Exceptional Fusogenicity of Chinese Hamster Ovary Cells with Murine Retroviruses Suggests Roles for Cellular Factor(s) and Receptor Clusters in the Membrane Fusion Process

DON C. SIESS,[†] SUSAN L. KOZAK,
AND DAVID KABAT*

*Department of Biochemistry and Molecular Biology, Oregon Health Sciences University,
Portland, Oregon 97201-3098*

Received 28 September 1995/Accepted 4 March 1996

Chinese hamster ovary (CHO) cells are naturally resistant to infection by amphotropic and ecotropic murine retroviruses, but they become susceptible after expressing corresponding receptors rRAM-1 and mCAT-1, respectively, and they then form abundant syncytia when exposed to these viruses. The fusogenic activities of CHO cell clones increase much more strongly with levels of receptor expression than do their susceptibilities to infection, suggesting that the assembly of receptor clusters may limit syncytium formation. However, other cell lines are not fusogenic, even if they express larger amounts of receptors. Our results suggest that a factor that is relatively abundant or active in CHO cells may functionally interact with rRAM-1 and mCAT-1 in a pathway that enables receptor-bearing membranes to fuse with membranes that contain viral envelope glycoproteins. In the case of CHO/rRAM-1 cells, syncytia form at foci of amphotropic 4070A virus infection by fusion-from-within of infected with uninfected cells. This fusogenic propensity is a sole property of the uninfected CHO/rRAM-1 cells, which fuse in cocultures with any cells infected with 4070A virus. With CHO/mCAT-1 cells, fusogenicity is even greater and involves fusion-from-without by ecotropic virion particles. In contrast to infection, which behaves as expected for a process limited by ecotropic virus attachment to single receptors, fusion-from-without increases dramatically for cells that express the highest levels of mCAT-1. We propose that infection and syncytium formation are limited at distinct steps of a common pathway that requires virus binding to a single receptor, assembly of multivalent virus-receptor complexes, structural changes in viral envelope glycoproteins, and membrane fusion. The limiting step in syncytium formation is a cellular process that depends on receptor clustering and is relatively active in CHO cells.

Although the mechanism(s) of retrovirus infection is poorly understood, it is initiated by the binding of the virus onto cell surface receptors (6, 42). This binding is followed by the fusion of the viral membrane with the receptor-bearing cellular membrane, either while the virus is on the cell surface or, perhaps for some retroviruses, after endocytosis of virus-receptor complexes (1, 6, 24, 25, 27, 29–31, 42, 43). Entry routes for murine retroviruses have not been definitively established.

In the case of human immunodeficiency virus type 1 (HIV-1), attachment onto cell surface CD4 is insufficient to trigger membrane fusion. The fusion step occurs when human CD4 is expressed in most human cells but not when it is expressed in the cells of other species (2, 8, 13, 42). Similarly, CD4-positive human cells form syncytia with any cells that express the HIV-1 envelope glycoprotein gp120-gp41, whereas cells of other species that contain human CD4 do not efficiently form syncytia. Studies of interspecies cell heterokaryons suggested that human cells contain an accessory factor that cooperates with CD4 in a postattachment step of HIV-1 infection that is essential for membrane fusion (7–9). Other studies indicated that monoclonal antibodies to the virus attachment site on CD4 can block infection even if they are added substantially after the initial virus adsorption onto the cells, suggesting that multivalent

HIV-1–CD4 complexes may be necessary for infection (11, 21, 22, 33, 34). It is not known whether a similar host-cell factor(s) and multivalent complexes contribute to infections by other retroviruses. However, multivalent virus-receptor complexes appear to be necessary for membrane fusions caused by influenza A virus and tick-borne encephalitis virus (3, 32, 37, 44).

Chinese hamster ovary (CHO) cells are naturally resistant to infection by amphotropic and ecotropic murine retroviruses. However, they become susceptible and able to bind the corresponding viral gp70 glycoproteins after they express receptors from susceptible species. The rat amphotropic receptor (rRAM-1) is a Na⁺-phosphate symporter (16, 20), whereas the mouse ecotropic receptor (mCAT-1) is a uniporter for cationic amino acids (17, 39). Recently, we found that infection of our CHO/rRAM-1 cultures with amphotropic murine leukemia virus (MuLV) 4070A resulted in initial syncytium formation and cytopathology that disappeared after the virus had spread throughout the culture (20). This outcome suggested that cytopathology might be caused by the interactions of infected and uninfected CHO/rRAM-1 cells and that recovery occurred after all the cells were infected and polyploid cells were overgrown. In striking contrast, infection of other cells with 4070A virus does not produce syncytia. During subsequent analysis, we observed that our CHO/mCAT-1 cells also form abundant syncytia after they are incubated with ecotropic murine retroviruses, implying that CHO cells may be promiscuously active in receptor-dependent membrane fusion reactions. Our investigations suggest that a host cell factor(s) functionally interacts

* Corresponding author. Phone: (503) 494-8442. Fax: (503) 494-8393.

[†] Present address: Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland, OR 97201-3098.

FIG. 1. Infection of CHO/rRAM-1 cells with amphotropic 4070A results in syncytium formation at foci of infection. CHO/rRAM-1 (B and C) or control CHO (A) cells were seeded into the wells of a 24-well plate (2.5×10^4 cells per well). The following day, the cells were infected with 4070A MuLV for 2 h in the presence of Polybrene. The medium was changed, and the cells were then incubated for an additional 48 h. The cells shown in panels A and C were fixed in 95% ethanol and then incubated sequentially with goat antiserum to p30 (1:1,000) followed by horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (1:500). The immune complexes were stained by incubation with a peroxidase substrate solution (0.2 mg of 3-amino-9-ethylcarbazole per ml, 0.00015% hydrogen peroxide, 50 mM sodium acetate [pH 5.0]). The cells were examined for stained foci by light microscopy. Dense staining occurred in the infected CHO/rRAM-1 culture (C), but no staining occurred in the CHO culture (A). CHO/rRAM-1 cells (B) were also infected with 4070A MuLV and were fixed and stained only with toluidine blue. The nuclei in the syncytia are more easily seen in this panel because of the absence of the dense immunoperoxidase staining. Panel A was photographed at a lower magnification to show the absence of syncytia in a large field.

with receptors in a multistep pathway that leads to membrane fusion and that receptor clustering may be an essential and possibly limiting step in this pathway.

