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Receptor Choice Determinants in the Envelope Glycoproteins of Amphotropic, Xenotropic, and Polytropic Murine Leukemia Viruses

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The envelope glycoproteins (SU) of mammalian type C retroviruses possess an amino-terminal domain of about 200 residues, which is involved in binding a cell surface receptor. In this domain, highly conserved amino acid sequences are interrupted by two segments of variable length and sequence, VRA and VRB. We have studied the role of these variable regions in receptor recognition and binding by constructing chimeric molecules in which portions of the amino-terminal domains from amphotropic (4070A), xenotropic (NZB), and polytropic (MCF 247) murine leukemia virus SU proteins were permuted. These chimeras, which exchanged either one or two variable regions, were expressed at the surface of replication-defective viral particles by a pseudotyping assay. Wild-type or recombinant *env* genes were transfected into a cell line producing Moloney murine leukemia virus particles devoid of envelope glycoproteins in which a retrovirus vector genome carrying an *Escherichia coli lacZ* gene was packaged. The host range and sensitivity to interference of pseudotyped virions were assayed, and we observed which permutations resulted in receptor switch or loss of function. Our results indicate that the determinants of receptor choice are found within the just 120 amino acids of SU proteins. Downstream sequences contribute to the stabilization of the receptor-specific structure.

The spikes present on the outside of retrovirus particles are formed by the multimerization of a protein heterodimer (11, 27, 41), composed of a transmembrane polypeptide (TM) and a surface glycoprotein (SU). Both polypeptides arise from the processing of a single precursor encoded by the virus *env* gene (18). The SU protein recognizes and binds the viral receptor on the cell surface (7, 19). Binding is thought to induce a conformational change in the spike structure that facilitates the fusion between viral and cellular membranes and allows the capsid to enter the cell (20).

Five functionally and structurally different SU proteins have been identified in murine leukemia viruses (MLVs). These viruses are classified into subgroups displaying distinct host ranges, depending on the type of SU protein present on their surface. When a virus from a given subgroup chronically infects cells, the continuous synthesis of the SU protein saturates the pool of receptor molecules and interferes with reinfection by a virus belonging to the same subgroup. Both interference and host range assays are currently used to characterize retroviral isolates (31, 32, 44). Ecotropic MLVs recognize a receptor on mouse and rat cells only, whereas amphotropic MLVs infect most mammalian cells (14). Xenotropic and polytropic mink cell focus-forming (MCF) viruses interact with yet another set of cell surface molecules in a variety of species (4). The 10A1 virus uses the amphotropic receptor as well as a different receptor expressed on mouse and hamster cells and represents a fifth MLV subgroup (32).

Biochemical studies (23, 29) and amino acid sequence comparisons (12, 21, 25, 34, 39) have shown that the SU proteins encoded by MLVs, and more generally by all mammalian type C retroviruses, are composed of three structurally distinct domains. The 200 to 230 amino-terminal residues show important sequence variations among the different viruses and are followed by an even more variable proline-rich region of 50 to 60 amino acids. The carboxy-terminal 160-amino-acid domain is very conserved and is believed to interact with the transmembrane protein (28). In the mouse, recombination can occur between exogenous ecotropic or amphotropic retroviruses and endogenous sequences, resulting in the emergence of replication-competent leukemogenic viruses with modified host ranges (5, 40). Recombination events invariably involve the replacement of *env* gene sequences encoding both the amino-terminal and the proline-rich domains by polytropic sequences which therefore appears to be responsible for the acquisition of new receptor specificities (2, 17, 21, 39). Such a strong selection for recombination events downstream of the proline-rich region suggested an important role for this hypervariable segment in receptor choice (21). Accordingly, Ott et al. proposed that in the 10A1 virus, the insertion of a polytropic proline-rich region within an amphotropic SU protein was responsible for the recognition of a novel receptor molecule (26). However, experiments using in vitro-generated recombinant viruses derived from either polytropic MCF viruses (42, 43) or feline leukemia viruses (33) indicated that a switch in receptor choice could be obtained by exchanging amino-terminal fragments of the SU protein which did not contain the proline-rich region. Furthermore, using an interference assay, we have recently shown that the amino-terminal domain of the Friend MLV ecotropic SU protein binds its receptor in the absence of the hypervariable proline-rich region (15). Therefore, a direct role for the proline-rich region in receptor recognition and binding is disputable.

