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Identification of Two Residues within the LDL-A Module of Tva That Dictate the Altered Receptor Specificity of Mutant Subgroup A Avian Sarcoma and Leukosis Viruses

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Avian sarcoma and leukosis virus subgroup A (ASLV-A) entry is mediated by interactions between the viral glycoprotein EnvA and its cognate receptor Tva. Previously, some interesting mutants of ASLV-A have been selected by others which can use chicken Tva, but not quail Tva, for efficient entry. The mutant phenotypes are caused by two point mutations within the surface subunit of EnvA (S. L. Holmen, D. C. Melder, and M. J. Federspiel, J. Virol. 75:726–737, 2001). In this study, we have shown that the altered receptor specificity maps to the LDL-A module of Tva. Further, we have identified two residues in the chicken LDL-A module that allow more efficient viral entry by the mutant viruses. These results demonstrate that the altered receptor specificity of the mutant viruses is determined by specific interactions with residues in the LDL-A module of Tva.

Avian sarcoma and leukosis viruses (ASLVs) are classified into subgroups A through J, depending upon their diverse envelope glycoprotein/receptor interactions, interference patterns, and host ranges (29). As with other retroviruses, ASLV entry into target cells is mediated by interactions between the envelope glycoprotein and the cognate receptors/coreceptors. The interaction between ASLV subgroup A (ASLV-A) glycoprotein EnvA and its receptor Tva in viral entry is the best-understood system among all of the ASLVs. The role of Tva in ASLV-A entry has been extensively studied by molecular, biochemical, and structural techniques. It has been shown that the viral interaction domain of Tva is determined by a 40-amino-acid-long motif called the LDL-A module within the extracellular region of Tva (1, 5, 10, 21, 31, 32). The LDL-A module of Tva contains six cysteines and five acidic residues which are highly conserved in all members of the LDL receptor superfamily (4). This sequence conservation dictates that the overall conformations of all of the LDL-A modules are very similar; that is, in each, the protein structure is stabilized by three pairs of disulfide bonds formed by the six invariable cysteines and by calcium coordination via the side chains of the conserved acidic residues and the carboxyl oxygen groups of two other residues (6–9, 11, 15, 17, 18, 24–28). In addition, several residues which are located at the C-terminal end of the module and are critical for ASLV-A entry have been identified (22, 23, 27, 33). Recently, our group has demonstrated that the spacing between cysteines 2 and 3 is important for Tva to efficiently mediate ASLV-A entry (19), indicating that the N-terminal region of the module of Tva also plays an important role in maintaining proper conformation and thus, in viral entry.

The genes encoding the Tva protein have been identified from both chicken and quail cells by a DNA transfer technique (2, 3, 30). Although there is a limited sequence divergence between chicken and quail Tva proteins both within and outside of the LDL-A module, particularly near the N-terminal region of the module, wild-type ASLV-A can efficiently infect both quail and chicken cells. Interestingly, others have reported several escape mutants of ASLV-A with altered receptor specificity. These mutant viruses can use chicken Tva, but not quail Tva, for efficient entry. Analysis of these mutants revealed that the mutant phenotypes are determined by specific substitutions in the viral glycoprotein EnvA, as expected, and that two amino acid substitutions, both of which mapped to the hr1 domain of the surface subunit (SU), determine the specificity (12–14, 16). Two of the mutants, Y142N and E149K, are single-amino-acid substitutions, and the third one is a double mutant, Y142N/E149K.

Based on the previous findings that the LDL-A module of Tva is necessary and sufficient to mediate efficient ASLV-A entry, we hypothesized that the differences in receptor specificity observed for the mutant ASLV-A viruses are determined by the sequence divergence within the LDL-A module of the quail and chicken Tva proteins, as suggested by others (13). To test this hypothesis, a chicken-quail chimeric construct of Tva (referred to as the chicken LDL-A module in Table 1), in which the quail LDL-A module is replaced by the corresponding module of the chicken Tva, was generated (Fig. 1), using the myc-tagged quail Tva as the backbone (22). Expression of this chimeric construct in 293T cells was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting after transient transfection, following a previous protocol (22), and it was found that it was expressed as well as the wild-type quail Tva (data not shown).

