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Identification and Characterization of the Viral Interaction Determinant of the Subgroup A Avian Leukosis Virus Receptor

KURT ZINGLER,[1,2] CAROLE BÉLANGER,[1,2] REUBEN PETERS,[3] DAVID AGARD,[3,4] AND JOHN A. T. YOUNG[1,2]*

Gladstone Institute of Virology and Immunology,[1] Department of Microbiology and Immunology,[2] Department of Biochemistry and Biophysics,[3] and Pharmaceutical Chemistry,[4] University of California School of Medicine, San Francisco, California 94141-9100

Received 10 January 1995/Accepted 6 April 1995

The cellular receptor for subgroup A avian leukosis viruses (ALV-A) has a small, 83-amino-acid extracellular domain containing a motif that is related in sequence to the ligand binding repeats of the low-density lipoprotein receptor. Extensive mutagenesis of the ALV-A receptor has identified two acidic amino acids (Asp-46 and Glu-47) and an adjacent aromatic amino acid (Trp-48) in the carboxy-terminal portion of this low-density lipoprotein receptor-related motif that are crucial for efficient viral entry. In addition, a 19-amino-acid peptide derived from this region efficiently and specifically blocked subgroup A viral infection when oxidized to form a disulfide bond previously predicted to form in the native receptor (C. Bélanger, K. Zingler, and J. A. T. Young, J. Virol. 69:1019–1024, 1995). Thus, the charged and aromatic amino acid determinants that are required for viral infection appear to lie on a small loop region of the ALV-A receptor. Previously, a single aromatic and one or more charged residues on the CD4 receptor for human and simian immunodeficiency viruses, and the MCAT receptor for ecotropic murine leukemia viruses, were shown to be important for viral entry. These results suggest that different retroviruses may recognize related determinants on structurally divergent cellular receptors.

Following binding to specific host cell surface receptors, retroviral envelope (Env) proteins are thought to undergo conformational changes that drive virus-cell membrane fusion and lead to viral entry (12, 27). The Env proteins of different retroviruses that mediate this process are structurally related, composed of both surface (SU) and transmembrane (TM) subunits (27). However, the cognate receptor proteins identified thus far include single and multiple membrane-spanning proteins from a diverse array of protein families (2, 4, 5, 15, 17, 21, 26). To date, no related receptor determinants that would suggest any conservation between different retrovirus-receptor interactions have been identified.

To better understand the process of retroviral entry, we are studying the interaction between subgroup A avian leukosis viruses (ALV-A) and their cellular receptor (5, 31). Protein products of the cloned quail receptor gene have a predicted 83-amino-acid extracellular domain (5), which contains the site of virus interaction (8, 10). This domain shows no obvious similarity to any previously characterized retrovirus receptor; rather, it contains a six-cysteine motif (amino acids 11 to 50) that is related in sequence to the ligand binding repeats of the low-density lipoprotein receptor (LDLR) (5). Two of the six cysteines (Cys-35 and Cys-50) within this domain were recently shown to be important for ALV-A infection, suggesting direct involvement of the LDLR-related motif in viral entry (6). In this study, we subjected the extracellular region of the ALV-A receptor to extensive mutagenesis and identified a three-amino-acid segment within this motif that is crucial for efficient viral entry.

* Corresponding author. Mailing address: Gladstone Institute and Department of Microbiology and Immunology, University of California School of Medicine, P.O. Box 419100, San Francisco, CA 94141-9100. Phone: (415) 695-3815. Fax: (415) 826-8449.

MATERIALS AND METHODS

Cells and virus. COS-7 cells (11), quail QT6 cells (20), and primary chicken embryo fibroblasts (CEFs) were grown and selected as previously described (8). RCASH-A, RCASH-B, and RCASH-C virus stocks, representing ALV subgroups A, B, and C, respectively, were also prepared as previously described (8, 31).

