Analysis of Dominant-Negative Effects of Mutant Env Proteins of Human Immunodeficiency Virus Type 1

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The Env protein of human immunodeficiency virus type 1 is assembled into a stable trimer, and oligomerization is required for maintenance of viral infectivity. This property of Env suggests that Env mutants may have a dominant-negative effect on virus infectivity. To investigate this possibility, we established a packaging cell line in which both wild-type and mutant Env proteins could be expressed simultaneously in a single cell. We analyzed the effects of two types of Env mutants: cytoplasmic tail-truncated TM mutants and a mutant defective in gp120/gp41 cleavage. The cytoplasmic tail-truncated proteins were found to be incorporated into virions by forming an oligomer with wild-type TM, but could not inhibit the wild-type function. In contrast, phenotypic mixing of cleavage-defective Env with the wild-type protein caused dramatic inhibition of infectivity, indicating that this mutant has a strong dominant-negative phenotype.

Key Words: HIV-1; TM protein; cytoplasmic tail; oligomerization; packaging cell line.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein is synthesized as a precursor, gp160, which is folded, N-linked glycosylated, and oligomerized. During transport to the cell surface, gp160 is proteolytically cleaved to gp120 (SU) and gp41 (TM), which are noncovalently linked. At the cell surface, mature Env proteins are preferentially incorporated into viral particles. The maturation of the Env protein is critical to the production of infectious viruses (Freed and Martin, 1995a; Swanstrom and Wills, 1997).

It has been reported that the mature Env protein consists of a stable trimer containing SU–TM heterodimers (Farzan et al., 1998; Lu et al., 1995; Tan et al., 1997; Weissenhorn et al., 1997; Yang et al., 2000). Prior to structural maturation of the TM domain (Ottenko et al., 1996), the Env precursor is oligomerized in the rough endoplasmic reticulum through an amphipathic α-helical sequence called a “heptad repeat” (Chan et al., 1997; Earl et al., 1991; Poubmabnors et al., 1995; Weissenhorn et al., 1997). Several studies demonstrated that an Env protein-containing mutation within the heptad repeat region was defective in mediating membrane fusion and virion entry, although the mutant Env could be transported to the cell surface and incorporated into virions. This indicates that Env oligomerization is required for virus entry (Chen et al., 1993; Freed et al., 1992; Layne et al., 1990). The fact that oligomerization is required for activity raises the possibility that some Env mutants could be dominant negative, i.e., by forming a hetero-oligomer with wild-type Env.

The TM protein of primate lentiviruses has a long cytoplasmic tail (CT) region and in the case of HIV-1, it consists of approximately 150 amino acid residues. Several studies indicated that the long CT region is important for virion infectivity and that the region is required for the efficient incorporation of Env into virions through an interaction with matrix (MA) protein (Dubay et al., 1992; Freed and Martin, 1996; Gabuzda et al., 1992; Yu et al., 1993). This result is supported by a variety of biochemical (Cosson, 1996; Vincent et al., 1999) and genetic studies (Dorfman et al., 1994; Freed and Martin, 1995b; Lee et al., 1997; Mammano et al., 1995; Murakami and Freed, 2000a; Ono et al., 1997; Yu et al., 1992). An interaction between MA and CT is also supported by the observation that a tyrosine-based targeting signal in CT directs the transport of Gag particles in polarized epithelial cells (Lodge et al., 1997; Owens et al., 1991). However, several other studies indicated that a large truncation of CT did not reduce either Env incorporation or virion infectivity (Freed and Martin, 1996; Murakami and Freed, 2000b; Reil et al., 1998), suggesting that Env incorporation is

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Construction of a packaging cell line

To order to analyze the phenotype of virions that contain both wild-type and CT-truncated Env proteins, both proteins must be expressed in a single cell. This is necessary since it is difficult to exclude cells expressing only a single species of Env protein by cotransfection techniques. To approach this issue, we established a new packaging cell line derived from HeLa cells which allows both wild-type and mutant Env to be coexpressed in a single cell. Using an infectious proviral DNA clone, pLAI, we constructed the pPACK plasmid, which was engineered as follows: (1) a packaging signal region (308–324 nt) was removed, (2) the first ATG codon of the rev ORF was converted to ACG (no Rev expression), (3) a hygromycin B phosphotransferase (hyg') gene was inserted into the nef ORF region (8491–8645 nt), and (4) the vif gene was disrupted by a small truncation (4706–4990 nt). HeLa cells were transfected with pPACK DNA and cell clones with an integrated copy of pPACK were obtained by hygromycin B selection of cells expressing the tat and hyg' genes. Complementation of rev gene expression (e.g., by transfection of prev-1; Adachi et al., 1996) in the cell clones triggers the expression of HIV-1 gene products (Gag, Pol, Env, and Vpr) from the integrated pPACK. One of the isolated clones with these properties was designated RIPH (Rev-inducible packaging HeLa) (Fig. 1).