If the aforementioned hypothesis was correct, we would expect that this chimeric receptor would be able to mediate more efficient viral entry for the mutant ASLV-A viruses than for the wild-type quail Tva. Here, we used a human immunodeficiency virus (HIV)-based pseudotyped virus as a surrogate system to test our hypothesis. As previously demonstrated, this surrogate system provides a quantitative and reliable measure

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TABLE 1. Luciferase activity of 293T cells expressing Tva or mutant Tva challenged by EnvA/HIV or mutant EnvA/HIV pseudovirions

Tva module or mutant	Luciferase activity resulting from challenge by[a]:			
	wt EnvA	Y142N	E149K	Y142N/E149K
LDL-A modules				
Quail	$5.9 (\pm 0.8) \times 10^6$	$5.0 (\pm 0.2) \times 10^3$	$6.1 (\pm 0.2) \times 10^4$	$4.2 (\pm 0.1) \times 10^2$
Chicken	$7.7 (\pm 0.5) \times 10^6$	$3.0 (\pm 0.2) \times 10^6$	$1.6 (\pm 0.5) \times 10^6$	$6.2 (\pm 0.1) \times 10^4$
Quail LDL-A Mutants				
P12S	$1.2 (\pm 0.1) \times 10^7$	$1.4 (\pm 0.1) \times 10^5$	$9.5 (\pm 0.2) \times 10^5$	$6.3 (\pm 0.1) \times 10^2$
R17H	$1.0 (\pm 0.2) \times 10^7$	$1.5 (\pm 0.1) \times 10^4$	$6.2 (\pm 0.1) \times 10^4$	$3.9 (\pm 0.1) \times 10^2$
P22R	$6.7 (\pm 0.6) \times 10^6$	$7.1 (\pm 0.2) \times 10^3$	$5.2 (\pm 0.3) \times 10^4$	$2.1 (\pm 0.1) \times 10^3$
G23D	$7.3 (\pm 0.9) \times 10^6$	$1.5 (\pm 0.1) \times 10^4$	$6.8 (\pm 0.1) \times 10^4$	$5.1 (\pm 0.2) \times 10^3$
A24P	$7.6 (\pm 0.1) \times 10^6$	$7.4 (\pm 0.2) \times 10^3$	$1.1 (\pm 0.1) \times 10^5$	$1.0 (\pm 0.2) \times 10^3$
H25Q	$5.8 (\pm 0.3) \times 10^6$	$8.0 (\pm 0.2) \times 10^3$	$5.4 (\pm 0.4) \times 10^4$	$9.2 (\pm 0.1) \times 10^2$
G26T	$6.4 (\pm 0.2) \times 10^6$	$6.1 (\pm 0.2) \times 10^3$	$8.5 (\pm 0.3) \times 10^4$	$3.8 (\pm 0.5) \times 10^2$
E27D	$6.8 (\pm 0.2) \times 10^6$	$2.1 (\pm 0.6) \times 10^4$	$8.5 (\pm 0.5) \times 10^4$	$5.4 (\pm 0.2) \times 10^2$
Q31L	$3.9 (\pm 0.8) \times 10^6$	$7.2 (\pm 0.9) \times 10^6$	$2.2 (\pm 0.4) \times 10^5$	$3.6 (\pm 0.3) \times 10^4$
D32E	$7.2 (\pm 0.2) \times 10^6$	$8.9 (\pm 0.1) \times 10^3$	$6.9 (\pm 0.4) \times 10^4$	$5.8 (\pm 0.3) \times 10^2$
P12S/Q31L	$7.2 (\pm 0.3) \times 10^6$	$1.9 (\pm 0.5) \times 10^6$	$9.8 (\pm 0.2) \times 10^5$	$1.5 (\pm 0.3) \times 10^5$
CKM	$1.1 (\pm 0.3) \times 10^7$	$3.6 (\pm 0.6) \times 10^4$	$1.4 (\pm 0.1) \times 10^5$	$1.4 (\pm 0.1) \times 10^3$
Chicken LDL-A mutant				
CKQ	$4.7 (\pm 0.9) \times 10^6$	$1.2 (\pm 0.2) \times 10^4$	$3.1 (\pm 0.7) \times 10^4$	$3.9 (\pm 0.5) \times 10^2$