Our interest here was to localize more precisely the determinants of receptor choice in MLV SU proteins. We have exchanged fragments between the MLV amphotropic, xenotropic, and polytropic proteins, and we have designed an assay to analyze the host range and sensitivity to interference of these chimeras. In this assay, we analyzed the properties of replication-defective retroviral vector particles in which the modified SU proteins were incorporated. We have isolated a cell line in which a retroviral vector carrying a *lacZ* reporter gene is packaged into noninfectious Moloney

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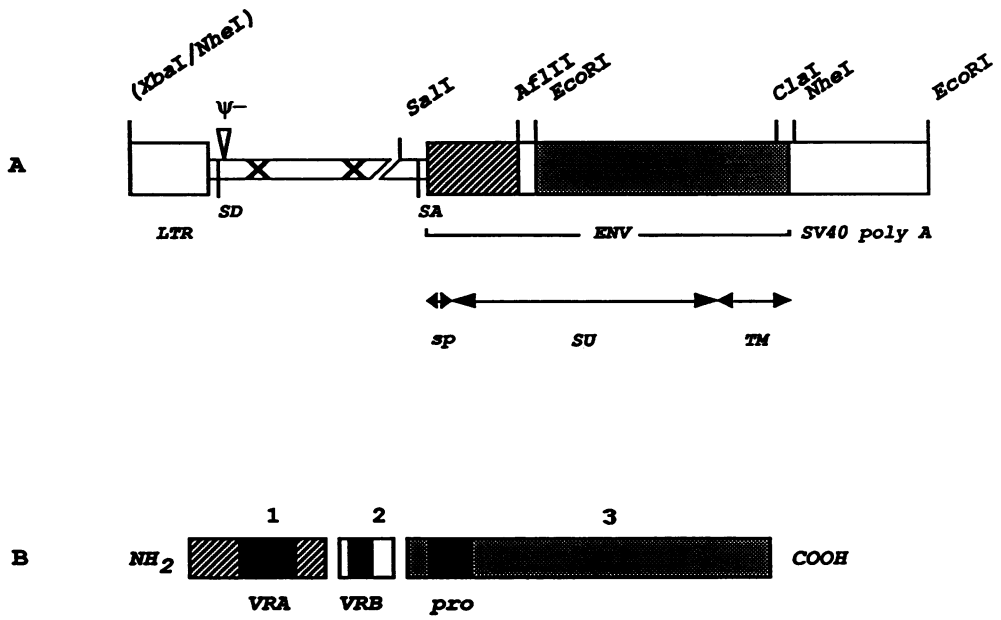

FIG. 1. (A) Structure of pCRUC *env* expression vector. The vector is derived from a Mo-MLV provirus which contains the Ψ-packaging mutation, two frameshift insertions in the *gag* gene (×), and the simian virus 40 (SV40) polyadenylation signal in place of the 3′ long terminal repeat (LTR) (6). Expression of the *env* gene is under the control of the 5′ LTR. The splice donor (SD) and acceptor (SA) are shown. Restriction sites used in the construction of the recombinant *env* genes are indicated. The three *env* gene segments used to assemble the recombinants are 5′ to *Afl*II (hatched), *Afl*II to *Eco*RI (white), and 3′ to *Eco*RI (grey). The *env* gene is composed of three coding regions: sp, signal peptide; SU, surface glycoprotein; and TM, transmembrane protein. (B) Schematic representation of the MLV SU protein. The three fragments exchanged in the chimeric proteins are shown. Black boxes represent the two variable regions (VRA and VRB) and the proline-rich region (pro).

MLV (Mo-MLV) particles devoid of envelope glycoprotein. Infectious pseudotyped virions were formed by transfecting the recombinant *env* genes into these cells and were used to transfer the *lacZ* reporter gene to a panel of cell lines from different species. Our analysis indicates that the main determinants of receptor choice are found within the first 120 amino acids of SU proteins. Sequences downstream of this amino-terminal fragment, including the proline-rich region, contribute to the stabilization of the receptor-specific structure at the surface of the viral particle.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Plasmids used as a source of *env* sequences were pCRIPAMgag^{-2} (6), NZB9.1 (25), and p247-W (16). The pCMV-LUC plasmid used as a control in transfection experiments was derived from pCMV-CAT (13) in which the firefly luciferase *luc* gene was introduced.

Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum (NIH 3T3 cells) or 10% fetal calf serum (*Mus dunni* and D17 cells). The ΨCRD polytropic packaging cell line was isolated by introducing pCRUCM (see below) into the *env-15* NIH 3T3 mouse fibroblasts which constitutively express the Mo-MLV *gag-pol* gene, as described for the isolation of the amphotropic ΨCRIP and the ecotropic ΨCRE packaging cell lines (6). D17.NZB and *Dunni*.NZB cells were obtained by infecting D17 and *M. dunni* cells with supernatant from CCL64 mink cells producing xenotropic NZB virus (a gift of S. Gisselbrecht, Institut National de la Santé et de la Recherche Médicale U152, Paris, France).

The EB8 cell line was derived from the *env-15* cells. These cells were infected with the BAG retroviral vector which contains both *neo* and *lacZ Escherichia coli* genes (30), and clones were isolated in the presence of G418. These clones were tested for their ability to produce infectious BAG pseudotypes when transfected with an ecotropic Mo-MLV envelope expression vector (pCRUCgag^{-2}; see below). Titers of BAG pseudotypes in culture supernatants were measured as described below. The EB8 clone yielded the highest titers (1×10^3 to 3×10^3 β-galactosidase-positive [β-Gal$^+$] CFU/ml) and was used throughout this study.