Mutagenesis of the ALV-A receptor. A synthetic processed quail receptor gene (6) was used as a vector for all mutations described. This synthetic gene contains several introduced restriction enzyme sites which allowed the introduction of site-specific mutations either by double-stranded oligonucleotide replacement as described previously (6) or by PCR. DNA sequences of all altered receptors were confirmed by using a PCR-based dideoxy sequencing protocol (Bethesda Research Laboratories). All of the mutant receptors were expressed under the control of the cytomegalovirus early-region promoter in the expression plasmid pCB6 (kindly provided by M. Stinski).

The glycosylphosphatidylinositol-anchored ALV-A receptor was generated by introducing an anchor signal sequence derived from decay-accelerating factor (19), two amino acids after the sixth cysteine (Cys-50) of the LDLR-related motif.

The expression of mutant proteins was monitored by using monoclonal antibody 12CA5, which is specific for the influenza virus hemagglutinin epitope tags (9, 25) located in the cytoplasmic domain of the synthetic receptor (6).

Transfection of COS-7 cells and infection with RCASH-A. COS-7 cells were transfected with 20 μg of plasmid DNA by the calcium phosphate precipitation method (28). After 36 h, cells were split to approximately 5% confluence on six-well plates and 24 h later infected with serial 10-fold dilutions of RCASH-A (10, 1, and 0.1 μl) (31); 24 to 36 h later, virus-infected cells were selected in medium containing 300 μg of hygromycin B per ml. Colonies were counted 11 to 14 days after infection.

Cell lysates and immunoblot analysis. Transfected COS-7 cells were lysed in Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris [pH 8.0]). Protein lysates (50 μg) were electrophoresed on sodium dodecyl sulfate–12% polyacrylamide gels and transferred to Hybond-ECL nitrocellulose membranes (Amersham). Membranes were blocked for 1 h in Tris-buffered saline containing 0.1% Triton X-100 (TBST) and 8% powdered milk and then incubated with a 1:3,000 dilution of monoclonal antibody 12CA5 (1 mg/ml; Babco) in TBST containing 2% powdered milk. After being washed with TBST, membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibodies (1:3,000; Amersham), and bound proteins were detected with the enhanced chemiluminescence system (Amersham). The filters were exposed to Kodak XAR-5 film for 5 min.

Biotinylation of cell surface proteins. Cell surface proteins were labeled with a membrane-impermeable amine-reactive biotin reagent (22). Approximately 5×10^5 transfected COS-7 cells were plated on 60-mm-diameter plates. Twenty-

four hours later, the cells were rinsed four times with ice-cold biotinylation buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 140 mM NaCl, 5 mM $MgCl_2$). Cell surface proteins were then biotinylated with 3 mg of NHS-LC-Biotin (Pierce) in 1.5 ml of biotinylation buffer for 45 min at 4°C. Cells were rinsed three times with biotinylation buffer containing 20 mM glycine and then lysed in NP-40 lysis buffer. The biotinylated proteins were precipitated overnight by incubation with avidin-agarose beads (Pierce). The beads were washed three times in NP-40 lysis buffer and once with 10 mM Tris-HCl (pH 8.0) and resuspended in Laemmli loading buffer, and one half of each sample was subjected to gel electrophoresis.

Peptide blocking. The W33-G51 peptide (WLCDGHPDADDGRDEWGCG), corresponding to residues Trp-33 to Gly-51, was synthesized on an Applied Biosystems peptide synthesizer by using fluorenylmethyloxycarbonyl chemistry. The W48A peptide (WLCDGHPDADDGRDEAGCG) was purchased from Research Genetics. Both peptides were purified by high-pressure liquid chromatography and were oxidized in 1 mM ammonium hydroxide (pH 8.0) under dilute peptide concentrations to favor intramolecular disulfide bonding. They were then dried and resuspended in 30 mM sodium acetate (pH 5.2) at a peptide concentration of approximately 0.5 mM. The W33-G51 peptide was 93% oxidized, and the W48A peptide was 70% oxidized, as determined by using Elman's reagent (Pierce). The reduced version of the W33-G51 peptide was prepared in 4.5 mM dithiothreitol, and the thiol groups were blocked with 45 mM iodoacetate. The reduced peptide was then purified by using a desalting column.