[a] All values are in RLUs. Standard errors of the means are in parentheses. Positive control: VSV-G/HIV, 1.8×10^7. Negative control: HIV alone (no Env), 3×10^2.

for dissecting the role of Tva in mediating ASLV-A entry (19). The HIV-pseudotyped viruses with either wild-type (wt), Y142N, E149K, or Y142N/E149K EnvA proteins incorporated on the virions were used separately to challenge the 293T cells transiently expressing the wild-type quail Tva or the chimeric Tva (chicken LDL-A), and each of the infected cell populations was assayed for luciferase activity, which was proportional to the level of the ASLV-A infection. As expected, the cells expressing either the wild-type quail Tva or the chimeric Tva gave comparable luciferase activities (5.9×10^6 and 7.7×10^6 relative light units [RLUs], respectively) when the HIV virions carrying the wild-type EnvA were used in the infection (Table 1). These results suggest that the chicken LDL-A module can mediate the wt ASLV-A viral entry as efficiently as does the wild-type quail Tva. In contrast, the chimeric Tva (chicken LDL-A) construct was markedly more efficient than the wt quail Tva in mediating entry for the EnvA mutants (Table 1). The cells expressing the chimeric Tva and challenged with the HIV/Y142N gave a much higher luciferase activity (3.0×10^6 RLUs) than the cells expressing the wt quail Tva (5.0×10^3 RLUs). Similarly, the HIV/E149K and the HIV/Y142N/E149K could use the chimeric Tva more efficiently (with luciferase activities of 1.6×10^6 and 6.2×10^4 RLUs, respectively) than the quail Tva (6.1×10^4 and 4.2×10^2 RLUs, respectively) for viral entry. It should be noted that the EnvA double mutant (Y142N/E149K) was more impaired

