Novel Monoclonal Antibody Directed at the Receptor Binding Site on the Avian Sarcoma and Leukosis Virus Env Complex

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We report here on the generation of a mouse monoclonal antibody directed against Rous sarcoma virus (RSV) subgroup A Env that will be useful in functional and structural analysis of RSV Env, as well as in approaches employing the RCAS/Tva system for gene targeting. BALB/c mice were primed and given boosters twice with EnvA-expressing NIH 3T3 cells. Resulting hybridomas were tested by enzyme-linked immunosorbent assay against RCANBP virions and SU-A-immunoglobulin G immunoadhesin. One highly reactive hybridoma clone, mc8C5, was subcloned and tested in immunofluorescence, immunoprecipitation (IP), and Western blotting assays. In all three assays, mc8C5-4 subgroup-specifically recognizes SR-A Env, through the SU domain, expressed from different vectors in both avian and mammalian cells. This multifunctionality is notable for a mouse monoclonal. We furthermore observed a preference for binding to terminally glycosylated Env over core-glycosylated Env precursor in IPs, suggesting that the epitope is at least partially conformational and dependent on glycosylation. Most importantly, we found mc8C5-4 inhibited Env function: in vitro, the monoclonal not only interferes with binding of the EnvA receptor, Tva, but it also blocks the Tva-induced conformational change required for activation of the fusion peptide, without inducing that change itself. Infection of Tva-expressing avian or mammalian cells by avian sarcoma and leukemia virus (ASLV) or EnvA-pseudotyped murine leukemia virus, respectively, is efficiently inhibited by mc8C5-4. The apparent interference of the monoclonal with the EnvA-Tva complex formation suggests that the epitope seen by mc8C5 overlaps with the receptor binding site. This is supported by the observation that mutations of basic residues in hr2 or of the downstream glycosylation site, which both impair Tva-binding to EnvA, have similar effects on the binding of mc8C5. Thus, anti-ASLV-SU-A mc8C5-4 proves to be a unique new immunoreagent that targets the receptor-binding site on a prototypical retroviral envelope.

Not only are the avian retroviruses (alpharetroviruses, or avian sarcoma and leukemia viruses [ASLV]) relevant pathogens in poultry with economic impact, but they also have long served as an important model system for studying the biological functions of retroviruses and other enveloped viruses. Attachment to and infection of target cells is mediated by the attachment to and infection of target cells is mediated by the attachment to and infection of target cells is mediated by the attachment to and infection of target cells is mediated by the attachment to and infection of target cells is mediated by the attachment to and infection of target cells is mediated by the EnvA envelope (Env) glycoproteins. Env is synthesized as a precursor molecule (Pr95) that is proteolytically processed in the Golgi into two disulfide-linked subunits, SU/gp85 (surface) and TM/gp37 (transmembrane) (18). The TM region is divided into an ectodomain involved in membrane fusion, a membrane-spanning domain anchoring the protein, and a cytoplasmic C terminus containing endocytosis and potentially other trafficking signals (39). The ectodomains of both SU and TM are extensively glycosylated. SU contains two hypervariable regions, hr1 and hr2, which are determinants of host range and thus of the interaction of SU with its respective receptor (4, 5, 14, 15, 35, 36). The receptor for ASLV subgroup A (Tva) (3, 52) has been well characterized, and receptors for subgroups B, D, and E have been recently described (1, 2, 49). A cluster of basic amino acids in hr2 of EnvA has been implicated as important for Tva binding (5, 8, 14, 35, 36, 46), as well as polar residues in hr1 (34). The EnvA-Tva interaction induces conformational changes in the ectodomain of TM, resulting in the activation and exposure of the fusion peptide (9, 26, 30), in the subsequent fusion of host cell and viral membranes, and in infection. Glycosylation of retroviral Env proteins is important for both folding and function (19, 29) and has also been implicated in immune evasion, especially in the case of human immunodeficiency virus (HIV) and simian immunodeficiency virus Env (44).

