The Spacing between Cysteines Two and Three of the LDL-A Module of Tva Is Important for Subgroup A Avian Sarcoma and Leukosis Virus Entry

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Rong et al. have demonstrated previously that with a few substitutions, the fourth repeat of human low-density lipoprotein (hLDL-A4) receptor can functionally replace the LDL-A module of Tva, the cellular receptor for subgroup A avian sarcoma and leukemia virus (ASLV-A), in viral entry (L. Rong, K. Gendron, and P. Bates, Proc. Natl. Acad. Sci. USA 95:8467-8472, 1998). Here we have shown that swapping the amino terminus of hLDL repeat 5 (hLDL-A5) with that of Tva, in addition to the corresponding substitutions made in human LDL-A4, was required to convert hLDL-A5 into an efficient ASLV-A receptor. These results substantiated our previous findings regarding the role of the specific residues in the viral interaction domain of Tva and demonstrated the critical role of the amino terminus of the Tva LDL-A module in ASLV-A infection. Furthermore, we have shown that the residues between cysteines 2 and 3 of the Tva LDL-A module in a Tva/LDL-A5 chimeric protein can be functionally replaced by the corresponding region of another LDL-A module, human LDL receptor-related protein repeat 22 (LDL-A22), to mediate efficient ASLV-A entry. Since the only conserved feature between the C2-C3 region of LDL-A22 and the Tva LDL-A module is that both contain nine amino acids of which none are conserved, we conclude that the spacing between C2 and C3 of the LDL-A module of Tva is an important determinant for ASLV-A entry. Thus, the present study provides strong evidence to support our hypothesis that one role of the N terminus of the LDL-A module of Tva is to allow proper folding and conformation of the protein for optimal interaction with the viral glycoprotein EnvA in ASLV-A entry.

Entry of subgroup A avian sarcoma and leukemia virus (ASLV-A) into its hosts is mediated by interactions between the viral glycoprotein EnvA and a small cell surface glycoprotein, Tva (1, 29). Direct interaction of EnvA with Tva is not only important for initial viral attachment but is also important for the postbinding steps in ASLV-A entry. Binding between Tva and EnvA in vitro can induce a series of conformational changes on EnvA that lead to exposure of the putative fusion peptide and its association with the lipid membrane (7, 11, 13, 19). These results provide direct biochemical evidence that receptor binding can trigger the structural rearrangements likely required for membrane fusion mediated by a viral glycoprotein. Since receptor triggering appears to be a common yet poorly understood mechanism for entry of various enveloped viruses, analysis of the Tva-EnvA interaction serves as an excellent model in elucidating the basic principles in viral entry.

One of the major advantages of using Tva/EnvA as a model to elucidate the viral entry mechanism is that the viral interaction domain of Tva is solely determined by a single low-density lipoprotein (LDL) receptor module within the extracellular domain of Tva (3, 21), making it a simple model amenable to molecular, biochemical, and structural analysis. The LDL-A module of Tva is 40 amino acids in length and includes six invariable cysteines and five highly conserved acidic residues found in other LDL-A modules such as human LDL receptor and LDL receptor-related proteins (1). Biochemical and structural analysis of different LDL-A modules demonstrates that the six conserved cysteines are involved in the formation of three pairs of disulfide bonds. Furthermore, structural analysis of several individual LDL-A modules (and, recently, of the entire ectodomain of human LDL receptor) by X-ray crystallography showed that the side chains of four conserved acidic residues near the C terminus and the carbonyl oxygen groups of two nonacidic residues of each LDL-A module coordinate calcium binding (9, 24, 25). These common structural features of LDL-A modules are important for proper folding and, thus, for their functions in ligand binding.

