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Modifications in the Binding Domain of Avian Retrovirus Envelope Protein To Redirect the Host Range of Retroviral Vectors

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On the basis of theoretical structural and comparative studies of various avian leukosis virus SU (surface) envelope proteins, we have identified four small regions (I, II, III, and IV) in their receptor-binding domains that could potentially be involved in binding to receptors. From the envelope gene of an avian leukosis virus of subgroup A, we have constructed a set of SU mutants in which these regions were replaced by the coding sequence of FLA16, a 16-amino-acid RGD-containing peptide known to be the target for several cellular integrin receptors. Helper-free retroviral particles carrying a *neo-lacZ* retroviral vector were produced with the mutant envelopes. SU mutants in which regions III and IV were substituted yielded normal levels of envelope precursors but were not detectably processed or incorporated in viral particles. In contrast, substitutions in regions I and II did not affect the processing and the viral incorporation of SU mutants. When FLA16 was inserted in region II, it could be detected with antibodies against FLA16 synthetic peptide, but only when viral particles were deglycosylated. Viral particles with envelopes mutated in region I or II were able to infect avian cells through the subgroup A receptor at levels similar to those of the wild type. When viruses with envelopes containing FLA16 peptide in region II were applied to plastic dishes, they were found to promote binding of mammalian cells resistant to infection by subgroup A avian leukosis viruses but expressing the integrins recognized by FLA16. Deglycosylated helper-free viruses obtained by mild treatment with *N*-glycosidase F have been used to infect these mammalian cells, and infections have been monitored by neomycin selection. No neomycin-resistant clones could be obtained after infection by viruses with wild-type envelopes. Conversely, colonies were obtained after infection by viruses with envelopes bearing FLA16 in region II, and the genome of the retroviral vector was found correctly integrated in cell DNA of these colonies. By using a blocking peptide containing the minimal adhesive RGD sequence contained in FLA16, we have shown that preincubation of target cells could specifically inhibit infection by viruses with FLA16.

Retroviruses use a discrete number of cell surface receptors to initiate infection (for a review, see reference 57). The retroviral component which mediates interaction with receptor and entry of the virus into the cell is the envelope glycoprotein (reviewed in references 36 and 57). Retroviruses that infect human cells have been categorized into eight distinct receptor subgroups (55). Avian retroviruses of the avian leukosis virus (ALV) group have also been divided into several envelope subgroups according to their host range and interference patterns (49), as were murine leukemia viruses (MLVs) (13, 51) and feline leukemia viruses (39, 54).

Today, six receptors for retroviruses have been cloned and identified. Different physiological functions have been assigned to some of these molecules, and no common structures or functions seem to be shared by all these cell surface receptors. It seems that any cell surface molecule may function as a virus receptor. However, given the high potential for adaptability of retroviruses, it is interesting that such a small number of cell surface molecule types are used as retroviral receptors. Indeed, retroviruses have evolved efficient mechanisms—by virtue of random integration in the host genome and reverse transcriptase-mediated recombination—that have allowed them to incorporate oncoproteins in their own genomes. Interestingly, during long-term replication in animals the only reported incorporations of host-derived sequences that may affect host ranges involve recombinations with endogenous retroviral envelopes. In vitro, retroviruses can be coated by the envelope proteins of unrelated enveloped viruses, thereby allowing the use of receptors of the pseudotyping viruses. These properties are well documented, and the phenomenon is known as phenotypic mixing (63). However, it is not known whether such events can occur in vivo.

Whether the retroviruses are able to use receptors other than those described so far is a question of considerable importance. For example, retroviruses are being used more and more widely for gene transfer. The envelopes used so far to package MLV-based retroviral vectors (from amphotropic MLV or from gibbon ape leukemia virus for those used for human gene therapy) can recognize a vast array of cell types with few tissue restrictions (44). It will be advantageous to use targeting retroviral vectors allowing delivery of transgenes in specific cell types either when these target cells are rare or when the transgene encodes a toxic product that should not be expressed in all cell types. Such vectors might be obtained by altering the viral envelope so that the modified product can recognize cell surface molecules specifically expressed on the target cells. These modifications should retain the other envelope functions, and after binding to this new receptor, the virus

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should also be able to achieve the other steps of viral infection, such as membrane fusion and uncoating, leading to cell uptake of the viral genetic material.

Few successful attempts to address the question of redirection of viral tropism have been reported to date. Hybrid retroviral particles consisting of retroviral core and envelopes of various viruses have been produced and shown to infect cells through the receptors recognized by the pseudotyping envelope (11, 22). Nonviral cell surface molecules can also be efficiently incorporated on retroviral core particles. For example, wild-type CD4 molecule as well as CD4 chimerized with the retroviral envelope cytoplasmic domain have been incorporated on Rous sarcoma virus (RSV) particles (60), opening a way to the design of retroviral vectors coated with virtually any ligand or receptor molecule. Practically, however, successes may be limited, as viral incorporation of nonviral constituents is rather unpredictable (20, 21). In addition, even if viral incorporation of such molecules could be efficient, coincorporation of a fusion protein such as those of paramyxoviruses would also be required to obtain fully infective viral particles.

Another approach for targeting gene delivery is to manipulate the retroviral SU (surface) protein itself so that it can recognize other receptor molecules. Bridging MLV virions to various receptors on cell types resistant to MLV infection with antibodies has provided a first argument that retroviral host range was manipulable. Human cell types expressing HLAs or epidermal growth factor receptors or others could be infected with ecotropic MLV vectors following the addition of molecular adaptors between the virion envelope and the cell receptors (26, 52). Neda et al. have chemically modified the carbohydrate moieties of ecotropic virions by grafting of lactose, which renders them competent for infection of human hepatocytes through the asialoglycoprotein receptor (46). A potentially more useful and versatile approach was recently reported by Russell et al. (53). They have inserted a fragment encoding a single Fv antibody chain at the N terminus of the Moloney MLV *env* gene. This 250-amino-acid insert did not impair the incorporation of the mutant SU on the virions, and the mutant viruses could recognize the corresponding epitopes. That such modified viruses can infect cells through cell surface antigens recognized by single-chain antibodies has not yet been demonstrated. However, the finding that large inserts can be added into the retroviral envelope is promising. Several groups, including ours, had tried to replace large fragments of the envelope with foreign protein domains without success. Problems were generally related to incorrect processing of the surface subunit, as well as lack of viral incorporation of the chimeric product.

We describe here a strategy consisting of minimal modifications introduced in naturally variable or flexible parts of the envelope. Such small modifications are likely to have less effect on SU processing, on viral incorporation of mutant SU, and on postbinding events required for infection than insertion or replacement with large fragments because the overall scaffold of the envelope is retained.