For our ongoing studies of intracellular targeting of Rous sarcoma virus (RSV) Env molecules (39), as well as of the molecular events involved in entry (11), we are using ASLV subgroup Schmidt-Ruppin A (SR-A). To elucidate steps in entry we are probing the conformational changes in the EnvA ectodomain during the fusion process. To assess intracellular targeting functions we are analyzing transmembrane (TM) cytoplasmic tail mutations and truncations. For these studies we needed a multifunctional immunoreagent(s) directed against the ectodomain of subgroup A Env. Since the generation of broadly applicable polyclonal antisera against SU or the ectodomain of TM has proven difficult in the past, we chose to attempt the generation of monoclonal antibodies. Not only might such reagents recognizing a single epitope be subgroup specific, but they could also be produced in large quantities once stable hybridoma cultures were established. Precedence...
for the usefulness of anti-SU monoclonal antibodies comes from extensive work on HIV-1 Env (reviewed in references 6, 17, and 38).

Here we report on the successful generation of anti-ASLV SR-A Env mAbcs5-4, a unique mouse monoclonal that can be used in a variety of applications including immunofluorescence, flow cytometry, IP, and Western blotting. The mcs5-4 antibody interferes with Tva receptor binding to EnvA in vitro and in cell culture. It efficiently inhibits infection of Tva-expressing cells with ASLV or EnvA-pseudotyped murine leukemia virus (MLV). Additionally, the ability of mcs5-4 to bind mutant EnvA proteins that have been described to display reduced receptor binding properties (8, 11, 46) parallels that of Tva.

**MATERIALS AND METHODS**

**DNA expression vectors.** The proviral RSV SR-A-AP expression vector RCANBP was described previously (43). In RCASBP-X-AP, X represents the coding region for the SU domain (between the XhoI and EcoRI sites) of either SR-A, SR-B, or Pr-C Env cloned into the SR-A env gene. The vector furthermore contains an alkaline phosphatase (AP) gene in the place of src, thus allowing virus titration determination by staining for AP activity (21).

**Generation of hybridoma cell clones.** Tva (PG950 cells) (26) have been described previously. The plasmid vector for the generation of SU-A-rabbit immunoglobulin G (SU-A-rbIgG) immunoadhesin has been described previously (53); plasmid vectors for Tva-rbIgG immunoadhesin were kindly provided by K. Zingler and J. Young (University of Minnesota, St. Paul), and was cultivated in DMEM–10%FCS. The chickenursalbroblast cell line Df-1 (32, 48) was kindly provided by D. Foster (University of Minnesota, St. Paul), and was cultivated in DMEM–10%FCS. NIH 3T3 cell lines stably expressing wild-type SR-A Env (27) or full-length Tva (PG950 cells) (26) have been described previously.

The myeloma line P3X63-Ag8.653 was used as fusion partner for the generation of hybridoma cell clones.

**Generation of mouse monoclonal directed against ASLV SR-A SU.** In order to generate antibodies directed against ASLV EnvA in its native form, BALB/c mice were immunized and then twice given boosters with NIH 3T3 cells stably expressing RSV SR-A Env glycoprotein (27). For each injection, each mouse received approximately 10⁷ cells that were removed from confluent culture dishes with 20 mM EDTA in phosphate-buffered saline (PBS), washed twice in PBS, and then resuspended in 100 µl of cold PBS. For the first injection the mice were emulsified in complete Freund’s adjuvant. Approximately 3 weeks later the mice received a booster consisting of the same number of cells in incomplete Freund’s adjuvant. The cells were injected subcutaneously into the thigh area of the rear legs. Approximately 2 weeks after the booster, the mice were bled and sera were tested for reactivity against gradient-purified RCANBP virus by enzyme-linked immunosorbent assay (ELISA). A virus titer of approximately 1:512 was determined. Approximately 1 month after the first booster, we administrated the second booster, which consisted of 10⁷ cells in PBS. The cells were once more injected subcutaneously into the rear thigh. Five days after the final booster, the mice were sacrificed and the popliteal and inguinal lymph nodes were removed. Lymph node cells were fused with cells of the P3X63-Ag8.653 myeloma line using polyethylene glycol. Fused cells were seeded into 24-well plates in HAT medium for selection following standard procedures. Six days after fusion, hybridomas were tested for reactivity against SR-A by ELISA, using gradient-purified RCANBP virions, or against chimeric SU-A-rbIgG immunoadhesin (53). Chimeric Tva-rbIgG (expression vector kindly provided by K. Zingler and J. Young) served as a negative control.