In the W33-G51 peptide titration experiment, different amounts of peptide were preincubated with approximately 200 infectious units of RCASH-A at 4°C for 90 min prior to infection of 2×10^5 quail QT6 cells. Cells were selected, and hygromycin B-resistant colonies were counted as previously described (8).

In the control peptide experiments, approximately 4,000 infectious units of RCASH-A was preincubated for 90 min at 4°C in the presence of 1 μM the different peptides in a total volume of 4 ml. Aliquots (1 ml and 100 μl) of these samples were then used to infect 2×10^5 QT6 cells (900 μl of medium containing 1 μM the appropriate peptide was added to the 100-μl aliquots immediately prior to infection). The numbers of colonies that were obtained (see the legend to Fig. 4C) after incubation with the oxidized or reduced W33-G51 peptide were the averages derived from three independent 1-ml infections and extrapolated from three independent 100-μl infections. For incubation with the oxidized W48A peptide or with no peptide, the numbers obtained were extrapolated from the 100-μl infections only, because too many colonies arose after the 1-ml infections to accurately count.

For infection of CEFs, approximately 100 to 500 infectious units of RCASH-A, RCASH-B, and RCASH-C (5, 31) viruses was preincubated with or without 0.5 μM oxidized W33-G51 peptide at 4°C for 1 h prior to infection. Medium was collected before the first cell passage and assayed for virus, using a colorimetric, enzyme-linked immunosorbent (ELISA)-based, quantitative reverse transcriptase assay (Boehringer Mannheim) as previously described (8). Neither peptide used demonstrated any cellular toxicity at the concentrations used (data not shown).

RESULTS

A quantitative infection assay is used to measure receptor function. To assess the relative importance of different amino acid side chains in the receptor for viral interaction, mutations were introduced into a synthetic ALV-A receptor gene (6). The products of this gene contain influenza virus hemagglutinin epitope tags in the cytoplasmic domain, which permit protein detection by monoclonal antibody 12CA5 (9, 25) regardless of the mutations placed in the extracellular domain (6). Mutations introduced into this gene were tested for their impact on ALV-A entry in transiently transfected monkey COS-7 cells by using a quantitative viral infection assay, which employed subgroup A viruses containing a gene that confers hygromycin B resistance (31). COS-7 cells are permissive for ALV-A infection only when transfected with a functional viral receptor gene (31). Following virus challenge, mutant and wild-type receptor function was measured by counting the drug-resistant colonies that arose as a consequence of individual infection events.

The LDLR-related domain contains the viral interaction site. To determine if amino acids outside the LDLR-related motif (residues 11 to 50) were necessary for viral interaction, we first replaced all of the residues after position 52 with a glycosylphosphatidylinositol membrane attachment sequence derived from the carboxy-terminal end of decay accelerating factor (19). This glycosylphosphatidylinositol-anchored, chimeric receptor allowed ALV-A entry at levels equivalent to those of the wild-type receptor (data not shown). Also, in a series of independent point mutations, 7 of the first 10 amino acids of the mature ALV-A receptor could be substituted without altering its ability to allow subgroup A virus infection (see the legend to Fig. 1). Together, these results demonstrated that specific amino acid residues outside the LDLR-related motif were not required for receptor function, suggesting that this motif alone contains the viral interaction site.

Substitution of amino acids between each cysteine identifies two regions within the LDLR-related domain that influence receptor function. To identify regions within the LDLR-related motif that were important for receptor function, each of the five regions bounded by cysteines was replaced independently with sequences found in the corresponding positions of the human LDLR, which is presumed not to function as an ALV-A receptor (Fig. 1A) (29). This approach was chosen, instead of random mutagenesis, to reduce the possibility that the mutant receptors would be misfolded during biosynthesis and not transported to the cell surface. Western blot (immunoblot) analysis of whole cell lystates and purified cell surface proteins (probed with monoclonal antibody 12CA5) confirmed that each of these mutant receptors was expressed both within the cell and at the cell surface at levels comparable to that of the wild-type receptor (Fig. 1B and C). Similar studies revealed that all of the other mutant receptors described in this report were expressed both within the cell and at the cell surface at levels comparable to that of the wild-type receptor (data not shown).

