JOURNAL OF VIROLOGY, Sept. 1997, p. 7012–7019 Vol. 71, No. 9
0022-538X/97/$04.00+0
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The Critical N-Linked Glycan of Murine Leukemia Virus Envelope Protein Promotes both Folding of the C-Terminal Domains of the Precursor Polyprotein and Stability of the Postcleavage Envelope Complex

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Received 30 January 1997/Accepted 29 May 1997

The infectivity of Friend ecotropic murine leukemia virus was previously shown to be highly sensitive to modification in its envelope protein (Env) at only one of the eight signals for N-linked glycan attachment, the fourth from the N terminus (gs4). In the present study, a set of six single-amino-acid substitutions in or near gs4 was used to determine the function of this region of Env and the role played by the glycan itself. One mutant that lacked the gs4 glycan was fully infectious, while one that retained this glycan was completely noninfectious, indicating that the gs4 glycan per se is not required for Env function. Infectivity correlated with the level of mature Env complex incorporated into virus particles, which was determined by the severity of defects in transport of the envelope precursor protein (gPrEnv) from the endoplasmic reticulum into the Golgi apparatus, in cleavage of gPrEnv into the two envelope subunits (the surface protein [SU] and the transmembrane protein [TM]), and in the association of SU with cellular membranes. All of the mutants induced the wild-type level of superinfection interference, indicating that the gs4 region mutations did not interfere with proper folding of the N-terminal domain of SU. These results suggest that the gs4 region mediates folding of the C-terminal domains of gPrEnv and stability of the interaction between SU and TM. Although the gs4 glycan was not essential for infectivity, processing of all mutant Envs lacking this glycan was significantly impaired, suggesting that efficient folding of gPrEnv requires a glycan at this position. The conservation of a glycosylation site homologous to gs4 across a broad range of retroviruses suggests that this sequence may play a similar role in many retroviral Envs.

Retroviral envelope proteins (Envs) perform multiple functions that are critical for both virus replication and pathogenicity (2, 22). They are synthesized as glycosylated polyproteins (gPrEnvs) that are proteolytically processed by host enzymes into surface protein (SU) and transmembrane protein (TM) subunits during passage through the Golgi apparatus. The primary determinants of receptor binding activity reside in SU while those for membrane fusion activity, necessary for penetration of the host cell, are believed to reside in TM. SU is a peripheral membrane protein that is attached to the virion via interactions with TM, which include intersubunit disulfide bonds in some retroviral Envs (11–13, 17, 23, 24, 25, 31).

The gPrEnvs of murine leukemia viruses (MuLV) contain six to eight N-linked glycans, all of which are attached to the SU domain. Studies using specific inhibitors have shown that N-linked glycan addition is required for correct folding and processing of MuLV gPrEnv (21, 28, 29) and that processing of high-mannose N-linked glycans is required for efficient incorporation of Env into virions (26). Thus, N-linked glycosylation of MuLV Env is important for production of infectious virus. Using site-directed mutagenesis to examine the impact of the removal of individual glycans, it was shown for both Friend and Moloney ecotropic *env* that only the loss of the glycan located at the beginning of the C-terminal domain of SU compromised infectivity (3, 9). For Friend ecotropic MuLV (Fr-MuLV), mutation of asparagine to aspartate at this fourth glycan attachment site (gs4) interfered with gPrEnv transport from the endoplasmic reticulum (ER) into the Golgi apparatus and severely inhibited its proteolytic processing into SU and TM. This critical glycosylation signal is widely conserved among the *env* genes of a broad range of retroviruses, coordinately with a nearby CWLC sequence in SU and a CX_6CC sequence in TM (9), which have recently been shown to be involved in the labile disulfide linkage between SU and TM in Fr-MuLV (25).

In order to better understand the role of this glycan and its surrounding region of SU, a panel of mutations in or near gs4 of Fr-MuLV was generated and characterized in detail. These studies indicated that a glycan at gs4 is not absolutely essential for the production of functional Fr-MuLV Env but that the gs4 glycan and the gs4 region in general modulate the efficiency of transport and processing of gPrEnv and affect interactions that are important for the stable association of SU with TM in the postcleavage Env complex.

MATERIALS AND METHODS

Viruses and cell lines. The MuLV *env* was from clone 57 Fr-MuLV (18). Ecotropic AKR623-MuLV (14) and dualtropic Friend mink cell focus-forming virus (Fr-MCF) (30) were used in the superinfection interference assay. Mutations were generated in the gs4 region of Fr-MuLV by Kunkel enrichment site-directed mutagenesis as previously described (9). Table 1 shows the nucleotide and amino acid sequences of gs4 region of each mutant used in this study; the mutant with an N-to-D substitution at position 302 (N302D mutant) has been described previously (9). The gs4 region mutant *env* genes were expressed from a chimeric Fr-MuLV colinear genome plasmid (pLRB303 for wild-type virus)

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TABLE 1. Sequences of gs4 region mutants of Fr-MuLV Env

Virus	Sequences of gs4 region[a]
Wild type	Tyr Gln Ala Leu Asn Leu Thr Asn Pro tac cag gca ctc aac ctt acc aac cct
L301R	Tyr Gln Ala **Arg** Asn Leu Thr Asn Pro tac cag *gcG cGc* aac ctt acc aac cct *Bss*HII
N302D	Tyr Gln Ala Leu **Asp** Leu Thr Asn Pro tac cag gca ct*A GaT ct*t acc aac cct *Bgl*II
N302Q	Tyr Gln Ala Leu **Gln** Leu Thr Asn Pro tac cag gca ctc *CaG ctG* acc aac cct *Pvu*II
L303S	Tyr Gln Ala Leu Asn **Ser** Thr Asn Pro tac cag gca ctc aac *AGt acT* aac cct *Sca*I
T304M	Tyr Gln Ala Leu Asn Leu **Met** Asn Pro tac cag gca ctc aac c*tC aTG a*ac cct *Bsp*HI
T304V	Tyr Gln Ala Leu Asn Leu **Val** Asn Pro tac cag gca ctc aac ctt *GTc Gac* cct *Sal*I

[a] Amino acids that differ from the wild-type sequence are in boldface type; bases that differ from the wild-type sequence are capitalized. The location of gs4 and the conserved leucine at position 301 is underlined. Restriction sites introduced during mutagenesis are italicized and labeled.

containing promoter and enhancer sequences from the FB29 clone in order to increase expression levels (10).