The role of the Tva LDL-A module in EnvA binding and ASLV-A entry has been extensively examined by molecular, biochemical, and structural analysis. It has been demonstrated that the LDL-A module of Tva efficiently mediated ASLV-A entry when it was appended to a heterologous membrane-spanning domain (21). Furthermore, this module of Tva can be functionally replaced by a modified human LDL-A4 module in mediation of ASLV-A entry (22). These studies, together with mutational analysis (23, 31, 32), have identified several putative viral interaction residues important for viral entry. Biochemical and structural analysis demonstrated that like other LDL-A modules, the correct in vitro folding of the Tva LDL-A module is calcium dependent (26-28). Surprisingly, however, calcium is not essential for EnvA binding once the protein is correctly folded (30).
One important structural difference between the Tva and other LDL-A modules is that the Tva LDL-A module does not have the signature anti-parallel β-sheet observed at the N-terminal C1-C3 region of other LDL-A modules (5, 6, 8, 9, 17, 20, 25, 26, 28). The C2-C3 region of the Tva LDL-A module contains nine residues instead of the four to five residues seen in the other LDL-A modules, and this region of Tva is responsible for the flexible conformation at the N terminus (28). In this study, we wanted to examine the role of this region in determining the receptor function of Tva. We found that the spacing between the C2 and C3 regions of the Tva LDL-A module is an important determinant for ASLV-A entry. The present study provides strong evidence that one role of the N-terminal region of the Tva LDL-A module is to allow proper folding and overall conformation of the protein for optimal interaction with EnvA in ASLV-A entry.

MATERIALS AND METHODS

Cells and viruses. Human embryonic kidney 293T cells were maintained as described previously (22). RCAS(A)AP (10) viral stocks were generated as described previously (21). The EnvA-pseudotyped human immunodeficiency virus (HIV) viral stocks were generated using the HIV vector pNL4-3.Luc.R-E (4, 12) cotransfected with ASLV-A glycoprotein EnvA into 293T cells. Briefly, 293T cells were transiently transfected with 10 μg of the HIV vector either alone or with 10 μg of EnvA DNA or vesicular stomatitis virus glycoprotein G (VSV-G) DNA by a modified CaPO4 method as previously described (21). The supernatants (viral stocks) were harvested 48 h posttransfection, filtered through a 0.22-μm-pore-size filter, and stored at -80°C.

Cloning, mutagenesis, and mutant nomenclature. A chimeric Tva construct containing the human LDL-A5 instead of the Tva LDL-A module was generated as follows. The PCR-amplified coding region for human LDL-A5 was digested with restriction endonucleases BamHI and SacII, and the digested DNA fragment was inserted into the BamHI/SacII-digested Myc-Tva vector (22). This construct is referred to as TL5, where T stands for Tva and L5 pertains to the LDL-A5 region. The other TL5 derivatives were generated using the TL5 construct as the template by a standard two-step PCR protocol. Four amino acids in the TL5 construct are similar to that used previously for the TL4 constructs.
R19, G23, E33, and G34, were replaced with the corresponding residues of the Tva LDL-A module either individually or in combination (Fig. 1B). Two additional TL5 chimeric constructs were generated. TL5 chimera 1 has the N-terminal C1-C3 region from the Tva LDL-A module and also contains the substitutions R19L, G23H, and E33W. TL5 chimera 2 is same as TL5 chimera 1 except that it also has the N34G substitution (Fig. 1B). In addition, constructs C5 and L5 were generated using TL5 chimera 2 as the template but contained the C2-C3 region from the chicken Tva LDL-A module and the C2-C3 region of the human LDL-A22 module of LRP2, respectively.

Analysis of protein expression. Human kidney embryonic 293T cells were transiently transfected with Myc-Tva and the TL5 constructs by a modified CaPO4 method. Cells were lysed 48 h after transfection, and protein expression was examined by Western blotting as previously described (22). Myc-tagged Tva and TL5 proteins were detected using anti-myc monoclonal antibody (MAB) 9E10 as the primary antibody.

Protein surface expression was examined by fluorescence-activated cell sorter (FACS) analysis. Briefly, 293T cells were transiently transfected with 10 μg of DNAs of myc-Tva and TL5 constructs by the CaPO4 method. At 48 h posttransfection, cells were resuspended in FACs buffer (phosphate-buffered saline containing 2% fetal calf serum) to 5 × 106 cells/ml. Cells were seeded into a U-bottom 96-well plate and were incubated with 50 μl of mouse MAB 9E10 (10 μg/ml stock) for 30 min. Cells were then washed twice with FACs buffer and incubated with 50 μl of fluorescein isothiocyanate-conjugated rat anti-mouse immunoglobulin G1 antibody at a final concentration of 10 μg/ml for 20 min. Cells were washed again and resuspended in 300 μl of FACs buffer. Cells were stained for viability with 0.5 μl of propidium iodide and were analyzed using a Becton Dickinson FACSCalibur flow cytometer and CellQuest software.