MATERIALS AND METHODS

Cells and viruses. Normal CHO cells, Swiss murine NIH 3T3 fibroblasts, and mink lung CCL 64 fibroblasts were obtained from the American Type Culture Collection (Rockville, Md.). Rat 208F fibroblasts (28) were a gift of A. Dusty Miller (Fred Hutchinson Cancer Center, Seattle, Wash.). The ecotropic host range packaging cell line ψ 2 (23) was obtained from Richard C. Mulligan (Massachusetts Institute of Technology, Cambridge, Mass.). The murine IP/IR erythroleukemia cell line has been described elsewhere (36). CHO clones that express rRAM-1 or mCAT-1 and the clone of CCL 64 mink cells that expresses a large amount of mCAT-1 have been previously described (20, 40). As was described previously, all CHO/rRAM-1 clones were derived from a clone of CHO cells that expresses mCAT-1 (20). Amphotropic MuLV 4070A was pseudotyped with an ecotropic envelope by passage through ψ 2 cells, and the resulting virus was used to infect the clone of CHO/mCAT-1 cells as described previously (20). CHO cells and their derivatives were grown in the α modification of minimal essential medium supplemented with 10% fetal bovine serum. All other cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

The 4070A isolate of amphotropic MuLV was obtained from the cell-free supernatant of infected murine BALB/c 3T3 fibroblasts. Rauscher MuLV and Friend MuLV (10) were obtained from infected cultures of NIH 3T3 fibroblasts. Helper-free ecotropic host range retrovirions that encode human growth hormone were obtained from a clone of ψ 2 cells transduced with retroviral expression vector pSFF-hGH (19, 40). Virus preparations were harvested by placing fresh culture medium on half-confluent monolayers of virus-producing cells for 16 to 24 h, removing the medium, and filtering it through 0.45- μ m-pore-size filters. In some cases, virus preparations were stored frozen at -80°C until use. Cells were infected by incubation with virus-containing medium at 37°C in the presence of Polybrene (8 $\mu\text{g}/\text{ml}$).

Analyses of viral infection and cell-to-cell fusion. Foci of 4070A MuLV infection of CHO/rRAM-1 cells were detected by an immunological assay (5). Briefly, cells that had been infected 48 h earlier were washed, fixed in 95% ethanol, and then sequentially incubated for 1 h at room temperature with goat antiserum to MuLV core protein p30 (1:1,000) (10) followed by horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (1:500; Organon Teknika, Durham, N.C.). The immune complexes were stained by incubation with a peroxidase substrate solution (0.2 mg of 3-amino-9-ethylcarbazole per ml, 0.00015% hydrogen peroxide, 50 mM sodium acetate [pH 5.0]). The cells were examined for stained foci by light microscopy.

Cocultures that contained 4070A MuLV-infected cells or uninfected control cells plus CHO/rRAM-1 cells or control CHO cells were prepared and analyzed as previously described (20). Unless otherwise mentioned, infected cells were seeded into culture plates and incubated overnight. The next day, CHO/rRAM-1 cells or control CHO cells were lifted with 8 mM EDTA in 0.9% saline, washed in complete medium, and then added to the cultures of infected cells. After different times of coculture, the adherent cells were fixed in methanol, stained with toluidine blue, and examined by light microscopy. The presence of amphotropic envelope glycoprotein on the surfaces of infected cells was verified by immunofluorescence specific for gp70 (40). In some instances, 4070A MuLV-infected cells were labeled for 18 h with bromodeoxyuridine (Amersham, Chicago, Ill.) prior to coculture with CHO/rRAM-1 cells or control CHO cells. Following coculture, the cells were fixed and then incubated simultaneously with DNase and a mouse monoclonal antibody to bromodeoxyuridine according to the manufacturer's protocol (Amersham) (12). Bromodeoxyuridine-labeled nuclei were detected by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse immunoglobulin (1:100; Organon Teknika).

Syncytium formation was assessed at various times after the exposure of CHO/mCAT-1 cells or control CHO cells to ecotropic host range viruses. The cells were fixed in methanol, stained with toluidine blue, and examined by light microscopy. Percentages of nuclei present in syncytia were determined by counting at least 500 nuclei. Quantitative analyses of ecotropic host range virus infections were performed as described previously with helper-free retrovirions that encode human growth hormone (19, 40). In this assay, the infected cells expressing human growth hormone are detected by immunofluorescence, and the multiplicity of infection is calculated from the fraction of uninfected cells. For each assay, at least 400 cells were counted.

Other methods. All other methods were previously described. These methods include assays for the measurement of basic amino acid transport by mCAT-1-expressing cells (38) and of gp70 envelope glycoprotein binding to cells (20, 40). Protein concentrations were measured by the Coomassie blue dye method (Bio-Rad, Richmond, Calif.).

