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# Insertion of the Human Immunodeficiency Virus CD4 Receptor into the Envelope of Vesicular Stomatitis Virus Particles

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**Enveloped virus particles carrying the human immunodeficiency virus (HIV) CD4 receptor may potentially be employed in a targeted antiviral approach. The mechanisms for efficient insertion and the requirements for the functionality of foreign glycoproteins within viral envelopes, however, have not been elucidated. Conditions for efficient insertion of foreign glycoproteins into the vesicular stomatitis virus (VSV) envelope were first established by inserting the wild-type envelope glycoprotein (G) of VSV expressed by a vaccinia virus recombinant. To determine whether the transmembrane and cytoplasmic portions of the VSV G protein were required for insertion of the HIV receptor, a chimeric CD4/G glycoprotein gene was constructed and a vaccinia virus recombinant which expresses the fused CD4/G gene was isolated. The chimeric CD4/G protein was functional as shown in a syncytium-forming assay in HeLa cells as demonstrated by coexpression with a vaccinia virus recombinant expressing the HIV envelope protein. The CD4/G protein was efficiently inserted into the envelope of VSV, and the virus particles retained their infectivity even after specific immunoprecipitation experiments with monoclonal anti-CD4 antibodies. Expression of the normal CD4 protein also led to insertion of the receptor into the envelope of VSV particles. The efficiency of CD4 insertion was similar to that of CD4/G, with approximately 60 molecules of CD4/G or CD4 per virus particle compared with 1,200 molecules of VSV G protein. Considering that (i) the amount of VSV G protein in the cell extract was fivefold higher than for either CD4 or CD4/G and (ii) VSV G protein is inserted as a trimer (CD4 is a monomer), the insertion of VSV G protein was not significantly preferred over CD4 or CD4/G, if at all. We conclude that the efficiency of CD4 or CD4/G insertion appears dependent on the concentration of the glycoprotein rather than on specific selection of these glycoproteins during viral assembly.**

The host range of viruses is initially determined by the interaction of the virus with the plasma membrane of the host cell (for a review, see reference 33). This interaction first involves a specific binding of the virus particle to cellular receptor molecules. The receptor can be a highly specific protein molecule like CD4 (30, 50), or it can consist of ubiquitous molecules like phosphatidyl serine or other negatively charged lipids which are found on many cell types (33), thereby contributing to the broad host range of the virus. With respect to the study of viral receptors and viral host range, enveloped viruses are particularly attractive because envelope proteins can be exchanged between different enveloped viruses. Such an exchange or mixing of envelope glycoproteins can take place, for instance, during a coinfection of two distinctly different enveloped viruses (48, 56, 57). It can give rise to phenotypically mixed viruses with a potentially altered and possibly broadened host range (26, 30, 34, 46, 58).

Phenotypic mixing of viral proteins is usually limited to enveloped viruses; however, at least experimentally, capsid proteins of nonenveloped viruses (12) have been genetically altered to carry partial structures of surface proteins of unrelated viruses. While the size of such additional structures is limited in this case, the insertion of cellular or heterologous viral proteins into the viral envelope during the budding process is less restricted. The efficiency of phenotypic mixing, or, in the extreme case, the generation of pseudotype virus during a coinfection, varies greatly with a particular combination of viruses (34, 48, 56, 57). In addition, low amounts of cellular membrane proteins can also be found in the envelope of virus particles (25, 57). Factors influencing the efficiency of insertion may therefore simply be the amount of the glycoprotein, its structural configuration, its location within the various cellular membranes, or, possibly, the specific interactions between its cytoplasmic tail region and the viral core proteins.

The ability to control the host range of an enveloped virus particle could potentially be exploited in the specific targeting of cells. Target cells could, for instance, be virally infected cells which exhibit the viral glycoprotein on the cell surface. For example, polarized Madin-Darby canine kidney cells are resistant to vesicular stomatitis virus (VSV) infection when the virus is presented to the apical surface. Fuller et al. (17) have shown that these cells become susceptible to VSV after a preinfection with influenza virus. They demonstrated that the influenza virus hemagglutinin protein itself functions as a receptor for VSV and thereby allows coinfection. When receptor and viral glycoprotein interact as strongly and specifically as the human CD4 and the human immunodeficiency virus (HIV) envelope protein do ($K_d$, approximately $10^{-9}$ M) (24), it represents an opportunity to test whether a role reversal of cell and virus during an infection could be facilitated. This may be achieved by placing the HIV receptor itself into the envelope of the targeting virus. Ultimately, such a pseudotype virus may acquire a new tropism for HIV-infected cells and could potentially be used in a targeted therapeutic approach.

With the study presented here, we asked what structural features are required for the efficient insertion of a foreign membrane protein into the envelope of a virus particle? We focused on the envelope of VSV, an enveloped animal

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rhabdovirus, and on insertion of the human CD4 receptor protein for HIV into the envelope of VSV.

The genetic manipulation of the RNA genome of a rhabdovirus on the DNA level and the generation of recombinant virus particles have not been accomplished for any rhabdovirus. Therefore, insertion of a foreign glycoprotein into the VSV envelope is dependent on the coexpression and subsequent phenotypic mixing of envelope proteins. VSV is a lytic virus which shuts off the macromolecular synthesis of the host cell (for a review, see reference 43). Cell lines which constitutively express the foreign glycoprotein of interest may therefore not be suitable. It has been reported earlier that, at least for VSV, there is no interference during coinfection of the two cytoplasmic viruses, vaccinia virus and VSV (49). Vaccinia virus recombinants expressing a great variety of foreign proteins and especially viral envelope proteins have been described during the past years (36). For these reasons it seemed attractive to coinfect VSV and vaccinia virus recombinants expressing the HIV receptor.

Initially, we assumed that for efficient insertion of the HIV receptor into the VSV envelope a chimeric receptor molecular may be required, and we generated a receptor molecule, consisting of the ectodomain region of the human CD4 molecule precisely fused to the transmembrane and cytoplasmic tail regions of the VSV glycoprotein G. This communication describes the establishment of methods and a quantitative comparison of the efficient insertion of a functional chimeric receptor (CD4/G) and a normal HIV receptor (CD4), respectively, into the envelope of VSV particles.

(Part of these data were presented at the VIIIth International Congress of Virology in Berlin, Germany, in 1990.)

## MATERIALS AND METHODS

**Cells and viruses.** Baby hamster kidney (BHK 21), HeLa, and HuTk$^-$143 cell lines were obtained from the American Type Culture Collection and were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, nonessential amino acids, glutamine, and penicillin and streptomycin according to standard procedures. Propagation and plaque-forming assays for VSV Indiana and New Jersey serotypes as well as the temperature-sensitive glycoprotein mutant *ts*O45 of VSV Indiana serotype (13, 14) have been described earlier. Vaccinia virus WR strain, vaccinia virus recombinant v37 (expressing VSV glycoprotein G) (29), and vaccinia virus recombinants vPE16 (expressing complete functional HIV envelope protein) (11) and vTF7-3 (expressing T7 RNA polymerase) (15, 16) as well as a mixture of vaccinia virus recombinants vTF7-3 and vEB-8 (expressing the human CD4 protein) (3) were all generously provided by Patricia L. Earl, Edward A. Berger, and Bernard Moss (National Institutes of Health, Bethesda, Md.). Vaccinia viruses were propagated and purified as previously described (27, 28).