ELISA-based binding test. To examine the ability of the TL5 chimeric proteins to bind EnvA, an enzyme-linked immunosorbent assay (ELISA)-based binding test, which was modified from a published protocol (22, 23), was developed. Human 293T cells were transiently transfected with gD-EnvA, myc-Tva, and the TL5 chimeric constructs shown in Fig. 1B. The transfected cells were induced with 10 mM sodium butyrate 24 h posttransfection and lysed 40 h posttransfection with 1% Triton xylene buffer. Protein expression was examined and semiquanti- tified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using MABs 9E10 (for myc-tagged Tva and TL5 constructs) and 1D3 (for gD-tagged EnvA). The ELISA binding test was performed as follows: 96-well plates were coated with MAB 9E10 for 24 h. After three washings, the plates were blocked for 30 min with phosphate-buffered saline containing 0.05% Tween 20, 0.5% bovine serum albumin, and 0.05% sodium azide. A total of 100 μl of lysates from cells transfected with wild-type (wt) Tva or TL5 constructs DNAs was added to each well for 1 h with shaking at 4°C. The plates were then washed three times with distilled water, and 100 μl of gD-EnvA lysate was added to each well for 1 h with shaking. The wells were washed and incubated with an anti-EnvA tail rabbit antibody for one more hour at 4°C. Finally, the plates were washed four times, ABTS [2,2'-azino-bis (3-ethylbenzthiazolesulfonic acid) and hydrogen peroxide were added to each well, and the absorbance at 405 nm was read after incubation. Each experiment was performed in triplicate, and the relative ability of each protein to bind EnvA was expressed as the percentage of wt Tva binding to EnvA.

Infectivity assays. Two different viral systems were employed in this study: a recombinant ASLV-A, RCAS(A)AP, which carries an alkaline phosphatase (AP) gene as the reporter (10), and the EnvA-pseudotyped HIV viruses, which carry a luciferase gene as the reporter (4, 12). The infectivity assay was carried out as previously described (21). Briefly, 293T cells were transiently transfected with DNAs of the Tva or TL5 constructs by the CaPO4 method. At 24 h posttransfection, cells were seeded in six-well plates and then challenged with RCAS(A)AP- or the EnvA-pseudotyped HIV viruses. The RCAS(A)AP-infected cells were stained for AP, and the AP-positive cells were enumerated under a microscope. The EnvA-pseudotyped HIV-infected cells were lysed, and luciferase activity was measured with a standard luminometer.

RESULTS

Human LDL-A5 cannot replace the Tva LDL-A module for viral receptor function. A chimeric Tva protein called TL5, in which the Tva LDL-A module was replaced with human LDL receptor repeat 5 (LDL-A5) (Fig. 1B), was created. To test its ability to mediate viral entry, TL5 DNA was transiently trans- fected into 293T cells and the transfected cells were challenged with a recombinant ASLV-A carrying an AP reporter gene [RCAS(A)AP]. As expected, TL5 was unable to mediate viral entry (data not shown). To ensure that the defect was not due to a lack of protein expression, the TL5 transfected cells were analyzed by Western blotting; it was found that total protein expression was comparable to that of a myc-tagged wt Tva (Fig. 2, lanes 1 and 2). The surface expression was measured by flow cytometry, and it was found indistinguishable from that of wt Tva (data not shown). In addition, TL5 was also examined, using an ELISA-based test (described later and in Materials and Methods), for its ability to bind the ASLV-A glycoprotein EnvA. As expected, TL5 was unable to bind EnvA (see Fig. 5). These results are similar to the previous findings with TL4, a chimeric protein between Tva and human LDL-A4 which was shown to be unable to bind EnvA and which could not mediate ASLV-A entry (22).

Human LDL-A5 with substitutions corresponding to the viral interaction residues of Tva cannot be converted into an effective viral receptor. Previously it was shown that a human LDL-A4 module with a few amino acid substitutions could functionally substitute for the Tva LDL-A module in media- tion of ASLV-A entry (22). This gain-of-function approach was instrumental in identifying several putative viral interaction
residues in Tva. Sequence comparison of human LDL-A5 with human LDL-A4 and the Tva LDL-A module indicated that none of the putative interaction residues of Tva, Leu34, His38, Trp48, and Gly49 are conserved in LDL-A5 (Fig. 1A). To test whether TL5 could be converted into a functional ASLV-A receptor, 11 TL5 substitution variants were generated in which the corresponding residues in TL5 were replaced with the four putative viral interaction residues of Tva either individually or in combination (Fig. 1B).

