Properties of the Naturally Occurring Soluble Surface Glycoprotein of Ecotropic Murine Leukemia Virus: Binding Specificity and Possible Conformational Change after Binding to Receptor

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Ecotropic murine leukemia virus (MuLV) infection is initiated by the interaction between the surface glycoprotein (SU) of the virus and its cell-surface receptor mCAT-1. We investigated the SU-receptor interaction by using a naturally occurring soluble SU which was encoded by the *envelope* (*env*) gene of a defective endogenous MuLV, *Fv-4'*. Binding of the SU to mCAT-1-positive mouse cells was completed by 1 min at 37°C. The SU could not bind to mouse cells that were persistently infected by ecotropic MuLVs (but not amphotropic or dualtropic MuLVs) or transfected with wild-type ecotropic *env* genes or a mutant *env* gene which can express only precursor Env protein that is restricted to retention in the endoplasmic reticulum. These cells were also resistant to superinfection by ecotropic MuLVs. Thus, superinfection resistance correlated with the lack of SU-binding capacity. After binding to the cells, the SU appeared to undergo some conformational changes within 1 min in a temperature-dependent manner. This was suggested by the different properties of two monoclonal antibodies (MAbs) reactive with the same C-terminal half of the *Fv-4'* SU domain, including a proline-rich motif which was shown to be important for conformation of the SU and interaction between the SU and the transmembrane protein. One MAb reacting with the soluble SU bound to cells was dissociated by a temperature shift from 4 to 37°C. Such dissociation was not observed in cells synthesizing the SU or when another MAb was used, indicating that the dissociation was not due to a temperature-dependent release of the MAb but to possible conformational changes in the SU.

At the initial step of virus infection, a virion binds to a specific cell-surface receptor. The receptor binding protein of a virion is the envelope glycoprotein of enveloped viruses or the capsid protein of nonenveloped viruses. After binding, the virion penetrates the cell by crossing the plasma membrane either by fusion between the viral and cellular membranes in enveloped viruses or by lysis or permeabilization of the cellular membrane in nonenveloped viruses. There are two alternative pathways of virus entry: entry occurs either at the outer cell surface membrane or at the endosomal membrane after endocytosis of a virion. These pathways are often referred to as the pH-independent and pH-dependent entries. For viruses using a pH-dependent pathway, the acidification of the endosome is essential to the penetration step. The decreased pH induces conformational changes in the envelope or capsid proteins of a virus to create its active form for penetration (24, 37).

Retroviruses have two envelope proteins, a surface glycoprotein (SU) and a transmembrane protein (TM), which are involved in receptor binding and fusion between a virus and a cell. The TM is anchored in the lipid bilayer of the virion. The complex of the SU and TM is held together by disulfide bonds and noncovalent interactions. Most retroviruses use a pH-in-

dependent entry pathway, with the exception of ecotropic murine leukemia viruses (MuLVs) (47) and possibly mouse mammary tumor virus (55). SU glycoprotein 70 (gp70) of ecotropic MuLV binds to the receptor mCAT-1, which was originally described as EcoR. mCAT-1 has 14 potential membrane-spanning domains (3) and functions physiologically as a cationic amino acid transporter (33, 65). The attachment and fusion steps of retroviruses have been extensively characterized in human immunodeficiency virus-1 (HIV-1) and simian immunodeficiency virus (SIV). The binding of soluble receptor CD4 to HIV-1 and SIV virions induces conformational changes in both SU and TM (57), leading to enhancement or inhibition of infection (4, 59) and to a high-affinity binding of the CD4-SU-TM complex to coreceptors (63, 64, 70). Recent reports describing the crystal structure of a protein complex containing fragments of CD4, SU, and a neutralizing antibody against a CD4-induced epitope confirmed the previously proposed interaction among these molecules (38). Influenza virus of *Orthomyxoviridae* has also been extensively studied at the early steps of infection and often serves as a model of a pH-dependent entry pathway. The envelope proteins of the influenza virus consist of the receptor binding protein HA1 and the fusion protein HA2, which are functionally equivalent to the SU and TM of retroviruses (24). The HA2 and TM fusion proteins show a striking structural similarity (12, 67). Decreased pH induces a conformational change in HA2 in the absence of HA1 (10, 11). Thus, the activation of fusion pro-

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teins is suspected to be triggered by the acidic environment for influenza virus and by the binding of SU to the receptor for HIV-1 (9, 67).

This paper presents evidence indicating a possible conformational change in SU of an endogenous ecotropic MuLV gene, termed *Fv-4'*, upon binding to the mCAT-1 receptor. A conformational change has been documented in the SUs of HIV-1, SIV (57, 58, 63, 70), and subgroup A avian leukosis and sarcoma virus (19, 25), all of which use a pH-independent entry pathway. However, this change has not been observed in HA1 of influenza virus or the SU of ecotropic MuLV, both of which penetrate via a pH-dependent pathway. Our results probably indicate that even in viruses using a pH-dependent entry pathway, a conformational change in a receptor binding protein may be important to the subsequent steps, such as movement to the endosomal compartment and activation of the fusion protein.

MATERIALS AND METHODS

Mice and cells. BALB/cAJcl mice were purchased from Clea Japan, Inc. C4W (BALB/c-*Fv-4'* w) is a partial congenic mouse strain carrying the *Fv-4'* gene on a BALB/c genetic background (51).

Cells of *Mus musculus* origin were NIH 3T3 cells, C182 cells which were persistently infected with a defective Moloney sarcoma virus but not helper MuLV (6), P19 embryonal carcinoma cells (30), and SC-1 cells which were derived from a feral mouse (21). L929 cells (ATCC CCL-1), 8313 cells (T-cell line) (2), and LE750 cells (B-cell line) (1) were derived from the laboratory mouse strain C3H/He (*M. musculus*). CC81 cells were derived from cat (18), XC cells were derived from rat (56), Mink cells were derived from mink (23), and SIRC cells were derived from rabbit (53). M. dunni cells (39) are of *Mus dunni* origin and are the gift of S. K. Chattopadhyay. AmpliGPE cells are an NIH 3T3-derived packaging cell line producing Moloney MuLV proteins (61) and are the gift of Y. Takahara. FL21 cells are NIH 3T3 cells transfected with a DNA construct combining the *Fv-4'* MuLV region and its putative promoter region (43).

