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A Domain of Murine Retrovirus Surface Protein gp70 Mediates Cell Fusion, as Shown in a Novel SC-1 Cell Fusion System

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Virus-induced cell fusion of the fusion-from-without type was observed in SC-1 cells infected with Moloney murine leukemia virus when grown in NIH 3T3 cells. Replication-competent virus mutants with altered surface protein gp70 were examined. Fusion mutations were found in the proline-rich region of gp70. They acted on a step after binding and before or during endocytosis. The fusion mutants had an altered gp70 isomer pattern, presumably caused by different glycosylation. Other mutants with deleted glycans were analyzed, and some which also showed defective fusion were found. The interrelationship of the proline-rich region, glycosylation, and fusion is discussed.

Enveloped viruses enter cells by membrane fusion. This occurs either by plasma membrane fusion, as seen for Sendai virus (22, 32) and human immunodeficiency virus (HIV) (39), or by fusion with the membrane of acidic vesicles after endocytosis, as seen for ecotropic murine leukemia virus (MLV) (3, 24) and influenza virus (22).

One aspect of membrane fusion is virus-mediated cell fusion (32). Two (or more cells) fuse to form a syncytium. This is either caused by virus particles (fusion from without), whereby it is believed that the viral envelope fuses with two cells, or by viral envelope proteins expressed on the cell surface, bringing two cells to fuse directly (fusion from within). HIV normally induces cell fusion (10, 11), whereas ecotropic MLV induces cell fusion only with certain cell lines such as the rat cell line XC or under certain circumstances (4, 17). Slight MLV-induced cell fusion has, however, been observed with the feral mouse cell line SC-1 (24). The process of virus-mediated cell fusion is thought to resemble virus-cell fusion during entry (32); however, experiments on pH dependency have questioned this analogy (43).

Retrovirus has two surface proteins. The first is the transmembrane protein TM (p15E in MLV), which contains a hydrophobic domain on its outer N-terminal end in analogy to a number of other enveloped viruses. This domain is believed to be involved in the actual membrane-membrane fusion (17, 42). The second protein is the outer surface protein SU (gp70 in MLV). gp70 is linked to p15E through disulfide bridges (31). gp70 binds the virus to the receptor (1, 15). It can be divided into a variable N-terminal end and a conserved C-terminal end linked by a variable proline-rich and probably flexible region (18) (amino acids 231 to 274 in Moloney MLV [MoMLV]). The C-terminal end contains five of seven glycans in MoMLV (8, 36). In the N-terminal end, two especially variable regions, VRA and VRB (amino acids 51 to 130 and 169 to 179 in MoMLV), are recognized. Through chimeric gp70 molecules, the VRA region was found to be responsible for receptor binding (5).

In the context of these findings, findings of two earlier studies are not accounted for. Zarling and Keshet (45) and Pinter et al. (29) found that cell fusion cannot be inhibited by polyclonal antibodies against p15E. This enigma can be explained by a steric protection of p15E by gp70 which needs to be released for fusion to occur. This is supported by our earlier results, showing that gp70 is cleaved during entry (2) and that protease treatment enhances fusion (4).

Antibodies against gp70 can inhibit fusion (45), which is expected if the receptor binding region is blocked. However, fusion was inhibited not only by monoclonal antibodies against the N-terminal part but also by antibodies against the C-terminal part of gp70 (29), indicating the importance for fusion of this part of the molecule.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells (16), SC-1 cells (14), and XC cells (37) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, nonessential amino acids, penicillin, and streptomycin.

Virus. The NIH 3T3 cells were transfected with a plasmid containing the MoMLV wild-type genome (pKA1558) or virus mutant plasmids as previously described (38). After a few passages, the virus from the positively transfected cells infected the entire culture. Overnight media from confluent or near-confluent virus producer cultures were used as a source of mutant virions. In addition, plasmids containing MoMLV mutants in which the glycosylation sites were altered one by one and the parental plasmid pNCAC containing the wild-type MoMLV genome (6) were obtained from Felkner and Roth (8).

Fusion assays. SC-1 cells were seeded in Nunc (Roskilde, Denmark) 96-well plates with 5,200 cells and 70 μl of culture medium (or, when noted, in 24-well plates with 30,000 cells in 400 μl of culture medium or in 0.013-cm^2 plates with 750 cells in 10 μl of medium). The following day, 5 μg of Polybrene (hexadimethrin bromide; Sigma, St. Louis, Mo.) per ml was added, after which virus was added; 8 to 15 h later, the cultures were stained with Giemsa stain.