Human 293T cells were transiently transfected with the TL5 construct DNAs and lysed. Protein expression was examined by Western blotting using 9E10, an antibody which specifically recognizes the myc tag portion of these proteins. All 11 TL5 constructs were expressed well in 293T cells (Fig. 2, lanes 3, 4, 5, 7, 8, 9, and 11 to 15). As was previously observed with wt Tva and TL4 constructs (22, 23), each of the TL5 proteins migrated as a smear of multiple bands on SDS-PAGE, indicating that these proteins were heavily modified by glycosylation in 293T cells.

To examine the ability of the TL5 proteins to mediate ASLV-A infection, the 293T cells transiently transfected with the TL5 construct DNAs were challenged with the RCAS(A)AP and the recombinant ASLV-A vector; the infected cells were enumerated postinfection. Since expression of Tva in native avian cells is extremely low, we were concerned that overexpression of the TL5 constructs in 293T cells by transient transfection might mask the defect of these proteins in mediation of viral entry. To avoid this potential problem, various amounts (1, 5, and 20 μg) of DNA of each TL5 construct as well as wt Tva were used in transfection prior to RCAS(A)AP challenges. Protein expression was detectable by Western blotting and flow cytometry when 5 or 20 μg of DNA was used in transfection (data not shown).

As shown in Fig. 3, challenging the wt Tva DNA-transfected 293T cells with RCAS(A)AP viruses resulted in high numbers of AP-positive cells (approximately 10^5 AP-positive cells per milliliter of viral stock used) when either a low or high amount of DNA was used in transfection, indicating that wt Tva can efficiently mediate ASLV-A entry. As a negative control, mock-transfected 293T cells were also challenged with RCAS(A)AP viruses; no background AP-positive cells were detected. Among the 11 TL5 constructs, only three (TL5LHG, TL5LHW, and TL5LHWG) mediated low levels of ASLV-A infection at a level at least 100-fold lower than that seen with wt Tva (Fig. 3). The rest of the TL5 constructs did not display any viral receptor function (data not shown). These results are in stark contrast with those seen with the TL4 chimeric constructs with respect to mediation of ASLV-A infection, as previously reported (22). For example, while TL5LHWG was approximately 100-fold lower than that seen with wt Tva, the similar TL4 construct (TL4G-A19LD23H) was able to mediate efficient viral entry. One plausible explanation is that human LDL-A4 is more conserved than human LDL-A5 with respect to the Tva LDL-A module between cysteines 3 and 6 (Fig. 1A). These results suggest that additional viral interaction determinants must be critical for maintaining the optimal viral receptor function of Tva.

The amino terminus of the Tva LDL-A module is required to convert human LDL-A5 into an efficient viral receptor. Previous reports suggest that the amino-terminal region of the Tva LDL-A module plays a subtle role in mediation of ASLV-A infection (22, 23). Thus, we generated two additional chimeric constructs (named TL5 chimera 1 and TL5 chimera 2) which replaced the human LDL-A5 C1-C3 region of TL5LHW and TL5LHWG with the corresponding region of Tva (Fig. 1B). These constructs were expressed well in 293T cells compared to the other TL5 proteins (Fig. 2, lanes 6 and 10). TL5 chimera 1 mediated low levels of ASLV-A infection, while TL5 chimera 2 was as efficient as wt Tva in mediation of viral infection in transfected 293T cells challenged with the RCAS(A)AP vi...
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FIG. 4. (A) Establishment (using a luciferase gene as the reporter) of the EnvA-pseudotyped HIV system. Human 293T cells were transiently transfectected with Tva or mock transfected, and the transfected cells were challenged with the EnvA-pseudotyped HIV viruses. The infected cells were lysed, and luciferase activity levels were measured and are expressed as RLU. EnvA, the ASLV-A glycoprotein-pseudotyped HIV; vector, HIV without any viral glycoprotein. Experiments (performed in triplicate) were repeated several times. The results of a representative experiment are shown here. (B) The abilities of TL5 constructs to mediate ASLV-A entry as measured using the EnvA-pseudotyped HIV viruses. Mock, mock transfected; TL5 Ch2, TL5 chimera 2; TL5 Ch1, TL5 chimera 1. Experiments (performed in triplicate) were repeated three times with consistent results. Bars, standard deviations.

ruses (Fig. 3). These results provide strong evidence that the region between cysteines 1 and 3 of the Tva LDL-A module plays a crucial role in the viral receptor function of Tva. In addition, since TL5 chimera 1 and TL5 chimera 2 differ by a single residue (Gln in TL5 chimera 1 versus Gly in TL5 chimera 2) (Fig. 1B), these results suggest that maintaining a small residue-like glycine at this position is important for the viral receptor function of Tva. In rats (Fig. 3). Therefore, we have established an easy and quantitative assay to measure ASLV-A entry; this assay will facilitate studies of Tva-EnvA interactions.