FIG. 2. In cocultures of amphotropic 4070A MuLV-infected CHO cells and uninfected CHO/rRAM-1 cells, infected cells fuse with uninfected cells. CHO cells infected with pseudotyped (20) amphotropic 4070A MuLV and no longer exhibiting virally induced cytopathology (5×10^3 cells per well of an eight-well chamber slide) were labeled with bromodeoxyuridine in complete medium for 18 h. The labeled cells were washed with fresh medium, and then unlabeled, uninfected CHO/rRAM-1 cells were added (2×10^4 cells per well). The cells were cocultured for 24 h and then fixed and stained to detect bromodeoxyuridine-labeled nuclei by immunofluorescence (A) as described in Materials and Methods. The same field is shown by phase-contrast microscopy (B).

RESULTS

Membrane fusion mediated by rRAM-1 amphotropic receptors in CHO cells. Figure 1 shows a culture of CHO cells compared with a culture of CHO/rRAM-1 cells (clone CEAR-13) infected with amphotropic MuLV 4070A (panels A and B, respectively). Syncytia were evident 30 h after infection of the CHO/rRAM-1 cultures, and they became abundant by 48 to 72 h. As seen by immunological staining specific for viral protein p30, the syncytia occur at foci of infection (Fig. 1C). After the infection had spread into all the cells, the cultures recovered morphologically within 3 to 4 weeks as polyploid cells were overgrown. Such recovered infected cultures formed syncytia after being mixed with uninfected CHO/rRAM-1 cells but not after being mixed with control CHO cells (20). Similarly, using an appropriately pseudotyped virus, we were able to infect CHO cells that lacked rRAM-1 with MuLV 4070A (reference 20 and also see Materials and Methods), and they also formed syncytia after being cocultured with CHO/rRAM-1 but not control CHO cells. When CHO/rRAM-1 cells were mixed with MuLV 4070A-infected CHO cells that had been labeled by incorporation of bromodeoxyuridine into DNA, the resulting multinucleated cells contained mixtures of labeled and unlabeled nuclei, confirming that they were indeed syncytia that formed by the fusion of infected cells with uninfected CHO/rRAM-1 cells (Fig. 2).

Cell lines other than CHO/rRAM-1 that we tested did not form syncytia after being infected with amphotropic MuLV 4070A. In addition to the cell lines described below, these lines included mouse NIH 3T3 and BALB/c fibroblasts and human HeLa cervical carcinoma cells. This difference in the fusogenicities of CHO/rRAM-1 and the other tested cells cannot be simply explained by a deficiency in the receptor level, because a gp70 binding study indicated that HeLa cells have an amount of amphotropic receptors similar to that of a clone of CHO/rRAM-1 (clone 40) that readily forms syncytia (see below).

Consistent with the evidence in Fig. 2 that the CHO/rRAM-1 polykaryons form principally by fusion-from-within (i.e., by fusion of infected with uninfected cells), the process required replication-competent MuLV 4070A and did not occur when the CHO/rRAM-1 cells were incubated with 4070A virus that had been inactivated by UV irradiation. For example, in one experiment, a CHO/rRAM-1 culture contained $20\% \pm 1\%$ of its nuclei in syncytia 48 h after incubation with 4070A MuLV. A parallel culture incubated with a 4070A

MuLV aliquot that had been irradiated with 0.4 J of 254-nm UV light per cm^2 had only $2\% \pm 0.2\%$ of its nuclei in syncytia, whereas the control CHO/rRAM culture that was not exposed to virions contained $1\% \pm 0.2\%$ of its nuclei in syncytia. This dose of irradiation is sufficient to inactivate more than 99% of retroviral infectivity without significantly reducing its envelope fusion activity (see below).

After a suspension culture of MuLV 4070A-infected mouse IP/IR erythroleukemia cells was overlaid onto a dispersed monolayer of uninfected CHO/rRAM-1 cells, cell aggregates formed and began to fuse within 2 h and syncytia became abundant by 5 to 10 h. This result indicated that CHO/rRAM-1 cells could fuse in cocultures with heterologous cells that had been infected with the 4070A virus. Table 1 shows representative coculture analyses of the cellular specificity of syncytium formation. Uninfected CHO/rRAM-1 cells fused extensively with all tested mammalian cells that had been infected with MuLV 4070A. However, these same infected cell lines did not fuse with other uninfected cells that had amphotropic receptors. The fact that rat 208F fibroblasts lacked a fusion propensity implies that fusion is not caused simply by rRAM-1 as opposed to homologous RAM-1 proteins of other species. These coculture studies suggest that the focal syncytia caused by 4070A MuLV infection of CHO/rRAM-1 cultures (Fig. 1) are due to an exceptional fusogenic propensity of uninfected CHO/rRAM-1 cells rather than to any specific property of the infected cells. These results also suggest that CHO cells do not secrete a factor that activates the fusion activity of amphotropic receptors on other cells.

Although all CHO/rRAM-1 clones prepared in this laboratory have similar fusogenic properties, the rates and extents of syncytium formation are markedly higher for clones that have the largest amounts of rRAM-1. For example, in one experiment, we cocultured 4070A-infected or uninfected IP/IR erythroleukemia cells with two clones of CHO/rRAM-1 (clones CEAR-13 and CEAR-40) (20) that express rRAM-1 in a ratio of 4.9 ± 0.3 to 1. Each of these clones contained only a small percentage of multinucleated cells after being grown alone or in cocultures with uninfected IP/IR cells. These control values were subtracted from the results observed in the cocultures that contained infected IP/IR cells. In this case, after 3 h of coculturing, the CEAR-13 monolayer had $6\% \pm 2\%$ of its nuclei in syncytia, whereas the CEAR-40 monolayer had none of its nuclei in syncytia. After 8 h, the monolayers had $15\% \pm 2\%$ and $7\% \pm 3\%$ of the nuclei in syncytia, respectively. In

TABLE 1. Fusion of CHO/rRAM-1 cells cocultured with cells infected with amphotropic MuLV