Mouse NIH 3T3 fibroblasts, the ecotropic packaging lines Ψ_2 (15) and PE501, and the amphotropic packaging line PA317 (16) were maintained as previously described (9). Mutant viruses were expressed by transfecting the plasmids into an ecotropic packaging cell line and the amphotropic packaging cell line by using Lipofectamine (GibcoBRL). The transfected ecotropic and amphotropic cell lines were then cocultured for 3 to 5 days to generate high-titer stocks of pseudotyped virus (1, 9). 3T3 cells were infected with these viral stocks for 2 h in the presence of 8 μg of Polybrene per ml. Twenty hours later, the infected cultures were cloned by limiting dilution. Positive clones were identified first by immunofluorescence with Fr-MuLV-specific monoclonal antibody (MAb) 10BA10, and then the absence of Envs derived from the packaging cell lines, which have electrophoretic mobilities distinct from that of Fr-MuLV Env, was confirmed by radioimmunoprecipitation (RIP) using goat anti-Rauscher gp70 serum. This procedure yielded cell lines expressing viral genomes that had not been subjected to selection for *env* function.

Immunoassays. Goat anti-Rauscher gp70 serum (lot 79S) and goat anti-Rauscher p30 serum (lot 77S) were obtained from Quality Biotech, Camden, N.J.). Rat MAbs 10BA10 (specific for Fr-MuLV $p12^{gag}$) (9), 35/56 (specific for AKR SU) (27), and 7C10 (specific for SU of MCF MuLV) (32) were previously described. Rat MAb 10CE11, specific for TM, was isolated in the Laboratory of Retroviral Biology, Public Health Research Institute, New York, N.Y.

Viral infection was detected by immunofluorescence assay (IFA) as previously described (9). Viral proteins were characterized by RIP assays (RIPAs) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) assays as previously described (9), except that cell lysate samples were often subjected to two rounds of immunoprecipitation in order to eliminate background bands. In this procedure, the precipitates of the first round of RIP were solubilized by resuspending in 10 μl of sample buffer containing 0.8% SDS for 5 min at 95°C, diluted in 10 volumes of RIP buffer, and then reprecipitated with the original antibody. Cells were generally radiolabeled for 1 h to assay cell-associated *env* products and for approximately 20 h to assay secreted *env* products as previously described (9). In pulse-chase labeling studies, cells were labeled for 30 min followed by chase with excess cold amino acids as previously described (9), and both cell-associated and secreted *env* products were analyzed. For analysis of association of Envs with virions, virus particles were purified from overnight-labeled supernatants by gel chromatography on Sepharose-CL4B columns (Pharmacia). Radioisotopes were obtained from New England Nuclear. Peptide *N*-glycosidase F (N-glycanase) digestion was performed as previously described (33).

For cell surface expression and secretion studies, 35-mm-diameter plates containing cells at approximately 75% confluence were labeled with $[^3H]$glucosamine for 3 h. Culture media were collected for analysis of secreted Env. Env present on the cell surface was specifically detected by washing intact cells once with phosphate-buffered saline and incubating at 4°C for 2 h with 0.6 ml of regular medium containing a 1:50 dilution of goat anti-Rauscher gp70 antiserum. After unbound antibody was removed by washing the cells three times with phosphate-buffered saline, the cells were lysed with RIP buffer, cell lysates were centrifuged to remove debris, and antibody-bound antigens were precipitated with Pansorbin (Calbiochem). Intracellular Env was then immunoprecipitated from the resulting supernatants by adding fresh goat anti-Rauscher gp70 antiserum (at a 1:150 dilution) to the remaining supernatant and precipitating with Pansorbin.

For Western blot analysis, proteins from purified virions were separated by SDS-PAGE, electroblotted onto nitrocellulose membrane, and incubated with appropriate primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibody as previously described (33). Signal was developed with a chemiluminescence reagent for HRP (catalog no. NEL-101; DuPont).

Superinfection interference assay. Uninfected 3T3 cells and cells expressing Fr-MuLV were infected with ecotropic AKR623-MuLV or dualtropic Fr-MCF in the presence of 8 μg of Polybrene per ml for 2 h. Cells were then refed with fresh complete medium, and the extent of infection was determined 24 h later by IFA using MAb 35/56, which detects an epitope present on AKR623 gp70 but not on Fr-MuLV gp70, or MAb 7C10, which detects an epitope present on Fr-MCF gp70 but not on Fr-MuLV gp70.

RESULTS

Infectivity of viruses carrying gs4 region mutations. In order to better understand the role of the gs4 region in Env function, a panel of mutants with different amino acid changes in the gs4 region was constructed and characterized (Table 1). In addition to the previously described N302D substitution, glycosylation at gs4 was blocked by N302Q, T304M, and T304V substitutions. Two mutations that were not expected to affect glycosylation, L301R and L303S, were also made. 3T3 cell lines expressing the mutant *env*s in the absence of other MuLV *env*s were isolated.

Infectivity of the mutant viruses was determined by mixing cells producing virus with uninfected cells at a ratio of 1:1,000 and monitoring viral spread by IFA (Fig. 1A). Two of the mutants (N302D, as reported previously [9], and L301R) were noninfectious, two (N302Q and T304M) spread more slowly than the wild type, and two (L303S and T304V) spread as efficiently as the wild type. To eliminate the possibility that the slow growth seen for the N302Q and T304M mutants was due to reversion, the growth of viruses recovered at the end of the experiment shown in Fig. 1A was compared with that of the original mutants. The kinetics of viral spread was indistinguishable for each pair of passaged and original viruses (Fig. 1B), and the sizes of intracellular and secreted Envs appeared unchanged by SDS-PAGE (data not shown). Thus, the range in growth phenotype seen for the gs4 region mutant viruses was due to intrinsic properties of the mutants.