The fusions were counted and expressed as a fusion index, defined as (N − S)/T (N is the number of nuclei in syncytia, S is the number of syncytia; and T is the total number of nuclei), and expressed as a percentage. At least 500 nuclei were counted. The expression N − S was used in order to have a measure of the number of fusion events (a syncytium with two nuclei is counted as one fusion event in this method). Titration curves showed a sigmoid pattern, defining a maximal fusion

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TABLE 1. Mutants tested

Mutant	Description[a]
K177I	Lys-177→Ile
R194G/R195Q	Arg-194→Gly, Arg-195→Gln
R208Q	Arg-208→Gln
R232A	Arg-232→Ala
K249Q/K251A	Lys-249→Gln, Lys-251→Ala
R280Q	Arg-208→Gln
K299Q	Lys-299→Gln

[a] Each mutant is denoted by its position in the gp70 molecule preceded by the amino acid replaced and followed by the amino acid replacing it. Two numbers denote that both amino acid exchanges are made.

index, F_{max}, and a virus concentration giving 50% of F_{max}, FC_{50} (usually expressed as multiplicity of infection, PFU per cell).

Virus titration. SC-1 cells were seeded in 24- or 96-well plates (30,000 or 5,200 cells, respectively). The following day, 5 μg of Polybrene per ml and virus were added. After 2 to 3 days, the cultures were fixed with methanol, and virus plaques were immunoperoxidase stained (25), using a primary rabbit antibody against MoMLV p30.

Reverse transcriptase. Medium samples were centrifuged for 5 min at 400 × *g* to remove cells and debris. The supernatants were then centrifuged for 1 h at 20,000 × *g*. Reverse transcriptase activity in the pellets was then measured as described by Roy-Burman et al. (35).

Analysis of viral proteins. The producer cultures (10 cm^2) were labelled overnight with 40 μCi of [^{3}H]glucosamine (40 Ci/mmol; NEN Research Products) in medium containing 1/100 of the normal glucose concentration. Virus particles were collected by centrifugation as for the reverse transcriptase assay described above. Cells were dissolved in detergent, precipitated with anti-gp70 (gift from B. A. Nexø), and collected on fixed *Staphylococcus aureus* (26). Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) or by isoelectric focusing as described by Giulian et al. (13). The protein bands were revealed by standard fluorographic techniques.

RESULTS

Seven gp70 mutants of MoMLV were examined. Their construction has previously been described (38). They all have one or two adjacent basic amino acids exchanged with small neutral amino acids (Table 1). The exchange of arginine and lysine was chosen both to make drastic changes by altering charges and because arginine and lysine are likely to occur on the surface of the protein as previously discussed (38). All mutants tested here produce replication-positive virions (38). Virus-producing cultures were obtained by transfection of the plasmids into NIH 3T3 cells. Only a fraction of the cells were originally transfected; however, after a few passages, the cultures were infected throughout. Supernatants from these producer cultures were used in this study as the source of virus. Representative values for virus production in terms of PFU and reverse transcriptase activity are given in Table 3.

Fusion mechanism. MoMLV from infected NIH 3T3 cells was observed to produce extensive fusion when added to SC-1 cells in the presence of Polybrene (2 to 10 μg/ml). Less than 1% fusion was observed in the absence of Polybrene and virus. In each titration of wild-type virus, a characteristic maximal level of fusion was reached (30 to 70%; see Fig. 2). No fusion was observed when virus was added to MoMLV-infected SC-1 cells (not shown), indicating that the normal receptor is used for the fusion pathway, nor was fusion observed when virus was added to NIH 3T3 cells, showing the importance of the cell line used. Other cell lines (BALB/3T3 and NZB) tested did not fuse upon virus addition.

The fusion was shown to be caused by the virus particles. The fusinogenic ability was totally removed from the culture supernatant by centrifugation (30,000 × *g*, 1 h). Furthermore, the fusinogenic ability was retained by the virus particles after isopycnic banding in Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) (results not shown).

The development of fusion is shown in Fig. 1. As seen, fusion occurred slowly until 1 to 2 h after virus addition and then occurred quickly until a maximal fusion level was reached 8 to 10 h after virus addition. As the fusion occurs faster than a replication cycle, it is concluded to be fusion from without. The following fusion experiments were, as a consequence of this

