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Proteinase-Resistant Factors in Human Erythrocyte Membranes Mediate CD4-Dependent Fusion with Cells Expressing Human Immunodeficiency Virus Type 1 Envelope Glycoproteins

TATJANA DRAGIC, LAURENT PICARD, AND MARC ALIZON*

Institut National de la Santé et de la Recherche Médicale U332, Institut Cochin de Génétique Moléculaire, Paris, France

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Murine CD4$^+$ cells are resistant to human immunodeficiency virus type 1 (HIV-1) entry and to fusion with cells expressing HIV-1 envelope glycoproteins (Env). The role of human-specific factors in Env/CD4-mediated fusion is shown by the ability of transient cell hybrids formed between CD4$^+$ murine cells and human HeLa cells to fuse with Env$^+$ cells. Fusion events were observed when other human cells, including erythrocytes, were substituted for HeLa cells in the hybrids. Experiments with erythrocyte ghosts showed that the factors allowing Env/CD4-mediated fusion are located in the plasma membrane. These factors were fully active after extensive digestion of erythrocytes with proteinase K or pronase. Nonprotein components of human plasma membranes, possibly glycolipids, could therefore be required for Env/CD4-mediated fusion and virus entry.

Human immunodeficiency virus type 1 (HIV-1) enters CD4$^+$ cells after fusion of its envelope with the plasma membrane. This process is mediated by the viral envelope glycoproteins gp120 and gp41 (Env) and initiated by the interaction of gp120 with the CD4 receptor (for recent reviews on HIV entry, see references 37, 44, 47, and 52). Other cellular components are probably required for membrane fusion, since expression of human CD4 in nonprimate cell lines allows neither their infection by HIV-1 nor their fusion with cells expressing HIV-1 envelope glycoproteins (4, 13, 31, 32, 36). In contrast, all human cell lines tested were permissive to Env-mediated fusion and/or to HIV-1 entry provided that CD4 was expressed on their surface, either constitutively or after gene transfer. The human glioma cell lines U87MG and U373 and the squamous cell carcinoma cell line SCL1 represent exceptions to this rule (12, 13, 23). The U87MG cell line, and more recently a mink and a cat cell line, was found to be permissive for CD4-dependent infection by HIV-2 (13, 36). Different cellular factors could therefore be required for HIV-1 and HIV-2 entry, even though both retroviruses use CD4 as their attachment receptor.

Over the past years, numerous cellular factors have been proposed to participate in cell fusion or virus entry mediated by CD4 and HIV-1 envelope glycoproteins. However, these observations are supported only by indirect or circumstantial evidence. None of these molecules were found to be sufficient or absolutely required for HIV entry. Among these are the LFA-1 adhesion molecule (26, 38), the Fc receptor (35), major histocompatibility complex class I and class II molecules (15, 33), CD7 (43), lectins (16), heparan sulfates (39), and unidentified molecules of diverse molecular weights (11, 19, 25, 41). Particular attention has been devoted to cell surface proteases, proposed to cleave gp120 in the V3 domain, thus allowing conformational changes and activation of membrane fusion (14, 24, 28). However, this model remains hypothetical. Also, gp120 cleavage was observed in hamster CHO cells, which are resistant to CD4-dependent HIV-1 entry, and is therefore not specific to human cells (13). More recently, dipeptidyl peptidase IV (CD26), a membrane protease, was proposed to be a cofactor sufficient for HIV-1 entry in murine CD4$^+$ cells (9). We and others have been unable to confirm this observation (1, 7, 10, 29, 40).

Attempts to identify human cellular factors involved in HIV-1 entry by genetic complementation of murine or simian cells have not been successful (30a; our unpublished results). To our knowledge, two groups obtained stable human-murine somatic hybrids apparently permissive for CD4-dependent HIV-1 infection (42, 51). Several other investigators could not detect permissivity for HIV-1 entry or Env-mediated fusion in collections of CD4$^+$ somatic cell hybrids even when they carried a complete set of human chromosomes (4, 31, 48; our unpublished results). In contrast, transient hybrids formed between murine CD4$^+$ NIH 3T3 cells and human HeLa cells were found to be permissive for fusion with cells expressing HIV-1 or HIV-2 envelope proteins (8, 17, 18). Permissivity for Env-mediated fusion and HIV-1 entry was observed for CD4$^+$ transient hybrids formed between HeLa cells and the HIV-1-resistant human cell lines U87MG and U373 (17, 23), but not for CD4$^+$ hybrids between murine or simian cells and U87MG cells. This type of assay can therefore be used to determine if the human-specific factors required for Env/CD4-mediated fusion are expressed by a given cell line. Here, we report evidence that these factors are present in human erythrocytes (RBC) and are associated with the plasma membrane. The permissivity of the hybrids was not affected by extensive digestion of RBC with proteinase K.

