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Receptor-Binding Properties of a Purified Fragment of the 4070A Amphotropic Murine Leukemia Virus Envelope Glycoprotein

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A 208-amino-acid amino-terminal fragment of the 4070A amphotropic murine leukemia virus envelope glycoprotein contains all of the determinants required to recognize cell surface amphotropic receptors. This fragment was fused with a streptavidin-binding tag, expressed in Sf9 insect cells by using a baculovirus vector, and purified to homogeneity. The ^{125}I-labeled purified fragment (AS208) specifically bound various cell lines susceptible to amphotropic murine leukemia virus infection. The number of AS208-binding sites was in the range of 7×10^4 to 17×10^4 per cell. Quantitative analysis of binding revealed that AS208-binding sites are heterogeneous with regard to ligand binding affinity or that cooperativity exists between receptors. Competition experiments showed that the concentration of AS208 required to inhibit virus entry was lower than that required to inhibit the binding of virus particles at the cell surface. Taken together, these data suggested that amphotropic envelope-binding sites present at the cell surface do not act independently and do not participate equally in virus infection.

Retrovirus entry into cells is mediated by a specific interaction between the surface component (SU) of the envelope glycoproteins and cell surface receptors. SUs of murine leukemia viruses (MuLVs), feline leukemia viruses, and the gibbon ape leukemia virus are partly homologous, and their structures are probably related (4). The receptor recognition domain has been assigned to the amino-terminal domains of the SUs of the ecotropic, amphotropic, xenotropic, and polytropic MuLVs (3, 4, 18, 31, 34) and of feline leukemia virus subgroups A and C (6, 38). Two variable regions, referred to as VRA and VRB, are located in the MuLV amphotropic envelope. VRA is likely to bear the major receptor recognition determinants, while VRB appears to have an accessory role in receptor recognition and binding (4, 16, 31).

Genes encoding the receptors for ecotropic MuLVs (*rec-1*) (2), amphotropic MuLVs (*ram-1*) (29, 44), and the gibbon ape leukemia virus (*glvr-1*) (32) have been identified. The protein products are multiple membrane-spanning, dual-function molecules, acting as a cationic amino acid transporter in the case of Rec-1 (25, 46) and as sodium-phosphate symporters in the cases of Ram-1 and Glvr-1 (30, 33). Alleles associated with susceptibility or resistance to infection have been isolated. Sequence comparison and chimeric receptor molecules led to the identification of determinants interacting with SU molecules in the third and fourth extracellular loops of the Rec-1 (1, 51) and Ram-1 (19, 35, 48) proteins, respectively.

Ecotropic envelope-receptor interactions have been analyzed by using radiolabeled purified SUs (7, 10, 12, 15, 20, 23) or by cytofluorimetric analysis of cells incubated with ecotropic virus particles (22, 27, 28, 52). Affinity constants were determined to be in the range of 10^9 M^{-1}. Amphotropic envelope-receptor interactions were investigated by using similar approaches. However, since purified preparations of the amphotropic envelope were not available, the binding parameters have not been determined (22, 26). We have purified to homogeneity a fragment of the amphotropic SU glycoprotein (referred to as AS208) containing the amphotropic receptor recognition domain, and its cell-binding properties on various cell types susceptible to amphotropic virus infection were characterized. Binding of the purified protein competed with that of amphotropic virus particles and led to complete resistance to the entry of amphotropic virus pseudotypes into cells.

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MATERIALS AND METHODS

Construction of recombinant baculovirus. A DNA fragment encoding the 20 amino acids of the amphotropic SU leader peptide, the 208 amino-terminal amino acids of the mature SU, a streptavidin-binding polypeptide (40), and a translational stop codon was synthesized by PCR with the primers 5′-CGCG GATCCATGGCGCGTTCAACGCTCTC-3′ and 5′-CGCGGATCCTTAACC ACCGAACTGCGGGTGACGCCAAGCTCCCACATTAAGGACCTGCCG GG-3′, which contained *Bam*HI restriction sites for convenient cloning. The 760-bp PCR amplification product was inserted downstream of a polyhedrin promoter into the *Bam*HI site of the Baculotransfer vector pVL1393 (Pharmingen Inc.), giving rise to pB.SU.S, and was sequenced. Sf9 cells were then cotransfected with pB.SU.S and linearized baculovirus genomic DNA (*Autographa californica* nuclear polyhedrosis virus Baculogold; Pharmingen Inc.). Cells and culture medium were harvested 24, 48, 72, and 120 h postinfection and analyzed for the expression of the AS208 protein by immunoblotting. After two plaque purifications, the B.SU.S vector was selected among 10 clones on the basis of high-level AS208 protein expression.

