The role of gp55 N-glycosylation in pathogenesis of Friend spleen focus-forming virus

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The product of the envelope gene (gp55) of Friend spleen focus-forming virus is responsible for the acute form of erythroleukaemia caused by this virus. In order to investigate the role that the four known N-linked carbohydrate side-chains of gp55 play in pathogenesis, we have inactivated the four N-glycosylation signals by mutating the asparagine residues of these four sites into serine. When glycosylation sites 1 and/or 2 were altered, the viruses remained fully pathogenic. However, mutation at either of glycosylation sites 3 or 4 rendered the virus apathogenic, independent of mutations at other sites. Furthermore, when site 3 was changed, a new product appeared which seemed to have acquired a carbohydrate chain at a position normally not glycosylated, presumably at position Asn378.

Introduction

Friend spleen focus-forming virus (F-SFFV) is a highly pathogenic, replication-defective retrovirus inducing acute erythroleukaemia in mice which is either associated with a polycythaemia (F-SFFVp) or a slight anaemia (for reviews, see Ruscetti & Wolff, 1984; Ostertag et al., 1987; Kabat, 1989). In both instances, the presence and expression of the viral envelope (env) gene was shown to be essential and sufficient for induction of the disease (Linemeyer et al., 1981, 1982; Machida et al., 1985; Wolff & Ruscetti, 1985, 1988; Li et al., 1986). It has been further demonstrated that the large deletion and the frameshift mutation near the 3' end of the SFFV env gene are the essential determinants for the high degree of pathogenicity of these viruses (Friedrich et al., 1991; Watanabe et al., 1991).

The precise mechanism by which SFFV causes erythroleukaemia remains unknown. The available evidence, however, indicates that the SFFV env gene product interacts with the cellular erythropoietin receptor (Li et al., 1990; Yoshimura et al., 1990; Casadevall et al., 1991) leading in the case of SFFV into an erythropoietin-independent proliferation of the cells (Ruscetti et al., 1990; Hoatlin et al., 1990). This factor-independent growth is primarily determined by the transmembrane domain of the envelope protein (Chung et al., 1989). The resulting erythroblastosis may be an important step in induction of the disease. At a later stage, erythroblast immortalization seems to occur as a consequence of specific integration of the provirus within the cellular genome (Spiro et al., 1988), activating the putative oncogene Spi-1 (Moreau-Gachelin et al., 1988; Paul et al., 1991).

The F-SFFV env gene encodes a glycoprotein with five potential glycosylation sites (Amanuma et al., 1983; Clark & Mak, 1983; Wolff et al., 1983). The primary gene product formed has an apparent Mr of 55 000 (gp55) and carries solely high-mannose type glycans (Ruta et al., 1982; Srinivas & Compans, 1983; Strube et al., 1988). The majority of gp55 accumulates intracellularly. Only a small proportion of the molecules is further processed in the Golgi apparatus yielding a glycoprotein with an Mr of about 65 000 (gp65) which carries complex-type carbohydrate chains (Srinivas & Compans, 1983; Strube & Geyer, 1989) as well as O-linked glycans (Pinter & Honnen, 1989; Gliniak & Kabat, 1989) and can be readily detected on the cell surface (Ruscetti et al., 1981). It has been suggested that the surface expression of gp65 (Li et al., 1987) and, possibly, also shedding of the molecule from the plasma membrane by proteolytic cleavage of the membrane anchor from the C terminus (Pinter & Honnen, 1985, 1989; Gliniak & Kabat, 1989) may be a prerequisite for the leukaemogenicity of F-SFFV env. Hence, intracellular transport and processing of the env gene product seem to be important for viral pathogenicity.