SIRC-NIH EcoR cells were established by transfection of SIRC cells with pcDNA-NIH EcoR (see below) by the electroporation method (Gene Pulser; Bio-Rad), and transformants were selected in a medium containing 1 mg of Geneticin (Sigma) per ml. A few SIRC-NIH EcoR transformant cell lines analyzed in this study were independently derived from single primary colonies. NIH 3T3-HmFCR cells were NIH 3T3 cells transfected with a plasmid, pHmFCR (46), with Lipofectamine reagent (Gibco BRL) and were selected with 100 µg of hygromycin-B (Wako Pure Chemical Industries, Ltd.) per ml. We confirmed that the NIH 3T3-HmFCR cell line we established produced precursor gp85env but not its processed gp70env and was resistant to ecotropic MuLV infection, as previously indicated (46).

Viruses and plasmids. Friend MuLV 57, AKVL1 (21), Moloney MuLV, AKR13 (13), and amphotropic MuLV (22) were provided by A. Ishimoto, Kyoto University.

The plasmid pJET which contains NIH 3T3-derived ecotropic MuLV receptor cDNA (3) was kindly provided by J. M. Cunningham. An EcoRI fragment (about 2.3 kb) of the cDNA was inserted into the EcoRI site of pcDNA3.1, a mammalian expression vector in which a cytomegalovirus promoter drives an inserted gene (Invitrogen). The vector was termed pcDNA-NIH EcoR (62).

For the epitope mapping of monoclonal antibodies (MAbs), we used MuLV DNAs carrying the chimeric *env* genes of *Fv-4'* MuLV and Moloney MuLV in the backbone of full-length Moloney MuLV (45). To express the chimeric Envs of these constructed MuLVs, we made a series of constructs in which approximately 6-kb *HindIII*-*EcoRI* fragments from each chimeric MuLV DNA, including a splicing acceptor site, *env* gene, long terminal repeat (LTR), and 3' cellular flanking sequences (5) (see Fig. 12), were placed downstream from a 2.3-kb fragment containing the putative nonviral promoter and the splicing donor site of the *Fv-4'* gene (43), termed FL in this paper. The FL region was previously used to make *Fv-4'* transgenic mice (43).

Antibodies. A hybridoma cell producing MAb282 was derived from a fusion of mouse myeloma cell line P3X63Ag8U.1 (ATCC CRL-1597) and spleen cells from a BALB/c mouse immunized with C4W spleen and thymus cells. The hybridoma was selected based on the reactivity of the secreted antibody with NIH 3T3 cells (as a negative control) and FL21 cells (*Fv-4'* *env*-transfected NIH 3T3 cells), both of which were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde at 4°C for 15 min. The screening was performed using an enzyme-linked immunosorbent assay system (H. Kitani, data not shown). Biotinylated MAb282 (immunoglobulin G1 [IgG1]) was kindly prepared according to the method (8) of Japan Immunoresearch Laboratories Co., Ltd. MAb4D2 is reactive with *Fv-4'* MuLV SU (32, 45) and was a kind gift from Hidetoshi Sato, Sapporo City General Hospital, Sapporo, Japan. Polyclonal BALB/c anti-C4W alloantiserum was produced by immunizing BALB/c mice

with C4W spleen and thymus cells (27). Goat anti-Rauscher MuLV gp70 was provided by the Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md.

Flow cytometric analysis. To measure the binding of soluble SU to cells, we performed a membrane immunofluorescence (IF) assay with a flow cytometer (Epics Profile II; Coulter). Various monolayer cell lines were trypsinized to make a single-cell suspension, and, in general, 1×10^5 to 10×10^5 cells were incubated in 500 µl of Dulbecco's modified Eagle medium (DMEM) containing various concentrations of C4W serum for 30 to 60 min at 7°C with rotation. After washing, cell-bound SU was detected by staining the cells with 2 µg of biotinylated MAb282 or biotinylated MAb4D2 for 30 min and then with 0.5 µg of streptavidin-coupled phycoerythrin (Streptavidin-PE; PharMingen) for 30 min. After further washing the cells were fixed in PBS containing 1% paraformaldehyde prior to the flow cytometric analysis. The staining and washing steps were done on ice and in a cold centrifuge with cold DMEM containing 1% fetal calf serum, 0.05% sodium azide, and 0.1 µg of kanamycin (Meiji Seika, Ltd.) per ml.

To examine the effect of lysosomal enzyme inhibitor on the loss of MAb282 fluorescence, NIH 3T3 cells were cultured for 1 h in DMEM containing 20 µM chloroquine (Nacalai Tesque, Inc., Tokyo, Japan) (41a). The chloroquine-treated cells were analyzed as untreated cells, except that the incubations with C4W serum, biotin-labeled MAbs, and Streptavidin-PE and the washes were done in medium containing 20 µM chloroquine.

Immunoprecipitation and Western blotting. SUs in serum were immunoprecipitated and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sera from BALB/cAJcl and C4W mice were treated three times with protein G-Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden) to remove Igs. MAb282 and anti-C4W alloantiserum (27) were bound to protein G-Sepharose. The precleared sera were incubated with MAb282-bound or anti-C4W-bound protein G-Sepharose for 1 h at 7°C with rotation. The Sepharose beads were extensively washed with digitonin buffer (1% digitonin, 10 mM triethanolamine, 150 mM NaCl, 10 mM iodoacetamide, 1 mM EDTA, and 10 µg of aprotinin [pH 7.8] per ml) and boiled for 7 min in protein sample buffer (187.5 mM Tris, 6% SDS, 30% glycerol, 125 mM dithiothreitol, and 0.03% phenol red). The immune precipitates were fractionated by SDS-PAGE on a 7.5 or 10% polyacrylamide gel. Proteins were transferred to an Immobilon polyvinylidene difluoride transfer membrane (Millipore, Bedford, Mass.). The membrane was probed with goat anti-gp70 serum for 1.5 h at room temperature and then treated with horseradish peroxidase (HRP)-conjugated anti-goat Ig (Amersham, Little Chalfont, England) for 1 h at room temperature. An HRP-mediated chemiluminescent reaction was performed with ECL Western blotting detection reagents (Amersham, Little Chalfont, England).