Purification of AS208. Eighteen roller bottles (850 cm^2) containing log-phase Sf9 insect cells were infected with B.SU.S (multiplicity of infection = 10). Four liters of culture supernatant was harvested at 48 h, cleared by centrifugation (30,000 rpm, 1 h [Beckman L-70 apparatus]), and concentrated 30-fold with an Amicon 30 membrane. Four columns of streptavidin bound to Sepharose (Biometra) were loaded with concentrated samples (30 ml), washed three times with 5 ml of 50 mM Tris (pH 7)–1 mM EDTA–0.02% sodium azide, and eluted in 40 ml of 5 mM diaminobiotin (Sigma). Fractions containing the partially purified AS208 fragment were pooled (total volume of approximately 140 ml [40 ml from each column]), and the Tris buffer was replaced by HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (30 mM, pH 7) by three cycles of concentration-dilution on an Amicon C10 membrane. Samples were then loaded onto a cation-exchange chromatography column (Resource S; Pharmacia). The column was washed with 15 ml of 30 mM HEPES (pH 7), and 1-ml fractions were collected during elution with a 0 to 2 M NaCl gradient in 30 mM HEPES (pH 7). AS208 eluted in one or two fractions at approximately 150 mM NaCl. The protein concentration was measured by spectrophotometry at 280 nm ($\varepsilon = 1.9$ [determined by amino acid analysis]). An average of 4 mg of pure AS208 was recovered from 4 liters of baculovirus supernatant.

^{125}I-labeling and -binding studies. Five micrograms of purified AS208 was labeled for 2 h at 4°C according to the method of Bolton and Hunter (5) and loaded on a Sephadex G-10 column to remove unincorporated ^{125}I-*N*-succinimide ester. The specific activity of the labeled protein was between 15,000 and 24,000 cpm/ng. ^{125}I-labeled or unlabeled preparations of AS208 competed equivalently with amphotropic pseudotype entry into cells, indicating that the recognition of amphotropic receptors was not altered by labeling. Binding assays were performed both on adherent monolayers and on single cell suspensions. Monolayers of adherent cells plated in 24-well culture plaques were incubated in 300 μl of Dulbecco's modified Eagle medium (DMEM) containing 1 mg of bovine serum albumin (BSA) per ml, 30 mM HEPES, and ^{125}I-AS208. After incubation, the supernatant was collected to measure unbound ligand, and the cells were washed with cold phosphate-buffered saline (PBS) and lysed with 20 mM HEPES–0.5% Triton X-100–150 mM NaCl to measure the incorporated radioactivity. Single-cell suspensions were obtained by detaching adherent cells in the presence of PBS–1 mM EDTA. Incubation with ^{125}I-AS208, washes, and lysis were performed as for adherent cells, except that cells were centrifuged between each step.

The number of binding sites per cell was calculated under conditions of receptor saturation as the ratio of the number of binding molecules (minimal fragment molarity giving maximal binding × Avogadro's number) to the number of cells per liter.

Electrophoresis and immunoblotting. The purity and integrity of the AS208 protein were examined at different stages of the purification process by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue or silver staining. For immunoblotting, AS208 proteins were digested or not with peptide N-glycosidase F (New England Biolabs) prior to SDS-PAGE. Electrophoretic transfer of AS208 proteins from the gel to nitrocellulose (Hybond C Super; Amersham) was performed in a Phast blot B33 (Biometra). The membrane was blocked for 1 h in PBS–5% milk powder–1% Nonidet P-40, incubated overnight at 4°C with goat anti-Rauscher leukemia virus gp70 (Quality Biotech, Camden, N.J.) or rabbit anti-AS208 serum diluted 1,000-fold in PBS–5% milk powder–0.1% Tween 20, washed three times, incubated with a peroxidase-conjugated antigoat or antirabbit serum, and detected with an ECL detection kit (Amersham).

Cells and viruses. Cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS) (wild mouse [*Mus dunni*] and human TE671 cells) or newborn calf serum (NCS) (NIH 3T3 cells). CHO cells were grown in Ham's F12 medium (Gibco BRL) supplemented with 10% FCS. Insect Sf9 cells were grown in TC100 cell culture medium with 10% FCS. Stocks of helper-free ecotropic and amphotropic pseudotypes of particles containing the *Escherichia coli lacZ* gene were obtained from Ψ-CRE and Ψ-CRIP clones, respectively, as previously described (4). Xenotropic pseudotypes were obtained by stable expression of the X-GP expression vector (3) in EB8 cells (4). Infectious titers were measured by exposing NIH 3T3 cells, or other cell types when indicated, at 37°C for 1 h to serial dilutions of the filtered culture supernatant of producer clones in the presence of 8 μg of Polybrene per ml. Titers were expressed as the number of β-galactosidase-positive foci induced on target cells per milliliter of infecting medium (focus-forming units [FFU] per milliliter). The following titers were determined for the stock of amphotropic particles used in the various experiments: 7.4×10^4 FFU/ml on NIH 3T3 cells, 4.6×10^4 FFU/ml on *M. dunni* cells, and 3×10^4 FFU/ml on TE671 cells.