**Phenotypic mixing and analyses of the virus progeny.** Coinfections with VSV and vaccinia virus recombinants were carried out in either BHK 21 or HeLa cells. The timing for the superinfections was originally determined as shown in Fig. 1. In most subsequent coinfections, the multiplicity of both infections was chosen to be at least 5. For the expression of CD4, cells were coinfected with both vTF7-3 and vEB-8. Cells were always first infected with vaccinia virus for at least 16 h followed by the VSV infection. Phenotypically mixed virus was harvested by a low-spin centrifugation to remove cell debris. Virus titers were determined with monolayers of BHK 21 cells and agarose overlay, followed by staining with neutral red. The permissive temperature of the *ts*O45 mutant is 32°C. Its glycoprotein could be inactivated by being heated for 30 min at 45°C (13, 14). Virus was neutralized by using high concentrations of serotype-specific rabbit antiserum to VSV Indiana or VSV New Jersey serotype, respectively (Microbiological Associates, Bethesda, Md.).

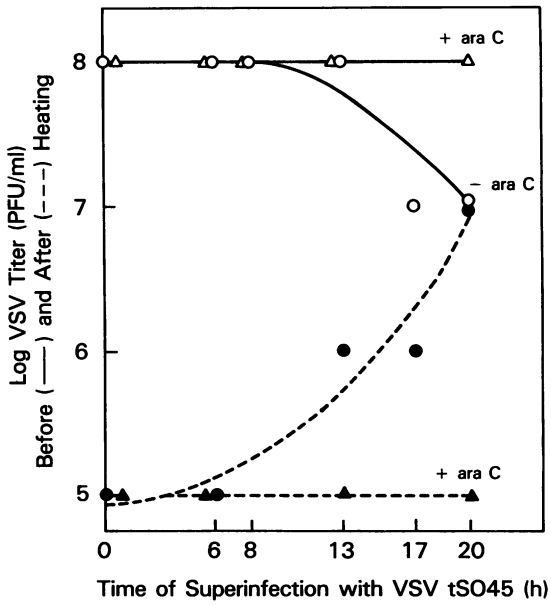


FIG. 1. Generation of heat-resistant VSV particles from a thermolabile mutant virus. BHK 21 cells were infected with the vaccinia virus recombinant which expresses a heat-resistant glycoprotein G of VSV (29). At different times after the vaccinia virus infection, the cells were superinfected at the permissive temperature (32°C) with the glycoprotein mutant of VSV *ts*O45 which encodes a thermolabile glycoprotein (14). At 20 h after VSV infection, released VSV virus titers were determined on BHK 21 cells in the presence of 1-β-D-arabinofuranosylcytosine (ara C) to prevent the formation of vaccinia virus plaques. Released virus was plaqued directly (open symbols) or after being heated for 30 min at 45°C (closed symbols). To prevent vaccinia virus replication as well as expression of the recombinant G protein during the initial coinfections, the experiments were carried out in the presence (triangles) and absence (circles) of ara C.

The method for the immunoprecipitation of VSV particles containing the HIV receptor was similar to the method described by Little et al. (25), with some modifications. Equal volumes of virus supernatants and primary mouse monoclonal antibodies, either OKT4 (Dakopatts A/S, Glostrup, Denmark) or Leu 3a-Leu 3b (Becton Dickinson, Mountain View, Calif.) (45) at a concentration of 0.5 μg of protein per ml, were mixed and incubated at 4°C for 1 h with continuous shaking. After the incubation, 0.1 ml of rabbit anti-mouse immunoglobulin G (IgG) (10 μg/ml) (Cappel, Organon Teknika Co., Durham, N. Car.) was added and incubated as above. The immunoprecipitates were then treated with 50 μl of 10% washed *Staphylococcus aureus* cells (GIBCO-BRL, Gaithersburg, Md.) for another hour at 4°C. The bacterial cells were pelleted, extensively washed, and diluted. Adsorbed virus as well as the supernatant fractions was titrated on BHK 21 cells.

**DNA fusion methods and construction of the CD4/G insertion vector.** The precise DNA fusion of the CD4 and the VSV G gene to form a chimeric CD4/G gene was carried out by

methods which have independently been developed in our laboratory and have in part been previously published by others (19, 53). The DNA fusions require the two starting DNAs, which contain the predetermined sites at which fusion will be carried out. Depending on the method used, three or four synthetic oligonucleotides are needed and all the components of a standard polymerase chain reaction (44), as well as a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, Conn.).

Each reaction mixture contained 1 μg of DNA template (each), 1 μM terminal oligonucleotide primers, various amounts (from 1 to 0.1 μM) of fusion primer(s), 200 μM deoxynucleoside triphosphate (each), 10 mM Tris HCl (pH 8.4), 2.5 mM $MgCl_2$, 50 mM KCl, 200 μg of gelatin per ml, and 2 U of *Taq* DNA polymerase (Perkin-Elmer-Cetus) in a final volume of 100 μl. The reaction experiments were carried out by using a Perkin-Elmer-Cetus DNA Thermal Cycler set at 30 repeated cycles of 1 min at 95°C, followed by 2 min at 45°C, followed by 3 min at 70°C. After 30 cycles, there was an extension for 7 min at 70°C. The fusion primers were 40 nucleotides in length, and the terminal primers contained unique restriction enzyme sites for convenient cloning of the fused DNA (32).

For the fusion of the CD4/G gene the following DNAs and primers were used: pT4B (31) for the human CD4 gene and pSVGL (41, 42) for the glycoprotein gene of VSV. The terminal oligonucleotides for the amplification of the fused DNA were OMS1 (5′ end), ATCTCGAGATGAACCGGG GAGTCCCTTTTAGGCACTTGCTT; OMS6 (3′ end), ACG CGCGCGAGTTACTTTCCAAGTCGGTTCATCTCTAT GTC; OMS2 (fusion primer), TAAAGAAAAAAGAGGCA ATAGAGCTCATTGGCTGCACCGGGGTGGACCAT; and OMS4 (fusion primer complement), ATGGTCCACCCCG GTGCAGCCAATGAGCTCTATTGCCTCTTTTTTCT TTA. The oligonucleotides were synthesized with an automated Applied Biosystems (Foster City, Calif.) oligonucleotide synthesizer. OMS1 contained a terminal unique *Xho*I site and OMS6 contained a terminal unique *Bss*H2 site for the asymmetrical cloning of the fused CD4/G gene into the *Xho*I and *Bss*H2 sites of a modified ET3a vector under control of the T7 RNA polymerase promoter. The CD4/G insert of this plasmid pCD4/G was amplified by a standard polymerase chain reaction by using the primers OMS1 and OMS21 (TAGTCGACGCGCGAGTTACTTTCCAAGT CGGTTCATCTCTA); the latter introduces a unique *Sac*I restriction site at the 3′ end of the gene. After cleaving the polymerase chain reaction product with *Xho*I and *Sac*I, the chimeric gene was inserted into the *Xho*I cloning site of the insertion vector pMM34 under control of the vaccinia virus 7.5-kDa protein promoter (29). The resulting plasmid pvCD4/G was used for the isolation of the vaccinia virus recombinant expressing the chimeric CD4/G protein.

