Effect of the Cytoplasmic Domain of the Simian Immunodeficiency Virus Envelope Protein on Incorporation of Heterologous Envelope Proteins and Sensitivity to Neutralization

A. N. VZOROV AND R. W. COMPANS*

Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, Georgia 30322

Received 6 August 1999/Accepted 10 June 2000

In addition to the viral envelope (Env) proteins, host cell-derived proteins have been reported to be present in human immunodeficiency virus and simian immunodeficiency virus (SIV) envelopes, and it has been postulated that they may play a role in infection. We investigated whether the incorporation of host cell proteins is affected by the structure and level of incorporation of viral Env proteins. To compare the cellular components incorporated into SIV particles and how this is influenced by the structure of the cytoplasmic domain, we compared SIV virions with full-length and truncated Env proteins. The levels of HLA-I and HLA-II molecules were found to be significantly (15- to 25-fold) higher in virions with full-length Env than in those with a truncated Env. Virions with a truncated Env were also found to be less susceptible to neutralization by specific antibodies against HLA-I or HLA-II. We also compared the level of incorporation into SIV virions of a coexpressed heterologous viral glycoprotein, the influenza virus hemagglutinin (HA) protein. We found that SIV infection of cells expressing influenza virus HA resulted in the production of phenotypically mixed SIV virions containing influenza virus HA as well as SIV envelope proteins. The HA proteins were more effectively incorporated into virions with full-length Env than in virions with truncated Env. The phenotypically mixed particles with full-length Env, containing higher levels of HA, were sensitive to neutralization with anti-HA antibody, whereas virions with truncated Env proteins and containing lower levels of HA were more resistant to neutralization by anti-HA antibody. In contrast, SIV virions with truncated Env proteins were found to be highly sensitive to neutralization by antisera to SIV, whereas virions with full-length Env proteins were relatively resistant to neutralization. These results indicate that the cytoplasmic domain of SIV Env affects the incorporation of cellular as well as heterologous viral membrane proteins into the SIV envelope and may be an important determinant of the sensitivity of the virus to neutralizing antibodies.

A critical step during human or simian immunodeficiency virus (HIV or SIV) assembly is the incorporation of viral Env proteins into mature virions. In addition to the viral Env proteins, host cell-derived molecules have been demonstrated to be present on the viral surface (1, 2, 26), and it has been postulated that these proteins may play a role in viral infection (4, 7, 17, 31). Incorporation of HLA molecules by SIV also has significant immunologic effects. Macaques immunized with uninfected human cells were protected against challenge with SIV grown in human cells (33). It has been suggested that the selective incorporation of cellular antigens within retrovirus envelopes may affect host range and influence the course of the disease (22, 23). Incorporation of the intercellular adhesion molecule ICAM-1 into HIV type 1 (HIV-1) has been reported to increase the avidity of virus-cell attachment and enhance virus entry (30). However, it is still controversial whether the incorporation of cellular membrane proteins by retroviruses is selective or not (1, 5, 22–24, 30). Evidence indicates that during replication or release of human T-cell leukemia virus, the virions become preferentially associated with the Tac antigen (21). Other findings indicated that feline leukemia virus specifically incorporated host-derived FLA antigens (23). Evidence has been obtained for a selective incorporation of HLA-DR over other HLA proteins into HIV-1 virions (2). However, others have reported that uptake of cellular proteins by the viral envelope is nonselective and depends on the type of cells and level of expression of host and Nef proteins (1, 7).

SIVmac239 is pathogenic molecular clone of SIV that encodes a TM (transmembrane) protein of 41 kDa with a cytoplasmic domain of 164 amino acids. SIVmac239 efficiently infects macaque peripheral blood lymphocytes, but infection of the human T-cell line HUT78 results in low levels of virus production. Continued passage of this virus in HUT78 cells resulted in the appearance of a virus encoding a 28-kDa TM protein with a truncated cytoplasmic domain of 18 amino acids (20). Similar truncations have been observed in other SIV isolates that were passaged in human cell lines (9, 14, 19, 20). Truncation of the cytoplasmic domain of the SIV Env glycoprotein was found to increase Env incorporation into virus particles (16, 35, 36) and also to enhance the cell fusion activity of the Env protein (32).

Because of the reported differences in density and conformation of Env glycoproteins on surfaces of particles with truncated versus full-length Env proteins, we have investigated whether the incorporation of host cell proteins could also be affected by the structure and level of incorporation of Env proteins. To compare the cellular components incorporated into SIV particles and how this is influenced by the structure of the Env cytoplasmic tail, we used SIV virions with full-length
or truncated Env proteins. We have also investigated the sensitivity of these virions to neutralization by antibodies to viral or cellular antigens.