To detect MAbs in NIH 3T3 cells which absorbed SU, biotin-MAb, and Streptavidin-PE, the cells were lysed in lysis buffer (10 mM Tris, 1% NP-40, 0.1% sodium deoxycholate, 18% SDS, 0.15 M NaCl, 1 mM EDTA, and 4 U of aprotinin per ml). One volume of the cell lysate was mixed with a one-half volume of protein-loading buffer (187.5 mM Tris, 6% SDS, 30% glycerol, 125 mM dithiothreitol, and 0.03% phenol red) and boiled for 10 min. The final concentration of SDS at boiling was 14%, which seemed essential to dissociate the biotin-avidin complex (E. Hunter, personal communication). Blotted membrane was probed with avidin-HRP (Amersham, Arlington Heights, Ill.).

RESULTS

Soluble SU protein in *Fv-4'* mouse serum. Soluble SU is encoded by the truncated endogenous MuLV *Fv-4'* gene and has been detected in sera from *Fv-4'* transgenic mice (49), C4W mice (36) (a partial congenic mouse strain carrying the *Fv-4'* gene [51]), as well as in culture supernatants of C4W cells, *Fv-4'* *env*-transfected cells, and *Fv-4'* transgenic mouse cells (49). Soluble *Fv-4'* SU of C4W serum was used in this study. To quantitatively measure the SU bound to the cells, flow cytometric analysis with MAb282 reactive with the *Fv-4'* SU was utilized. The SU was allowed to bind to cells at 4 to 10°C for 1 h. The cells were then treated with biotinylated MAb282 followed by Streptavidin-PE. The mean fluorescence intensity was proportional to the concentration of C4W serum (Fig. 1A). With this assay system, C4W sera from individual mice were analyzed to determine the variation in the amount of the SU protein. The soluble SU bound to NIH 3T3 cells was detected at a constant level in sera from C4W mice but not from BALB/cAJcl mice of both sexes, aged 3 weeks to 18 months (Fig. 1B). For further analysis, several pooled C4W sera were used, and no obvious difference in titers or binding properties was found among them, even when they were stored at 4°C for a few months.

FIG. 1. Quantitative assay for binding the serum *Fv-4'* MuLV SU to NIH 3T3 cells. (A) NIH 3T3 cells were incubated at 7°C for 1 h in medium containing the indicated concentrations of a pooled serum of C4W mice, a partial congenic mouse strain carrying the *Fv-4'* gene on a BALB/c background. The cells were sequentially treated with biotin-labeled MAb282 reactive with *Fv-4'* SU and Streptavidin-PE. After each treatment, the cells were washed three times to remove unbound SU, antibodies, or Streptavidin-PE. The samples were kept cool (about 7°C) throughout the experiment. The cells were fixed in PBS containing 1% paraformaldehyde prior to flow cytometric analysis. The fluorescent intensity of each sample was presented as the mean immunofluorescence (IF) intensity. (B) Variation in the soluble *Fv-4'* SU concentration in the serum of an individual C4W mouse. NIH 3T3 cells were incubated in medium containing 2% serum from individual BALB/cA/Jcl and C4W mice of different ages.

Binding specificity of the soluble SU to various cells. There are several lines of evidence which suggest that the *Fv-4'* SU belongs to the ecotropic MuLV group: (i) the interference pattern of the *Fv-4'* Env-expressing cells (28), (ii) the high nucleotide sequence homology to other ecotropic MuLV *env* genes (45), and (iii) the virological properties of an ecotropic MuLV strain Cas-Br-M (21), whose *env* region is highly homologous to the *Fv-4'* *env* gene (45, 52). Thus, we anticipated that the soluble *Fv-4'* SU would bind to cells susceptible to ecotropic MuLVs.

The crucial role of the mCAT-1 receptor molecule in the binding of soluble SU to the cell surface was revealed with SIRC-NIH EcoR cells, which are SIRC cells transfected with the mCAT-1 gene. Rabbit SIRC cells were resistant to infection by ecotropic MuLVs, while SIRC-NIH EcoR cells were susceptible (62), as previously reported with human cells (3, 66). SIRC-NIH EcoR (clone 10) cells were also susceptible to SU binding (Fig. 2 and 3). Four other SIRC-NIH EcoR cell clones were analyzed. They showed identical susceptibility to ecotropic MuLV infection but variable SU-binding capacities; one exhibited a high absorbing capacity similar to clone 10 (Fig. 2 and 3), two absorbed slightly or heterogeneously, and one was completely resistant to SU binding (data not shown). Wang et al. (66) reported that the susceptibility of various EcoR-transfected human cells to ecotropic MuLV infection was unrelated to the amount of cell surface EcoR which was measured by the binding of soluble Moloney MuLV SU. Thus, in certain cells, quantitative differences in SU binding may not be directly correlated to the susceptibility of these cells to ecotropic MuLV infection.

Fv-4' SU did not significantly bind to cells of species other than *M. musculus*, i.e., cat CC81 cells, rat XC cells, mink cells, rabbit SIRC cells, and *M. dunni* cells (Fig. 3). The lack of absorbing capacity of XC cells and *M. dunni* cells was unexpected because they are susceptible to infection by ecotropic MuLVs. XC cells form syncytium when infected with ecotropic MuLVs (47, 56) or bound by purified soluble SU of ecotropic Moloney MuLV (69). *M. dunni* cells were susceptible to many, but not all, strains of ecotropic MuLVs (39). However, the susceptibility of these cells to the ecotropic Moloney MuLV

FIG. 2. mCAT-1 specific binding of the SU protein. NIH 3T3, SIRC, and SIRC-NIH EcoR cells were incubated at 7°C for 1 h in medium without (A) or with (B) 10% pooled serum of C4W mice. Rabbit SIRC-NIH EcoR (clone 10) cells are SIRC cells transfected with the expression vector pcDNA-NIH EcoR containing the ecotropic MuLV receptor gene (encoding mCAT-1) derived from NIH 3T3 cells (3).