Construction of recombinant envelope glycoproteins. The pCRUCgag^{-2} vector (Fig. 1) contains the ecotropic Mo-MLV *env* gene and was derived from pCRIPgag^{-2} (6), in which the sequences of bacterial (pBR322) or mouse genomic origin (*Eco*RI to *Nhe*I at the beginning of the 5′ long terminal repeat) were replaced by pUC19 (*Eco*RI to *Xba*I). The following DNA fragments containing MLV *env* genes were purified from cloned MLV proviruses: amphotropic 4070A (*Sal*I to *Cla*I), polytropic MCF 247 (*Sal*I to *Nhe*I), and xenotropic NZB (*Sal*I to *Nhe*I). These fragments were used to replace the corresponding ecotropic sequences in pCRUCgag^{-2} (Fig. 1). The resulting expression vectors for amphotropic, polytropic, and xenotropic *env* genes were called pCRUCA, pCRUCM, and pCRUCX, respectively.

Two conserved restriction sites (*Afl*II or *Eco*RI) in the 5′ variable region of each of the three MLV *env* genes were used to construct recombinants (Fig. 1). First, six recombinants at the *Eco*RI site were obtained by exchanging, in all possible combinations, the *Eco*RI fragment encompassing the 3′ *env* region and the simian virus 40 sequences between pCRUCA, pCRUCM, and pCRUCX. Another set of six recombinants at the *Afl*II site was obtained by permuting the *Afl*II-*Nhe*I fragment encompassing the 3′ *env* region between pCRUCA, pCRUCM, and pCRUCX. Finally, double recom-

TABLE 1. Host range analysis of BAG pseudotypes bearing native or chimeric envelope glycoproteins

Substitution site(s)	BAG pseudotype[a]	Normalized titer[b] on:		
		NIH 3T3 cells	D17 cells	*M. dunni* cells
None	E	376	<0.21[c]	11
	A	139	55	68
	M	146	<0.76	50
	X	<0.33	96	23
*Eco*RI	AAM	111	42	48
	AAX	107	74	221
	MMX	36	<0.5	95
	XXM	<0.16	34	6
*Afl*II	MXX	170	<1	258
	XMM	<0.69	155	39
*Afl*II and *Eco*RI	AMA	26	<0.49	11
	AXA	27	<0.65	10
	MAM	64	<0.49	28
	MXM	10	<0.74	4
	XAX	<1.56	109	28
	XMX	<0.96	256	140

[a] Pseudotypes are designated with a three-letter code according to the nature of the three fragments assembled in the chimeras (A, amphotropic; M, polytropic; and X, xenotropic).

[b] Values in arbitrary units are the ratio of the number of β-Gal$^+$ CFU per milliliter measured in EB8 cell supernatants to the luciferase activity measured in EB8 cell extracts and multiplied by 10^4. Values shown are from a representative experiment.

[c] In the absence of infection, data are expressed as the ratio of lowest detectable titer (6 CFU/ml) to the luciferase activity, multiplied by 10^4.

binants at the *Eco*RI and *Afl*II sites were derived by permuting *Afl*II-*Nhe*I fragments between *Eco*RI recombinants. Recombinants were designated according to a three-letter code which indicated the origin of the three successive fragments (5′ to *Eco*RI, *Eco*RI to *Afl*II, and 3′ to *Afl*II; Fig. 1) used to assemble the hybrid *env* gene (A for amphotropic, M for polytropic, and X for xenotropic).

Transfection, infection, and quantification of pseudotype activity. *env* gene expression vectors were introduced into EB8 cells by calcium phosphate coprecipitation. Three micrograms of expression vector DNA and 0.3 μg of the pCMV-LUC plasmid DNA were added onto 10^5 cells in a 35-mm-diameter plate. Culture medium (2 ml) was changed 48 h after transfection, harvested 16 h later, and filtered through a 0.45 μm-pore-size filter. The transfected cells were lysed, and luciferase activity was measured as described previously (37). Filtered culture medium was diluted three times, and 0.5 ml was used to infect target cells in the presence of 8 μg of Polybrene per ml, as previously described (15). Following a transfection experiment, infection of all target cells was performed simultaneously. Two days after infection, cells were fixed with 0.5% glutaraldehyde and stained to reveal the presence of β-Gal activity (35). Infectious titers were expressed as the number of blue, β-Gal$^+$ cell clusters (CFU) obtained per milliliter of medium from transfected EB8 cells. Since a single infectious event (i.e., one blue cluster) was detectable in our assay, and taking into account the dilution of the EB8 supernatant, the lowest detectable titer was 6 β-Gal$^+$ CFU/ml. Interference values (see Table 2) were expressed as the ratio of the titer measured on cells expressing a retroviral *env* gene to the titer measured on normal cells. Titers shown in Table 1 were normalized to the luciferase activity measured in extracts from transfected EB8 cells. All experiments were repeated several times.

Computer analysis of amino acid sequences. Amino acid sequences were aligned by the VIZ program (36) in which conserved regions are defined by looking for the most complex key words that can be extracted from a collection of related sequences. The resulting sequence alignment was optimized manually, and a consensus was derived.

