Binding of equine infectious anemia virus to the equine lentivirus receptor-1 is mediated by complex discontinuous sequences in the viral envelope gp90 protein

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The identification and characterization of a functional cellular receptor for equine infectious anemia virus (EIAV), designated equine lentivirus receptor-1 (ELR1), a member of the tumour necrosis factor receptor protein family, has been reported previously [Zhang, B. et al. (2005). Proc Natl Acad Sci U S A, 102, 9918–9923]. The finding of a single receptor for EIAV is distinct from feline, simian and human immunodeficiency viruses, which typically utilize two co-receptors for infection, but is similar to avian and murine oncoviruses, which use single receptors. This study sought to determine ELR1-binding domains of EIAV gp90. Towards this goal, a GFP-tagged gp90 fusion protein (gp90GFP) expression vector was constructed and a specific cell–cell binding assay was developed to measure EIAV gp90 binding to ELR1. Using these assays, the receptor-binding properties of 41 gp90GFP mutants were evaluated, each with a sequential replacement 11 aa linear epitope peptide from the vesicular stomatitis virus glycoprotein (VSV-G tag), as well as eight mutants containing individual gp90 variable-domain deletions. The results of these studies demonstrated that, in general, gp90 constructs containing substitutions or deletions in the N-terminal third of gp90 retained their receptor-binding activity. In contrast, segment substitutions or deletions in the C-terminal two-thirds of gp90 eliminated receptor-binding activity. Thus, these results reveal for the first time that the ELR1-binding domains of EIAV gp90 are located in the C-terminal two-thirds of EIAV gp90, apparently as a complex of discontinuous determinants.

INTRODUCTION

Adsorption and entry of retroviruses into target cells requires the interaction of viral envelope (Env) glycoproteins with specific cellular receptor proteins. Retroviral envelope glycoproteins are synthesized as polyprotein precursors that are cleaved, during transport to the surface of infected cells, into the surface (SU) subunit and transmembrane (TM) subunit. The SU protein is bound at the virion surface through interaction with the TM protein and is responsible for the initial binding to receptor proteins on the target cell surface. Studies of the specificity of functional receptors for various retroviruses have indicated a general pattern of single receptor protein use by oncoviruses (such as murine and avian leukemia viruses) that is in distinct contrast to the dual co-receptor usage observed with the lentiviruses human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) (Eiden et al., 1993; de Parseval et al., 2004; Douglas et al., 1997; Barnard et al., 2006). Whilst the general assumption has been that all lentiviruses may use dual co-receptors for infection of target cells, the recent identification of a single functional receptor for equine infectious anemia virus (EIAV) has revealed an unexpected diversity in lentivirus receptor specificity and interactions (Zhang et al., 2005).

Murine leukemia virus (MuLV) Env SU proteins and their interactions with receptors are the best defined among the simple oncoviruses and represent the prototypic model for retrovirus SU binding to a single functional receptor. The receptor-binding domain has been located in the N-terminal third of the subunit, and two variable regions, VRA and VRB, have been shown to contribute to receptor recognition for the amphotropic and ecotropic MuLVs (Heard & Danos, 1991; Battini et al., 1992; Ott & Rein, 1992; Morgan et al., 1993; Battini et al., 1995). In contrast to MuLV receptor usage, HIV, SIV and FIV have been shown to recognize the CD4 (HIV-1, HIV-2 and SIV) or CD134 (FIV) proteins as a primary binding receptor, with
either CXCR4 or CCR5 chemokine receptor as a secondary co-receptor for infection of target cells (Douglas et al., 1997; de Parseval et al., 2004, 2005; Shimojima et al., 2004; Gomez & Hope, 2005). The detailed structural characterizations of HIV-1 gp120 and its co-receptors provide the prototypic model for the functional binding of lentivirus SU proteins to dual co-receptors, distinct from that of MuLV (Fass et al., 1997; Kwong et al., 1998; Poignard et al., 2001). The extensively glycosylated gp120 has five variable regions (V1–V5) interspersed with five conserved regions (C1–C5) (Modrow et al., 1987; Leonard et al., 1990). The first four variable regions form surface-exposed loops that contain disulfide bonds at their bases (Leonard et al., 1990; Moore et al., 1994). The conserved gp120 regions fold into a core that contains many discontinuous structures important for interactions in receptor binding (Cordonnier et al., 1989; Kwong et al., 1998; Wyatt et al., 1998). HIV-1 gp120 binding to co-receptors requires that gp120 first binds CD4 (Wyatt et al., 1995; Rizzuto et al., 1998; Kwong et al., 1998). The gp120 residues involved in the co-receptor CCR5-binding site have been characterized by analysis of the binding of a panel of gp120 mutants to CCR5. A highly conserved gp120 structure that is located adjacent to the V3 loop and contains neutralization epitopes induced by CD4 binding is critical for CCR5 binding. Experimental data have suggested that binding to CD4 leads to the repositioning of the V1–V2 loop and to the exposure or formation of the co-receptor binding site (Wyatt et al., 1995).