MATERIALS AND METHODS

Cell culture, viruses, and plasmids. The recombinant monkey cell line sMAGI was provided by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID, NIH) (Rockville, Md.). sMAGI cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. p239SpSp3 and p239SpE3 were provided by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The SIVmac239 provirus was generated by restriction digestion of p239SpSp3 and p239SpE3 (18, 29) and religation of a mixture of the two plasmids. SIVmac1A11 virus, which was produced by continued passage of infected HUT78 cells, was kindly provided by C. Miller. For preparation of virus stocks, virus-infected cell supernatants were precleared by low-speed centrifugation, filtered through a 0.45-μm-pore-size filter (Nalge Company), and used as stock, or they were pelleted by centrifugation for 2 h at 20,000 rpm in an SW28 tube, resuspended, frozen in aliquots at −80°C, and used as concentrated stock.

The titer of the virus stock was quantified by performing an endpoint dilution assay (35).

DNA transfaction and virus infection. Infectious SIVmac239 DNA was transfected into HUT78 cells by a DEAE-dextran procedure (27); during a 1-month period of virus growth, we passed the virus twice. For SIVmac1A11 infection, 107 cells were pelleted and diluted in 3 ml of complete RPMI 1640 medium containing approximately 105 infectious particles. After overnight incubation, the medium was removed and fresh complete RPMI 1640 was added. Virus replication was analyzed by measurement of RT activity in the culture supernatant (11).

Monoclonal antibodies, antisera, and plasma samples. SIV-specific plasma samples from SIV-exposed monkeys infected with SIVmac239, SIVmac239/17E, or SIVsmm9 were kindly provided by S. O'Neil; samples from monkeys infected with SIVmac251 were provided by P. Marx. Monoclonal antibodies against SIVmac p27 and SIVmac251 gp41 (KK15) were provided by the NIAID AIDS Research and Reference Reagent Program; antibodies against cellular proteins HLA-ABC and HLA-DR were obtained from Immunotech, and HLA-DR antibodies (L-243) was obtained from the American Type Culture Collection. Rabbit anti-BSA and anti-influenza virus polyclonal antibodies were previously described (25).

Mouse anti-A/PR/8/34 (H1N1) influenza virus protein was kindly provided by Harriet Robinson (12).

Virus-binding ELISA. A virus-binding enzyme-linked immunosorbent assay (ELISA) modified from that of Orentas and Hildreth (28) was used to quantify the capture of SIV by anti-HA antibodies. Briefly, 96-well plates were coated overnight at 4°C with 0.75 μg of goat anti-rabbit IgG (Fc fragment specific) or rabbit anti-mouse IgG (Fc fragment specific) (Jackson Laboratory, West Grove, Pa.) well in carbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, 3 mM NaN3, pH 9.6). The plates were blocked for 1 h at 37°C with 200 μl of 3% BSA in PBS well per well; then murine or rabbit antibodies against HA were added at a 1:50 dilution (100 μg/ml in PBS) (8). As a positive control, murine anti-HA antibodies were added at 2 μg/ml in DMEEM containing 5% heat-inactivated newborn calf serum and 0.02% NaN3 (Sigma), and the mixture was incubated for 2.5 h at 37°C. This was followed by the addition of 100 μl of 2% (2 or 35 ng of p27) of purified virus in PBS. The virus was allowed to bind to the anti-HA antibodies overnight at 4°C. After six washes with RPMI 1640 to remove unbound virus, the bound virus was lysed with 250 μl of 1% Triton X-100 (Sigma) per well for 1 h at room temperature, and p27 was quantitated by ELISA (Immunotech) according to the protocol provided by the manufacturer. All experiments were run in duplicate. The results for the experimental groups were compared to the results for controls in which virus was exposed to secondary rabbit anti-mouse IgG or goat anti-rabbit IgG in the absence of anti-gp41, and anti-HLA antibody.

Neutralization assays. Neutralization assays were performed on SIVmac1A11 cells (8). Briefly, SIVmac cells were added to a 96-well plate 24 h prior to infection. Antibody samples were diluted in complete DMEM to a final volume of 25 μl and incubated with an equal volume of supernatant cells or infected inoculum per well. After 4 h, 4% dialyzed serum was added. Supernatants from samples were collected, and virus titers were determined by the plaque assay. Antibody samples were preincubated with SIV for 20 min before addition of virus to 96-well plates. After 24 h, the cells were overlaid with agar and incubated for 7 days. The agar with infected cells was then stained to score plaques.

