seen in the *gs4⁻* lysate are gPr90 and gPr80.

above, no gp70 was detected by [³H]glucosamine labelling. Thus, the *gs4⁻* mutation has two effects on *env* processing: a decrease in maturation of gPr80 to gPr90 and a stringent blockage of gPr90 proteolytic processing.

The lack of proteolytic processing of *gs4⁻* gPr90 might be due either to its failure to be translocated into the subcellular compartment where proteolysis occurs or to an inherent resistance of the mutant precursor to the protease involved. These possibilities were tested by using the drug brefeldin A (BFA) to bypass the requirement for translocation. BFA inhibits transport of proteins from the endoplasmic reticulum into the Golgi apparatus (26) and induces collapse of Golgi apparatus membrane structures and enzymes into the endoplasmic reticulum (4, 20). In the presence of BFA, MuLV *env* products were not incorporated into virions or secreted from cells (data not shown). Wild-type *env* precursor was slowly cleaved into SU and TM domains, with approximately 50% of the labelled *env* protein found as cleavage product by 8 h after the beginning of the chase period (Fig. 7). This result indicates that at least a fraction of the processing protease is present and active in the combined endoplasmic reticulum-Golgi apparatus compartment in BFA-treated cells. Thus, treatment with BFA bypasses whatever translocation steps are normally required for *env* precursors to be available to the processing protease. In contrast to what is seen for wild-type *env* products, no processing of *gs4⁻* precursor into SU and TM domains was detected within 8 h of the start of the chase. Since translocation is not required for proteolytic processing of envelope precursor in the presence of BFA, these data argue that the processing protease does not recognize the mutant protein.

To determine whether the partial block in *gs4⁻* gPr80 processing to gPr90 and/or the failure of *gs4⁻* gPr90 to be processed to gp70 and Pr15(E) is mediated or accompanied by gross perturbation of glycan structures beyond the loss of

FIG. 7. Proteolytic processing of *env* products in BFA-treated cells. BFA-treated wild-type and *gs4⁻* virus-infected cells were labelled with [³H]glucosamine for 1 h and then chased for the times indicated above each lane in hours. The cell lysates were immunoprecipitated with hyperimmune anti-gp70 serum and analyzed by SDS-PAGE and autoradiography. The migration positions of wild-type gPr80 and gp70 from cells not treated with BFA are indicated.

FIG. 8. Endoglycosidase analysis of glycan processing of $gs4^{-}$ *env* products. Wild-type and $gs4^{-}$ virus-infected cell lysates, labelled for 1 h with [3 H]glucosamine, were immunoprecipitated with hyperimmune anti-gp70 serum and treated with various endoglycosidases prior to visualization by SDS-PAGE and autoradiography. In the wild-type lysate, gPr80, gPr90, and gp70 were detected; in the $gs4^{-}$ lysate, gPr80 and gPr90 were detected, although they were not clearly resolved from each other in the untreated control lane. The deglycosylation product of gPr80 was not visible after treatment with PNGase F because of the removal of all carbohydrate radiolabel from this precursor. The migration positions of wild-type *env* products are indicated. The arrowheads indicate the migration positions of gPr90 (open; detected on exposures longer than that shown) and gp70 (closed) after removal of N-linked glycans with PNGase F. Lanes: A and D, no enzyme treatment; B and E, endo H treatment; C and F, PNGase F treatment.