To verify the response of RIPH cells to rev expression, the cells were transfected with various amounts of a mutant proviral DNA (pΔCT-104), which encodes CT-truncated Env protein and provides the rev gene product in trans. Gene expression derived from pPACK was assessed by analyzing Env precursors in the cell lysate: pPACK produces the wild-type gp160 precursor, whereas pΔCT-104 produces a truncated form of gp160 (Fig. 2A). Without rev complementation, RIPH cells expressed very little HIV-1 gp160; however, when the amount of pΔCT-104 was increased, the integrated copy of pPACK was activated for expression of wild-type Env. When transfected with 3–4 μg of pΔCT-104, the RIPH cells expressed almost equivalent amounts of Env proteins from both pPACK and pΔCT-104 (Fig. 2B). As a control for this experiment, we transfected RIPH cells with a rev expression plasmid, prev-1, and observed wild-type gp160 expression as shown in lane C (Fig. 2A). In addition, expression of other virus proteins, e.g., Gag and Pol, could be induced by transfection of prev-1 alone (data not shown).

Comparison of virus infectivity in HeLa and RIPH cells

CT truncations have been reported to severely reduce the virion infectivity of HIV-1. To evaluate the infectivity of CT-truncated mutant viruses, a reporter proviral DNA encoding the CAT gene, pLAI CAT(rev+), or its derivative encoding the CT-truncated Env protein, was transfected into HeLa cells; the resulting virions were used for infection of A3.01 cells. At 48 h after infection, viral infectivity was determined by analyzing CAT activity in infected cells. An env-deficient mutant, pLAI CAT(rev+), was employed as a negative control. As shown in Table 1 (HeLa), a small truncation of up to 15 amino acids (aa) did not affect infectivity, while large truncations of 42–139 aa severely impaired virion infectivity. The mutant viruses having these large truncations failed to replicate in T cell lines, such as A3.01, H9, and CEMx174 cells (data not shown) (Dubay et al., 1992; Yu et al., 1993). It was observed, however, that truncation of almost the entire CT region (ΔCT-143) restored the infectivity to 66.3% of the wild-type level.

We next analyzed the phenotypes of virions produced from RIPH cells transfected with a series of pLAI CAT(rev+) derivatives. In contrast to virions containing a single species of mutant or wild-type Env protein, virions derived from RIPH cells, in which wild-type and CT-truncated Env proteins were coexpressed, maintained a level of infectivity comparable to that of wild type (Table 1, RIPH). This indicates that the CT-truncated Env protein functioned as a recessive factor and did not interfere with virion infectivity in the phenotypic mixing experiment.

A mutant virus defective in gp120/gp41 cleavage,
envR515A, was reported to be deficient in infectivity, because the mutant Env was devoid of membrane fusion activity (Freed et al., 1989; Guo et al., 1990; McCune et al., 1988). The mutant virus produced from transfected HeLa cells showed only 0.8% of wild-type infectivity (Table 1). In phenotypic mixing experiments using RIPH cells, incorporation of R515A mutant Env almost completely blocked infectivity even though the wild-type Env protein was expected to be expressed on the virion surface. This result suggested that the precursor form of Env protein acted as a dominant-negative mutant for virion infectivity. A similar dominant-negative effect of the cleavage-defective Env could be observed with virus obtained from HeLa cells cotransfected with pLAInCAT(rev+) and penvR515A (data not shown).

Analysis of Env incorporation into virions

In view of the above results, it was next of interest to analyze the mechanism by which the Env mutants affected virion infectivity. The CT truncation had no apparent effect on cell-associated viral protein expression in transfected HeLa cells, but it severely impaired the incorporation of Env into virions (Fig. 3, AIDS serum). This suggests that reduction in Env incorporation may be a major mechanism for regulation of virion infectivity by the CT region. In contrast to the effect observed with HeLa cells, incorporation of Env into the virus particles produced by RIPH cells was not significantly affected by CT (Fig. 4, AIDS serum). These results indicated that the hetero-oligomer containing wild-type and CT-truncated

FIG. 1. Scheme for phenotypic expression in RIPH cells. pPACK DNA integrated in RIPH cells has mutations in the packaging signal region (Ψ) and rev gene and an insertion of a hyg' gene in the nef gene region. In the absence of rev expression, the RIPH cells express only tat and hyg' (top). The expression of structural viral proteins can be triggered by complementation of rev function, for example, through the transfection of HIV-1 proviral DNA (bottom). The produced virions contain the viral components derived from both the transfected provirus DNA and the integrated pPACK DNA.
Env proteins retained Env functions, including virion incorporation, attachment, membrane fusion, and penetration.

