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Influence of Transmembrane Domains on the Fusogenic Abilities of Human and Murine Leukemia Retrovirus Envelopes

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The envelopes of two highly divergent oncoviruses, human T-cell leukemia virus type 1 (HTLV-1) and Friend murine leukemia virus (F-MuLV), have distinct patterns of cellular receptor recognition, fusion, and syncytium formation. To analyze the influence of the transmembrane envelope subunit (TM) on fusogenic properties, we substituted either the entire TM or distinct domains from F-MuLV for the corresponding domains in the HTLV-1 envelope. Parental, chimeric, and truncated envelopes cloned into a eukaryotic expression vector were monitored for fusogenic potential in human, rat, and murine indicator cell lines by using a quantitative assay. This highly sensitive assay allowed us to assess the fusogenic properties and syncytium-forming abilities of the HTLV-1 envelope in murine NIH 3T3 cells. All chimeric envelopes containing extracellular sequences of the F-MuLV TM were blocked in their maturation process. Although deletions of the HTLV-1 cytoplasmic domain, alone and in combination with the membrane-spanning domain, did not prevent envelope cell surface expression, they impaired and suppressed fusogenic properties, respectively. In contrast, envelopes carrying substitutions of membrane-spanning and cytoplasmic domains were highly fusogenic. Our results indicate that these two domains in F-MuLV and HTLV-1 constitute structural entities with similar fusogenic properties. However, in the absence of a cytoplasmic domain, the F-MuLV membrane-spanning domain appeared to confer weaker fusogenic properties than the HTLV-1 membrane-spanning domain.

Retroviral envelopes are synthesized as precursor proteins which are cleaved in the late Golgi apparatus to yield two major subunits: the entirely extracellular amino-terminal surface glycoprotein (SU) and the carboxy-terminal transmembrane protein (TM) (15). Retroviral SU show little homology in their sequences and predicted spatial structure, whereas TM display a relatively more conserved framework (9, 24). From the extracellular amino terminus to the intracellular carboxy terminus, at least four functional domains are present in all TM: (i) a highly hydrophobic sequence composed of the 13 to 24 amino-terminal-most amino acids, known as the fusion peptide, which is directly involved in the membrane fusion process (15); (ii) a more downstream sequence comprising two or three cysteine residues thought to form a loop which serves as a major B-cell epitope in some lentiviruses and which is described as an immunosuppressive peptide in oncoviruses (24); (iii) a membrane-spanning hydrophobic region of 19 to 27 amino acids allowing anchorage to the cell; and (iv) a cytoplasmic domain for which no clear general structure or function(s) is established. The envelope is the first element to interact with the cellular receptor expressed on the target cell, leading to specific virus-to-cell fusion and subsequent infection. The envelope is also responsible for cell-to-cell fusion and syncytium formation, and determinants influencing cell-type-dependent fusion through direct or indirect interaction with the receptor and accessory factors have been identified in the SU (38).

Whereas cellular specificity of envelope-driven fusion is attributed to the SU, the TM appears to drive cell fusion. The envelope cytoplasmic domain of TM is often involved in the global modulation of cell fusion and syncytium formation. For example, the naturally occurring late cleavage performed by the viral protease of a small carboxy-terminal peptide in the TM of type C ecotropic murine leukemia viruses (MuLV) and type D Mason-Pfizer monkey virus considerably enhances cell-to-cell fusion (1, 30, 31). Also, truncations introduced in the TM cytoplasmic domain of human T-cell leukemia virus type 1 (HTLV-1) lead to cell-type-dependent alterations of the syncytial effect (27). In addition to the cytoplasmic domain, the membrane-spanning and extracellular domains of the TM have also been shown to influence global fusion efficiency (2, 6, 14, 16). In the present study, we took advantage of structural analogies between retroviral TM to derive envelopes which are chimeric between two functional retroviral envelopes in order to evaluate the influence of various TM domains on cell-specific fusion and syncytium formation. For this purpose, we used HTLV-1 and the ecotropic Friend MuLV (F-MuLV), which are two highly divergent oncoviruses differing in genomic organization, cell receptor distribution, and target cell types for fusogenic and syncytium-forming properties. Functional modified parental envelopes allowing convenient domain swapping were derived. Fusogenicity and syncytium formation of parental, chimeric, and truncated envelopes were monitored in cell lines of different origins upon coculture with envelope-expressing Cos cells. HTLV-1 envelopes with parts or all of the extracellular region of the F-MuLV TM were nonfunctional, whereas envelopes with substitutions of the membrane-spanning and cytoplasmic domains were functional. Our results, obtained with different functional chimeric envelopes, indicated that optimal fusion involves interactions between the anchor and the cytoplasmic domain which are likely to constitute a structural entity. Fusion efficiency of an HTLV-1 chi-

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meric envelope, in which such a structural entity was derived from a highly divergent retrovirus such as F-MuLV, was similar to that of the parental HTLV-1 envelope.

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MATERIALS AND METHODS

DNA, cells, and antibodies. Plasmid pBEH contains the MT-2 HTLV-1 envelope (11) cloned in the polylinker of the pBS(+)KS vector (Stratagene) from the *Hin*dIII site to the *Pst*I site. Plasmid pBSE contained the F-MuLV 57 (21) envelope and a fragment of the 3′ long terminal repeat (LTR) cloned in the *Xba*I and *Kpn*I sites of pBS(+)KS. The envelope expression vector (pCEL) used in this study contains the cytomegalovirus promoter, the pX region of HTLV-1 with the *env*, *tax*, and *rex* sequences, and the 3′ LTR of HTLV-1 as the terminator sequence. This vector was derived from a vector based on the CR HTLV-1 strain (4). Mutated, chimeric, and truncated envelopes described below were reintroduced into this expression vector as a cassette, using two unique restriction sites 5′ (*Sph*I) and 3′ (*Pst*I) of the envelopes. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere. Cos cells stably expressing the LacZ gene under the control of the human immunodeficiency virus type 1 (HIV-1) LTR (CosLTR LacZ cells) were a gift from T. Dragic and M. Alizon (5) and were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin (complete DMEM) plus hygromycin B (300 μg/ml; Calbiochem); the Tat-expressing cell lines used were HeLa-Tat (5), which was grown in complete DMEM with 2 mM (final concentration) methotrexate, and XC-Tat and Dunni-Tat, which were obtained by infection of the cells with a cell-free supernatant of the Ψ CRIP packaging cell line (3) transfected with an HIV-1 Tat-expressing retroviral vector (pLXSN), carrying the neomycin resistance gene (gift from O. Schwartz). After 7 days of selection in G418 (400 μg/ml), the resistant cell population was frozen and used for fusion experiments. Dunni-Tat and XC-Tat cells were cultured in presence of G418 in complete DMEM and RPMI, respectively. The NIH 3T3-Tat cells contain the HIV-1 *tat* gene placed under the control of the simian virus 40 promoter and the histidinol dehydrogenase gene (*hisD*) as a selection marker and were routinely cultured in complete DMEM in absence of selection. The antibodies against HTLV-1 envelope used were monoclonal antibody 1C11 (23), kindly provided by R. M. Scearce, and a serum pool from HTLV-1-infected patients (gift of N. Monplaisir).