The *env* genes of ALVs represent a group of allelic sequences that encode proteins capable of interacting with different cell receptors and thus are particularly suitable for this strategy. ALVs form a group of avian tropic viruses, with the exception of subgroup D ALVs and some subgroup C strains which can penetrate mammalian cells (56, 57). Recent experiments suggest that the ability of the latter viruses to enter some mammalian cells might be due to amino acid changes resulting in an unstable envelope that is fusogenic in the absence of the normal receptor (10). In the work reported here, we have used the envelope gene of a subgroup A ALV which is strictly avitropic (49, 57). The subgroup A receptor has recently been cloned (61) and belongs to the low-density lipoprotein receptor family (4). ALV envelope proteins of the different subgroups display a high degree of sequence homology, except for five stretches of sequence heterogeneity which are clustered in the same relative positions along the various ALV envelopes and which contain the determinants of host ranges of the different virus subgroups (8, 9, 23, 24). The two larger determinants, named *hr*1 and *hr*2, are 50 and 30 amino acids long, respectively, and display the greatest sequence variability. However, they contain conserved amino acids such as proline, glycine, cysteine, and hydrophobic amino acids which play key roles in protein folding. This suggests similar folding for the binding domains of the various envelope subgroups. Within this binding domain scaffold, sequences variable in length or in amino acid content may contribute to defining the host ranges of the envelopes by directly interacting with the receptor. Their replacement by foreign amino acid sequences of similar sizes and structural characteristics may therefore affect only envelope-receptor interactions and not the overall conformation of the SU. On the basis of that postulate, we identified by computer modelling four regions which may have these characteristics. We then questioned whether the replacement of these regions by foreign peptides with new binding specificities would affect the tropism of mutant viruses. In the work reported here, we describe the biological properties of ALV subgroup A mutant envelopes in which we have inserted a small RGD-containing peptide, FLA16 (CQGATFALRGDNPQGC) (1), which is a ligand for cell surface receptors of the integrin superfamily (37). The viral particles produced with some mutant envelopes bearing FLA16 were shown to specifically use integrins as receptors to initiate infection of mammalian cells. Subsequently, the retroviral vector genome carried by these viral particles was stably integrated and expressed in host cell DNA.

MATERIALS AND METHODS

Cell lines. QT6 cells susceptible to infection by subgroup A ALVs were kindly provided by C. Moscovici (45). The HNL cell line (18) was derived from the QT6 line and was designed to produce Rous-associated virus type 1 (RAV-1) *gag-pol* proteins, as well as RNAs of the NLB retroviral vector which carries the Neo^r and *lacZ* reporter genes (15, 17). HNL cells, devoid of *env* gene, could not produce infectious retroviral particles. QT6/A cells were Isolde cells (16). These QT6-derived cells have been rendered nonpermissive to subgroup A infection by expressing subgroup A envelopes after transfection of a psi-deleted ALV provirus. Human fibrosarcoma (HT1080, ATCC CCL 121) and rat glioblastoma (Rugli) cells have been previously described (3, 33). They were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL) supplemented with 10% fetal calf serum and 2 mM glutamine. These mammalian cells were chosen for their expression of integrin at their surface and for their nonpermissiveness to subgroup A ALV infection.

Plasmids and construction of chimeric *env* genes. pPhEAδ (Fig. 1A) has been described previously (18) and was designed to express the RAV-1 subgroup A *env* gene. This plasmid, and also the derived plasmids described below, contains the Phleo^r selectable marker in the same transcriptional unit (in the *gag-pol* intron), thereby allowing an efficient and reproducible expression of envelopes in stable transfectants. pPhEEδ (18) allows the expression of subgroup E envelope proteins.

Chimeric envelope proteins were expressed by the pPhE

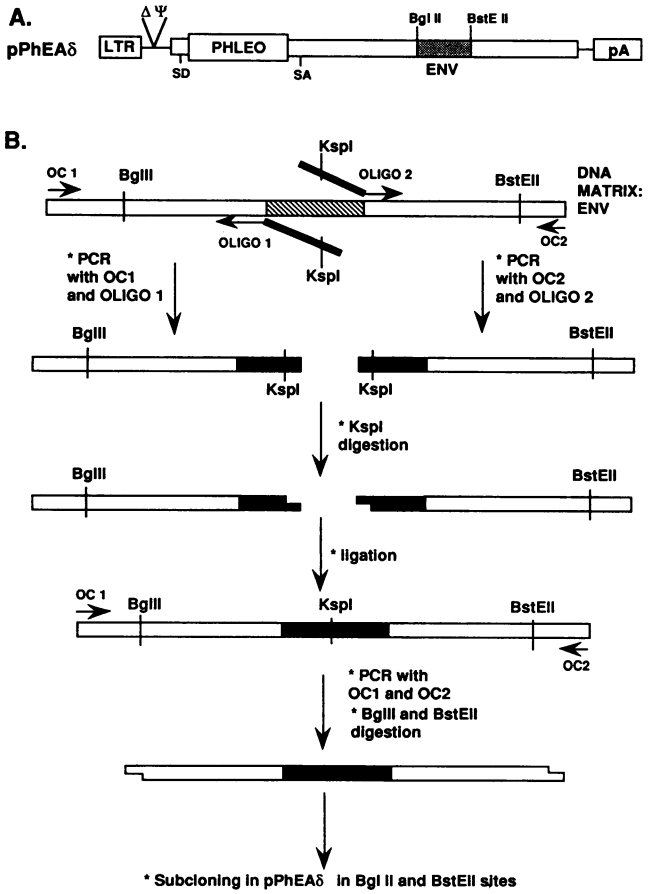

FIG. 1. General strategy for construction of envelope mutants. (A) Genetic structure of pPhEAδ expressing the ALV subgroup A envelope and the Phleo selectable marker. The shaded box (between the *Bgl*II and *Bst*EII restriction sites) represents the portion of the *env* gene which encodes the receptor-binding domain and which was modified by using PCR for construction of mutants. SD and SA are splice donor and acceptor sites, respectively. pA is the simian virus 40 polyadenylation sequence, and LTR is the RAV-1 long terminal repeat. Δψ, deletion of the packaging signal in the leader region. (B) Strategy used for mutagenizing regions between *Bgl*II and *Bst*EII. The shaded box represents the region of the *env* gene which is replaced by the new sequence (black box). See Materials and Methods for details.

plasmids (derived from pPhEAδ) in which portions of the wild-type *env* gene contained within the *Bgl*II and *Bst*EII restriction sites were replaced by fragments encoding the FLA16 ligand. The following mutagenesis strategy was adopted (Fig. 1B). Two oligonucleotides (OC1, CTACAGCT GTTAGGTTCCC; OC2, AATGTCAGTGGGTGTTGC) were located upstream of the *Bgl*II and downstream of the *Bst*EII restriction sites, respectively. For each *env* mutant, two PCR fragments were generated by using the subgroup A *env* gene as the template. A 5′ fragment was generated by using OC1 and a 5′ mutagenesis oligonucleotide (OLIGO 1 in Fig. 1) containing the 30 nucleotides belonging to the *env* gene immediately upstream of the mutation introduced and also some nucleotides coding for the 5′ half of the insert with a *Ksp*I restriction site. A 3′ PCR fragment was generated by using OC2 and a 3′ mutagenesis oligonucleotide (OLIGO 2 in Fig. 1) containing the 30 nucleotides corresponding to the *env* gene immediately downstream of the mutation and also some nucleotides coding for the 3′ half of the insert with a *Ksp*I restriction site. Both PCR fragments were digested by either *Bgl*II and *Ksp*I (for the 5′ PCR fragment) or *Ksp*I and *Bst*EII (for the 3′ PCR fragment). Both fragments were then coligated in pPhEAδ from which the *Bgl*II-*Bst*EII *env* fragment encoding the wild-type binding domain had been removed. All PCR-derived fragments were sequenced in the resulting plasmids by using either OC1 or OC2 as the sequencing primer.