We have previously observed that the CT region is involved in selective incorporation of mature Env proteins into virions and that truncations in CT induce the incorporation of Env precursor into virions (Iwatani et al., 2001). To detect the incorporation of the Env precursor in the current study, we used a monoclonal antibody (C8 mAb), obtained from Chessie 8 hybridoma cells, which specifically recognizes the CT region of TM protein. When the CT-truncated mutants were expressed in HeLa cells, the incorporation of Env precursor into the virions was stimulated (Fig. 3, Chessie 8). However, a much smaller effect was observed with virions from RIPH cells. Note that since the Env products derived from ΔCT-139 are missing the epitope for C8 mAb, mutant Env could not be detected in the ΔCT-139 lane. The results of Fig. 3 raise the possibility that the full-length CT functions to exclude gp160 precursor protein from virions.

Hetero-oligomeric Env is incorporated into virions from RIPH cells

It has been reported that the ectodomain of TM protein directs the oligomerization of HIV-1 envelope glycoprotein (Bernstein et al., 1995; Mcinerney et al., 1998; Pombourios et al., 1995, 1997; Weissenhorn et al., 1997; Yang et al., 2000); this leads to the prediction that the CT-truncated TM is incorporated into virions as a hetero-oligomer with the wild type when produced in RIPH cells. To confirm this possibility, we analyzed the virion-associated TM proteins produced from RIPH cells by an immunoprecipitation assay under conditions that maintain the oligomeric structures of TM proteins (see Materials and Methods). To identify the hetero-oligomeric TM proteins, we constructed a proviral DNA, pLAIΔC8, which had two amino acid substitutions at the epitope recognized by C8 mAb, and derivatives having CT truncations, pLAIΔC8 · ΔCT-42, pLAIΔC8 · ΔCT-61, and pLAIΔC8 · ΔCT-104. The infectivities of these ΔC8 viruses produced from transfected HeLa cells appeared to be similar to those of the parental LAI viruses, suggesting that the mutation at the epitope did not significantly modify TM function (data not shown). By using these ΔC8 clones in the RIPH cell system, the oligomers containing wild-type TM protein could be precipitated with C8 mAb, but homo-oligomers of the ΔC8 TM protein could not. As shown in Fig. 5, the truncated TM proteins (ΔCT-42, ΔCT-61, and ΔCT-104) having the ΔC8 mutation could be coimmunoprecipitated with the wild type. These results suggested that the CT-truncated TM proteins could be incorporated in virions by forming hetero-oligomers with wild-type TM. However, further biochemical studies will be required to exclude the possibility that there was some interaction among different homo-oligomers that could result in coimmunoprecipitation.

DISCUSSION

In the present study, we investigated the effect of phenotypic mixing of Env proteins on both virus infectiv-

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<th>Mutant</th>
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<th>Virus infectivity (%)</th>
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<tr>
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<td>0.8 ± 3.0</td>
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<td>Δenv</td>
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*To analyze virus infectivity, the CAT-expressing reporter virus (LAI/NCAT[rev+]) having CT mutation was used to infect A3.01 cells. CAT activity within the infected cells was monitored. A Δenv mutant (LAI/NCAT[rev+]Δenv) served as a negative control. The data represent the averages of three independent experiments with the standard deviation noted.

*ND, not determined.
ity and Env incorporation into virions, by using a novel packaging cell line. The CT region of HIV-1 has been reported to be important for virus infectivity. Thus, we found that CT-truncated Env protein in the form of a homo-oligomer severely impaired Env incorporation in accord with earlier reports (Dubay et al., 1995; Freed and Martin, 1996; Yu et al., 1993); however, such an effect was not observed for hetero-oligomers containing wild-type protein. In contrast to the results obtained with the CT-truncated mutants, phenotypic mixing of a cleavage-defective Env (R515A) with wild-type Env markedly suppressed viral infectivity, suggesting that R515A Env is a dominant-negative protein.

