

Cell Fusion Induced by the Murine Leukemia Virus Envelope Glycoprotein

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To determine whether ecotropic murine leukemia virus (MuLV) envelope glycoproteins are sufficient to cause cell-to-cell fusion when expressed in the absence of virus production, we used an ecotropic MuLV, AKV, to construct *env* expression vectors that lack the *gag* and *pol* genes. The rat cell line XC, which undergoes cell-to-cell fusion upon infection with ecotropic MuLV, was transfected with wild-type *env* expression vectors, and high levels of syncytium formation resulted. Transfection of the murine cell line NIH 3T3 with expression vectors containing the wild-type or mutated *env* region did not result in syncytium formation. Immunoprecipitation analysis of the envelope glycoproteins expressed in NIH 3T3 and XC cells showed that the mature surface glycoprotein expressed in XC cells was of a much lower apparent molecular weight than that expressed in NIH 3T3 cells. Further characterization showed that most if not all of this difference was the result of differences in glycosylation. Finally, site-directed mutagenesis was used to introduce several conservative and nonconservative changes into the amino-terminal region of the transmembrane protein. Analysis of the effect of these mutations confirmed that this region is a fusion domain.

Retroviruses are enveloped viruses that carry virally encoded glycoprotein spikes on their surfaces. These envelope glycoproteins (*env*-gp) are synthesized as polyprotein precursors (*pre-env*) that are cleaved by an unidentified cellular protease(s) into the mature surface protein (SU) and transmembrane protein (TM) (39). *Pre-env*, SU, and TM of murine leukemia viruses (MuLVs) have apparent molecular masses of 85, 70, and 15 kDa, respectively (38). Retroviral *env*-gp are critically involved in the entry of the viruses into host cells, since they recognize and bind cell surface receptors and are required for fusion of the viral envelope and host membranes. The fusion event can take place at the cell surface or in cellular vesicles following endocytosis of the virion. In either case, the fusion of cellular and viral membranes deposits the nucleocapsid into the cytoplasm of the cell. In addition to allowing viral entry, fusion mediated by viral surface proteins can, under various conditions, cause cell-to-cell fusion, resulting in the formation of multinucleate giant cells (also referred to as polykaryons or syncytia). Syncytium formation is thought to arise by at least two routes. A single virion can simultaneously fuse with two cells (fusion from without), or an infected cell that expresses viral *env*-gp on its cell surface can fuse with an adjacent cell (fusion from within) (40).

Fusion mediated by the surface proteins of orthomyxoviruses and paramyxoviruses has been studied for a number of years, with influenza virus in particular being widely regarded as the model system for viral fusion (for reviews, see references 25, 37, 40, 41). Retroviruses reported to induce cell-to-cell fusion include feline viruses (feline leukemia virus and RD-114 [18]), mouse mammary tumor virus (only at an acidic pH [31]), reticuloendotheliosis virus, an avian virus (only transiently following transfection of *env*-gp expression plasmids into dog cells [6]), and human and simian immunodeficiency viruses (see Discussion). Ecotropic

MuLVs only induce syncytium formation under altered growth conditions, such as those following the addition of amphotericin B (28), following protease treatment (3), or in certain rat cell lines (33, 42). The advent of the AIDS epidemic has resulted in increased interest in the mechanism of viral entry and cell-to-cell fusion induced by human immunodeficiency virus type 1 (HIV-1) and other retroviruses (for a review, see reference 11). While the mechanism by which viral *env*-gp induce membrane fusion has yet to be elucidated, the study of these systems has resulted in the identification of proteins involved in viral fusion (hemagglutinin of influenza virus and gp41 of HIV-1) as well as the characterization of functional domains of these proteins (4, 7-9, 14, 22). Additionally, this previous work has identified several general properties of viral entry and virus-induced cell fusion. Specifically, a correlation between the route of viral entry and conditions necessary for syncytium formation has been noted. Viruses, such as HIV-1, that enter cells via a pH-independent route are able to induce syncytia at a neutral pH, but those, such as influenza virus, that enter via a low-pH-dependent pathway (endosomal route) require a low pH to induce syncytium formation (16, 37).

To study the route of MuLV entry, Anderson and Nexø (2) used drugs that alter the pH of endosomal vesicles. This study showed that the entry of MuLVs into murine SC-1 cells is inhibited by lysosomotropic agents, suggesting a low-pH requirement for MuLV cell entry. A study with a mouse (*Mus dunni*) tail skin cell line suggested that MuLV entry is inhibited by a low pH (29). Recently, another study with lysosomotropic agents concluded that MuLV entry is pH dependent in several murine cell lines but that in the rat cell line XC, MuLV entry is pH independent, suggesting that alternate routes can be used for cell entry (26). MuLVs are capable of causing fusion from without in several rat cell lines, even when the virions have been rendered noninfectious, and this fusogenic function can be blocked by the addition of antisera specific for the *env*-gp (43, 44). Since most MuLV studies that use fusion as an assay use replication-competent virus, it is not possible to conclude from

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them whether MuLVs can induce fusion from within. Reports of a defective MuLV that expresses a fully functional env-gp (35) and is capable of fusing XC cells only in the presence of a helper virus (32) and of a temperature-sensitive Moloney MuLV that is capable of initiation of virus budding but not particle release or syncytium formation at the nonpermissive temperature (43) are consistent with an inability of ecotropic MuLVs to cause fusion from within. However, virus-induced cell-to-cell fusion was only an assay used in these studies of complex cell systems and not the focus of investigation, so this inference may not be valid.