FIG. 3. Binding specificity of SU for various cell lines. The indicated cell lines were incubated at 7°C for 1 h in medium with (■) or without (□) 10% pooled serum of C4W mice. SIRC-NIH EcoR cells are SIRC cells transfected with the ecotropic MuLV receptor gene. AmpliGPE is an NIH 3T3-derived packaging cell line (61) which expresses ecotropic Moloney MuLV proteins, including envelope glycoprotein. NIH 3T3-HmFCR cells are NIH 3T3 cells transfected with the expression vector pHmFCR containing a mutant *env* gene of ecotropic Friend MuLV (46). SC-1/Friend, SC-1/Moloney, and SC-1/AKV are SC-1 cells persistently infected with ecotropic Friend, Moloney, and AKV MuLV strains, respectively. SC-1/AKV13 and SC-1/Ampho are SC-1 cells persistently infected with dualtropic MuLV strain AKV-13 and amphotropic MuLV strain 4070A.

strain is controversial (48). Soluble SUs derived from ecotropic Moloney and Rauscher MuLVs did not bind to *M. dunni* cells. In addition, transfection of the mCAT-1 receptor homologue cDNA isolated from *M. dunni* cells into receptor-negative cells did not confer SU-binding capacity (17).

C-182 cells, which were infected by *env*-deficient mouse sarcoma virus but not MuLV (6), embryonal carcinoma P19 cells, NIH 3T3 cells, and SC-1 cells were susceptible to SU binding (Fig. 3). In contrast, 8313, L929, and LE750 cells of C3H mouse origin were resistant to SU binding. Reasons for the resistance are not known. One possibility is that since C3H mice carry one endogenous ecotropic MuLV gene termed the *Env-1* gene, which can express infectious viruses or Env proteins in aged mice and cultured cells, the ecotropic Env protein expressed by the endogenous virus could interfere with binding.

In contrast to NIH 3T3 cells, NIH 3T3 cells transfected with *env* genes of ecotropic MuLVs such as AmpliGPE and NIH 3T3-HmFCR were resistant to both SU binding (Fig. 3) and infection by ecotropic MuLVs (data not shown). AmpliGPE is a packaging cell line (61) expressing all the viral proteins of Moloney MuLV, including SU, on the cell surface. The FCR gene is a variant of the Friend MuLV *env* gene, which expresses only the precursor gp85 protein but not its processed proteins SU (gp70) and TM (p15E) (46). The gp85 protein was retained in the cytoplasm and could not be expressed on the cell surface. NIH 3T3-HmFCR cells were resistant to ecotropic MuLV infection, so it was speculated that the gp85 might trap the mCAT-1 receptor in the cytoplasm. Therefore, the absence of the mCAT-1 receptor on the cell surface may be responsible for the resistance to exogenous virus infection (46). Our data is consistent with this speculation.

When SC-1 cells were persistently infected by ecotropic MuLVs (Friend, Moloney, and AKV), they became resistant to superinfection by ecotropic MuLVs. These cells were also resistant to SU binding (Fig. 3). SC-1 cells are susceptible to dualtropic and amphotropic MuLVs, which use different re-

ceptors. As expected, SC-1 cells infected with dualtropic MuLV (AKR13) or amphotropic MuLV (4070A) were susceptible to SU binding, as were the parental SC-1 cells (Fig. 3).

These results indicated that the binding specificity of the soluble *Fv-4'* SU appeared to roughly correlate with the infection specificity of ecotropic MuLVs and supported the general anticipation that superinfection resistance may be restriction at the binding step of the virion's envelope glycoprotein to the cell surface. However, we observed several cell lines that were expected to be susceptible to ecotropic MuLVs but actually did not absorb the soluble SU. This discrepancy could be due in part to the amount of the mCAT-1 receptor molecules, as in the cases of some SIRC-NIH EcoR transformants, the possible expression of endogenous Env protein, or the properties of the soluble SU used in this study.

Kinetics of binding of soluble SU. We examined the binding efficiency of the serum SU at various temperatures with NIH 3T3 cells. Within 5 min, the binding efficiency was dependent on temperatures ranging from 0 (kept in ice-cold water) to 37°C. At 30 min, the total amounts of SU bound to cells were identical at temperatures greater than 9°C. At 37°C, the binding had almost reached a plateau by 1 min (Fig. 4).

Temperature-dependent release of MAb282 reacting with the membrane-bound SU. We further followed the soluble SU after binding to NIH 3T3 cells. The NIH 3T3 cells were incubated for 1 h in medium containing 5% C4W serum and treated with either biotin-labeled MAb282 or biotin-labeled MAb4D2 and then Streptavidin-PE. All the steps were carried out with the samples kept cool. The cells were then incubated at either 4 or 37°C. At various intervals, aliquots of the cell suspension were immediately fixed with paraformaldehyde. The two MAbs gave different results (Fig. 5). In the MAb282-treated cells, the mean fluorescence intensities remained at the initial intensity when the cells were kept at 4°C, while the temperature shift to 37°C caused a rapid decrease in intensity. By 1 min after the shift to 37°C, intensity decreased to a point 0.41 log unit lower than the initial intensity, which was 1.43 log

FIG. 4. Effect of temperature on binding of SU to NIH 3T3 cells. Ten microliters of a 4×10^5 NIH 3T3 cell suspension was mixed with 750 μ l of medium containing 2% C4W serum that had been preheated at the temperatures indicated. One, 5, or 30 min after mixing and incubation, aliquots of the cell suspension (250 μ l) were quickly washed to remove unbound SU. Membrane-bound SU was detected by incubating the cells with biotin-labeled MAb282 followed by Streptavidin-PE. NIH 3T3 cells untreated with C4W serum were also examined.

unit higher than the background level, and then continued to decrease slowly during the 60-min observation. In contrast, the MAb4D2-treated cells did not change intensities at 37°C as drastically as did the MAb282-treated cells.