Monoclonal and polyclonal antibodies. The 83A25 monoclonal antibody to MuLV envelope glycoprotein has been previously described (14) and was kindly provided by Leonard H. Evans (Rocky Mountain Laboratories Hamilton, Mont.). Goat anti-Rauscher leukemia virus gp70 serum was obtained from Quality Biotech. Polyclonal anti-AS208 fragment antisera were raised in New Zealand White rabbits immunized with approximately 100 μg of affinity-purified AS208 in complete Freund's adjuvant. After 15 days, rabbits were boosted three times at 2-week intervals with 100 μg of AS208 in incomplete Freund's adjuvant.

Interference assay. NIH 3T3, *M. dunni*, or human TE671 target cells (2×10^5 cells) plated in 35-mm-diameter dishes were infected for 1 h at 37°C with 1 ml of DMEM containing 10% FCS or NCS, 300 FFU of amphotropic or control pseudotypes, various concentrations of AS208, and 8 μg of Polybrene per ml. The viral supernatant was then replaced by fresh medium. Cells were grown to confluence and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). β-Galactosidase-positive foci were scored and expressed as percentages of the values scored for control cells.

Virus-binding assay. Direct binding of amphotropic or control pseudotypes to cells was analyzed by fluorescence-activated cell sorting. NIH 3T3, *M. dunni* or TE671 cells (2×10^5 cells) were detached with PBS–1 mM EDTA and washed with PBS. Cells were incubated for 1 h at 37°C in 1 ml of DMEM containing 10% FCS or NCS, 300 FFU of viral pseudotypes, various concentrations of AS208, and 8 μg of Polybrene per ml. The cells were washed twice with ice-cold PBS and resuspended for 30 min on ice in a 48-h hybridoma supernatant containing the rat monoclonal antibody 83A25. Cells were washed twice, labeled with phycoerythrin-conjugated goat anti-rat immunoglobulin G (Southern Biotechnology Associates) in PBA (1% BSA, 0.1% sodium azide in PBS), and fixed in PBA–1% paraformaldehyde before analysis of fluorescence intensity with a FACScan cytofluorometer (Becton Dickinson).

For the virus-binding kinetic assay, 10^5 FFU was added to target cells suspended in 1 ml of DMEM supplemented with 10% FCS or NCS. The reaction was stopped by adding ice-cold PBS to virus-cell mixtures and centrifugation. Labeling of cell-virus complexes was performed as described above.

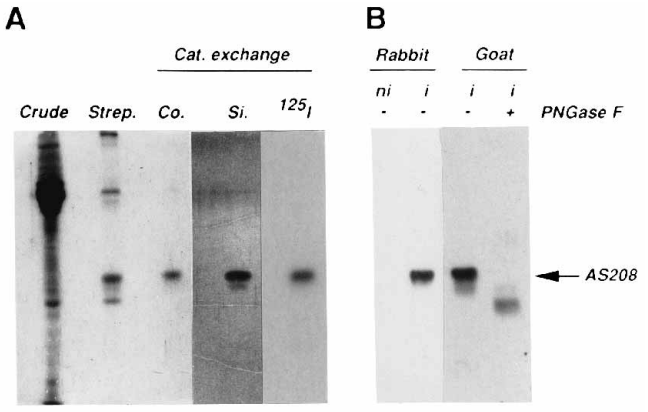

FIG. 1. Purification of AS208. (A) Analysis by SDS-PAGE of crude supernatant from infected insect cells (Crude), pooled fractions harvested after elution of the streptavidin column (Strep.), purified AS208 obtained after cation-exchange chromatography (Cat. exchange) and stained by Coomassie blue (Co.) or silver nitrate (Si.), and autoradiography of ^{125}I-labeled AS208 (^{125}I). (B) Immunoblotting of purified AS208 treated (+) or not treated (−) with Peptide N-glycosidase F (PNGase F). Blots were revealed either by a rabbit serum raised against AS208 (Rabbit) or by a goat anti-Rauscher leukemia virus gp70 serum (Goat). ni, preimmune serum; i, immune serum.