System for coexpression of wild-type and mutant Env proteins in a single cell

To analyze the effect of mutant Env proteins present in hetero-oligomers containing wild-type protein, it was necessary to express both Env proteins in a single cell. With conventional cotransfection, it is difficult to exclude the cells transfected with only a single species of DNA, which would affect the interpretation of the results. For this purpose, we developed a novel packaging cell line, designated RIPH. Only RIPH cells transfected with proviral DNA clones encoding mutant env could express both wild-type and mutant Env proteins simultaneously. As illustrated in Figs. 1 and 2, it was observed that the amount of mutant gp160 protein relative to that of the wild-type increased in proportion to the amount of transfected DNA.

We could also induce Gag protein by transfection of prev-1 into RIPH cells (data not shown). In a similar manner, the RIPH cell will be applied for studying the effects of other viral mutant proteins on the interference of virus infectivity and virus assembly, since both wild-type and mutant proteins can be expressed simultaneously in a single RIPH cell but not by conventional cotransfection technique.

CT-truncated mutant shows a recessive negative mutant phenotype

In an earlier report, it was found that the hetero-oligomeric complex of wild-type and CT-truncated Env proteins in virions, which was produced by cotransfection experiments, appeared to be severely defective in the post-CD4 binding step in the virus replication (Chen et al., 1996). However, our result indicates that the infectivity of viruses produced from RIPH cells, which express both wild-type and CT-truncated Env proteins, was not reduced (Table 1). Even with a 61-residue truncation, virus infectivity was reduced to only 7.4% of the wild-type level. This small reduction in infectivity can be explained by the reduced incorporation of Env proteins in virions as shown in Fig. 4, rather than by a defect in an early phase of viral replication caused by a dominant-negative effect of the ΔCT-61 mutant. The discrepancy between the paper by Chen et al. and our results might be caused by a difference in the assay systems. Chen et al. used a cotransfection assay, whereas we have developed the RIPH cell system, in which the two Env proteins

![Radioimmunoprecipitation analysis of viral proteins in transfected HeLa cells. HeLa cells were transfected with 10 μg of the indicated proviral DNAs. At 48 h after transfection, the cells were metabolically labeled with [35S]methionine and cysteine. The cell-associated and virion-associated viral proteins were immunoprecipitated with AIDS patient serum (top) or C8 mAb (bottom) and analyzed by SDS–PAGE. The arrows indicate the positions of the viral proteins. C8 mAb could not bind to ΔCT-139 mutant Env, since it lacks the epitope for the mAb.](image-url)
are expressed at a ratio of nearly 1:1. It is also possible that higher levels of mutant protein expression are needed to interfere with infectivity.

Cleavage-defective gp160 functions as a dominant-negative mutant for virion infectivity

The precursor form of Env has been reported to be able to bind to CD4 molecules, but not to induce membrane fusion. Thus, it would appear that the mutant virus, having an R515A substitution, lost the infectivity due to a defect in virus entry process (Bosch and Pawlita, 1990; Dubay et al., 1995; Freed et al., 1992; Iwatani et al., 2001; McCune et al., 1988). In this study, the virions obtained from the RIPH cells transfected with penvR515A showed only 4.7% of the infectivity of the wild type, suggesting that the cleavage-defective gp160 (R515A) has a dominant-negative effect on virion infectivity. However, it was reported that a mutation at the cleavage site of gp160 reduced by 57% the syncytium formation induced by wild-type Env in a membrane fusion assay (Freed et al., 1992). This difference may be due to the fact that the target of the mutant Env in the dominant-negative effect would be different between the penetration of virus entry and the cell–cell fusion.

The infectivity of ΔCT-104 virus produced from HeLa cells was severely impaired even though the virion-associated gp120 of ΔCT-104 virions from HeLa cells was at a level similar to that of wild type (Table 1). We found that the CT truncation increased the incorporation of Env precursor into virions produced from transfected HeLa cells (Fig. 3, virion-associated proteins detected with C8 mAb).

FIG. 4. Radioimmunoprecipitation analysis of viral proteins in transfected RIPH cells. RIPH cells were transfected with 10 μg of the indicated proviral DNAs. At 48 h after transfection, the cells were metabolically labeled with [35S]methionine and cysteine. By using RIPH cells, viral proteins from both the integrated pPACK copy and the input plasmid DNA could be simultaneously expressed in the transfected cells, resulting in the release of phenotypically mixed virions (see schematic diagram in Fig. 1). The cell-associated and virion-associated proteins were immunoprecipitated with AIDS patient serum (top) or C8 mAb (bottom) and analyzed by SDS–PAGE. The positions of the viral proteins are indicated by arrows. The Env proteins derived from pΔCT-139, which lacks the epitope for C8 mAb, could be detected very faintly with C8 mAb (indicated with asterisks), indicating the formation of a hetero-oligomer between wild-type and ΔCT-139 Env proteins.