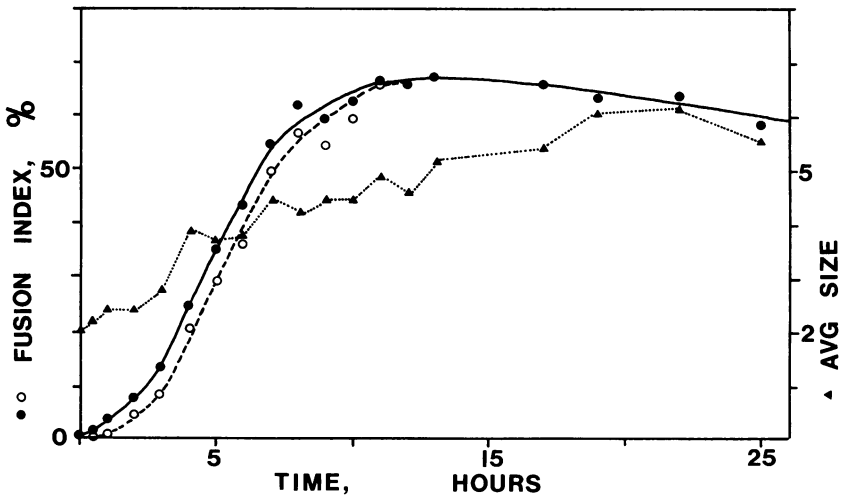

FIG. 1. Development of fusion. SC-1 cultures were grown in a 24-well plates; 300 μl of medium (5 × FC_{50}) from pKA1558 (wild-type) NIH 3T3 culture was added, cultures were stained after the indicated times, and fusions were counted. ●, fusion index; ○, fusion index of fusions with three or more nuclei (calculated as fusion index minus fusion index of fusions with two nuclei); ▲, average size of fusions.

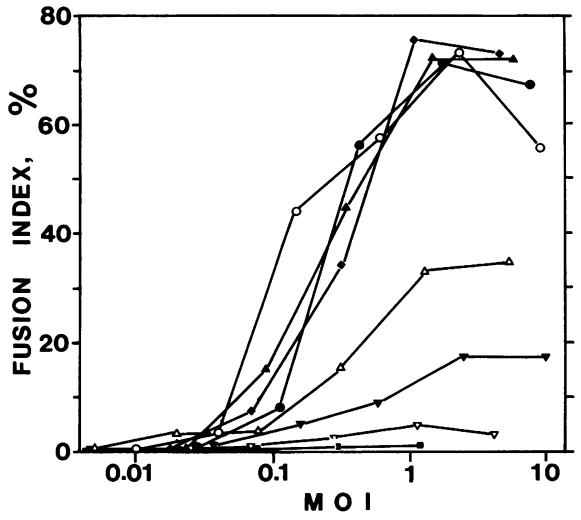

FIG. 2. Fusion of mutant virions. Supernatants from mutant virus NIH 3T3 producer cultures were added to SC-1 cultures, the cultures were stained after 15 h, and fusion was determined. The highest addition for each curve corresponds to 160 μl of culture medium. The virus titer was determined by immunoperoxidase staining in parallel cultures. The fusion index is expressed against the multiplicity of infection (MOI; PFU added per cell). ○, wild type; ●, K177I; △, R194G/R195Q; ▲, R208Q; ▼, R232A; ▽, K249Q/K251A; ■, R280Q; ◆, K299Q.

result, scored 10 to 16 h after virus addition. Fusion proceeded with the same kinetics, independent of the virus amount added, though the fusion level differed (experiment not shown).

The number of syncytia with two nuclei and the average number of nuclei in the syncytia were also determined. Two-nuclei syncytia were essentially seen only while the fusion index increased, showing that the newly formed syncytia rapidly grew larger. The mean lifetime of the two-nuclei syncytia was approximately 30 min. Growth of the syncytia is also seen from their average size, which increased to over five nuclei after 15 h. Up to 30 nuclei were seen in the syncytia after this time. The syncytia were monitored for several days. After 20 h, the nuclei often fused together, fragmented, and loosened from the culture wells. The fusion index then decreased, also as a result of growth of nonfused cells.

Details of the fusion system will be published elsewhere.

Fusion of mutants. The SC-1 fusion of the arginine/lysine mutants was tested and compared with their infectious titers, as measured by immunoperoxidase plaques in SC-1 cultures (25) (Fig. 2). The titration curves showed typically sigmoid patterns defining F_{max} and FC_{50}. For the wild type, F_{max} was 70% and FC_{50} was 0.2 PFU per cell. Mutants K177I, R208Q, and K299Q showed approximately the same F_{max} as that of the wild type. Mutants R194A/R195Q and R232A showed lower F_{max}s of 35 and 15%, respectively. Mutant K249Q/K251A showed an F_{max} of only 5%, whereas R280Q did not show any significant fusion. The low-fusinogenic or nonfusinogenic mutants K249Q/K251A and R280Q were concentrated by banding in Percoll. The fusion followed the virus band, but the F_{max} did not change (results not shown), indicating that the low fusion is inherent to these virus particles and not caused by inhibitory substances separated from the virus particles in the gradient. It is interesting that all mutants with measurable fusion showed the same FC_{50} as that of the wild type (approximately 0.2 PFU per cell), independent of their F_{max}. Though the F_{max} for the wild type differed between different experiments, the relative fusion values did not differ greatly.

It should be noted that the three most defective fusion mutants are neighbors, indicating that the domain from 232 to 280 is important for fusion.