Some of the histograms of the flow cytometric analysis presented in Fig. 5 are shown in Fig. 6. In the MAb282-treated cells, the entire cell population lost fluorescence 1 min after the temperature shift to 37°C, resulting in a decreased mean intensity (Fig. 5). In the MAb4D2-treated cells, only a small fraction of the cell population lost fluorescence 10 min after the temperature shift. In the experiment shown in Fig. 6, the decreased intensity appeared to accompany an apparent heterogeneity of the fluorescence intensities among the cell population. However, such heterogeneity was not consistently observed; in identical experiments, the histogram showed a homogeneous loss of fluorescence (data not shown).

The decreased intensity of the MAb282 fluorescence was

observed under various temperature conditions (Fig. 7). At 0 and 9°C, the intensities were not altered. The most apparent decrease was seen at 37°C, while intermediate changes were detected at 18 and 27°C. The loss of MAb282 fluorescence could be detected within 5 min at temperatures above 18°C. These results suggested that once the soluble SU bound to cells, it was retained in a temperature-independent manner and that, of the two MAbs reactive with the same SU, only MAb282 fluorescence rapidly disappeared from cells in a temperature-dependent manner.

The decrease of fluorescence was detected in SU-bound cells pretreated with biotin-labeled MAb282 and Streptavidin-PE before the temperature shift. However, when the treatment with MAb282 was performed after the temperature shift, the total amount of SU on the cell surface did not decrease as much as on the pretreated cells during the 60-min observation (data not shown). Thus, the majority of the SU retained on the cell surface at 37°C and during the temperature shift did not induce a loss of the MAb282 and MAb4D2 epitopes. The pretreatment of biotin-labeled MAb282 and Streptavidin-PE before the temperature shift may be important for the drastic decrease in fluorescence.

To determine whether the loss of MAb282 fluorescence resulted from dissociation or degradation of the antibody or from other reasons such as quenching of the fluorescence in endosome, NIH 3T3 cells were incubated in medium containing chloroquine, a lysosomal enzyme inhibitor (41a). The inhibitor treatment did not affect the decrease or retention of fluorescence, as demonstrated with normal NIH 3T3 cells (Fig. 8). The cell-bound biotinylated MAbs were detected in the cell lysates by Western blot analysis using biotin-HRP. Cell-bound MAb282 rapidly disappeared by the temperature shift, while cell-bound MAb4D2 was retained (Fig. 8). Based on the intensities of heavy-chain Ig, 78 and 83% of the cell-bound MAb282 were lost at 1 and 5 min. An identical result was obtained with normal NIH 3T3 cells (data not shown). Thus, chloroquine did not block the loss of MAb282 and fluorescence.

Lack of MAb282 dissociation from Fv-4' SU-expressing cells. The loss of fluorescence in the MAb282-treated cells at 37°C could be accounted for either by a temperature-dependent conformational change of the soluble SU when it binds to

FIG. 5. Dissociation of fluorescence from the cell-SU-MAb282-biotin-Streptavidin-PE complex. NIH 3T3 cells were incubated for 1 h at 7°C with 2% C4W serum and then with biotinylated MAb282 (A) or biotinylated MAb4D2 (B) and Streptavidin-PE. The cell suspensions were further incubated at 4 or 37°C in medium containing 1% fetal calf serum and 0.05% NaN_3 . After the time indicated, aliquots of the cell suspension were immediately mixed with a large volume of PBS containing 1% paraformaldehyde.

FIG. 6. Histograms of the flow cytometric analysis shown in Fig. 5. Histograms of immunofluorescence (IF) intensities are from the samples before (0 min) and after (1, 3, and 10 min) the shift to 37°C. The fluorescence intensity started to decrease in MAb282-treated cells 1 min after the shift to 37°C.

a cell, leading to the dissociation of the MAb from the cell-bound SU, or by the binding properties of MAb282. MAb282 may react with the cell-bound SU at 4°C but may dissociate from the antigen at 37°C. To test these possibilities, we analyzed the FL21 cell line, which was NIH 3T3 cells transfected with the *Fv-4'* gene. The FL21 cells expressed SU and TM on the cell surface and also released soluble SU into the culture supernatant (data not shown). In our preliminary experiments, brief treatment of the cells absorbing soluble SU with 0.25%

trypsin readily removed SU from the cell surface (data not shown). This was in contrast to our general observation that Env proteins (SU and TM) expressed on MuLV-infected cells or MuLV *env*-transfected cells were resistant to trypsin treatment. The FL21 cells were trypsinized to make the cell suspension and to remove the soluble SU from the cell surface and were then incubated with either MAb282 or MAb4D2. Both antibodies detected a large amount of trypsin-resistant *Fv-4'* SU. After a temperature shift to 37°C, no gross change in fluorescence intensity was observed in either of the MAb-treated cells (Fig. 9). These results indicated that MAb282 did not lose affinity for the SU at 37°C, and that the dissociation of MAb282 was only evident with the soluble SU which was bound to the cell membrane but not with the SU which was newly synthesized, trypsin resistant, and expressed on the cell surface, probably in association with TM.

Loss of MAb282 fluorescence via interaction between SU and mCAT-1 receptor. In the previous experiments, the loss of MAb282 fluorescence has been observed with mouse NIH 3T3 cells. To determine whether the mCAT-1 receptor or some other factors unique to NIH 3T3 cells are involved in the phenomenon, we carried out identical flow cytometric experiments using SIRC-NIH EcoR cells. Binding the soluble SU to SIRC-NIH EcoR cells was detected by both MAb282 and MAb4D2. In MAb282-treated cells, a temperature shift to 37°C induced a loss of fluorescence in two-thirds of the total cell population within 5 min (Fig. 10). Such a change was not observed in MAb4D2-treated cells. Although the loss of fluorescence was not as clear as that observed with NIH 3T3 cells, the majority of the cell population displayed it, as did NIH 3T3 cells. Thus, binding to the mCAT-1 receptor may be necessary for the potential conformational change in the SU, and other NIH 3T3 cell-specific factors are not likely to be essential for this event.