**Isolation of vaccinia virus recombinant expressing CD4/G protein.** The vaccinia virus recombinant vvCD4/G was generated by homologous recombination of the genome of the WR strain of vaccinia virus with the insertion vector DNA which contained the chimeric CD4/G gene under control of the vaccinia virus 7.5-kDa protein gene promoter flanked by regions of the thymidine kinase gene for selection. Recombinant virus was selected in the presence of bromodeoxyuridine in HuTk$^-$143 cells as previously described (27, 28). The virus was plaque purified three times and was analyzed by dot blot hybridizations, using $^{32}$P-RNA transcripts of the CD4 gene in pCD4/G. Viral stocks were generated in HeLa cells and purified according to standard procedures, including sucrose gradient centrifugation. The virus was sonicated prior to infection of cells.

**Antibodies and immunoprecipitations.** BHK 21 or HeLa cells were infected with the vaccinia virus recombinant vvCD4/G at a multiplicity of infection of 10. At 20 h after the infection, the medium was replaced with methionine-free minimal essential medium for 30 min, after which 150 μCi of [$^{35}$S]methionine was added to label about $10^6$ cells for 4 h. The cells were scraped from the dish, washed with phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay buffer according to published procedures (22). Cell debris was removed by centrifugation for 45 min at 30,000 rpm in an SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.).

Labeled CD4/G protein was immunoprecipitated with a mixture of monoclonal antibodies Leu 3a and Leu 3b (Becton Dickinson) or a rabbit antibody directed against the carboxyl-terminal tail region amino acid (positions 501 to 511) of the VSV glycoprotein G (1). The latter antibody was a generous gift of Heinz Arnheiter (National Institutes of Health). The antibodies were added to the cell extracts at 4°C for 1 h, and then either affinity-purified anti-mouse or anti-rabbit IgG antibody (Cappel, Organon Teknika) was added for 30 min, followed by the addition of protein A agarose (GIBCO-BRL) for 30 min. The immunoprecipitates were washed three times, electrophoresis sample buffer was added, and the proteins were separated after being boiled on sodium dodecyl sulfate (SDS)-polyacrylamide gels according to standard procedures (21).

**Western immunoblot analyses of cell extracts and virus pellets.** Cell extracts of vaccinia virus (expressing CD4/G and CD4 and T7 RNA polymerase) and VSV-infected cells were prepared at the times when the pseudotype viruses were harvested from the supernatants. Approximately $10^7$ infected cells were washed, pelleted, and disrupted with radioimmunoprecipitation assay buffer according to standard procedures (22). After removal of the cell debris by centrifugation, equivalent amounts of the cell extracts were boiled in SDS, followed by polyacrylamide gel electrophoresis (21).

The supernatants of the cells were centrifuged at low speed to remove cell debris, and the virus was pelleted onto a glycerol cushion at 38,000 rpm for 90 min in an SW41 rotor. The small pellets were suspended in PBS, and equal portions of parallel preparations were solubilized with Nonidet P-40 and denatured, followed by SDS-polyacrylamide gel electrophoresis.

For Western blot analyses, the separated proteins of cell extracts and virus were blotted onto nylon membrane according to standard procedures. Monoclonal anti-CD4 (a mixture of Leu 3a and Leu 3b or OKT4) or a monospecific antibody directed against the carboxyl-terminal tail region of the VSV glycoprotein G was used for specific binding. The protein bands were detected on parallel gel blots, using either anti-mouse IgG (for CD4) or anti-rabbit IgG (for G tail) antibodies labeled with $^{125}$I, respectively. After autoradiography, the respective protein bands as well as background control lanes were excised from the membrane and quantitated by liquid scintillation counting.

## RESULTS

Vaccinia virus recombinants have earlier been employed to insert the glycoprotein of VSV into the envelope of temperature-sensitive glycoprotein-mutant viruses (51). Despite the important demonstration of genetic complementa-

TABLE 1. Phenotypic mixing of VSV by coexpression of vaccinia virus recombinants expressing various glycoproteins[a]

| Phenotypic mix | Vaccinia virus recombinant | Total virus titer (PFU/ml) | Virus titer (PFU/ml) after treatment | | | |
|---|---|---|---|---|---|---|
| | | | 30 min at 45°C | Anti-VSV Ind | Anti-VSV NJ | Anti-VSV Ind + NJ |
| VSV $^{tsG}_{Ind}$ | | $10^8$ | $10^5$ | $10^2$ | $10^6$ | ND[b] |
| VSV $^{tsG}_{Ind}$ + Vacc $^{wtG}_{Ind}$ | | $10^7$ | $10^7$ | <10 | ND | ND |
| VSV $^{tsG}_{Ind}$ + Vacc $^{HIV}_{env}$ | | $10^7$ | $<10^5$ | <10 | ND | ND |
| VSV $^{wtG}_{NJ}$ | | $10^8$ | $10^7$ | $10^7$ | <10 | ND |
| VSV $^{wtG}_{NJ}$ + Vacc $^{wtG}_{Ind}$ | | $10^7$ | $10^7$ | $10^6$ | $10^4$ | <10 |

[a] BHK 21 cells were infected with vaccinia virus (Vacc) recombinants expressing the wild-type G protein (wtG) of the Indiana (Ind) serotype of VSV (29) or the HIV envelope (env) protein (11), respectively. At 20 h after vaccinia virus recombinant infection, the cells were superinfected with either a temperature-sensitive glycoprotein mutant of VSV (*ts*O45) (14) or with wild-type VSV of the New Jersey (NJ) serotype. The infections were carried out at 32°C, and the titers of released virus were determined and tested for heat resistance and neutralization by anti-VSV Indiana and anti-VSV New Jersey sera, respectively.
[b] ND, not determined.

tion, the yield of virus particles carrying the recombinant wild-type glycoprotein was low, on the order of $10^5$ PFU/ml. Our first goal was, therefore, to increase the yield of phenotypically mixed virus particles during a vaccinia virus-VSV coinfection. Conditions were established by using a vaccinia virus recombinant expressing the VSV wild-type glycoprotein (29) together with the recipient temperature-sensitive glycoprotein mutant of VSV (*ts*O45) which has a thermolabile glycoprotein G (13, 14). BHK 21 cells were infected with the vaccinia virus recombinant at a minimum multiplicity of infection of 5 PFU per cell. At different times after the vaccinia virus infection, the cells were superinfected at the permissive temperature (32°C) with the temperature-sensitive G protein mutant of VSV at a multiplicity of at least 5 PFU/ml.

At 20 h after the VSV infection, supernatants were collected and the titer of released VSV particles was determined. Figure 1 shows the amounts of VSV particles which were released after preinfection of the cells for various times with vaccinia virus. Only at late times after vaccinia virus infection was some interference with the replication of VSV detectable. To our surprise, at times when the cytopathic effect caused by vaccinia virus was very severe (20 h) the cells could still be infected with VSV and the yield of released VSV particles was decreased by a factor of approximately 10, still giving rise to $10^7$ PFU/ml. This finding is consistent with the earlier observation that there is no or only limited interference with VSV replication.