EIAV is an exclusively macrophage-tropic lentivirus that causes a uniquely rapid and episodic disease in horses, providing a dynamic animal model for studying lentiviral replication, pathogenesis and immune control. EIAV Env evolution studies have indicated that EIAV SU protein (gp90) is the predominant site of EIAV antigenic variation, and distinct conserved and variable domains in gp90 have been defined (Leroux et al., 1997, 2001; Zheng et al., 1997). The general structural organization of EIAV gp90 has been predicted to be similar to the analogous Env proteins of both HIV-1 and MuLV (Gallaher et al., 1989, 1995).

A functional cellular receptor for EIAV, designated equine lentivirus receptor-1 (ELR1), has recently been identified (Zhang et al., 2005). ELR1 is a member of the tumour necrosis factor receptor (TNFR) protein family with typical cysteine-rich domains. ELR1 appears to be sufficient for mediating EIAV infection in transduced cells in the absence of any apparent co-receptor (Jin et al., 2005; Zhang et al., 2005), in marked contrast to HIV, SIV and FIV, which typically require co-receptors.

The observation of a single cellular receptor mediating infection of target cells raises a number of interesting questions about the nature of the interaction between EIAV gp90 and ELR1, including the fundamental question of whether the gp90 receptor-binding domain is a complex of discontinuous segments, as in HIV-1. In the present study, we examined this question by mapping the ELR1-binding domains of EIAV gp90 by measuring the effect of a series of segment substitutions and selected deletions on the ability of gp90 to bind ELR1 in cell–cell binding assays. The results of these studies revealed for the first time that the ELR1-binding domain of EIAV gp90 is a complex of discontinuous sequences located in the C-terminal two-thirds of the Env protein.

**METHODS**

**Cells.** Cf2Th cells (ATCC CRL-1430) were grown in Eagle’s minimal essential medium (EMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin. Stably transduced Cf2Th cells stably expressing haemagglutinin-tagged EIAV receptors (Cf2Th/ELR1) (Zhang et al., 2008) were cultured in EMEM with 10 % FBS, 1 % penicillin/streptomycin and 800 μg G418 ml⁻¹. 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10 % FBS and 1 % penicillin/streptomycin.

**Monoclonal antibodies and anti-EIAV polyclonal sera.** The reference EIAV-specific polyclonal immune serum (Lady) was obtained from a naturally infected horse (Montelaro et al., 1984). The reference monoclonal antibodies (mAbs) to EIAV gp90 were specific for epitopes B, E and F, as described previously (Hussain et al., 1987, 1988; Ball et al., 1992). Anti-ELR1 rabbit polyclonal serum was produced commercially (Washington Biotechnology) and has been characterized previously (Zhang et al., 2008). The anti-VSV-G mAb (clone P5D4) recognizes an 11 aa peptide epitope derived from the vesicular stomatitis virus glycoprotein (Roche Applied Science).