Oligonucleotide-directed mutagenesis and DNA manipulation. To construct the chimeric envelope genes, three allelic restriction sites were introduced in the coding sequence of HTLV-1 and F-MuLV *env* by site-directed mutagenesis, using a previously described (8) adaptation of the method of Kunkel (19). In both *env* genes, we introduced a *Bgl*II site at the SU/TM cleavage site, a *Hin*dIII site at the amino terminus of the membrane-spanning domain of the TM, and an *Mlu*I site immediately 3′ of the *env* stop codon. These restriction sites were chosen in part because of their absence in the two wild-type envelopes. The three corresponding mutations were combined in each envelope, and nucleotidic sequences of the fragments containing the mutations were determined before being substituted into the original pBEH and pBSE plasmids. The mutated HTLV-1 and F-MuLV envelopes containing the *Bgl*II, *Hin*dIII, and *Mlu*I restriction sites were designated H and F, respectively. All mutations and chimeric constructions are depicted schematically in Fig. 1 and 2A, respectively. As shown in Fig. 1, the *Bgl*II site introduced in the envelope cleavage site of HTLV-1 did not modify the predicted amino acid sequence of this region (Arg-Ser-Arg-Arg), whereas introduction of this site in F-MuLV changed the predicted Arg-His-Lys-Arg sequence to that of HTLV-1. The *Hin*dIII restriction site introduced into the membrane-spanning region substituted a Ser-Phe dipeptide for a Thr-Leu at residues 448 to 449 of the H Env, counting from the AUG initiation codon. In the mutated F Env, the equivalent mutation substituted a Ser for a Thr at residue 619 (Fig. 1). The *Mlu*I site was introduced either by substituting two nucleotides (gt for aa) 12 nucleotides downstream of the HTLV-1 envelope stop codon or by adding four nucleotides (cgcg) between the two successive stop codons of the F-MuLV envelope (18). To obtain additional hybrids in which smaller fragments of the extracellular transmembrane were substituted, two additional allelic sites were independently added. A *Pst*I site, allelic to the one that includes *env* codon 549 in F-MuLV, was introduced in codons 376 to 377 of the H *env* and resulted in the substitution of a Leu for Ala. An *Ssp*I site was introduced in both H and F *env* within codons 404 to 405 and 576 to 577, respectively. This mutation was synonymous in H, whereas in F this mutation substituted the corresponding H codons (Leu-Asn-Ile) for the original F-MuLV codons (Tyr-Ala-Asp) (Fig. 1). Chimeric HF envelopes containing F-MuLV sequences were designated HFTM, HFTMe, HFTMi, HFTM312-377, HFTM 404-447, HFTMi377, and HFTMi404, with the last letters and numbers identifying the F-MuLV sequence present in the HTLV sequence (Fig. 2A). We also derived two truncated HTLV-1 envelopes lacking either the anchoring and cytoplasmic domains (HdAC) or only the cytoplasmic domain (HdC). The corresponding mutations were generated by introduction of linkers with in-frame stop codons and new restriction sites for easier screening. Linkers were composed of two complementary oligonucleotides. Linker HN9/10, containing a *Pst*I site (HN9 [5′AGCTAACCCTGCAG3′] and HN10 [5′AATTCTGCAGGGT T3′]), was introduced into the *Hin*dIII site. Linker HN1/2, containing an *Nhe*I site (HN1 [5′TCTGATGAGCTAGCTGCA3′] and HN2 [5′GCTAGCTCAT CAGATGCA3′]), was introduced in the *Nsi*I site of the H *env* sequence. The predicted *env* gene products have either a membrane-spanning domain limited to four amino acids or a complete membrane-spanning domain and no cytoplasmic domain, respectively (Fig. 2B). We also deleted all but two codons of the cytoplasmic domain in the HFTMi chimera (HFTMidC) by introduction of linker HN11/12, containing the *Pst*I site (HN11 [5′CGATAACTGCAGTCAT3′] and HN12 [5′CGATGACTGCAGTTAT3′]), into the F-MuLV *Cla*I site. The TM region of this envelope mutant was limited to the extracellular TM (TMe) of HTLV-1 and the membrane-spanning domain of F-MuLV (Fig. 2B). All linker insertions were verified by DNA sequencing.

Transfections and envelope fusion assay. Metabolic labeling and flow cytometry analyses (see below) were performed after transfection of 5×10^5 CosLTR LacZ cells with 10 μg of DNA in 10-cm-diameter plates, using the DEAE-dextran/chloroquine and dimethyl sulfoxide technique (28). Fusogenicity of the different envelopes was tested upon transfection of 0.5×10^5 to 1×10^5 CosLTR LacZ cells in six-well plates with either 1 μg or 25 ng of the corresponding expression vector DNA. Either 600,000 XC-Tat, 400,000 HeLa-Tat, 400,000 Dunni-Tat, or 600,000 NIH 3T3-Tat cells were added as indicator cells 48 h posttransfection. Confluent cocultures were washed 16 h later with phosphate-buffered saline (PBS), fixed for 10 min with 0.5% glutaraldehyde at room temperature, washed twice in PBS, stained by incubation for 2 h at 37°C with a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution, and washed twice with PBS. All blue foci visible under a stereomicroscope at a ×40 magnification were counted, regardless of size and morphology, and numbers exceeding 1,000 were recorded as too numerous to be counted (noted as >1,000).

Flow cytometry analysis of envelope cell surface expression. Fifty to sixty hours posttransfection, CosLTRLacZ cells were trypsinized at 37°C in the shortest time necessary for detachment, and 5×10^5 live cells were stained for 30 min at 4°C with a 1/100 dilution of monoclonal antibody 1C11 diluted in FACS (fluorescence-activated cell sorting) flow buffer (ICN Flow) containing 0.5% (wt/vol) bovine serum albumin. Cells were washed twice in FACS flow buffer and incubated for 30 min at 4°C with a 1/100 dilution of a goat anti-mouse immunoglobulin serum labeled with phycoerythrin (Southern Biotech). Cells were fixed with 0.5% paraformaldehyde, and integrated fluorescence was measured. Data from 5,000 events were collected, and surface value expression was determined as the product of mean fluorescence intensity times the percentage of fluorescent cells.