Expression of gs4 region mutant Envs. To determine the basis for these different growth phenotypes, the biochemical properties of the Envs produced by the gs4 region mutants were examined. Lysates of $[^{35}S]$cysteine-labeled cells producing mutant or wild-type Env products were immunoprecipitated with goat anti-gp70 serum and analyzed by SDS-PAGE (Fig. 2A). Wild-type Env (lane 1) was processed very efficiently: the majority of Env in lysates of cells labeled for 1 h was in the form of mature SU (gp70), with a large amount of the primary translation product, gPr80, and a trace of the intermediate Env precursor, gPr90, also present. gPr90 is formed in the Golgi apparatus by conversion of the N-linked glycans of gPr80 to complex forms and addition of O-linked glycans and is then processed by proteolytic cleavage into SU and TM (22). All of the mutant Envs were processed less efficiently than the wild type, although the stringency of the defect varied with the mutation. In contrast to the wild type, gPr80 was the major *env* product seen for each mutant after

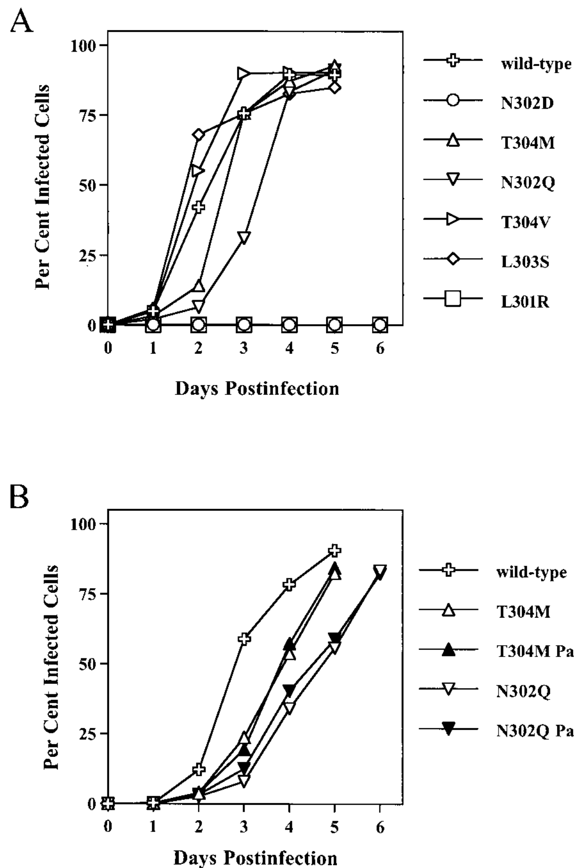

FIG. 1. Infectivity of gs4 region mutant viruses. Cells producing wild-type or mutant viruses were mixed with uninfected NIH 3T3 cells at a ratio of 1:1,000. Viral spread was monitored by immunofluorescence by using anti-p12 MAb 10BA10. (A) Cells expressing mutant viruses prepared without selection for functional Env were used to initiate the infections. (B) Viral spread from the cells resulting from selected growth curves shown in panel A (passaged [Pa] viruses) was compared to spread from the cells used to initiate those cultures (original mutants).

labeling for 1 h, indicating a delay in transport of the mutant precursors from the ER to the Golgi apparatus. Each of the mutant cell lysates contained a small amount of the intermediate precursor, gPr90, but only the highly infectious L303S mutant (lane 6) had a significant amount of cell-associated SU. The cell lysate of the other highly infectious mutant, T304V (lane 5), contained only a trace of SU, whereas SU was not detectable under these conditions for the mutants with attenuated infectivity, T304M (lane 3) and N302Q (lane 4), and for the noninfectious mutants, N302D (lane 2) and L301R (lane 7). The concomitant presence of normal or elevated amounts of gPr90 and decreased amounts of SU indicated that the gs4 region mutations also reduced the rate of proteolytic processing of the intermediate Env precursor, gPr90, to SU and TM.

Comparison of protein mobilities on SDS-PAGE indicated that all of the gPrEnvs that had lost the gs4 glycosylation signal were similar to each other in size and smaller than wild-type gPr80 by an amount consistent with the loss of one N-linked glycan. The L303S (lane 6) and L301R (lane 7) mutant gPrEnvs comigrated with wild-type (lane 1) gPr80, indicating that they carried the gs4 glycan. The small mobility differences caused by the absence of the gs4 glycan were highly reproducible. Removal of N-linked oligosaccharides by digestion with N-glycanase resulted in the comigration of all mutant gPrEnvs with wild-type gPrEnv (lanes 8 to 14), confirming that the migration differences among the gPr80s were due to differences in the number of N-linked glycans present.

Despite the highly defective processing of many of the gs4 region mutant Envs, each mutant secreted at least a small amount of an SU-like product, i.e., a secreted Env product lacking detectable p15E sequence and similar in size to wild-type SU. These products were detected in the culture media of infected cells metabolically labeled for 24 h by immunoprecipitation using anti-gp70 serum (Fig. 2B). In order to simultaneously visualize all of the mutant proteins, the volumes of culture supernatant medium precipitated for each virus were adjusted in the experiment shown to compensate for the large differences in the amount of Env secreted. The L303S mutant SU (lane 6) comigrated with secreted wild-type gp70 (lane 1), consistent with a fully glycosylated and otherwise normal SU. All of the other mutant SUs were smaller. The sizes of the T304M (lane 3), N302Q (lane 4), and T304V (lane 5) mutant SUs were consistent with the absence of one glycan. The noninfectious N302D mutant (lane 2) secreted a protein that was slightly smaller than the other SUs missing one glycan, along with a larger protein that was the size of gPr90 lacking the gs4 glycan and low levels of smaller proteins that appeared to be proteolytic fragments. The secreted products of the noninfectious L301R mutant (lane 7) included a band similar in size to wild-type gPr90, an SU intermediate in size between the N302D SU and wild-type SU, and proteolytic fragments. Comparison of the secreted Env proteins after N-glycanase treatment showed that most of the deglycosylated mutant SUs comigrated with deglycosylated wild-type SU, indicating that they differed from the wild type only by the absence of the gs4 glycan. However, the N302D (lane 9) and L301R (lane 14) SU-like proteins remained smaller than wild-type SU after deglycosylation, suggesting that the SU-like proteins of the two noninfectious mutants were produced by aberrant proteolytic processing.