Insertion of the wild-type G protein into the envelope of the temperature-sensitive mutant was measured by heat inactivation of the released virus particles. Heating the mutant virus, which has a heat-labile glycoprotein, for 30 min at 45°C generally decreased the infectious virus titer by 1,000- to 10,000-fold. As can be seen in Fig. 1, VSV particles, which were released from vaccinia virus coinfections, became increasingly heat resistant, depending on the time of superinfection with the mutant VSV. When the cells were superinfected with VSV at 20 h after vaccinia virus infection, virtually all released VSV particles, which were harvested from the cell supernatant, were resistant to heat inactivation. These data demonstrate that the recombinant wild-type glycoprotein expressed by the vaccinia virus recombinant was inserted into the envelope of virtually every VSV particle.

Efficient insertion was dependent on the replication of vaccinia virus. The addition of 1-β-D-arabinofuranosylcytosine to the medium, which specifically inhibits vaccinia virus but not VSV replication, abolished the accumulation of heat-resistant VSV in the supernatant. Because the recombinant G protein is expressed under the early/late 7.5-kDa promoter of vaccinia virus, its high level of expression is also dependent on vaccinia virus replication. The VSV G gene also contains early transcriptional termination signals (42, 55) which presumably do not allow expression without prior genome replication. We conclude that the efficient insertion of homologous G protein into the envelope of released VSV particles required replication of vaccinia virus and accumulation of the glycoprotein in the membrane before budding from the cell occurred. In all subsequent vaccinia virus-VSV coinfections we have used these conditions, which involve a preinfection with vaccinia virus for about 20 h before VSV superinfection and a harvest of the supernatant after an additional 20 h.

Table 1 shows the results of phenotypic mixing, using two different serotypes of VSV (Indiana and New Jersey serotypes) and two different vaccinia virus recombinants, one expressing the wild-type G protein of the Indiana serotype of VSV (29) and one expressing the HIV envelope protein (11). Phenotypically mixed viruses were analyzed for heat resistance and for neutralization by using specific antibodies directed against the two viral serotypes. To demonstrate that vaccinia virus proteins did not confer heat resistance to the heat-labile VSV mutant, cells were first infected with a vaccinia virus recombinant expressing the HIV envelope protein. Released virus showed the same heat sensitivity as the mutant itself. In contrast, virtually all virus particles released after coinfection with the vaccinia virus, which expressed the wild-type G protein, were heat resistant.

Wild-type G protein from the Indiana serotype of VSV was also inserted into the envelope of the VSV New Jersey serotype (42). Its presence was readily detected by using high concentrations of serotype-specific, neutralizing antibodies. Antibodies directed against the VSV New Jersey serotype completely neutralized the VSV New Jersey strain (Table 1). In contrast, when VSV New Jersey was coinfected with vaccinia virus expressing the G protein of the Indiana serotype, $10^4$ PFU/ml resisted neutralization by the anti-VSV New Jersey antibody. This resistant fraction, however, could be completely neutralized by using antibodies directed against the VSV Indiana serotype. These data demonstrate insertion of the Indiana G protein into the envelope of the New Jersey wild-type VSV particle. In fact, this resistant fraction represents a pure pseudotype population which has Indiana serotype properties with respect to its surface antigen and neutralization but has a New Jersey serotype genome. The frequency of such pure pseudotypes, however, was low, approximately $10^{-3}$.

We have also tried to generate and to identify VSV

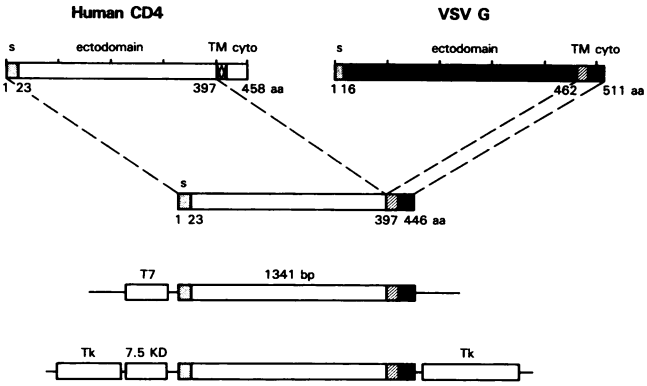


FIG. 2. Fusion of CD4 and VSV G genes. The chimeric CD4/G gene was constructed by gene fusion as described in Materials and Methods. The fused gene contains the entire ectodomain of the human CD4 gene, including the signal peptide precisely fused in frame with the transmembrane and cytoplasmic tail region of the VSV glycoprotein. This gene, which encodes a 446-amino-acid-long CD4/G protein, was cloned behind a T7 RNA polymerase promoter (pvCD4/G). It was also cloned behind the vaccinia virus 7.5-kDa protein gene promoter flanked by the thymidine kinase (Tk) gene fragments (pvCD4/G) to allow insertion of the gene into the vaccinia virus genome.

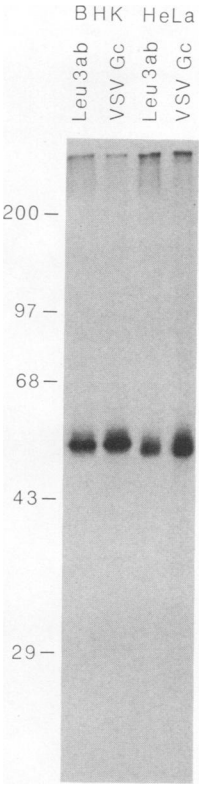


FIG. 3. Expression of the chimeric CD4/G protein. The CD4/G protein was expressed in BHK 21 and in HeLa cells after isolation of a vaccinia virus recombinant. The protein was isotopically labeled with [$^{35}$S]methionine, and the protein was immunoprecipitated by using a mixture of Leu 3a and Leu 3b antibodies (lanes Leu 3ab) as well as a specific antibody directed against the carboxyl terminus of the VSV glycoprotein G (lanes VSV Gc) (1). The proteins were separated on an SDS-polyacrylamide gel next to molecular weight standards.

pseudotypes which had the HIV envelope protein inserted into the VSV envelope. These particles may have properties of HIV with respect to binding and entry into host cells. In fact, pseudotype viruses of the VSV(HIV) have been described and used by others previously (30, 34). The efficiency of their generation, however, had always been very low, using a coinfection with HIV and VSV, and was in the order of $10^{-4}$ to $10^{-5}$ particles. In collaboration with Patricia L. Earl and Bernard Moss (National Institutes of Health, Bethesda, Md.), we have also employed the conditions described above for insertion of the HIV envelope protein (Table 1). After neutralization using anti-VSV antibodies or after heat inactivation, the virus was screened for its tropism toward CD4-positive cell lines such as MT4, CEM, and HeLa T4 cells. We have not been able to detect any VSV pseudotypes which entered the cells via direct membrane fusion caused by the HIV envelope protein (data not shown). Our level of detectability with this functional assay was approximately $10^{-5}$ particles. The reason for the absence of recombinant VSV(HIV) pseudotypes using the vaccinia virus expression system is unclear. The relative instability of the HIV envelope protein during 20 h of preinfection with vaccinia virus may have been a significant contributing factor.