FIG. 5. Detection of the hetero-oligomer of TM proteins in the virions produced by RIPH cells. RIPH cells were transfected with 10 μg of the indicated proviral DNAs. Virions produced by HeLa cells transfected with pLAIΔ8 served as a control. At 48 h after transfection, the cells were metabolically labeled with [35S]methionine and cysteine. The virions produced in culture medium were lysed with 1% digitonin lysis buffer, as described under Materials and Methods. The lysates were immunoprecipitated with C8 mAb and analyzed by SDS–PAGE. Note that C8 mAb could not directly recognize the ΔC8 TM proteins. As a control, RIPH cells were transfected with pLAIΔgagΔenv, which is defective in production of gag and env gene products. The positions of gp160 and gp41 are shown with arrows.
mAb). Therefore, the reduced infectivity by CT truncation might be responsible for the interference by nonspecifically incorporated precursor Env proteins as well as the inhibition of gp120/gp41 incorporation.

CT-truncated Env protein could be incorporated into virions as a form of oligomer with the wild type

The results shown in Fig. 4 clearly indicate that CT-truncated TM proteins are incorporated into virions produced by RIPH cells expressing both wild-type and CT-truncated TM proteins. However, it was difficult to determine whether the mutant protein formed a hetero-oligomer with the wild type. To investigate whether the hetero-oligomer of wild-type and CT-truncated Env proteins was incorporated into virions, we performed an immunoprecipitation assay, as described under Materials and Methods. The assay indicated that the truncated forms of TM proteins could be coprecipitated with the wild type from virions produced by RIPH cells. This suggests that the hetero-oligomer form of TM protein can be incorporated into virions. The amount of CT-truncated TM protein coprecipitated with wild type appeared to be less than that of wild type alone (Fig. 5). This might be due to incorporation of the homo-oligomer of wild-type TM proteins. It has been shown that the function of the CT region in Env incorporation was cell-type specific (Murakami and Freed, 2000b). In that report, HeLa cells were described as a permissive cell for CT truncation. In our system with HeLa-derived cells, the CT-truncated mutant oligomerized with the wild type did not significantly affect Env incorporation, although it may be possible that the incorporation of the hetero-oligomeric Env is modified in a cell-type-dependent manner.

In conclusion, in this report we have introduced a new system, RIPH cells, to analyze the phenotype of viruses containing both wild-type and mutant Env proteins. We have also described the effects of CT-truncated and gp120/gp41 cleavage-site mutants. The results obtained with the cleavage-defective mutant showing a dominant-negative effect on infectivity suggest that other hetero-oligomeric viral proteins may also be exploited for developing inhibitors of virus infectivity. The RIPH cell system will be useful for the research of such molecules.

MATERIALS AND METHODS

Plasmid construction

Full-length infectious DNA clone HIV-1 LAI, designated pLAI (kindly provided by Dr. Keith Peden), was used in this study. The infectious DNA clones encoding mutant Env with serial truncations in the CT region were constructed using PCR-based oligonucleotide-directed mutagenesis (Table 1). The sequences for the 5′ primers were 5′(7948)-GGATATTTTCTGATAT-TAGTTTCAG-3′ (for pΔCT-143), 5′(7957)-CCATTATCGT-