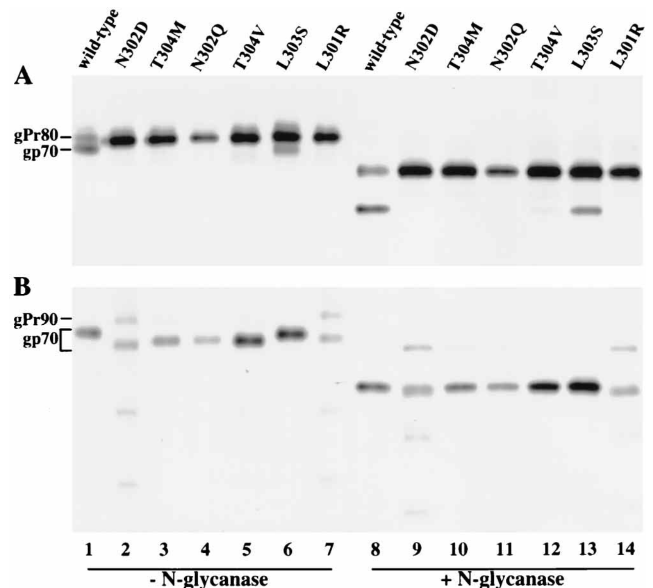

FIG. 2. Expression of gs4 region mutant Envs. (A) Cell lysates labeled with [^{35}S]cysteine for 1 h were precipitated with goat anti-gp70 serum and analyzed by SDS-PAGE with (lanes 8 through 14) or without (lanes 1 through 7) N-glycanase digestion to remove all N-linked glycans. (B) Cell culture media labeled overnight with [^{35}S]cysteine were analyzed as described for panel A except that the amount of sample was adjusted to compensate for secretion levels.

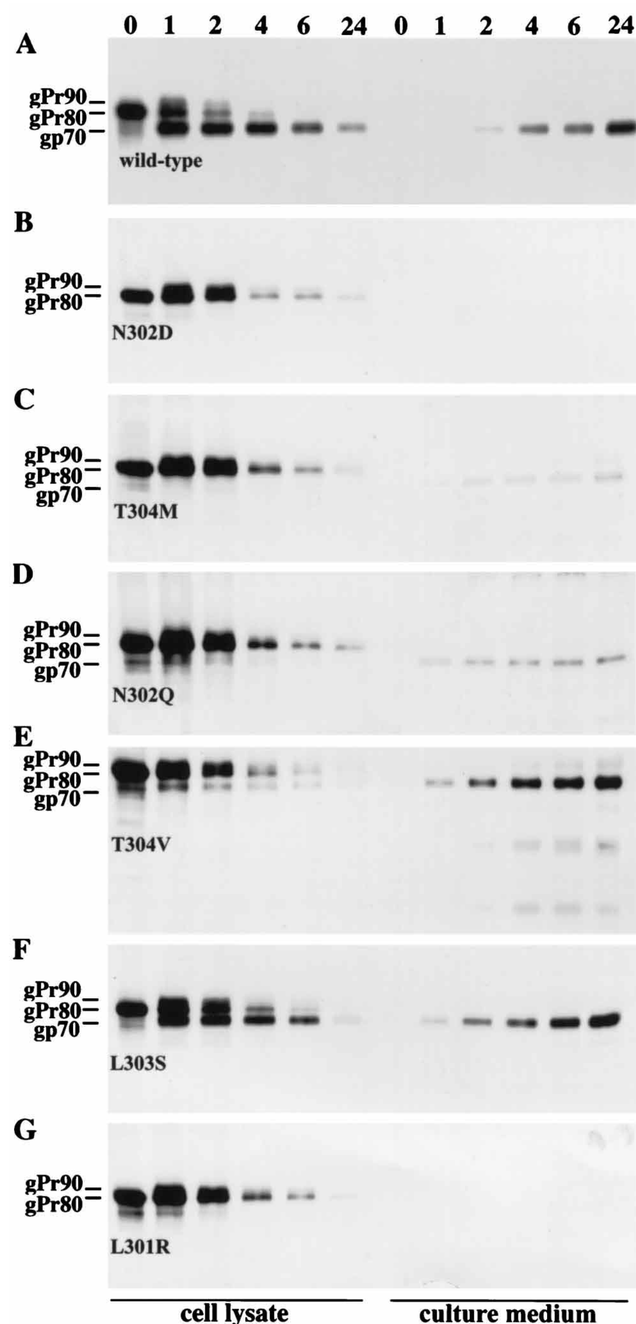

FIG. 3. Pulse-chase analysis of processing and secretion of gs4 region mutant Envs. Cells were labeled with [^{35}S]cysteine for 30 min, and then chased for the periods as indicated (in hours) above each lane. Cell lysates and culture media were precipitated with goat anti-gp70 serum and analyzed by SDS-PAGE. (A) wild type; (B) N302D; (C) T304M; (D) N302Q; (E) T304V; (F) L303S; (G) L301R.

More-precise information about the defects caused by the gs4 region mutations was obtained by examining the kinetics of the processing and secretion of the mutant Envs. Infected cells were pulse-labeled for 30 min with [^{35}S]cysteine, followed by further incubation with excess cold cysteine for various times (Fig. 3). For all viruses, the major component seen after the 30-min labeling period corresponded to gPr80. For the wild type, most of gPr80 had been converted to gp70 by 1 h of the chase, and a small amount of gPr90 was also detected at this time point. Extracellular gp70 first appeared after 2 h and increased in concentration over the 24-h chase period, while cell-associated gp70 decreased gradually during this time. For all of the mutants, loss of gPr80 was slower and gPr90 accumulated to an abnormally high level. The level of SU present in the extracellular medium varied with the ability of the mutants to grow in cell culture: the fully infectious L303S and T304V mutants secreted an amount of SU similar to that of the wild type; the partially growth-defective N302Q and T304M mutants secreted substantially less SU than did the wild type; the noninfectious L301R and N302D mutants did not produce sufficient SU to be detected under these experimental conditions. For those mutants for which gp70 was seen in the culture medium, the secreted gp70 represented a larger fraction of the total gp70 than for wild-type Env, and it was secreted more rapidly. This pulse-chase analysis confirmed that all of the gs4 region mutant Envs were processed less efficiently than wild-type Env and that the degree of defect varied widely depending on the mutation, ranging from mild for the fully infectious L303S mutant to severe for the noninfectious L301R and N302D mutants.

