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Requirement of N-Terminal Amino Acid Residues of gp41 for Human Immunodeficiency Virus Type 1-Mediated Cell Fusion

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An expression vector was designed to test the structural requirements of the gp41 N terminus for human immunodeficiency virus type 1-induced membrane fusion. Mutations in the region coding for the N terminus of gp41 were found to disrupt glycoprotein expression because of deleterious effects on the Rev-responsive element (RRE). Insertion of an additional RRE in the 3′-noncoding sequence of *env* made possible efficient glycoprotein expression, irrespective of the mutations introduced into the RRE in the natural location. This permitted the insertion of the unique restriction site *Spe*I within the N-terminal sequences of gp41, allowing convenient and efficient mutation of the gp41 N terminus by using double-stranded synthetic oligonucleotides. Mutants with deletions of 1 to 7 amino acids of the N terminus were constructed. Expression and cleavage of all mutants were confirmed by Western immunoblot analysis with anti-gp41 antibodies. The capability of mutants to induce membrane fusion was monitored following transfection of HeLa-T4$^+$ cell lines with wild-type and mutant expression vectors by electroporation and microinjection. The efficiency of cell-fusing activity decreased drastically with deletion of 3 and 4 amino acids and was completely lost with deletion of 5 amino acids. Cotransfection of the parent and mutant expression vectors resulted in reduced cell-fusing activity. The extent of this dominant interference by mutant glycoprotein paralleled the decrease in cell-fusing activity of the mutants alone. This suggests the existence of a specific N-terminal structure required for fusing activity. However, there does not appear to be a stringent requirement for the precise length of the N terminus. This finding is supported by the length variation of this region among natural human immunodeficiency virus type 1 isolates and is in contrast to the apparent stringency in the length of analogous N-terminal structures of influenza A virus and paramyxovirus fusion glycoproteins.

The entry of human immunodeficiency virus (HIV) into cells involves fusion of the viral envelope with the plasma membrane of the target cells. Membrane fusion requires cleavage of the inactive precursor gp160 (42) and is thought to be mediated by the hydrophobic N terminus of one of the cleavage products, i.e., the transmembrane protein gp41. Single substitutions with polar amino acids within this hydrophobic region, as well as nonconservative mutations in the neighboring polar region, reduce or abolish the capacity to induce membrane fusion (21–23). In coexpression experiments, such mutant glycoproteins dominantly interfere with wild-type glycoprotein, indicating that formation of oligomeric structures is necessary for membrane-fusing activity (22). Within the hydrophobic region, there is a tandem repeat of FLG beginning 7 or 8 residues from the N terminus depending on the virus isolate. On the basis of a homology of this motif with the very N termini of several fusion proteins of paramyxoviruses (7, 8), it has been proposed that the fusogenic site of HIV is located 7 or 8 amino acids from the proteolytic cleavage site (24, 26). This hypothesis is further based on the high degree of sequence conservation within the FLG motifs, contrasting with the high degree of sequence variation in the first 7 or 8 amino acids (44). It has been found that the efficiency in membrane fusion is not affected by such variations in natural isolates (1a). However, insertion of 4 to 6 amino acids into this region strongly interferes with the ability to induce syncytia (35, 36).

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The coding region of the gp41 N terminus also serves as the 5′ part of a recognition site for the transacting protein Rev, i.e., the Rev-responsive element (RRE). The RRE has been mapped to a region that comprises 240 nucleotides (nt) and has the propensity to form extensive secondary structure (9, 12, 40). The interaction of Rev and the RRE has been shown to regulate the expression of the *env* gene at the posttranscriptional level (12, 19, 28, 29, 40, 48, 56). For Rev-mediated transport to the cytoplasm of unspliced and singly spliced mRNAs possessing the RRE (18, 20, 34, 39, 40), direct binding of Rev to the RRE is required (40, 48). Although transactivation is possible with a minimal RRE covering less than 90 nt (10, 30, 33), its efficiency can be reduced by mutations in the flanking regions (9, 13, 14, 30, 33, 41, 44). The secondary structure of the Rev-binding domain has been reported to be more important than the nucleotide sequence (41, 44). However, extensive regions of sequence specificity have recently been identified throughout the RRE (13). Thus, mutations directed against the N terminus of gp41 concomitantly can be deleterious to the function of the RRE, obviating the analysis of mutational effects on the fusogenic properties of gp41.

On the basis of the above findings, we developed an *env* expression vector with an additional RRE downstream of the *env* gene that allows the introduction of mutations within the coding region of the gp41 N terminus despite their inhibitory effect on the natural RRE. Using this construct, we obtained a series of deletion mutants to assess the length of the hydrophobic N terminus of gp41 required for the induction of membrane fusion. The results indicate that deletions lead to a gradual loss of fusing activity and that N-terminal amino acids preceding the FLG motif are indispensable for fusing activity.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were synthesized on an automated DNA synthesizer (model 381A; Applied Biosystems, Weiterstadt, Germany) with final removal of the trityl group, desalted on NAP-25 columns (Pharmacia, Freiburg, Germany), eluted with 10 mM Tris-HCl–1 mM EDTA (TE), and used without further purification.