**Preparation of purified RSV virions and of SU-A-immunoadhesins.** For large-scale production of RCANBP virions, DF-1 cells were infected with high-titer virus stocks and cultured in roller bottles for 6 days with daily medium changes. Virus-containing culture supernatants were kept on ice until a total of 2 liters had been collected. Virions were concentrated by ultracentrifugation. Pellets were resuspended in PBS and subjected to mild sonication, and the nonaggregated portion (∼50%) was further purified by Optiprep gradient centrifugation. The virus fraction was washed, pelleted again, resuspended in PBS, and kept on ice until further use. Protein concentration was quantitated by spectrometry.

Large-scale production of purified SU-A or Tva immunoadhesins over protein A columns was performed essentially as described previously (53).

**ELISA screening of hybridoma culture supernatants.** For antibody capture assays, purified RCANBP virus dilute was loaded to 20 µg/ml in borate-buffered saline (BBS) pH 8.5, and with a 96-well ELISA plate (Dynex) were coated with 100 µl of the virus dilution overnight at 4°C. Plates were blocked for 1 h with 1% BSA in BBS. After blocking, wells were washed with BBS and incubated with hybridized supernatants for 4 h at RT. Plates were washed in BBS and incubated with alkaline phosphatase-labeled goat anti-mouse Ig (1:4,000; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After washing, plates were developed by addition of p-nitrophenyl phosphate (104 phosphatase substrate; Sigma). ELISAs using SU-A-rbIgG to capture antibody were performed essentially the same way, by coating plates with 100 µl of 5-µg/ml immunoadhesin per well.

**Purification of mc8C5 from hybridoma supernatants.** Antibody was purified from culture supernatants by affinity chromatography on recombinant protein G columns (Gamma Bind Plus; Pharmacia/LKB) according to the manufacturer’s instructions, using a Shimadzu automated preparative/analytical high-performance liquid chromatography system. Briefly, culture supernatants were filter sterilized and loaded directly onto the equilibrated column. The column was washed with equilibration buffer (0.01 M sodium chloride, 0.1 M sodium chloride; 0.01 M EDTA), and antibody was eluted with 0.5 M acetic acid, adjusted to pH 3.0 with ammonium hydroxide. The antibody peak was immediately adjusted to pH 7.0 by the addition of 2 M Trizma base. Antibody purity was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining using a Pharmacia/LKB PhastSystem according to the manufacturer’s instructions.

**Generation of titrated virus stocks and infectivity assay.** DF-1 cells were transplanted with proviral RCASBP-A-AP DNA, and the titers of virus supernatants collected 4 days posttransfection were determined on DF-1 cells. For this, the target cells were infected in quadruplicate in 24-well plates (4 x 10⁴ cells per well) with serial 10-fold virus dilutions. After 48 h in culture, cells were fixed in paraformaldehyde (PFA) and stained for exogenous alkaline phosphatase activity. For flow cytometry measuring infection, a total of 1,000 infectious units were preincubated with increasing concentrations of purified mc8C5-4 for 1 h at 4°C in a total of 200 µl of DMEM–10%FCS. The preincubated virus samples were then used to infect quadruplicate fresh DF-1 cells grown on coverslips. At 4 h after infection, 400 µl of DMEM–10%FCS was added to a final volume of 600 µl. At 48 h postinfection, cells were fixed and stained for AP activity. Infectivity was determined by counting blue cells on bright-field photographs taken from four to five random fields for each of the four coverslips per sample, at a magnification of ×20 or ×10, using a Zeiss microscope.

Infectivity assays using EnvA-pseudotyped MLV and PG950 cells were performed as previously described (12).

**Polyclonal antiserum.** Rabbit sera directed against C-terminal peptides of the TM cytoplasmic domain (rb-anti-A-tail and -C-tail) have been described previously (25). rb-Ngp37 is directed against the N-terminal ectodomain of EnvA TM (31).