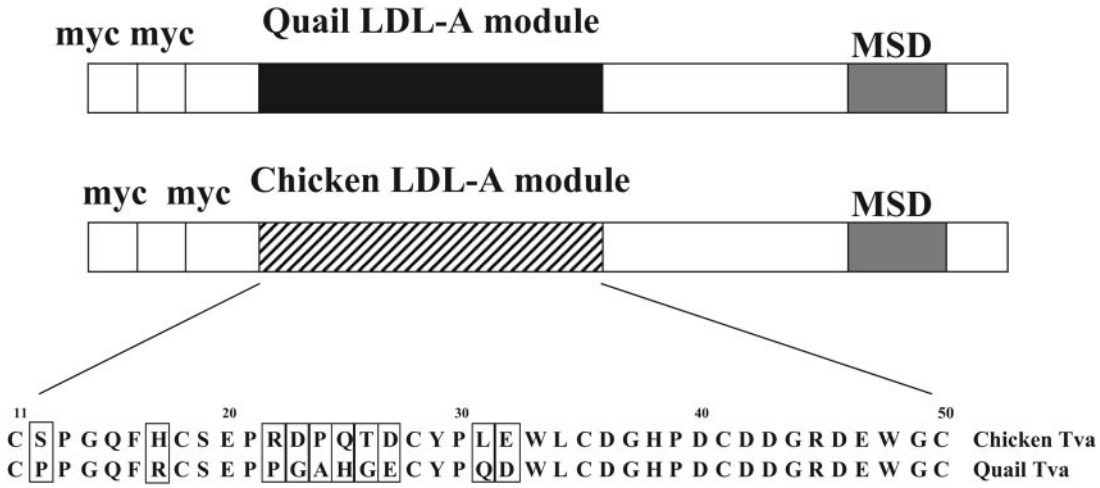

FIG. 1. Mapping the altered receptor specificity of the mutant ASLVs to the LDL-A module of Tva. The diagram depicts a chicken-quail chimeric construct of Tva generated in this study. In this construct, the LDL-A module of quail Tva (top) is substituted for by the corresponding region of chicken Tva (middle). Divergent residues (10 total) between the chicken and quail LDL-A modules are shown in the boxes (bottom). The Tva residues are numbered according to the mature Tva sequence (2). MSD, membrane-spanning domain.

than the EnvA single-point mutants (Y142N or E149K), so the double mutant was using the chimeric Tva or quail Tva very inefficiently in viral entry. In these experiments, the HIV pseudotyped with vesicular stomatitis virus G glycoprotein (luciferase activity, 1.8×10^7 RLUs) and the HIV alone (no viral glycoprotein; luciferase activity, 3×10^2 RLUs) were used as the positive and negative controls, respectively. These results demonstrated that these EnvA mutants can use the chicken LDL-A module more efficiently than that of quail in mediating viral entry, consistent with the previous report (13). Therefore, we can conclude that the receptor specificity of these ASLV-A mutants is determined by the LDL-A module of Tva, as we hypothesized.

Sequence comparison between the quail and chicken LDL-A modules indicates that there are ten residue variations, all of which are clustered at the N terminus of the module (Fig. 1). To identify the potential residues in this region which determine the receptor specificity for the ASLV-A mutants, each of the ten residues in the LDL-A module of quail Tva was substituted for by the corresponding residue of the chicken module, thus generating ten mutants of quail Tva (labeled as quail LDL-A mutants in Table 1). To test whether any of these quail Tva mutants could mediate more efficient entry for the EnvA mutants, the 293T cells expressing each of the ten Tva mutants (by transient transfection) were challenged by the HIV-pseudotyped viruses carrying either wild-type or mutant EnvA proteins, using the aforementioned luciferase entry assay. It was found that substitution for either one of the two residues P12S and Q31L in the LDL-A module of Tva by the corresponding residues of the chicken module could markedly enhance the ability of quail Tva to mediate entry for the EnvA mutants. In contrast, other substitutions did not greatly affect the ability of quail Tva to mediate viral entry (Table 1). A single substitution at position 31 (Q31L) rendered the quail Tva fully functional to mediate viral entry for the EnvA mutant Y142N (luciferase activity, 7.2×10^6 RLUs). In addition, substitution at position 12 (P12S) appeared to enhance the ability of quail Tva to mediate viral entry for mutant E149K (luciferase activity, 9.5×10^5 RLUs). In contrast, other substitutions did not markedly enhance the ability of quail Tva in viral entry. For EnvA mutant Y142N/E149K, a substitution only at position 31 (Q31L), and not at any other positions, caused the quail Tva to mediate viral entry at a relatively high level (luciferase activity, 3.6×10^4 RLUs).