Cell type ^{a,b}	RAM-1-expressing or control cell type ^{b,c}	Approximate % nuclei in syncytia ^d
Hamster		
4070A-CHO	CHO/rRAM-1	15
4070A-CHO	(CHO)	<1
(Uninfected CHO)	CHO/ram-1	<1
4070A-CHO	CCL 64	<1
4070A-CHO	NIH 3T3	<1
4070A-CHO	IP/IR	<1
4070A-CHO	Rat 208F	<1
Mink		
4070A-CCL 64	CHO/rRAM-1	30
4070A-CCL 64	(CHO)	<1
(Uninfected CCL 64)	CHO/rRAM-1	<1
Mouse		
4070A-NIH 3T3	CHO/rRAM-1	20
4070A-NIH 3T3	(CHO)	<1
(Uninfected NIH 3T3)	CHO/rRAM-1	<1
4070A-IP/IR	CHO/rRAM-1	60
4070A-IP/IR	(CHO)	<1
(Uninfected IP/IR)	CHO/rRAM-1	<1

^a The presence or absence of amphotropic envelope glycoprotein on the surfaces of 4070A MuLV-infected or uninfected control cells was verified by gp70-specific immunofluorescence (40). CHO cells are of hamster origin, CCL 64 cells are mink cells, NIH 3T3 and IP/IR erythroleukemia cells are from mice, and rat 208F cells are from rats. The 4070A-infected CHO cells were prepared as described previously (20). Control uninfected cells are shown in parentheses.

^b Unless indicated otherwise, 4070A MuLV-infected cells or their controls from column 1 were seeded into culture plates and incubated overnight. The following day, the cells in column 2 were lifted from cultures with 8 mM EDTA in 0.9% saline, washed in complete medium, and overlaid onto the cells from column 1. The plates were generally fixed with methanol after 18 to 24 h of coculturing, and the cells were then stained with toluidine blue and examined by light microscopy. IP/IR suspension cultures were not treated with EDTA but were overlaid onto the adherent cells, in which case fusion was evident within 2 h and was extensive by 5 to 10 h. The remaining nonadherent cells were removed by rinsing prior to fixation.

^c The cells with RAM-1 include CHO/rRAM-1 (clone CEAR-13) and non-CHO cells that are susceptible to 4070A MuLV infection and that express endogenous RAM-1 receptors. Control CHO cells, which are not susceptible to amphotropic MuLVs and do not bind amphotropic gp70 (20), are listed in parentheses in this column.

^d The approximate percentages of nuclei present within syncytia were estimated in intervals of $\pm 5\%$ by counting at least 500 nuclei in random fields of cells. Cocultures that did not show fusion all contained less than 1% of their nuclei in syncytia, as recorded.

contrast, these CHO/rRAM-1 clones were infected at equal multiplicities when they were incubated for 2 h with preparations of amphotropic viruses (20). Compatible results were obtained with other CHO/rRAM-1 clones that express distinct amounts of rRAM-1.

Membrane fusion mediated by mCAT-1 ecotropic receptors in CHO cells. When ecotropic virions were incubated with CHO/mCAT-1 cells, cell-cell fusion began within 2 h and became extensive by 4 to 5 h (Fig. 3). Interestingly, syncytia formed with different isolates of ecotropic MuLV and even with helper-free SFF-hGH ecotropic virions that encode human growth hormone or with genome-free ecotropic virion particles harvested from $\psi 2$ packaging cells (Fig. 3 and Table 2). Furthermore, the extents of syncytium formation increased substantially with CHO/mCAT-1 cells that express high levels of mCAT-1 (Table 2).

The murine fibroblasts employed in this laboratory (i.e., NIH 3T3 and BALB/c 3T3 cells) have mCAT-1 expression levels, as determined by gp70 binding, similar to those on the CERD.10 clone of CHO/mCAT-1 cells (40), but they do not form syncytia after being incubated with ecotropic MuLVs.

Moreover, mink CCL 64/mCAT-1 cells (clone CEN), which contain approximately 2.5 ± 0.2 times more mCAT-1 than the highest-expressing CHO/mCAT-1 cells (clone CERD.9) (40), also do not fuse after being incubated with ecotropic MuLVs. For example, in one experiment, a CHO/mCAT-1 (CERD.9) culture contained $26\% \pm 2\%$ of the nuclei in syncytia 18 h after incubation with a preparation of ecotropic Rauscher MuLV, whereas the control CHO culture contained $1\% \pm 0.6\%$ of its nuclei in syncytia. In the same study, the CCL 64/mCAT-1 (clone CEN) and control CCL 64 cultures contained $1\% \pm 0.4\%$ and $1\% \pm 0.6\%$ of their nuclei in syncytia, respectively. Therefore, the fusogenicity of CHO/mCAT-1 cells cannot be explained simply by their quantity of mCAT-1.

Rapid formation of syncytia by virion particles that had an ecotropic envelope but did not encode ecotropic MuLV envelope glycoproteins strongly suggested that syncytia form by fusion-from-without, a process in which single virions fuse to two adjacent cells, thereby unifying their cell surface membranes (1, 24, 41–43). The results suggest that infectious virus is not required for syncytium formation. Consistent with this interpretation, 254-nm UV light inactivated SFF-hGH ecotropic virus infectivity approximately 50- to 100-fold more rapidly than it did the syncytium-forming activity of the virion preparation (Fig. 4).

An intriguing aspect of the results discussed above was the strong correlation between syncytium formation and the levels of mCAT-1 expression on CHO/mCAT-1 cells (Table 2). Although ecotropic virus infections occur in our cell cultures under adsorption-limiting conditions, the infections are independent of mCAT-1 expression levels above a low trace threshold (40), consistent with theoretical expectations for monovalent ligand adsorption to cell surface receptors and for results with phages and other viruses (4, 14, 35) (see Discussion). As is shown in Table 3, a preparation of ecotropic SFF-hGH virions was equally infectious after being incubated for 2 h with CHO/mCAT-1 cells that had widely different amounts of mCAT-1. In the same study, this virus preparation caused many more syncytia in the cells that had more mCAT-1 (Table 3).