Double-stranded DNA carrying the desired mutations covered 36 codons around the cleavage site of gp160 (13 codons of gp120 and 23 codons of gp41) and was flanked by *Sty*I and *Spe*I restriction sites. The 3′-coding region was contained in the antisense oligonucleotide 76 (5′-CAT ACT aGt TGC GCC CAT AGT GCT TCC TGC TGC TCC CAA GAA CCC tAa GAA). In this and the following oligonucleotides, restriction sites are indicated by underlining and silent mutations are indicated by lowercase letters. The 5′-coding region was provided by oligonucleotides with different codons for the N terminus of gp41 flanked by identical 5′ ends (5′-CC ACC AAG GCA AAG AGA AGA GTG GTG CAG AGA GAA AAA AGA) and by identical 16-nt stretches at the 3′ end that were complementary to the 3′ end of oligonucleotide 76. The different codons for gp41 were 79 (env0; 5′-GCA GTG GGA ATA GGA GCT TTG), 85 (env−1; 5′-GCA GTG ATA GGA GCT TTG), 87 (env−2; 5′-GCA ATA GGc GCc TTC), 89 (env−3; 5′-GCc GGA GCT TTG), 72 (env−4; 5′-GCA GCT TTG), 73 (env-5; 5′-GCA TTG), and 88 (env−6; 5′-GCA). Pairs of single-stranded oligonucleotides were converted to double-stranded DNA by a cyclic "fill-in" method based on the PCR technique. Oligonucleotides (200 pmol each) were incubated with 0.5 U of *Taq* polymerase and standard PCR reagents (49) for 30 or 35 cycles (1 min at 40°C, 2 min at 60°C, and 1 min at 91°C). Reagents were obtained from Biozym, Hameln, Germany, and Cetus-Perkin-Elmer, Überlingen, Germany. PCR products were extracted with phenol and chloroform-isoamyl alcohol, ethanol precipitated, and resuspended in TE for further enzymatic digestion with *Sty*I and *Spe*I.

For amplification of the RRE sequence, PCR primers 109 (5′-GGG CTC GAG TCT AGA TGC GCA GTG GGA ATA GGA GCT) and 108 (5′-GGC CTG CAG GGT ACC TAG GTA TCT TTC CAC) were used.

Recombinant plasmids. HIV sequences were derived from plasmid pNLA1 (58), which is a cDNA derivative of pNL4-3 (1). The nucleotide numbering system used in the following is that of the parent sequence HIVNL43 in the HIV databank of Myers et al. (43).

In construct pNLA1-RRE, an additional copy of the RRE was introduced into the *nef* coding region. RRE sequences of pNLA1 were amplified by PCR with primers 109/108, yielding nt 7747 to 7975 flanked by 5′ *Xho*I and 3′ *Kpn*I restriction sites. This fragment, the *Eco*RI(nt 5743)-*Xho*I(nt 8887) fragment of pNLA1, and pNLA1 lacking nt 5743 (*Eco*RI) to nt 9005 (*Kpn*I) were used to constitute pNLA1-RRE.

The construction of the parent vector for deletion mutants, penv0RRE, involved insertion of synthetic sequences (nt 7710 to 7816) between *Sty*I and *Spe*I and the PCR-derived sequences (nt 7817 to 8131) between *Spe*I and *Hin*dIII contained in a different subclone (pUH-L41; unpublished results). The seven deletion mutants from penv−1RRE through penv−7RRE were obtained by insertion of synthetic sequences (nt 7710 to 7816 [see above]).

DNA sequencing. All plasmid sequences whose construction involved synthetic oligonucleotides or PCR products were verified by DNA sequencing by the dideoxy-chain termination method (50). Reagents were obtained from Pharmacia (t7Sequencing Kit).

Transfection. HeLa-T4$^+$ cells (38) and HeLa-T4 pBKLTR lac cells (2) were propagated in Dulbecco's minimal essential medium (MEM) supplemented with 10% fetal calf serum and 100 μg of geneticin (Gibco) per ml and additional hygromycin B (100 μg/ml) for HeLa-T4 pBKLTR lac cells. Plasmid DNA was introduced into these HeLa cell lines by high-electric-field-mediated gene transfer (electroporation). Subconfluent monolayers were removed from 175-cm^2 culture flasks by treatment with trypsin-EDTA. For transfection, 130 μl of Eagle's MEM supplemented with 10 mM sodium bicarbonate and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (HMEM) containing 10% fetal calf serum, 3×10^6 viable cells (trypan blue exclusion) in 200 μl of HMEM, and 20 μl of DNA (1 μg/1 μl of TE) were mixed, and the cells were exposed to a single pulse with a setting of 550 V/cm and 500 μF (Bio-Rad Gene Pulser with capacitance extender). Cells were kept at room temperature for an additional 5 min and transferred to 25-cm^2 culture flasks, and 5 ml of medium supplemented with 5 mM sodium butyrate (27) was added. For immunofluorescence analysis, aliquots of 400 μl of cell suspension were grown in eight-chamber Labtek slides (Nunc). After 24 h, the medium was changed to butyrate-free medium.