These mutant receptors were next tested for function in the quantitative viral infection assay described above. Using this approach, amino acids between Cys-11 and Cys-18, Cys-18 and Cys-28, and Cys-35 and Cys-41 did not appear to be critical for receptor function, because all of the residues between these cysteines could be replaced without impairing ALV-A entry (mutant receptors M101, M102, and M104 [Fig. 1A and D] and additional mutants described in the legend to Fig. 1). In contrast, replacing five of six residues between Cys-28 and Cys-35 or replacing five of eight residues between Cys-41 and Cys-50 caused complete loss of receptor function (mutant receptors M103 and M105; Fig. 1D).

Identification of specific residues between Cys-28 and Cys-35 and between Cys-41 and Cys-50 that influence ALV-A entry. Because amino acids between Cys-28 and Cys-35 and between Cys-41 and Cys-50 appeared to be important for receptor function, the residue requirements at these positions were studied in more detail. In the analysis of the Cys-28–Cys-35 region, the function of the M103 receptor was restored to wild-type levels by mutating residue 34 back to the wild-type residue (Leu), indicating that residues 29 to 32 are unlikely to play a direct role in viral entry (Fig. 2A, M106). Surprisingly, changing Leu-34 to either Arg or Ala in the context of the wild-type receptor had only 38- and 6-fold effects on entry, respectively (Fig. 2A, M108 and M109), suggesting that a leucine at this position can influence the efficiency of receptor function but is essential only in the context of the M103 receptor. Furthermore, the only amino acid that was not exchanged in the M103 receptor, Trp-33, could be replaced with Leu without noticeably impairing ALV-A entry (Fig. 2A, M107).

In the Cys-41–Cys-50 region, function was partially restored to the M105 receptor by changing Glu-48 back to Trp (Fig. 2B, M111) and fully restored by the additional change of Asn-49 back to Gly (Fig. 2B, M110). Although Gly-49 contributed to the efficient function of the M110 receptor, this residue alone was unable to confer function on the M105 receptor (Fig. 2B,