RESULTS

Transfection assay for envelope glycoprotein activity. A pseudotyping system for testing the activity of SU proteins was designed. In this assay, *env* genes were introduced into an expression vector (Fig. 1) and transfected into the EB8 cell line which produced retroviral vector particles (see Materials and Methods) lacking envelope glycoproteins. The BAG retroviral vector (30) which carries the *E. coli lacZ* gene was used. The production of infectious replication-defective BAG pseudotypes in the culture medium of the transfected EB8 cells was measured by the transfer of β-Gal activity to different target cells. To account for variations in transfection efficiencies, a luciferase reporter gene was co-transfected into the EB8 cells.

Four different MLV *env* genes (E, ecotropic Mo-MLV; A, amphotropic 4070A; X, xenotropic NZB; and M, polytropic MCF 247) were tested for their ability to pseudotype BAG retroviral vector particles. Supernatants from transfected EB8 cells were harvested 64 h after transfection, and the host ranges of pseudotypes were identified by infecting laboratory mouse (NIH 3T3), wild mouse (*M. dunni*), and dog (D17) cells. Pseudotype titers varied according to the efficiency of the transfection, the nature of the transfected *env* gene, and the target cells. They ranged between 4,500 and 130 β-Gal$^+$ CFU/ml. Normalized values, calculated as the ratio of the number of β-Gal$^+$ CFU/ml to the amount of luciferase activity detected in transfected EB8 cell extracts (see Materials and Methods), are shown in Table 1. These corrected values allowed comparison of the performances of the different SU proteins on target cells. The expected host range was observed for each pseudotype, as shown in Table 1. Amphotropic, xenotropic, and polytropic virions exhibited a distinct pattern of infectivity and were readily identified. Ecotropic and polytropic virions were distinguished by infecting CCL64 mink cells (data not shown) or according to their sensitivity to interference (see below).

We examined whether the constitutive expression of a given envelope glycoprotein at the surface of target cells interferes with the entry of the corresponding BAG pseudotype, as expected if a competition for receptor binding occurs. ΨCRE and ΨCRIP are packaging cell lines which express the Mo-MLV ecotropic and 4070A amphotropic SU proteins, respectively (6), and ΨCRD is a similar cell line in which the polytropic MCF 247 *env* gene is expressed (see Materials and Methods). Dog D17 and *M. dunni* cells chronically infected with the xenotropic NZB virus were also used. Interference was expressed for each BAG pseudotype as the ratio of the number of β-Gal$^+$ CFU per milliliter measured on SU protein-expressing cells to that measured on parental cells (Table 2). Constitutive SU expression was associated with a nonspecific two- to fourfold reduction of pseudotype titers. However, a higher titer reduction was observed when the same SU protein was both present at the surface of infecting retrovirus vector particle and expressed in target cells. The effect ranged between 16-fold (polytropic

TABLE 2. Interference sensitivities of BAG pseudotypes

Substitution site(s)	BAG pseudotype[a]	Interference ratio[b] with given SU proteins				
		Ecotropic ΨCRE/NIH 3T3	Amphotropic ΨCRIP/NIH 3T3	Polytropic ΨCRD/NIH 3T3	Xenotropic D17.NZB/D17	Xenotropic *Dunni*.NZB/*M. dunni*
None	E	0.06	0.52	0.66	—	1.9
	A	0.4	0.017	0.78	0.35	0.25
	M	0.72	0.33	0.06	—	≤0.015
	X	—	—	—	≤0.003	≤0.014
*Eco*RI	AAM	0.43	≤0.003	0.65	ND	0.15
	AAX	0.7	≤0.005	0.47	ND	0.19
	MMX	0.64	0.42	≤0.01	—	≤0.005
	XXM	—	—	—	≤0.005	≤0.028
*Afl*II	MXX	ND	1.08	0.2	—	≤0.004
	XMM	—	—	—	≤0.004	≤0.017
*Afl*II and *Eco*RI	AMA	ND	≤0.019	0.38	—	ND
	AXA	ND	≤0.024	0.43	—	ND
	MAM	ND	0.55	0.1	—	≤0.017
	MXM	ND	0.45	0.07	—	≤0.2
	XAX	—	—	—	≤0.014	≤0.05
	XMX	—	—	—	≤0.004	≤0.007

[a] Pseudotypes are designated as in Table 1.
[b] Values are the ratio of titers in β-Gal$^+$CFU measured on SU-expressing cells to titers measured on parental cells. Underlined values indicate interference. Data are from a representative experiment. ND, not determined; —, cells resistant to infection.

pseudotype on ΨCRD cells) and more than 330-fold (xenotropic pseudotype on D17.NZB). The interference data were always consistent with the host range determination. The previously described cross-interference between polytropic and xenotropic viruses on *M. dunni* cells (3) was also observed here.

We concluded that the EB8 transfection assay provides a rapid and reliable test for retrovirus entry into target cells in the absence of virus replication and propagation. It offers a way of testing the ability of SU proteins to be incorporated into MLV virions and to recognize their specific cellular receptor.