The weak fusion can be explained as inherent to the virus particles or by the presence of inhibitory components in the virus medium, e.g., shed gp70. To investigate this possibility, virions were centrifuged from the medium (30,000 × *g*, 1 h). All fusinogenic activity was then removed (Table 2). The virus-free supernatants were added to SC-1 cells, and later wild-type virus was added to the cells. As seen in Table 2, the supernatants from the mutant virions did not inhibit the wild-type fusion. As a control, a freeze-thaw supernatant of MoMLV could inhibit fusion fully (Table 2, experiment 3). gp70 is known to be released by the freeze-thaw procedure (40). The freeze-thaw supernatant inhibited infection more than 1,000-fold (not shown). Anti-gp70 also inhibited fusion (Table 2, experiment 4). The latter experiments indicate that fusion occurs after a normal binding of the virions to the receptor.

All of the tested mutants are replication competent, though R280Q and R194A/R195Q replicated slowly in NIH 3T3 cultures (38). All producer cultures except the R280Q culture produced almost identical amounts of physical virus particles (as pelletable reverse transcriptase) (Table 3). These values, however, differed between experiments.

The infectivity of the mutants varied up to 25-fold, as measured by the ratio between infectious and physical particles (Table 3). It correlated generally with F_{max}, but in terms of infectivity, the differences were only small.

The fusion defect does not appear to be caused by weak binding of virus to the cells. In that case, larger virus addition would cause larger binding and hence fusion, in contrast to the results. To examine the binding ability of the mutants further, a competition experiment was performed (Table 4). The fusion mutants were added to SC-1 cells, and 1.5 h later, wild-type virus giving submaximal fusion was added. As seen, K249Q/K251A and R280Q both inhibited fusion in comparison with media from which the virions were removed by centrifugation. This result shows that the two mutants bind to the cells and can interfere with fusion.

The rat cell line XC is known for its ability to fuse in the presence of murine retrovirus (34). XC and the SC-1 fusion were compared. The wild type gave up to 90% fusion of XC cells (measured under the same conditions as the SC-1 fusion). Of the seven mutants, only mutants K249Q/K251A and R280Q differed from the wild type. They both gave low but significant fusion (approximately 5 and 2%, respectively). On basis of both SC-1 and XC fusion, the fusion mutants can be arranged according to increasing defect on the fusion: R194G/R195Q < R232A < K249Q/K251A < R280Q.

The results presented above raised the question of which part of the gp70 molecule is important for fusion. An experiment was performed to compare fusion of wild-type MoMLV propagated in NIH 3T3 and SC-1 cells. The FC_{50} values were identical, approximately 0.3 PFU per cell, as also seen for the mutants. However, whereas the F_{max} for NIH 3T3 MoMLV was 48%, it was only 15% for SC-1 MoMLV. As seen for the virus mutants, the lower fusion was followed by a lower infectivity (30% of that of NIH 3T3 MoMLV). Also, MoMLV propagated in BALB/3T3 and NZB cells gave low maximal fusion upon addition to SC-1 cells (not shown).

For MoMLV propagated in different cell lines, the virus genomes are alike, but components obtained from the host cell (i.e., membrane components or postranscriptional modifica-

TABLE 2. Effects of supernatants from transfected cultures on wild-type virion-induced SC-1 fusion[a]

Expt	1st addition	Fusion index (%) with 2nd addition	
		None	Wild-type virus
1	Fresh medium	0.2	53
	Centrifuged culture supernatant from:		
	NIH 3T3	0.2	54
	Wild-type NIH 3T3	0.1	54
	K177I-NIH 3T3	0.1	51
	R194G/R195Q-NIH 3T3	0.1	54
	R208Q-NIH 3T3	0.1	56
	R232A-NIH 3T3	0.2	47
	K249Q/K251A-NIH 3T3	0.1	51
	R280Q-NIH 3T3	0.1	52
2	Centrifuged culture supernatant from:		
	NIH 3T3	0.0	55
	K299Q-NIH 3T3	0.7	58
3	MoMLV freeze-thaw supernatant	0.0	0.5
	Mock (NTE buffer)	0.6	54
4	No addition	0	35
	Rabbit nonimmune serum	0	36
	Rabbit anti-MoMLV gp70	0	0
	Rabbit anti-MoMLV p30	0	42