To further examine the roles of the individual residues of Tva in determining the receptor specificity for the EnvA mutants, two more quail Tva mutants were created, P12S/Q31L and CKM. P12S/Q31L is a double mutant of quail Tva in which both residues Pro (position 12) and Leu (position 31) are replaced with the corresponding residues of the chicken module. CKM contains six residue substitutions of quail Tva (PGAHGE) by the corresponding residues of the chicken module (RDPQTD) between cysteines 2 and 3. Our previous work has demonstrated that the spacing of this region of Tva is essential for optimal viral entry (19). Here we wanted to examine whether the individual residues of this region together could enhance viral entry for the mutant ASLV-A viruses. Again, as expected, the HIV-pseudotyped viruses carrying the wt EnvA protein could use P12S/Q31L and CKM as efficiently as wt quail Tva and the chicken LDL-A chimeric construct (Table 1). In contrast, the HIV-pseudotyped viruses carrying the EnvA mutants Y142N, E149K, or Y142N/E149K could infect the cells expressing either the chicken LDL-A chimeric Tva, as shown above, or the cells expressing P12S/Q31L more efficiently than the cells expressing CKM (Table 1). Again, the HIV-pseudotyped viruses carrying the EnvA double mutant Y142N/E149K were less efficient at infecting the target cells than was that carrying the single EnvA mutants, which was observed above. Together, these results demonstrated that the receptor specificity of Tva for the three EnvA mutants tested in this study is determined by residue variations at position 12 and 31. In contrast, the residue variations between cysteines 2 and 3 of Tva are not responsible for this specificity. Furthermore, it seems that the defect of one EnvA mutation (Y142N) can be compensated for by substitution for Gln at position 31 by Leu, while the defect of another EnvA mutation (E149K) can be compensated for by substitution for Pro at position 12 by Ser of Tva.

Based on the results described above, we predicted that a substitution for the residues at position 12 and 31 of the chicken LDL-A module with the corresponding residues of the quail Tva (creating a mutant named CKQ) would impair its ability to mediate efficient entry for the ASLV-A mutants. To test this, the 293T cells expressing either wt quail Tva, the chicken LDL-A chimeric construct, or CKQ were challenged with the HIV-pseudotyped viruses carrying either wt EnvA or the mutant EnvA proteins. As predicted, CKQ behaved like the wt quail Tva, not the chicken LDL-A chimeric construct, in mediating viral entry for all of the HIV-pseudotyped viruses tested (Table 1). These results demonstrated that residues at 12 (Ser) and 31 (Leu) of the chicken LDL-A module determine the receptor specificity for the mutant ASLV-A viruses, further supporting the overall conclusion of the current study.

The results from the current work have further demonstrated the importance of the N-terminal region of the Tva LDL-A module in mediating viral entry; that is, subtle differences in the amino acid sequence in this region of Tva can dictate altered receptor specificity for ASLV-A mutants. Furthermore, we have demonstrated previously that the LDL-A module of Tva can be functionally substituted for by the LDL-A4 but not the LDL-A5 of the human LDL receptor with minor residue modifications in the C-terminal end, suggesting that subtle differences in the receptor can exert profound effects on viral entry (19, 22). Together with the work by others on EnvA, the current work has important implications in our understanding of how ASLV-A viruses interact with the cognate receptor Tva in viral entry.

It appears that the aforementioned leucine (position 31) of the chicken LDL-A module is most critical for efficient viral entry by ASLV-A EnvA mutant Y142N, while the serine (position 12) in the chicken module is more responsible for the altered specificity by the E149K mutant EnvA (Fig. 2). These residue variations between chicken and quail Tva proteins are not drastic substitutions, either in size or charge. Thus, there does not seem to be a simple explanation why such subtle amino acid variations between chicken and quail LDL-A modules exert such profound effects on these escape ASLV-A mutants in viral entry. Nevertheless, we can speculate that a more hydrophobic residue at position 31 in the chicken LDL-A module (a leucine instead of a glutamine in