To analyze MuLV-induced fusion, we exploited the ability of many ecotropic MuLVs to cause syncytium formation at a neutral pH in XC cells. In this study, we investigated the fusion properties of the env-gp of the ecotropic MuLV AKV, an endogenous virus isolated from AKR mice (24), by using a virus-free fusion assay to characterize the effect of mutations in a region of TM that has been identified as a putative fusion domain. We also investigated differences in the env-gp expressed in NIH 3T3 (murine) and XC (rat) cells. Our results show that the expression of the AKV env-gp in the absence of other viral proteins is sufficient for induction of syncytium formation (fusion from within) in XC cells but not in NIH 3T3 cells. We show that mutations within the putative fusion peptide of AKV alter the fusogenicity of the env-gp, suggesting that the amino-terminal region of TM is a fusion domain. We have also detected a large difference in the apparent molecular weight of SU in XC cells versus NIH 3T3 cells and show that the difference is the result of differences in glycosylation in the two cell types.

MATERIALS AND METHODS

Plasmids. Recombinant DNA techniques were carried out by standard methods (34) with enzymes obtained from either Boehringer Mannheim or New England BioLabs. AKV envelope expression plasmids were constructed by blunting the ends of a 2.9-kb *NcoI-BssHII* fragment of p623 (19), containing the *env* gene, with T4 DNA polymerase. The *env*-containing fragment was fused to the long terminal repeat (LTR) promoter of Moloney MuLV by insertion into the unique *SalI* site of pDol (21), which had also been blunted by T4 DNA polymerase treatment. The *env*-containing fragment was also fused to the human cytomegalovirus (HCMV) immediate-early promoter-enhancer by insertion into the unique *XhoI* site of p763 (27). The approximately 500-bp *KpnI-XbaI* fragment of p623, which spans the precursor cleavage site, was cloned into M13mp18 as a substrate for mutagenesis. Site-directed mutagenesis was carried out with *dut ung Escherichia coli* RZ1032 for the preparation of uracil-containing M13 single-stranded DNA templates (23). Oligonucleotides for mutagenesis were obtained from Genosys Biotechnologies. Following mutagenesis, the approximately 300-bp *ScaI-XbaI* internal fragment used to reconstruct *env* expression vectors containing mutations was sequenced (Sequenase sequencing kit [United States Biochemicals] or T7 sequencing kit [Pharmacia]) to confirm the presence of the desired mutation and the absence of unintended changes.

Cells and virus. NIH 3T3 cells were grown in Dulbecco modified Eagle medium (DMEM) with a high glucose concentration and supplemented with penicillin-streptomycin, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.3), sodium pyruvate (GIBCO Laboratories, Grand Island, N.Y.), and 5% calf serum (HyClone, Logan, Utah). XC cells were cultured in McCoy 5a medium supplemented

with 5% fetal bovine serum. Cells were maintained in 5% CO₂ at 37°C. Sixty-millimeter plates containing 5 × 10⁵ to 8 × 10⁵ cells were transfected with 15 μg of DNA per plate by the calcium phosphate method (17), including a 4-min shock with a 15% glycerol solution to increase efficiency (20). The optimal transfection efficiency was achieved by (i) replacing McCoy 5a medium (XC cultures only) with DMEM prior to transfection (McCoy 5a medium forms excessive precipitates after the addition of the calcium phosphate-DNA mixture), (ii) allowing the DNA-calcium phosphate mixture to incubate on the cells for at least 6 and up to 8 h, (iii) carefully timing the glycerol shock, and (iv) rinsing the cells extensively but gently following the shock. At 2 to 3 days posttransfection, XC cultures used for fusion assays were fixed and stained, and 20 fields were scored microscopically for syncytia (cells containing four or more nuclei). AKV stocks were obtained by transfecting NIH 3T3 cells with proviral clone p623 (24), passaging the cells for several weeks to allow viral spread, and determining the titer of the virus pools by the UV XC assay (33). The titer of the virus pools was approximately 5 × 10⁵ PFU/ml.

Cell labeling and immunoprecipitations. Cell labeling and immunoprecipitations were carried out as previously described in detail (10). Approximately 48 h posttransfection or postinfection, the medium was changed to RPMI 1640 lacking methionine or cysteine (supplemented with 10% dialyzed fetal bovine serum) and cells were labeled with 250 μCi of [³⁵S]methionine or 250 μCi of [³⁵S]cysteine (Amersham) for 12 to 16 h. Labeling to detect the incorporation of L-[5,6-³H]fucose, D-[6-³H(N)]glucosamine hydrochloride (NEN DuPont), or D-[2-³H]mannose (Amersham) was carried out in complete RPMI 1640 supplemented with 10% dialyzed fetal bovine serum for approximately 8 h. Cells were then lysed, and the lysates were immunoprecipitated (twice if the background was too high) with polyclonal goat anti-SU (anti-gp70) antisera (National Cancer Institute lot 79S-00834) and formalin-fixed *Staphylococcus aureus* (Boehringer). The precipitates were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels, which were treated with En³Hance (NEN DuPont) and fluorographed. Note that under these conditions, the anti-SU antisera recognized and precipitated both pre-env and SU but not TM. Endoglycosidase H and *N*-glycosidase F were obtained from Boehringer and were used in accordance with the instructions of the manufacturer. When used, the mannosidase inhibitor deoxymannojirimycin (DMM; Boehringer) was added to a final concentration of 1 mM prior to the addition of label.