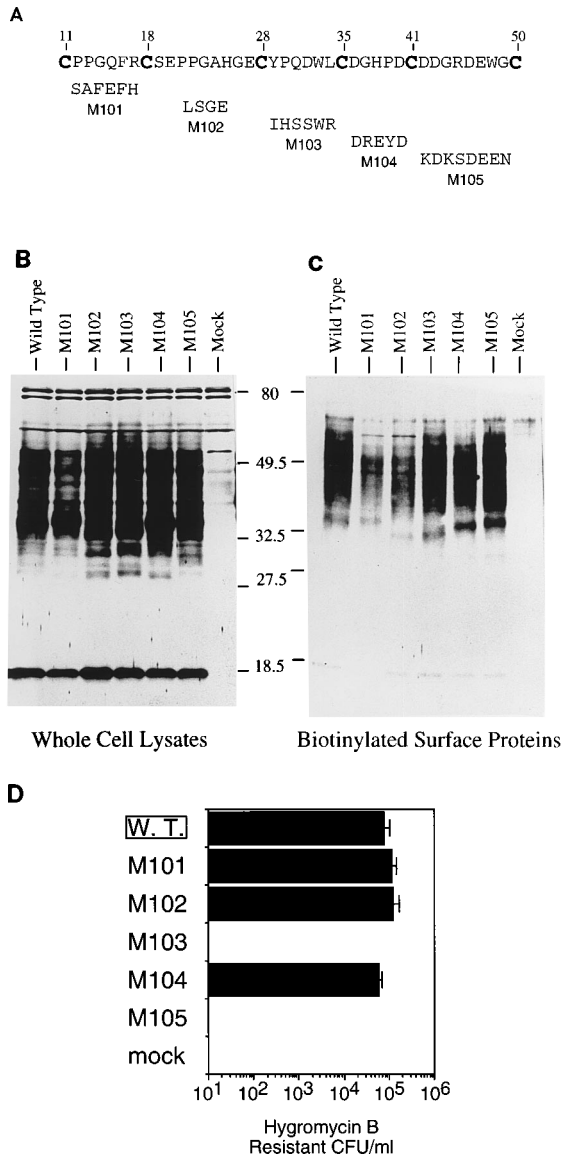

FIG. 1. Mutations in the LDLR-related domain that inhibit ALV-A receptor function. (A) Amino acid sequences of the mutant receptors. Specific regions of a synthetic ALV-A receptor gene (6) were replaced with the corresponding regions of the fifth (M101, M102, M103, and M105) or sixth (M104) ligand binding repeat of the human LDLR (29). (B and C) Western blot analysis of receptor proteins expressed in COS-7 cells. (B) Whole cell lysates from transfected cells expressing wild-type or mutant receptors, or cells transfected with a control plasmid (mock), were probed with monoclonal antibody 12CA5 raised against the epitope tags in the cytoplasmic tail region. All receptors were expressed at similar levels. (C) Biotinylated cell surface proteins were probed with monoclonal antibody 12CA5. All of the mutant receptors were able to reach the cell surface. (D) Mutant receptor function. COS-7 cells expressing altered receptors were challenged with RCASH-A, a subgroup A-ALV containing a hygromycin B resistance gene (31). Resultant hygromycin B-resistant colonies were counted as CFU per milliliter as a measure of ALV-A receptor function (6, 8, 31). These results represent a minimum of three separate experiments, with error bars indicating standard deviations. In addition, receptors bearing the following independent mutations functioned at approximately wild-type levels: N1A, S3A, G4A, N5A, G6A, S7A, S9A, F16I, Y29I, P30H, D32S, W33A, D36N, D40N, and G51A. R10, G26, E27, and T52 were not changed because these residues differ in the chicken receptor. G2 and L8 were not tested. W. T., wild-type receptor.

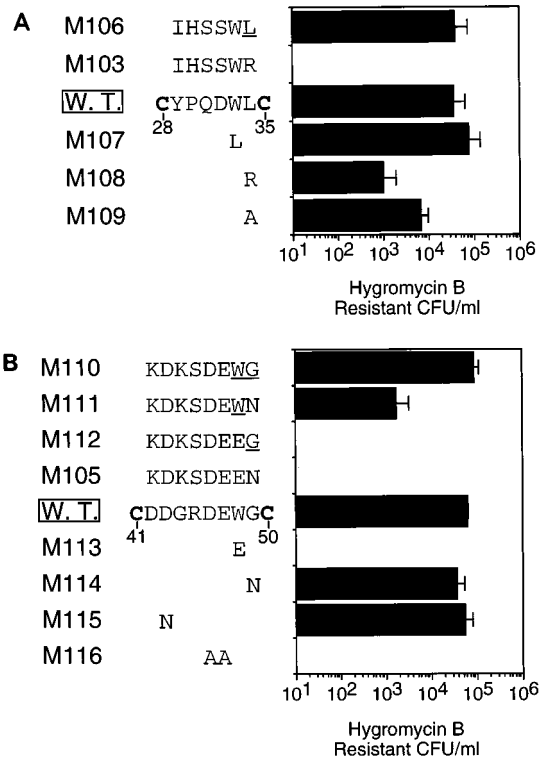

FIG. 2. Specific residue requirements between Cys-28 and Cys-35 and between Cys-41 and Cys-50. COS-7 cells expressing mutant receptors were assayed for receptor function as described in the legend to Fig. 1. These results represent a minimum of three separate experiments, with error bars indicating standard deviations. (A) Although no single substitution between Cys-28 and Cys-35 abrogated receptor function, Leu-34 exerted some influence over ALV-A infection (compare M103 and M106). (B) Between Cys-41 and Cys-50, mutation of Asp-46, Glu-47, and Trp-48 blocked receptor function.