DISCUSSION

The CHO cell model for membrane fusion by type C retroviruses. It is often assumed that the attachment of enveloped viruses to cell surface receptors is directly followed by a change in the organization of viral envelope glycoproteins that allows the transmembrane subunit to insert its hydrophobic amino terminus into the target cell membrane and to catalyze membrane fusion. According to this idea, receptors might have only a passive attachment role in the overall process. However, assuming that the CHO cell model of syncytium formation is indicative of events during infection (see below), our results imply that the process may be more complex and that receptors may be important in several steps of the membrane fusion pathway. These inferences derive from the following observations. First, CHO cells are extraordinarily permissive to retrovirus-induced syncytium formation, suggesting that cellular factors might be limiting for the membrane fusion process in other cells. According to this reasoning, a putative CHO cell factor could facilitate membrane fusion or, alternatively, CHO cells might be deficient in a factor that inhibits membrane fusion in other cells. Since CHO cells are infected at approximately the same efficiency as other cells that have amphotropic or ecotropic receptors (16, 20, 38, 40), it is likely that their fusogenicity derives from a quantitative difference in the

FIG. 3. Syncytium formation mediated by mCAT-1 ecotropic receptors in CHO cells. CHO/mCAT-1 cells incubated with ecotropic virions develop many multinucleate cells (A to C), in contrast to control CHO cells that lack mCAT-1 (D). CHO/mCAT-1 cells were incubated with Rauscher MuLV (A), helper-free ecotropic SFF-hGH virions that encode human growth hormone (B), or genome-free virions obtained from ψ 2 packaging cells (C). Control CHO cells incubated with ecotropic SFF-hGH virions did not form syncytia (D). After 18 h, the cells were fixed in methanol and stained with toluidine blue. Syncytia were observed in CHO/mCAT-1 cultures as early as 2 h after exposure to ecotropic virions (data not shown).

rate of a process that occurs in other cells. Secondly, our coculture analyses of syncytium formation in CHO/rRAM-1 cells imply that the CHO cell fusogenic propensity affects the receptor-bearing membrane of the target cells rather than the membrane that expresses the viral envelope glycoproteins (Table 1). Thirdly, the fusogenicity of both CHO/rRAM-1 and CHO/mCAT-1 cells requires a relatively high concentration of receptors for its full manifestation (see Results) (Tables 2 and 3). As described below, these observations imply that the putative CHO cell factor may activate a membrane fusion pathway that requires multiple receptor molecules.

The considerations discussed above suggest that retrovirus-mediated membrane fusion may require a multistep pathway. During infection, this process must be initiated by the monovalent binding of virus onto a receptor, and it is this step that seems to be limiting for the rates and efficiencies of infection by ecotropic and amphotropic MuLVs (20, 38, 40) and HIV-1 (14) (see below). We propose that subsequent events occur in the receptor-bearing cells that have different rates or efficiencies in distinct cells. These events probably include the clustering of receptors at the virus attachment site. It is intriguing that these inferences are consistent with evidence for a host cell factor in HIV-1 infection (2, 7–9) and with recent indications that receptor clustering may be necessary for infections with HIV-1 (11, 21, 22, 33, 34) and other membrane-enveloped viruses (3, 32, 37, 44). For example, studies of syncytium for-

mation by cells that express different amounts of influenza A virus hemagglutinin have suggested that multivalent complexes with receptors may be essential for the formation of a fusion pore in the infection pathway (44).

It is interesting that the CHO cell propensity for membrane fusion is evident with either rRAM-1 or mCAT-1 receptors. This result implies that a putative common factor of CHO cells might act promiscuously to enhance membrane fusion that involves different receptors and retroviruses. Previous evidence appears to be compatible with this hypothesis, because rat XC fibroblasts form syncytia after being infected not only with mouse ecotropic MuLVs but also with simian sarcoma-associated virus and feline leukemia virus (18, 41). XC cells do not fuse after being infected with amphotropic MuLVs, but this outcome could possibly result from a low level of rRAM-1 expression by these cells. Previous evidence that oncogenic transformation can enhance fusogenicity by different cells and retroviruses (15, 26, 45) supports the idea that common cellular changes can influence syncytium formation mediated by distinct receptors.

The fusogenic properties of CHO/rRAM-1 and CHO/mCAT-1 cells are similar but not identical. In both cases, the fusogenicity involves a specific activation of the receptor-bearing membranes that is enhanced by high levels of receptor expression. However, the CHO/rRAM-1 process principally but not necessarily exclusively involves fusion-from-within

TABLE 2. Fusion of CHO cells bearing receptors for ecotropic MuLV (mCAT-1) by incubation with ecotropic retrovirions

CHO/ mCAT-1 cell clone ^a	Relative $V_{\max}$ (nmol/ min-mg of protein) ^b	Amt of gp70 bound (cpm/mg) ^c	% Nuclei in syncytia ^d				
			R-MuLV	F-MuLV	SFF-hGH	ψ 2	No virus
CERD.9	3.8 $\pm$ 0.09	735 $\pm$ 144	66 $\pm$ 5	30 $\pm$ 3	56 $\pm$ 4	34 $\pm$ 2	<1
CERD.10	1.7 $\pm$ 0.11	421 $\pm$ 26	29 $\pm$ 2	13 $\pm$ 2	31 $\pm$ 2	17 $\pm$ 2	<1
CER 18.11	0.39 $\pm$ 0.22	281 $\pm$ 89	11 $\pm$ 1	9 $\pm$ 1	4 $\pm$ 1	10 $\pm$ 1	<1
CHO	0	0	<1	<1	<1	<1	<1

^a The CHO cell clones expressing various levels of mCAT-1 have been previously described (40).