β-Galactosidase syncytium assay. Forty-eight hours after transfection, HeLa-T4 pBKLTR lac cells were fixed at room temperature with acetone-ethanol (50:50, vol/vol), washed twice with phosphate-buffered saline (PBS), and incubated for 90 min at 37°C in 3 ml of staining solution (1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] per ml of PBS, 5 mM potassium ferricyanate, 5 mM potassium ferrocyanate, 2 mM $MgCl_2$). The reaction was stopped by replacing the staining solution with PBS, and syncytia were scored in a light microscope.

Western immunoblot analysis. Two days after transfection, cells were scraped from the culture flasks, sedimented at $1,000 \times g$ for 5 min at 4°C, and suspended in 300 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (37). Proteins (40-μl aliquots) were separated on a 0.1% SDS–10% polyacrylamide gel and electrotransferred to an Immobilon-P membrane (pore size, 0.45 μm; Millipore). Blots were blocked with PBS–10% bovine serum albumin (BSA)–10% Tween 20 for 1 h. The following steps were performed in PBS–1% BSA–1% Tween 20. Blots were allowed to react with two monoclonal antibodies to gp41 (NEA-9303 [DuPont], 1:100; and RV100020 [Olympus, Biomol, Hamburg, Germany], 1:100) for 1 h, washed twice, incubated with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (1:3,000; Promega) for 30 min, washed four times, and rinsed with water. The bands were visualized with the Western immunoblot ECL system on ECL hyperfilm (Amersham). The anti-Nef monoclonal antibody (NEA-9302) was obtained from DuPont and used in a 1:100 dilution.

Immunofluorescence. After 48 h, cells were fixed for 2 min at room temperature in acetone-ethanol (80:20, vol/vol). All subsequent steps were performed with PBS. Cells were washed twice and allowed to react with monoclonal antibodies to gp41 (NEA-9303 1:100) and gp120 (NEA-9305 [DuPont], 1:500) for 30 min. After two washes, the cells were reacted with goat anti-mouse immunoglobulin G $F(ab')_2$ fragments conjugated with fluorescein isothiocyanate (FITC; Sigma). Cells were washed twice and covered with PBS (pH 8.5) and a coverglass. Fluorescence was visualized with a Zeiss IM35 microscope equipped for microphotography.

Microinjection. Plasmid DNA was dissolved in PBS lacking Ca^{2+} and Mg^{2+}. Prior to microinjection, samples were mixed with equal volumes of 2% FITC-coupled dextran (molecular weight, 150,000; Sigma). Approximately 10^4 cells were seeded on glass coverslips (26 by 21 mm) in 60-mm petri dishes and used after 1 day. Microinjection was performed under phase-contrast microscopy (IM35 microscope) with a microinjector (model 5242; Eppendorf) and glass capillaries (Femtotip, Eppendorf [3, 5]). Injections were aimed at a nucleus in a small cluster of cells. Coinjection of FITC-coupled dextran permitted the identification of injected cells over observation periods of up to 5 days. In some experiments, all nuclei were additionally stained with Hoechst dye 33342 (10 μM final concentration in PBS lacking Ca^{2+} and Mg^{2+}) for 30 min at 37°C.

Average hydrophobicity values. Average hydrophobicity values were calculated with the program HELIXMEM (PC/GENE software package; IntelliGenetics, Inc., Mountain View, Calif. [17]).

RESULTS

Construction of an *env* expression vector that allows mutations within the RRE. A cloning strategy was designed to allow introduction of synthetic mutant DNA into pNLA1 by using the natural *Sty*I restriction site at codons 11 to 13 upstream from the proteolytic cleavage site and a newly introduced *Spe*I site at codons 22 and 23 downstream in gp41. The latter site was selected because it maintained the amino acids (TS) of the published sequence of the parent strain HIVNL43 (43). By subsequent sequence determination of pNLA1, codons for amino acids AS were found in this location. Thus, along with the *Spe*I site, we had introduced the mutation AS→TS into pNLA1. However, this is not likely to affect the functionality of the N terminus, because amino acids -TS- are also found in this location in natural variants of HIV, e.g., HIVSC and HIVCDC4 (43). By using these *Sty*I and *Spe*I restriction sites, a series of constructs was made including penv0, which differs from pNLA1 only with regard to the *Spe*I site at codons 22 and 23 in gp41 and a silent mutation eliminating a *Sty*I site at codons 8 to 10 of gp41. After transfection into HeLa-T4$^+$ cells, this vector consistently failed to result in syncytium formation. By Western blot analysis of transfected cells, gp41 was found in drastically reduced quantity compared with pNLA1, even though gp160 can be detected with penv0 and pNLA1 (Fig. 1A, lanes 1 and 4).