MATERIALS AND METHODS

Cell lines. Adherent cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin and streptomycin, 2 mM glutamine, and either 10% newborn calf serum (for NIH 3T3 derivatives) or 10% fetal calf serum (for HeLa derivatives). Suspension-growing cells were propagated in RPMI 1640 plus antibiotics, glutamine, and 10% fetal calf serum. The HeLa-LTRlacZ (Z24) and 3T3-CD4-LTRlacZ (SC6) cell lines have been described before, as have the H9/IIIB and CEM/NDK cell lines, chronically infected with HIV-1 LAI and NDK isolates, and the uninfected Jurkat-*tat* cell line (18). The K562 (3) and HEL (34) human erythroleukemia cell lines were obtained from S. Gisselbrecht (Institut Cochin de Génétique Moléculaire).

Preparation of RBC and RBC ghosts. RBC and leukocytes from different healthy donors were separated on Ficoll-Paque (Pharmacia). The RBC pellet

* Corresponding author. Mailing address: INSERM U332, Institut Cochin de Génétique Moléculaire, 22 rue Méchain, 75014 Paris, France. Phone: 33-1-40 51 64 86. Fax: 33-1-40 51 77 49.

was washed three times in cold, serum-free RPMI 1640. RBC could be stored in RPMI 1640 for several days at 4°C.

Ghosts were prepared by a modified version of the method of Steck and Kant (45). Briefly, $\sim 10^{10}$ RBC were washed twice with cold phosphate-buffered saline (PBS) and then lysed in 100 ml of cold 5 mM sodium phosphate buffer (pH 8)–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride. The ghosts were pelleted by centrifugation and washed twice in lysis buffer and then twice in PBS. They could be stored in PBS for several days at 4°C.

Proteinase treatment of RBC. RBC (2×10^8/ml) were digested with proteinase K (100 μg/ml; Boehringer, Mannheim, Germany) for 1 h at 37°C in serum-free DMEM and then washed three times in cold serum-free DMEM. Intracellular proteinase digestions were carried out under identical conditions except that Nonidet P-40 (0.005%, final) was added to digestion and washing media. Treated cells were resuspended in serum-free DMEM.

Analysis of RBC protein digestions. Intact or treated RBC were labelled for fluorescence-activated cell sorting (FACS) analysis with anti-CD35 (CR1) monoclonal antibody at a dilution of 1:100 (Becton Dickinson), antiglycophorin α plus β at 1:100 (Sigma Immunochemicals), or anti-human β_2-microglobulin at 1:100 (Serotec) as a negative control, followed by a phycoerythrin-conjugated goat anti-mouse immunoglobulin G (IgG) at 1:100 (Immunotech). Analysis was carried out with a Becton Dickinson FACScan.

For Western blotting (immunoblotting), 10^6 RBC were lysed in gel loading buffer (50 mM Tris-Cl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol [final]), boiled for 10 min, and stored at −80°C. Sample proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Filters were incubated in TBS–5% milk protein–0.1% Tween 20 with antispectrin monoclonal antibody at 1:1,000 (Sigma Immunochemicals) or anti-band 3 intracellular epitope at 1:5,000 (Sigma Immunochemicals) for 30 min at room temperature, followed by a sheep anti-mouse Ig coupled to horseradish peroxidase (HRP) diluted to 1:10,000 (Amersham) for 30 min at room temperature.

For total protein digestion profiles, 2×10^8 RBC were surface labelled with 0.5 mg of sulfo-*N*-hydrosuccimide-biotin (sulfo-NHS-biotin; Pierce) per ml for 1 h at 4°C, followed by proteinase K treatment. Protein separation and blotting were carried out as above with HRP-conjugated streptavidin 1:15,000 (Dako). HRP activity was revealed by using the enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham).