pPhE2 was generated by using OL2 (TGTACCGCGG AGCGCGAAGG TACCGGCTTG GGGGCGTCTA AGG AGAAACC GCGTGGC) and OU2 (CGCGCCGCGG CG ATAATCCC CAAGGATTCT CTAACTCCTC GAAACC GTTT ACAGTG) as 5′ and 3′ mutagenesis oligonucleotides, respectively. pPhE2C was generated by using OL2C (CACTC CGCGG AGTGCAAAGG TGCCAGCTTG ACAGGGGC GT TAAGGAGAA ACCGCGTGGC) and OU2C (CCAC CCGCGG TGACAATCCA CAAGGCTGCT TCTCTAAC TC CTCGAAACCG TTTACAGTG). pPhE3 was generated by using OL3 (GCCACCGCGG AGCGCAAACG TCCCA GCTTG GCAGTTATAT ATGTTCCAAA ATCTGTAGCC) and OU3 (GCTCCCGCGG AGATAATCCG CAAGGCT GTG GCAATGCACG CCGCCCCCGC CCGGGT). pPhE4 was generated by using OL4 (CTGTCCGCGG AGGGC-GAAGG TTCCAGCTTG ACAGCGGTAC TGCTGCCCCA CCTGTGAGC) and OU4 (ATGCCCGCGG CGACAAC-CCT CAAGGTCCTG AAACCCAGTG TACAAGGAGA GGAGGC). pPhE4C was generated by using OL4 and OU4C (CCCTCCGCGG TGATAATCCA CAAGGGTGTA CAAG-GAGAGG AGGCAAATGG G).

Rescue of viral particles. HNL cells were transfected with DNA of the various pPhEAδ-derived constructs by using cationic liposomes (lipofectin; Gibco-BRL). Transfected cells were selected with phleomycin (50 μg/ml; CAYLA), and phleomycin-resistant clones were pooled. Bulk populations of more than 100 Phleo$^+$ clones were grown to limit individual variations. This strategy ensured reproducible levels of envelope protein expression after the various transfection experiments. As a control, pPhEAδ expressing the wild-type *env* gene was also transfected in parallel. Producer cells were grown to confluence, and viral particles were harvested after overnight incubation of the cells in a medium containing 1/3 normal QT6 medium and 2/3 Optimem-1 medium (Gibco-BRL). After being cooled on ice, viral supernatant was centrifuged for 10 min at 3,000 rpm at 4°C to remove cell debris and ultracentrifuged for 1 h at 30,000 rpm at 4°C in a Beckman 50.2 Ti rotor. Pelleted viral particles were suspended in 1/100 of the initial volume in cold phosphate-buffered saline (PBS) and centrifuged for 5 min at 13,000 rpm at 4°C to pellet aggregates. This protocol ensured a rough purification of virions from other components of the culture media. For infection of cells, after treatment with *N*-glycosidase F (PNGase F) (Boehringer Mannheim), viruses were diluted with normal medium and filtered through 0.45-μm-pore-sized acrodiscs.

Binding assays. Ninety-six-well plates for tissue culture (Costar) were coated at 4°C overnight with dilutions of virus supernatants harvested in serum-free medium or with collagen IV as a positive control. Plates were postcoated for 1 h with 1% bovine serum albumin (BSA) in PBS to block nonspecific cell adhesion to plastic. Cells, suspended in serum-free DMEM, were seeded with cells at a density of 1×10^5 to 5×10^5 cells per ml (100 μl per well), and the culture was incubated on the coated wells for 40 min at 37°C. Nonattached cells were removed by PBS washes. Adherent cells were fixed with 1% glutaraldehyde, stained with crystal violet, and quantitated by color readings done with an enzyme-linked immunosorbent assay reader as previously described (2). Each experimental

point was carried out with triplicate wells, and nonspecific binding to wells coated with BSA only was automatically subtracted.

Deglycosylation of viral particles. Concentrated viral particles suspended in PBS were incubated for 1 h at 37°C with 1 U of PNGase F (Boehringer Mannheim) per 100 μl of viral suspension. Efficacy of trimming of $gp85^{SU}$ carbohydrates was checked by Western blots (immunoblots).

Infection assays. Viral particles produced with the different envelope mutants were titrated by using the *lacZ* and Neo^r reporter genes carried in their genomes as previously described (17). Subgroup A receptor tropism of the viruses was assayed by using QT6 (subgroup A permissive) or Isolde cells as the target. To assay for integrin receptor tropism of the viruses, 100-mm-diameter plates were seeded with 10^6 Rugli cells per plate. The day after, plates were washed with serum-free medium and cells were, or were not, incubated at room temperature for 20 min in serum-free medium containing 0.2 mM either RGDS (competitor for binding to integrin) or RGES (negative control, but structurally similar to RGDS) synthetic peptide [BACHEM (UK), Ltd.]. After removal of the conditioning media, cells were incubated overnight with the viruses diluted with 4 ml of normal medium. Infected cells were trypsinized the day after and were plated on 170-cm^2 plates. After adhesion, cells were selected in normal medium supplemented with 2 mg of G418 (Gibco-BRL) per ml. Neomycin-resistant clones were scored 2 weeks later.

Western blots. Transfected or untransfected HNL cells were suspended with 0.02% EDTA in PBS and lysed with radioimmunoprecipitation assay buffer. Lysates were clarified by centrifugation, and the supernatants were mixed with Laemmli buffer containing β-mercaptoethanol (5% final concentration), boiled, and stored at −20°C until further analysis. Viruses obtained as described above were lysed by being boiled for 3 min in the same Laemmli reducing buffer and stored at −20°C.

The various samples were subjected to sodium dodecyl sulfate–9% polyacrylamide gel electrophoresis followed by blotting onto nitrocellulose filters. Filters were blocked with PBS-milk and incubated either with a monoclonal antibody (CVI-ALVgp85-61.6) (47) against RAV-1 SU (kindly provided by G. Koch) or with anti-FLA16 rabbit antiserum. After washes, filters were incubated with horseradish peroxidase-conjugated antibodies to mouse or rabbit immunoglobulin Gs. Blots were revealed by electrochemiluminescence (Amersham Life Science).