M112). Furthermore, replacement of Gly-49 with Asn did not impair wild-type receptor function (Fig. 2B, M114). Asp-43 also appeared to be unimportant, because replacement with Asn did not inhibit infection (Fig. 2B, M115). The importance of Trp-48 in the wild-type receptor was confirmed by a Glu substitution, which abolished activity (Fig. 2B, M113). Similarly, replacing Asp-46 and Glu-47 with two alanines completely blocked receptor function (Fig. 2B, M116).

Amino acid side chain requirements at positions 46, 47, and 48. Because residues Asp-46, Glu-47, and Trp-48 appeared to be critical for receptor function, we further examined the specific amino acid side chain requirements at these positions. At Asp-46, a nonconservative Ala substitution abrogated receptor activity entirely (Fig. 3, M117). Even simply removing the charge of Asp-46 by replacement with Asn also reduced activity 890-fold (Fig. 3, M118). Glu-47 appeared less important for virus entry, since Ala or Gln substitutions were significantly less inhibitory than the substitutions made at Asp-46 (Fig. 3, M120 and M121). In contrast, receptors bearing mutations which retained acidic side chains at positions 46 and 47 functioned well in ALV-A infection (Fig. 3, M119 and M122). This finding suggested that the negative charges associated with these residues are necessary for efficient receptor function.

At the third position, substitution of the aromatic Trp-48 with Ala abolished receptor function entirely (Fig. 3, M123), while replacing Trp-48 with large hydrophobic amino acids had less impact, reducing activity 40- to 200-fold (Fig. 3, M124, M125, and M126). In this assay, only replacement of Trp-48 with another aromatic residue (Tyr or Phe) maintained full receptor function (Fig. 3, M127 and M128). Thus, while receptors bearing bulky, hydrophobic residues at position 48 could

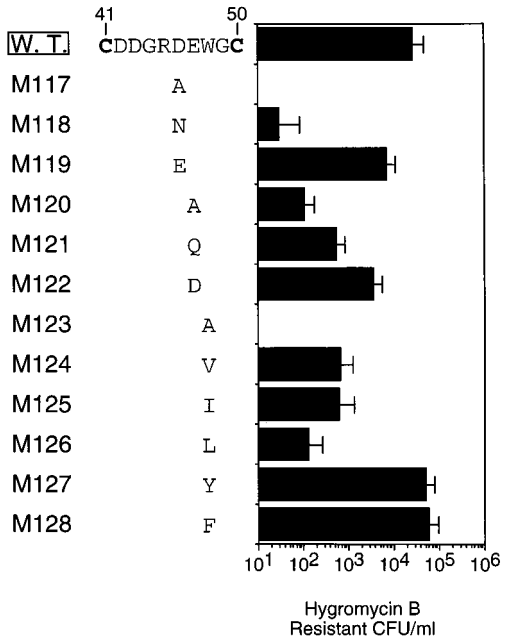

FIG. 3. Acidic residues at positions 46 and 47 and an aromatic residue at position 48 are essential for efficient ALV-A entry. COS-7 cells expressing mutant receptors were assayed as described in the legend to Fig. 1. These results represent a minimum of three separate experiments, with error bars indicating standard deviations. An acidic residue at either Asp-46 or Glu-47 preserved near-wild-type (W. T.) receptor function. Similarly, replacement of Trp-48 with other aromatic residues maintained wild-type activity, although receptors with bulky hydrophobic amino acids at this position had partial activity.