Examination by an EnvA-pseudotyped HIV infection assay of ASLV-A entry for TL5 constructs. The assay described above, which uses a recombinant ASLV-A virus for infection followed by AP staining and counting under a microscope for infectivity, is a reliable method for measurement and comparison of the abilities of Tva and its derivatives or mutants to mediate viral entry. However, one major drawback of this assay is that counting under a microscope is time consuming and error prone. Thus, we used the luciferase gene as the reporter to establish an HIV-based pseudotyping system to quantitatively measure ASLV-A entry.

To test the feasibility and specificity of this system, the DNA of HIV vector pNL4-3.Luc.R-E (4, 12) was cotransfected with the DNAs of either the ASLV-A glycoprotein EnvA or VSV-G to generate the pseudotyped HIV viral stocks (see Materials and Methods for the details). The produced viral stocks were used to challenge mock-transfected human 293T cells or the 293T cells transiently expressing Tva, and the luciferase activity of the cells was determined with a luminometer. The cells infected by the VSV-G-pseudotyped HIV viral stock, as expected, gave a high level of luciferase activity (approximately 8 logs of relative light units [RLU]) regardless of whether Tva was expressed or not (Fig. 4A), indicating that VSV-G mediated entry is Tva independent. In contrast, the cells challenged with the EnvA-pseudotyped HIV viruses only gave a high level of luciferase activity (also about 8 logs of RLU) when the cells expressed Tva. However, the cells challenged by the HIV virions lacking a glycoprotein, or the cells without Tva expression challenged by the EnvA-pseudotyped HIV virions, only gave background luciferase activity (Fig. 4A). These results indicate that EnvA-mediated HIV entry is Tva dependent, as expected, demonstrating the feasibility and specificity of the EnvA-pseudotyped HIV system to measure ASLV-A entry.

The abilities of the TL5 chimeric constructs (shown in Fig. 3) to mediate ASLV-A entry were also tested using the EnvA-pseudotyped HIV viruses. TL5 chimera 2 could mediate ASLV-A entry as efficiently as wt Tva, while TL5 chimera 1 was at least three logs less efficient than Tva. Furthermore, TL5LHWG was better than TL5LHW, while TL5LHG was unable to mediate ASLV-A entry (Fig. 4B). Thus, the results were remarkably consistent with that seen with RCAS(A)AP infection (Fig. 3). Therefore, we have established an easy and quantitative assay to measure ASLV-A entry; this assay will facilitate studies of Tva-EnvA interactions.

Most TL5 proteins do not efficiently bind to ASLV-A glycoprotein EnvA. To further investigate the interaction between the TL5 proteins and EnvA, the binding properties of these proteins to EnvA were examined by an ELISA-based binding test. This assay was modified from a previous protocol (23) which allows quick assessment of a large number of Tva mutants for their ability to bind EnvA. As shown in Fig. 5, among the 14 TL5 proteins only TL5 chimera 2 displayed a relatively high level of EnvA binding, giving approximately one-third of that of wt Tva, while the remaining 13 TL5 proteins displayed little or no detectable EnvA binding (less than 10% of that of wt Tva). These results suggest that the failure of these TL5 constructs to mediate efficient ASLV-A infection is due to a defect in high-affinity binding to EnvA. It is interesting that although three TL5 constructs (TL5LHW, TL5LHWG, and TL5 chimera 1) supported a low level of ASLV-A infection (Fig. 3 and 4), they did not display detectable EnvA binding (Fig. 5). Rong et al. have previously observed similar discrepancies (measured by ELISA-based test) between the ability of many Tva mutants and TL4 proteins to mediate viral entry and
their ability to bind EnvA (22, 23). A plausible explanation for these discrepancies is that the ELISA-based binding test used in this and previous studies is not sensitive enough to distinguish the binding differences among the TL5 proteins that have low-level binding affinities to EnvA.