Protein modelling studies. The protein sequence analysis was performed with the help of the ANTHEPROT suite of programs (29, 31). The multiple alignment was carried out with the Clustal program (35) with default gap penalties of 8. The substitution matrix was that of Dayhoff et al. (19). The secondary structures were predicted by using statistical methods (14, 28) or homology-based algorithms (30, 42). The hydropathy profile was generated by the method of Kyte and Doolittle (41). The solvent accessibility profile was obtained as described by Boger et al. (6). The flexibility profile was derived from the temperature B factor (40) measured from a set of crystallographic data of 19 protein structures.

RESULTS

Theoretical considerations, supporting the construction of envelope mutants. In order to rationalize the approach of changing virus tropism by insertion of peptides into ALV envelopes, we have used physicochemical profiles and the combination of multiple alignments with secondary-structure predictions. Potentially interesting regions to mutate should be (i) poorly conserved within the different subgroups of envelopes, (ii) poorly structured to avoid altering or losing important regular secondary structures, and (iii) easily accessible (hydrophilic) and/or flexible.

The multiple alignment of four subgroups (A, B, C, and E) of ALV envelopes is shown in Fig. 2a. These proteins appeared very well conserved, with 247 matches among 353 aligned residues (70% global identity). As demonstrated by others using genetic recombinants, the multiple alignment suggested that the regions conferring specificity in receptor recognition are located within two poorly conserved patches named *hr*1 and *hr*2 (8, 9, 23, 24). Yet, some amino acids likely to be important for secondary structures are conserved between the different envelope subgroups within *hr*1 and *hr*2. To delineate more precisely the regions best suited for mutation, we have undertaken a study aimed at predicting the structures of ALV envelope proteins.

Secondary-structure predictions (displayed in Fig. 2b for subgroup A SU envelope protein only) suggested that all ALV SU proteins belong to the all-β structural class. This property was confirmed by the structural class prediction algorithm based on amino acid composition (data not shown). Moreover, the β-sheets appeared to be mostly antiparallel, since sheet-turn-sheet patterns are predominant along the sequences. In addition, almost no helices were predicted and the helical contents were below 10% regardless of the subgroup of the envelope considered. We have delineated four regions that are interesting to replace, according to the above-mentioned criteria, in *hr*1 and *hr*2. Region I, located in *hr*1, has been identified on the basis of a high level of solvent accessibility and low probabilities of being a structured region. It is also poorly conserved between subgroups. Region II, also located in *hr*1, is one of the most flexible regions of subgroup A SU protein and may form a β-turn. Region III and region IV, located in *hr*2, are the most hydrophilic and are likely to be folded in aperiodic conformational states (probably large loops).

The sequence of FLA16, a 16-mer peptide (CQGATF ALRGDNPQGC) (1) that binds RGD-dependent integrins, was inserted in various configurations in place of either region II, III, or IV of subgroup A envelope by PCR-mediated mutagenesis. Plasmid pPhEAδ (Fig. 1A), expressing the wild-type envelope, was used as the backbone for construction of mutants. FLA16 was inserted in place of region II with its two cysteines at each end, resulting in mutant envelope E2C. A second mutant altered in region II, E2, was designed to take advantage of the putative β-turn of that region. In this mutant, FLA16 was inserted without flanking cysteines. For mutant E3, the 14 amino acids of FLA16 were flanked with the cysteines that bound region III itself (Fig. 2a). Two mutants altered in region IV were designed. For each, only the 14-amino-acid core of FLA16 was used because region IV is already bordered by conserved cysteines. For mutant E4C, FLA16 was inserted between these two cysteines (Fig. 2a), whereas for mutant E4, four amino acids (PETQ) which are more-or-less conserved between the four envelope subgroups were retained between the two cysteines of region IV, together with the 14 amino acids of FLA16.

Expression of chimeric envelopes. HNL cells, which provide ALV *gag* and *pol* proteins and genomic RNAs of the Neo^r *lacZ* NLB retroviral vector, were transfected with constructs. Pools of stably transfected cells were grown for each pPhE plasmid and were assayed for envelope expression by Western blotting (Fig. 3) (summarized in Table 1) with an anti-SU monoclonal antibody. As expected, cells transfected with construct pPhEAδ were found to express the wild-type envelope