Surface expression and particle association of mutant Envs. Cells expressing each of the gs4 region mutants contained higher levels of gPr90 than was found in wild-type infected cells (Fig. 3). To determine the location of the mutant gPr90s, the intracellular, cell surface, and extracellular distributions of *env* products were examined. Cells were labeled for 3 h to allow a significant amount of labeled Env to be processed into various compartments, and [^{3}H]glucosamine was used to enhance detection of processed Env relative to gPr80 (Fig. 4). Cell surface Env was detected by incubating intact cells with anti-gp70 serum before washing, lysis, and precipitation (lanes 2). Intracellular Env was detected by adding additional anti-gp70 serum to the cell lysates obtained after the above-described treatment for precipitation of residual Env (lanes 1). Secreted Env was detected by immunoprecipitating culture media (lanes 3).

gp70 was the major intracellular component detected under these conditions for wild-type Env, and a small amount of gPr80 and a trace of gPr90 were also seen. All of the gPr80 and gPr90 and most of the gp70 were recovered in the intracellular fraction; only a small fraction of the gp70 was detected on the cell surface, and even less was detected in the supernatant medium. The fully infectious L303S mutant had a similar overall pattern, except that the level of gPr90 was elevated and the secreted SU/cell surface SU ratio was somewhat higher. For all of the other mutants, including the fully infectious T304V mutant, gPr80 was the major intracellular form of Env and an appreciable level of gPr90 was also present. For the infectious N302Q, T304V, and T304M mutants, only gPr90 was detected on the cell surface under this labeling condition, while gp70 was found only intracellularly and in the culture medium. The

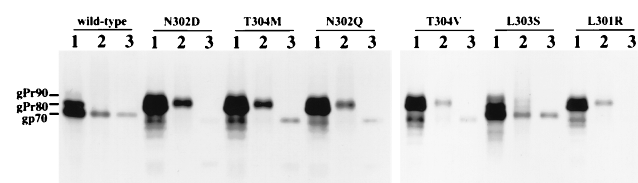

FIG. 4. Surface expression and secretion of gs4 region mutant Envs. Cells were labeled with [^{3}H]glucosamine for 3 h and samples were precipitated with goat anti-gp70 serum as described in Materials and Methods. Precipitates were analyzed by SDS-PAGE. Lanes 1, intracellular Env; lanes 2, cell surface Env; lanes 3, secreted Env.

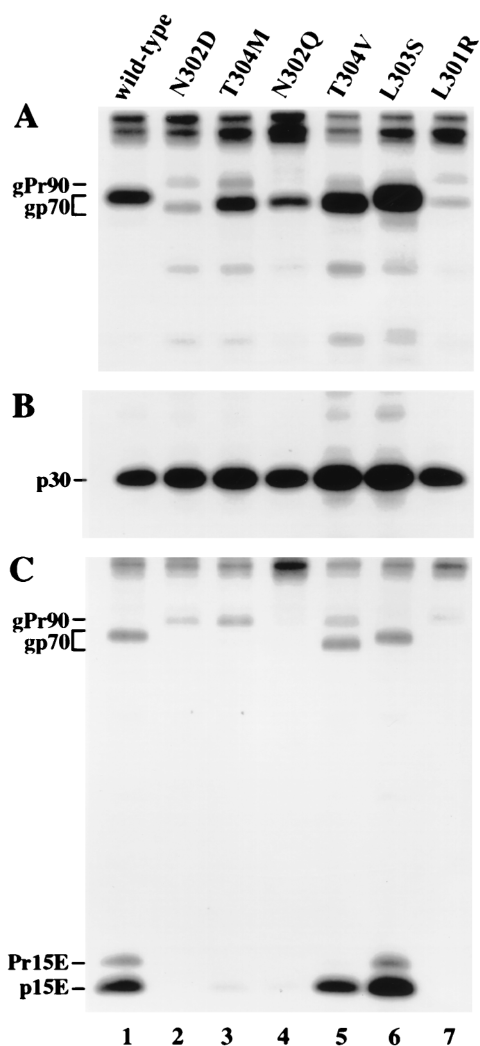

FIG. 5. Secretion levels of gs4 region mutant Envs. Culture media from cells labeled overnight with [^{35}S]cysteine and [^{35}S]methionine were precipitated with goat anti-gp70 (A), goat anti-p30 (B), or rat anti-p15E MAb 10CE11 (C) and analyzed by SDS-PAGE. For each mutant, the same volume of medium was used in each panel.

two noninfectious mutants (L301R and N302D) also had only gPr90 on the cell surface, but differed from the other mutants in that little if any secreted Env was detected. These results indicated that cleavage of gPr90 into SU and TM is not required for transport of these mutant Env proteins to the cell surface, consistent with previous reports of transport of other cleavage-defective mutant gPrEnvs to the cell surface (4, 20).