FIG. 7. Effect of temperature on the dissociation of fluorescence from the cell-SU-MAb282-biotin-Streptavidin-PE complex. NIH 3T3 cells treated with 10% C4W serum, biotinylated MAb282, and Streptavidin-PE were incubated at the temperatures indicated. Aliquots of the cell suspension were removed 5 and 30 min after the temperature shift and fixed in PBS containing 1% paraformaldehyde. The background indicates NIH 3T3 cells not treated with C4W serum but treated with biotinylated MAb282 and Streptavidin-PE. IF, immunofluorescence.

FIG. 8. Dissociation of MAb282 from chloroquine-treated, SU-bound NIH 3T3 cells. NIH 3T3 cells were cultured for 1 h in DMEM containing 20 μ M chloroquine. The cells were treated with C4W serum, biotinylated MAb282 or MAb4D2, and Streptavidin-PE in medium containing 20 μ M chloroquine. (A) The cell-bound immunofluorescence (IF) was measured 1 and 5 min after the temperature shift to 37°C. (B) The cell lysates were run on SDS-10% PAGE and blotted onto membrane. The membrane was probed with Streptavidin-HRP. MAb282 and MAb4D2 had the same size of Ig light-chain IgL but different sizes of Ig heavy-chain IgH.

Reactivity of MAb282 with the *Fv-4'* SUs in serum. MAb282 appeared to recognize some conformations of *Fv-4'* SU, because MAb282 did not work in our standard Western blot assay for cell lysates or in an immunoprecipitation assay for metabolically-labeled cell lysates but did work well in the membrane immunofluorescence assay for various live cells. In contrast, MAb4D2 can be used in all of these assays (32, 36, 49). We tested various experimental conditions to prove that MAb282 indeed reacts with the soluble *Fv-4'* SU present in serum and found that a digitonin buffer which has been commonly used to immunoprecipitate weakly interacting protein complexes could be used in the immunoprecipitation assay. C4W serum was mixed with MAb282 or anti-C4W alloantiserum coupled with protein G-Sepharose in digitonin buffer. The immunoprecipitates were washed with the buffer and subjected to SDS-PAGE and Western blot analysis. Both MAb282

and anti-C4W alloantiserum precipitated two proteins (approximately 75 and 80 kDa) that were reactive with goat anti-MuLV gp70 serum (Fig. 11). Two identical SUs in C4W serum have been previously detected with MAb4D2 (36). The *Fv-4'* SUs with various apparent molecular weights have been found in cells of various organs (27, 36). The origin of the SUs in serum was unknown, but both bound to NIH 3T3 cells (data not shown).

Epitope mapping of MAb282 and MAb4D2. We attempted to locate antigenic determinants recognized by MAb282 and MAb4D2 with chimeric MuLV DNAs. Masuda and Yoshikura (45) constructed a series of MuLV DNAs carrying the chimeric Envs of *Fv-4'* MuLV and Moloney MuLV in the backbone of full-length, infectious Moloney MuLV DNAs. Replacement of almost an entire *env* region (*AccI-EcoRV*; A-R region in Fig. 12) of Moloney MuLV with the corresponding region of *Fv-4'*

FIG. 9. Lack of dissociation of MAb282 from NIH 3T3 cells synthesizing *Fv-4'* env protein. FL21 cells are NIH 3T3 cells transfected with the *Fv-4'* gene that includes both the truncated MuLV region and its putative promoter region (43) and expresses the *Fv-4'* env protein on the cell surface. FL21 cells treated with (○ and ●) or without (□ and ■) biotin-labeled MAb282 (left) or biotin-labeled MAb4D2 (right) and then Streptavidin-PE were incubated for 5 or 30 min at 4°C (○ and □) or 37°C (● and ■). FL21 cells untreated with MABs (□ and ■) were saved as negative controls.

FIG. 10. Dissociation of MAb282 from soluble SU bound to SIRC-NIH EcoR cells. SIRC-NIH EcoR cells, which are SIRC cells transfected with the pcDNA-NIH EcoR expression vector containing the ecotropic MuLV receptor gene (3), were treated sequentially with 10% C4W serum, biotin-labeled MAb282 or MAb4D2, and Streptavidin-PE. The cells were then shifted to 4 or 37°C. After the time indicated, the cells were fixed with paraformaldehyde. IF, immunofluorescence.

did not produce an infectious virus. However, among the chimeric MuLVs tested, three (Mo-AB, Mo-BBa, and Mo-BaN) could produce infectious viruses, suggesting that the entire *env* region of *Fv-4'* is inactive for producing an infectious virus but that parts of the *env* region are competent (45).

We transfected the parental Moloney MuLV DNA and the three chimeric MuLV DNAs into NIH 3T3 cells and confirmed that all of them can produce XC-positive infectious viruses. SC-1 cells were infected with these viruses, and the Env proteins expressed on the cell surface were analyzed by flow cytometric assay. Both MAb282 and MAb4D2 were reactive with cells infected with the Mo-BBa virus and, as a positive control, cells transfected with the *Fv-4'* DNA (Fig. 13A). These MABs were not reactive with cells infected with Moloney, Mo-AB, or Mo-BaN viruses, although Western blot analysis showed that the three cells expressed as much Env protein as did the Mo-BBs infected cells (data not shown).

We further examined cells expressing the chimeric Env proteins under the control of a nonviral promoter. Because transfection of NIH 3T3 cells with the original chimeric MuLV DNAs did not induce sufficient Env proteins to be detected by our flow cytometric analysis, we made a series of constructs in which approximately 6-kb *HindIII-EcoRI* fragments from each chimeric MuLV DNA, including a splicing acceptor site, *env* gene, LTR, and 3' cellular flanking sequences (5), were placed downstream of a 2.3-kb fragment containing the putative non-

FIG. 11. Reactivity of MAb282 with the serum *Fv-4'* SUs. Sera from BALB/cAJcl (lanes 1 and 2) and C4W (lanes 3 and 4) mice were immunoprecipitated with MAb282 (lanes 1 and 3) or anti-C4W alloantiserum (lanes 2 and 4) in digitonin buffer. The immunoprecipitates were subjected to SDS-PAGE and Western blotting. The membrane was probed with goat anti-gp70 serum and HRP-conjugated anti-goat Ig. The HRP-mediated chemiluminescent reaction was performed with ECL reagents.

viral promoter and the splicing donor site of the *Fv-4'* gene (43).