RESULTS

The MuLV env-gp induces fusion from within. A number of laboratories have reported that expression of the HIV-1 or simian immunodeficiency virus env-gp is sufficient for induction of syncytium formation in the absence of expression of other retroviral structural genes (4, 7-9, 22). We investigated whether ecotropic MuLV env-gp are capable of causing syncytium formation in the absence of free virus. Expression vectors were generated (as described in Materials and Methods) to express the wild-type AKV env-gp, but not other viral proteins of AKV, from the Moloney MuLV LTR promoter or the immediate-early promoter-enhancer of HCMV. Additionally, expression vectors were constructed by use of the *env* region from pEF38, which was shown previously to be fusion defective as a result of a single amino acid substitution at the pre-env cleavage site (10). The expression vectors and proviral clones were separately

TABLE 1. Syncytium formation in cultures of XC cells transfected with *env*-expressing vectors

DNA transfected	Expression vector	<i>env</i> region	Relative no. of syncytia formed ^a in expt:		
			1	2	3
pJJ422	HCMV	Wild type	100	100	100
pJJ426	HCMV	Cleavage and fusion defective ^b	<1	4	2
pJJ412	pDol (MuLV LTR)	Wild type	33	82	47
pJJ416	pDol (MuLV LTR)	Cleavage and fusion defective ^b	<1	2	<1
pJJ450	Proviral clone	Wild type	12	17	17
pJJ449	Proviral clone	Cleavage and fusion defective ^b	<1	3	2
None (mock)		None	<1	3	2

^a In each experiment, the score from the plates with the largest number of syncytia (pJJ422, containing the HCMV promoter-enhancer driving the expression of wild-type AKV *env*) was set at 100, and the number of syncytia detected in the other cultures was expressed relative to this value. A total of 637, 318, and 502 syncytia per μg were detected in cultures transfected with pJJ422 in experiments 1, 2, and 3, respectively.

^b The *env* region used for these constructions was derived from pEF38 (10).

transfected into XC cells. At 2 to 3 days posttransfection, cells were fixed and stained and syncytia were counted (Table 1). Cell-to-cell fusion was observed in the XC cell cultures transfected with plasmids and proviral clones containing the wild-type *env* region but not in those transfected with vectors expressing the fusion-defective *env* region. The highest level of fusion was observed in cultures transfected with plasmids containing the HCMV promoter-enhancer driving the wild-type *env* gene. Fewer but significant numbers of syncytia were observed in cultures transfected with the MuLV LTR expression vector or the AKV proviral clone. The absolute numbers of syncytia in cultures transfected with the expression vectors containing the wild-type *env* gene varied, but the HCMV expression vector always yielded the largest number of syncytia, the LTR expression vector yielded an intermediate number, and the proviral clones yielded the smallest number. The level of syncytium formation observed in cultures transfected with expression vectors or proviral DNA clones containing the fusion-defective pEF38 *env* region was comparable to the background level observed in mock-transfected cultures (Table 1). Further experiments comparing the three types of expression vectors or various pairwise combinations of the expression vectors revealed the same relative efficiency of syncytium induction as the results presented in Table 1. Attempts to transfer the "fusogenic agent" by transferring the medium from transfected cultures to fresh cultures of XC cells at various times after transfection were unsuccessful (data not shown). This result is consistent with the idea that virus particles or free *env*-gp are not responsible for the syncytium formation observed in these experiments. These results suggest that the expression of fusion-competent MuLV *env*-gp in the absence of other viral proteins is sufficient for the induction of syncytia in XC cells. Thus, MuLV *env*-gp are capable of causing fusion from within. Although immunoprecipitation experiments showed that *env*-gp were expressed in NIH 3T3 cells (data not shown), syncytium formation above background levels was not observed in cultures of NIH 3T3 cells transfected with the expression vectors.

The pattern and extent of SU glycosylation are different in XC and NIH 3T3 cells. To demonstrate directly that *env*-gp are expressed in XC cells, we used polyclonal goat anti-SU antisera to immunoprecipitate pre-*env* and SU from XC cells. The mature SU (gp70) immunoprecipitated from XC cells migrated at an apparent molecular mass of approxi-

mately 50 to 55 kDa on SDS-polyacrylamide gels, rather than the 70 kDa usually observed for murine cell lines (see below). As mentioned above, ecotropic MuLV *env*-gp are able to induce syncytium formation in rat cell line XC but not in most murine cell lines under normal culture conditions, so this difference in apparent molecular mass of SU in the XC cells versus the murine cell line NIH 3T3 was particularly interesting. The difference in the apparent molecular mass of SU isolated from the two cell types could result from differences in the proteolytic processing of pre-*env* or from differences in the glycosylation of SU. Differences in the pattern of complex glycosylation of a polytropic MuLV *env*-gp in NIH 3T3 cells versus mink lung cells have been reported (15); however, the SU isolated from mink lung cells in that study was still approximately 70 kDa in mass. We postulated that the large difference in molecular mass that we observed might contribute to the increased fusogenicity of XC cells, so we further characterized SU in XC and NIH 3T3 cells.