RESULTS

Incorporation of full-length and truncated Env into SIV particles. We have compared the incorporation of Env proteins into SIVmac239 virions which contain a full-length TM-
protein and SIVmac1A11 virions which possess a truncated Env protein. SIVmac1A11 virions were found to be produced by HUT78 cells at a level about 30-fold higher than that of SIVmac239. Comparison of the distribution of SIVmac239 and SIVmac1A11 proteins showed that for both viruses, Gag was broadly distributed in sucrose gradients (Fig. 1). However, the amount of SIVmac1A11 Gag in the top fraction was about 14-fold higher than the amount of SIVmac239 Gag (Fig. 1, lanes 3 and 6). Env proteins were found in only the lower fractions (Fig. 1, lanes 1 and 4). We did not observe a significant difference in the levels of Env incorporation into SIVmac1A11 or SIVmac239 virions (Fig. 1, lanes 1 and 4), as indicated by the Env/Gag ratio. Therefore, both truncated Env and full-length Env were incorporated with high efficiency into SIV particles.

To compare the morphologies of SIVmac239 and SIVmac1A11 virions, we used electron microscopy. A more prominent layer of spikes was identified on SIVmac1A11 particles containing truncated Env (Fig. 2A) than on SIVmac239 particles containing full-length Env (Fig. 2B). These results are similar to those previously obtained for virus-like particles (VLPs) having truncated or full-length Env proteins (35). We suggest that this morphological difference may be due to changes in the conformation of the Env proteins.

Incorporation of cellular proteins into SIV particles. We compared the incorporation of HLA-I and HLA-II molecules in SIV virions by surface labeling with \( ^{125}\text{I} \). SIVmac1A11 or SIVmac239 virions with full-length or truncated Env proteins were purified, and similar amounts of proteins were estimated by core antigen ELISA) were labeled either by the membrane-permeable reagent SHPP-\(^{125}\text{I} \) to analyze Gag proteins or by the surface-labeling reagent sulfo-SHPP-\(^{125}\text{I} \) to analyze proteins on the viral surface (Fig. 3A). We observed labeling of the full-length TM protein in SIVmac239 (Fig. 3A, lane 2) and truncated TM in SIVmac1A11 (Fig. 3A, lane 4). The levels of TM proteins in the two samples were similar. We observed that much higher levels of HLA-I and HLA-II proteins were incorporated into SIVmac239: about a 25-fold-higher level of HLA-I proteins was observed in SIVmac239 (Fig. 3A, lane 5) compared with SIVmac1A11 (Fig. 3A, lane 7), and about a 15-fold-higher level of HLA-II proteins was found in SIVmac239 (Fig. 3A, lane 6) compared with SIVmac1A11 (Fig. 3A, lane 8).

When we used equal amounts of cells to prepare virus samples (Fig. 3B), the levels of Gag proteins determined by ELISA were found to be about 30-fold higher in the case of SIVmac1A11 compared to SIVmac239 (data not shown). The ratio of TM in SIVmac239 to TM in SIVmac1A11 was also about 30:1 (Fig. 3B, lanes 1 and 2). However, we observed that levels of HLA-I and HLA-II proteins in both of these samples were similar (Fig. 3B, lanes 3 to 6). Taken together, these results show that HLA-I and HLA-II proteins are present at about 15- to 25-fold-higher concentrations in SIVmac239 particles compared to SIVmac1A11 particles which have truncated Env proteins.
The determination of a physical association between cellular proteins and retrovirus particles can be complicated by the presence of cellular debris containing host proteins (6, 13). To control for such possible contamination, we carried out several experiments. First, we carried out virus purification using medium after culture of SIVmac239-transfected HUT78 cells for 3 or 11 weeks. Released virus as detected by RT assay was present only in the 11-week sample. SIV as well as host (HLA-I and HLA-II) proteins were found only in the samples obtained after 11 weeks, and were not detected after 3 weeks, providing evidence that cellular HLA proteins are not released in cellular components which cosegregate with virions (not shown). In the second experiment, VSV particles were purified from VSV-infected HUT78 cells. We observed only VSV-specific proteins in these particles, which indicated that HLA proteins were not incorporated in detectable amounts into VSV virions and that such virions were not contaminated by cellular vesicles (not shown). As an additional experiment we used virus-binding ELISA to determine the profile of HLA molecules on the viral envelope. When we used equal amounts of virus particles with full-length or truncated Env, according to the estimated level of Gag proteins, similar amounts of intact virions were captured by anti-gp41 antibodies (not shown). HLA-II molecules were about threefold more abundant than HLA-I proteins in both SIVmac239 and SIVmac1A11 virions. These data are consistent with our observation that higher levels of HLA molecules are incorporated into SIVmac239 than SIVmac1A11.