Secondary antibodies for immunofluorescence and fluorescence-activated cell sorting (FACS) were purchased from Molecular Probes, Eugene, Ore.

**Metabolic labeling, IP, and quantitation of autoradiographs.** Cells were metabolically pulse-labeled with [35S]methionine-cysteine for 30 min (500 µCi/ml) in deficient medium and chased for 2 h in complete medium essentially as previously described (39). Cells were lysed in buffer containing 1% Triton X-100 and 0.5% deoxycholate and subjected to different conditions of IP as specified in the text and figure legends. After SDS-PAGE, autoradiographs were quantitated using a Cyclone scanner and OptiQuant software (Packard Instrument Company, Meriden, Conn.).
Indirect immunofluorescence and Western blotting. All immunofluorescence analyses were performed essentially as previously described (39, 46). To detect intracellular steady-state distribution of wild-type and mutant Env, protein-expressing cells grown on glass coverslips were fixed in ice-cold acetone and then probed with rb-anti-A-tail or -C-tail, or with mc8C5-4 ascites, using goat-anti-rb-Alexa488 or rb-anti-mouse Alexa488 as secondary antibody (all primary and secondary antibodies used at 1:200 in PBS–5% BSA). For surface immunofluorescence, unfixed cells on coverslips were incubated with mc8C5-4 antibody on ice for 30 min, washed in PBS, fixed with ice-cold ethanol-acetic acid (95:5), and stained with rb-anti-mouse Alexa488. All samples were observed and photographed with a Zeiss fluorescence microscope.

For analysis of mc8C5-4 in enhanced chemiluminescence (ECL)-Western blotting, EnvA, RCANBP, or SU-A-rblgG-expressing cells were lysed in 2× sample buffer (4% SDS, 0.125 M Tris-HCl [pH 6.8], 10% glycerol, 10% β-mercaptoethanol, 0.002% bromophenol blue) and boiled for 5 min. Samples were subjected to SDS-PAGE, and mini-gels were soaked in blotting buffer (20% methanol, 200 mM glycine, 25 mM Tris) prior to blotting onto nitrocellulose in the semidry apparatus at 12 V for 24 h or overnight. The blots were washed twice as above, and the blotted membranes were blocked briefly with PBSA solution and incubated 30 min on ice. The cells were washed twice in PBSA and then pelleted at 300 g (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

mc8C5-4 was able to recognize the SU-A domain in a subclass SU-A domain were detected (Fig. 1a to d), demonstrating the subgroup A specificity of the monoclonal antibody.

Use of mc8C5-4 for detection of SU-A in Western blot and IP, mc8C5-4 also demonstrated activity in ECL-Western blotting against SU-A-rblgG immunoadhesin (53) expressed in 293T cells (Fig. 2A), as well as the gp85 subunit of EnvA expressed from RCANBP in Df-1 cells (Fig. 2B). Tva-rblgG, EnvA gp37, and EnvC were not detected (not shown), indicating again specificity for SU-A. When purified SU-A-rblgG was completely N deglycosylated by PNGase F digestion, subjected to SDS-PAGE, and blotted onto nitrocellulose in SDS-free, but 20% methanol-containing buffer (see Materials and Methods), mc8C5-4 was still able to detect the antigen (not shown). Thus, the monoclonal reagent is not directed against a carbohydrate epitope. However, after purified, PNGase F-treated SU-immunoadhesin was boiled in 1% SDS and then applied directly (dot blotted) onto nitrocellulose presoaked in blotting buffer, mc8C5-4 did not bind to the N-deglycosylated SU-A

RESULTS

A mouse monoclonal was generated against ASLV EnvA and reacts with SU-A in ELISA. In order to generate antibodies directed against ASLV EnvA in its native form, BALB/c mice were immunized and then given booster doses with NIH 3T3 cells stably expressing RSV SU-A Env glycoprotein (27). Approximately 2 weeks after the first booster, the mice were bled and sera were tested for reactivity against gradient-purified RCANBP virus by ELISA. A virus titer of approximately 1:512 was determined. The mice were sacrificed 5 days after the second boost, and the popliteal and inguinal lymph nodes were removed. Lymph node cells were fused with the P3X63-Ag8.653 myeloma line using polyethylene glycol. Fused cells were seeded into 24-well plates in HAT medium for selection following standard procedures (28).