Metabolic labeling and endoglycosidase H treatment. Forty-eight hours after transfection, cells were labeled either overnight or for 6 h prior to endoglycosidase H (endo H) treatment of the lysates, as previously described (28), in 3 ml of cysteine-free RPMI containing 100 μCi of [^{35}S]cysteine (ICN) per ml and 2% dialyzed fetal calf serum. Supernatants were collected and spun down at 4°C to eliminate cell debris. These clarified supernatants were treated with 300 μl of 10× lysis buffer (500 mM Tris HCl [pH 7.5], 150 mM NaCl, 50 mM $MgCl_2$, 50 mM KCl, 10% Triton X 100). Cells were washed once in PBS and lysed in situ with 2 ml of 1× lysis buffer in the presence of 0.5% sodium deoxycholate and a cocktail of protease inhibitors. Cleared lysates were enriched in glycoprotein content by overnight incubation with lentil lectin-Sepharose (Pharmacia) at 4°C. After centrifugation, the lectin-bound proteins in the pellet were eluted twice with 200 μl of 0.5 M α-methylmannoside (Sigma) in lysis buffer. This step eliminated the otherwise considerable background signals from Cos cells with no qualitative alteration of the envelope-specific signals (not shown). Lysates were then precleared with a normal human serum and a rabbit anti-actin serum (Sigma) and immunoprecipitated either with a pool of sera from HTLV-1-infected patients or in combination with an anti-MuLV TM. The immunoprecipitates from the 6-hour-labeling lysates were treated for 1 h at 37°C in the absence or presence of 1,000 IU of endo H in the buffer provided by the manufacturer (Biolabs). Immunoprecipitates were electrophoresed in sodium dodecyl sulfate–12% polyacrylamide gels under reducing or nonreducing conditions. Nonreducing conditions maintain disulfide bonding of the immunoglobulin and thus exclude light immunoglobulin chains from the 46-kDa region on the gels; this allowed a better visualization of the cleaved HTLV-1 SU component.

RESULTS

Chimeric HTLV/F-MuLV envelopes were derived from modified functional HTLV and F-MuLV *env* genes. Synonymous and nonsynonymous mutations were introduced into wild-type HTLV-1 and F-MuLV as described in the Materials and Methods, resulting in the H and F mutated *env* genes, respectively (Fig. 1). These mutations allowed exact substitutions of different domains of the TM. In vitro translation of the parental H and F and the chimeric HFTMi, HFTM, and HFTMe envelopes (Fig. 2A) was efficient in the absence or presence of microsomal membranes. The observed corresponding nonglycosylated and glycosylated products had apparent molecular masses which were compatible with modifications of the po-