the $gs4$ glycan, the glycan composition of these proteins was examined by digestion with endoglycosidases (Fig. 8). After wild-type-infected cells were labelled for 1 h with [3 H]glucosamine, gPr80, gPr90, and gp70 were detected by immunoprecipitation with hyperimmune anti-gp70 serum (Fig. 8, lane A). Digestion of the wild-type sample with endo H (lane B), which cleaves between the first and second *N*-acetylglucosamine residues of high-mannose glycans, caused a large increase in the mobility of gPr80, consistent with cleavage of all eight N-linked glycans. Endo H digestion caused only small increases in the mobilities of gPr90 and gp70, consistent with the expected cleavage of only the $gs2$ glycan from each of these *env* products. The deglycosylated product of gPr80 was not detected after treatment with PNGase F (lane C), which cleaves between the Asn residue and the first *N*-acetylglucosamine of high-mannose and complex forms and thus removes all label present in N-linked carbohydrates. The product of gp70 was visualized after PNGase F treatment because of its O-linked glycans (lane C). Longer exposures detected the deglycosylated gPr90 from the wild-type sample, also by virtue of its O-linked glycans. $gs4^{-}$ virus-infected cells, which had been labelled similarly, contained gPr80 and gPr90 but no gp70 (lane D). Treatment with endo H caused a large increase in $gs4^{-}$ gPr80 mobility and a small increase in $gs4^{-}$ gPr90 mobility (lane E) similar to the changes seen for wild-type precursors. Treatment with PNGase F removed all label from $gs4^{-}$ gPr80, consistent with the absence of O-linked glycan, while $gs4^{-}$ gPr90 was converted to a product that was larger than endo H-digested $gs4^{-}$ gPr80 and retained a fraction of its radiolabel, indicating the presence of O-linked glycan (lane F). Thus, glycan processing of $gs4^{-}$ *env* precursors is not distinguishable from that of wild-type products by endoglycosidase sensitivity criteria.

DISCUSSION

The functions of the N-linked glycans carried on MuLV *env* proteins have previously been studied by using drugs that block the addition of these structures to proteins or that inhibit processing of high-mannose forms. Tunicamycin, which blocks transfer of N-linked glycans to nascent polypeptides, inhibits the processing of MuLV *env* precursors into gp70 and Pr15(E) and the incorporation of envelope

proteins into virus particles (40, 42, 48). A study using monoclonal antibodies against a conformational epitope on gp70 suggested that tunicamycin interferes with proper folding of the *env* precursor (35). 1-Deoxynojirimycin, which inhibits the glycosidases that remove the three glucose residues present on the initially transferred glycans, also interferes with transport and proteolytic processing of the *env* precursor (40). This observation indicates that at least some of the N-linked glycans need to undergo processing for proper function of the *env* protein. Taken together, these data suggest that N-linked glycans function at multiple steps in *env* precursor folding and processing.

The characterization of glycosylation signal mutations of Friend MuLV *env* proteins reported here provides a more precise picture of the role of N-linked glycans in envelope protein function. Only one glycosylation mutation in *env* had sufficient impact on protein function to interfere with viral growth. Such attachment site specificity for the effect of glycosylation mutations has been observed for a number of other proteins (e.g., as described in references 9, 23, 28, and 64). The Asn-302→Asp mutation at $gs4$ interferes with the maturation of gPr80 to gPr90 and causes accumulation of gPr90 because of a stringent block in its conversion to gp70 and Pr15(E). It is possible that the $gs4$ glycan is a critical target for one or more of the inhibitors that block attachment and processing of N-linked glycans. gPr80 has been localized to the endoplasmic reticulum by its characteristic endo H-sensitive glycans (36). The reduced processing of $gs4^{-}$ gPr80 to gPr90 may be due to an increased percentage of improperly folded or oligomerized subunits that are not competent for translocation into the Golgi apparatus, as has been observed for a number of other glycosylation mutant proteins (8, 11, 28). The $gs4^{-}$ cleavage defect was not suppressed by BFA, which bypasses the protein transport normally required for proteolytic processing of wild-type gPr90. In addition, the N-linked and O-linked glycans found on $gs4^{-}$ gPr90 are normal in their sensitivity to endoglycosidases, consistent with it being located in the proper subcellular compartment. It thus appears that $gs4^{-}$ gPr90 is probably transported into the Golgi compartment in which proteolytic cleavage normally occurs, and it is resistant to the processing protease.