Fourteen days after fusion, hybridoma supernatants were tested for reactivity against SU-A by ELISA, using gradient-purified RCANBP virions or chimeric SU-A-rblgG immunoadhesin (53). Chimeric Tva-rblgG (7) (expression vector kindly provided by K. Zingler and J. Young) served as a negative control. Of the four positive hybridoma clones identified, one clone, mc8C5, which is a mouse IgG1 subtype, was highly reactive in both assays (data not shown) and could be successfully subcloned. mc8C5-4 hybridoma cells were used to generate ascites fluid, and antibody was also purified from hybridoma cell culture supernatants. Subclone mc8C5-4 was used for a majority of the characterizations described below.

mc8C5 reacts with SU-A in immunofluorescence. To test whether mc8C5-4 could be used in immunofluorescence applications, EnvA or chimeric molecules containing the SU-A domain were expressed from various expression vectors in either avian Df-1 cells (32, 48) or a variety of mammalian cells. mc8C5-4 was able to recognize the SU-A domain in a subgroup-specific manner using various indirect immunofluorescence approaches: EnvA could be detected after expressing cells were incubated with mc8C5-4 without prior fixation on ice or at 37°C, after fixation with ice-cold acetone or ethanol-acetic acid (95:5) or after fixation with 1 to 4% PFA. Figure 1 shows an example of Df-1 cells which were transfected with proviral vectors RCANBP or RCASBP-X-AP encoding A, B, and C subgroup SU domains (21–23). When acetone-fixed cells were incubated with an anti-cyttoplasmic tail peptide antisera (rb-anti-A-tail; [25]) detecting the C terminus of the SR-A TM protein shared by all constructs, Env expression was seen in all samples (Fig. 1e to h). However, when parallel samples were incubated with mc8C5-4, only those glycoproteins containing the SR-A SU domain were detected (Fig. 1a to d), demonstrating the subgroup A specificity of the monoclonal antibody.

To test inhibition of s47 binding to surface-expressed EnvA by mc8C5-4, cells were incubated with mc8C5-4 without prior fixation on ice or at 37°C, after fixation with ice-cold acetone or ethanol-acetic acid (95:5) or after fixation with 1 to 4% PFA. Figure 1 shows an example of Df-1 cells which were transfected with proviral vectors RCANBP or RCASBP-X-AP encoding A, B, and C subgroup SU domains (21–23). When acetone-fixed cells were incubated with an anti-cyttoplasmic tail peptide antisera (rb-anti-A-tail; [25]) detecting the C terminus of the SR-A TM protein shared by all constructs, Env expression was seen in all samples (Fig. 1e to h). However, when parallel samples were incubated with mc8C5-4, only those glycoproteins containing the SR-A SU domain were detected (Fig. 1a to d), demonstrating the subgroup A specificity of the monoclonal antibody.
domain, while glycosylated antigen was still detected under such conditions (not shown). These observations suggest that the presence of carbohydrate side chains and/or electroblotting in the presence of methanol allows the partial conservation and/or refolding of epitope conformation, while such conformation is completely lost in the presence of 1% SDS when N glycosylation is absent.