[a] In experiments 1 and 2, to each SC-1 culture well was added 100 μl of the indicated centrifuged (30,000 × *g*, 1 h) supernatant of virus medium and, 30 min later, 30 μl of wild-type virus (5 × FC_{50}). The first added medium corresponded to 0.2 to 10 PFU per cell, as measured before centrifugation of medium. After 15 h, the cultures were stained and fusion was counted. The standard deviation between separate determinations was 10% of the values. Experiments 3 and 4 were performed in 0.013-cm^2 wells; 0.5 mg of MoMLV (gift from the National Cancer Institute, Bethesda, Md.) was pelleted and repeatedly frozen and thawed for 20 cycles in 20 μl of NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) followed by centrifugation (30,000 × *g*, 1 h). Then 2.5 μl of supernatant was added to the wells, followed by addition of 5 × FC_{50} of wild-type virus. In the control experiment, NTE buffer was added in place of the freeze-thaw supernatant. In experiment 4, 0.5 μl of immune serum was added, followed by 10 × FC_{50} of wild-type virus. The rabbit immune serum was a gift from B. A. Nexø.

tions) possibly differ. Glycosylation is known to differ in different cell types (9).

Chemical differences of mutants. gp70 is the only glycosylated protein in ecotropic MLV. It was labelled with [^{3}H]glucosamine. The sizes of the mutant gp70s were examined by SDS-PAGE (Fig. 3 and 4C). Mutant K249Q/K251A was seen to differ. gp70 in its virions and the precursor $pr80^{env}$ in infected cells were approximately 3 kDa larger than those of the wild type. The size difference can be explained by differences in the glycans, each of which normally contributes 4 to 5 kDa to the size of gp70 (8). gp70 of R280Q was seen to streak in the top of the gel (Fig. 4C), indicating a strong aggregation and making it difficult to determine its size.

Even though the virions were metabolically labelled with glucosamine, p30 was also labelled, although very weakly (to approximately 1/100 the intensity of gp70). This label is presumably due to slight metabolic conversion of the glucosamine. The p30 bands were used as an internal standard against which to compare the gp70 intensities. As seen in Fig. 3A, the intensity of the mutant and wild-type gp70 bands correlated with that of p30, showing that the gp70 density on the examined mutants did not alter considerably from that of the wild type.

The glycans add several negative charges to gp70 (as sialic acids) (9, 31). The charges were examined by isoelectric focusing of gp70 (Fig. 4). As with other glycoproteins, many isoforms of gp70 are seen, as a result of heterogeneity of the glycans. The mutants did not show new isoforms. However, many had differing distributions of isoforms. K299Q had stronger basic isoforms, whereas K177I was like the wild type.

TABLE 3. Infectivity and Polybrene stimulation of infection[a]

Mutant virions from cultures	Reverse transcriptase relative to wild type (%)	Infectivity relative to wild type (with Polybrene) (%)
K177I-NIH 3T3	86	121
R194G/R195Q-NIH 3T3	107	5
R208Q-NIH 3T3	105	38
R232A-NIH 3T3	72	26
K249Q/K251A-NIH 3T3	85	15
R280Q-NIH 3T3	35	4
K299Q-NIH 3T3	115	53

[a] Virus was collected from producer cultures. Pelletable reverse transcriptase and immunoperoxidase titers were measured. Results are means of at least two independent measurements. The average wild-type titer was 250 PFU/μl. The standard deviations were 1.1- and 1.7-fold, respectively, for the two columns (calculated from the logarithms to the values).

TABLE 4. Inhibition of wild-type fusion by mutants[a]

1st addition of culture supernatant from:	Fusion index (%) with 2nd addition	
	None	Wild-type virus
Fresh medium	0.3	17
NIH 3T3	0.3	18
K249Q/K251A-NIH 3T3	1.9	2.9
K249Q/K251A-NIH 3T3, centrifuged	0.3	18
R280Q-NIH 3T3	0.7	2.9
R280Q-NIH 3T3, centrifuged	0.3	19

[a] Virus was collected from producer cultures; 150 μl of medium (15,000 PFU of K249/K251 or 3,000 PFU of R280Q) was added to SC-1 cells. When noted, the collected medium was centrifuged (30,000 × *g*, 1 h). After 1.5 h, 10 μl of wild-type virus was added as indicated (approximately 2 × FC_{50}). The cells were stained after an additional 10 h.