Functional roles of specific N-linked glycosylation signals have previously been reported for the SU proteins of human immunodeficiency virus type 1 (HIV-1) (gp120) and HIV-2 (gp105). Unlike the results reported here for the $gs4^{-}$ mutation of gp70, no effects on protein maturation were found. In HIV-1, elimination of one of the four glycosylation signals of gp120 examined by site-directed mutagenesis interferes with viral growth because of a defect in a postbinding step in the infection cycle (64). In HIV-2, elimination of one of the three glycosylation signals near or within the region believed to comprise the CD4-binding site of gp105 markedly decreased its affinity for CD4 (27).

Glycosylation signals homologous to those of Friend MuLV *env* are largely conserved among MuLV and feline leukemia virus (FeLV) *envs* (Table 2), suggesting that there has been selective pressure for their retention. It was therefore somewhat surprising that viral growth in cell culture is insensitive to the individual elimination of seven of the eight glycosylation signals in the SU domain of Friend MuLV. The presence of an endo H-sensitive glycan in the N-terminal domain of ecotropic and polytropic MuLV gp70s (37, 38) also argues for selective pressure on this structural feature, particularly since it is not attached at precisely the same site in the two classes of *envs*. This glycan was found to be

TABLE 2. Conservation of glycosylation signals in viral surface proteins

Virus isolate (reference) ^a	MuLV class	Presence of homologous glycosylation signal(s) ^b							
		1	2	3	4	5	6	7	8
Friend MuLV (15)	Ecotropic	+	+	+	+	+	+	+	+
Moloney MuLV (49)	Ecotropic	+	+		+	+	+	+	+
Akv MuLV (19)	Ecotropic	+	+	+	+	+	+	+	
Cas-Br-E MuLV (44)	Ecotropic	+	*	+	+		+	+	+
Radiation MuLV (25)	Ecotropic	+	+	*	+	+	+	+	+
Ho MuLV (62)	Ecotropic	+	+	+	+	+	+		+
NZB 9-1 MuLV (32)	Xenotropic	+	*		+	+	+	+	+
MCF 247 (13)	Polytropic	+	+		+	+	+	+	
Moloney MCF (3)	Polytropic	+	+		+	+	+	+	+
Friend MCF Nx (1)	Polytropic	+	+		+	+	+	+	+
Friend MCF 54B (16)	Polytropic	+	+		+	+	+	+	+
Rauscher MCF (61)	Polytropic	+	+		+	+	+	+	+
4070A MuLV (33)	Amphotropic	+	+	*	+	+	+	+	+
FeLV type A (54)		+	+	*	+	*	+	+	*
FeLV type C (45)		+	+	+	+	+	+		+
FeLV type B(GA) (10)		+	+	*	+	+	+	+	+

^a MCF, mink cell focus-forming virus.

^b The glycosylation signals of Friend MuLV gp70 are indicated in the column headings by sequential numbering from the N terminus. The presence of the signals in Friend MuLV and of homologous signals in other envelope proteins is indicated by a plus sign. The presence of a glycosylation signal lacking a homolog in the Friend MuLV protein is indicated by an asterisk positioned sequentially according to its location in the protein. A plus sign in a column containing an asterisk is used to indicate that such a signal is also present in other proteins, while an asterisk is used to indicate that the additional glycosylation signals in two or more other proteins are not homologous.

attached at gs2 of Friend MuLV, which is conserved among ecotropic *envs* but is not found in polytropic *envs* (Table 2). The glycosylation signal in this domain that is conserved among polytropic *envs* but is not found in ecotropic *envs* may be the attachment site of the N-terminal endo H-sensitive glycan carried by polytropic SU proteins. There are several plausible ways to reconcile the expectation of selective pressure on many of these glycosylation signals with the observation that seven out of eight are dispensable for growth in NIH 3T3 fibroblasts. First, N-linked glycans may possess functions that are relevant in host animals but not in cell culture, such as masking of epitopes (51, 66) or protecting against proteolytic degradation. Second, small effects on growth rate that were not detected in cell culture may be important in animals, where selection for faster-growing variants should occur. Third, the mutations may have larger effects on growth in other cell types that are important targets in mice.