^b The initial rates of uptake of L-[³H]arginine by the CHO/mCAT-1 clones and by the parental CHO cell line were analyzed, and the data were fitted to the Michaelis-Menten equation. The $V_{\max}$ values (in nanomoles per minute per milligram of protein) were as follows: CERD.9, 13 $\pm$ 0.59; CERD.10, 7.4 $\pm$ 0.36; CER 18.11, 3.8 $\pm$ 0.08; and CHO, 2.74 $\pm$ 0.20 (means $\pm$ standard deviations). The relative $V_{\max}$ for each clone was determined according to the equation

$$\frac{V_{\max} \text{ of the clone} - V_{\max} \text{ of CHO}}{V_{\max} \text{ of CHO}}$$

This equation expresses the ratio of the transport due to mCAT-1 relative to that which occurs in the control CHO cells.

^c Cells were incubated with or without ecotropic gp70 and then with antiserum to gp70 and finally with ¹²⁵I-protein A as described previously (20, 38, 40). The reported values normalized to milligrams of protein (means $\pm$ standard deviations) have been corrected for nonspecific binding to cells in the absence of gp70 and for binding to CHO cells lacking mCAT-1 expression.

^d Cells were incubated with virus-containing medium for 8 h. Eighteen hours following the initiation of these incubations, the cells were fixed in methanol, stained with toluidine blue, and examined by light microscopy. Percentages of nuclei in syncytia (means $\pm$ standard deviations) were determined by counting at least 500 nuclei in several fields. Control cultures incubated without virus were all estimated to contain fewer than 1% multinucleated cells, as indicated. R-MuLV, Rauscher isolate of MuLV; F-MuLV, Friend isolate of MuLV. SFF-hGH is a helper-free ecotropic virus that encodes human growth hormone (19). ψ 2 is cell-free conditioned medium from ψ 2 ecotropic packaging cells containing genome-free retrovirions. Unused culture medium was used as the virus-negative control.

(e.g., Fig. 2), whereas that of CHO/mCAT-1 occurs by fusion-from-without. Presumably, this difference could be caused by differences in the rates of formation or the structures of the receptor clusters or in receptor affinities for the corresponding viral glycoproteins or for host cell factors. It is likely that amphotropic viruses could cause fusion-from-without in other cell lines. We consider it unlikely that these viruses use different pathways or mechanisms for membrane fusion. Understanding these issues will require additional investigations.

Cell-cell fusions appear to require multivalent retrovirus-receptor complexes. Infection and fusion-from-without both involve ecotropic virion particles, but they have distinct dependencies on mCAT-1 expression levels in cultured CHO/mCAT-1 cells (Tables 2 and 3). The multiplicities of infection that occur when CHO/mCAT-1 cells are incubated for 2-h periods with ecotropic viruses are independent of mCAT-1 expression levels above a trace threshold (Table 3), and identical results occur with mink or human cell clones that express different amounts of mCAT-1 (40). The same relationship between levels of receptor expression and rates of infection occurs with amphotropic MuLV (20), HIV-1 (14), and bacteriophage λ (35). Moreover, in all of these cases, the multiplicities of infection are limited by a slow process of virus adsorption from the culture medium (14, 20, 35, 40). The fact that infections in adsorption-limited conditions are independent of receptor expression levels above a low trace threshold is consistent with theoretical and experimental evidence for monovalent ligand adsorptions onto cell surface receptors. As was shown by Berg and Purcell (4), the probability that a ligand that diffuses to a cell surface will encounter a receptor is $NS/(NS + \pi a)$, where N is the number of receptors per cell, S is the receptor radius, and a is the cell radius. This behavior occurs because ligands that diffuse to cells move over a substantial patch of membrane and rapidly rotate to expose their surfaces to the cell. If the receptor density is sufficient to occupy such a patch, encounter will occur and adsorption can be very efficient, so further increases in the number of receptors per cell will not enhance adsorption. The consequence is that rates of ligand adsorption often reach half-maximum values when the number of receptors is only $\pi a/S$, a number that typically occupies less than 0.5% of the cell surface (4). These considerations are compat-

FIG. 4. Inactivation of ecotropic retrovirions by UV light. Virus infectivity is inactivated more rapidly than is syncytium-forming activity. SFF-hGH virions were irradiated at 254 nm with the indicated doses in a Stratalinker (Stratagene, La Jolla, Calif.). Irradiated virus was used to infect CHO/mCAT-1 or control CHO cells (data not shown) growing on eight-well chamber slides seeded the previous day at 1.5×10^4 cells per well. Two hours after the virus was added, fresh complete medium was added and the cells were incubated overnight. The cells were then fixed and stained with toluidine blue to determine syncytial formation or stained for human growth hormone-specific immunofluorescence to assess infectivity (19). The ratio of nuclei in syncytia to total nuclei was calculated after representative fields were counted. The fraction of cell fusion activity remaining after irradiation was calculated by comparison with the fraction of nuclei in syncytia following infection of the cells with unirradiated virus. Infectivity following virus irradiation was quantified by human growth hormone-specific immunofluorescence. The fraction of fluorescent cells was determined by analyzing at least 400 cells in several different fields. Multiplicity of infection (M.O.I.) was calculated from the binomial distribution as $-\log P/0.44$, where P is the fraction of nonfluorescent cells (19). The fraction of infectivity remaining after irradiation of the virus was calculated by comparison with the multiplicity of infection of unirradiated virus.