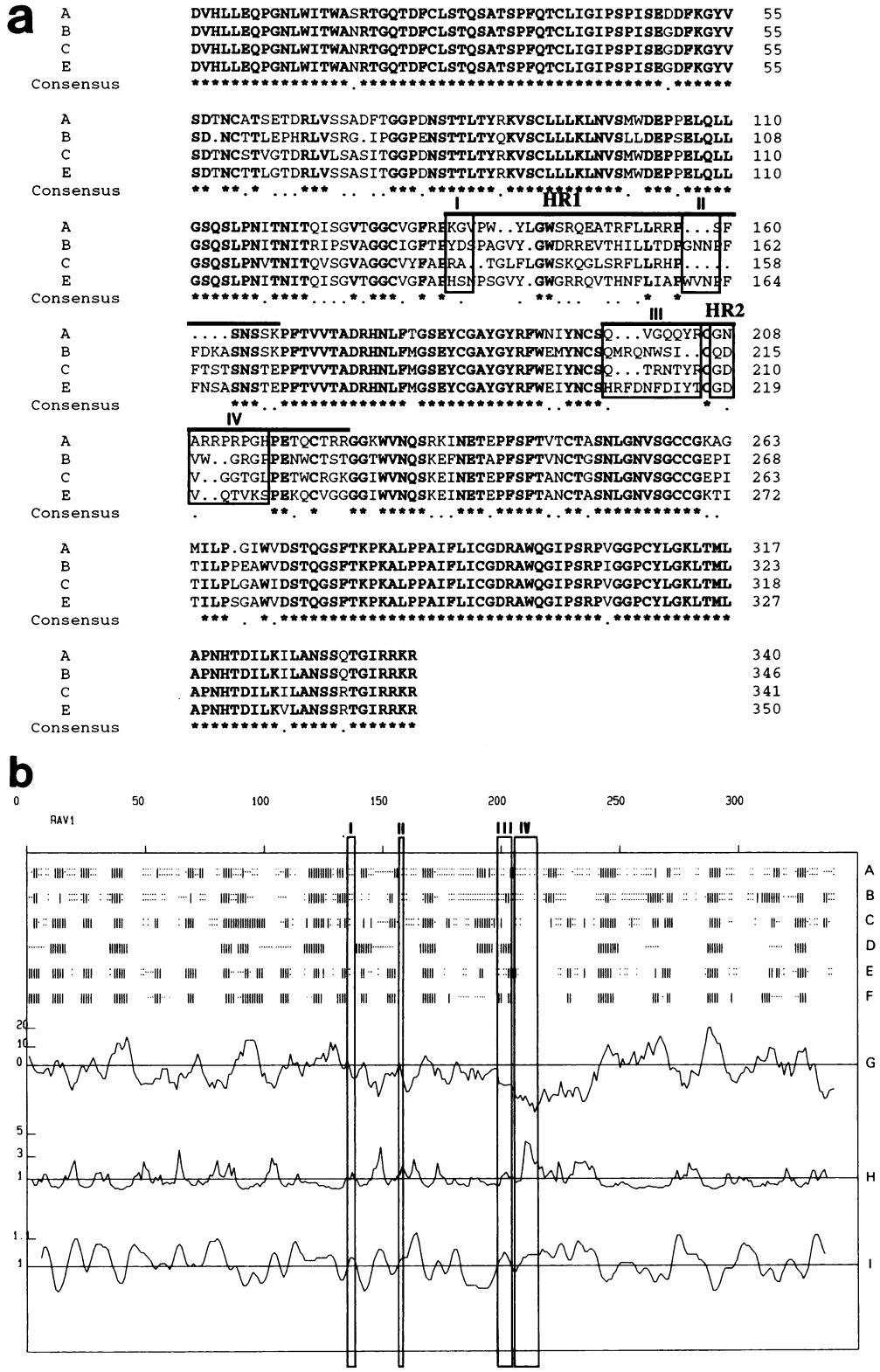

FIG. 2. Delineation of sites of insertion in SU. (a) Multiple alignment of the deduced amino acid sequences of the surface envelope proteins from subgroup A (RAV-1) (9), B (RAV-2) (8), C (Prague RSV-C) (9), and E (RAV-0) (9, 24) ALVs. Regions I, II, III, and IV, which were replaced by FLA16 adhesive peptide, are indicated by small boxes. *hr*1 and *hr*2 host range determinants (23, 24) are indicated. The asterisks indicate identical residues, and the points indicate conserved residues (values greater than 17 in the substitution matrix). (b) Predicted secondary structures and physicochemical profiles of RAV-1 subgroup A SU protein. The secondary structures (rows A through F) have been predicted according to the GOR II (32), GOR I (28), double prediction (31), Chou and Fasman (14), HOMOLOGUE (42), and SOPM (30) methods, respectively. ·····, α-helices; ::::::::, β-turns; |||||||, β-sheets. Blanks in the sequence represent coil segments. The numbered scale indicates the position of residues in the sequence. Lines G to I are, respectively, the hydropathy, the solvent accessibility, and the flexibility profiles.

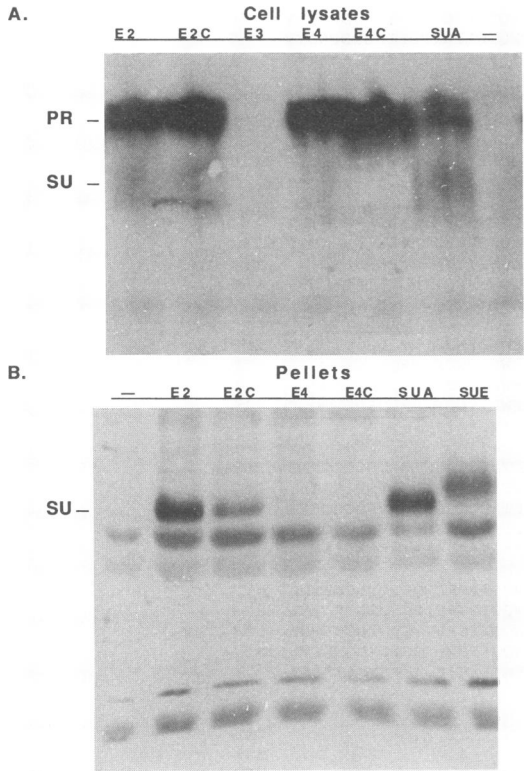

FIG. 3. Western blots of cell lysates or of viral pellet. Detection of envelope proteins from cell lysates (A) or from viral pellets (B) is shown. Samples are from nontransfected HNL cells (−) and from HNL cells transfected with pPhE2 (E2), pPhE2C (E2C), pPhE3 (E3), pPhE4 (E4), pPhE4C (E4C), pPhEAδ (SUA), and pPhEEδ (SUE). PR, p95 envelope precursor; SU, gp85 surface protein. Blots were stained with the anti-SU monoclonal antibody (CVI-ALVgp85-61.6).

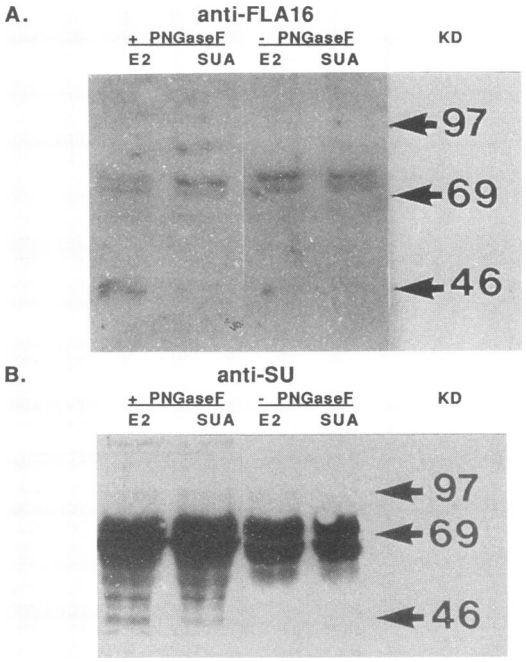

FIG. 4. Detection of FLA16 peptide in viral envelope. Immunoblots of viruses NLB(A) (SUA) and NLB(E2) (E2) treated (+) or not treated (−) with PNGase F. Blots were stained with an antiserum against FLA16 synthetic peptide (A) or with the anti-SU monoclonal antibody to check the efficiency of deglycosylation (B).

precursor p95 as well as the processed $gp85^{SU}$ product. In addition, subgroup A SU was detected on viral particles produced in the supernatant of the same cells. Subgroup E SU (from pPhEEδ-HNL-transfected cells) also could be detected with the monoclonal antibody, showing that the recognized epitope was not located in the binding domain of the envelope.

Except with HNL cells transfected with pPhE3, precursors of all chimeric envelopes were found in cell lysates and were detected at levels similar to those of the wild type (Fig. 3A). However, depending on the mutant tested, variable levels of SU processing were found. No SU could be found in cells transfected with pPhE4C, and envelope precursor in cells transfected with pPhE4 was inefficiently cleaved. By contrast, precursors of envelope mutants altered in region II (from pPhE2 and pPHE2C constructs) were efficiently converted into mature SU. SU conversion in E2C was reduced only twofold compared with that in the wild type. No envelope precursor could be detected on viral particles released by all categories of pPhE-transfected HNL cells, including those transfected with pPhEAδ (Fig. 3B). Incorporation of chimeric SU could be demonstrated only for envelopes mutated in region II, with an efficiency similar to (mutant E2) or slightly weaker than (mutant E2C) that of the wild-type SU incorporation. By contrast, SU from envelope mutants altered in regions III and IV could not be detected on viral particles.