**EIAV gp90 mutagenesis.** Construction of gp90 mutants for mapping receptor-binding domains was based on the GFP-tagged EIAV gp90 (gp90GFP) expression vector (Zhang et al., 2008). An overlapping PCR strategy was used for the construction of the series of EIAV gp90 segment substitution mutants containing the VSV-G tag, an 11 aa epitope from the cytoplasmic tail of the VSV-G protein (Rose & Gallione, 1981; Kreis, 1986). The two hybrid primers were constructed to contain VSV-G tag sequences at the 5’ ends and EIAV gp90-specific sequences at the 3’ ends of both of the primers used for the substitutions. For construction of deletion mutants in the variable domains of EIAV gp90, primer pairs with a specific deletion in each variable region of EIAV gp90 were used for PCR. The final overlapping PCR products were then subcloned into the p2CI-gp90GFP construct. All mutants were verified by DNA sequencing.

**Transfection procedures.** 293T cells were plated in 10 cm culture dishes or six-well plates and transfected with GFP-labelled EIAV Env expression vectors using FuGENE 6 transfection reagent, as recommended by the manufacturer (Roche Applied Science).

**Flow cytometry.** To monitor expression of the GFP-tagged gp90 fusion proteins on the surface of transfected 293T cells, antibody staining of intact transfected cells was performed using standard FACS procedures. In brief, the transfected 293T cells were harvested with 10 mM EDTA in PBS, collected by centrifugation, washed three times with FACS wash buffer (5 % FBS, 0.5 mg sodium azide 1⁻¹ in PBS) and resuspended in FACS wash buffer with either the individual anti-gp90 mAbs or the anti-VSV-G mAb and incubated at 4 °C for 30 min. The cells were then washed three times with FACS wash buffer, reacted with the corresponding Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) in the same wash buffer and fixed with 1 % paraformaldehyde in PBS before analysis using a FACScalibur (Becton Dickinson). A total of 1 x 10⁶ cells per sample was acquired and analysed using CellQuest 3.0 software. Total Env protein expression was based on detection of GFP-positive cells, and...
surface expression of Env proteins was determined by antibody labelling of intact cells.

**EIAV gp90–ELR1 binding assay.** The cell–cell binding system used here to measure the binding of ELR1 to gp90 and its derivative constructs was adapted from procedures described previously by Zhang et al. (2008). Briefly, Cf2Th/ELR1 cells expressing ELR1 were seeded at 2 x 10^5 cells per well in six-well plates and grown to a confluent monolayer (about 1 x 10^6 cells per well) to serve as a target cell in the binding assay. In parallel, individual 293T cells, transfected for 48 h with equal amounts of the appropriate EIAV gp90GFP construct, were suspended in 10 mM EDTA in PBS and pelleted by centrifugation. The transfected 293T cells were then resuspended in DMEM with 10 % FBS and 1 % penicillin/streptomycin. Samples of each transfected 293T cell were taken for FACS analysis of expression levels of each gp90 construct. The transfected 293T cell suspensions and the target Cf2Th/ELR1 cells were pre-incubated separately at 4 °C for 30 min. About 1 x 10^7 ligand 293T cells expressing the test gp90 were then added to each well of the target Cf2Th/ELR1 cell monolayer at 4 °C and incubated at 4 °C for 2 h. The incubating plates were shaken gently every 30 min. After incubation, the cells were washed at 4 °C once with PBS, three times with PBS containing 500 mM NaCl and once again with PBS to remove the non-specifically bound 293T cells. Plates were then moved to room temperature and the attached cells were removed by pipetting using wide-bore tips with 0.5 ml PBS in each well. The mixed cells, including ligand 293T-bound Cf2Th/ELR1 cells and unbound Cf2Th/ELR1 cells, were then resuspended and fixed with 1 % paraformaldehyde in PBS for subsequent analyses by flow cytometry. A total of about 1 x 10^4 events per sample was acquired and analysed using CellQuest 3.0 software. The percentage of GFP-positive events (gp90GFP ligand 293T cell-bound Cf2Th/ELR1 cells) was calculated in the gated population events (including the ligand 293T-bound Cf2Th/ELR1 cells and unbound Cf2Th/ELR1 cells) as a measure of the binding of the gp90 mutants to ELR1 receptors.