The nature of mutant Envs secreted from cells was further investigated by immunoprecipitation of total culture supernatant media (Fig. 5) and purified virions (Fig. 6) with antibodies to either SU (Fig. 5A and 6A) or TM (Fig. 5C and 6C). For this experiment the cells were labeled with a mixture of [^{35}S]cysteine and [^{35}S]methionine to allow efficient detection of both SU and TM, and sample volumes were normalized for each mutant according to the amount of secreted capsid protein (p30) detected by immunoprecipitation with anti-p30 serum (Fig. 5B and 6B). Anti-gp70 serum precipitated only SU from the culture media of cells producing wild-type virus (Fig. 5A, lane 1), while an SU-like protein and, in most cases, a larger product and several smaller products were precipitated from the culture media of cells producing the gs4 region mutants. The level of TM-related proteins present in culture medium as determined by immunoprecipitation with an anti-p15E MAb correlated well with the infectivity of the viruses (Fig. 5C). The fully infectious mutants, T304V (lane 5) and L303S (lane 6), had wild-type levels (lane 1) of the mature form of TM, p15E. The L303S mutant also secreted a wild-type level of the immature form of TM, Pr15E, whereas Pr15E was only marginally detectable in the T304V sample. The mutants that grew with delayed kinetics, T304M (lane 3) and N302Q (lane 4), had very low but detectable levels of p15E, while TM proteins were not detected in the culture media from cells expressing either of the noninfectious mutants, N302D (lane 2) and L301R (lane 7). Although loosely correlated with infectivity and level of secreted TM, the amount of secreted SU appeared to be in large excess over TM, especially for the mutants with growth defects. The mutant products larger than SU that were precipitated by the anti-gp70 serum were also recognized by the anti-p15E MAb and therefore were secreted forms of gPr90. Secreted gPr90 was not seen for any of the gs4 mutants in Fig. 4 due to the shorter labeling period used in that experiment, suggesting that mutant gPr90 is incorporated only slowly into virus particles (see below). The smaller products were not precipitated by the anti-p15E MAb and therefore were presumably proteolytic fragments of SU.

The amount of secreted Env products associated with virions was determined by performing similar RIPs on purified virus particles (Fig. 6). Purified virions differed from unfractionated supernatant media in that the level of SU in virions more closely paralleled the level of TM (Fig. 6A and C). The fully infectious T304V (lane 5) and L303S (lane 6) mutant virions

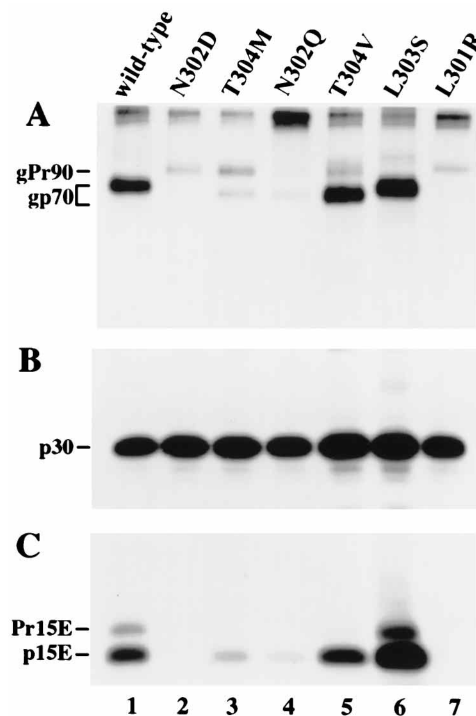

FIG. 6. Particle association of gs4 region mutant Envs. Virions labeled with [^{35}S]cysteine and [^{35}S]methionine were purified from culture media by gel chromatography, lysed with Nonidet P-40, and precipitated with goat anti-gp70 (A), goat anti-p30 (B), or rat anti-p15E MAb 10CE11 (C) and analyzed by SDS-PAGE. For each mutant, the same amount of viral sample was used in each panel.

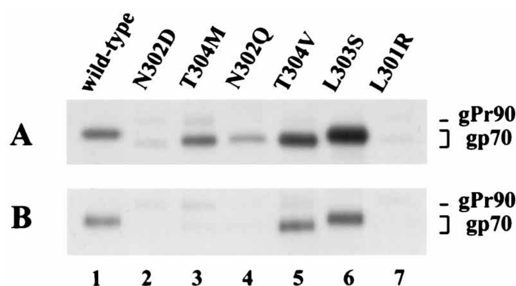

FIG. 7. Secretion level and particle association of gs4 region mutant SUs. Culture media from cells labeled overnight with [^{35}S]cysteine and [^{35}S]methionine (A) or virions purified from these culture media by gel chromatography and lysed with Nonidet P-40 (B) were precipitated with goat anti-gp70 and analyzed by SDS-PAGE. These are shorter exposures of the same SDS-polyacrylamide gels shown in Fig. 5A and 6A.

TABLE 2. Results of superinfection interference assay

Virus expressed in target cell	Level of infection with MuLV	
	Fr-MCF[a]	AKR623[b]
None	0.124 ± 0.020	0.209 ± 0.013
L301R	0.145 ± 0.010	<0.0002[c]
Wild type	0.146 ± 0.010	<0.0002[c]
N302D	0.148 ± 0.015	<0.0002[c]
N302Q	0.162 ± 0.022	<0.0002[c]
L303S	0.155 ± 0.006	<0.0002[c]
T304M	0.144 ± 0.009	<0.0002[c]
T304V	0.170 ± 0.009	<0.0002[c]

[a] Mean ± standard deviation of the fraction of fluorescent cells stained with MAb 7C10.
[b] Mean ± standard deviation of the fraction of fluorescent cells stained with MAb 35/56.
[c] No fluorescent cells were found in slide wells containing at least 5,000 cells.

contained levels of SU and TM similar to those in wild-type virions (lane 1), although Pr15E was not detected in the T304V virions as noted above for the unfractionated culture supernatant medium. The partially infectious T304M (lane 3) and N302Q (lane 4) mutant virions had very low levels of both SU and TM in virions, while the noninfectious N302D (lane 2) and L301R (lane 7) mutant virions did not contain detectable SU or TM. By comparing Fig. 5 and 6, it can be seen that supernatant media of the partially infectious and noninfectious mutants contain a large excess of soluble SU or SU-like protein that is not associated with virions. Because the long exposures of the RIPAs necessary to visualize secreted Envs from some of these mutants resulted in overexposure of the samples from the wild type and the fully infectious mutants (Fig. 5 and 6), shorter exposures of these anti-gp70 precipitates are shown in Fig. 7. Under these conditions, it can be seen that the ratio of secreted SU to particle-associated SU is larger for both T304V (lane 5) and L303S (lane 6) than for the wild type (lane 1), although to a lesser degree than for the growth-defective mutants. The mutant gPr90s found in the culture media were largely if not exclusively associated with the virus particles. In contrast to gPr90, the smaller SU-related proteins seen in the culture media of most of the mutant-expressing cell lines were absent from the purified virions, supporting the interpretation that these were soluble proteolytic fragments of gp70.