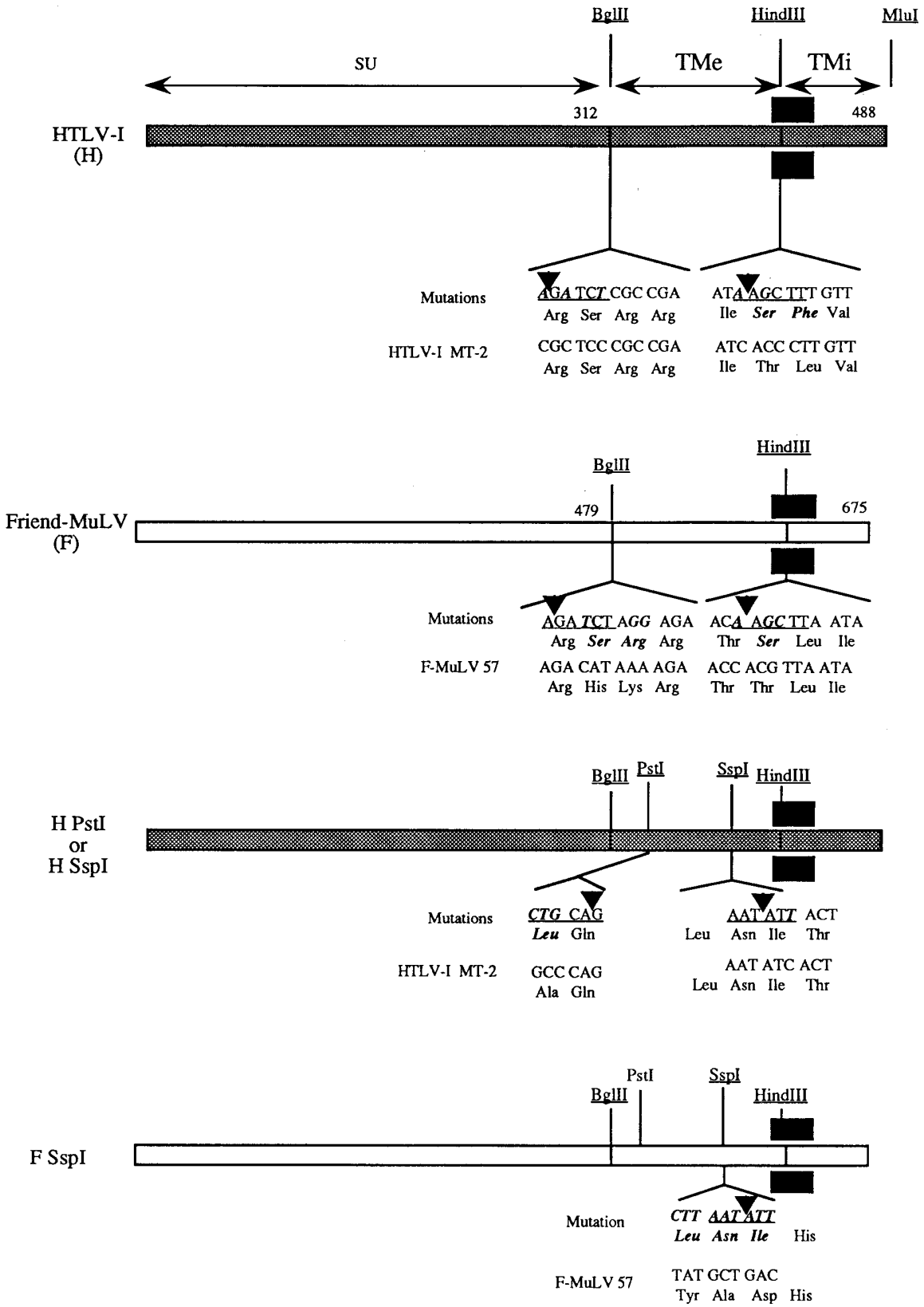
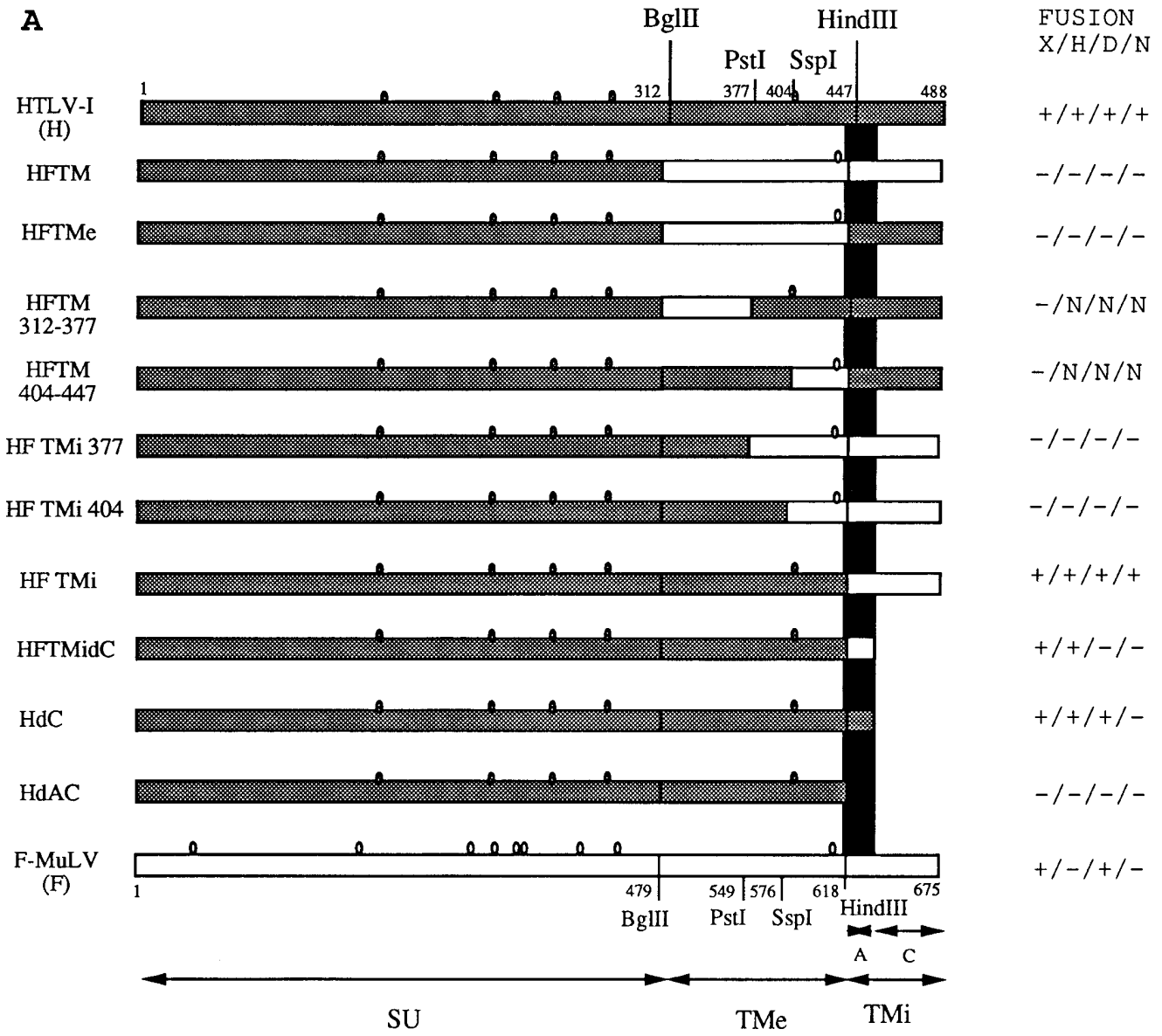


FIG. 1. Modifications of HTLV-1 and F-MuLV envelope genes to introduce new restriction sites. HTLV-1 and F-MuLV full-length envelopes are shown and numbered as amino acid precursors including the signal peptide. The three allelic restriction sites introduced in HTLV-1 MT-2 and F-MuLV 57 envelope sequences were a *Bgl*II site located in the first codon of the SU/TM cleavage recognition sequence, a *Hin*dIII site located at the amino terminus of the putative membrane-spanning region of the HTLV and F-MuLV TM, and an *Mlu*I site located immediately downstream of the *env* stop codon. The resulting mutated parental HTLV-1 and F-MuLV envelopes are designated H and F, respectively. Nucleotide sequences and corresponding amino acids are indicated, with substitutions shown in boldface and italics. Newly introduced restriction sites are underlined, and arrowheads indicate the location of the enzymatic cut. The *Pst*I and *Ssp*I sites were introduced independently within the envelope external part (TMe) to obtain some of the hybrids (see Fig. 2A); note that the *Pst*I site occurs naturally in the TMe of F-MuLV.



B

	Membrane-spanning region	Cytoplasmic region
F-MuLV WT	FTTLISTIMGPLIILLLILLFGPCIL	NRLVQFVKDRISVVQALVLTQQYHQLKPLEYEP
HTLV-I WT	QTGITLVALLLLVILAGPCIL	RQLRHLPSRVRYPHYSLINPESSL
H	QTGISFVALLLLVILAGPCIL	RQLRHLPSRVRYPHYSLINPESSL
F	FTSLISTIMGPLIILLLILLFGPCIL	NRLVQFVKDRISVVQALVLTQQYHQLKPLEYEP
HdC	QTGISFVALLLLVILAGPCS*	
HdAC	QTGIS*	
HFTMi	QTGISLISTIMGPLIILLLILLFGPCIL	NRLVQFVKDRISVVQALVLTQQYHQL
HFTMidC	QTGISLISTIMGPLIILLLILLFGPCIL	NR*

FIG. 2. (A) Structures and fusogenic properties of parental, chimeric, and truncated envelopes. HTLV-1 (shaded boxes) and F-MuLV (open boxes) envelopes are represented as uncleaved protein precursors anchored in the plasma membrane, symbolized by the vertical solid box. Locations of the SU and TM components are indicated at the bottom, with the TM comprising the external (TMe) and intracellular (TMi) domains. The TMi domain is further divided into membrane-spanning anchor (A) and cytoplasmic (C) domains. Potential N-linked glycosylation sites are indicated by small circles. A summary of the envelope fusogenic properties upon coculture is shown on the right. Indicator cell lines stably expressing HIV-1 Tat protein were: rat XC-Tat (X), human HeLa-Tat (H), mouse Dunni-Tat (D), and mouse NIH 3T3-Tat (N). The envelope was considered fusogenic (+) when more than 10 fusion events, identified as blue foci, were observed; nonfusogenic combinations are indicated by −. N, not performed. (B) Amino acid sequences of membrane-spanning and cytoplasmic domains of homogeneous and chimeric truncated envelopes. The amino acids introduced by the mutagenesis procedure and originally absent from both HTLV-1 MT-2 and F-MuLV 57 envelopes are underlined. Locations of stop codons introduced to derive truncated envelopes are indicated (*). Envelopes are designated as shown in panel A.