PEG fusions and X-Gal assay. Trypsinized adherent cells and suspension-growing cells or RBC were washed with warm DMEM. Cells to be fused were mixed and pelleted together in 15-ml round-bottomed polypropylene tubes. K562, HEL, or CEM cells were mixed in a 3:1 ratio with either Z24 or SC6 LTRlacZ cells; RBC or ghosts were mixed in a 10:1 ratio with LTRlacZ cells. Cell pellets were resuspended in 1.5 ml of a 50% solution of polyethylene glycol 6000 (PEG-6000; Merck) in DMEM warmed to 37°C and then immediately in 10 ml of warm serum-free DMEM. After centrifugation, cells were washed once more in serum-free DMEM and plated in 35-mm wells (six-well plates) at a density of $\sim 10^6$ adherent cells per well. Cells were allowed to adhere for 4 h, washed once, and placed in coculture with $\sim 2 \times 10^6$ effector cells per well for the next 20 h. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) assays were carried out overnight at 4°C, and blue syncytia were scored as previously described (17, 18).

RESULTS

CD4$^+$ murine cells and human RBC hybrids fuse with HIV-1-infected cells. We have established a murine cell line, SC6, bearing human CD4 and the *Escherichia coli* β-galactosidase gene (*lacZ*) under the transcriptional control of the HIV-1 long terminal repeat (LTR) sequences (18). Because they are of murine origin, SC6 cells are not infected by HIV-1, nor do they fuse to form syncytia with HIV-1 envelope-expressing cells. We have previously shown that transient hybrids (heterokaryons) formed by PEG treatment of SC6 and human HeLa cells fused with chronically HIV-1-infected cells, expressing Env and Tat proteins (18). These fusion events can be specifically detected by the induction of β-galactosidase activity in syncytia, due to Tat-induced transactivation of the LTRlacZ transgene. Blue-stained multinucleated structures are detected after fixation of cells and incubation with the X-Gal substrate (Fig. 1). By forming PEG hybrids with SC6 cells, it is therefore possible to determine if a given cell line is resistant or permissive to Env/CD4-induced fusion.

Using this type of assay, we observed that human RBC conferred fusion permissivity on hybrids formed with murine cells (Fig. 1b). Hybrids formed with sheep, rabbit, or murine RBC and SC6 cells did not fuse above background levels with HIV-1-infected cells (Table 1; other data not shown). Hybrids of human RBC and CD4-negative HeLa-LTRlacZ cells did not fuse with Tat$^+$/Env$^+$ effectors, and CD4$^+$ hybrids did not fuse with Jurkat-*tat* cells, used as Tat$^+$/Env$^-$ effectors (Table 1). Fusion was therefore dependent upon the interaction of CD4 and HIV-1 envelope glycoproteins and upon factors present in human RBC.

RBC ghosts complement murine cells for Env/CD4-induced fusion. To pinpoint the cellular location of the fusion factor, RBC were lysed by osmotic shock and washed extensively to free them of their cytoplasmic contents. Hybrids formed by PEG treatment of murine SC6 cells and human RBC ghosts fused with Tat$^+$/Env$^+$ cells (Fig. 1c). The higher numbers of fusion events detected in this experiment (Table 1) could be due to a better efficiency of PEG fusion of murine cells and ghosts, which are less rigid than intact RBC. Fusion was Env- and CD4-dependent and was not detected in hybrids formed with ghosts from sheep RBC (Table 1). These experiments indicate that the factors allowing human cells to fuse with HIV-1 envelope-expressing cells are associated with the plasma membrane.

Effect on Env-induced fusion of proteinase K treatment of RBC. To address the biochemical nature of the factors allowing fusion of CD4$^+$ hybrids with Env$^+$ cells, RBC were treated with proteolytic enzymes. Their lack of a nucleus or a translation machinery makes any modification caused by this treatment irreversible. RBC were treated for 1 h with a relatively high amount of proteinase K (100 μg/ml), which has no cleavage site specificity. The efficiency of the proteolytic treatment was indicated by the loss of high-molecular-weight proteins after SDS-PAGE and Coomassie blue staining of RBC extracts (data not shown). For more sensitive detection, we also analyzed cell surface sulfo-NHS-biotin-labelled proteins by Western blotting with streptavidin-HRP, revealed by enhanced chemiluminescence (Amersham). No biotinylated proteins could be detected after proteinase K treatment of RBC (Fig. 2). This technique detects picogram amounts of antigen and should theoretically allow the detection of about 10 molecules of a 50-kDa protein per RBC. Also, proteinase K treatment eliminated cell surface CD35 and glycophorin α plus β, as shown by FACS analysis and Western blotting (Fig. 3a and data not shown). PEG hybrids formed with proteinase-treated RBC and murine CD4$^+$ cells were able to fuse with Env$^+$ cells (Fig. 1d and Table 2). Similar results were obtained after pronase digestion of RBC under identical conditions (data not shown).