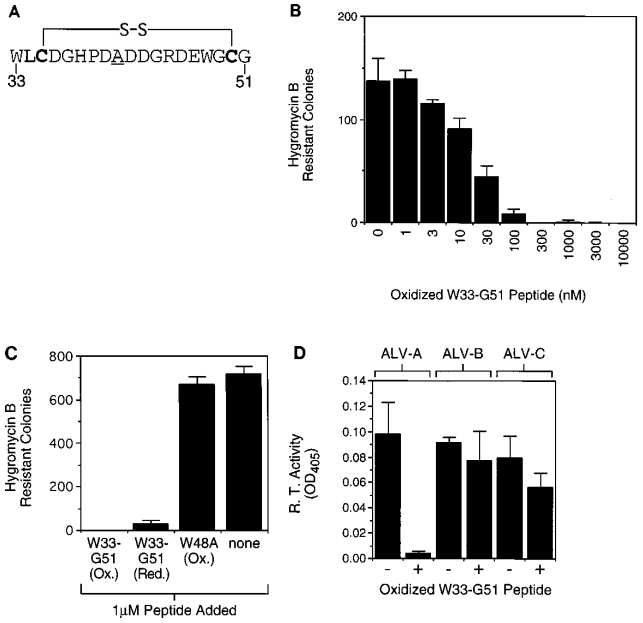

FIG. 4. An oxidized 19-amino-acid peptide specifically blocked ALV-A infection. (A) The W33-G51 peptide, representing amino acids 33 to 51 of the ALV-A receptor. To ensure Cys-35–Cys-50 disulfide bonding (S-S), Cys-41 was changed to Ala (underlined). (B) The oxidized W33-G51 peptide blocks RCASH-A infection of quail QT6 cells, which express endogenous ALV-A receptors (5) in a dose-dependent manner. From the results for peptide concentrations of 1 to 100 nM, the peptide had a 50% inhibitory concentration of approximately 20 nM. (C) Infection of QT6 cells by RCASH-A was blocked efficiently by the oxidized (Ox.) W33-G51 peptide but not by the oxidized version of a similar peptide, W48A, in which Trp-48 was substituted with Ala. A reduced (Red.) version of the W33-G51 peptide was significantly less inhibitory. The average numbers of hygromycin B-resistant colonies (derived from approximately 1,000 infectious units of RCASH-A) were as follows: 0.6, 27, 667, and 717, from left to right. (D) The oxidized W33-G51 peptide blocked infection of CEFs after preincubation with ALV-A but not after preincubation with ALV-B or ALV-C. A colorimetric, ELISA-based, quantitative reverse transcriptase (R. T.) assay was used to assess viral spread, and thus results are expressed in units of optical density at 405 nm (OD_{405}). The results shown in panels B to D represent the means of three separate experiments; error bars indicate standard deviations.

facilitate ALV-A infection, receptor function was most efficient with an aromatic residue at this position.

A 19-amino-acid peptide derived from the carboxy-terminal end of the LDLR-related domain specifically blocks ALV-A infection. The results of the mutagenesis suggested that ALV-A might interact with Asp-46, Glu-47, and Trp-48 in the context of a short disulfide-bonded loop, previously postulated to form between Cys-35 and Cys-50 at the carboxy-terminal end of the LDLR-related region (6). To test this hypothesis, a 19-amino-acid peptide spanning this region (W33-G51) was synthesized and oxidized to form the Cys-35–Cys-50 disulfide bond (Fig. 4A). When preincubated with ALV-A, this peptide significantly inhibited viral infection in a dose-dependent manner, virtually eliminating infection at a concentration of 300 nM (Fig. 4B; 50% inhibitory concentration of 21 nM). This activity appears to depend at least in part on the structural constraints imposed by the disulfide bond, because virus preincubated with a reduced version of this peptide gave rise to approximately 45-fold more hygromycin B-resistant colonies than were obtained from virus preincubated with the oxidized peptide (Fig. 4C).