TCTAGACCCACCTC-3′ (for pΔCT-139), 5′(8064)-GAA CGGATCTGAGC-CACT-3′ (for pΔCT-104), 5′(8191)-GCCC-TCAAATATTAGTGGAAT-3′ (for pΔCT-61), 5′(8249)-CTGTTAGCTAGCTCAATGCC-3′ (for pΔCT-42), 5′(8336)-TTCTGCTAGATACTGAGAATAAG-3′ (for pΔCT-15), and 5′(8365)-GGCTTGAAAGGATCTAGTATAAGATGG-3′ (for pΔCT-2). The mutant proviral HIV-1 DNA with a mutation at the cleavage site between gp120 and gp41 was constructed by oligonucleotide-directed mutagenesis. The sequence for the 5′ primer was 5′(7338)-GAGAGAAAAAGCTGCAGTGG-3′ (for pΔCT-61). The resulting construct, designated penvR515A, could express a mutant Env protein having a single amino acid change, R515A. This mutation inactivates cleavage of the gp160 precursor to gp120 and gp41, as previously reported (Bosch and Pawlita, 1990; Dubay et al., 1995). pLAInCAT(rev+) is similar to pNLnCAT (Adachi et al., 1995; Sakai et al., 1993), which has an intact rev gene and a nef gene replaced by the CAT gene. pLAInCAT(rev+)Δgag has a frame-shift mutation at the ClaI restriction site (378 nt) in the gag coding region. pLAInCAT(rev+)Δenv has a frame-shift mutation at a KpnI restriction site (5930 nt) in the env coding region. The rev expression plasmid, prev1, was described in an earlier report (Sakai et al., 1993). The pLAIΔC8 plasmid has a mutation in the epitope recognized by a monoclonal antibody (C8 mAb), which is located in the CT region (the epitope sequence PDRPEG was substituted with PARPER) (Abacioglu et al., 1994). The primer for the mutagenesis was 5′(7987)-CCGAGGGGACCCGCCGCGAGGAAATGAA-GAA. To establish packaging cells in which the virus structural proteins were induced by rev expression, we constructed a plasmid of HIV-1 proviral DNA, pPACK. pPACK is described under Results.

Antibodies

Monoclonal antibody (C8 mAb) was affinity purified from a hybridoma cell (Chessie 8) (Abacioglu et al., 1994), which was obtained through the AIDS Research and Reference Reagent Program, National Institute for Allergy and Infectious Diseases. AIDS patient serum was kindly provided by Dr. M. W. Cloyd (University of Texas Medical Branch).

Cells and transfection

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The human CD4+ cell line A3.01 was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. To establish a packaging cell, HeLa cells were transfected with a plasmid of HIV-1 proviral DNA, pPACK, that was linearized by XbaI. The transfected cells were selected in D-MEM with 10% FBS plus 0.4 mg/ml hygromycin B.
Several randomly picked hygromycin B-resistant clones were transfected with prev-1 and then checked for the expression of viral proteins both by indirect immunofluorescence analysis and by radioimmunoprecipitation assay with AIDS patient serum. One of the highly expressing cells was chosen to use as a packaging cell in this study. Routinely, the packaging cells, termed RIPH cells, were maintained in D-MEM with 10% FBS plus 0.4 mg/ml hygromycin B. We have occasionally observed that long-term passage of RIPH cells reduces their ability to express viral proteins (data not shown). Therefore, cells passaged more than 20 times were not used. As previously reported, transient transfections of adherent cell lines were performed by the calcium phosphate method (Adachi et al., 1996).

Single-round replication assay

HeLa or RIPH cells were transfected with 10 μg of pLANiCAT (rev+) encoding wild-type or mutant env. Two days after transfection, RT production in the culture supernatant was determined. An equivalent amount of virions as measured by RT activity (corresponding to approximately 10 ng of p24 by ELISA (Cellular Products)) was used for infection of 2 × 10^6 A3.01 cells. At 48 h after infection, CAT activity in the infected cell lysate was determined by a standard assay. The activities obtained with env-deficient virus derived from pLANiCAT (rev+)Δenv were used as negative controls to eliminate the activity of Env-independent uptake of virions.

Metabolic labeling and immunoprecipitation

At 48 h posttransfection of proviral DNA clones into HeLa or RIPH cells, cells were labeled for 4 h with [35S]methionine and [35S]cysteine (Redivue PRO-MIX L-[35S] in vitro cell labeling mix (Amersham Pharmacia Biotech)). To study virion-associated Env, the supernatants were filtered through a 0.45-μm pore size filter and the cell-free virus particles were pelleted in 1.5-ml microtubes (4°C, 120 min, 16,000 g). The cell-free virus particles were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris·HCl (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, and 50 μg/ml phenylmethylsulfon fluoride (PMSF)). For the analysis of Env oligomers (Fig. 5), digitonin buffer (100 mM triethanolamine, 150 mM NaCl, 1 mM EDTA, 1.0% digitonin, 10 mM iodoacetamide, 1 μg/ml leupeptin, and 50 μg/ml PMSF) was used to lyse cells and virions. Immunoprecipitated proteins from cell and virion lysates with AIDS patient sera or C8 mAb plus protein G-Sepharose were separated on 8 or 10% polyacrylamide gels. The gel image was obtained by exposure to BioMax MS films (TranScreen was used for detection of virion-associated viral proteins) (Eastman Kodak).

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