To test the possibility that low gp41 expression of penv0 was due to the disruption of the RRE by the introduced mutations, a second RRE was inserted into the *nef* gene, 3′ to the *env* gene. In the resulting plasmid, penv0RRE, the capacity for syncytium formation is restored, although with a delay compared with pNLA1, and gp41 expression is increased compared with that when penv0 was used (Fig. 1A). The same insertion of a second RRE into pNLA1 did not interfere with syncytium formation or glycoprotein expression (Fig. 1A, lane 3). Thus, neither the presence of the second RRE nor the concomitant disruption of *nef* expression (Fig. 1B, lanes 2 and 3) noticeably interferes with *env* expression. This independence of *env* ex-

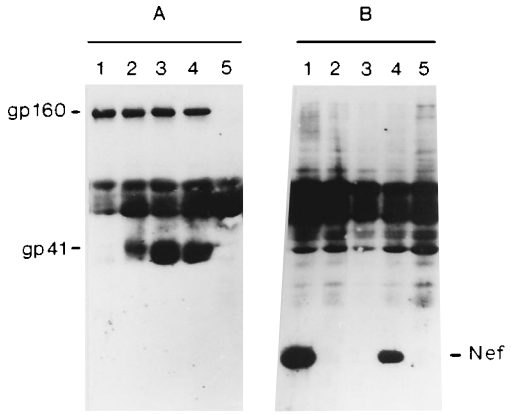

FIG. 1. Immunoblot analysis of glycoproteins expressed in HeLa-T4$^+$ cells transfected with plasmids carrying the *env* gene. For each plasmid, transfection was done with 20 μg of DNA prepared by CsCl density gradient centrifugation and 3×10^6 cells. Cells were cultured for 48 h, lysed, and subjected to SDS-PAGE. The cells were transfected with penv0 (lane 1), penv0RRE (lane 2), pNLA1-RRE (lane 3), and pNLA1 (lane 4) and mock transfected (lane 5). Immunoblots were performed with two monoclonal antibodies to gp41 (A) and a monoclonal antibody to Nef (B), as described in Materials and Methods.

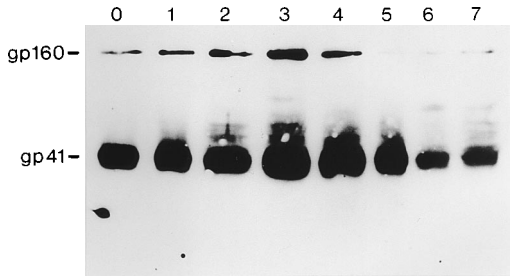

FIG. 3. Immunoblot analysis of glycoproteins expressed in HeLa-T4$^+$ cells transfected with penv0RRE and deletion mutants. Transfection and immunoblot analysis were performed with anti-gp41 monoclonal antibodies, as described in the legend to Fig. 1 and Materials and Methods.

pression from Nef makes it unlikely that *nef* disruption accounts for the restoration of *env* expression in penv0RRE.

Deletions in the gp41 N terminus. Deletions were introduced into the glycoprotein between the REKR motif at the C terminus of gp120 and the FLG motif starting at residue 8 of the gp41 N terminus in some natural HIV isolates including HIVNL43 (the parent of pNLA1) and at residue 9 in others. Sequential reduction in the length of the N terminus by 1 residue led to the series of plasmids termed penv−1RRE through penv−7RRE, containing N termini with decreasing lengths (Fig. 2). Deletions were placed so that the N-terminal alanine that is invariably found in all HIV isolates and residues GA in positions 6 (A) and 5 (G) were maintained in that order of priority. The deletion in penv−7RRE leads to a gp41 N terminus that is homologous to the N terminus of the F_1 glycoprotein of paramyxoviruses (24, 26, 52). The deletions changed the average hydrophobicity calculated for the N-terminal 18 amino acid residues of gp41 from 0.70 in penv0RRE to 0.71, 0.67, 0.63, 0.60, 0.56, 0.53, and 0.50 in the mutants penv−1RRE through penv−7RRE, respectively.

To test penv0RRE and the deletion mutants for glycoprotein expression, DNA was transfected into HeLa-T4$^+$ cells and accumulated HIV glycoprotein was assayed by immunoblot analysis after 48 h with two monoclonal antibodies with specificity for gp41 (Fig. 3). With these antibodies, gp41 and gp160 could be detected in all mutants. In several experiments with anti-gp41 as well as anti-gp120 antibodies, there was no consistent difference in the level of glycoprotein expression or cleavage between penv0RRE and the deletion mutants, indicating that processing and cleavage are not significantly affected by the mutations.