Another possibility may be that during the viral budding process a sorting of membrane proteins occurs which may exclude or include certain glycoproteins for envelope insertion. Since our goal was to insert foreign glycoproteins into the VSV envelope, we hoped that our chances might be increased by inserting a chimeric glycoprotein. Such a chimeric protein may consist of the ectodomain of a foreign glycoprotein precisely fused to the transmembrane and cytoplasmic domains of the VSV glycoprotein. The latter domains may possibly promote the sorting and insertion of the glycoprotein into the VSV envelope (7, 9, 10, 35). It has earlier been shown that the binding domain for HIV can be mapped within the ectodomain and that the protein exists as a monomer in the cell membrane (2, 23). This is in contrast to findings with the VSV glycoprotein, which is inserted as a trimer (9, 20).

Figure 2 shows maps of the two genes which were used for the fusion into a chimeric CD4/G protein gene. The precise boundaries of the two protein portions are indicated. The chimeric gene was fused by using the rapid and precise gene fusion method described in Materials and Methods. The fused gene was initially cloned into a plasmid under the control of the T7 RNA polymerase promoter (pCD4/G) followed by cloning into a vaccinia virus insertion vector under control of the vaccinia virus 7.5-kDa protein promoter (pvCD4/G). The gene was sequenced from close to the carboxyl end of the CD4 ectodomain to the last nucleotide of the cytoplasmic carboxyl-terminal tail region derived from the VSV G protein, and it was found without changes from the published sequences (31, 42) (data not shown).

The insertion vector plasmid was used to generate vaccinia virus recombinants by homologous recombination and bromodeoxyuridine selection as previously described. A vaccinia virus recombinant was isolated and plaque purified. To demonstrate expression of the chimeric CD4/G glycoprotein, BHK 21 and HeLa cells were infected with the recombinant vaccinia virus for approximately 20 h followed by isotopic labeling of the proteins for 4 h. Cell extracts were prepared, and the chimeric receptor protein was precipitated

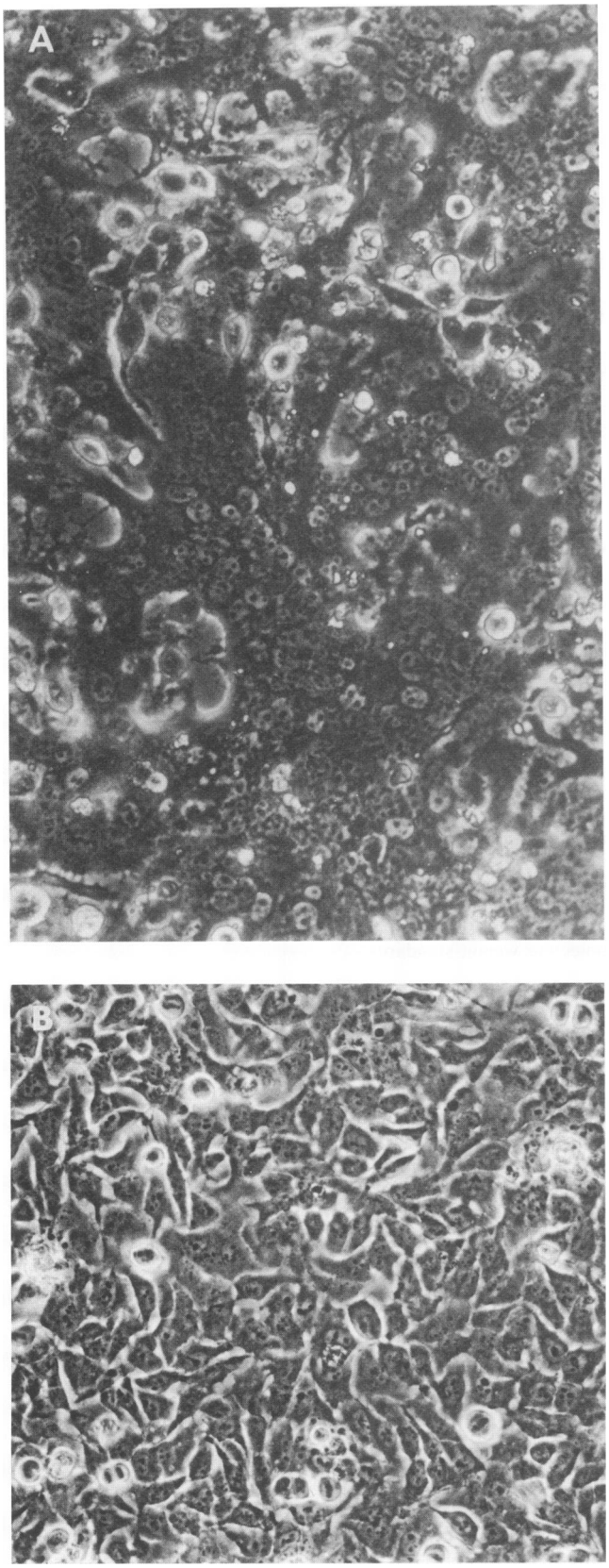


FIG. 4. Syncytium formation by coexpression of the chimeric CD4/G and HIV envelope proteins in HeLa cells. HeLa cells were infected with a mixture of two vaccinia virus recombinants, one expressing the chimeric CD4/G protein (vvCD4/G) and the other expressing the HIV envelope protein (vPE16) (11), respectively. After 20 h of infection, a very dramatic fusion of the cells was visible, resulting in the formation of large numbers of multinucleated cells, often with every cell on the plate affected (panel A). Fusion was dependent on the presence of both the chimeric protein and the HIV envelope protein. Infecting the cells with only one of the vaccinia virus recombinants did not result in fusion (panel B, infection with vvTF7-3 is shown). For optimal cell fusion, the ratio of both vaccinia viruses needed adjustment. HeLa cells were the most efficient in syncytium formation. Overexpression of CD4/G significantly delayed syncytium formation by 1 or 2 days (data not shown). No fusion was observed with BHK 21 cells.

by using monoclonal antibodies directed against either the ectodomain of the CD4 molecule (Leu 3a-Leu 3b) or the cytoplasmic tail region of the VSV G protein (VSV Gc) (1), respectively. As shown in Fig. 3, both monoclonal antibodies immunoprecipitated the same-sized protein whether it was synthesized in BHK 21 cells or in HeLa cells, demonstrating precise fusion of the chimeric gene also on the protein level. The size of the fusion protein was approximately 55 kDa, as expected from the predicted amino acid sequence. The protein migrated slightly faster when expressed in HeLa cells than in BHK 21 cells, suggesting a potential difference in the posttranslational modification in the two different cell types, possibly due to a different glycosylation pattern.