TABLE 1. Fusogenic properties of parental, chimeric, and truncated envelopes[a]

Envelope	Amt transfected	Expt	No. of blue foci			
			XC[b]	HeLa	Dunni	NIH 3T3
H	25 ng	a	>1,000	>1,000	880	100
	1 μg	b	>1,000	>1,000	>1,000	600
HFTMi	25 ng	a	>1,000	>1,000	290	70
	1 μg	b	>1,000	>1,000	>1,000	500
HdC	25 ng	a	90	60	30	<10
	1 μg	b	>1,000	690	180	40
HFTMidC	25 ng	a	<10	16	<10	<10
	1 μg	b	130	120	<10	<10
F	1 μg	c	>1,000	<10	190	<10

[a] Each envelope-to-cell combination was performed at least twice within eight independent transfection series. a, b, and c are independent experiments whose results are representative of these transfection series.
[b] Indicator Tat cell line.

tential N-glycosylation sites similar to that of the unmodified wild-type sequences (17, 26) (not shown). Furthermore, introduction of the F *env* in the entire F-MuLV genome yielded a virus with ex vivo and in vivo spreading kinetics and pathogenic hemolytic and leukemogenic abilities (32, 33) indistinguishable from those of wild-type F-MuLV (not shown).

The fusogenic properties of H and F Env were monitored upon transfection in CosLTRLacZ cells and coculture with four indicator cell lines of either human (HeLa), rat (XC), or murine (Dunni and NIH 3T3) origin. In our assay, the H envelope was fusogenic with all four cell lines, exhibiting the strongest fusogenic effect on XC and HeLa cells and the weakest, although significant, fusogenic effect on NIH 3T3 cells (Table 1 and Fig. 3). Of the three indicator cell lines expressing the ecotropic MuLV receptor (XC, Dunni, and NIH 3T3), only the first two gave detectable fusion when cocultured in presence of cells expressing the F envelope. As expected, the human HeLa cells, which lack the ecotropic MuLV receptor, did not fuse with cells expressing the F envelope (Table 1). Altogether, these results indicated that both the H and F modified envelopes were functional.

Generation of envelopes with distinct fusogenic properties by exchange of different TM domains. Substitution of either the entire TM (HFTM) or the TM extracellular domain (HFTMe) yielded envelopes which were nonfusogenic for any of the four cell lines tested. Even more limited substitutions of either the amino-most amino acids (HFTM312-377) or the carboxy-most amino acids (HFTM404-447) in the extracellular domain of the TM also yielded nonfusogenic envelopes (Fig. 2A). In contrast, substitution of both intracellular domains, comprising the membrane-spanning and cytoplasmic sequences, yielded the HFTMi envelope, which had a fusogenic efficiency similar to that of the HTLV-1 envelope (Fig. 2A and Table 1). We also tested limited substitution of TM extracellular sequences in association with substitution of the F-MuLV intracellular domains (HFTMi377 and HFTMi404) in order to provide the F-MuLV TMe sequences present in the corresponding chimeric envelopes with homologous adjacent intracellular domains. These combinations resulted in the conservation of the amino-most sequence of the HTLV TM extracellular domain, either up to residue 377 (HFTMi377), including the region corresponding to the so-called fusion peptide (9, 38), or further up to residue 404 (HFTMi404) (Fig. 2A). Neither of these chimeric envelopes yielded detectable fusogenic effects on any of the our cell lines tested.

Since the simultaneous substitution of F-MuLV membrane-spanning and cytoplasmic domains for those of HTLV, such as in TMi, yielded an envelope with fusogenic properties similar to those of the parental H envelope (Fig. 2A and 3), we examined whether the latter domains were indeed necessary for fusion. Simultaneous deletion of the membrane-spanning and cytoplasmic domains (HdAC) abolished detectable fusion in all cell types. Deletion of the cytoplasmic domain of the nonchimeric H envelope (HdC) reduced fusogenicity by approximately 10-fold in the four cell types tested (Fig. 2A and Table 1). This deletion resulted in no detectable fusion on NIH 3T3 cells after transfection with the lowest amount of HdC DNA (25 ng). Upon transfection of 1 μg of HdC DNA, significant although reduced numbers of foci were observed in both NIH 3T3 and Dunni cells (Table 1). These results indicated that the presence of a cytoplasmic domain was essential for full fusogenicity of the HTLV envelope. Although chimeric HFTMi and parental H envelopes had very similar fusogenic abilities, deletion of the cytoplasmic domain had a more profound effect on the HFTMi envelope (HFTMidC) than on the H envelope. For instance, HFTMidC had only marginal or no fusogenic effects on the rat (XC) and human (HeLa) cell lines and no detectable fusogenic effects on either Dunni or NIH 3T3 mouse cells (Table 1). Therefore, although the F-MuLV membrane-spanning domain is less fusogenic than the HTLV membrane-spanning domain in the absence of a cytoplasmic domain (compare HdC with HFTMidC), the presence of the F-MuLV cytoplasmic domain efficiently compensated for this difference (compare H with HFTMi).

Envelopes with distinct fusogenic properties are diversely processed and expressed at the cell surface. We used flow cytometry to monitor the presence of several of the envelopes at the surface of transfected CosLTRLacZ cells. Truncated envelopes were more abundant at the cell surface than complete envelopes, independent of their fusogenic ability. Thus, the extensively truncated and nonfusogenic HdAC envelope was present at the surface of almost all cells, whereas the partially truncated and distinctively fusogenic HdC and HFTMidC envelopes were detected in a large proportion of but not all cells (Fig. 4). The complete and fully fusogenic H and HFTMi envelopes were less efficiently detected, and the complete but nonfusogenic HFTM and HFTMe envelopes were detected in an even lower proportions of cells (Fig. 4). We examined the shedding of these different envelopes by immunoprecipitation from cell-free supernatants. Mature envelope products from the extensively truncated HdAC envelope were massively detected in cell-free supernatant, whereas no envelope products were detected in the supernatant of cells transfected with any of the other truncated or complete envelopes (not shown). Therefore, high levels of detection at the cell surface of the HdAC truncated envelope, which lacks all but four amino acids of the membrane-spanning domain, was most likely due to attachment of released envelopes to cell surface receptors. In contrast, partially truncated HdC and HFTMidC envelopes appeared stably cell associated.