For qualitative characterization of wild-type *env*-gp in XC and NIH 3T3 cells, we used high-titer AKV pools for infection of the cultures because infection with replication-competent virus pools is more efficient than transfection and results in higher protein yields following immunoprecipitation. We confirmed that the SUs observed in cell lysates were the mature proteins being released from the cells by immunoprecipitating the proteins in the medium of radiolabeled infected XC or NIH 3T3 cultures with anti-SU antisera. We found that the 50- to 55-kDa protein expressed in XC cells accounts for the vast majority of detectable anti-SU antiserum-reactive protein released (data not shown), so we conclude that it is the mature SU produced in this cell type.

Cultures of XC or NIH 3T3 cells were mock infected or infected with high-titer AKV pools and immunoprecipitated with anti-SU antisera as described in Materials and Methods. The precipitates were incubated with or without endoglycosidase H prior to SDS-polyacrylamide gel analysis and fluorography. Pre-*env* from XC cells but not NIH 3T3 cells was partially resistant to endoglycosidase H (data not shown). Since endoglycosidase H cleaves high-mannose side chains but not hybrid or complex side chains, this result suggests that the pattern of glycosylation in the two cell types is different. This analysis was repeated with *N*-glycosidase F, which is able to remove a broader range of oligosaccharides from glycoproteins. Following *N*-glycosidase F digestion, pre-*env* and SU from both cell types

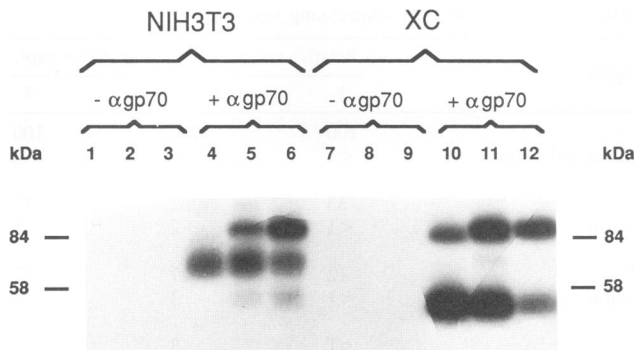


FIG. 1. env-gp produced in XC or NIH 3T3 cells labeled with tritiated sugars. Parallel cultures of XC or NIH 3T3 cells were infected with high-titer stocks of AKV, labeled with tritiated fucose, glucosamine, or mannose, lysed, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Specific bands were detected in the lanes corresponding to NIH 3T3 or XC cell lysates immunoprecipitated with goat anti-SU antisera (lanes 4 to 6 and 10 to 12, respectively). Cultures of either NIH 3T3 or XC cells were labeled with tritiated fucose (lanes 1, 4, 7, and 10), tritiated glucosamine hydrochloride (lanes 2, 5, 8, and 11), or tritiated mannose (lanes 3, 6, 9, and 12). Lanes 1 to 3 and 7 to 9 correspond to lysates from NIH 3T3 and XC cells, respectively, that were immunoprecipitated with nonspecific normal goat serum.

migrated significantly faster on SDS-polyacrylamide gels (data not shown), indicating the release of oligosaccharides. Pre-env from both cell types comigrated at approximately 70 kDa, as expected. While *N*-glycosidase F-treated SU from both cell types migrated faster than untreated SU, SU from XC cells still migrated slightly faster than SU from NIH 3T3 cells. It is possible, however, that some oligosaccharides on SU isolated from NIH 3T3 cells are resistant to *N*-glycosidase F digestion. Further characterization of glycosylation patterns was done by immunoprecipitating lysates from parallel infected cultures of XC or NIH 3T3 cells labeled for approximately 8 h with the tritiated sugar L-[5,6-³H]fucose, D-[6-³H(N)]glucosamine hydrochloride, or D-[2-³H]mannose (Fig. 1). Immunoprecipitations with anti-SU antisera of lysates from XC or NIH 3T3 cultures labeled with either tritiated glucosamine hydrochloride (lanes 5 and 11) or tritiated mannose (lanes 6 and 12) showed that both pre-env and SU incorporated the label, indicating the presence of this sugar on both forms in both cell types. Consistent with previous reports (10), tritiated fucose was not detected in the pre-env (85-kDa) band in lysates isolated from NIH 3T3 cells (lane 4). In contrast, fucose was incorporated into pre-env in XC cells (lane 10), showing that the two cell types do have different patterns of glycosylation. The similarity in the apparent mass of the pre-env band (85 kDa) in both cell types and the large difference in the apparent mass of the SU band (70 kDa in NIH 3T3, lanes 4 to 6, or 50 to 55 kDa in XC, lanes 10 to 12), apparent in Fig. 1, are discussed further below.