The spacing between the second and third cysteines of the Tva LDL-A module is important for receptor function. Previously we have shown that the overall conformation of the Tva LDL-A module is different from the structures of the other reported LDL-A modules, including human LDL-A5. The N terminus of the Tva LDL-A module does not contain the signature antiparallel β-sheet observed in other LDL-A modules and is more flexible than other reported LDL-A modules (28). Sequence alignment shows that there are nine residues between the second and third cysteines for the Tva LDL-A module and that the other LDL-A modules with reported structures contain only four to six residues. The fact that the TL5 chimera 2 can mediate ASLV-A infection efficiently and TL5LHWG cannot do so prompted us to examine whether the overall conformation of the N terminus of the Tva LDL-A module contributes to the receptor function of Tva. As previously reported, LDL-A22 is the only other known LDL-A module that (just like that of Tva) contains nine residues between C2 and C3, thus conserving the spacing between cysteines 2 and 3. Wang et al. proposed that LDL-A22 adopts a structure similar to that of the Tva LDL-A module (28). However, none of the nine amino acids in the C2-C3 regions are conserved between human LDL-A22 and the quail Tva LDL-A module, while four of the nine residues are conserved between quail and chicken Tva proteins.

Two TL5 variants, C5 and L5, which are identical to TL5 chimera 2 except that the residues between C2 and C3 of TL5 chimera 2 have been replaced with the corresponding residues of chicken Tva (C5) or LDL-A22 of LDL receptor-related protein (L5), respectively, were constructed (Fig. 6A). When they were examined by FACS analysis, both constructs were expressed well on the surface of the transfected 293T cells (data not shown). The EnvA-pseudotyped HIV viruses were used to challenge the transfected 293T cells as described above to examine the TL5 variants for their ability to mediate ASLV-A entry. The cells transiently expressing C5 gave a level of luciferase activity comparable to that of wt Tva and TL5 chimera 2 (Fig. 6B), suggesting that C5 is as efficient as wt Tva and TL5 chimera 2 in mediation of ASLV-A infection. Surprisingly, the cells transiently expressing L5 gave a level of luciferase approximately 100-fold higher than the cells expressing TL5LHWG and only about 6-fold lower than the Tva-expressing cells (Fig. 6B). Since the amino acid sequences of TL5LHWG and L5 are identical between C3 and C6 and are very similar between C1 and C2, we can conclude that the amino acids between C2 and C3 of L5 play an important role in mediation of ASLV-A entry. Furthermore, since only the spacing between C2 and C3 of human LDL-A22 is conserved with respect to that of Tva from quail and chicken, we can conclude that the proper spacing (and thus, likely the proper conformation) of this region plays an important role for the viral receptor function.

**DISCUSSION**

In this report we have shown that the putative viral interaction residues of Tva that were previously identified by a gain-of-function approach and mutational analysis are indeed critical for the conversion of human LDL-A5 into a functional ASLV-A receptor. Furthermore, our results have revealed a critical role of the N terminus between cysteines 2 and 3 of the
The role of Tva in ASLV-A entry has been extensively examined by molecular, biochemical, and structural analysis. However, most work has concentrated on the C-terminal region of the Tva LDL-A module (C3-C6); this region was found to be essential for viral entry. This has led to the identification of individual residues in this region which are either ligand interaction residues or involved in stability and conformation of the protein (2, 22, 23, 31, 32). For example, Leu34, His38, Trp48, and Gly49 of Tva were identified as being important for viral receptor function. These results are substantiated by the results of the present study. We have shown that replacements of the corresponding residues in human LDL-A5 by these four amino acids, in addition to the amino terminus of Tva, were required to convert human LDL-A5 into a functional viral receptor. Interestingly, the side chains of His38 and Trp48 of Tva were shown to be exposed on the surface, in consistency with the notion that these residues are EnvA contact residues. However, the side chain of Leu34 is buried in the interior of the module together with Phe16 and Pro21, forming the hydrophobic core. These data indicate that the role of Leu34 is to maintain the correct folding of Tva; thus, it is unlikely to be a ligand contact residue (28). We speculate that conversion of the corresponding residue of human LDL-A5 (Arg19) to a leucine (as described in the previous report) can help maintain the correct folding in TL5 and TL4 chimeras, respectively.