Biological activity of chimeric envelope glycoproteins. A comparative analysis of amino acid sequences from type C retrovirus SU proteins revealed a succession of conserved and highly variable segments in their amino-terminal domains, upstream of the proline-rich region (Fig. 2). We delineated two variable segments, here referred to as VRA and VRB, which displayed characteristic length and amino acid sequences in each protein. Conserved restriction sites on the amphotropic (A), xenotropic (X), and polytropic MCF (M) *env* genes were used to construct chimeric SU proteins in which either both VRA and VRB (*Eco*RI recombinants) or only VRA (*Afl*II recombinants) or VRB (*Eco*RI and *Afl*II double recombinants) was permuted (Fig. 1). Pseudotypes carrying these chimeric envelope glycoproteins were produced in EB8 cells and analyzed for host range and interference sensitivity.

(i) *Eco*RI recombinants. Six permutations were generated between the amino-terminal domains of the amphotropic, xenotropic, and polytropic SU proteins by using the conserved *Eco*RI site (Fig. 1 and 2). In these chimeras, the recombination sites between the heterologous amino- and carboxy-terminal fragments were located at amino acid positions 167 (amphotropic), 158 (xenotropic), and 157 (polytropic). The proline-rich region begins 42 or 43 amino acids downstream on the carboxy-terminal fragment, and both VRA and VRB are exchanged in these recombinants. When transfected into EB8 cells, the two recombinant *env* genes with amphotropic sequences 3′ of the *Eco*RI site (XXA and MMA) did not yield infectious virus. This suggested that complementarity between different portions of the molecule may be required for SU function. The four other recombinants (AAM, AAX, MMX, and XXM) generated functional pseudotypes as efficiently as the parental *env* genes. Table 1 shows that virion host ranges were determined by the nature of the SU amino-terminal fragment. Interference assays (Table 2) confirmed that the chimeric SU proteins recognized cell surface receptors depending upon their amino-terminal region. Indeed, interference patterns were identical for pseudotypes obtained with A and AAM or AAX, M and MMX, or X and XXM. We concluded from these data that all of the determinants of receptor choice were located within the first 155 to 165 amino acids of the SU proteins. The AAM and AAX constructs mimicked the SU organization previously described for the 10A1 SU protein, in which an amphotropic amino-terminal domain is linked to a polytropic proline-rich region (26). However, unlike 10A1, these pseudotypes could not infect Chinese hamster ovary cells. This suggested that the proline-rich region did not have a direct role in receptor recognition but rather influences determinants located in the amino-terminal domain.

(ii) *Afl*II recombinants. An additional set of recombinant *env* genes was constructed in order to examine the respective contributions to receptor recognition of fragments containing VRA or VRB. Recombinant *env* genes were produced by using the conserved *Afl*II restriction site located at the 5′ border of the VRB coding sequence (Fig. 1 and 2). In the SU proteins encoded by these recombinants, a 120-amino-acid-long fragment containing VRA was linked to a heterologous carboxy-terminal fragment containing VRB and the proline-rich region. The four chimeras containing amphotropic sequences (AXX, AMM, XAA, and MAA) were inactive, implying again that complementarity between the different envelope regions was required for function. In contrast, the two reciprocal recombinants between xenotro-