We also examined the env-gp produced in the presence of DMM. DMM prevents mannosidases from trimming the high-mannose side chains of glycoproteins, thereby preventing complex glycosylation (12, 13). Parallel infected or mock-infected cultures of XC and NIH 3T3 cells were incubated in the presence or the absence of DMM. [³⁵S]cysteine was added to all the cultures, which were then incubated overnight. Immunoprecipitations of lysates obtained from the cultures were carried out with anti-SU antisera, and

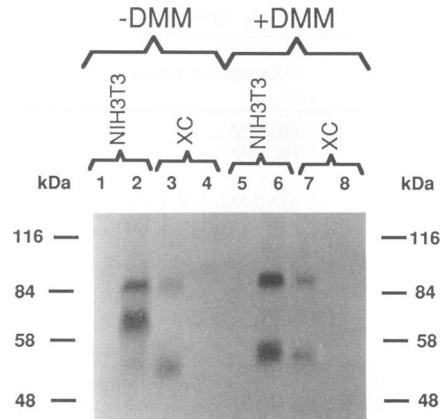
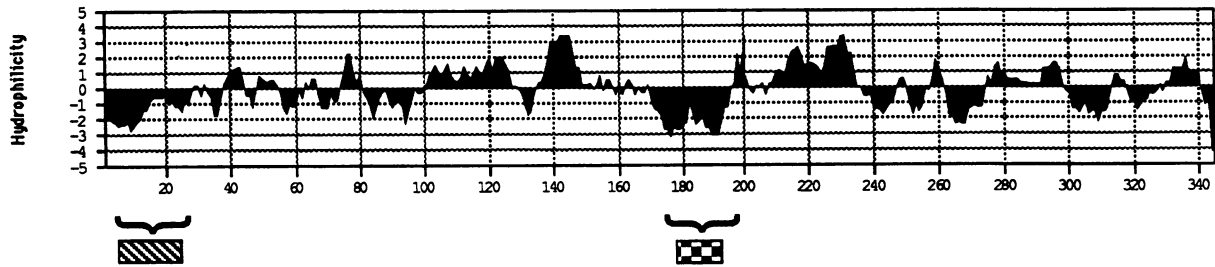


FIG. 2. Effect of DMM on the apparent molecular mass of env-gp expressed in XC or NIH 3T3 cells. Parallel XC or NIH 3T3 cell cultures were mock infected or infected with concentrated stocks of AKV. Cells were labeled with [³⁵S]cysteine and incubated overnight in the absence (-DMM) or the presence (+DMM) of the mannosidase inhibitor DMM. Proteins were analyzed as described in the legend to Fig. 1. Immunoprecipitated material from infected NIH 3T3 or XC cells incubated in the absence of DMM (lanes 2 and 3, respectively) and immunoprecipitated material from infected NIH 3T3 or XC cells incubated in the presence of DMM (lanes 6 and 7, respectively) are shown. Comigration of the SU bands derived from cultures incubated with DMM (lanes 6 and 7) suggests that the difference in the masses of the bands under normal conditions (lanes 2 and 3) is due to differences in glycosylation. No bands were detected in the lanes corresponding to lysates from mock-infected NIH 3T3 cells (lanes 1 and 5) or XC cells (lanes 4 and 8), regardless of the presence or the absence of DMM in the cultures.

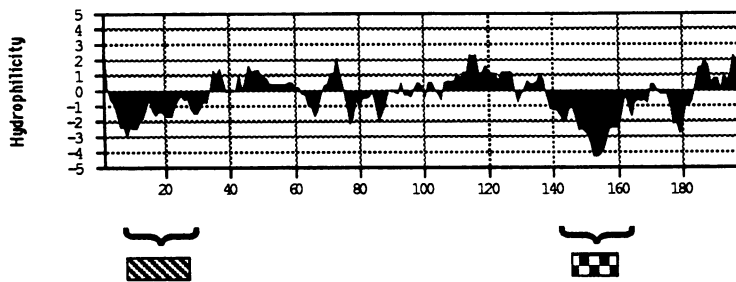
fluorograms were obtained (Fig. 2). Immunoprecipitation analysis of cultures incubated in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of DMM showed that no proteins were detected in uninfected cultures (lanes 1, 4, 5, and 8). The proteins immunoprecipitated from the AKV-infected NIH 3T3 cell cultures incubated in the absence of DMM showed the expected pattern, pre-env and SU at approximately 85 and 70 kDa, respectively (lane 2). The proteins immunoprecipitated from the parallel AKV-infected XC cell cultures incubated in the absence of DMM migrated to 85 kDa and approximately 50 to 55 kDa (lane 3), as described above. In contrast, the proteins from the AKV-infected NIH 3T3 or XC cultures incubated in the presence of DMM (lanes 6 and 7) comigrated at approximately 85 and 57 kDa (pre-env and SU, respectively). These results confirm that the difference in the apparent molecular mass of SU in XC versus NIH 3T3 cells is due mostly, if not completely, to differences in the pattern and extent of glycosylation in the two cell types. Since the mass of SU expressed in AKV-infected XC cells in the absence of mannosidase trimming (+DMM, lane 7) is higher than that in untreated AKV-infected XC cells (-DMM, lane 3), SU expressed in XC cells is probably minimally glycosylated under normal conditions.