To address the possible role of proteins located on the cytoplasmic surface of the plasma membrane, RBC were digested with proteinase K in the presence of a small amount of the Nonidet P-40 detergent. Intact spectrin, an abundant protein attached to the inner leaflet of the RBC membrane, and band 3 (intracytoplasmic epitope), an RBC transmembrane protein, were no longer detectable by Western blot analysis (Fig. 3b). In spite of such extensive proteolysis of RBC, hybrids formed with murine cells were still permissive for Env/CD4-mediated fusion. More fusion events were actually detected than in parallel experiments with RBC treated only with Nonidet P-40 (Table 2), probably because PEG fusion is more efficient on protein-free membranes (27).

Human erythroleukemia cell line K562 is resistant to Env/CD4-mediated fusion. Two human cell lines with pre-RBC characteristics, K562 and HEL, were tested for their ability to complement murine SC6 cells for Env/CD4-mediated fusion. Interestingly, complementation was not detected with the K562 cell line, while SC6-HEL heterokaryons readily fused with HIV-1-infected cells (Table 3). As could be expected,

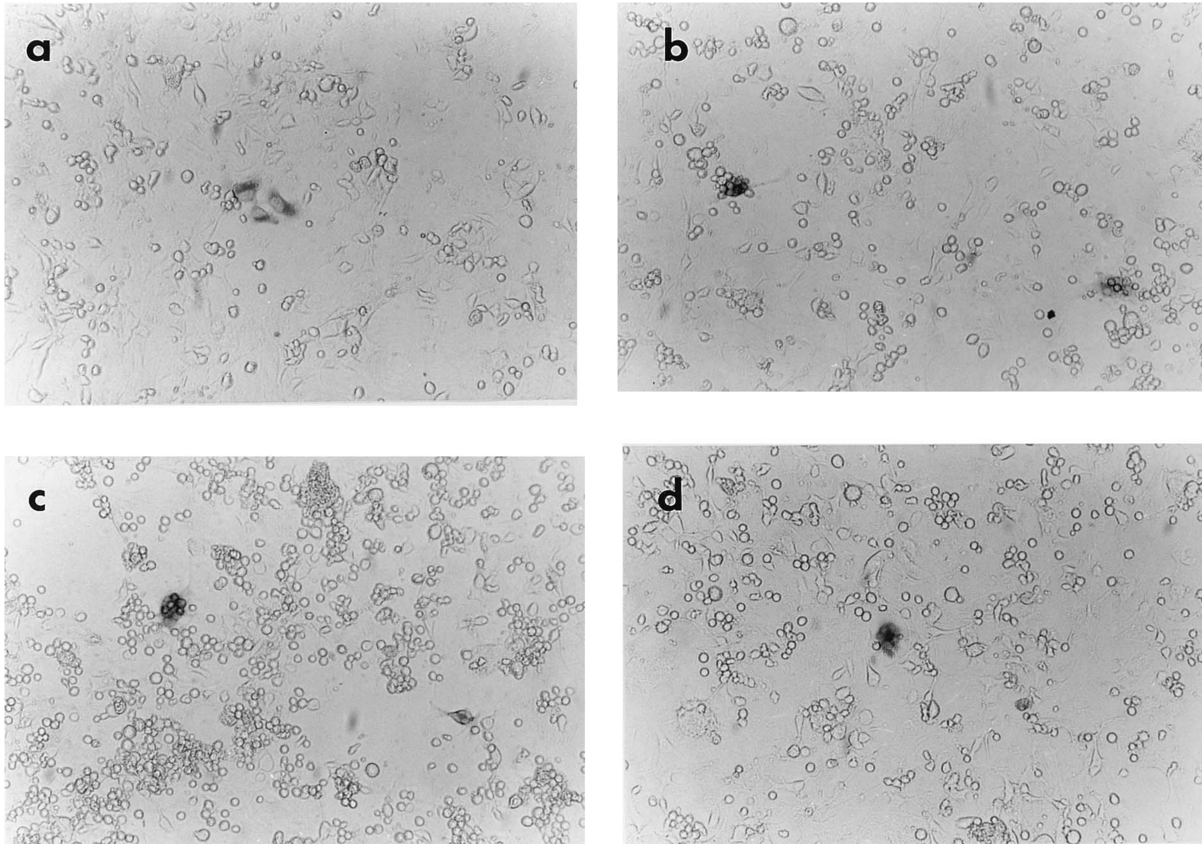

FIG. 1. Detection of fusion between CD4$^+$ cell hybrids and cells expressing HIV-1 envelope glycoproteins. Hybrids were formed by PEG fusion of 3T3-CD4-LTRlacZ (SC6) and either CEM cells (a), human RBC (b), human RBC ghosts (c), or proteinase K-treated human RBC (d). Hybrids were cocultured for 20 h with chronically HIV-1-infected H9/IIIB cells. Photographs were taken under 40× magnification after an overnight X-Gal assay at 4°C.

CD4$^+$ K562 cells (a gift from T. Valère, ICGM) were resistant to HIV-1 infection and syncytium formation with HIV-1-infected cells (data not shown). Similar observations have been made by D. Klatzmann and coworkers (CHU Pitié, Paris) with another CD4$^+$ K562 cell line derivative (28a). Like other human cell lines resistant to HIV-1 infection, K562 cells do not express dominant-acting inhibitors of Env/CD4-mediated fusion, since hybrids formed from CD4$^+$ K562 and HeLa-LTRlacZ readily fused with Env$^+$ cells (Table 3).