Detection of FLA16 in envelopes carried by viral particles. Proteins of viral particles released from HNL cells transfected with plasmid pPhEAδ or pPhE2 were analyzed on Western blots by using an antiserum raised against FLA16 synthetic peptide (Fig. 4). Unexpectedly, bands the size of SU (85 kDa) could be found for both viruses, although the wild-type subgroup A SU does not contain the RGD tripeptide which would explain the cross-reaction with the antibodies. Alternatively, that no difference in the patterns of bands between the two viruses was observed could be due to a masking of FLA16 in the context of the envelope glycoproteins. To address this question, viral particles were treated with PNGase F, which

TABLE 1. Infection of QT6 cells

env mutant tested	Result by Western blot[a]			Titer on QT6[b]		Titer on QT6/A[c]
	p95	SU	INC	−PNG	+PNG	
E2	+++	+++	+++	5×10^4	5×10^4	$<10^1$
E2C	+++	++	++	2×10^4	2×10^4	$<10^1$
E3	−	−	−	<1	<1	<1
E4	+++	+	−	<1	<1	<1
E4C	+++	−	−	<1	<1	<1
SUA	+++	+++	+++	10^5	10^5	10^1
SUE	+++	+++	+++	10^5	ND[d]	10^5
None[e]	−	−	−	<1	<1	<1

[a] A summary of Fig. 3 is given. The presence of p95 and SU in cell lysates and of SU incorporated (INC) on viral particles is shown. +, hardly detectable; ++, readily detectable; +++, wt signal; −, not detectable.

[b] Titers as *lacZ* CFU/ml of viral supernatant determined on QT6 cells. Viruses were treated (+PNG) or not treated (−PNG) with PNGase F (PNG).

[c] Titers as *lacZ* CFU/ml of viral supernatant determined on subgroup A-resistant QT6 cells.

[d] ND, not determined.

[e] Nontransfected HNL cells were tested.

cuts N-linked carbohydrates. Mild conditions of incubation were used to retain infectivity of the viruses. Proteins of PNGase F-treated viruses were analyzed on immunoblots by comparison with proteins of intact (non-PNGase F-treated) viruses. Deglycosylation was checked by using the monoclonal antibody against SU. Bands migrating between 85 and 40 kDa (the size of the nonglycosylated subgroup A SU) were detected (Fig. 4B). When Western blots were stained with the FLA16 antiserum, a low-molecular-mass band (at ca. 45 kDa) could be detected only for proteins of viruses from pPhE2-transfected cells (Fig. 4A). This band indicated the presence of FLA16 epitope that became fully accessible to antibodies after complete deglycosylation of the protein. These data suggested that FLA16 was, at least partly, masked by SU carbohydrate chains.

Cell binding to virus substrates. The FLA16 peptide has been shown to promote binding of Rugli and HT1080 cells on plastic (1). We have asked whether the supernatants of HNL cells transfected with pPhE2 or pPhE2C, or pPhEAδ as a control, had such an activity (Fig. 5A). Both mutants E2 and E2C induced adhesion of Rugli cells in a dose-dependent manner and at a level well over the binding observed with substrates made of viral particles with wild-type envelopes (Fig. 5A, SUA). At plateaus, cell adhesion to mutant E2 and mutant E2C represented 80 and 60%, respectively, of the maximal cell adhesion recorded on collagen IV used as a positive control.

Qualitative differences in cell adhesion were observed between the different substrates tested (Fig. 5B). On substrate made from supernatant of viruses with wild-type envelopes (Fig. 5B, SUA), most of the few adhered cells remained round. Conversely, when supernatants containing viruses with FLA16 mutant envelopes (E2 and E2C) were used to coat wells, not only were a higher proportion of the cells adherent, but they were also well spread, as was particularly obvious for HT1080 cells. The unexpected binding observed with the wild-type substrate (30% at the plateau) was probably due to some extracellular matrix proteins released by the producer cells which could induce some level of cell adhesion.

Taken together, these data suggested that FLA16 inserted in the context of a viral envelope could promote cell adhesion by providing a new cellular target receptor.

Infection assays. QT6 cells were used to assay for subgroup A receptor tropism of viral particles with the different chimeric envelopes (Table 1). The NLB retroviral vector contained in the viral particles was used to monitor infection. Infected cells were assayed after 2 days for β-galactosidase expression by using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. No β-galactosidase-positive colonies were found following infection with viral particles released by HNL cells alone or released by HNL cells transfected with constructs expressing envelopes modified in regions III and IV [viruses NLB(E3), NLB(E4), and NLB(E4C)]. In contrast, β-galactosidase-positive colonies were detected after infections of QT6 cells with viral particles carrying envelopes mutated in region II [viruses NLB(E2) and NLB(E2C)] and, as expected, with virus NLB(A) coated with nonmutated envelopes. Titers of viruses with mutations in region II were found to be reduced two- to fivefold in comparison with those of NLB(A).

To check the subgroup A receptor specificity of infection, Isolde cells, derived from the QT6 line but resistant to subgroup A ALV infection, were used for infection with viruses (Table 1). A strong interference, reducing the titers of all three viruses by about 4 orders of magnitude, was observed, showing that the viruses had a subgroup A tropism on avian cells. By contrast, viral particles from HNL cells transfected with pPhEEδ [producing virus NLB(E) with subgroup E envelopes] could easily infect Isolde cells, demonstrating the specificity of subgroup A receptor interference. Taken together, these data suggested that modifications in region II did not affect the initial tropism of the viruses.

TABLE 2. Infection of Rugli cells

Envelope tested[a]	Result for PNGase F-treated virus preincubated with[b]:			−PNGase F result[c]
	No peptide	RGES	RGDS	
SUA	<1	<1	<1	2 ± 2
E2	78 ± 9	80 ± 10	7 ± 5	16 ± 4
Am	10^2 ± 10	95 ± 8	98 ± 9	ND

[a] Envelopes used to coat the Neo[r] retrovirus vectors were the ALV subgroup A envelope (SUA), the subgroup A envelope with FLA16 in region II (E2), and the MLV amphotropic envelope (Am).

[b] Number of neomycin-resistant colonies per 50 μl of deglycosylated viruses coated with SUA or E2 envelopes (the Am-coated vector was not treated with PNGase F). Rugli cells were incubated without peptides or with 0.2 mM either RGDS or RGES synthetic peptide before infection.

[c] Results of infection with non-PNGase F-treated viruses are shown. ND, not determined.

To address the question of an additional tropism dependent on FLA16, mammalian cells resistant to ALV subgroup A infection but expressing high levels of integrins recognized by FLA16 were used as target cells (Table 2). Rather than using the *lacZ* gene of the NLB retroviral vector as the reporter for infectivity, infected cells were selected with G418 and resistant colonies were scored. Since deglycosylation of viral particles was shown to increase the accessibility of FLA16 to antibodies (Fig. 4), for these experiments we used viral particles treated with PNGase F. When the titer of PNGase F-treated viruses was determined with QT6 cells, no obvious differences could be seen in comparison with titers of nontreated virions (Table 1), suggesting that carbohydrates may not be required for subgroup A ALV envelope-receptor interactions, as already shown for other viruses. A very low and nonreproducible background of neomycin-resistant colonies was observed when Rugli cells were infected with viral particles coated with wild-type envelope [virus NLB(A)]. In contrast, viruses coated with FLA16-containing envelopes [virus NLB(E2)] gave rise to about 80 G418-resistant colonies per 50 μl of deglycosylated viral suspension (Table 2). Consistent with data shown in Fig. 4, infection with non-PNGase F-treated NLB(E2) viruses gave rise to significantly fewer neomycin-resistant colonies.