Gp55 as well as gp65 have been shown to form disulphide-bonded homooligomers inside the cell (Gliniak & Kabat, 1989; Kilpatrick et al., 1989; Yang et al., 1990; Gliniak et al., 1991). Further evidence indicates that gp55 folds into different disulphide-linked components, whereas only one specific dimer is formed in the case of gp65, suggesting that only a distinct
homodimer of gp55 is competent for transport to the plasma membrane (Gliniak & Kabat, 1989; Gliniak et al., 1991). Thus, it has been concluded that the defective transport of this molecule results from improper folding and/or oligomerization of gp55 caused by the 585 bp deletion found in the SFFV env gene (Kilpatrick et al., 1989; Srinivas et al., 1992). Pinter & Honnen (1989), however, provided evidence that transfer of the SFFV Env protein to the plasma membrane is blocked in the presence of 1-deoxynojirimycin, an inhibitor of oligosaccharide trimming, indicating that proper glycosylation and processing of the high-mannose type glycans of gp55 are also prerequisites for its intracellular transport. Similar evidence was obtained in the case of Env proteins from replication-competent murine leukaemia viruses (Polonoff et al., 1982; Pinter et al., 1984), glycoproteins of lymphocytic choriomeningitis virus (Wright et al., 1990) and vesicular stomatitis virus (HSV) (Machamer et al., 1985; Kotwal et al., 1986). Studies on the HSV glycoprotein further revealed that its carbohydrates do not act as a transport signal per se but play an indirect role by influencing polypeptide folding and oligomerization (Machamer & Rose, 1988a,b). It should be noted that mutant proteins lacking oligosaccharides at the normal positions were found to form aberrant disulphide-bonded complexes accumulating in the endoplasmic reticulum.

In order to study the possible biological functions of the carbohydrate moieties of SFFV Env proteins for viral pathogenicity, we have created nine mutant viruses encoding glycoproteins that lack distinct potential N-glycosylation sites. Consensus sequences (Asn-X-Thr/Ser) were destroyed by replacing AsnX, AsnX, AsnX, and/or AsnX by Ser. Since the potential glycosylation site at AsnX is generally considered not to be glycosylated (Kabat, 1989), this part of the molecule was not mutated. Pathological properties of mutant and wild-type viruses were compared.

**Methods**

Construction of glycosylation mutants of the SFFV env gene. To alter the consensus sites for N-glycosylation of the SFFV env gene product we used either site-directed mutagenesis (AsnX and AsnX) or (AsnX, and AsnX) (positions are counted from the initiator methionine; subtract 32 for positions in the mature gp55). The plasmid pSAX encoding a molecular clone of the F-SFFV has been described previously (Friedrich et al., 1991). Thus, it has been concluded that the defective transport of this molecule results from improper folding and/or oligomerization of gp55 caused by the 585 bp deletion found in the SFFV env gene (Kilpatrick et al., 1989; Srinivas et al., 1992). Pinter & Honnen (1989), however, provided evidence that transfer of the SFFV Env protein to the plasma membrane is blocked in the presence of 1-deoxynojirimycin, an inhibitor of oligosaccharide trimming, indicating that proper glycosylation and processing of the high-mannose type glycans of gp55 are also prerequisites for its intracellular transport. Similar evidence was obtained in the case of Env proteins from replication-competent murine leukaemia viruses (Polonoff et al., 1982; Pinter et al., 1984), glycoproteins of lymphocytic choriomeningitis virus (Wright et al., 1990) and vesicular stomatitis virus (HSV) (Machamer et al., 1985; Kotwal et al., 1986). Studies on the HSV glycoprotein further revealed that its carbohydrates do not act as a transport signal per se but play an indirect role by influencing polypeptide folding and oligomerization (Machamer & Rose, 1988a,b). It should be noted that mutant proteins lacking oligosaccharides at the normal positions were found to form aberrant disulphide-bonded complexes accumulating in the endoplasmic reticulum.

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Oligonucleotide-directed mutagenesis. A BamH1/AatII fragment (572 bp) containing the sequences encoding AsnX and AsnX was inserted into M13mp18. Mutagenesis was carried out by the method of Taylor and Eckstein (Taylor et al., 1985) utilizing the Amersham oligonucleotide-directed in vitro mutagenesis system. Two synthetic oligonucleotide primers were used to mutate the Asn-X-Thr/Thr N-glycosylation signals, each having a single base mismatch to change asparagine into serine [primer for glycosylation site 1 (AsnX): CAAGTAAACACTGAGAC; site 2 (AsnX): GGTTAGGCTAGCTTGTG].