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                    ⇒ Signal  Peptide                        ⇒ SU
4070A      MArSTlSK..............PpQDKiNPWkPLIVmGVLlgvg...MaE..SPHQVFNVTWkVTNLmTGkTANATSlLGTVQDAFPALYF  42
MCF247     MEgPAfSK..............PlKDKiNPWgPLIVlGILirag..vSvRhdSPHQVFNVTWrVTNLmTGqTANATSlLGTMTDAFPKLYF  45
NZB.IU.6   MEgSAfSK..............PlKDKiNPWgPLIVmGILvragas.VqRd.SPHQIFNVTWrVTNLmTGqTANATSlLGTMTDTFPKLYF  43
Moloney    MArSTlSK..............PlKNKvNPRgPLIPlILLmlrgvstAsPgsSPHQVYNITWeVTNG.DReTVWATSgNHPLWTWWPDLTP  43
SEATO      MVlLPgSMlltsnlhhlrhqmsPgSWKrLIIlLSCVfGGGgt.....SlQnkNPHQPMTLTWqVLSQ.TGdVVWDTKaVQPPWTWWPTLKP  43
F6A        MEsPThPK..............PsKDKtLSWnLAFLvGILftidigmAnP..SPHQIYNVTWvITNVqTNtQANATSmLGTLTDAYPTLHV  42
FB.GA      MEsPThPK..............PsKDKtLSWnLVFLvGILftidigmAnP..SPHQVYNVTWtITNLvTGtKANATSmLGTLTDAFPTMYF  42
FSC        MEsPThPK..............PsKDKtFPWnLVFLvGILfqidmgmAnP..SPHQVYNVTWvITNVqTNsRANATSmLGTLTDAYPTLYV  42
consensus  M+-++-+K--------------P-KDK-++W-++++-G+L-------+-+--SPHQ++NVTW-+TN+-T+-+ANATS-LGT++D++P+L++
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                                                    VRA
4070A      DLCDLVG  ...EeWDPsdqepyvgy..........................GCkYPagRqRtrtfd......FYVCPGHtvksG.....  90
MCF247     DLCDLIG  ...DdWDEtgl................................GCrTPggRkRartfd......FYVCPGHtvptG.....  87
NZB.IU.6   DLCDLVG  ...DyWDDpepdigd............................GCrTPggRrRtrlyd......FYVCPGHtvpiG.....  89
Moloney    DLCMLAH  hgpSyWGLeyqspfssppgppccsggsspgcsrdceepltsltpRCnTAwnRlKldqtthksnegFYVCPGPhrprEsks..  130
SEATO      DVCALAA  .slEsWDIpgtdvssskrvrppdsdytaaykqitwgai......GCsYPraRtRmasst......FYVCPRDgrtlSearr.  118
F6A        DLCDLVG  ...DtWEPivlnptnvkhgaryssskyky...............GCkTTd.RkKqqqtyp.....FYVCPGHapslGpkgth  105
FB.GA      DLCDIIG  ...NtWNPsdqepfpgy..........................GCdQPm.RrWqqrntp.....FYVCPGHanrkQ.....  90
FSC        DLCDLVG  ...DtWEPiapdprswarysssth...................GCkTTd.RkKqqqtyp.....FYVCPGHapsmGpkgty  102
consensus  DLCDL+G  ---+-W++-----------------------------------GC-++--R-+-----------FYVCPGH----+-----
             *                                                 *                     *
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                                                   AflII             VRB                   EcoRI
                                                     ⬆                                      ⬆
4070A      CGGPgeGYCGkWGCETTGqAYWKPTSSWDLISLKRGnT  pwdtgcskvacgp.CYdlskvsnsfqgatrg  GRCNPLVLeFTDAGKk.AN.  176
MCF247     CGGPreGYCGkWGCETTGqAYWKPSSSWDLISLKRGnT  pqnqgp........CYdssavssdikgatpg  GRCNPLVLeFTDAGKk.AS.  166
NZB.IU.6   CGGPgeGYCGkWGCETTGqAYWKPSSSWDLISLKRGnT  pkdqgp........CYdss.vssgvqgatpg  GRCNPLVLeFTDAGRk.AS.  167
Moloney    CGGPdsFYCAyWGCETTGrVYWKPSSSWDFITVNNNlT  sdqavqv.......CKdn.............  KWCNPLVIrFTDAGRrvTS.  198
SEATO      CGGLesLYCKeWDCETTGtGYWLSKSSKDLITVKWDqN  sewtqkfqq.....CHqt.............  GWCNPLKIdFTDKGK..LSk  187
F6A        CGGAqdGFCAaWGCETTGeAWWKPTSSWDYITVKRGsS  qdns..........CE...............  GKCNPLVLqFTQKGRq.AS.  167
FB.GA      CGGPqdGFCAvWGCETTGeTYWRPTSSWDYITVKKGvT  qgiyqcsgggwcgpCYdkavhsstt.gaseg  GRCNPLILqFTQKGRq.TS.  176
FSC        CGGAqdGFCAaWGCETTGeAWWKPTSSWDYITVKRGsN  qdns..........CK...............  GKCNPLVLqFTQKGRq.AS.  164
consensus  CGG+--G+C+-WGCETTG-+YWKP+SSWD+I++K+G-+  --------------C+---------------  G+CNPLVL-FT++G+--+S-
           *     *   *                                           *                  *
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                                   ⇒ pro
4070A      .WDGPKsWGLRLYRTGt.DPItmFSltRQVlNVGPRVP   212
MCF247     .WDGPKvWGLRLYRSTgiDPVtrFSltRQVlNIGPRVP   203
NZB.IU.6   .WDAPKvWGLRLYRSTgaDPVtrFSltRQVlNVGPRVP   204
Moloney    .WTTGHyWGLRLYVSGq.DPGltFGirLRYqNLGPRVP   234
SEATO      dWITGKtWGLRFYVSG..HPGvqFTirLKI.TNMPAVA   222
F6A        .WDGPKmWGLRLYRTGy.DPIalFTvsRQVsTITPPQA   203
FB.GA      .WDGPKsWGLRLYRSGy.DPIalFSvsRQVmTITPPQA   212
FSC        .WDRPKmWGLRLYRSGy.DPIalFSvsRQVmTITPPQA   200
consensus  -WD+PK-WGLRLYRSG--DP+--F+--RQV-+++P+++
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FIG. 2. Alignment of amino-terminal sequences from the SU proteins of mammalian type C retroviruses. Amino acids upstream of the proline-rich region in the SU proteins from the following viruses are shown: amphotropic MLV 4070A (26), polytropic MLV MCF 247 (17), xenotropic MLV NZB.IU.6 (25), ecotropic Mo-MLV (38), gibbon ape leukemia virus SEATO (8), feline leukemia virus type A F6A (9), feline leukemia virus type B FB.GA (12), and feline leukemia virus type C-SARMA FSC (34). The standard one-letter code for amino acids is used. The first residue of each mature SU protein is shown in boldface, and amino acid positions are indicated on the right. Conserved amino acids appear in capital letters, variable positions appear in small characters, and gaps appear as dots. A consensus sequence was deduced from the alignment, and residues which are found in at least six of the eight sequences are shown. Positions with fewer than six and at least four occurrences of the same residue are indicated (+). Dashes indicate nonconserved positions. Conserved cysteine residues are indicated by stars. The starts of the envelope precursor, SU protein, and proline-rich region (pro) are indicated by arrows. Locations of the recombination sites used to construct the chimeras are shown (*Afl*II and *Eco*RI).