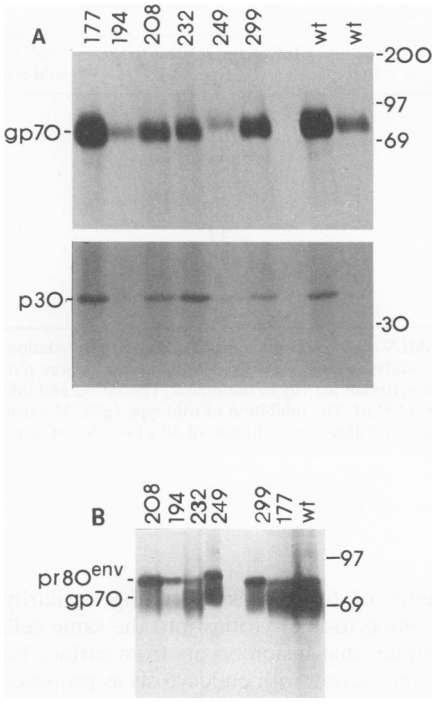

FIG. 3. SDS-PAGE of mutant gp70 and cellular precursor. Mutant virus producer cultures were labelled with [^{3}H]glucosamine. Virions were collected by precipitation (A), and cells were immunoprecipitated with anti-gp70 (B) and then electrophoresed on SDS–10% polyacrylamide gels. Mutants are denoted by the number of the first exchanged amino acid. wt, wild type. The lower portion of panel A shows the lower part of the gel, exposed 10 times longer in order to show p30 bands. Sizes are indicated in kilodaltons.

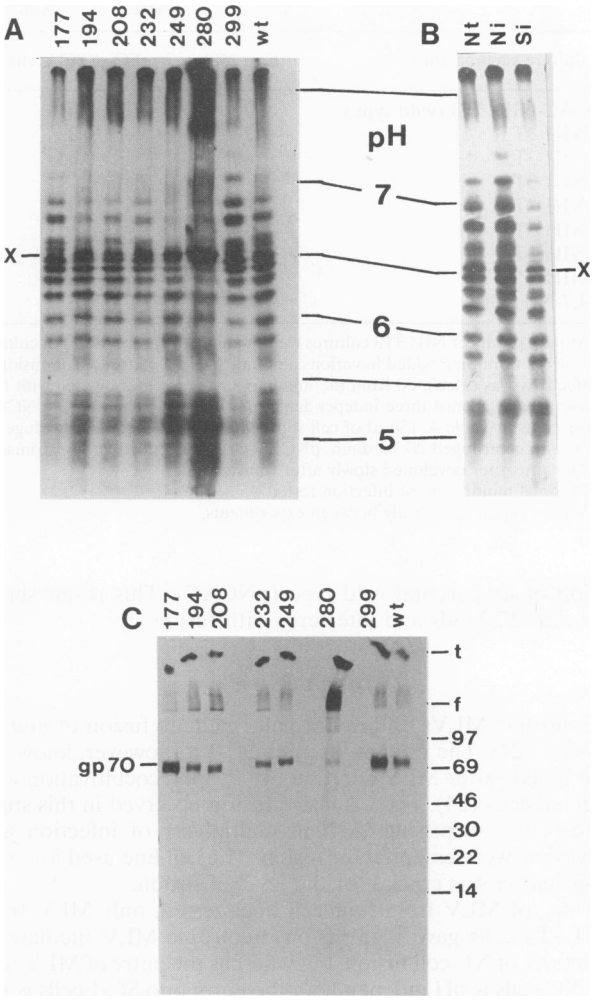

FIG. 4. Isoelectric focusing of mutant gp70. Virions were labelled with [^{3}H]glucosamine and electrophoresed on isoelectric focusing gels. The pHs of the gel slices were measured after equilibration in 20 mM KCl and are shown between panels A and B. (A) Mutants are denoted by the number of the first exchanged amino acid. wt, wild type. (B) Wild type. Nt, virions from pKA1558-NIH 3T3 culture; Ni, virions from NIH 3T3 cells infected with medium obtained as for Nt; Si, virions from SC-1 cells infected with medium obtained as for Nt. (C) SDS-PAGE (4 to 20% gradient gel) of the mutant virions in panel A in order to show purity. Sizes of molecular weight markers are given in kilodaltons. f, front of the running gel; t, top of the stacking gel.

The mutants in the range of 194 to 280 had weaker basic isoforms above pI 6.5 and a stronger isoform at pI 6.5 (X in Fig. 4). This was especially true for K249Q/K251A, for which the basic isoforms above 6.5 were almost absent. The same was possibly true for R280Q; however, its gp70 did not focus well, presumably because of aggregation. It is striking that the same shift toward less basic isoforms was also seen when the wild type was propagated in SC-1 cells in comparison to NIH 3T3 cells (Fig. 4B).

In summary, for all tested virions, low amounts of basic gp70 isoforms, weak virus-induced cell fusion, and weak infectivity correlated. However, R208Q did not show any fusion defects despite moderately weak basic isoforms.

It is interesting that the removal of one or two positive charges of arginine or lysine in the mutants did not just give a shift of the entire isoform pattern toward more acidic pIs. A more acidic pI can be explained by a deamination. This is, however, unlikely, since the entire isoform pattern then would be shifted. The selectively altered pattern thus indicates different glycosylation trimming.