Treatment of K562 cells with differentiating agents, such as sodium butyrate and heme, did not modify their resistance to HIV-1. However, in our hands, differentiation was relatively inefficient and did not go beyond the normoblast phase, judging by the morphology of the cells after May-Grünwald-Giemsa staining (data not shown). Systematic comparison of K562 and HEL cell lines for surface markers could provide clues to the identity of the factors responsible for permissivity for HIV-1.

DISCUSSION

Hybrids formed between CD4$^+$ murine cells and human RBC were permissive for fusion with cells expressing HIV-1 envelope proteins (Env). Human RBC, like most human cell lines, apparently carry a factor(s) that is necessary and sufficient for Env/CD4-mediated membrane fusion. We assume

TABLE 1. Fusion of effector cells with hybrids formed between LTRlacZ cells and RBC

LTRlacZ cells	No. of blue-stained foci[a] with fusion partner:				Effector cells	HIV-1 envelope expression
	Human RBC	Sheep RBC	Human RBC ghosts	Sheep RBC ghosts		
3T3-CD4 (SC6)	38 ± 11	2 ± 2	73 ± 19	3 ± 2	H9/IIIB	+
	31 ± 8	3 ± 2	68 ± 21	4 ± 2	CEM/NDK	+
	3 ± 2	3 ± 1	4 ± 2	2 ± 1	Jurkat-*tat*	−
HeLa (Z24)	3 ± 2	ND[b]	4 ± 2	ND	H9/IIIB	+
	4 ± 1	ND	3 ± 2	ND	CEM/NDK	+
	3 ± 2	ND	3 ± 1	ND	Jurkat-*tat*	−

[a] Per 35-mm plate, after overnight X-Gal incubation at 4°C. Each value is the average of four independent experiments.
[b] ND, not done.

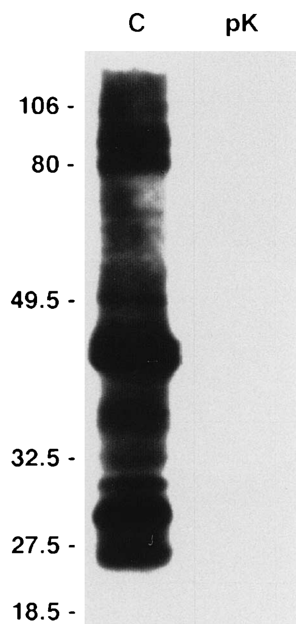

FIG. 2. Analysis of human RBC proteins after proteinase K (pK) treatment. Streptavidin-HRP detection of biotinylated RBC surface proteins after SDS-PAGE separation and membrane transfer. HRP activity was revealed by enhanced chemiluminescence. Lane C, intact, biotinylated RBC; lane pK, proteinase K-treated, biotinylated RBC. The positions of size markers are shown (in kilodaltons).