pic and polytropic *env* genes (XMM and MXX) led to the synthesis of active SU protein. The exchange of the 120-amino-acid N-terminal fragment between xenotropic and polytropic SU proteins resulted in an exact inversion of the pseudotype host ranges (compare MXX with M and XMM with X in Table 1). The interference patterns (Table 2) were also consistent with a switch in receptor recognition, although the interference observed for the MXX pseudotype on NIH 3T3 cells expressing the polytropic SU protein (ΨCRD) was only fivefold. Yet, these data did not offer a definitive demonstration that the determinants of receptor choice were solely contained in VRA, since polytropic and xenotropic VRB are closely related and could arguably replace one another.

(iii) *Afl*II-*Eco*RI double recombinants. To further examine the role of VRB, a series of double recombinants was created by permuting the internal *Afl*II-to-*Eco*RI fragment between *env* genes (see Materials and Methods). This resulted in the exchange of VRB only between SU proteins. All of these recombinants generated infectious virions in the EB8 assay. The replacement of VRB resulted in a 5- to 15-fold reduction in titers for AMA, AXA, and MXM, suggesting an influence of this variable region on the stability of the receptor binding structure. When VRB was replaced in the context of a xenotropic or polytropic SU protein (MAM, MXM, XAX, and XMX), the host range and interference properties of the pseudotypes were not modified (Tables 1 and 2). Therefore, xenotropic and polytropic VRBs can be substituted for each other or replaced with amphotropic sequences without affecting receptor choice, provided that the downstream SU protein fragment is of xenotropic or polytropic origin. In contrast, when VRB was replaced in the amphotropic SU proteins (AXA and AMA), a change in host range was observed. We were unable to infect dog D17 cells with the AXA or AMA pseudotype in repeated experiments. Compared with that of the wild-type amphotropic pseudotype, the titers of these recombinants were decreased at least 100-fold on D17 cells (Table 1). However, the chimeric AXA and AMA SU proteins still interacted with the amphotropic receptor, as shown by the 40- and 50-fold reduction in pseudotype infectivity on ΨCRIP cells relative to that on NIH 3T3 cells (Table 2). We concluded that VRB can be replaced without affecting receptor choice in the amphotropic SU protein. However, VRB substitutions certainly affect the efficiency of the interaction between the SU protein and its receptor.

DISCUSSION

The amino-terminal half of the ecotropic MLV envelope glycoprotein can fold into an autonomous domain that recognizes a receptor at the cell surface (15). From the amino acid sequence alignment shown in Fig. 2, it can be assumed that the amino-terminal domains of SU proteins encoded by all mammalian type C retroviruses have a common structure. All SU proteins share eight conserved cysteine residues and display highly homologous stretches of amino acid sequences. These mostly invariant segments are interrupted by two regions of variable length (VRA and VRB) in which each virus subtype accumulates variation. VRA is 35 to 80 amino acids long and bordered by two cysteines and starts about 50 residues from the amino-terminal extremity. It includes a peptide of variable length (44 residues for Mo-MLV versus 8 residues for MCF 247), followed by a highly hydrophilic segment that contains two conserved cysteines. VRB is separated from VRA by 38 conserved residues and also contains an invariant cysteine followed by a series of polar amino acids. The data reported here indicated that in the SU proteins of amphotropic, xenotropic, and polytropic MLVs, VRA is the major determinant which specifies the choice of the receptor but is not the only region involved in receptor interaction.

Experiments were performed with recombinant *env* genes assembled with sequences from amphotropic 4070A, xenotropic NZB, and polytropic MCF 247 MLVs. The SU proteins encoded by these *env* genes are closely related but display characteristic variations in both VRA and VRB and recognize different receptors at the cell surface (26). A conserved *Eco*RI site was used to obtain recombinant *env* genes in which the fragment encoding the amino-terminal one-third of the SU proteins, including both VRA and VRB, was exchanged. Then, with a conserved *Afl*II site, a shorter fragment encoding about 120 amino acids and encompassing VRA alone was permuted. Finally, *Eco*RI-*Afl*II double recombinants were made and resulted in the replacement of VRB alone. The three types of recombinant *env* genes were expressed in the EB8 cell line which constitutively produces a defective retrovirus vector packaged into Mo-MLV particles without envelope proteins. The nature of the cell surface receptor recognized by the chimeric SU proteins was then determined by analyzing the host range and sensitivity to interference of the pseudotyped virions.