For the functional analysis of the chimeric receptor molecule, we have coinfected HeLa cells with vaccinia virus recombinants expressing the CD4/G protein and the HIV envelope protein (11), respectively. When CD4-positive cells are infected with HIV, one can often detect a fusion of the infected cell with neighboring cells, resulting in the typical formation of giant multinucleated cells. This cell fusion in tissue culture is similar to the membrane fusion which occurs when the envelope of a single HIV particle fuses with the cellular membrane during viral entry (3, 6, 34). Figure 4 shows the dramatic result of such a coexpression of HIV envelope protein together with the chimeric receptor molecule CD4/G. Overnight we detected massive cell fusion affecting virtually every cell on the dish. This result clearly demonstrates that the chimeric HIV receptor protein was properly inserted into the cell membrane and that it was recognized by the HIV envelope protein as a functional receptor.

To see whether the chimeric receptor protein can be inserted efficiently into the envelope of VSV particles, the CD4/G protein was expressed in either BHK 21 or HeLa cells, using the timing of superinfection determined in Fig. 1. Cells infected with recombinant vaccinia virus for 20 h were infected with the temperature-sensitive mutant of VSV (*ts*O45) at the permissive temperature. At 24 h later, supernatants were tested for the presence of VSV particles carrying the chimeric CD4/G molecule in their envelope. VSV particles were immunoprecipitated in separate reactions, using two different antibodies directed against the ectodomain of the CD4 molecule. After binding to secondary antibody as well as to *S. aureus* cells, the virus was pelleted by low-speed centrifugation. The pellet was washed extensively with buffer, diluted, and directly plaqued onto BHK 21 cells (25). Virus which did not pellet during the immunoprecipitation, as well as the initial virus supernatant, was titrated. The results are shown in Table 2.

Two virus supernatants from two different vaccinia virus-

TABLE 2. Immunoprecipitations of VSV particles containing the chimeric CD4/G protein in their envelopes[a]

| Total titer (PFU/ml) of phenotypic mix | Primary antibody | Virus titer (PFU/ml) | | % Pellet of phenotypic mix |
|---|---|---|---|---|
| | | Supernatant | Pellet | |
| VSV(CD4/G) ($6 \times 10^6$) | Leu 3a-Leu 3b | $7 \times 10^5$ | $5 \times 10^5$ | 42 |
| | OKT4 | $7 \times 10^5$ | $5 \times 10^5$ | 42 |
| | None | $3 \times 10^6$ | $5 \times 10^3$ | 0.2 |
| VSV(−) ($5 \times 10^6$) | Leu 3a-Leu 3b | $3 \times 10^6$ | $4 \times 10^3$ | 0.1 |
| | OKT4 | $3 \times 10^6$ | $4 \times 10^3$ | 0.1 |
| | None | $4 \times 10^6$ | $4 \times 10^3$ | 0.1 |

[a] Vaccinia virus and VSV coinfections were carried out in BHK 21 cells under the conditions established in Fig. 1 and as summarized in the Material and Methods section. Vaccinia virus recombinants expressing either the chimeric CD4/G protein VSV(CD4/G) or an unrelated cytoplasmic protein, VSV(−), were used. In both coinfections, titers of VSV found in the medium were determined on BHK 21 cells. The virus was immunoprecipitated with or without neutralization in the presence of the primary antibody directed against the ectodomain region of the HIV receptor (Leu 3a-Leu 3b or OKT4) as described in the Materials and Methods section. The VSV titers of the supernatant and pellet fractions are indicated, and the pellet fraction is also expressed as the percentage of both pellet and supernatant fractions. Note that omitting the primary anti-CD4 antibody from the reactions or the absence of CD4/G expression did not drastically reduce the virus titer of the initial medium. Similar results were obtained by using HeLa cells as the host, except that the virus yield was 10- to 100-fold lower than with BHK 21 cells.

TABLE 3. Immunoprecipitations of VSV particles containing the normal CD4 protein[a]

| Total titer (PFU/ml) of phenotypic mix | Primary antibody | Virus titer (PFU/ml) | | % Pellet of phenotypic mix |
|---|---|---|---|---|
| | | Supernatant | Pellet | |
| VSV(CD4/G) ($8 \times 10^6$) | OKT4 | $2 \times 10^6$ | $5 \times 10^5$ | 20 |
| | None | $9 \times 10^6$ | $4 \times 10^3$ | <0.1 |
| VSV(CD4) ($4 \times 10^6$ | OKT4 | $3 \times 10^5$ | $8 \times 10^4$ | 21 |
| | None | $5 \times 10^6$ | $1 \times 10^3$ | <0.1 |
| VSV(−) ($6 \times 10^6$)[b] | OKT4 | $1 \times 10^7$ | $5 \times 10^3$ | <0.1 |
| | None | $7 \times 10^6$ | $2 \times 10^3$ | <0.1 |

[a] Coinfections were carried out as described in the Materials and Methods section, using BHK 21 cells. Vaccinia virus recombinants expressing the chimeric CD4/G protein under the 7.5-kDa protein promoter were used during the coinfections. Alternatively, the normal CD4 protein was expressed using a combination of vaccinia virus recombinants expressing the T7 RNA polymerase and the CD4 gene under control of the T7 promoter (3). In both cases, immunoprecipitations of the VSV particles were carried out in the presence or absence of the primary anti-CD4 antibody as described in footnote *a* of Table 2.

[b] Control.

VSV coinfections were generated for analysis. One coinfection involved a vaccinia virus recombinant expressing the chimeric CD4/G protein, the other coinfection was carried out with a vaccinia virus recombinant expressing an unrelated cytoplasmic protein. The initial VSV titers of both coinfections were basically the same ($5.5 \times 10^6$ to $6 \times 10^6$ PFU/ml). Differences in virus titers were clearly observed after immunoprecipitations. As can be seen, both CD4-specific antibodies immunoprecipitated VSV when it was derived from a coinfection with the vaccinia virus recombinant expressing the chimeric receptor CD4/G. Only background levels of virus were detected in the pellet when the virus was harvested from a coinfection with a vaccinia virus recombinant expressing an unrelated protein. Of the total virus which was recovered after specific immunoprecipitation with anti-CD4 antibody, about 42% was found in the pellet fraction. When anti-CD4 antibody was omitted, generally less than 1% was found in the pellet fraction, demonstrating specific precipitation with both antibodies. Precipitation was, therefore, dependent on the presence of the chimeric receptor in the particle. Usually only about 0.1% of the virus was precipitated when the virus was grown in the absence of CD4/G expression. As an additional control for the envelope insertion of CD4/G protein and to rule out nonspecific coprecipitation, we have mixed the heat-labile *ts*O45 particles, which carry the CD4/G protein, with the heat-resistant wild-type virus of the Indiana and New Jersey serotypes, respectively. Immunoprecipitation procedures were carried out using OKT4 antibody. As expected, although both viruses were found in the supernatants, only the heat-labile *ts*O45 which carried the HIV receptor was present in the pellet fraction (data not shown).