**Mutagenesis by the polymerase chain reaction.** To eliminate the two consensus sites for N-glycosylation at AsnX and AsnX, we employed the PCR using the following pair of primers, each having a single base mismatch to change the asparagine into a serine residue: glycosylation site 3 (AsnX): GCTCTAGCTCCACCACAC and TGGTGGGCTGAGGCTTGG; glycosylation site 4 (AsnX): TATTTTAGTCTACCATTTGGGCTG and GCAATGGTATGACTAAAAATAAG. For amplification of the PCR fusion product, two external primers were used (P1: ATCATCATCATGGGCACCTAATT; P2: TTGGGATTTTTTCATGCCTTGC). The plasmid pUSAX was linearized with PspI. Amplification of the DNA fragments from the plasmid template was achieved by adding 1 ng of template DNA, 0.25 μM of each primer and 2.5 units Taq polymerase (Perkin-Elmer Cetus) to a final volume of 50 μl, 50 mM-KCl, 10 mM-Tris-HCl pH 8.6, 1.5 mM-MgCl2, and 200 μM of each dNTP. These samples were overlaid with 100 μl paraffin and subjected to 25 cycles of denaturation (1 min, 95 °C), annealing (1 min, 56 °C) and extension (3 min, 72 °C). Reaction products were analysed on gels containing 1.5 % agarose and 1 lag/ml ethidium bromide in Tris borate buffer (45 gM-Tris-borate, 1 laM-EDTA). Samples of 5 μl (or dilutions thereof) of the three PCR reaction mixtures containing the overlapping fragments were mixed and subjected to PCR amplification using the external primers (P1 and P2) which were complementary to sequences located approximately 100 bp (for P2 300 bp) outside of the unique EcoRI and AsuII restriction sites, thus making it possible to ligate the mutated fusion product from the overlap extension reaction into the expression vector. DNA fragments were purified from agarose by using glass powder according to Vogelstein & Gillespie (1979).

**Analysis of the DNA constructs.** Mutations at amino acid positions 58, 297 and 329 created or destroyed restriction enzyme sites which facilitated screening of the mutants. All mutations and all sequences of DNAs synthesized by PCR were confirmed by sequencing with the dideoxynucleotide method (Sanger et al., 1977).

Transfection procedure and cell culture. The Sphl fragment of pSAX containing the whole viral genome was ligated into concatemers which were used for transfection of Rat-1 cells essentially as described previously (Friedrich et al., 1991). Transfection with plasmid DNA was performed by lipofection (Felgner et al., 1987), using 5 μg of viral DNA in the presence of 0.1 μg of a plasmid DNA containing the gene for neomycin resistance. The transfection reagent DOTMA was obtained from Boehringer. Rat-1 cells were propagated in Dulbecco's MEM supplemented with 5 % fetal calf serum. Two days after transfection, 600 μg/ml G418 (Gibco) was added to the culture medium. After 2 to 3 weeks, individual G418-resistant colonies were isolated and tested by hybridization and immunofluorescence, as described previously (Friedrich et al., 1991). Positive cell lines were superinfected with Friend murine leukaemia virus 22N (F-MuLV; Ostertag et al., 1980), a highly effective helper virus for SFFV.

**Immune precipitation.** Cells were labelled in 35 mm dishes with 100 μCi [35S]methionine (1000 Ci/mmol, Amersham) for 1 h. After labelling, the cells were washed twice with PBS and incubated on ice for 15 min in 250 μl extraction buffer (Friedrich et al., 1991). The extracted supernatants were clarified by centrifugation at 13000 g for 10 min at 0 °C. Samples of 50 μl of labelled cell extracts were mixed with anti-gp70 rabbit serum (kindly provided by H. Bauer) and heat-inactivated Staphylococcus aureus (Calbiochem) for 3 h at 4 °C. Immune precipitates were isolated by centrifugation, washed twice with...
extraction buffer and resuspended in 50 μl buffer containing 125 mM-Tris–HCl pH 6.8, 4% SDS, 2% 2-mercaptoethanol, 10% glycerol and bromophenol blue. The samples were boiled for 5 min before they were loaded onto 10% SDS–polyacrylamide gels.

Digestion of carbohydrates with glycopeptidase F. For the removal of N-linked oligosaccharides, immunoprecipitates were, after washing with extraction buffer and 10 mM-Tris–HCl pH 7.2, resuspended in 100 μl of buffer containing 25 mM-sodium phosphate pH 7.2, 0.6% Nonidet P40 and incubated for 48 h at 37 °C with 15 mU glycopeptidase F (EC 3.5.1.52, N-glycosidase F; Boehringer).