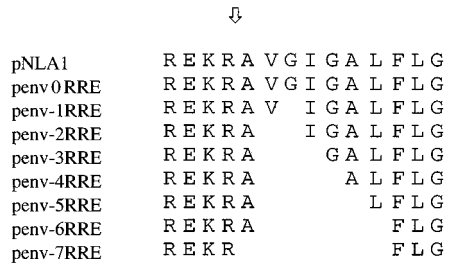

FIG. 2. Amino acid sequences of penv0RRE and deletion mutants at the cleavage site of gp160. The vertical arrow indicates the point of cleavage generating the N terminus of gp41. Deletions are indicated by blank spaces. Outside of the region depicted here, the penv vectors are identical (see Materials and Methods). The shaded areas indicate regions that are highly conserved among HIV-1 isolates.

Syncytium induction in HeLa-T4$^+$ cells transfected with penv0RRE and the −1 and −2 deletions was readily detectable in transfected cultures. Immunofluorescence staining with antibodies against HIV glycoproteins indicated that syncytia contain HIV glycoprotein (Fig. 4). With penv−3RRE and mutants with larger deletions, single cells contained glycoprotein with strong staining intensity; however, syncytia could not be safely identified. The uncertainty is in part because fluorescent cells with two nuclei could rarely be observed with all mutants. It cannot be excluded that these are the result of HIV glycoprotein-induced cell-cell fusion, even though many of them are likely to be the result of spontaneous events, because cells with two or few nuclei were present in untransfected cell cultures as well.

To also investigate glycoprotein-induced fusion events that are less prominent than those induced by wild-type glycoprotein, transfections were performed by the microinjection technique with a modification that allows the follow-up of individual transfected cells (3). penv0RRE and all deletion mutants were microinjected into nuclei of subconfluent HeLa-T4$^+$ cells. The injection contained, in addition to plasmid DNA, FITC-conjugated DEAE-dextran, which remains in the injected nucleus so that it can be readily detected among the nuclei of a syncytium (Fig. 5). Thus, a glycoprotein-induced syncytium detected in phase-contrast microscopy can be safely attributed to an injection event because it contains a fluorescent nucleus. By using this technique, a quantitative assessment of the membrane-fusing potential of mutant glycoproteins was performed (Table 1). Irrespective of the plasmid injected, 82 to 85% of injection events could be identified after 21 h by fluorescence microscopy. The stepwise reduction in the length of the N terminus from penv0RRE to penv−4RRE was accompanied by a stepwise drop in the number of syncytia and the average number of nuclei per syncytium. Mutant penv−4RRE results in cells containing only two nuclei (Fig. 5). Because of the predominance of fluorescent dye in only one of the two nuclei, such fusion events can be safely attributed to glycoprotein-mediated cell fusion rather than a late stage of mitosis. This is further supported by the fact that such small fluorescent syncytia were absent from cultures transfected with penv−5RRE and shorter mutants.

Dominant interference of mutant glycoproteins with the membrane-fusing activity of wt-Env. To assess the effects of