The glycosylation signal that did prove to be sensitive to mutation, gs4, is one of two that are fully conserved among the closely related MuLV and FeLV genes. Examination of known sequences of retroviral *envs* shows that a homolog of gs4 is retained even in genes with very little sequence similarity in SU domains. The gs4 motif consists of a hydrophobic glycosylation signal (usually) seven amino acids N terminal to an invariant Cys-Trp-Leu-Cys sequence (Table 3). Biochemical data have demonstrated that, for the Friend MuLV *env* product, one or both of these cysteines are involved in disulfide bonding between gp70 and p15(E) (unpublished data). The evolutionary association of gs4 with these cysteine residues suggests that this glycan plays a role in establishing correct interdomain disulfide bonding in gPr80. Data from a number of systems suggest that N-linked glycans affect protein tertiary structure by controlling the

pattern of disulfide bonds formed during folding and oligomerization (28, 52, 56, 60, 66), and two observations indicate that the gs4⁻ mutation affects overall *env* precursor structure, in particular in or near the p15(E) domain. First, the gs4⁻ mutation exerts an effect at a distance on the gp70-Pr15(E) cleavage site (amino acid 302 versus 445), suggesting a change in tertiary structure. Second, anti-p15(E) monoclonal antibody 9E8 recognizes gs4⁻ gPr80 much more efficiently than wild-type gPr80, demonstrating that the structure or accessibility of p15(E) sequences within gPr80 is affected by the gs4⁻ mutation. If the Friend MuLV gs4 glycan is involved in regulation of interdomain disulfide bonding, the evolutionary retention of a homologous site implies that this structural motif may be used in a surprisingly diverse group of retroviral *envs*. It will be interesting to determine whether mutations in the gs4 homologs of these other *envs* yield similar phenotypes.

The data presented above do not distinguish between effects due to the absence of an N-linked glycan at position 302 and those due to the presence of an aspartic acid residue at this position. Caution is needed in interpreting phenotypes of glycosylation mutations as due to the loss of the glycan per se. For example, the phenotypes of glycosylation mutations in HLA-2A depend on the specific amino acid substitution introduced (46), suggesting a major role for the amino acid sequence at the glycosylation site. This is also suggested by the analysis of the glycosylation site in HIV-1 gp120 discussed above, where mutations adjacent to or within the glycosylation signal that would not be expected to affect glycan addition had phenotypes similar to those of mutations that destroy the glycosylation signal (64). In addition, the phenotypes of glycosylation mutations in HIV-1 gp120 (64) and vesicular stomatitis virus G protein (41) were suppressed by amino acid changes that do not