Importance of individual glycans. Felkner and Roth have removed the N-linked gp70 glycans one by one from MoMLV by mutation of the asparagines providing the links (8). The mutants are named Δ1 to Δ7 according to the glycan deleted. Glycans 1 and 2 are located to the N-terminal side of the proline-rich region at positions 12 and 166. The following glycans are located to the C-terminal side of the proline-rich region at positions 293, 325, 332, 365, and 401. A three-glucan deletion, Δ1,4,7 was also made. In our hands, virion Δ3 was not viable as reported previously (8). Δ2 infected the cultures slowly. The fusion and infectivity in SC-1 cells were tested (Table 5). As seen, the fusion of mutant Δ5 was slightly decreased, whereas no fusion of mutant Δ7 was observed. As the titer of Δ7 was low, it was concentrated by Percoll banding; however, no significant fusion was observed (not shown). Interestingly, Δ1,4,7 showed a higher fusion than Δ7, even if more glycans were removed. Furthermore, its fusion varied between experiments. As it was seen for the arginine/lysine mutants, the FC_{50} values expressed as PFU were identical for the wild-type and the low-fusion mutants. Likewise, low fusion correlated with low infectivity.

Whether the fusion and infection defect of Δ7 could be explained by poor binding was tested by a competition experiment similar to that of Table 4. Δ7 was able to inhibit the

TABLE 5. Fusinogenic activity of glycan mutants[a]

Culture supernatant from:	F_{max} (%)	FC_{50} (PFU/cell)	Reverse transcriptase relative to wild type (%)	Infectivity relative to wild type (%)	Inhibition relative to wild-type fusion (%)
pNCAC-NIH 3T3 (wild type)	70	0.4	100	100	
Δ1-NIH 3T3	70	0.3	141	101	
Δ2-NIH 3T3	60	0.35	150[b]	123	
Δ3-NIH 3T3			<0.2		
Δ4-NIH 3T3	60	0.4	90	60	
Δ5-NIH 3T3	10	1	48	49	
Δ6-NIH 3T3	70	0.4	176	92	
Δ7-NIH 3T3	0	0.4[c]	20	0.5	66
Δ1,4,7-NIH 3T3	10[d]	0.4	53	29	

[a] Mutant producer NIH 3T3 cultures were obtained by propagation of cultures transfected with MoMLV plasmids containing altered gp70 glycosylation sites (8). The virus media were then added in various amounts to SC-1 cultures. The fusion indices and immunoperoxidase titers were scored, and the results were plotted as in Fig. 3. Infectivity was calculated from the immunoperoxidase titer divided with the pelletable reverse transcriptase activity in the media. The fusion and infectivity values are averages of at least three independent measurements. The titer of pNCAC was on average 1,700 PFU/μl. The inhibition of wild-type (pNCAC) fusion by Δ7 was measured as in Table 4; 150 μl of cell supernatant (600 PFU) or centrifuged cell supernatant was added, followed by addition of 30 μl of pNCAC virus, giving 14% fusion with centrifuged Δ7 medium. pNCAC virus was added at approximately 2 × FC_{50}.

[b] The virus titer developed slowly after transfection.

[c] Maximal multiplicity of infection tested.

[d] Values varied extensively between experiments.

fusion of its parental wild type (pNCAC). This result shows that also Δ7 binds and interferes with fusion.

DISCUSSION

Ecotropic MLVs do not normally mediate fusion of murine cells (17, 24). The feral mouse line SC-1 is, however, known to fuse weakly after MLV addition (4) or upon cocultivation with infected cells (24). The extensive fusion observed in this study is therefore surprising. A high multiplicity of infection and Polybrene were essential for fusion. The cell line used for virus propagation was crucial for the level of fusion.

Thus, of MLV from four cell lines tested, only MLV from NIH 3T3 cells gave high fusion. Ecotropic MLV mediates a high level of XC cell fusion, but whereas the entry of MLV into the XC cells is pH independent, the entry into SC-1 cells is pH dependent (24). In comparison with the XC fusion system, the SC-1 fusion system was seen to be more sensitive, detecting slight differences in fusion.

Polybrene and other polycations have been used in previous fusion from without studies of MLV (4, 43) and is generally used in infection studies, in which it helps bind virus to the cells (21, 25). Polybrene and other polycations are also known to enhance cell fusion by other viruses (23). It is believed to bring negative charges close together. In contrast to the effect on cell-cell fusion, Polybrene was not obligate for infection, showing that cell-cell fusion is not obligate for infection as also reported for other cell lines (43). Polybrene, however, stimulated the infection approximately fivefold (results not shown), and a correlation between F_{max} and infectivity was observed, showing that cell-cell fusion presumably shares steps with the infectious route.