The major difference between the present study using human LDL-A5 and the previous study using LDL-A4 is that LDL-A5 with the aforementioned four substitutions was not able to mediate efficient viral infection unless its N terminus was also replaced with the corresponding region from Tva. This finding is in contrast to that of the previous study with LDL-A4, which demonstrated that LDL-A4 could be converted into a functional viral receptor with merely the aforementioned substitutions and without replacement of the N terminus by that of Tva (22). This may be explained by a higher level of sequence homology between Tva and LDL-A4 than between Tva and LDL-A5 at the C terminus. For example, there are two lysine residues in LDL-A5 (K27 and K29) (Fig. 1A) that are absent in the Tva module and LDL-A4. Perhaps these positively charged residues adversely affect the binding affinity between LDL-A5 and EnvA, thus making it a less efficient viral receptor. It should be pointed out that the modified human LDL-A4 protein (TL4G-A19LD23H) was also somewhat defective in EnvA binding compared to that of wt Tva (22). This defect in EnvA binding could be corrected by replacing the N terminus of LDL-A4 with the corresponding region of Tva (data not shown), again suggesting the important role of the N-terminal region of Tva in mediation of efficient EnvA binding and ASLV-A infection.

Sequence alignment between Tva and other LDL-A modules indicates that the N terminus of the Tva LDL-A module (between cysteines 1 and 3) is more divergent than the C terminus of other LDL-A modules (Fig. 1A). Indeed, the structure of the LDL-A module of Tva reveals that compared to the other LDL-A module structures reported to date, the N terminus of the Tva module adopts a unique conformation. The Tva LDL-A module does not have the signature anti-parallel β-sheet at the N terminus observed in other LDL-A modules; thus, it has an even less recognizable secondary structure (28). Comparisons of quail and chicken Tva protein sequences indicate that the C1-C2 regions are highly similar except for two conserved variations and that the C3-C6 regions are identical or conserved except for one residue. However, there is significant sequence divergence in the Tva LDL-A
module between quail and chicken in the region between the second and the third cysteines—only four of the nine residues are conserved (Fig. 7). This sequence divergence may dictate subtle differences in this region and determine specificity in receptor usage. Indeed, it was reported that several ASLV-A variants which were genetically selected for a soluble form of quail Tva could not use quail Tva but could still efficiently use chicken Tva as the receptor in viral entry (14–16). These results seem to suggest that specific residues in the N terminus of Tva play an important role in specificity in receptor usage.

In contrast, our results have shown that the spacing between the second and third cysteines also plays a critical role in receptor function. The C2-C3 regions of both chicken Tva and LDL-A22 have the same number of residues as the quail Tva module. However, the individual amino acids are more conserved between quail and chicken Tva than between quail Tva and LDL-A22. As mentioned above, four out of nine amino acids between C2 and C3 are conserved between chicken and quail Tva proteins, while not a single residue is conserved between LDL-A22 and quail Tva. Nevertheless, both C5 and L5 were able to mediate viral entry at a level that was at least 100-fold higher than that seen with TL5LHWG (Fig. 6B). Since the C2-C3 regions of both LDL-A4 and LDL-A5 have only four residues (Fig. 7), these results illustrate the importance of maintaining the proper spacing between C2 and C3 of Tva for optimal receptor function. These results may appear to be inconsistent with those of a previous report by Hong et al. demonstrating that deletion of the C1-C3 region of Tva reduced the viral receptor function of Tva by only approximately 50 to 60%. One possible explanation is that the defective phenotype of the C1-C3 deletion mutant of Tva was masked due to overexpression of the protein in the previous study (23). Another possibility is that there are other viral interaction residues in the C3-C6 region of Tva which are critical for the viral receptor function but which are not conserved in human LDL-A5. Thus, the defective phenotype of TL5 LHWG became more apparent than that of the C1-C3 deletion mutant of Tva in mediation of ASLV-A infection.

In conclusion, the results of the present study have demonstrated that the spacing between cysteines 2 and 3 of the Tva LDL-A module plays a crucial role in mediation of ASLV-A entry. We hypothesize that this region can help maintain the proper conformation of Tva upon EnvA binding, thus optimizing Tva-EnvA interactions in ASLV-A entry. It is important to point out that the results reported here do not exclude the possibility that some individual residues in this region play a role in viral entry. We have shown that C5 is approximately 10-fold more efficient than L5 in mediation of viral entry (Fig. 6B), suggesting that certain residues of chicken and quail Tva in this region are directly involved in ligand contact. Therefore, the N-terminal region of the Tva LDL-A module is responsible for both correct protein conformation and ligand recognition for optimal receptor function in mediations of ASLV-A entry.

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