Next, we wanted to determine whether the VSV-derived transmembrane and cytoplasmic portions of the chimeric CD4/G protein actually helped to increase the efficiency of insertion into the VSV envelope. Therefore, we have used a mixture of two recombinant vaccinia viruses, one expressing the T7 RNA polymerase and the other expressing the normal human CD4 protein under control of a T7 promoter (3). This virus mixture was generously provided by Edward Berger (National Institutes of Health, Bethesda, Md.). We coinfected cells with this mixture of vaccinia viruses, followed 20 h later by VSV infection as described above. Again, the virus was immunoprecipitated from the cell supernatant by using anti-CD4 antibodies. Table 3 shows a comparison of the virus titers after immunoprecipitations when VSV was propagated in the presence of CD4/G and of CD4 expression or when an unrelated cytoplasmic protein was expressed. As can be seen, except for the control, approximately the same fraction of the virus could be precipitated, suggesting that there is no obvious preference for the insertion of either the CD4 or CD4/G molecules. Both proteins were inserted into roughly the same number of VSV particles.

These data indicate that the transmembrane and cytoplasmic tail regions of the VSV glycoprotein are not required for insertion of the HIV receptor into the VSV envelope, which demonstrates that the corresponding regions of the normal CD4 molecule can substitute for these regions under the conditions of viral assembly described here. These conditions, of course, also include coexpression of the VSV G protein.

For an evaluation of the efficiency of CD4 and CD4/G protein insertion, the pools of G, CD4, and CD4/G in the cell extracts at the time of viral assembly have to be taken into account; these are then compared with the amounts of these proteins inserted into released virus particles. The precise comparison of the amounts of CD4, CD4/G, and G cannot rely on isotopic labeling because of the difference in the timing of expression through the double infection. A comparison has to rely on a Western blot analysis. Antibodies, however, have different affinities, and simple staining for CD4 and G would not reveal the precise relative concentrations. Fortunately, we were able to use an antibody which overcomes this problem. The epitope for this monospecific antibody is directed against the carboxyl-terminal domain of the VSV G protein which is present in both the chimeric CD4/G protein as well as G. This allows a direct comparison of the relative amounts of G and CD4/G, whereas the antibody directed against CD4 allows a quantitation of CD4

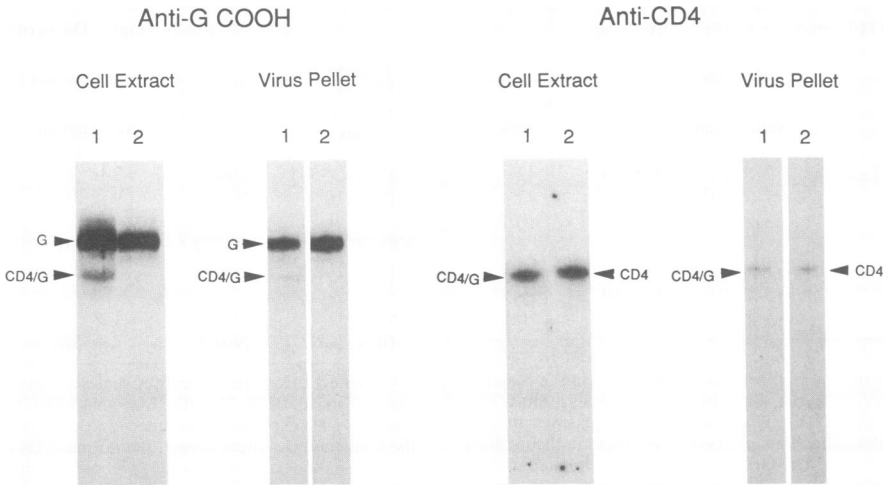


FIG. 5. Quantitation of CD4, CD4/G, and G protein in cell extracts and virus. BHK cells were infected with vaccinia virus(es) to express CD4 or CD4/G proteins. The cells were superinfected 20 h later with VSV for 16 h. Released virus particles were purified from the cell supernatants, and the cells were harvested and cell extracts were prepared. Equal amounts of cell extracts and virus pellets from both parallel preparations were denatured, and the proteins were separated by SDS-polyacrylamide gel electrophoresis. The proteins were blotted onto nylon membranes and visualized by using a specific antibody directed against the carboxyl terminus of the VSV G protein (anti-G COOH), which also binds to the chimeric CD4/G protein, or the CD4-specific antibody (anti-CD4), which binds to CD4 and to CD4/G. The proteins were subsequently identified with a $^{125}$I-labeled secondary antibody. The protein bands identified with anti-G COOH antibody were excised and quantitated as listed in Table 4.

and CD4/G. Western blot analyses of the cell extracts and virus pellets using these antibodies are shown in Fig. 5.

The use of this combination of antibodies allows a close approximation of the ratios of CD4, CD4/G, and G in both cell extracts and virus particles. Since the average number of G proteins per virus particle is about 1,200 molecules (47), we can calculate the average number of receptor molecules per virus particle. The protein bands marked G and CD4/G in the detection using anti-G COOH were excised, and the radioactivity was determined for each. Table 4 shows that the total amount of CD4/G in the cell extract was about one-fifth of the total amount of G protein during the time of viral budding. The amount of G protein in the cells which express the CD4 protein was roughly the same. In contrast, the total amount of CD4/G relative to G protein in the virus pellet was approximately 20-fold less, whereas slightly more G protein was found in the supernatant from CD4-expressing cells.

TABLE 4. Quantitation of G and CD4/G in infected cell extracts and released virus[a]

| Protein | Radioactivity (cpm) in protein bands from Fig. 5[b] | | | |
|---|---|---|---|---|
| | Cell extract | | Virus pellet | |
| | No. 1 | No. 2 | No. 1 | No. 2 |
| G | 161,322 (5.3) | 151,592 (5.0) | 79,089 (19.6) | 137,338 (34.0) |
| CD4/G | 30,277 (1.0) | 0 | 4,034 (1.0) | 0 |

[a] The protein bands marked G and CD4/G as shown in Fig. 5 were labeled by using antibody directed against the carboxyl-terminal region of G together with a secondary $^{125}$I-labeled anti-rabbit IgG antibody. The bands were excised from the membrane, and the radioactivity was determined by liquid scintillation counting. Background radioactivity bound to the membrane was subtracted from each sample.

[b] The relative amounts of CD4/G and G are calculated respectively for cell extract and for virus pellet and are listed in parentheses.

Anti-CD4 can be used to quantitate both CD4 and CD4/G in the same preparations of both cell extracts and virus pellets. Figure 5 shows that the amounts of CD4/G and CD4 were the same in both virus particle preparations, whereas there was only about twofold more CD4 than CD4/G in the cell extracts. CD4/G appears to migrate slightly ahead of CD4. From this we can conclude that CD4 and CD4/G are expressed to about the same extent and they are inserted into the viral membrane at about the same frequency. The exchange of the transmembrane and cytoplasmic domain of CD4 with the respective domains of G did not significantly affect the efficiency of integration into the VSV membrane.

From the quantitation of the amounts of G and CD4/G and since CD4/G and CD4 are present to the same amounts, we can calculate that each VSV particle has about 60 molecules of CD4/G (or CD4 within a factor of two to three) compared with 1,200 molecules of G protein (or 400 G protein trimers). Considering that the CD4/G and CD4 concentrations in the cell extracts were fivefold less than the concentration of G protein, the chance for the monomeric CD4/G protein to be inserted was also about fivefold less on a random basis. VSV G protein, however, is inserted as a trimer. Taking these two points into account, we conclude that the functional G protein trimer surprisingly does not have an advantage during viral assembly.