Since differences in cell surface detection could be due to different levels of expression, we examined the levels of cell-associated precursor envelopes. Low levels of cell surface detection and altered or suppressed fusogenicity were not due to low levels of envelope precursor production. Thus, compared with complete parental (H) and chimeric (HFTMi) envelopes, larger amounts of precursors were detected for complete but nonfusogenic chimeric envelopes containing partial (HFTM 312-377 and HFTMi404) or extensive (HFTM and HFTMe) substitutions in the TM extracellular domain (Fig. 5A). Also, there were lower levels of precursors of the truncated envelopes, which were the forms most abundantly detected at the

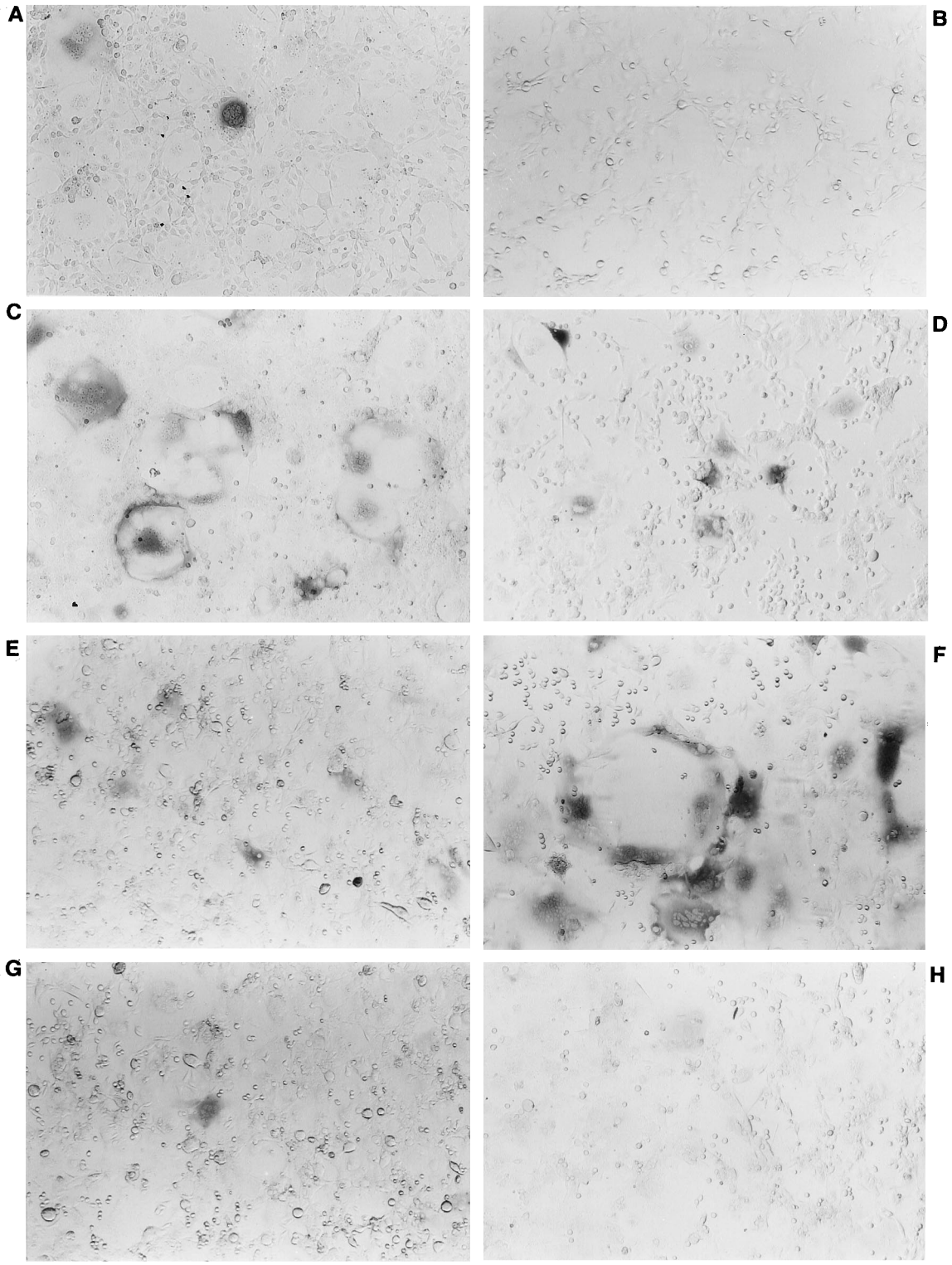


FIG. 3. Fusion and syncytium formation in NIH 3T3-Tat and XC-Tat cells exposed to chimeric and truncated envelopes. After transfection with different envelope-expressing vectors, CosLTRLacZ cells were cocultured with either NIH 3T3-Tat (A and B) or XC-Tat (C to H) indicator cells. Each blue focus represents a specific fusion event between at least one envelope-presenting cell and one Tat-carrying indicator cell. Representative fields of cocultures upon transfection with either a complete HTLV (A and C) or F-MuLV (B and D) homogeneous envelope, truncated HdC homogeneous envelope (E), complete HFTMi (F) or truncated HFTMidC (G) chimeric envelope, or envelope-less mock plasmid (H) are shown.