To assay for specificity of infection via the integrin receptor, Rugli cells were incubated with either of the synthetic RGDS or RGES peptides at 0.2 mM prior to infection with the deglycosylated viruses NLB(A) and NLB(E2) (Table 2). Incubation with peptides had no side effects, since cells infected with an MLV-based retroviral vector (BAG) (12) coated with amphotropic MLV envelope gave rise to similar numbers of neomycin-resistant colonies whatever the preinfection conditions (with or without peptides). Infection with NLB(E2) viruses gave rise to equivalent numbers of neomycin-resistant colonies when the cells were preincubated with RGES (nonblocking) or without peptide. In contrast, 10-fold fewer neomycin-resistant colonies were obtained when cells were incubated with the RGDS blocking peptide prior to infection with NLB(E2). Taken together, these results suggested that viruses with FLA16 in the envelopes could infect cells by using integrins as receptors. G418-resistant clones were stained with X-Gal to reveal in infected Rugli cells the expression of *lacZ*, the second gene carried by the NLB retroviral vector. *lacZ* gene expression could be detected in these colonies, suggesting

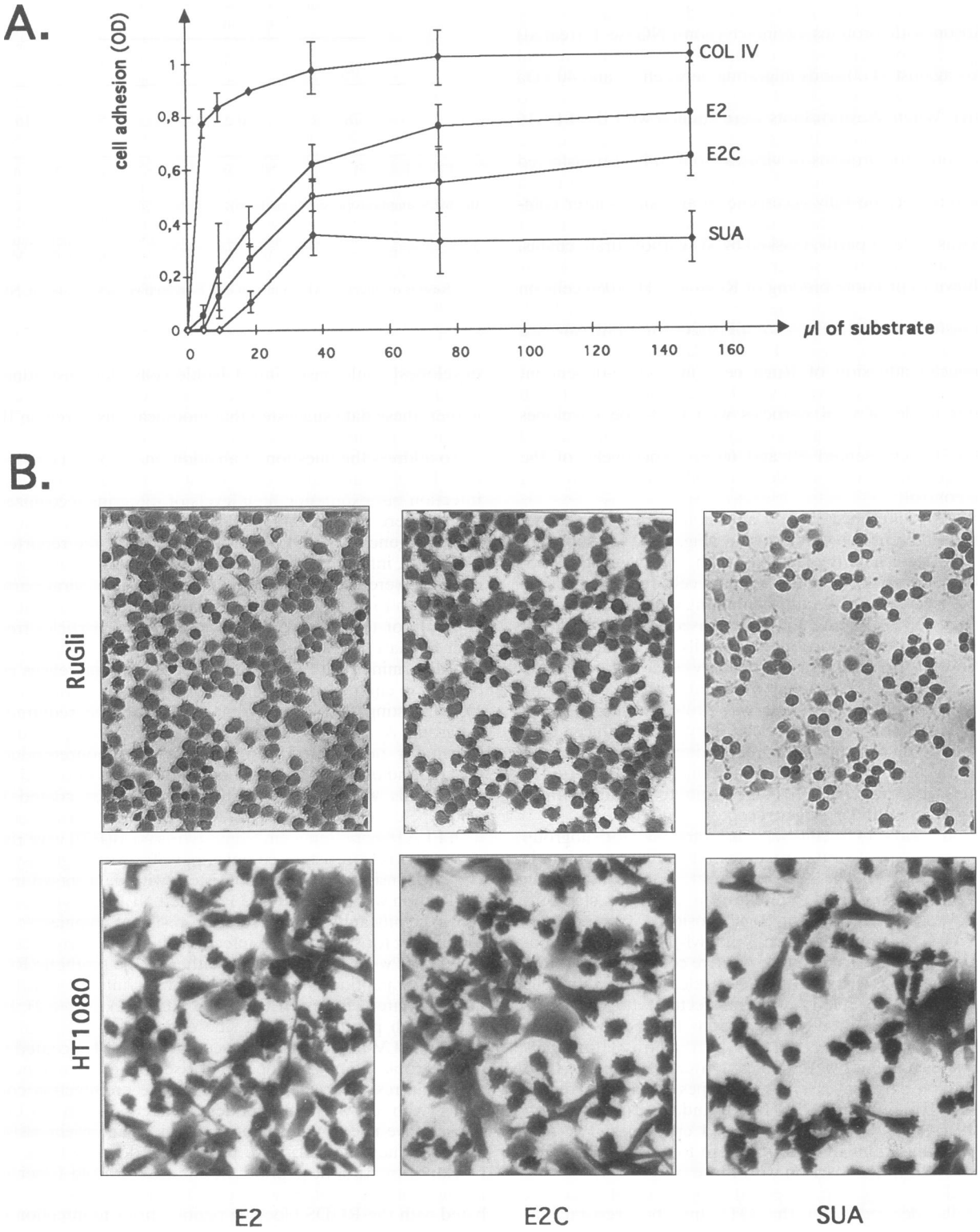

FIG. 5. Binding assays of HT1080 or Rugli cells. (A) Dose-response curves for the binding of Rugli cells to substrates made of collagen IV (COL IV) or of viruses NLB(A) (SUA), NLB(E2) (E2), and NLB(E2C) (E2C). The number of adherent cells was measured by a colorimetric assay and is given as optical density (OD). (B) Phase-contrast photographs of Rugli or HT1080 cells on virus substrates. The same substrate dilutions were used for all three viruses. Abbreviations for viruses are as described in the legend to panel A.

that infection with NLB had occurred as expected. To check that NLB was normally integrated in these infected cells, DNA from the population of neomycin-resistant cells was prepared. The presence of NLB retroviral vector was demonstrated by Southern blots by using *lacZ* or the Neo^r gene as the probe. Bands at sizes consistent with a correctly integrated NLB provirus could be detected. Junction fragments were also found, demonstrating that NLB vector was stably inserted in cell DNA (data not shown).

DISCUSSION

The determinants of the host range of ALVs are located in a central portion of the envelope (*hr*1-*hr*2) which is highly variable among the different subgroups of ALVs (8, 9, 23, 24). By using computer modelling, we have identified four small regions in *hr*1-*hr*2 which may represent flexible parts of a binding domain structurally related among the different envelope subgroups. A set of envelope mutants was generated by replacement of these regions with foreign peptides of similar sizes in a subgroup A envelope gene backbone.