RESULTS

Purification and labeling of an amino-terminal fragment of the amphotropic MuLV envelope glycoprotein (AS208). We have previously shown that a 208-amino-acid amino-terminal fragment of the amphotropic envelope glycoprotein specifically interferes with the entry of virus particles bearing the amphotropic envelope into various cell types, including mouse NIH 3T3, *M. dunni*, dog D17, and human HOS cells (3). A tag of nine amino acids (AWRHPQFGG), which specifically binds streptavidin (40), was added to the C-terminal extremity of this fragment, and the fusion protein (AS208) was expressed in Sf9 insect cells by using a baculovirus vector. AS208 secreted in an Sf9 culture supernatant (Fig. 1A, Crude) was purified by affinity chromatography through a column of streptavidin bound to Sepharose followed by cation-exchange chromatography. Contaminants present after the streptavidin column step (Fig. 1A, Strep.) were removed by the cation-exchange chromatography (Fig. 1A, Cat. exchange). Figure 1A shows purified AS208 stained with Coomassie blue (Co.), treated with silver nitrate (Si.), or labeled with ^{125}I. These data indicated that the recovered protein was more than 95% pure. AS208 appeared as two bands of 32 and 30 kDa, both of which were recognized by a goat anti-Rauscher leukemia virus gp70 serum in a Western blot (immunoblot) analysis (Fig. 1B). Removal of oligosaccharide chains by peptide N-glycosidase F resulted in a single 28-kDa band, which agreed with the calculated molecular mass (Fig. 1B, PGNase F). The loss of the 32-kDa protein indicated that the two species corresponded to differently glycosylated forms of AS208, as previously observed when AS208 was expressed in mouse NIH 3T3 cells (3). Immune sera obtained by immunizing rabbits with AS208 produced in insect cells also detected the fragment (Fig. 1B).

AS208 binding to cells susceptible to amphotropic virus infection. Monolayers of mouse NIH 3T3 cells, which are susceptible to amphotropic virus infection, or of hamster CHO cells, which are resistant to amphotropic virus infection (11, 17, 36), were incubated at 37°C for various periods of time in the

presence of 0.5 nM ^{125}I-labeled AS208. Figure 2A shows that the binding of AS208 to NIH 3T3 cells rapidly increased with time, reaching a plateau value after 20 min of incubation, which then was stable for at least 2 h. The concentration of free ligand was maintained at the same level over the incubation period. The amount of bound material increased with the number of cells. Cell-bound radioactivity on CHO cells was low and increased linearly with time, indicating nonspecific binding. AS208 binding to NIH 3T3 cells was abolished in the presence of a rabbit anti-AS208 antiserum and increased with temperature and pH (not shown). These data showed that AS208 specifically bound to cells susceptible to amphotropic virus infection.

A quantitative analysis of AS208 binding was performed with a single-cell suspension of NIH 3T3 cells. Cells were detached with PBS–1 mM EDTA, and 2×10^5 nonadherent cells were incubated with increasing concentrations of ^{125}I-labeled AS208 for 1 h in order to reach equilibrium. Unbound and, after extensive washing, cell-bound radioactivity, were counted. The amount of bound material showed a plateau value for 0.75 nM labeled ligand, indicating that binding was saturable (Fig. 2B). Representation of these data on a Scatchard plot showed a regression curve with downward concavity ($r = 0.995$) (Fig. 2C). Scatchard plots performed with different cell numbers showed a similar concavity (not shown). These data suggested that AS208-binding sites are heterogeneous with regard to ligand-binding affinity or that cooperativity exists between receptors. The estimated number of AS208-binding sites was 7.7×10^4 per NIH 3T3 cell.

We studied the binding of AS208 on other cell types susceptible to amphotropic virus infection. Wild mouse (*M. dunni*) cells and human TE671 cells were incubated at 37°C and pH 7.4 for increasing periods of time with 0.5 nM ^{125}I-labeled AS208. We first determined that this ligand concentration was high enough to saturate binding sites on a monolayer of 6×10^4 target cells (not shown). Nonspecific binding was measured on CHO cells. The amount of AS208 bound to susceptible cells increased during the first hour, after which it reached a plateau value (Fig. 3A). The variation in free ligand concentration over time was lower than 10% of the initial value. The number of AS208-binding sites per cell calculated according to plateau values of cell-bound material was 17×10^4 and 14×10^4 on *M. dunni* and TE671 cells, respectively. For NIH 3T3 cells, the calculated value agreed with the one determined by Scatchard analysis. With the aim to compare the binding behaviors of AS208 and virus particles, the same cell lines were incubated for various lengths of time in 1 ml with 10^5 FFU of an amphotropic MuLV pseudotype. The amounts of virus particles bound at the cell surface were quantified by flow cytometry with the monoclonal antibody 83A25 (14). CHO cells were used as a negative control. Virus particles rapidly bound to cells, with a maximal intensity at 20 to 30 min and a slow decrease over the next 2 h (Fig. 3B). Comparable data were previously reported by Kadan et al. (22). The persistence of bound particles for long periods of time indicated that the interaction of the amphotropic envelope with cell surface receptors was stable, as previously observed for AS208. Whether the early decrease of bound particles corresponded to virus dissociating from its receptor or to particle internalization by a fraction of the receptors has not been determined. The peak values for bound particles on *M. dunni* and TE671 cells were 121 and 71%, respectively, of that on NIH 3T3 cells.