Two control experiments were performed to confirm the specificity of virus interaction with the W33-G51 peptide. First, a mutation that abolished native receptor function, Trp-48 to Ala (Fig. 3, M123), was introduced into the peptide to determine whether the blocking activity of this reagent required the same critical aromatic residue as is required for the function of the full-length, membrane-bound receptor. The inability of this oxidized peptide to block infection (Fig. 4C) indicated that Trp-48 was crucial for peptide inhibition. This result also demonstrated that the peptide and the wild-type receptor most probably interact with virus in similar manners. In a second test of specificity, the peptide was assayed for its ability to block infection of CEFs by other ALV subgroups, which use different cellular receptors (27). In this experiment, the oxidized W33-G51 peptide efficiently blocked subgroup A-ALV infection of CEFs but not infection of these cells by other ALV subgroups (Fig. 4D). Thus, the peptide interaction was specific for ALV-A.

DISCUSSION

Using site-directed mutagenesis, we have shown that the ALV-A receptor is able to tolerate a large number of amino acid substitutions without loss of function. However, two cysteine residues, Cys-35 and Cys-50 (6), and three additional residues (Asp-46, Glu-47, and Trp-48) are important determinants for ALV-A entry. Specifically, ALV-A infection was most efficient with acidic residues at positions 46 and 47 and an aromatic residue at position 48. Also, we have found that a 19-amino-acid peptide spanning the region including Cys-35, Cys-50, Asp-46, Glu-47, and Trp-48 specifically blocked ALV-A infection. This subgroup A-specific blocking activity was significantly enhanced when the peptide was oxidized to form the disulfide bond between Cys-35 and Cys-50, which is predicted to form in the native receptor (6). Several lines of

evidence imply that the oxidized peptide contains all of the structural and functional information of the viral interaction site. Specifically, the ability of the peptide to block viral infection was dependent on Trp-48, a residue required for wild-type receptor function, and was also specific for subgroup A virus. These observations suggest that the viral interaction site of this protein may be contained in a short loop domain. Indeed the location of this disulfide bond might explain why Leu-34 had an influence on mutant receptor function (Fig. 2A, M106, M108, and M109). Since this residue is adjacent to Cys-35, mutations at this position might influence disulfide bond formation or influence viral interaction because of their proximity to the predicted interaction site (residues 46 to 48) following oxidation. Structural studies will be needed to differentiate between these possibilities.

Although our results do not distinguish between involvement of residues 46 to 48 in viral binding and/or fusion, previously it was found that an aromatic residue and additional charged residues within loop domains of the CD4 receptor of human and simian immunodeficiency viruses and of the MCAT receptor of ecotropic murine leukemia viruses are also important for viral entry. These loop domains form the C′C″ ridge of the CD4 receptor and the third extracellular loop in the MCAT receptor (1a, 3, 7, 18, 30). As in the ALV-A receptor, the critical aromatic residues in both the CD4 and MCAT receptors can be replaced with other aromatic or certain hydrophobic amino acids without abolishing function (1, 18). These results demonstrate a striking similarity between determinants important for viral recognition on three otherwise unrelated retroviral receptors (2, 5, 15). Interestingly, the cellular receptor for gibbon ape leukemia virus contains a tyrosine (at position 549) which is immediately adjacent to a charged amino acid required for infection, but the importance of this aromatic residue remains to be determined (14, 21, 24).

While the precise interactions between retroviral envelope and receptor proteins are unknown, the critical aromatic residue in CD4 (Phe-43) has been proposed to fit into a large hydrophobic pocket of human immunodeficiency virus gp120 to initiate receptor binding (18). This proposed receptor-binding pocket may be similar to that contained in the antigen-binding groove of major histocompatibility complex proteins. In this case, the hydrophobic pocket is filled by a single aromatic side chain of the bound peptide, an interaction which is essential for efficient peptide binding (13, 16, 23). Given the importance of an aromatic residue for the function of the CD4, MCAT, and ALV-A receptors, it is possible that their cognate viral Env proteins also contain a hydrophobic receptor-interaction pocket. This feature may in fact be generally conserved between different retroviral Env proteins, indicating a common mechanism of retrovirus-receptor interactions.

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