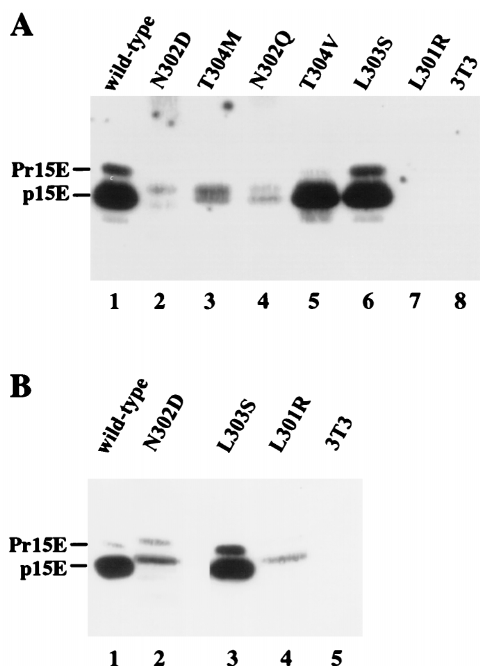

FIG. 8. Western blot analysis of TM in virions. Viral particles were partially purified from culture media of infected cells by centrifugation. Viral proteins were separated by SDS-PAGE, transferred to nitrocellulose paper, incubated with rat anti-p15E MAb 10CE11 followed by HRP-conjugated anti-rat immunoglobulin G serum, and visualized with chemiluminescence reagent. (A) Similar amounts of viral proteins were used for each virus. Lane 8 contains an uninfected NIH 3T3 control. (B) Twentyfold excess protein was used for N302D (lane 2) and L301R (lane 4) relative to wild-type (lane 1) and L303S (lane 3). Lane 5 contains an uninfected NIH 3T3 control.

The more sensitive Western blot technique showed that virions of most of the gs4 region mutants contained altered TM proteins. Consistent with the RIPA data, the sizes and amounts of Pr15E and p15E in virions were similar to those found in wild-type particles (lane 1) for only the fully infectious mutant with the most efficiently processed Env, L303S (Fig. 8A, lane 6), while virions of the equally infectious T304V mutant, whose Env was processed inefficiently, had the wild-type level of p15E but only a trace of Pr15E (lane 5). Virions of the mutants with reduced infectivity, T304M (lane 3) and N302Q (lane 4), had low levels of a doublet, consisting of one band that comigrated with wild-type p15E and one that was slightly larger but was still smaller than wild-type Pr15E. This upper band comigrated with the top of the broad band of wild-type p15E, and thus wild-type virions might contain a similar protein that is obscured by the large amount of p15E present. TM proteins were at best marginally detectable for the two noninfectious mutants, N302D (lane 2) and L301R (lane 7), when matched amounts of virus particles were used. However, when 20-fold more viral protein from these mutants was analyzed (Fig. 8B), two forms of TM, which were approximately 1 kDa larger than wild-type Pr15E and p15E (lane 1), were detected (lanes 2 and 4). The size differences and ratios of the mutant proteins, together with the observation that the SUs of these two mutants were smaller than wild-type SU by about 1 kDa, suggested that the aberrant *env* products resulted from cleavage of the mutant gPr90s at a site N terminal to the site normally used to generate SU and TM. Since this aberrant cleavage would add hydrophilic residues to the normally hydrophobic N terminus of TM, it may contribute directly to the extreme instability of the Env complex and the severe growth defect of the N302D and L301R mutants.

Interaction of mutant Envs with the MuLV receptor. Although the N302D and L301R mutants were noninfectious, small amounts of gPr90 and secreted SU-like proteins were

produced, suggesting that these Env precursors fold into sufficiently native conformations to allow some transport to occur. The conformational integrity of the N-terminal domain of the mutant Envs was therefore examined by determining whether these proteins interacted with the cell surface receptor for viral binding, by performing a superinfection interference assay (Table 2). In this assay, the ability of the immunologically distinguishable AKR623 ecotropic MuLV to superinfect cells expressing Fr-MuLV was detected by immunofluorescence with MAb 35/56, which recognizes AKR ecotropic gp70 but not Friend ecotropic gp70. As a control, superinfection with dualtropic Fr-MCF, which uses a different receptor, was assayed with an MCF-specific MAb, 7C10. Cell lines expressing either wild-type or mutant virus and uninfected NIH 3T3 cells were superinfected with Fr-MCF to a similar extent. The wild type and all of the gs4 region mutants conferred more than 3 logs of resistance to superinfection with AKR virus, indicating that all of these mutant Envs interacted efficiently with the ecotropic receptor.

DISCUSSION

Previous mutagenesis studies have shown that one particular glycosylation signal of ecotropic MuLV Env, gs4 in Fr-MuLV, is critical for protein function, while the other glycosylation signals are not (3, 9). Consistent with this finding, a gs4-like site is the most highly conserved glycan attachment signal among a large group of retroviral *env* genes, including many with little other similarity to MuLV in the SU domain (9). One of the earlier reports identified blockages in transport of gPr80 and in proteolytic cleavage of gPr90 and an increase in exposure of an epitope in the TM domain of gPr80 for the N302D gs4 mutation (9), but the phenotypes of this mutation were not fully explored. In addition, earlier reports did not address whether the gs4 glycan itself is required for Env function and/or whether the region immediately surrounding this glycan is particularly sensitive to modification. In the present study, a set of six mutants encoding different amino acid substitutions in the gs4 region of Fr-MuLV Env was used to clarify these issues.