Development of an env-gp assay system. The direct induction of syncytium formation in XC cells by transfection with a construct that expressed the AKV env-gp allowed us to assess the effect of mutations in the putative fusion peptide of the env-gp. The predicted positioning of the putative fusion peptide of AKV is based on the relative position and general hydrophobic nature of this region compared with those of the fusion peptides of other fusogenic viruses (Fig. 3A) (11 and references therein). As described in Materials

A HIV-1 HXB2



MuLV AKV



B

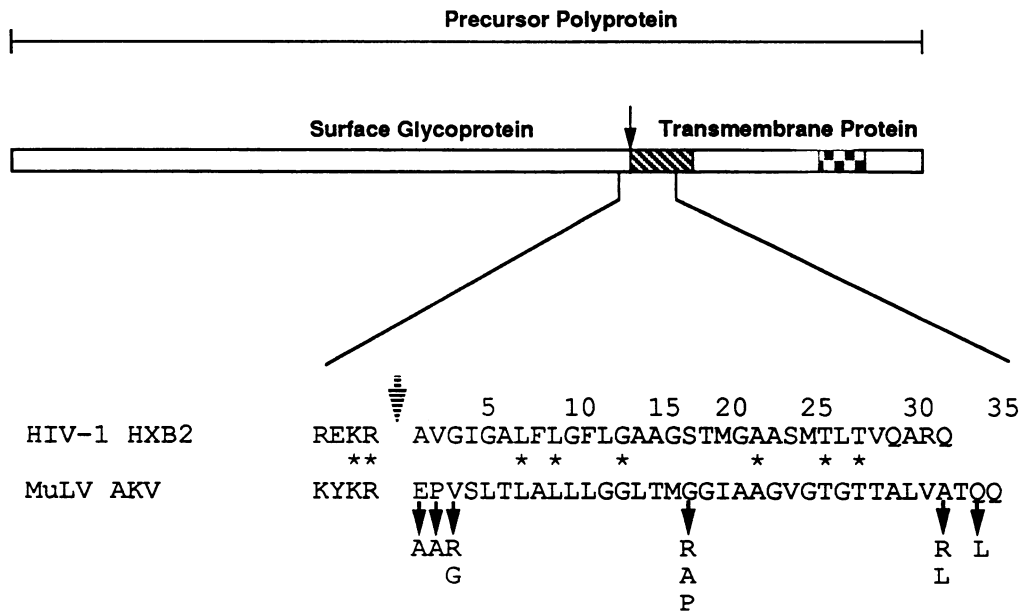


FIG. 3. Comparison of TMs of HIV-1 HXB2 and MuLV AKV and mutations introduced into the putative fusion peptide of AKV. (A) Hydrophobicity profiles of the TMs of HIV-1 HXB2 and MuLV AKV are shown to emphasize, as summarized by Freed and Risser (11), that charge profile analysis of these proteins has identified two regions that contain hydrophobic amino acids and that correspond to the putative fusion peptide (hatched boxes) and the transmembrane domain (checkered boxes). (B) Alignment of the amino acid sequences of the putative fusion peptides of the env-gp of AKV and HIV-1 indicates that little or no sequence identity exists. The protein sequence corresponding to the SU-TM cleavage site (indicated by the striped arrow) and the single-letter symbols corresponding to the amino acids at the amino terminus of the TMs of a representative HIV-1 isolate (30) and AKV are shown. Identical residues are emphasized by inclusion of an asterisk between the sequences. Although there is relatively little sequence conservation, the regions extending carboxy terminal to the cleavage point are generally hydrophobic, and no charged residues are present, with the exception of the first amino acid of the TM of MuLV AKV, which is glutamic acid (E). A series of conservative and nonconservative mutations were introduced into the AKV TM by site-directed mutagenesis (indicated by the solid arrows and single-letter amino acid symbols below the AKV sequence). The mutations are identified by the amino acid position in mature TM followed by the single-letter code of the amino acid in the wild-type env-gp (deduced from the published nucleotide sequence of p623 [19]) and the single-letter code of the amino acid in the mutated construct. For example, the glutamic acid residue at position 1 was changed to an alanine residue, and the mutation is referred to as 1EA.