TABLE 3. The extents of syncytium formation, but not infection efficiencies, correlate with the levels of mCAT-1 expression on CHO/mCAT-1 clones

mCAT-1 CHO clone	Level of mCAT-1 (nmol/min-mg of protein) ^a	Multiplicity of infection ^b	Percentage of nuclei in syncytia ^c
CERD.9	3.8 ± 0.09	0.64 ± 0.04	38 ± 3
CERD.10	1.7 ± 0.11	0.66 ± 0.04	24 ± 2
CER 18.11	0.39 ± 0.22	0.74 ± 0.05	13 ± 2
CHO	0	<0.001	<1

^a The level of mCAT-1 expressed on each clone is expressed as the relative $V_{\max}$ (mean ± standard deviation) for [³H]arginine uptake. See footnote *b* of Table 2.

^b Cells were infected for 2 h with ecotropic SFF-hGH virions (diluted 1:20 in fresh complete medium), and infectivity (in mean multiplicities of infection ± standard deviations) was determined by human growth hormone-specific immunofluorescence as described in the legend to Fig. 4.

^c Cells were incubated for 2 h with ecotropic SFF-hGH virions (undiluted), and then fresh complete medium was added and the cells were incubated for an additional 16 h. The cells were fixed in methanol, stained with toluidine blue, and examined by light microscopy. To determine the percentage of nuclei in syncytia (mean ± standard deviation), at least 500 nuclei in several different fields were counted.

ible with the idea that ecotropic and amphotropic MuLV infections are limited by the monovalent attachment of virus onto their receptors.

In contrast, fusion-from-without of CHO/mCAT-1 cells by ecotropic virions is clearly much more dependent on mCAT-1 expression levels (Tables 2 and 3). It is difficult to infer a precise order for the mCAT-1 dependency of cell-cell fusion, because the process obviously requires close cellular contacts that occur to various extents in the monolayers. For example, cells with many neighbors would fuse more easily than isolated cells. Nevertheless, the controlled comparison with infectivity (Table 3) suggests that fusion-from-without is not limited by the initial monovalent binding of virus onto a cell and that it probably requires multivalent virus-receptor linkages that bridge adjacent cells. Similarly, rRAM-1-mediated syncytium formation also seems to be highly dependent on the cell surface levels of receptor.

ACKNOWLEDGMENTS

We thank our colleagues Emily Platt, Greg Allen, and Michael Kavanaugh for their interest and encouragement.

This work was supported by NIH grant CA25810.