MAb282 and MAb4D2 reacted with NIH 3T3 cells transfected with FL/Mo-BBa (Fig. 13B) as they did with cells infected with the Mo-BBa virus (Fig. 13A). Other cells transfected with the constructs containing the *Fv-4'* B-Ba region, such as FL/Mo-BR and FL/Mo-AR, were reactive with both MABs, while cells without the *Fv-4'* B-Ba region, such as FL/Mo-AB, FL/Mo-BaN, FL/Mo-BaR, and FL/Mo-AB/BaR, were not (Fig. 13B). Thus, we conclude that both MAb282 and MAb4D2 recognized the 201-amino-acid (aa) SU region encoded by the *Fv-4'* B-Ba, which includes a hypervariable proline-rich motif and a TM-interacting carboxyl-terminal region (Fig. 12).

DISCUSSION

We characterized the soluble SU of ecotropic MuLV at the step of binding to the cellular receptor and immediately after binding. Binding was temperature dependent within 5 min, and at 37°C, binding was completed within 1 min (Fig. 4). After binding, some temperature-dependent changes, probably in the cell-bound SU, were observed. This was suggested by the release of MAb282 fluorescence from the cell-bound SU at high temperatures (above 18°C) (Fig. 5 and 7). Another monoclonal antibody, MAb4D2, was stably retained in the cells under the same conditions. The release of MAb282 fluorescence was not due to a possible characteristic of MAb282 to lose affinity for the antigen at these temperatures (Fig. 9). The two MABs recognized the C-terminal half of *Fv-4'* SU, which is located downstream from the amino-terminal receptor binding regions of the SU domain and includes the hypervariable proline-rich region and the TM-interacting carboxyl-terminal region (Fig. 12). MAb282 appears to recognize conformational or discontinuous epitopes because, in contrast to MAb4D2, it did not react with the SU in a Western blot assay or an im-

FIG. 12. Schematic representation of chimeric MuLVs and reactivities of Mab282 and Mab4D2 with the chimeric Env proteins. The upper figure shows the basic structure of the 3' half of MuLV: Pol, polymerase gene; SU, surface domain of env gene; TM, transmembrane domain of env gene; I, II, and III, disulfide-bonded structural elements (44); Pro, hypervariable proline-rich region. Restriction enzyme sites used for the construction of chimeric MuLVs (45) are: H, HindIII; A, AccI; B, BamHI; Ba, BstI; N, NcoI; R, EcoRV. SA, splicing acceptor site. White and black boxes indicate regions derived from Moloney MuLV (5) and endogenous Fv-4' MuLV (26), respectively. The right part of the figure shows a summary of the reactivities of Mab282 and Mab4D2 with SC-1 cells infected with chimeric MuLVs (Fig. 13A) and with NIH 3T3 cells transfected with nonviral expression vectors containing chimeric env regions (Fig. 13B). NA, not assayed.

munoprecipitation assay with standard buffers containing NP-40, sodium deoxycholate, or SDS (data not shown) but did react in Western blot assay with the digitonin buffer (Fig. 11).

The loss of Mab282 fluorescence should result from some change in receptor-bound SU, biotin-labeled Mab282, or Streptavidin-PE. The loss of fluorescence was associated with the disappearance of cell-bound Mab282 (Fig. 8), so that certain changes (conformation or degradation) in the SU appear to be the most probable reason. Initially, we considered

various other possibilities for the decreased immunofluorescence intensity (Fig. 5), rather than the conformational change in the SU. For example, internalization of the SU-Mab282-biotin-Streptavidin-PE complex into endosome might lead to its degradation or dissociation by low pH or lysosomal proteases in a way similar to that generally believed to cause pH-induced dissociation of a receptor-ligand complex. The temperature shift from 4 to 37°C will also initiate internalization of the SU-antibody complex. In flow cytometric analysis,

FIG. 13. Reactivities of Mab282 and Mab4D2 with chimeric envelope proteins expressed on the cell surface. (A) SC-1 cells infected with the indicated chimeric MuLVs (45) (Fig. 12) were stained with biotinylated Mab282, biotinylated Mab4D2, or goat anti-MuLV gp70 and then incubated with Streptavidin-PE or fluorescein isothiocyanate-labeled anti-goat IgG. (B) NIH 3T3 cells were cotransfected with pSVHygro⁺ and DNA constructs consisting of a 2.3-kb FL fragment of an Fv-4' promoter region (43) and an approximately 6-kb fragment of each chimeric MuLV DNA spanning from the HindIII (H) site of the pol gene (Fig. 12) to an EcoRI site of 3' cellular flanking sequences (5) (not shown in Fig. 12). About 50 to 200 Hygro⁺ cell colonies were pooled and passed several times and then assayed by flow cytometric analysis. The transfected cells were stained with the antibodies as in panel A. IF, immunofluorescence.

even if fluorescence that has initially bound to the cell surface is internalized into the endosomal vesicles, the detectable intensity of the fluorescence should be almost the same as with the initial fluorescence value. Our experiment with cells treated with chloroquine, a lysosomal protease inhibitor, gave a result identical to that seen with normal cells (Fig. 8). This result, together with the rapidity of MAb282 dissociation (within 1 min) after the temperature shift, seems to favor the possibility of the conformational change in the receptor-bound SU, rather than the degradation of the protein complex.

Our experiments could not determine where the dissociation of MAb282 or the possible conformational change occurred. A recent study indicated that an SU antigen of ecotropic MuLV virions colocalizing with mCAT-1 receptor molecules began to appear inside cells 5 min after virus binding (41). Compared with these results, MAb282 dissociation was rapid. To test the possibility that a low-pH condition, as in endosome particles, induces the dissociation of MAb282 from the cell-bound SU, we briefly (60 s) treated cells, which bound SU, biotinylated MAb282, and Streptavidin-PE, with buffers of various pHs ranging from 5.5 to 8.0 at 4°C. No decrease in fluorescence was observed (data not shown), indicating that a low-pH environment is not the sole trigger of MAb282 dissociation.