We next tested hybridoma supernatants of mc8C5 and its subclones mc8C5-4, -9, -33, and -40 in an immunoprecipitation (IP) assay (Fig. 3). In the initial experiment, we employed a chimeric ASLV Env molecule, similar to those expressed from RCASBP-X-AP, in which the coding region for SU-A between the XhoI and EcoRI site was cloned into the background of the Pr-C Env gene (designated SU-A/TM-C). This molecule also contains a cleavage site mutation (S19) (39, 42) and is therefore expressed on the cell surface as a terminally glycosylated precursor molecule, Prgp120. CV-1 cells expressing the chimeric protein Env-SU-A/TM-C-S19 were metabolically labeled and lysed in non-denaturing detergent. Aliquots of the lysate were then incubated with hybridoma supernatants, RPMI, or rb-anti-C-tail (25), respectively. All hybridoma supernatants immunoprecipitated Prgp120, as did the polyclonal serum control under these same IP conditions. mc8C5-4 was also successfully used for IP of nonchimeric EnvA and EnvA-S19 from Df-1, CV-1, 293T, NIH 3T3, and MDCK cell lysates, as well as of soluble monomeric EnvA ectodomain fragments (see Fig. 7, and data not shown), but did not react with subgroup C Env (not shown). It is important to note that the selection of protein G-Sepharose beads (protein G-agarose...
from Roche Diagnostics, Mannheim, Germany, or Protein G Gamma Bind Plus from Amersham Pharmacia Biotech, Piscataway, N.J.) used in IP was found to be critical for successful pull down of the monoclonal antibody.

The formation of EnvA-Tva complexes, and the Tva-induced activation of EnvA to fusion competence are inhibited by mc8C5-4. We next asked whether the SU-A specific monoclonal antibody could interfere with functions of EnvA, such as Tva binding. This was first investigated using a flow cytometry assay (Fig. 4A). NIH 3T3 cells and 293T cells expressing EnvA, either stably or transiently, were incubated in suspension with different concentrations of purified mc8C5-4 on ice. This was followed by addition of biotinylated s47, a peptide fragment of the Tva receptor that has previously been shown to bind to EnvA and to induce the conformational changes necessary for fusion peptide activation (9, 30). s47 bound to cells was detected after incubation with avidin-Oregon green 488 and quantitated by FACS. Mean fluorescence values indicative of s47 binding to EnvA were expressed as a percentage of s47-only fluorescence and plotted over the concentration of mc8C5-4 present during incubation. The resulting exponential inhibition curves for both cell types are nearly identical, even though EnvA was expressed to different levels on the cell surface of the different cell lines (mean fluorescent values differed by more than sixfold between the two cell lines; data not shown). Nevertheless, inhibition of s47 binding by mc8C5-4 is reproducibly concentration dependent. This suggests that the epitope recognized by mc8C5-4 is adjacent to or partially overlaps with the Tva binding site, resulting in steric hindrance of receptor binding.

Inhibition of Env function by the monoclonal antibody was next analyzed in a liposome flotation assay (30) (Fig. 4B). A soluble form of EnvA (which includes the fusion subunit ectodomain of TM [gp37]) was incubated in PBS with or without mc8C5-4 prior to addition of s47 or PBS. The formation of EnvA-s47 complexes able to interact with liposomes was probed by sucrose gradient centrifugation as described in Materials and Methods. Six fractions were taken from the top (fraction 1) to the bottom (fraction 6) of the gradient and probed by Western blotting with an antibody which recognizes gp37. Env proteins associated with liposomes are expected to float to the top of the gradient.