The gs4 glycan and adjacent residues were found to comprise a region of *env* that strongly influences the efficiency with which gPrEnv is processed into functional Env complexes. All of the mutants, including those that retained the gs4 glycan, exhibited decreases in the rate and extent of processing of the gPr80 and gPr90 precursors and in the stability of the association between SU and TM after cleavage. The severity of these defects varied among the mutants, resulting in characteristic levels of mature Env complex in virions that correlated well with the mutants' growth rates. This correlation suggests that the gs4 region mutations affect only the production of mature Env complexes, while the activity of mutant Env complexes in virions is normal. One of the four mutations that eliminated the addition of the gs4 glycan (T304V) grew as well as the wild-type virus, showing that this glycan per se is not essential for infectivity. However, this mutant did exhibit severe processing defects, indicating that the gs4 glycan may be required for efficient maturation of gPrEnv into active Env complex. The observation that a normal amount of mature Env complex was incorporated into virions by the T304V mutant, despite the severely decreased rate and extent of its gPrEnv processing, indicates that mature Env complex is normally produced in large excess over that needed for assembly into virions.

The primary evidence that the gs4 region mutations affected the efficiency of gPrEnv folding was their inhibition of transport of gPr80 from the ER to the Golgi apparatus; such retention in the ER of proteins that are normally transported is generally due to failure to achieve a compact globular conformation (8, 19). Also consistent with defective folding, elevated levels of fragments of SU were present in the supernatant medium for most of the gs4 region mutants. These soluble fragments presumably reflected proteolysis of incorrectly folded gPrEnv in the ER or perhaps later during transport. The increased accessibility of an epitope in TM on the N302D gPr80 reported previously (9) suggested that the folding or exposure of the TM domain in mutant Env molecules was also affected to some degree. However, the folding of the N-terminal globular domain of SU appeared to be normal, since superinfection interference assays showed that even the most defective mutant Envs bound efficiently to the cell surface receptor for virus attachment, a function of the N-terminal domain (6). This indicated that the folding defects induced by the gs4 region mutations were largely restricted to the C-terminal domain of SU and/or the TM domain within gPrEnv.

All of the gs4 region mutations resulted in elevated levels of gPr90 in infected cells, despite the decreased transport of gPr80 into the Golgi apparatus, where it is processed into gPr90. This demonstrated that a second effect of the gs4 region mutations was a reduction in the rate and extent of proteolytic cleavage of gPr90 into SU and TM. Since the gs4 region is not near the cleavage site in the linear sequence, this presumably reflected a conformational change that resulted in decreased accessibility of the normal SU-TM cleavage site in gPr90 to the processing protease, suggesting that even the mutant gPr80 molecules that were transported out of the ER were not uniformly native in conformation. Virions of most of the gs4 region mutants contained an appreciable amount of gPr90, unlike wild-type virions in which gPr90 was not detectable. This confirmed that at least a fraction of the mutant gPr90 was highly resistant to the processing protease and indicated that cleavage into SU and TM was not necessary for incorporation of these mutant Env precursors into virions. The complete lack of infectivity of the L301R and N302D mutant virions, which did not contain mature Env complexes but had levels of gPr90 equal to or greater than that of Env complex in the virions of the slow-growing mutants, indicated that these mutant gPr90s were unable to mediate a function(s) required for infection. Another indication of misfolded mutant gPr90s was the increased amount of altered forms of TM in many of the mutant virions, in particular those of the most defective mutant Envs (L301R and N302D). The larger TM-like proteins and smaller SU-like proteins seen for these mutants indicated that cleavage occurred at a site N-terminal to that used in wild-type gPr90.

A third phenotype of each of the gs4 region mutants was decreased stability of the SU-TM interaction, as indicated by increased shedding of SU from Env complexes. For the fully infectious mutants, L303S and T304V, this shedding was seen as an earlier appearance of secreted SU (Fig. 3), an increased secreted SU/cell surface SU ratio (Fig. 4), and a decreased ratio of SU in virions versus total supernatant compared to that in wild-type virus (Fig. 7). For the slowly replicating N302Q and T304M mutants, the decrease in the ratio of SU in virions (Fig. 6) to that in total culture supernatant medium (Fig. 5) was more extreme. For the noninfectious mutants that processed very little SU-like protein, L301R and N302D, cleaved Env products were not detected in virions or on the cell surface, indicating that for these mutants essentially all of the SU-like protein produced dissociated from TM. This increased shedding of SU from mutant Env complexes occurred primarily from cellular membranes, since the ratio of SU to TM in virus particles was close to normal for all of the replicating mutants. This difference in stability between mutant Env complexes on cellular and viral membranes might result from a

passive selection process in which cell-associated Env complexes that are not fully native shed their SU rapidly after cleavage of gPr90, so that only native complexes in which the interaction between SU and TM is normal are available for incorporation into virions. Alternatively, the maturation of Pr15E to its fusion-competent form, p15E, by a C-terminal proteolytic cleavage event that occurs during or after incorporation of Env complexes into virions (5, 7) may increase the stability of the interaction between SU and TM.

Given the destabilization of the interaction between SU and TM caused by gs4 region mutations, it is striking that the cysteine sequence motif (^{312}CWLC), six residues C terminal to the gs4 site and coordinately conserved with a gs4-like site in a broad range of retroviruses (9), is involved in a labile cystine bridge between ecotropic MuLV SU and the conserved CX_6CC motif in TM (25). The combination of the evolutionary relationship between gs4 and these cysteine motifs and the observations that both are involved in the stability of intersubunit interactions in Env complexes argues that these three coordinately conserved sequences constitute a single structural or functional unit within Env, with the gs4 region and glycan influencing the formation and/or stability of the intersubunit disulfide bond between the CWLC and CX_6CC sequences. This concordance further suggests that although these studies on the function of the gs4, CWLC, and CX_6CC sequences were carried out using a particular ecotropic MuLV Env (this report and [25]), the observations and conclusions are likely to apply to the wide range of retroviral Envs that contain these sequence motifs, including those of all classes of MuLV and feline leukemia virus as well as of a number of avian and primate retroviruses (9).

ACKNOWLEDGMENTS

This work was supported by PHS grant AI-23884 to A.P. and New York Community Trust grant 10330-1-014986 to S.C.K.; S.C.K. was a Scholar of the American Foundation for AIDS Research.

We thank Karl Drlica for a critical reading of the manuscript.

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