Competition of AS208 with amphotropic particles for receptor binding and cell infection. The capacity of AS208 to compete with the binding of amphotropic virus particles to cell surface receptors was examined by flow cytometry (Fig. 4A and

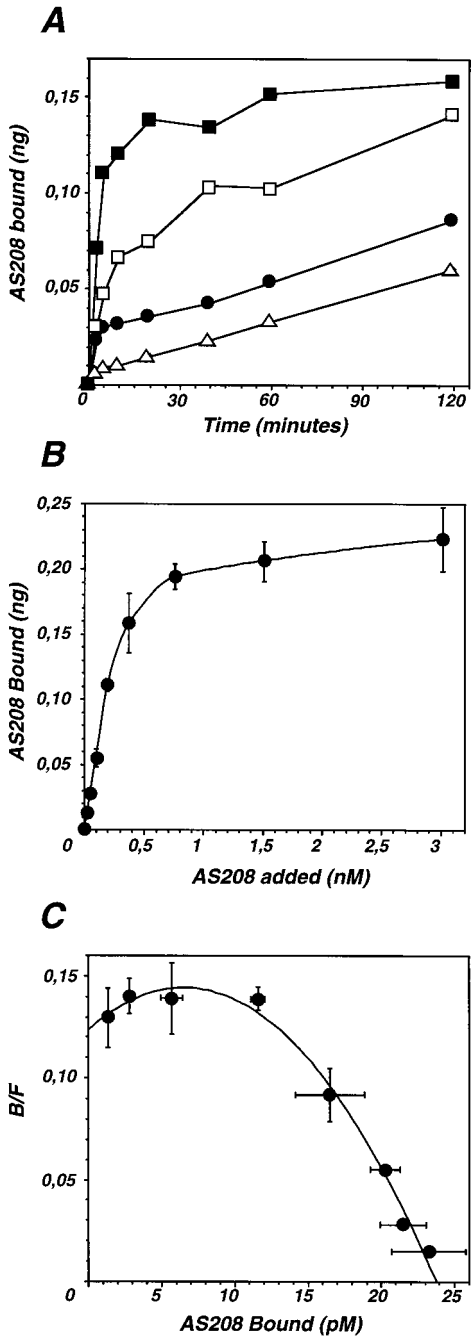

FIG. 2. Characteristics of AS208 binding on NIH 3T3 cells. (A) Monolayers of 2×10^5 CHO cells (triangles) or 6×10^4 (closed squares), 3×10^4 (open squares), or 6×10^3 (circles) NIH 3T3 cells were incubated for various periods of time at 37°C in 300 μl of culture medium at pH 7.4 in the presence of 0.5 nM ^{125}I-AS208. The cells were then washed with PBS, trypsinized, and counted, and the bound radioactivity was measured. (B) Quantitative analysis of ^{125}I-AS208 binding to NIH 3T3 cells. Cells were detached with PBS–1 mM EDTA and counted; 2×10^5 cells were incubated for 1 h at pH 7.4 with increasing concentrations of ^{125}I-AS208 in 300 μl of culture medium, and after extensive washing with PBS, cell-bound and free radioactivity were counted. Values are the means ± standard deviations for triplicate experiments. (C) Scatchard plot of data obtained in the experiment shown in panel B. B/F, bound radioactivity/free radioactivity.