REFERENCES

- Andersen, K. B. 1994. A domain of murine retrovirus surface protein gp70 mediates cell fusion, as shown in a novel SC-1 cell fusion system. *J. Virol.* **68**:3175–3182.
- Ashorn, P. A., E. A. Berger, and B. Moss. 1990. Human immunodeficiency virus envelope glycoprotein/CD4-mediated fusion of nonprimate cells with human cells. *J. Virol.* **64**:2149–2156.
- Bentz, J., H. Ellens, and D. Alford. 1993. Architecture of the influenza hemagglutinin fusion site, p. 164–199. *In* J. Bentz (ed.), *Viral fusion mechanisms*. CRC Press, Boca Raton, Fla.
- Berg, H. C., and E. M. Purcell. 1977. Physics of chemoreception. *Biophys. J.* **20**:193–219.
- Chesebro, B., K. Wehrly, J. Metcalf, and D. E. Griffin. 1988. Development of a sensitive quantitative focal assay for human immunodeficiency virus. *J. Virol.* **62**:3779–3788.
- Cunningham, J. M., and J. W. Kim. 1994. Cellular receptors for type C retroviruses, p. 49–59. *In* E. Wimmer (ed.), *Cellular receptors for animal viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Dragic, T., and M. Alizon. 1993. Different requirements for membrane fusion mediated by the envelopes of human immunodeficiency virus types 1 and 2. *J. Virol.* **67**:2355–2359.
- Dragic, T., P. Charneau, F. Clavel, and M. Alizon. 1992. Complementation of murine cells for human immunodeficiency virus envelope/CD4-mediated fusion in human/murine heterokaryons. *J. Virol.* **66**:4794–4802.
- Dragic, T., L. Picard, and M. Alizon. 1995. Proteinase-resistant factors in human erythrocyte membranes mediate CD4-dependent fusion with cells expressing human immunodeficiency type 1 envelope glycoproteins. *J. Virol.* **69**:1013–1018.
- Evans, L. H., S. Dresler, and D. Kabat. 1977. Synthesis and glycosylation of polyprotein precursors to the internal core proteins of Friend murine leukemia virus. *J. Virol.* **24**:865–874.
- Frey, S., M. Marsh, S. Gunther, A. Pelchen-Matthews, P. Stevens, S. Ortlepp, and T. Stegmann. 1995. Temperature dependence of cell-cell fusion induced by the envelope glycoprotein of human immunodeficiency virus type 1. *J. Virol.* **69**:1462–1472.
- Gratzner, H. G. 1982. Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* **218**:474–475.
- Harrington, R. D., and A. P. Geballe. 1993. Cofactor requirement for human immunodeficiency virus type 1 entry into a CD4-expressing human cell line. *J. Virol.* **67**:5939–5947.
- Kabat, D., S. L. Kozak, K. Wehrly, and B. Chesebro. 1994. Differences in CD4 dependence for infectivity of laboratory-adapted and primary patient isolates of human immunodeficiency virus type 1. *J. Virol.* **68**:2570–2577.
- Kaufman, S. J., and D. M. Ehrbar. 1980. Transformation of rat fibroblasts and formation of virus-induced syncytia. *Nature (London)* **285**:484–485.
- Kavanaugh, M. P., D. G. Miller, W. Zhang, W. Law, S. L. Kozak, D. Kabat, and A. D. Miller. 1994. Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. *Proc. Natl. Acad. Sci. USA* **91**:7071–7075.
- Kim, J. W., E. I. Closs, L. M. Albritton, and J. M. Cunningham. 1991. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature (London)* **352**:725–728.
- Klement, V., W. P. Rowe, J. W. Hartley, and W. E. Pugh. 1969. Mixed culture cytopathogenicity: a new test for growth of murine leukemia viruses in tissue culture. *Proc. Natl. Acad. Sci. USA* **63**:753–758.
- Kozak, S. L., and D. Kabat. 1990. Ping-pong amplification of a retroviral vector achieves high-level gene expression: human growth hormone production. *J. Virol.* **64**:3500–3508.
- Kozak, S. L., D. C. Siess, M. P. Kavanaugh, A. D. Miller, and D. Kabat. 1995. The envelope glycoprotein of an amphotropic murine retrovirus binds specifically to the cellular receptor/phosphate transporter of susceptible species. *J. Virol.* **69**:3433–3440.
- Layne, S. P., M. J. Merges, M. Dembo, J. L. Spouge, and P. L. Nara. 1990. HIV requires multiple gp120 molecules for CD4-mediated infection. *Nature (London)* **346**:277–279.
- Lu, S., S. D. Putney, and H. L. Robinson. 1992. Human immunodeficiency virus type 1 entry into T cells: more-rapid escape from an anti-V3 loop than from an antireceptor antibody. *J. Virol.* **66**:2547–2550.
- Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**:153–159.
- Marsh, M., and A. Helenius. 1989. Virus entry into animal cells. *Adv. Virus Res.* **36**:107–151.
- McClure, M. O., M. A. Sommerfelt, M. Marsh, and R. A. Weiss. 1990. The pH independence of mammalian retrovirus infection. *J. Gen. Virol.* **71**:767–773.
- Ocho, M., H. Ogura, T. Tanaka, and T. Oda. 1980. Induction of syncytia by simian sarcoma virus type I (SSV-I/SSAV-I) in several human transformed cell lines. *Exp. Cell Biol.* **48**:421–428.
- Pinter, A., T.-E. Chen, A. Lowy, N. G. Cortez, and S. Silagi. 1986. Ecotropic murine leukemia virus-induced fusion of murine cells. *J. Virol.* **57**:1048–1054.
- Quade, K. 1979. Transformation of mammalian cells by avian myelocytomatosis virus and avian erythroblastosis virus. *Virology* **98**:461–465.
- Ragheb, J. A., and W. F. Anderson. 1994. pH-independent murine leukemia virus ecotropic envelope-mediated cell fusion: implications for the role of the R peptide and p12E TM in viral entry. *J. Virol.* **68**:3220–3231.
- Redmond, S., G. Peters, and C. Dickson. 1984. Mouse mammary tumor virus can mediate cell fusion at reduced pH. *Virology* **133**:393–402.
- Rein, A., J. Mirro, J. G. Haynes, S. M. Ernst, and K. Nagashima. 1994. Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus *env* protein. *J. Virol.* **68**:1773–1781.
- Rey, F. A., F. X. Heinz, C. Mandi, C. Kunz, and S. C. Harrison. 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature (London)* **375**:291–298.
- Rieber, E. P., C. Federle, C. Reiter, S. Krauss, L. Gurtler, J. Eberle, F. Denhardt, and G. Riethmuller. 1992. The monoclonal CD4 antibody M-T413 inhibits cellular infection with human immunodeficiency virus after viral attachment to the cell membrane: an approach to postexposure prophylaxis. *Proc. Natl. Acad. Sci. USA* **89**:10792–10796.
- Sattentau, Q. J. 1992. CD4 activation of HIV fusion. *Int. J. Cell Cloning* **10**:323–332.
- Schwartz, M. 1976. The adsorption of coliphage lambda to its host: effect of variations in the cell surface density of receptor and its phage-receptor affinity. *J. Mol. Biol.* **103**:521–536.
- Spiro, C., B. C. Gliniak, and D. Kabat. 1989. Splenic accumulation of inter-

- leukin-3-dependent hematopoietic cells in Friend erythroleukemia. *J. Virol.* **63**:4434–4437.
37. **Stegman, T., and A. Helenius.** 1993. Influenza virus fusion: from models toward a mechanism, p. 90–106. *In* J. Bentz (ed.), *Viral fusion mechanisms*. CRC Press, Boca Raton, Fla.
 38. **Wang, H., E. Dechant, M. Kavanaugh, A. North, and D. Kabat.** 1992. Effects of ecotropic murine retroviruses on the dual-function cell surface receptor/basic amino acid transporter. *J. Biol. Chem.* **267**:23617–23624.
 39. **Wang, H., M. P. Kavanaugh, R. A. North, and D. Kabat.** 1991. Cell surface receptor for ecotropic murine retroviruses is a basic amino acid transporter. *Nature (London)* **352**:729–731.
 40. **Wang, H., R. Paul, R. E. Burgeson, D. R. Keene, and D. Kabat.** 1991. Plasma membrane receptors for ecotropic murine retroviruses require a limiting accessory factor. *J. Virol.* **65**:6468–6477.
 41. **Weiss, R.** 1984. Experimental biology and assay of RNA tumor viruses, p. 209–260. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *RNA tumor viruses*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 42. **Weiss, R. A.** 1994. Human receptors for retroviruses, p. 15–31. *In* E. Wimmer (ed.), *Cellular receptors for animal viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 43. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* **52**:675–697.
 44. **White, J. M.** 1994. Fusion of influenza virus in endosomes: role of the hemagglutinin, p. 281–301. *In* E. Wimmer (ed.), *Cellular receptors for animal viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 45. **Wilson, C. A., J. W. Marsh, and M. V. Eiden.** 1992. The requirements for viral entry differ from those for virally induced syncytium formation in NIH 3T3/DTras cells exposed to Moloney murine leukemia virus. *J. Virol.* **66**:7262–7269.