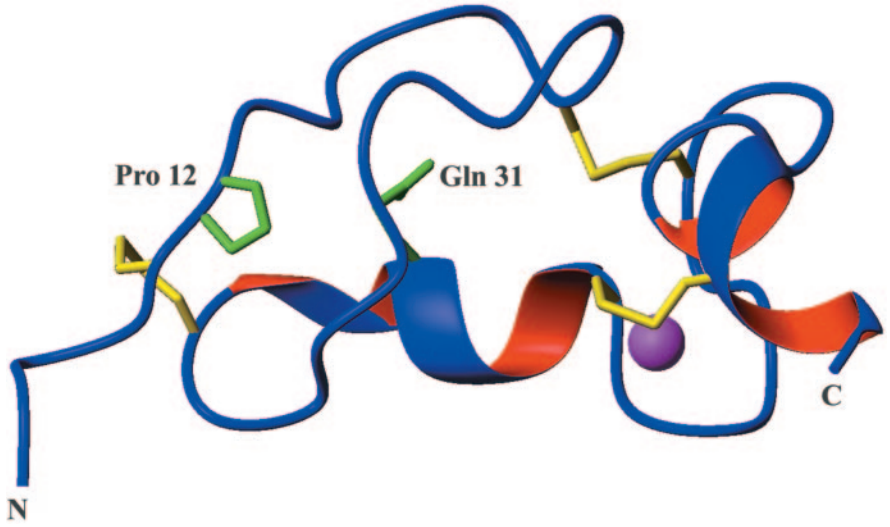

FIG. 2. The Tva LDL-A structure, highlighting the two residues which dictate the altered receptor specificity. The quail Tva LDL-A module structure shows the locations of the two corresponding residues of the chicken LDL-A module which are critical for the altered receptor specificity for mutant ASLVs. Side chains of proline 12 (serine 12 in chicken) and glutamine 31 (leucine 31 in chicken) are shown in green. Three pairs of disulfide bonds are shown in yellow, and a calcium ion is shown in purple. The amino terminus (N) and the carboxyl terminus (C) are labeled. The structure is displayed using the program MOLMOL with the coordinates deposited in the Protein Data Bank by Wang et al. (27).

quail Tva) can somehow compensate for the mutant with a substitution in the hr1 region of EnvA, Y142N, a change from an aromatic to a much less hydrophobic residue in the glycoprotein. Similarly, a proline-to-serine substitution at position 12 in the LDL-A module may provide a more optimized interaction between the mutant EnvA E149K and its cognate receptor. Further studies on "compensatory substitutions" on both EnvA and Tva proteins should provide a basis for biochemical and structural analyses of Tva/EnvA interactions in the future.

Studies by others on host extension in ASLVs suggest two nonexclusive modes. One strategy is likely via high-affinity binding between the viral glycoproteins and the receptors. In contrast, another strategy is that mutations in viral glycoproteins allow the infection of diverse host cells without an evident increase in receptor binding (20). In this report, we have shown large differences in viral entry between quail and chicken LDL-A modules for the mutant viruses Y142N, E149K, and Y142N/E149K (see Table 1), but we were unable to observe corresponding differences in binding between quail and chicken LDL-A with the mutant envelope proteins (data not shown). This discrepancy between the binding affinities of EnvA/Tva and viral infectivities has been previously observed by us; that is, there is not always a strong correlation between receptor binding and the ability of Tva to mediate ASLV-A infection (22, 23). These results are consistent with the second aforementioned mode of ASLV Env/receptor interactions. However, in the previous study by others, differences in viral entry between chicken and quail Tva were found to correlate with differences in binding between the quail and chicken Tva receptors and the mutant viral envelopes (13). Although the underlying reason is not clear at present, two plausible explanations could account for the discrepancy between the current report and the previous study. (i) The assays employed in the current study are different from those in the previous study. In the current report, the SUA-rabbit immunoglobulin G (rIgG) proteins, which contain only a single subunit of SUA, were used in the binding analysis in both f luorescence-activated cell sorting and an enzyme-linked immunosorbent assay, while others used virus-associated envelope proteins which exist as trimers of SU/TM dimers and thus may not have the same characteristics as SUA-rIgG proteins in Tva binding. (ii) The identified residues on SU of the mutant ASLV-A EnvA responsible for the altered receptor specificity may play a role in the postbinding steps, such as triggering conformational changes of EnvA during viral entry. If the residues of chicken Tva identified in this report which dictate the altered specificity for the mutant ASLVs are indeed involved in the postbinding steps, we would not expect to observe a difference in binding affinities among different Tva and SUA-rIgG proteins in the assays. However, further analysis is needed to clarify this issue.

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