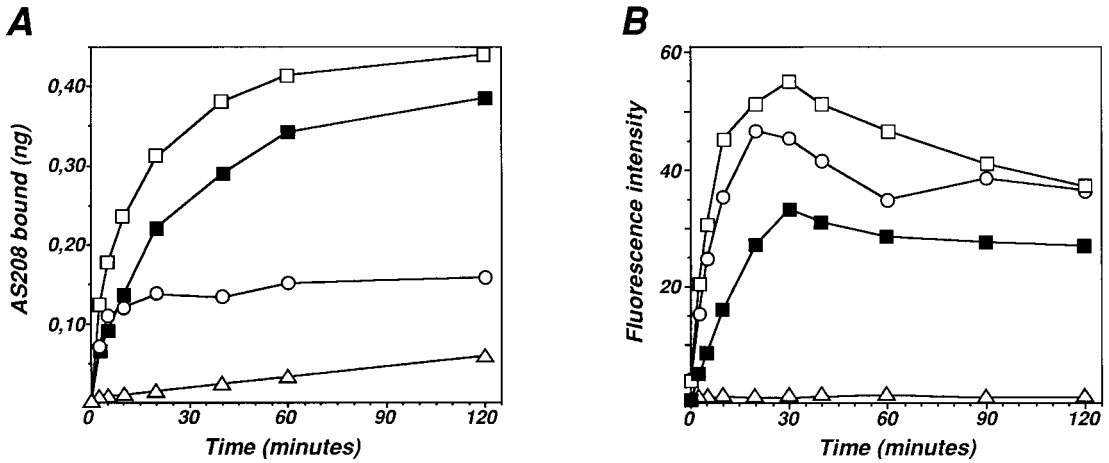

FIG. 3. Binding of AS208 and amphotropic particles to different cell types. (A) Monolayers of 6×10^4 cells were incubated for various periods of time at 37°C and pH 7.4 in 300 μl of DMEM containing 0.5 nM ^{125}I-AS208. After extensive washing with PBS, cell bound and free radioactivity were counted. (B) Cells (2×10^5) detached with PBS–1 mM EDTA and suspended in 1 ml of DMEM–10% FCS were incubated for various periods of time at 37°C and pH 7.4 with 10^5 FFU of an amphotropic pseudotype of a Moloney MuLV-derived retroviral vector containing the *E. coli lacZ* gene. Binding was terminated by adding ice-cold PBS, and cells were labeled with the rat 83A25 monoclonal antibody prior to flow cytometry analysis. The values are the means from triplicate experiments. Standard deviations never exceeded 10% of the indicated values. Closed squares, TE671 cells; open squares, *M. dunni* cells; circles, NIH 3T3 cells; triangles, CHO cells.

B). Ecotropic or xenotropic MuLV pseudotypes which do not bind the amphotropic receptor were used as controls. NIH 3T3, *M. dunni*, and TE671 cells (2×10^5 cells) were incubated in 1 ml of culture medium for 1 h with 300 FFU of the MuLV pseudotypes in the presence of increasing amounts of AS208. Bound virus particles were revealed with the monoclonal antibody 83A25 (14). This antibody recognizes a carboxy-terminal epitope common to the various SU subgroups which is not present in AS208. Binding of the ecotropic and xenotropic MuLV pseudotypes was not affected by AS208. Binding of amphotropic pseudotypes decreased in a dose-dependent manner in the presence of AS208. We estimated that a 50% decrease required 3 nM AS208 on NIH 3T3 cells and 1.8 nM AS208 on *M. dunni* and TE671 cells.

The capacity of AS208 to interfere with the entry of amphotropic particles was examined by a similar approach (Fig. 4C). Ecotropic or xenotropic MuLV pseudotypes which infect target cells through a different receptor were used as controls. NIH 3T3, *M. dunni*, and TE671 cells (2×10^5 cells) were incubated in 1 ml of culture medium for 1 h with 300 FFU of MuLV pseudotypes containing the *E. coli lacZ* gene. Increasing amounts of AS208 were added to the incubation medium. The number of β-galactosidase-positive cell foci was scored 3 days later, when cells reached confluency. AS208 did not interfere with the entry of control pseudotypes, but it decreased the susceptibility to amphotropic pseudotype infection in a dose-dependent manner. We estimated that a 50% decrease in the number of β-galactosidase-positive foci required 1 nM AS208 on NIH 3T3 cells and 0.5 nM AS208 on *M. dunni* and TE671 cells.

Free SU protein is known to contaminate amphotropic virus stocks (3). Since cell-bound SU can be recognized as a virus particle by 83A25 in cytofluorimetry experiments, whereas it acts synergistically with AS208 in infection interference assays, high concentrations of free SU in virus stocks might explain why more AS208 was required for inhibiting virus binding than for inhibiting virus entry. In order to estimate the free SU concentration in reaction mixtures, we assumed a number of 300 SU monomers per particle (52) and a relative proportion of particle-bound to free SU of 1 to 1,000. With these assumptions, the concentration of free SU in reaction mixtures was estimated to be in the range of 10^{-5} nM. We concluded that the trace amounts of free SU present in virus stocks did not account for the observed results.

DISCUSSION

The entry into cells of retroviruses particles coated with the envelope glycoprotein of the 4070A amphotropic MuLV is mediated by an interaction with cell surface receptors encoded by the *ram-1* gene (29, 44). Experiments performed with truncated amphotropic envelope glycoproteins have shown that a 208-amino-acid amino-terminal fragment (here referred to as AS208) interferes with virus entry as efficiently as the wild-type envelope glycoprotein, indicating that this protein contains all of the determinants required for efficient interaction with the amphotropic receptor (3). We show here that highly purified AS208 specifically binds to cells susceptible to amphotropic virus infection. AS208 competed with the binding of retrovirus particles coated with the amphotropic envelope glycoproteins and interfered with the entry of these particles into cells. These data indicated that AS208 binds the amphotropic receptor.

Investigations were performed with three cell lines, i.e., mouse NIH 3T3, wild mouse (*M. dunni*), and human TE671 cells. These cells express receptors for various MuLVs, and each cell line is susceptible to amphotropic virus infection. Although there were differences in the susceptibilities to infection with an amphotropic pseudotype of a *E. coli lacZ* retrovirus vector (see Materials and Methods), in the amounts of virus particles required to saturate binding sites, in the numbers of AS208-binding sites, and in the concentrations of AS208 required to inhibit virus particle binding or virus infection, the differences between the three cell lines examined were not much larger than twofold. It is nevertheless noticeable that no strict correlation between these various parameters could be established. For example, TE671 cells, which appeared to be less susceptible to infection than NIH 3T3 cells, bound almost twice as many AS208 molecules at the cell surface but a lower number of virus particles, and they required a lower AS208 concentration to inhibit virus binding and infection. It is not yet clear whether these apparent discrepancies