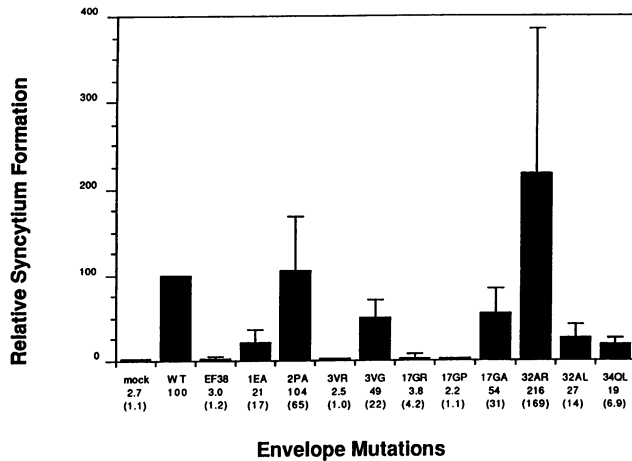


FIG. 4. Relative syncytium formation in XC cells transfected with AKV env-gp expression vectors containing wild-type (WT) and mutated *env* genes. Expression constructs were generated by placing the *env*-containing *NcoI*-*Bss*III fragment of AKV proviral clone p623 under the control of the HCMV promoter-enhancer. Following transfection of the expression plasmids into XC cells, env-gp-induced XC cell fusion was assayed in the absence of the *gag* and *pol* gene products. Thus, viral replication and viral spread could not influence the analysis of env-gp function. Similar constructs containing mutations of the putative fusion peptide, as shown in Fig. 3, were generated. XC cell cultures transfected with the various constructs were scored for syncytium formation as described in Materials and Methods. The results of each assay were normalized by setting the score obtained for the wild type at 100. Several preparations of DNA were obtained, and at least eight assays were performed for each of the wild-type or mutated HCMV-driven expression plasmids. The error bars represent the standard deviations. Mock-transfected cells and cells transfected with an HCMV expression plasmid containing the *env* region of pEF38 (10) were included so that the level of nonspecific giant cell formation could be monitored. The relative numeric scores, with standard deviations in parentheses, are shown along the *x* axis. The average number of syncytia per microgram in cells transfected with the wild-type *env*-containing expression plasmid DNA was 622.5 ± 324.8 .

and Methods, directed mutations were introduced at several positions in the putative fusion peptide of the AKV env-gp (Fig. 3B). In general, the mutations were intended to change the hydrophobic nature of the region; however, conservative changes were also introduced at several positions. Since the largest number of syncytia was observed with the HCMV promoter-enhancer *env* combination, the mutated *env* genes were cloned into the HCMV promoter-enhancer expression vector (Table 1). Fusion assays were carried out as described above (Fig. 4).

Mock-transfected cultures as well as those transfected with the expression vector containing the pEF38 *env* region, which was previously shown to be fusion defective because of a cleavage defect (10), showed background levels of syncytium formation (Fig. 4). A nonconservative substitution at position 1 (1EA) made the region more hydrophobic and thus more like the fusion peptides of other fusogenic viruses. This mutation, however, reduced syncytium formation somewhat. We also made a change at position 2 that was subtle with respect to charge but might be expected to alter secondary structure (2PA). As shown in Fig. 4, mutation 2PA conferred essentially wild-type levels of syncytium formation. The introduction of a highly charged arginine

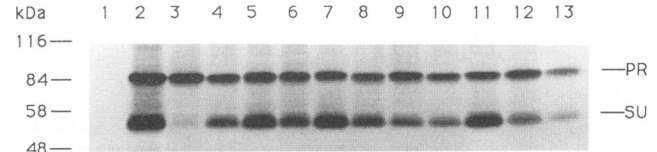


FIG. 5. Immunoprecipitation of lysates from XC cells transfected with AKV env-gp expression vectors containing wild-type and mutated *env* genes. Cells were incubated with [³⁵S]cysteine overnight and lysed. Lysates were immunoprecipitated twice with anti-SU antisera, resolved by SDS-polyacrylamide gel electrophoresis, and fluorographed. The lanes correspond to XC cell cultures transfected with expression vectors containing the following *env* genes: 1, mock transfection; 2, wild type; 3, EF38; 4, 1EA; 5, 2PA; 6, 3VR; 7, 3VG; 8, 17GR; 9, 17GP; 10, 17GA; 11, 32AR; 12, 32AL; and 13, 34QL.

residue at position 3 or 17 (3VR or 17GR, respectively), as well as the introduction of proline at position 17 (17GP) resulted in background levels of syncytium formation. More subtle mutations at these positions (3VG and 17GA) resulted in modest reductions in syncytium formation, compared with wild-type levels. Transfection of XC cells with mutant 32AR resulted in levels of fusion comparable to or higher than those observed with the wild type. A conservative mutation at this position (32AL) resulted in a moderate decline in syncytium formation, as did a change from a polar to a hydrophobic residue at position 34 (34QL).

To confirm that these results were not due to gross alterations in the level of expression of the env-gp or to defects in proteolytic cleavage or glycosylation, we immunoprecipitated proteins from transiently transfected, [³⁵S]cysteine-labeled XC cultures with anti-SU antisera. The result of a representative experiment is shown in Fig. 5. The *env* expression constructs expressed comparable levels of env-gp (lanes 2 to 13), and the fluctuations in the amounts of protein detected in various lanes for this experiment and two repeat experiments do not seem to be reproducible. Additionally, the fluctuations in syncytium formation and pre-env or SU immunoprecipitated in a given experiment do not seem to be correlated, so we conclude that the fluctuations are not significant. With the exception of the EF38 env-gp, which, as expected, was not proteolytically cleaved (lane 3), all pre-env and SUs comigrated, indicating that proteolytic processing and glycosylation were not significantly affected by the introduction of the TM point mutations. It is possible that 32AR (lane 11), which showed high levels of fusion (Fig. 4), reproducibly exhibits a slightly elevated ratio of SU to PR. It is also formally possible that some of the mutations affect SU-TM association or receptor binding. The cell surface expression of the env-gp containing point mutations potentially could have been compromised; however, since a cleavage-defective AKV env-gp was incorporated into virus particles only fourfold less efficiently than the wild-type env-gp (10) and we presented biochemical evidence that protein production was not grossly compromised in our experiments, a defect in cell surface expression seems unlikely. Thus, the measured level of syncytium formation for the various mutant env-gps is likely to reflect the specific alteration of a fusion function rather than a more general perturbation of protein processing or maturation or a gross alteration of secondary structure caused by the introduction of the mutations. These results are consistent with the idea that this region plays a role in cell-to-cell fusion induced by MuLV env-gp.