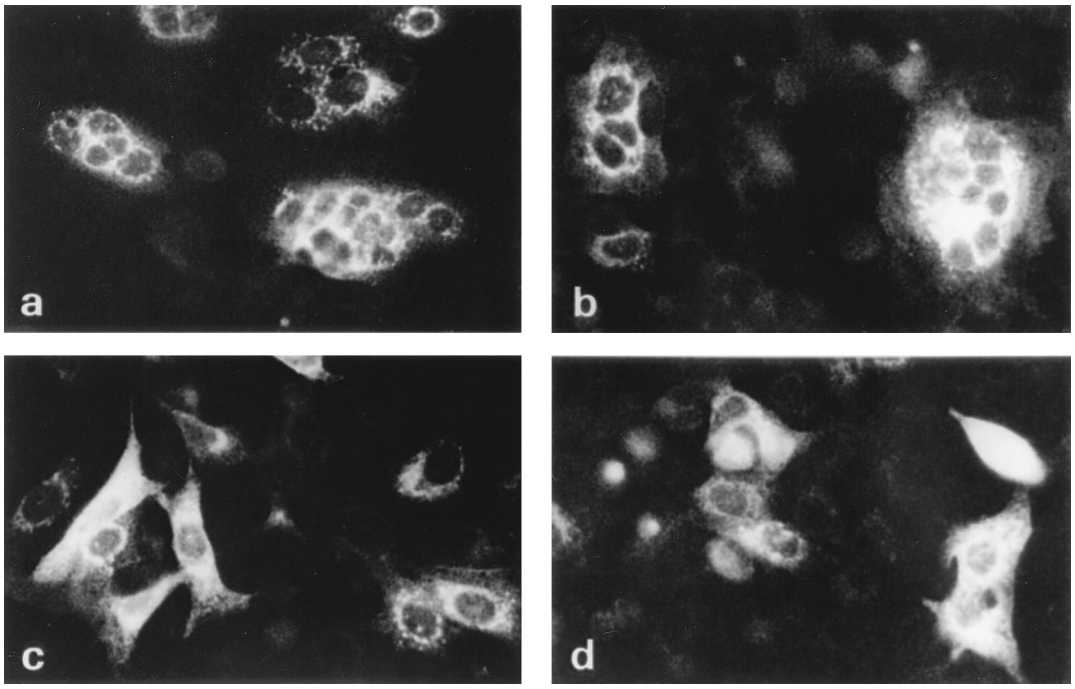

FIG. 4. Glycoprotein expression and cell-cell fusion in HeLa-T4$^+$ cells transfected with penv0RRE and deletion mutants. Cells were transfected with penv0RRE (a), penv−2RRE (b), penv−3RRE (c), and penv−7RRE (d), seeded onto chamber slides, and cultured for 48 h. The cells were fixed and stained with monoclonal antibodies to gp41 and gp120 and FITC-conjugated anti-mouse antibody as described in Materials and Methods.

Env deletions, mutant glycoproteins were also analyzed for their capability to inhibit the fusion activity of coexpressed wild-type (wt) glycoprotein (22). HeLa-T4 pBKLTR lac cells (2) were transfected by electroporation with 20 μg of penv0RRE, and after 2 days of incubation and staining of cells with X-Gal, the number of blue syncytia and the number of nuclei per syncytium were scored. Since *lac* expression of these cells is controlled by Tat, staining is restricted to cells transfected with Env expression vectors.

Coexpression of env−7RRE results in a quantity-dependent

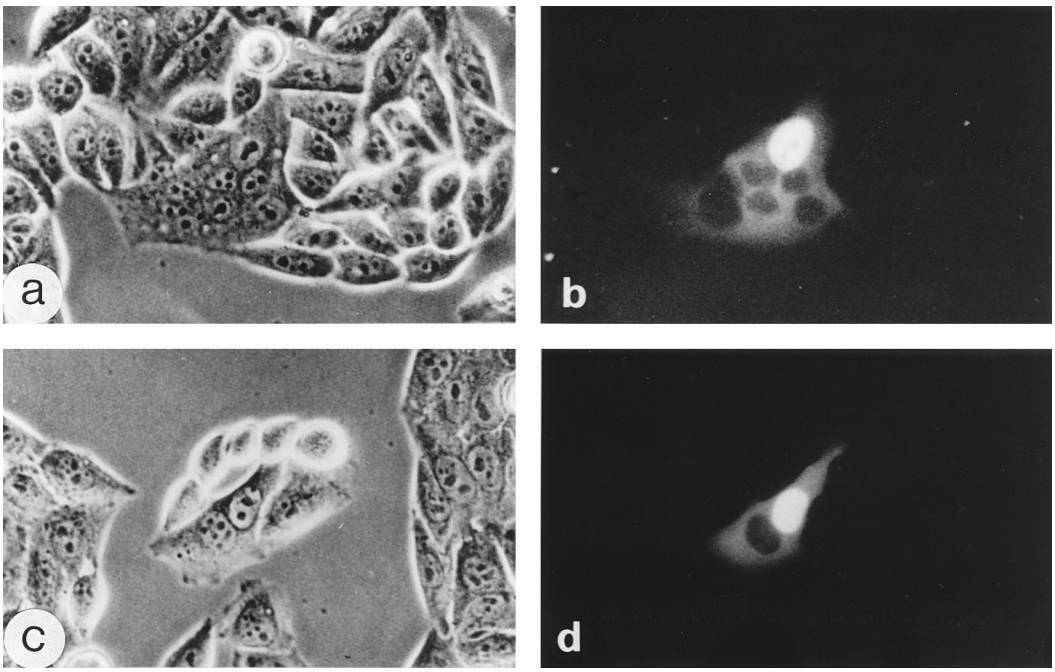

FIG. 5. Cell-cell fusion in HeLa-T4$^+$ cells transfected by microinjection with penv−3RRE (a and b) and penv−4RRE (c and d). Nuclei were coinjected with FITC-dextran (molecular weight, 150,000), allowing identification of injected nuclei up to 5 days after microinjection. Microphotography was done 48 h after transfection, with phase-contrast microscopy (a and c) and fluorescence microscopy (b and d) of identical fields at the same magnification.