## DISCUSSION

The mechanism for the efficient insertion of foreign glycoproteins into the envelope of viruses is not completely understood. It is not clear which portions of these complex membrane-spanning proteins are responsible for efficient transport and for the sorting of the glycoprotein during assembly of virus particles at the site of budding (9, 40). Interaction of the cytoplasmic portions of the proteins with the viral matrix protein and/or the viral nucleocapsid is suspected to be involved in assembly of the VSV particle (4,

10, 37–40, 51). Deletions of the cytoplasmic tail regions abolish VSV particle formation, whereas the ectodomain of the viral glycoprotein can be spared, at least, during late stages of the viral assembly process of VSV, resulting in the generation of spikeless, noninfectious particles (7, 35). These particles, however, have still retained the transmembrane and cytoplasmic portions of the protein. In the mature virion, the VSV glycoprotein exists as a trimer which was assembled while still associated with cytoplasmic membranes (9, 20). Similar to the HIV envelope protein, the ectodomain of the VSV G protein may in part determine oligomerization and transport (9). It is unclear whether trimerization of G is required for efficient assembly into virus particles. But it is conceivable that trimerization may affect presentation of the cytoplasmic portion of the protein and consequently the efficiency of its postulated interaction with the viral nucleocapsid and/or matrix protein.

In contrast to VSV, it has been shown for many retroviruses that only expression of the viral Gag proteins is required for the formation and release of empty virus particles (8, 18, 52) which lack both a genomic RNA as well as the envelope glycoprotein. We anticipated that the requirements for the bullet-shaped rhabdovirus VSV would be more stringent with respect to protein insertion during particle formation. Although not detected with VSV (38), there appears to be a specific accumulation of matrix protein by the related pleomorphic paramyxoviruses at the inner surface of the plasma membrane near the site of budding (5). This accumulation is correlated with a clustering of the viral glycoproteins at the external site, suggesting a specific interaction of both proteins, which may favor efficient insertion of the glycoprotein specifically into the envelope of the budding virus.

The study described here focuses on insertion of a foreign glycoprotein into the envelope of a VSV particle. For the reasons outlined above, we anticipated that a specific interaction of the cytoplasmic tail region of the chimeric CD4/G molecule with the nucleocapsid may enhance the insertion of the protein into the virus particle. Our data show, however, that the cytoplasmic tail region and the transmembrane region of the VSV G protein were not required. Both domains could be replaced by the corresponding portions of the CD4 molecule. Therefore, we can conclude that, at least when the normal G protein is coexpressed, there is no substantial discrimination in the insertion of either CD4 or CD4/G molecules. In fact, our quantitation of CD4, CD4/G, and G protein insertion into VSV particles revealed that the VSV G protein is surprisingly not favored during viral assembly (Fig. 5 and Table 4). This also explains the large fraction of VSV particles which could be immunoprecipitated with anti-CD4 antibodies (Tables 2 and 3). We do not know whether VSV particles can assemble in the absence of G protein expression. It is possible that the trimerization of G protein is essential to present the cytoplasmic tail in a correct configuration for assembly. The signal for trimerization, however, may in part be located in the ectodomain region of G.

Efficient insertion of the CD4 molecule or a chimeric CD4/Env protein into the envelope of avian leukosis virus was reported by Young et al. (54) shortly before this report was submitted. Interestingly, Young et al. find that the ratio of CD4 per Env protein was approximately 1/18. This is also what we have determined for VSV particles (1/20). These authors also conclude that the receptor is inserted very efficiently but that the chimeric receptor CD4/Env is inserted less well, possibly because it may, unlike CD4, compete with the avian leukosis virus Env protein. Our data does not provide any evidence for a competition with G or a preference or a disadvantage for the insertion of either protein.

It is interesting to note that the expression of wild-type G protein in our system did not lead to a genetic complementation of the G protein mutant *ts*O45 at the nonpermissive temperature (data not shown). We did not observe an increase in the amount of mutant virus which was released at the nonpermissive temperature, however, as was the situation at the permissive temperature, virtually all released particles were resistant to heating for 30 min at 45°C. This demonstrated again that the recombinant wild-type glycoprotein was inserted into the envelope of a vast majority of released virus particles, if not in all. We suspect that the recombinant wild-type G protein may have formed heterotrimers with the mutant G protein in each case. The number of wild-type homotrimers may be too low to allow for genetic complementation at the nonpermissive temperature, as would be obvious from an increase in the number of released infectious particles. While in the form of a heterotrimer, however, the wild-type G protein may stabilize the mutant G protein in the released particles and give them the typical heat resistance which we have observed.

We have demonstrated by specific immunoprecipitations that the CD4 as well as the chimeric CD4/G molecule could be inserted into up to 42% of the envelopes of VSV particles that we could detect by plaque formation. This number is most likely an underestimation and could be substantially higher. The bivalence of the antibody and the multivalence of the *S. aureus* cells together could trap multiple PFU onto the same bacterial cell, and they would consequently be scored as a single plaque. This is supported by our frequent observation that the number of infectious units in the pellet and supernatant fractions did not equal the total number of infectious units in the initial virus suspension. Most importantly, a substantial decrease in the total virus titer was only detected when anti-CD4 antibody was used.

The relatively high number of receptor-carrying VSV particles was very promising and suggested that these viruses may be able to specifically infect HIV envelope protein-expressing cells. Numerous attempts to demonstrate a specific tropism for HIV envelope-expressing cells were not successful so far. We have clearly shown that our chimeric CD4/G protein (Fig. 4) and the CD4 protein (data not shown) are both functional when expressed on the surface of a cell. In the environment of a viral membrane, however, the receptor may possibly not be functional. It has earlier been shown, for example, that the simple presence of a CD4 receptor in the plasma membrane of a mouse cell does not render this cell susceptible to HIV infection (3, 30). This strongly suggests that either a specific presentation of the receptor may be necessary for infectivity or that additional membrane-associated factors may positively or negatively affect infectivity. For this purpose we have generated the pseudotypes also in HeLa cells, which can be infected by HIV when the receptor is presented on the surface; but again, we have not been able to infect HIV envelope protein-expressing HeLa cells. We have also tried to generate VSV (CD4/G) virus particles by using *ts*O45 at the nonpermissive temperature, with the same result.

A convincing demonstration of a functional HIV receptor in the envelope of VSV particles would clearly be a productive VSV infection through a specific, possibly pH-independent fusion of viral and cellular membranes in some sort of an exchange in the positions of both the CD4 and HIV envelope glycoproteins. This may require either a breakthrough in the generation of recombinant VSV particles or,

alternatively, a highly efficient and selective inactivation of the glycoprotein of VSV. Studies on the multiple interactions of viral glycoproteins as well as on the cellular receptor proteins will surely help our understanding of the mechanisms of viral assembly and viral entry. This fundamental knowledge may eventually be exploited for the specific targeting of cells and the delivery of genes or gene products.

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