Minimal modifications introduced in the envelope genes generally did not affect the synthesis of an envelope precursor which, except for that of mutant E3, was the expected 95-kDa size. Two constructs not reported here and consisting of chimeric *env* genes mutated in region III by replacement with peptides different from FLA16 have been shown to express normal levels of p95. That no precursor could be found for mutant E3 might be due to a specific effect of FLA16—when inserted in region III—resulting in an unstable envelope precursor with a very short half-life or, alternatively, unable to be recognized by the anti-SU monoclonal antibody used in this work. Assuming that the case of mutant E3 is an exception, our data show that even if these small modifications had no effect on synthesis of the precursors, mutation of regions III and IV may have important consequences on the maturation process of the altered SU. Most of the mutants altered in these two regions, including others not reported in this work, did not maturate into SU and could not be incorporated in viruses. It could be that such mutants remain blocked in the endoplasmic reticulum because they are not correctly folded and hence cannot form the oligomers required for intracellular transport and expression at the cell surface (25, 36). In contrast, replacement of regions I (data not shown) and II located in *hr*1 with peptides up to 16 amino acids long impaired neither SU processing nor incorporation on viral particles. Moreover, all of these latter mutants retained the subgroup A tropism and fusogenic capacities of the envelope. As shown by the multiple alignment of the sequences of the different envelope surface proteins, *hr*2 is the most variable region of the binding domain. Predictions of secondary structures of the protein show that *hr*2 contains mostly turns or bends and, at least in the subgroup A envelope, is highly hydrophilic. Conversely, *hr*1, albeit variable, contains more structured regions which, in addition, are conserved between envelope subgroups. This suggests that *hr*1 is required to induce a general conformation of the binding domain, whereas *hr*2 may act by finely defining interactions with the envelope-binding site of the receptor. That replacement of regions III and IV in *hr*2 results in the loss of subgroup A tropism whereas *hr*1 seems to tolerate small modifications while retaining the initial subgroup A tropism is therefore not very surprising. It should be noted that peptide FLA16 is rather simple, from the point of view of protein folding, as it does not contain amino acids able to disrupt secondary structures. It remains to be demonstrated that region I or II can accept more complex peptides, i.e., induction of loops or regular secondary structures. We are currently testing envelope mutants in which region I or II is replaced by such complex ligand peptides. Conversely, that *hr*2 is not as flexible as *hr*1, despite a high level of variability between subgroups for both primary and secondary protein structures, is surprising and may suggest a role of ALV envelope binding domains in envelope folding and/or in envelope oligomerization.

To demonstrate the feasibility of redirection of viral tropism, we have chosen the integrins as the receptor candidate. Several of these receptors recognize an RGD sequence on extracellular matrix proteins (37, 59). Integrins also promote adhesion of bacteria (38) and can serve as receptors to initiate infection with a number of viruses (recently reviewed in reference 58). Interaction between these ligands and integrins usually involves the RGD tripeptide, as shown first for fibronectin (50), but amino acid sequences adjacent to or further away from RGD may play a critical role in modulation of binding. FLA16 is a 16-amino-acid-long RGD-containing peptide corresponding to a sequence of the A chain of laminin fragment P1, which promotes cell adhesion (1). In laminin fragment P1, the peptide is probably in a cyclic, disulfide-linked loop, but the synthetic peptide has been shown to display the same inhibitory activity in both cyclic and linear forms (1).

Region II of ALV SU is predicted to fold in a β-turn as the secondary structure. Hydrophilic peptides inserted in that region may therefore be constrained in that β-turn, a situation which has been shown to favor the biological activities of various RGD-dependent ligands (43, 62). When FLA16 was inserted in region II of the ALV subgroup A envelope, its biological activity could be demonstrated both by cell-binding assays and by infection assays of mammalian cells. However, the efficiency of infection of mammalian cells with mutant E2 was rather low compared with the number of input viruses. One possible explanation could be that, as for human immunodeficiency virus or other viruses, after ALV binds to the cell surface receptor, a second factor or a coreceptor may be required which may not be present on Rugli cells. Alternatively, the poor infectivity of viruses with FLA16 in the envelope may be due to a low affinity of this peptide when inserted in the context of a viral envelope. This affinity may be high enough to allow a reasonably efficient binding of viruses but too low to induce the conformational changes of the envelope complex required to fuse viral and cell membranes. A possible way to overcome this problem would be to insert more than one copy of FLA16 in the envelope to increase its affinity. An alternative way would be to replace the recombinant FLA16 envelope in the genome of a replication-competent virus. After transfection of mammalian cells with this modified ALV, viruses which would replicate at high levels would be not those with subgroup A tropism because of the nonpermissiveness of mammalian cells toward these viruses, but rather those which would have selected some amino acid changes in the binding domain of the envelope that would allow FLA16 to be optimally folded for receptor recognition. Envelope genes of these viruses could then be cloned and used for the design of packaging cells with new specificities.

A second problem arising from our strategy to modify viral tropism could be the effect of surrounding carbohydrate chains. The mature SU protein is heavily glycosylated and contains about 60% sugar. Our results suggest that when FLA16 is inserted in region II, its recognition by an antiserum against the synthetic peptide is dependent on the deglycosylation of the envelope. Non-PNGase F-treated virions can bind to and can infect cells expressing FLA16 receptor, but both the efficiency and the specificity of infection are increased when virions are deglycosylated with that enzyme. This probably

reflects the fact that after deglycosylation, a higher proportion of FLA16 peptides become available for binding to receptors because of their unmasking and thus enhance the biological activity of the mutant envelope. That antibodies against FLA16 cannot easily detect the peptide in the context of the intact viral envelope whereas the same envelope can promote the binding of cells expressing integrins might be explained by a difference between the two methods used to demonstrate the presence of FLA16. Hence, it could be worthwhile to reduce the envelope glycosylation in order to improve our strategy for modifying envelope tropism. There are 12 putative N-linked glycosylation sites in the subgroup A SU, and it is likely that most of them are actually used because of the apparent molecular weight of the protein. Inhibiting the cellular glycosylation pathway with drugs like tunicamycin has been shown to severely impair transport and cell membrane insertion of glycoproteins and thus impair their viral incorporation (7). It may be better to mutate some of the glycosylation sites, particularly those located in the vicinity of region II. It has been shown for the influenza virus hemagglutinin that not all N-linked glycosylation sites are required for efficient translocation and membrane insertion of the envelope glycoprotein and that a minimum of five of seven sites are necessary for these processes (27). However, that this is also true for the ALV envelope remains to be shown.

Because of the low efficiency and the drawbacks of our FLA16 envelopes, the data reported here should be considered only as a general principle for redirection of viral tropism by minimal modifications of the receptor-binding domain of the retrovirus SU. A similar approach with MLV-derived retrovirus vectors, which are probably more fitting for human gene therapy, is worth considering. Detailed mapping of receptor determinants among MLV strains has recently been accomplished with recombinant envelopes (5, 34, 48). Like the *hr*1-*hr*2 regions identified in avian SU, MLV envelopes have two discontinuous regions in the N-terminal portion of $gp70^{SU}$ which determine recognition of receptors. It would be interesting to insert adhesive peptides in selected parts of these regions to determine whether this can redirect the host ranges of MLV-based retroviral vectors.

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