The MAb282 release was detected in NIH 3T3 cells absorbing soluble SU (Fig. 5) but not in NIH 3T3 cells expressing SU (Fig. 9). In the former, the SU probably binds to the receptor, while in the latter, the SU binds to TM anchored to the cell membrane, which is considered to be equivalent to an MuLV virion. We speculate that, in the latter, the SU did not bind to the receptor during incubation at 37°C for 30 min, possibly because of a lack of the receptor expressed. Therefore, in a natural infection, the SU present on MuLV virion binds to the receptor and is expected to undergo such a conformational change.

Like MAb282 and MAb4D2, the polyclonal anti-C4W alloantiserum was also reactive with the C-terminal half of the SU domain encoded by the *Fv-4'* B-Ba region (data not shown), suggesting the existence of strong immunogenic epitopes in that region. These MAbs reacted with a limited number of MuLV Envs including those expressed by endogenous *Fv-4'* MuLV and a few ecotropic Cas E-type MuLVs that we isolated from wild mice but not Cas-Br-E or AKV (data not shown). Thus, the epitopes seem unique among MuLVs. The B-Ba region contains the hypervariable proline-rich motif and the relatively conserved C-terminal SU region. In the proline-rich region (274 to 319 aa), *Fv-4'* shows 70 to 71% homology with AKV and Cas-Br-E MuLVs and 34 to 49% homology with various eco-, dual-, and amphotropic MuLVs, while in the conserved C-terminal SU region (320 to 456 aa), the *Fv-4'* shows 78 to 91% homology with these MuLVs. Thus, the proline-rich motif seems the most probable candidate for the unique epitopes.

The C-terminal domain of SU binds to TM through covalent and noncovalent interaction (20, 54), and the proline-rich region is essential for the stability of the SU-TM heteropolymer (20). The relatively protease-resistant nature of the proline-rich region suggested an important role in the interaction between the domains of SU (44). Genetic modification in the proline-rich region of amphotropic MuLV altered the fusogenic phenotype of the virus and the stability of the SU protein (40). According to these studies, the phenomenon of the MAb282 dissociation from the *Fv-4'* B-Ba region also implies a structural change of the region immediately after receptor binding and may be related to an early event of virion attachment and fusion to the cell.

For HIV-1 SU, the conformational change seems to be a

trigger for the activation of TM under neutral conditions and for the exposure of V3 and other coreceptor binding domains of SU (38, 63, 64, 70). By contrast, influenza virus HA1, the equivalent to retroviral SU, appears to be unnecessary for the activation of fusion protein HA2 because the fusion-active form of HA2 could be generated at low pH without the presence of HA1 (10, 11). Because ecotropic MuLV and influenza virus use a pH-dependent entry pathway and retroviral TM and influenza virus HA2 have striking structural similarity, ecotropic MuLV can be expected to adopt an entry-and-fusion mechanism that is likely to be similar to that of influenza virus. Nonetheless, despite these similarities, it is not known how much these viruses share in their mechanism of entry.

In viruses using a pH-dependent entry, such as Semliki Forest virus, influenza virus, and vesicular stomatitis virus, brief treatment of virion attaching to susceptible cells with a mildly acidic medium enhanced the fusogenic activity of virus (68), while in ecotropic MuLV, no enhancing effect was observed (50), implying that low pH is not sufficient to trigger the fusion-active formation of TM in the case of ecotropic MuLV. Thus, the temperature-dependent, rapid conformational change in ecotropic MuLV SU might lead to activation of TM, signal transduction for endocytosis, a tighter interaction with the receptor, or a new interaction with putative coreceptors.

Interaction between SU and a receptor is crucial for superinfection resistance (or retroviral interference). The results of the binding specificity of the soluble SU to cells infected with MuLV or transduced with *env* genes (Fig. 2) largely confirmed the early studies using isotope-labeled SU (15) and agreed with the general concept that interference is mostly determined during virus attachment to cells. Interestingly, FCR-transfected cells which are resistant to MuLV infection were also resistant to SU binding (Fig. 3). FCR is the mutant Friend MuLV *env* gene which could not be expressed on the cell surface but was retained in the endoplasmic reticulum because of a point mutation responsible for the uncleaved precursor Env polypeptide. The resistance to virus infection was speculated to be due to interaction of the receptor with the FCR *env* in the cytoplasm and blocking of the transport to the cell surface (46). Similar examples were shown in other *env* gene mutants of avian reticuloendotheliosis virus (16) and HIV-1 (14) (29). Consistent with the above hypothesis, our results indicate that FCR-expressing cells are devoid of SU-absorbing capacity. However, in cells infected by wild-type MuLVs, it is not yet clear how and where the SU and the receptor interact and how the interference can be induced. Although intracellular localization of the receptor molecules and the SU of ecotropic MuLV virions has been characterized at an early stage of infection (41), such study needs to be done for persistently infected cells.

The soluble SU we analyzed in this study was derived from the truncated endogenous MuLV *Fv-4'* gene, which functions as a host resistance gene against exogenous infection by ecotropic MuLVs (28, 31, 43, 60). The *Fv-4'* SU is expressed in a variety of tissues and is also detectable in serum (27, 36, 49). The resistance mechanism is thought to be similar to retrovirus interference, in which cells infected by a retrovirus become resistant to superinfection by another retrovirus that has the same receptor binding specificity. However, several findings suggested that the soluble *Fv-4'* SU may also play an important role in the resistance. (i) Radiation chimera mice consisting of a mixture of *Fv-4'* gene-positive and -negative bone marrow cells were resistant to exogenous ecotropic MuLV infection (35, 42). (ii) In these chimera mice, cells not carrying the *Fv-4'* gene absorbed soluble SU (35). (iii) Binding of the serum SU protein to the cells blocked absorption of ecotropic MuLV

virions (34). Blocking of MuLV infection by soluble SU proteins has also been demonstrated in ecotropic, amphotropic, dualtropic, and xenotropic MuLVs (7).

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