TABLE 3. Conservation of a gs4-like glycosylation signal and related cysteines

Virus (reference) ^a	MuLV class	Sequence of gs4 homologous region ^b																									
Friend MuLV	Ecotropic	A	Y	Q	A	L	<u>N</u>	<u>L</u>	<u>T</u>	N	P	D	K	T	Q	E	-	<u>C</u>	<u>W</u>	<u>L</u>	<u>C</u>	L	V	S	G	(143)	
Moloney MuLV	Ecotropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	A	+	(143)	
Akv MuLV	Ecotropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	+	(143)	
Cas-Br-E MuLV	Ecotropic	+	+	L	T	+	<u>+</u>	<u>M</u>	<u>+</u>	D	+	T	R	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	E	(143)	
Radiation MuLV	Ecotropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	R	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	+	(143)	
Ho MuLV	Ecotropic	+	+	+	+	+	<u>+</u>	<u>A</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	S	(142)	
NZB 9-1 MuLV	Xenotropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	+	(143)	
MCF-247	Polytropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	+	(143)	
Moloney MCF	Polytropic	+	+	R	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	A	+	(143)	
Friend MCF Nx	Polytropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	+	(143)	
Friend MCF 54B	Polytropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	+	(143)	
Rauscher MCF	Polytropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	S	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	A	E	(143)	
4070A MuLV	Amphotropic	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	+	+	+	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	+	(143)	
FeLV type A		T	+	L	+	+	<u>+</u>	<u>A</u>	<u>+</u>	D	+	N	+	+	K	D	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	R	(143)	
FeLV type C		T	+	L	+	+	<u>+</u>	<u>A</u>	<u>+</u>	D	+	N	+	+	K	D	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	R	(143)	
FeLV type B(GA)		T	+	L	+	+	<u>+</u>	<u>A</u>	<u>+</u>	D	+	N	R	+	K	D	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	+	+	R	(143)	
GALV (7)		+	F	L	T	+	<u>+</u>	<u>A</u>	<u>+</u>	+	+	G	A	+	E	S	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	A	M	+	(145)	
SRV-1 (43)		V	H	S	L	+	<u>+</u>	<u>V</u>	<u>S</u>	S	Q	R	Q	L	A	+	D	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	R	+	+	(158)	
SRV-2 (58)		T	+	S	L	+	<u>+</u>	<u>K</u>	<u>S</u>	+	+	N	L	A	N	+	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	P	+	+	(149)	
MPMV (53)		V	H	S	L	+	<u>+</u>	<u>A</u>	<u>S</u>	Q	+	S	L	A	E	D	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	Q	+	+	(157)	
BaEV (14)		<u>L</u>	<u>N</u>	<u>I</u>	<u>+</u>	<u>+</u>	<u>A</u>	<u>+</u>	<u>Y</u>	<u>N</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>M</u>	<u>S</u>	<u>N</u>	<u>T</u>	<u>S</u>	<u>L</u>	<u>V</u>	<u>D</u>	<u>D</u>	<u>+</u>	<u>K</u>	<u>L</u>	<u>+</u>	(157)
SMRV-H (29)		T	H	+	+	+	<u>+</u>	<u>I</u>	<u>S</u>	+	+	S	L	A	+	N	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	+	N	Q	+	(147)	
Rev-A (63)		T	H	+	V	+	<u>+</u>	<u>A</u>	<u>+</u>	+	+	Q	L	A	E	N	-	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	M	T	L	+	(151)	

^a Where references are not given, they may be found in Table 2. Abbreviations: GALV, gibbon ape leukemia virus; SRV-1, simian retrovirus type 1; MPMV, Mason-Pfizer monkey virus; BaEV, baboon endogenous virus; Rev-A, reticuloendotheliosis virus strain A.

^b Sequences are grouped according to approximate similarity to Friend MuLV in the SU domain. The *env* of gibbon ape leukemia virus is the most divergent sequence that can be generally aligned with that of Friend MuLV in the SU domain. Numbers in parentheses indicate the distance in amino acids between the signal asparagine of the gs4 homolog and the C terminus of SU. Underlining indicates the location of the gs4 homolog and characteristic CWLC sequences, plus signs indicate the same amino acid as found in Friend MuLV, and minus signs indicate the introduction of a gap to align the sequences.

restore the glycosylation signal, suggesting that the presence of N-linked glycans at these sites is not stringently required for protein function. Examination of other mutations in and near gs4 of gp70 and isolation and characterization of revertants of gs4⁻ mutants are under way to determine the relative contributions of the absence of the glycan and the presence of specific amino acids to the phenotypes of gs4 mutations.

This work has shown that all but one of the N-linked glycans of Friend MuLV gp70 are not essential for viral growth, although mutation of at least one of these nonessential sites (gs5) does have a significant effect on protein maturation. Examination of mutants carrying multiple glycosylation mutations is under way to determine the structure(s) of the minimally glycosylated *env* product(s) that have sufficient protein function for normal viral growth and to investigate ways in which combinations of N-linked glycans contribute to protein function.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA-41229.

We thank Furong Shang for providing monoclonal antibodies, R. Friedrich for making the complete nucleotide sequence of Friend MuLV available, and Karl Drlica and Ellen Murphy for helpful comments on the manuscript.

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