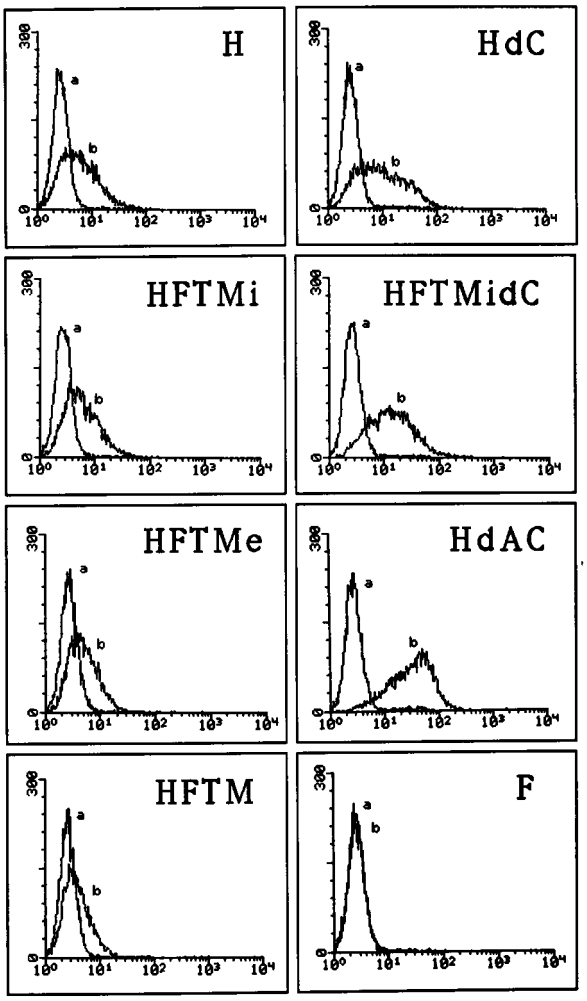


FIG. 4. Cell surface expression of chimeric and truncated envelopes. Forty-eight hours after transfection, CosLTRLacZ cells were stained with either monoclonal antibody 1C11, which recognizes the HTLV-1 SU (curve b), or a control nonreactive monoclonal antibody (curve a). Surface expression of HTLV-1 SU was monitored by flow cytometry. Envelopes are as diagrammed in Fig. 2A.

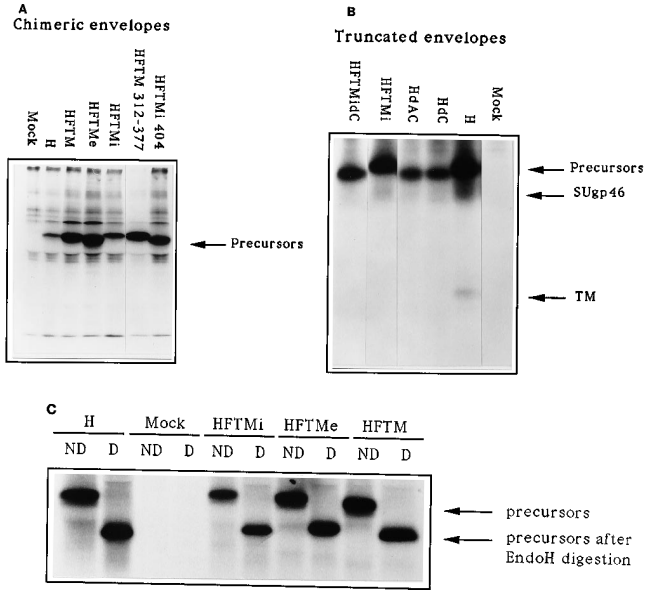


FIG. 5. Metabolic labeling and endo H treatment of chimeric and truncated envelope products. Forty-eight hours after transfection, CosLTRLacZ cells were incubated overnight with [^{35}S]cysteine, and cell lysates were immunoprecipitated as described in Materials and Methods. (A) Synthesis of complete parental and chimeric envelopes. Immunoprecipitates from mock-transfected cells or from cells transfected either with the parental HTLV homogeneous envelope (H) or with different chimeric envelopes (diagrammed in Fig. 2A) were electrophoresed under reducing conditions. Migration of the uncleaved SU/TM precursors is indicated. (B) Synthesis of parental and truncated envelopes. Electrophoresis was performed under nonreducing conditions to eliminate potential masking of the SU by the reduced immunoglobulin heavy chains. Migration of precursors, SU gp46, and TM envelope products is indicated. SU gp46 and the corresponding complete or truncated TM products were detected in all envelope-transfected cells after a longer film exposure. The corresponding envelope structures and partial amino acid sequences are shown in Fig. 2. (C) Endo H treatment of chimeric envelope precursors. Endo H-digested (D) or nondigested (ND) immunoprecipitated cell lysates were electrophoresed under reducing conditions. Positions of migration of digested and nondigested envelope precursors are indicated.

cell surface (HdAC, HdC, and HFTMidC), than of the complete H and HFTMi envelopes (Fig. 5B). Indeed, accumulation of envelope precursors appeared inversely related to detection of envelopes at the cell surface (Fig. 4, 5A, and 5B). We assessed the localization of some of these envelope precursors by treatment of the cell extracts with endo H, which removes the N-linked oligosaccharides of glycoproteins residing in the endoplasmic reticulum (ER). Since all detectable precursor molecules of both fusogenic and nonfusogenic envelopes were endo H sensitive (Fig. 5C), it is likely that ER export was a rate-limiting step in the maturation of all envelopes and that envelopes absent at the cell surface were accumulating in this compartment.

DISCUSSION

We derived chimeric retroviral envelopes in which all or part of the F-MuLV transmembrane component was substituted for that of HTLV-1. An HTLV-1 chimeric envelope containing heterologous membrane-spanning and cytoplasmic domains (TMi) was highly fusogenic for different cell lines, whereas hybrids containing all or part of the extracellular domain of the transmembrane (TMe) were not. The lack of detectable fusion observed with the TMe-containing envelopes was due not to a defect in production of the precursor but rather to weak expression at the cell surface with generally no detectable cleaved SU/TM products. Extensive endo H susceptibility of these precursors indicated that they localized in the ER. Immunofluorescence labeling with polyclonal antibodies to ER-resident proteins (20) also showed that HFTM and HFTMe colocalized mainly with ER-specific markers (not shown). This finding was consistent with the fact that SU/TM cleavage occurs late in the Golgi apparatus (15). However, in a very few cells, weak colocalization with Rab6 (10), a Golgi stack-specific marker, was observed (not shown). Therefore, TMe-containing chimeras were inefficiently processed during transport from the ER to the Golgi apparatus, and the rarely observed presence of envelope in the Golgi apparatus was most likely due to leakage in the ER quality control, as observed in overexpression systems (37).