TABLE 1. Enumeration of cell-cell fusion events in HeLa-T4$^+$ cells transfected by microinjection with penv0RRE and the deletion mutants

Vector	Efficiency of microinjection[a]		No. (%) of cells positive for syncytia	Avg no. of nuclei per syncytium
	No. of cells used	No. (%) found positive		
penv0RRE	225	188 (83)	172 (91)	14.5
penv−1RRE	272	222 (82)	141 (63)	10.3
penv−2RRE	229	189 (83)	96 (51)	7.0
penv−3RRE	345	291 (85)	85 (29)	3.6
penv−4RRE	544	453 (83)	38 (8)	2.2
penv−5RRE	456	380 (84)	0 (0)	
penv−6RRE	205	173 (85)	0 (0)	
penv−7RRE	208	175 (84)	0 (0)	

[a] The efficiency of the microinjection was scored as the number of cells containing a FITC-dextran-stained nucleus per number of cells injected.

inhibition of syncytium formation (Table 2, experiment 1). Thus, a deletion mutant of Env that by itself is incapable of syncytium formation dominantly interferes with the membrane-fusing activity of wt-Env, as has been shown with substitution mutants by Freed et al. (22). Coexpression of deletion mutants penv−2RRE through penv−5RRE also interferes with the wt-Env fusion activity, with the extent of inhibition being dependent on the size of the deletion (Table 2, experiment 2). This indicates that mutant glycoproteins that by themselves display a lowered fusion activity convey their lowered fusion activity on co-oligomers with wt-Env glycoprotein. The loss or inhibition of syncytium formation in these cotransfection experiments is not due to an overload of DNA or to overexpression of a regulatory protein such as Tat, because increasing amounts of transfected wt expression vector result in a quantity-dependent increase in syncytium formation (Table 2, experiment 3), ruling out nonspecific inhibitory effects of cotransfected plasmid DNA.

DISCUSSION

For convenient mutational analysis of the hydrophobic N terminus of gp41, two restriction sites were introduced into the *env* gene that were designed to be silent but de facto resulted in a A-to-T mutation at residue 22 from the cleavage site (see Materials and Methods). Unexpectedly, this construct, penv0, was not suitable as a parent vector, because transfection of HeLa-T4$^+$ cells did not result in syncytium formation or the formation of gp41 and gp120 even though synthesis of gp160 was demonstrable. One of the conceivable explanations for the failure to express functional glycoprotein is the disturbance of the secondary structure of the RRE. If this were the case, not only the introduction of restriction sites but also any other mutation in the N-terminal region of gp41 would be difficult to interpret because of possible side effects on the RRE, even though this has not been a point of concern in several mutational analyses of this region (21–23, 35, 36).

Therefore, in a derivative vector, i.e., penv0RRE, the entire RRE sequence of the original vector was duplicated outside the *env* gene in exchange for part of the *nef* coding region. This restores the formation of cleaved and functional glycoprotein. Thus, the deficit in penv0 can be attributed to a regulatory effect at the RNA level, and other conceivable possibilities, e.g., an effect of the A-to-T mutation on cleavability of gp160, can be excluded because the *env* genes in penv0 and penv0RRE are identical. This conclusion is further supported by the striking increase in *nef* expression in penv0 over that of the parent vector pNLA1 (Fig. 1B), which is compatible with the requirement of the Rev-RRE interaction for (i) suppression of multiply spliced mRNA species (such as that of Nef) and (ii) export from the nucleus of *env* mRNA (18, 20, 40, 48). The observed lack of gp160 cleavage in penv0 could conceivably be due to the paucity of gp160 expression. Thus, it has been shown with other viral glycoproteins that a reduction in expression can disproportionately interfere with oligomerization and transport to the Golgi compartment (vesicular stomatitis virus G protein [11], influenza A virus HA protein [6]), and, by analogy, a reduction in gp160 could conceivably reduce glycoprotein oligomerization (16, 46, 51), as well as transport and cleavage in the *cis* or medial regions of the Golgi apparatus (15, 57).

The resulting parent vector, penv0RRE, appears to be suitable for mutational analysis of the N terminus of gp41 because the nucleic acid sequence (including the defective RRE) coding for this region does not seem to interfere with the function of the added RRE. In combination with the previously demonstrated position independence of the RRE (40), this is a prerequisite for the study of RRE-independent mutational effects in the N terminus. In a very strict sense, this point would have to be individually established with every mutant under study. However, an interference by other defective RRE structures is unlikely, because efficient expression of functional gly-

TABLE 2. Effect of coexpression of wt-Env and mutant Env on fusion capability

Expt	Transfected DNA (μg)[a]	No. of syncytia with[b]:	
		3–5 nuclei	>5 nuclei
1	penv0RRE (20)	29	6
	penv0RRE (20) + penv−7RRE (10)	11	3
	penv0RRE (20) + penv−7RRE (20)	6	1
	penv0RRE (20) + penv−7RRE (30)	1	0
2[c]	penv0RRE (20)	16.3	6.7
	penv0RRE (20) + penv−2RRE (30)	10.0	2.0
	penv0RRE (20) + penv−3RRE (30)	10.7	2.0
	penv0RRE (20) + penv−4RRE (30)	5.3	0
	penv0RRE (20) + penv−5RRE (30)	3.7	0
3	penv0RRE (20)	13	3
	penv0RRE (30)	17	3
	penv0RRE (40)	22	7
	penv0RRE (50)	28	7

[a] HeLa-T4 pBKLTR lac cells (3×10^6) were transfected by electroporation with penv0RRE with or without additional DNA as indicated.