Injection of mice. To test the biological activity, virus stocks containing recombinant virus and the helper virus F-MuLV 22N were injected into the tail veins of 6 to 10 week old DBA/2J mice. At least six animals were infected with each viral construct. To ensure that equal amounts of virus were injected, SFFV RNAs prepared from cellular supernatants were quantified by hybridization according to the method of Paeratakul et al. (1988). Three to 6 weeks after infection, mice were killed and their spleen weights and haematocrit levels were determined. Spleens were fixed in Bouin’s solution and then examined for foci.

Results

Construction of glycosylation mutants

To determine the role of gp55 N-glycosylation in pathogenicity, we modified the four known N-glycosylation sites, replacing the asparagine residues with serine residues. In addition to viruses with mutations at glycosylation sites 1 to 4 (gm1 to gm4), multiple mutants (gm1.2, gm3.4, gm1.2.3, gm1.2.4 and gm.all) were constructed. Fig. 1 illustrates the various Env-derived proteins investigated.

Biochemical characterization of mutated env gene products

The env gene products of the wild-type and the various mutated viruses were characterized by immunoprecipitation after the cells had been labelled with [35S]-methionine. Fig. 2 and Table 1 show the size determination of the Env-derived proteins after separation on polyacrylamide gels. The products of gm1 to gm4 (all missing one glycosylation site) migrated with an apparent $M_r$ of about 52.5K compared to 55K for the wild-type virus-encoded glycoprotein. (The weak band above gp55 represented a cellular protein also found in uninfected cells; data not shown.) In the case of gm3, an additional protein with an apparent $M_r$ of 55K was seen. The Env-derived protein of the double mutant gm1.2 migrated with an apparent $M_r$ of about 50K. Double mutant gm3.4 revealed two protein bands, one with an apparent $M_r$ of about 50K similar to that of mutant gm1.2 and another one with an $M_r$ of about 52.5K. The Env-derived protein of the triple mutant gm 1.2.3 was again separated into two bands, one with an apparent $M_r$ of 47.5K and one of 50K. Triple mutant gm1.2.4, however, produced a single protein band migrating with an apparent $M_r$ of about 47.5K. Mutant gm.all, in which all four N-glycosylation sites known to be glycosylated in wild-type virus were destroyed, produced Env-derived proteins with an $M_r$ of about 45K and 47.5K.

To determine whether the additional protein bands observed in the case of mutants lacking glycosylation site 3 (Asn$_{297}$) carried an additional N-linked oligosaccharide side-chain, the immunoprecipitates of gm1.2.3-, gm1.2.4- and gm.all-transfected cells were treated with glycopeptidase F prior to SDS–PAGE. Although removal of the sugar side-chains by this enzyme was usually incomplete the results revealed products missing all four N-linked oligosaccharide side-chains (marked ‘−4’ in Fig. 3) in all cases. In the case of the Env-derived protein of gm.all, a complete conversion of the 47.5K band into the 45K polypeptide was observed.

Pathogenicity of the glycosylation mutants

All molecular clones were tested in DBA/2J mice for pathogenicity. Mice were sacrificed either 3, 4 or 6 weeks after infection and their spleen weights, haematocrit levels and the number of spleen foci were determined. The mutants gm1, gm2 and gm1.2 showed disease parameters very similar to those of wild-type virus. All
mutants involving glycosylation sites 3 or 4, however, were non-pathogenic (see Fig. 4 for spleen weights and foci). Haematocrit levels of diseased animals were 72 to 80%, whereas those of uninfected control animals or animals infected with non-pathogenic mutants were 47 to 55%.

### Discussion

Little is known about the roles of individual N-glycosylation sites of the Env-related product of SFFV in viral pathogenicity. To determine whether the N-linked carbohydrate side-chains exert an influence on the biological properties of this protein, we constructed virus mutants encoding glycoproteins in which asparagine residues of Asn-X-Ser/Thr glycosylation sequences were
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References
In agreement with corresponding studies on gp71 of F-MuLV (Kayman et al., 1991), our data suggest that N-glycosylation is required for pathogenicity and that the carbohydrate side-chains are important for correct folding and/or processing of the Env-related protein. It is also conceivable that the amino acid sequence at these sites may itself be critical for the pathogenic properties of the protein. Preliminary results with a construct carrying an Asn-to-Asp exchange at site 3 show that this mutation does not have such a striking effect on viral pathogenicity.

The presence of a carbohydrate side-chain at one site and the correct amino acid sequence at a second site may be important for the proper folding or processing of the protein. Further work is in progress to investigate whether incomplete glycosylation of the Env-related protein may impair its intracellular transport.

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References


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