The mechanism of virus-induced cell-cell fusion is not known in detail. Results presented above showing sigmoidal titration curves and competition of the fusion mutants indicate that both wild-type and mutant virions can saturate the cells. This conclusion is supported by the ability of cultures infected with the fusion mutants to resist superinfection with wild-type virus (results not shown). The fusion defects thus do not appear to be caused by poor binding. Accordingly, FC_{50} can be interpreted as the virus concentration giving half-maximal binding, occurring at approximately 0.3 PFU per cell. It should be noted that more virus particles are bound to the cell as the ratio of successful infections to physical particles is low (3).

The kinetics of fusion has a striking similarity with the kinetics of endocytosis of virions into the same cells (3). This similarity implies that fusion occurs from surface-bound virus, perhaps in conjunction with endocytosis as proposed by Wong et al. (44) for MoMLV-induced fusion of myoblasts. In summary, the examined mutations affect F_{max}, which appears to reflect a fusion step occurring after binding but before or during endocytosis.

The requirements for fusion were analyzed. Fusion was low for a series of different conditions or mutants: virus propagated in different cell lines, and certain arginine/lysine and glycan mutants of gp70. In all cases, the glycosylation of gp70 was apparently altered.

Because of heterogeneous glycosylation, gp70 molecules are not equally charged. Thus, 12 or more isoforms are seen in two-dimensional gels (9). Sialic acid residues account for most of the unequally distributed charges on gp70, as neuraminidase changes gp70 to one or few isoforms with a pI of at least 7.6 (9). From the sequence of gp70 (36) and the pK values of the amino acids, the pI of deglycosylated and reduced gp70 is estimated to be 7.8. By comparison of this value with the pIs of the isoforms, it is estimated that gp70 molecules contain in the range of 4 to 16 sialic acid residues (calculations not shown). For ecotropic MLVs, the C-terminal part of gp70 contains most glycans (often five), contains the most sialic acids, and is, as expected, most acidic (31). The N-terminal part only contains few glycans (often two), the second of which appears to be a high-mannose structure. The N terminus is, as expected, more basic (19, 31).

The same patterns of low amounts of basic gp70 isoforms were observed both for virions propagated in SC-1 cells and for the low-fusinogenic arginine/lysine mutants, indicating that the basic (presumably weakly sialated) isoforms are important for fusion. It is conceivable that a certain charge distribution on gp70 is important for fusion, as the charges of Polybrene and other polycations also appear to be essential for fusion.

Mutational analysis showed (i) that of the arginines and lysines tested, the greatest fusion defective mutants lie in or just around the proline-rich region (residues 232 and especially 249/251 and 280) and (ii) that mutants in a larger region in and around the proline-rich region showed the altered isomer pattern, with the most changes seen for K249Q/K251A and perhaps R280Q. The larger size of K249Q/K251A gp70 and

the nature of aggregation of R280Q gp70 also indicate their widely different glycosylation patterns. The role of the proline-rich region has been considered to be structural (18). Our results indicate that it also is important for glycosylation. It is known, for example, that in yeast cells, the protein conformation can affect glycan processing (41). In summary, our results show that the mutations in and next to the proline-rich region influence fusion, most likely through altering glycosylation.

The V3 loop of HIV gp120 has been shown to be important for fusion. Some of the most potent mutations were exchanges of arginine in the middle of the V3 loop with small neutral amino acids (10, 11, 12, 27). From these similar results, it can be speculated that the proline-rich region and the V3 loop serve analogous functions during fusion and infection. Whether the V3 loop has an influence on glycosylation remains to be shown.

The glycan mutants showed that glycans 5 and 7 are important for fusion, but whether these correspond to the glycans giving the altered isomer pattern is not known. Secondary effects of glycans 1 and/or 4 were furthermore seen, as the three-glycan mutant Δ1,4,7 showed higher fusion than Δ7. The influence of glycan 3 (next to the proline-rich region) and O-linked glycans (30) was not tested (in the first case because of the inviability of this mutant). Mutational removal of certain N-linked glycans from HIV gp41 (TM) gave a phenotype of low infectivity and fusion (7) similar to that observed in this study.

As mentioned in the introduction, gp70 is believed to provide a steric hindrance of the actual fusion protein p15E. It can be speculated that the charge alterations on gp70 influence its attachment to p15E and thus the steric hindrance. The fusion mutants of the glycan type fit well with this model, as glycans 5 and 7 are located in the C-terminal part of gp70, which provides the attachment to p15E (31).

Two monoclonal antibodies against the N-terminal (35/299) and C-terminal (35/56) ends of gp70 have been shown to inhibit fusion (29). 35/299 is presumably directed against the VRA region, as it distinguishes between different tropisms (5, 29). It apparently inhibits fusion by inhibiting receptor binding. In the context of the present data, 35/56 is interesting. It binds to a region masked by glycan 7, corresponding to the G_{IX} marker (8, 28, 29, 33), supporting our finding that glycan 7 is important for fusion. 35/56 presumably inhibits the same step as identified in this study.

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