[b] Cells were fixed and analyzed for β-galactosidase 48 h after transfection. Syncytia were counted in an area of 1 cm^2.

[c] Average of three experiments.

coprotein was possible with numerous recombinants containing penv0RRE in conjunction with sequences of HIV isolates resulting in hybrid RREs and because the lack of functional glycoprotein in a few of those recombinants could be explained by alterations in the amino acid sequence (1a).

Early work with fusion-inducing glycoproteins resulted in the conclusion that a specific amino acid sequence at the hydrophobic N terminus released by proteolytic activation is necessary for biological activity (7, 8). Thus, with widely different paramyxoviruses, the N termini of F_1 are homologous and at identical locations (52). With influenza A virus, the corresponding sequence of HA_2 (54) is similarly well conserved and homologous to that in paramyxoviruses, with one additional glycine residue (25). Additional evidence in support of the importance of the N-terminal structures of F_1 and HA_2 for biological activity stems from early work with peptide inhibitors whose activity is dependent on the similarity of their amino acid sequence with that of the F_1 and HA_2 N termini (47). Recently, synthetic peptides with amino acid sequences corresponding to the N terminus of the transmembrane glycoprotein gp41 of HIV type 1 (HIV-1) were also found to inhibit HIV glycoprotein-induced membrane fusion (45, 55). In contrast to the inhibitory effect of such N-terminal peptides, similar di- and tripeptides surprisingly exert an enhancer effect in bovine leukemia virus glycoprotein-mediated syncytium formation (59).

In view of the sequence specificity of synthetic peptides, it is surprising that the N terminus of the functionally analogous gp41 of HIV-1 (42) is subject to considerable variation (43). On the basis of the discovery of a strong homology between the paramyxovirus N termini and a region in gp41 7 or 8 residues removed from the N terminus, it has been suggested that this sequence (FLGFLG) is the fusion peptide in HIV-1 and that it may not be necessary for the fusion peptide to be terminal, raising the implicit possibility that the tailing amino acids may be dispensable (24, 26). Our deletion mutant penv−7RRE, which contains the FLG motif at the very N terminus, totally lacks membrane-fusing activity, indicating that additional amino acid residues are important for protein function.

To define more precisely the length of the N terminus that is necessary for functionality in membrane fusion, we reduced its length sequentially. The efficacy in cell fusion decreases with decreasing length of the N terminus. Fusing activity is barely detectable with deletion of 4 amino acids and is totally lost with deletion of 5. These findings support the compatibility of structural variation of the N terminus with fusion activity observed by sequence analysis of wt viruses. In addition, they define the limits of the length variation in this region.

Previous findings by Freed et al. (22) support a model that depicts the fusogenic structure as a multimer of equal N termini. The dominant interference of inactive mutant with coexpressed active wt glycoprotein is thought to involve formation of oligomers whose functionality is obliterated by one changed amino acid residue such as an glutamic acid at position 2 (22). In view of such a model, it is surprising that our mutants with lowered functionality also dominantly interfered with wt glycoprotein. This indicates that the first 7 amino acids are dispensable for the process of oligomerization; however, it also suggest that perfect identity of the constituents in an oligomer may be required for optimal fusion activity. On the other hand, the results clearly indicate that differences among the constituents of the fusogenic oligomer can be tolerated within limits that are similar to those found with fusogenic homooligomers.

Since the hydrophobic region is bordered by a polar region that might be involved in fusion peptide function (23), a deletion within the N terminus results in a shift of this polar region toward the N terminus. As a consequence, the average hydrophobicity of the N termini of the deletion mutants decreases continuously, beginning with deletion of the second residue (Fig. 2). Significant fusing activity is still observed with deletion of 1 and 2 residues. N termini equal in length to the −1 mutant are observed among natural variants (43), indicating that this structure is compatible with efficient virus replication. Since the fusion activity is completely abolished with a deletion of more than 4 amino acids, it is worth considering the possibility that the corresponding average hydrophobicity value of 0.6 represents a cutoff for function in membrane fusion. This would be in agreement with the average hydrophobicity values of fusion peptides of other virus glycoproteins, such as the F protein of paramyxoviruses (0.66 in Sendai virus) and the HA protein of influenza virus (0.64 in X-31). However, it is unlikely that the average hydrophobicity is the only parameter that determines fusion efficiency in the present series of mutants, because other structural requirements such as the formation of α-helices (32, 53) and the orientation of the fusogenic peptide in the lipid bilayer (4, 31, 60) have also been shown to be important determinants of membrane fusing activity.

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