**Incorporation of heterologous viral glycoproteins into SIV particles.** To further test the hypothesis that the length of the cytoplasmic tail of the SIV Env protein can affect the incorporation of heterologous proteins into virus particles, HUT78 cells producing SIVmac239 or SIVmac1A11 virions were transfected with plasmid pCMV/H1 expressing the HA protein of A/PR/8/34 (H1N1) influenza virus. After 7 days, the medium from cells was collected, and phenotypically mixed SIV particles containing influenza virus HA proteins were purified and analyzed by surface iodination. We found the presence of the HA protein in samples of SIV particles with either full-length or truncated Env (not shown). To compare the incorporation of HA proteins into virions, we used a virus-binding ELISA. As shown in Fig. 4, SIVmac239 was captured efficiently by anti-HA (rabbit) and anti-HA (mouse) antibodies. The two antibodies had similar abilities to interact with virus. However, compared to SIVmac239 virus with full-length Env, SIVmac1A11 particles showed about a threefold reduction in binding to anti-HA antibody. No virus capture was observed in wells with no anti-HA (rabbit) or anti-HA (mouse) antibodies or in control wells containing vesicles or SIV prepared from cells not coexpressing HA proteins. These results show that mouse or rabbit antibody against HA proteins specifically recognized HA molecules associated with released SIVmac239 but were able to detect HA in SIVmac1A11 with much lower efficiency.

**Sensitivity of SIV to neutralization by specific antibodies.** We initially examined whether the level of incorporation of HLA proteins into virions would influence the sensitivity of SIV to neutralization by anti-HLA antibodies. First, we found that anti-HLA-I and -II had a very limited neutralization effect. Only about 10% neutralization was observed after treating SIVmac239 virions with anti-HLA antibodies, and no neutralization by anti-HLA sera was detected with SIVmac1A11 (Fig. 5). We then determined whether treatment with secondary antibodies would enhance the neutralization effect. We found that secondary anti-mouse antibodies substantially increased the neutralization effect; when SIVmac239 virions were treated with anti-HLA-I plus anti-mouse serum, about 30% of the virus was neutralized, and 56% was neutralized after treatment with anti-HLA-II plus anti-mouse serum (Fig. 5). In contrast, with SIVmac1A11, no reduction in infectivity was observed after treatment with anti-HLA-I plus anti-mouse serum, and only about 10% reduction was found with anti-HLA-II plus anti-mouse serum (Fig. 5). We also used anti-VSV as an irrelevant antibody which did not have a neutralization effect on SIV virions. These results demonstrate that in contrast to SIVmac239, SIVmac1A11 virions were almost completely resistant to the neutralization effect of anti-HLA antibodies.

To extend these results, we also compared neutralization by anti-HA antibodies. In an attempt to increase incorporation of
HA proteins into SIV particles and the sensitivity to neutralization, we infected HUT78 cells with SIVmac1A11 or SIVmac239 at an MOI of 0.001; after a 2-h adsorption period, cells were transfected with plasmid pCMV/H1. Supernatants were collected after 3 days and used for neutralization assays. We observed that about 84% of SIVmac239 was neutralized after treatment with anti-HA serum (Fig. 6). In contrast, with SIVmac1A11, about 65% of infectivity was neutralized. Taken together, these results are consistent with the result that higher levels of cellular or heterologous viral proteins were incorporated into virus particles with full-length Env. Further, the HA protein is a more effective target for neutralization than the HLA proteins.

We also compared the abilities of antisera to SIV to neutralize the three viruses: SIVmac239 virions, which possess a full-length TM protein; SIVmac1A11, with a truncated TM protein; and SIVmac239(t) (five passages), with similar amounts of truncated and full-length TM proteins. We found that antisera to SIVmac239 almost completely neutralized SIVmac1A11 and neutralized 66% of SIVmac239(t) and about 60% of SIVmac239 (Fig. 7). We also compared the abilities of different anti-SIV antisera and found that anti-SIVmac239/17E and anti-SIVmac251 antisera had neutralization effects similar to those observed above with anti-SIVmac239 sera: SIVmac1A11 was highly sensitive to anti-HA antibody, SIVmac239 was relatively resistant, and SIVmac239(t) had intermediate sensitivity. Anti-SIVsmm9 serum neutralized 27% of SIVmac239, 56% of SIVmac239(t), and 86% of SIVmac1A11. These results indicate that SIV with a truncated Env is more sensitive to neutralization by polyclonal antisera to various SIV isolates, whereas SIV having a full-length Env protein is relatively resistant to neutralization.