Each recombinant *env* gene assembled from polytropic and xenotropic sequences produced a functional envelope glycoprotein which efficiently pseudotyped the retroviral vector particles produced in EB8 cells. The host range of the pseudotypes was determined by the nature of VRA (Table 1, MXX and XMM). In turn, receptor choice by the chimeric SU proteins was not influenced by the origin of VRB (MXM and XMX) or by the downstream sequences containing the proline-rich region and the carboxy-terminal domain (MMX and XXM). The interference patterns observed with these xenotropically and polytropically based recombinants were consistent with their host range. However, the titer reductions on cells expressing the polytropic SU protein were often low (ΨCRD, Table 2). The same result was obtained with pseudotypes bearing the wild-type polytropic protein (M), and the reason for this phenomenon is unknown. Yet, all the SU proteins with an amino-terminal fragment originating from the MCF virus could be identified as polytropic because they infected NIH 3T3 cells and were sensitive to NZB interference on *M. dunni* cells (3).

Our results are consistent with previous reports in which *Eco*RI chimeras, constructed by recombining Rauscher and Moloney MCF *env* genes at the *Eco*RI site, were shown to switch host ranges (42, 43). We show here that a shorter amino-terminal fragment of 120 amino acids carries the determinants which allow the SU protein to select a receptor at the cell surface. Also in agreement with our experimental findings, it has been noticed elsewhere (39) that the SU protein encoded by the Rauscher MCF virus (42, 43), which is unique in its inability to infect NIH 3T3 cells, contains xenotropic sequences encompassing VRA and the first half of VRB (amino acids 49 to 141). We interpret the peculiar phenotype of this naturally occurring MCF virus as resulting from the presence of a xenotropic VRA in a polytropic SU protein background.

Only subtle structural variations are expected between the xenotropic and polytropic SU proteins which are likely to interact with different allelic forms of the same receptor (22). Only 23 amino acid substitutions or deletions are found in the amino-terminal domain, while the proline-rich regions

have the same size and contain few amino acid changes (25, 26, 39). Although exchanging limited fragments between these closely related molecules led us to the conclusion that VRA specifies receptor selection, it was not possible to assess the contribution of other domains of these SU proteins in receptor interaction. Chimeras in which amphotropic sequences were included allowed us to address this issue, since they introduced more extensive perturbation in the overall protein structure. Indeed, the amino-terminal domain of the amphotropic SU protein diverges significantly from its polytropic or xenotropic counterparts, with a total of 42 changes, including larger insertions in the amphotropic VRA and VRB (Fig. 2 and reference 26). In addition, the amphotropic proline-rich region is markedly different from the two others. Three conclusions can be drawn from the analyses of the functional chimeras containing amphotropic sequences. First, both amphotropic VRA and VRB are required for the formation of a stable structure which binds the amphotropic receptor and mediates viral entry (AAX and AAM), whereas chimeras containing VRA alone are inactive (AXX and AMM). Second, when an amphotropic VRB is introduced in a xenotropic or polytropic SU protein, no receptor switch is observed (XAX and MAM). Reciprocally, VRB can be replaced within the amphotropic SU protein (AXA and AMA) without affecting receptor specificity. Third, the amphotropic VRB contributes to the stabilization of the receptor recognition structure. This is shown by the ability of AXA and AMA pseudotypes to interact with the amphotropic receptor expressed on mouse cells but not with the allelic form present on dog cells.

We do not know whether the absence of infectious pseudotype formation observed with a number of chimeric SU proteins containing amphotropic sequences resulted from incorrect processing, transport, or incorporation into virions or whether these molecules failed to recognize a cell surface receptor. However, the existence of incompatible permutations further suggests that the SU protein fitness is conditioned by structural constraints between VRA, which acts in selecting the receptor, and the rest of the molecule. We assume that functional folding of the amino-terminal domain involves a tripartite interaction among VRA, VRB, and downstream sequences. The proline-rich region could be the third partner in this interaction. Amino acids in VRA and VRB may form loops on the surface of the amino-terminal domain and either directly contact the receptor molecule or define the accessibility to a binding site. Whether it directly interacts with the receptor or not, each component of the binding structure must be optimally adapted to the others, with little tolerance to variation. For instance, polytropic and xenotropic VRAs do not tolerate an amphotropic proline-rich region, as shown by the inactivity of XXA, MMA, XAA, and MAA chimeras. Accordingly, recombination events selected in naturally occurring MCF viruses always result in the coexistence of an amino-terminal domain with its cognate proline-rich region.

The situation described here for MLV SU proteins is reminiscent of avian sarcoma-leukemia virus envelope glycoproteins in which host range determinants have been mapped to two short hydrophilic regions of variable sequence (10). Although no obvious amino acid sequence homologies can be found between murine and avian SU proteins, it is likely that common three-dimensional features are operating in receptor recognition. Cell surface molecules involved in type C retrovirus binding and entry are currently being characterized (1, 24). Future progress may lead to the discovery of common structural themes involved in the recognition of these molecules by retroviruses.

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