The formation of EnvA-Tva complexes, and the Tva-induced activation of EnvA to fusion competence are inhibited by mc8C5-4. (A) For fixed in PFA, and the fluorescence intensity of Oregon green 488 as a measure of the (relative) amount of s47 bound to cells was quantitated. (B) Liposome flotation assay. A soluble form of EnvA (which includes the fusion subunit ectodomain of TM [gp37]) was incubated in PBS with or without mc8C5-4 prior to addition of s47 or PBS. The formation of EnvA-s47 complexes able to interact with liposomes was probed by sucrose gradient centrifugation as described in Materials and Methods. Six fractions were taken from the top (fraction 1) to the bottom (fraction 6) of the gradient and probed by Western blotting with an antibody which recognizes gp37. Env proteins associated with liposomes are expected to float to the top of the gradient.
range-determining region hr2 affected recognition by mc8C5-4. hr2 constitutes part of the receptor-binding domain for EnvA, and a cluster of basic residues within hr2 (R210, R213, R223, R224, and R227), has been implicated in receptor recognition and efficient entry. We chose a set of five mutants (sM5, sM12, sM20, sM21, and sM28) with alterations in those residues, which have been characterized in detail previously (8, 46). sM5, sM12, sM20, and sM21 were previously found to be greatly reduced both in their Tva binding capability and infectivity. M28 (R213S) differed since here Tva binding nearly reached wild-type levels, while infectivity was still impaired by 95%. In order to assay mc8C5 binding, we expressed the mutant EnvA proteins, and wild-type EnvA, from pCB6-EnvA plasmids in Df-1 cells, which were then either prepared for immunofluorescence (Fig. 6A to C), or flow cytometry (Fig. 6D). Immunostaining of acetone-fixed cells with rb-anti-A-tail (Fig. 6A) revealed comparable transfection efficiencies and a similar overall intracellular and surface staining pattern and intensity for each of the constructs. This is consistent with the previous observation regarding overall and surface expression levels compared to wild type EnvA. To analyze, by immunofluorescence, the ability of mc8C5-4 to bind surface-resident EnvA, cells were incubated with mc8C5-4 on ice prior to fixation (Fig. 6B). Clear differences in the ability of the mutants to bind mc8C5-4 became apparent: While M28 showed strong surface fluorescence similar to wild type, M5 and M12 showed near-background levels, M20 showed weak but discernible levels, and M21 showed intermediate levels of fluorescence. In order to quantitate these differences, transfected Df-1 cells were prepared for flow cytometry analysis (Fig. 6D); incubation with mc8C5-4 and secondary antibody were performed on ice prior to PFA fixation. When background-corrected mean fluorescence levels of the gated populations were compared to the wild-type EnvA antibody binding level, the values reflected the results of the immunofluorescence analysis. Thus, the mutations that affected Tva binding in the previous studies (with somewhat-pleiotropic effects) also affect mc8C5-4 binding in a similar manner. Our results presented in Fig. 6 indicate that the structural requirements for efficient binding of mc8C5-4 to SU-A are related to those necessary for efficient receptor binding. As shown below, this interpretation can be extended to a critical role of glycosylation as well.

The effect of the SU-A glycosylation state on mc8C5-4 binding reflects the effect on Tva binding. When performing IPs of cleavage mutant EnvA-S19 with mc8C5-4 and rb-anti-A-tail in parallel (Fig. 7A), we repeatedly observed (Fig. 7B) that the ratio of core-glycosylated precursor, Pr95, to fully glycosylated precursor, Prgp120, was different for the different antibodies. mc8C5-4 preferentially precipitated Prgp120, not only when EnvA-S19 was expressed from Df-1 cells (shown) but also when it was expressed from CV-1 cells (not shown). This suggested that the glycosylation of EnvA influenced recognition by mc8C5-4, which is supported by our findings regarding detection of SU-A by mc8C5-4 in Western blotting (see above).

To further investigate this matter, we analyzed, by flow cytometry, a set of SU-A glycosylation mutants that have recently been characterized with regard to s47 (Tva) binding and virus infectivity (11). The mutants are designated EnvA/H9004N-g1, -g7, -g10, and -g11 and harbor mutations T19A, S232A, S256A, and S333A, respectively. The N-glycosylation sites (NXS/T) altered in the chosen mutants, with the exception of the g1 mutant, are conserved among all ASLV Env sequences examined. While EnvA/H9004N-g1, -g7, and -g11 are proteolytically cleaved, incor-
porated into virions, and result in virus infectivity similar to wild-type levels, EnvAΔN-g10 was found to be very poorly processed, and though cleaved Env was found in virions, infectivity was reduced approximately 1,000-fold. All mutants but EnvAΔN-g10 were found to bind s47 near wild-type levels in an in vitro assay, while EnvAΔN-g10 did not interact with s47 at all.

For analysis of mc8C5-4 binding to the glycosylation mutants by flow cytometry (Fig. 7C), the respective mutants were transiently expressed in 293T cells in duplicate. Cells were fixed with PFA and stained with either rb-anti-Ngp37 or mc8C5-4 and the appropriate secondary antibody. Since the N terminus of gp37 is identical in all cases, the staining with the rb-anti-Ngp37 serum (31) could be used to adjust for differences in surface expression levels. When corrected mean fluorescence values for mc8C5-4 binding were compared to wild-type levels, EnvAΔN-g10 showed the most dramatic reduction (~55%), while, interestingly EnvAΔN-g7 and -g11 displayed an increased mc8C5-4 affinity.