**DISCUSSION**

One goal of this study was to determine whether the incorporation of cellular membrane proteins into SIV virions is affected by the cytoplasmic domain of the Env proteins. Our results using surface iodination clearly indicated that the levels of HLA-I and HLA-II molecules were significantly (15- to 25-fold) lower in virions with truncated Env proteins than in those with full-length Env proteins. We extended these results by analysis of the incorporation of expressed influenza virus HA proteins into SIV particles and found that HA incorporation was also much lower in SIVmac1A11 virions than in SIVmac239 virions with full-length Env. These results indicate that the incorporation of cellular as well as heterologous viral proteins into virions or VLPs depends on the structure and level of incorporation of the SIV Env proteins. SIV virions with truncated Env proteins exhibit more clearly defined spikes on the viral envelope, and we suggest that they may be packed into a more regular arrangement in the virion which results in more effective exclusion of heterologous membrane proteins.

Our previous observations with recombinant VLPs revealed that glycoproteins with full-length cytoplasmic tails were incorporated into such particles at a much lower density than Env proteins containing truncated cytoplasmic domains (35). In contrast to truncated Env, increasing the expression level of full-length Env did not enhance incorporation of these proteins into VLPs. In the present study using SIV virions, we found that both forms of Env proteins are incorporated into virions at similar levels. These results point to possible differences between the assembly of SIV proteins expressed by recombinant expression vectors and assembly of virions during virus infection. It is possible that the higher level of expression obtained with vaccinia virus recombinants results in a more rapid assembly process and that truncated Env proteins are more efficiently incorporated into recombinant VLPs under these conditions.

We found that SIVmac239 virions with full-length Env could be partially neutralized by specific antibodies against HLA-I or HLA-II proteins, but SIVmac1A11 particles were almost completely resistant to neutralization by HLA antibodies. Neutralization of SIVmac239 and SIVmac1A11 was more effective when anti-HLA-II antibodies were used. This may be due to higher levels of incorporation of HLA-II than HLA-I proteins into virions. Anti-HLA-I and anti-HLA-II antibodies them-
selves had a very low neutralization activity with SIVmac239, but the addition of a secondary antibody was found to increase this effect. The mechanism of virus neutralization under these conditions could be due to the ability of the secondary antibody to bind to the virus and form virus aggregates. Phenotypically mixed SIVmac239 virions containing influenza virus HA proteins were partially neutralized with anti-HA antibody (without secondary antibody), whereas virions with truncated Env proteins were more resistant to neutralization. Previous studies reported that HLA proteins are weaker immunogens than influenza virus HA proteins (3), and this may contribute to differences in neutralization.

We also compared the susceptibilities to neutralization of SIV with full-length or truncated Env proteins by specific SIV antibodies. While both SIVmac1A11 and SIVmac239(t) with truncated Env proteins were susceptible to neutralization, SIVmac239 with a full-length Env protein was found to be more resistant to neutralization by SIV-specific antibodies. The high susceptibility of virions with truncated Env proteins to neutralization by anti-SIV antisera clearly demonstrates that the lack of neutralization of such particles by antisera to heterologous proteins reflects the reduced level of incorporation of such proteins into virions rather than insensitivity of the virions to neutralization per se. The low-level HA proteins in SIV virions may be able to function as targets for neutralization for two reasons: high neutralization activity of antibodies to HA (3) and space between full-length Env subunits in the SIV envelope. The neutralization of SIV depends on the ternary structure of the Env protein (15). Previous studies provided evidence that truncation of the SIV Env cytoplasmic tail changes the conformation of the external domain. Our electron microscopy studies support this result, in that a more prominent layer of spikes was seen in virions with truncated Env than in those containing full-length Env. The finding that SIVmac239 virions with full-length Env and those with truncated Env show differences in susceptibility to neutralization indicates that these differences result from the differences in the cytoplasmic tails of the Env protein. It will be of interest to determine the mechanism by which such changes affect sensitivity to neutralizing antibody and whether such differences could play a role in the differences in the pathogenic potential of SIV isolates.

Acknowledgments

This study was supported by NIH grants AI 28147 and AI 45883 from NIAID, NIH.

We thank Frank Novembre for assistance with RT assays and use of his laboratory facility, Lawrence Melson for assistance in preparing the figures, Tanya Cassignham for assistance in preparing the manuscript, and Dahnide Taylor for technical assistance.

References

29. Saarloos, M. N., B. L. Sullivan, M. A. Czernecki, K. D. Parameswar, and...