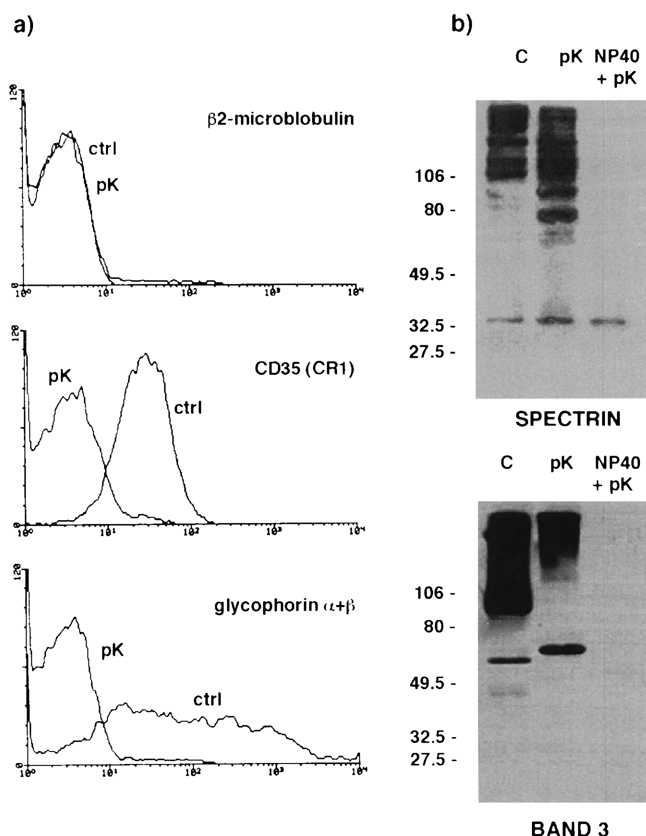

FIG. 3. Analysis of human RBC antigens after treatment by proteinase K or proteinase K and Nonidet P-40. (a) FACS analysis of intact or proteinase K-treated RBC with anti-β_2-microglobulin, anti-CD35, and antiglycophorin (control [ctrl]) monoclonal antibodies. (b) Immunological detection of spectrin and band 3 intracellular epitope after membrane transfer. Lanes C, untreated RBC; lanes pK, proteinase-treated RBC; lane NP40 + pK, Nonidet P-40- and proteinase K-treated RBC.

that this factor(s) is ubiquitously expressed in most, if not all, human tissues. Experiments with RBC ghosts allowed us to localize these fusion factors to the plasma membrane, as could be expected from their role. Hybrids formed with human RBC fused less efficiently with Env^+ cells than hybrids formed with human nucleated cells (Table 3). This may reflect differences in the level of expression of the fusion factor(s), but also the relative resistance of RBC to PEG-induced fusion (27). Their abundant glycocalyx may hamper close apposition of membranes, and their rigid cytoskeleton may limit diffusion of membrane factors.

Env/CD4-mediated membrane fusion is a complex process, and its restriction to human cells was naturally considered to be due to the interaction of HIV-1 with human-specific proteins. Our experiments with $CD4^+$ hybrids composed of murine cells and human RBC showed that the factors allowing fusion with Env^+ cells were fully active after extensive digestion of human RBC by proteinase K or pronase. The extent of proteolysis is shown by the loss of detectable biotinylated cell surface proteins in a very sensitive assay, as well as the loss of detectable, specific membrane-associated antigens. Differences exist in the accessibility of membrane proteins to proteases (and possibly to biotinylation), and it cannot be absolutely ruled out that the fusion factor is a highly protected protein—for example, by hyperglycosylation—or one required in trace amounts. We note that apparently milder conditions of proteolysis were usually sufficient to establish the protein or nonprotein nature of viral receptors (6, 46, 49). The role of nonprotein components of the plasma membrane in Env/CD4-mediated membrane fusion should therefore be considered.