**DISCUSSION**

Here we report on the generation of a monoclonal antibody, mc8C5-4, directed against the SU domain of the ASLV subgroup A glycoprotein. The antibody is specific for subgroup A Env expressed in avian as well mammalian cells in immunofluorescence and IP assays. Our results indicate that it recognizes an epitope that is adjacent to or overlaps with the binding site for the EnvA receptor, Tva, since it can interfere with Tva-EnvA complex formation in vitro and in cell culture. Consequently, mc8C5-4 also prevents Tva-induced conformational changes necessary to activate the fusion peptide located near the N terminus of the EnvA TM subunit. While binding sites
for Tva and mc8C5-4 may overlap, the monoclonal antibody does not induce such conformational changes on its own.

With all observations taken into account, it is plausible that the structural requirements for efficient binding of mc8C5-4 to SU-A are related to those necessary for efficient receptor binding. It appears that, even though mc8C5-4, by Western blotting, recognizes Env that was denatured during SDS-PAGE, the epitope is partially conformational and not merely linear. While it was determined that mc8C5-4 does not bind to carbohydrate moieties, glycosylation does influence antibody recognition, most likely by affecting overall conformation of the protein, and thus epitope structure. A single glycosylation site mutation (S256A), approximately 25 amino acids downstream of the host range-determining region hr2, significantly reduces EnvA interaction with mc8C5-4. The same mutation, we recently reported, abrogates Tva binding in an in vitro assay, suggesting that removal of a distal, but crucial glycosylation site might result in a structurally significant rearrangement of protein domains (11). Furthermore, mutations of basic residues in the host range-determining region hr2 itself that impair Tva binding to EnvA (8, 46) also affect mc8C5-4 binding. Tva is related to the low density lipoprotein (LDL) receptor, and basic amino acids in the receptor binding site of LDL have been implicated in interacting with acidic residues in the ligand binding site of LDL receptor. Similar interactions have been suggested for SU-A and Tva (for an overview, see reference 46), and basic residues have also been implicated as structurally important in interactions between antibodies and viral antigens (50, 51).

The observation that glycosylation affects the binding of the monoclonal anti-SU-A mc8C5-4 is further supported by the observation that terminally glycosylated EnvA is immunoprecipitated more efficiently than the core-glycosylated precursor, Pr95. While deglycosylated SU-A-rbIgG is not detected after dot blotting in the presence of SDS, it is detected after electrophoretic conditions that allow partial removal of SDS and thus limited refolding. In contrast, mc8C5-4 can bind to fully glycosylated SU-A-rbIgG under both conditions, raising the possibility that the sugar side chains limit the access of SDS to the peptide backbone and thus prevent complete denaturation.

The generation of antibodies in response to retroviral infection often fails to yield high-titer neutralizing antibodies. However, mc8C5-4 has the ability to potently inhibit infection of avian or Tva-expressing mammalian cells by ASLV- or EnvA-pseudotyped MLV. Inhibition of RCASBP-A-AP infection of avian Df-1 cells is very efficient, with an IC50 of 0.04 μg/ml.
consistent with the antibody blocking the binding of receptor by the Env protein.

The EnvA function-inhibiting characteristics and the fact that mc8CS-4 seems to interact with the receptor binding site without triggering the activation cascade of conformational changes induced by Tva make this novel monoclonal antibody a unique reagent for structural and functional studies of the ASLV glycoprotein. A similar monoclonal antibody, monoclonal antibody (MAb) b12, recognizes a conformational epitope that overlaps the CD-4-binding site of the human immunodeficiency virus type 1 (HIV-1) envelope protein. MAb b12 neutralizes a broad range of HIV-1 primary isolates and protects against primary virus challenge in animal models (37, 41, 45). The crystal structure of this monoclonal antibody has recently been determined, and together with peptides selected by phage display against Tva which we recently generated and will describe in addition, mc8C5-4, and monoclonal antibodies directed against Tva which we recently generated and will describe elsewhere, may prove useful in the research against Tva which we recently generated and will describe.

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