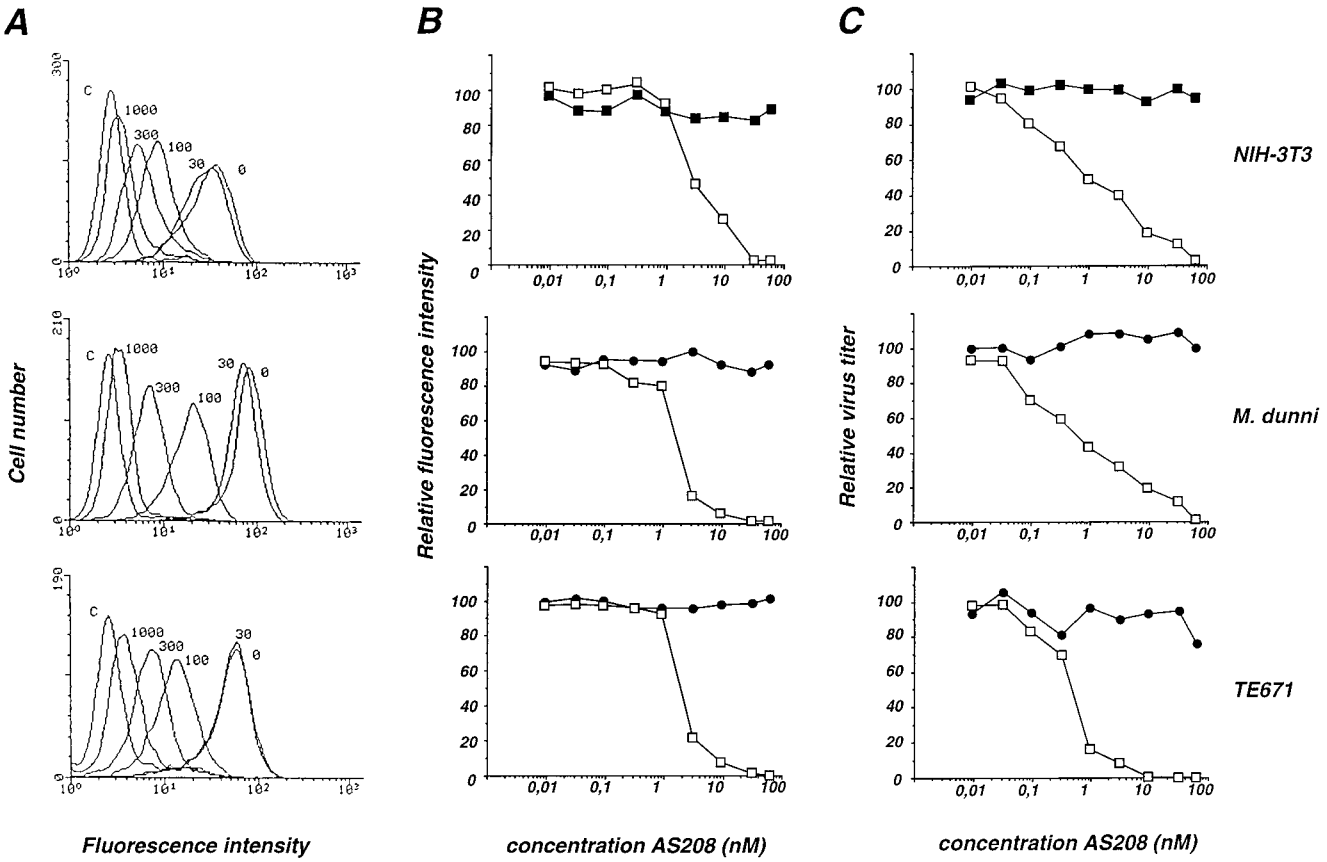

FIG. 4. Competitive inhibition of amphotropic virus pseudotype binding and entry by AS208. Target cells (2×10^5) were exposed to 300 FFU of a Moloney MuLV-derived retroviral vector containing the *E. coli lacZ* gene in the presence of increasing concentrations of the AS208 competitor. Cell surface binding (A and B) and cell infection (C) were quantified. Incubation was performed for 1 h at 37°C in 1 ml of DMEM with 10% FCS or NCS and 8 μg of Polybrene. Open squares, amphotropic virus; closed squares, control ecotropic pseudotype; circles, control xenotropic pseudotype. Virus dilutions were calculated according to the infectious titer measured with the various cell lines (see Materials and Methods). (A and B) Cells were detached with PBS–1 mM EDTA, exposed to virus particles, and labeled with the rat 83A25 anti-SU monoclonal antibody, and the surface levels of 83A25-amphotropic virus complexes were measured by flow cytometry after staining with a phycoerythrin-conjugated goat anti-rat immunoglobulin G. (A) Concentrations of AS208 are indicated above the curves in nanomolar. C, background staining with the phycoerythrin-conjugated anti-rat immunoglobulin G. (B) Same data as in panel A expressed as the percentage of the fluorescence intensity measured in the absence of AS208. The values are the means from triplicate experiments. Standard deviations never exceeded 8% of the indicated values. (C) Cell monolayers were grown for 2 days to reach confluency and stained with X-Gal. Data are expressed as the percentage of β-galactosidase-positive foci scored in the absence of AS208. Values are the means from triplicate experiments. Standard deviations did not exceed 8% of the indicated values.

resulted from experimental errors or from different receptor behaviors depending on the cell type.