Besides proteins, plasma membranes contain phospholipids,

TABLE 2. Effect of treatment of human RBC with detergent and/or proteinase on CD4/Env-mediated fusion

LTRlacZ cells	No. of blue-stained foci[a] after fusion with human RBC treated with:			Effector cells	HIV-1 envelope expression
	Nonidet P-40	Proteinase K	Nonidet P-40 + proteinase K		
3T3-CD4 (SC6)	40 ± 13	66 ± 16	62 ± 18	H9/IIIB	+
	36 ± 14	58 ± 14	52 ± 15	CEM/NDK	+
	3 ± 2	3 ± 2	4 ± 2	Jurkat-*tat*	−
HeLa (Z24)	4 ± 2	3 ± 2	2 ± 2	H9/IIIB	+
	ND[b]	ND	ND	CEM/NDK	+
	ND	ND	ND	Jurkat-*tat*	−

[a] Per 35-mm plate, after overnight X-Gal incubation at 4°C. Each value is the average of three independent experiments.
[b] ND, not done.

TABLE 3. Fusion of effector cells with hybrids formed between LTRlacZ cells and human cell lines

LTRlacZ cells	No. of blue-stained foci[a] with fusion partner:				Effector cells	HIV-1 envelope expression
	K562	K562-CD4	HEL	CEM		
3T3-CD4 (SC6)	2 ± 1	2 ± 2	214 ± 34	136 ± 21	H9/IIIB	+
	3 ± 2	ND[b]	254 ± 36	111 ± 18	CEM/NDK	+
	2 ± 1	ND	2 ± 1	2 ± 1	Jurkat-*tat*	−
HeLa (Z24)	2 ± 1	111 ± 14	2 ± 1	134 ± 19	H9/IIIB	+
	ND	163 ± 23	ND	129 ± 19	CEM/NDK	+
	ND	3 ± 2	ND	3 ± 1	Jurkat-*tat*	−

[a] Per 35-mm plate, after overnight X-Gal incubation at 4°C. Each value is the average of three independent experiments.
[b] ND, not done.

neutral lipids, cholesterol, and glycolipids. This last class of molecules exhibits considerable diversity because of carbohydrate residue sequence, linkage anomerity, and branching position (2, 21, 50). It seems conceivable that a particular glycolipid exists only or mostly in human cells. Increasing numbers of biological functions are being attributed to glycolipids, in particular in cell adhesion (reviewed in reference 50). They also serve as receptors for a variety of parasites, bacteria, and viruses (50). The specific interaction of HIV-1 envelope glycoproteins with lipid-anchored sugar residues could initiate lipid mixing between viral and cellular membranes, thereby allowing formation of the fusion pore (53).

The surface glycoprotein of HIV-1 (gp120) has been shown to bind galactosyl ceramide (GalCer) with high affinity (5, 20). A number of reports suggest that this glycolipid mediates HIV-1 infection in certain CD4-negative human cell lines (20, 22, 30). However, this process is inefficient compared with CD4-mediated infection of most human cell lines (22, 30). Also, the $GalCer^+$ human glioma cell line U373 was found to be resistant to CD4-dependent entry of HIV-1 (23). GalCer is a simple glycolipid and is not human specific. Therefore, it seems unlikely that it alone could be sufficient for HIV-1 entry, and other human-specific lipids should be sought for. Alternatively, Env/CD4-mediated fusion could require several types of lipids, all present in different species but found in a certain ratio only in human cells.

If a complex lipid is required for Env/CD4-mediated fusion, transfer of human genomic DNA or cDNA libraries would be unlikely to complement $CD4^+$ murine cells for HIV-1 entry. If more than one human-specific enzyme is required for the synthesis of such a lipid, this approach would not allow the simultaneous transfer of the necessary genes. Furthermore, a stoichiometry of lipid precursors may be required, as well as feedback signals from other lipid synthesis pathways unlikely to be found in murine cells. This could explain the resistance to HIV-1 entry of most human-murine somatic cell hybrids, which usually retain all murine chromosomes and have a murine cell phenotype. In contrast, transient hybrids share the membrane and cytoplasmic components of both cell types. They may retain enough human membrane components, or functional human metabolic chains, to mediate fusion with Env^+ cells for a short period of time. We have noted that the permissivity of human-murine hybrids for Env/CD4-mediated fusion was maintained for several days but decreased rapidly with time when selection was applied to generate hybrid cell lines (unpublished results). The human factors controlling HIV-1 entry remain to be identified, but novel strategies taking into account the role of nonprotein components of the human membrane should be considered.

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