DISCUSSION

In this study, we directly addressed the ability of the env-gp of MuLV AKV expressed on the cell surface to induce syncytium formation (fusion from within). env-gp expression vectors were constructed so that the other viral proteins would not be expressed. We showed that transfection of cultures of permissive (XC) cells with vectors that express *env* (but not the other viral genes) resulted in efficient syncytium formation. Similar experiments with NIH 3T3 cells, which are nonpermissive for the induction of syncytium formation by ecotropic env-gp under normal culture conditions, did not result in the induction of syncytia, consistent with the results of previous studies (32, 35). We were unable to transfer a fusogenic agent between cultures of susceptible cells by transferring the culture medium; therefore, virus particles or released env-gp were probably not responsible for the syncytium formation that we observed. In our experiments, XC cell syncytia were induced by the expression of the AKV env-gp at a neutral pH. Since virus-induced syncytium formation at a neutral pH is correlated with pH-independent cell entry, our results offer indirect support, derived from an alternative experimental approach, for the conclusion of McClure et al. (26) that the route of entry is pH independent in XC cells.

A virus-free MuLV fusion assay system was developed and used to characterize a proposed functional domain of the MuLV AKV env-gp. We believe that this system is superior to systems that use replication-competent virus for the characterization of *env* functions. Introduced *env* mutations may affect many aspects of the viral replication cycle, such as viral spread, resulting in an amplification of the differences detected between wild-type and mutated clones. Additionally, the high rate of mutation associated with retroviral replication (5) results in the rapid reversion of a point mutation at the SU-TM junction of *env* in a closely related virus (36). We used the virus-free assay system to characterize the effects of several mutations in the putative fusion peptide of the TM portion of the AKV env-gp. Several nonconservative changes in this region resulted in the loss of fusion activity. A relatively small reduction in syncytium formation was observed following the introduction of conservative changes in this region. These results are consistent with the results reported for mutations in the fusion peptides of simian immunodeficiency virus and HIV-1 (4, 7, 9). A series of polar residues are located adjacent to the fusion peptide of HIV-1. An alteration in this region has been shown to affect fusion induced by the HIV-1 env-gp (9). We report here that several mutations in the corresponding region of the MuLV env-gp reduced but did not abolish fusion activity. Interestingly, a radical change in this region (32AR) resulted in levels of fusion at least as high as those observed for wild-type cultures. Since the amino-terminal hydrophobic region of AKV TM is quite long, this mutation may have simply shifted the position of the polar border without compromising fusion activity. Finally, we present biochemical evidence that protein production was not grossly compromised in our experiments. Our data support the identification of this series of hydrophobic amino acids at the amino terminus of p15E as the fusion peptide of AKV.

Examination of env-gp expressed in transfected XC cells indicated that mature SU expressed from these cells was much smaller than expected. We showed that the level and pattern of glycosylation of the AKV env-gp were different in XC and NIH 3T3 cells. We also showed that the difference in the apparent molecular mass of SU in the two cell types was

largely, if not entirely, due to differences in glycosylation rather than differential proteolytic processing.

The observed correlations between the decreased glycosylation of MuLV env-gp and the increased induction of syncytium formation in XC cells and between the increased glycosylation of MuLV env-gp and the decreased fusogenicity in NIH 3T3 cells are intriguing, particularly in the context of several previous reports on MuLV-induced cell fusion. Anderson (1) reported that a brief trypsin treatment of virions resulted in the release of peptides and "high molecular weight material containing glucosamine." Protease treatment enhanced syncytium formation in a cell line that normally did not show ecotropic MuLV-induced cell fusion, leading to the speculation that the proteolytic cleavage of viral surface components during cell entry plays a role in infection (3). MuLV pools isolated from murine cell lines that were rendered noninfectious by UV irradiation could still induce syncytium formation in XC cell cultures (43, 44). It is tempting to speculate that some cell lines, such as XC, may express a cell surface enzyme, such as a protease or a glycosylase, that could modify MuLV env-gp and thereby enhance its fusogenicity. However, many other factors could be responsible for the ability of ecotropic MuLV env-gp to induce syncytium formation in XC cells but not most murine cell lines under normal conditions. These include differences in cell membrane composition, level of the viral receptor expressed at the cell surface, levels of other interfering cell surface components, the presence of other modifying enzymes on the surface of XC cells but not NIH 3T3 cells, or species-specific differences in the sequence or modification of the receptor in the two cell types. Further work is required to determine exactly what causes (or perhaps allows) fusion to occur in XC cells.

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