Any partial substitution introduced in the TMe of HTLV-1 appeared dramatically deleterious to proper structure and maturation, even when spanning a relatively small (chimera HFTMe404-447) or conserved region (chimera HFTMe312-377). We cannot exclude the possibility that more restricted substitutions within the HTLV-1 TMe are tolerated as previously reported for a functional substitution restricted to the

12-amino-acid-long fusogenic peptide located at the amino terminus of TMe of bovine leukemia virus and simian immunodeficiency virus (37). The apparent blockade in the ER of Env precursors harboring heterologous SU and TMe suggested that a rather specific interaction between these domains is required for the precursor to proceed to the Golgi apparatus. In contrast, substitution of the entire TMi region, including both membrane-spanning and cytoplasmic domains, had no detectable effect on cell surface expression and cell fusion in our model. It may be of interest that in the corresponding functional hybrids, the membrane-spanning domain is seven amino acids longer than in the original HTLV-1, envelope with no difference in charged residue content. The MuLV cytoplasmic tail includes the so-called R peptide, which is 16 amino acids long and is located at the carboxy terminus of the TM (12). Cleavage of the R peptide by the viral protease takes place in the released virions and activates the membrane fusion capability of MuLV envelopes for NIH 3T3 cells (29, 31). Cleavage of the R peptide in HFTMi does not take place, since our expression system does not include the viral protease and does not lead to release of viral particles. Despite this lack of cleavage, HFTMi, which contains the R peptide, appeared as efficiently fusogenic for NIH 3T3 cells as the HTLV-1 envelope. Thus, the fusion-inhibitory effect of the R peptide might be less dramatic in the presence of the HTLV-1 SU. Alternatively, our overexpression system may overcome this inhibitory effect. Thus, it remains to be determined whether cleavage of the R peptide in this context would enhance fusion of NIH 3T3 cells.

Interestingly, we observed that envelopes lacking the cytoplasmic domain (HdAC, HdC, and HFTMidC) were more abundantly present at the cell surface and had the lowest accumulation of precursor in the ER. This observation is in agreement with other findings which showed that the cytoplasmic domain is not necessary for envelope maturation and transport (7, 25, 39). Moreover, our results suggested that the presence of a cytoplasmic tail, be it homologous or heterologous, decreases the rate of ER export of the envelope precursor. Nevertheless, with this series of envelopes we observed no direct relationship between levels of detection at the cell surface and fusogenicity. As previously reported by others, our results indicated that the cytoplasmic domain per se is not required for fusion. However, we observed that envelopes which lack this cytoplasmic domain lost their fusogenic capacity for some cell lines and were less fusogenic for other cell lines. An HTLV-1 envelope, also with a deletion in the cytoplasmic domain but six amino acids longer than the HdC mutant described here and harboring two different substitutions at the carboxy terminus, has been shown to increase fusogenicity for XC cells (27). Since Cos cells were used in both cases, the apparent contradiction between these results and ours regarding the positive or negative modulation of fusion by the cytoplasmic domain is not due to the presenting cells. Rather, this difference is most likely due to the different locations of the truncations and to the two-amino-acid substitution at the carboxy terminus. Nevertheless, both sets of results underscore the role of the cytoplasmic domain in fusion modulation.

The importance of the membrane-spanning domain in fusion efficiency has been shown for HIV-1 by using mutations which decrease or abolish cell fusion without disrupting membrane anchor and cell surface transport (14). It has also been shown that substitution of the membrane-spanning domain of the nonfusogenic vesicular stomatitis virus envelope for that of the fusogenic HIV-1 altered HIV fusion abilities while preserving anchorage (22). We showed that in the context of a complete envelope, the membrane-spanning domain of the fusogenic HTLV-1 envelope could be efficiently replaced by that of the fusogenic F-MuLV (HFTMi). This result argued in favor of interchangeable and similarly efficient fusogenic membrane-spanning domains for F-MuLV and HTLV-1. Surprisingly, this was contradicted by our other results obtained with HdC and HFTMidC, which harbor a truncated cytoplasmic domain and which differ only in their membrane-spanning domains. Indeed, the truncated envelope containing the F-MuLV membrane-spanning domain (HFTMidC) was significantly less fusogenic than HdC, which contained the HTLV membrane-spanning domain. Together, these results indicated that the membrane-spanning and cytoplasmic domains form an entity which modulates fusion and that the less efficient fusogenicity conferred by the F-MuLV membrane-spanning domain can be overridden by adjunction of the F-MuLV cytoplasmic domain (compare HFTMidC with HdC and HFTMi in Table 1).

The influence of the membrane-spanning and cytoplasmic domains on fusion may be exerted at several levels. (i) The cytoplasmic domain may directly interact with the cell membranes, and intriguingly, it has been claimed that the long cytoplasmic tail of HIV-1 may even be expressed at the cell surface (13). (ii) Modifications of the cytoplasmic or membrane-spanning domains may in turn modify the conformation of the extracellular components of the envelope such as to increase or reduce fusion. Thus, it has been suggested that truncations of the cytoplasmic domain of the simian immunodeficiency virus envelope which modulate fusogenicity for several cell lines induce conformational changes in the extracellular domain of the TM protein (35). Also, a correlation between fusogenicity and the angle of insertion of the membrane-spanning domain has been suggested for the HTLV-related bovine leukemia virus (37). (iii) Differences in expression and envelope stability at the cell surface may also be involved. The latter differences were not responsible for the distinct fusogenic abilities of truncated and full-length simian immunodeficiency virus envelopes (35). In addition, oligomers of both simian immunodeficiency virus and mink cell focus-forming MuLV lacking the cytoplasmic domain or the entire TMi, respectively, were more stable than the corresponding full-length envelopes (35, 36).

The envelope-driven fusion assay described in this report is based on blue coloration occurring upon cell-to-cell fusion. Hence, this assay allowed us to detect single-cell fusion, and spontaneous background syncytia observed with many cell lines could be easily disregarded from their lack of coloration. Contrary to other previously described assays (34), the present assay revealed the fusogenic potential of the HTLV-1 envelope toward the mouse NIH 3T3 cells, demonstrating that these cells harbor an HTLV-1 receptor. The observation that under certain conditions an envelope can be efficiently fusogenic for only the most sensitive indicator cells (see results for HFT MidC in Table 1) underscores the fact that differences in fusogenic abilities between envelopes might go unnoticed when only highly susceptible cell lines are used. The four cell lines used in this study had different susceptibilities to fusion and subsequent syncytium formation upon coculture with homogeneous or chimeric HTLV-1 envelope-expressing Cos cells. Differences in receptor availability or other parameters acting at the surface of these cell lines, rather than the strict presence or absence of a receptor, are likely to play a role in these differences. Thus, under our experimental conditions, cell-specific fusion appeared to depend on thresholds resulting from intrinsic susceptibility to fusion of a given cell line and fusogenic potential of the envelope. Regarding the latter, we showed that the F-MuLV membrane-spanning domain, and not the cyto-

plasmic domain, seemed to confer a decreased fusogenic ability compared with the corresponding HTLV-1 domain.

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