There are several lines of evidence which suggest that, in living cells, the interaction between amphotropic envelopes and cell surface receptors, which mediates virus entry, is more complex than a bimolecular, two-order, reversible reaction. This hypothesis is supported by AS208-binding studies with NIH 3T3 cells. Scatchard plots of the AS208 interaction with binding sites were curvilinear with a downward concavity. This suggests that binding sites are heterogeneous with several classes of order differing in binding affinity. It is consistent with the hypothesis of a positive cooperativity between receptors. A second argument for receptor heterogeneity comes from competition experiments. The concentrations of AS208 required to inhibit the binding or the entry of amphotropic virus particles into cells were determined. A reduction of roughly 50% in the number of cell-bound virus particles required 1.8 to 3 nM AS208, whereas a reduction of roughly 50% in cell infection was observed with 0.5 to 1 nM AS208. Therefore, the inhibition of cell infection appeared with lower AS208 concentrations than the inhibition of receptor binding. For example, in the presence of 0.6 nM AS208, infection of TE671 cells was reduced fivefold, whereas particle binding was not affected, indicating that the number of available binding sites was sufficient to bind significant amounts of virus particles, whereas the number of functional receptors was drastically reduced. This finding suggests that only a fraction of the envelope-binding sites participated in the internalization of virus particles and actually functioned as virus receptors. Virus-binding kinetics studies were also consistent with the hypothesis of receptor heterogeneity. Whereas AS208 binding progressively increased over time until a plateau was reached, virus particle binding showed a peak followed by an early decrease in the number of attached particles and then a plateau. Since this experiment was performed with a large excess of unbound virus, one possible interpretation is that a fraction of the receptors may have internalized bound particles rapidly whereas others did not. We obtained additional data supporting the hypothesis of receptor heterogeneity by studying CHO cell clones transduced with various copy numbers of the *ram-1* cDNA (38a). In these cells, susceptibility to infection with an amphotropic *lacZ* retrovirus pseudotype appeared to be independent of the number of AS208-binding sites. Similar observations have previously been reported by Wang et al. for cells transduced with *rec-1*

(47). They suggest the existence of an accessory factor or process which is limiting for virus entry into cell.

It is conceivable that only a fraction of the amphotropic envelope-binding sites present at the cell surface are located in a suitable subcellular environment allowing efficient processing of the postbinding events required for infection. This would result in a heterogeneity regarding the function of Ram-1 proteins as retrovirus receptors. Efficient binding and internalization of retrovirus particles may also require cooperation between several Ram-1 molecules, which may not be necessary for the natural function of these molecules as phosphate transporters. Data on the stochiometry of envelope-receptor interactions are required to elucidate these points. Binding and penetration are dynamic processes which seem to require very specific features of both the envelope glycoprotein and the cell surface receptor. Refolding of cell-bound envelope molecules presumably plays a crucial role in the unmasking of epitopes involved in the fusion process. The motility of receptor molecules and/or their capacity to aggregate may be relevant to this process. Such features may be difficult to maintain in modified envelope molecules. On the other hand, it is presumable that only certain types of cell surface molecules have the capacity to manage retrovirus entry into cells. This would account for the usual low efficiency of infection observed after retargeting the binding of retrovirus envelope glycoproteins to cell surface molecules which have not been naturally selected as retrovirus receptors (9, 13, 21, 24, 39, 41–43).

The expression of the *rec-1* gene has been shown to vary significantly depending on the differentiation and proliferation states of hepatocytes (8, 49) and lymphocytes (45, 50). It is likely that cell differentiation, and presumably cell proliferation, also influences the expression of the amphotropic receptor, as suggested by the absence of detectable receptors on mouse fetal liver cells whereas adult hematopoietic tissues express significant levels of the molecule (37). Study of AS208 binding will be useful to investigate the expression of amphotropic envelope-binding sites on potential target cells for gene therapy, as, for example, in purified subpopulations of hematopoietic progenitors. As reported for Glvr-1 (19), Ram-1 appeared to be polymorphic between mammalian species, with only certain alleles coding for functional virus receptors. The molecular basis of receptor function could be conveniently studied by using AS208 binding for a rapid screening of functional and nonfunctional mutant or chimeric molecules.

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