**RESULTS**

**Validation of the cell–cell binding assays**

To map the sites on EIAV Env that mediate binding to ELR1 receptor, we employed a cell–cell binding assay system in which we used flow cytometry to measure the level of binding of a suspension of 293T cells transfected with a particular gp90GFP construct (‘ligand cells’) to a target monolayer of Cf2Th/ELR1 cells stably expressing ELR1 on the cell surface, essentially as described by Zhang et al. (2008), for mapping the ELR1 determinants of binding to EIAV gp90.

The data in Fig. 1 demonstrate the specificity of the binding assay. In the cell–cell binding assay, only 293T cells expressing gp90GFP bound to the target Cf2Th/ELR1 cells in flow cytometry (Fig. 1a, panel i). In contrast, there was no significant binding of 293T cells transfected with gp90GFP to Cf2Th cells lacking ELR1 expression (Fig. 1a, panel ii), and 293T cells expressing GFP only also failed to bind the target Cf2Th/ELR1 cells significantly (Fig. 1a, panel iii). The specificity of the binding observed between the ligand 293T (gp90GFP) cells and the target Cf2Th/ELR1 cells was tested further by determining the ability of a reference polyclonal immune serum from an EIAV-infected horse (Lady) or a gp90-specific mAb to block the cell–cell binding under the standard assay conditions. As summarized in Fig. 1(b), both the reference polyclonal immune serum and the mAb completely inhibited cell–cell binding, in contrast to normal horse serum, which failed to reduce the level of cell–cell binding. The observed inhibition of cell–cell binding by the reference anti-ELR1 rabbit polyclonal immune serum was further shown to be directly related to the dilution of the serum added to the binding assay (Fig. 1c). Thus, these

![Figure 1](http://vir.sgmjournals.org)
data indicated that the conditions used for the cell–cell binding assay specifically measured interactions mediated by EIAV gp90 and its receptor ELR1 expressed on the cell surface.

**Design and expression of EIAV gp90GFP mutants for binding-domain mapping studies**

A sequential segment substitution strategy has been used successfully to map the receptor-binding domain of the MuLV Env SU protein gp70 (Battini *et al.*, 1998). Applying this strategy to map the ELR1-binding domains of EIAV gp90, we constructed a panel of mutants in gp90GFP that contained sequential substitutions of approximately 10 aa segments of the gp90 sequence with a replacement 11 aa linear epitope peptide from the VSV-G protein (VSV-G tag). A total of 41 substitution mutants were produced, as summarized in Fig. 2. Segment substitutions were designed to conserve important structural features, such as the N-terminal signal peptide and all of the cysteine residues proposed to be involved in the formation of disulfide loops in the protein structure. To complement this sequential substitution strategy, we also constructed a series of mutants in which a single variable domain was deleted from the gp90 sequence.

To determine the expression levels of the segment-substituted gp90GFP protein mutants, each of the 41 substituted constructs was used individually to transfect 293T cells and mutant protein expression was measured by flow cytometry analysis of the intact cells. As summarized in Fig. 3(a), all 41 of the substituted gp90GFP constructs were successfully produced in transfected 293T cells at expression levels that ranged from 50 to 100% of the expression levels detected for the parental gp90GFP transfection. As a measure of functional structure of the substituted gp90GFP mutants, we next assayed the level of surface expression of each construct in intact transfected 293T cells after labelling either with mAbs specific for EIAV gp90 or with a mAb specific for the VSV-G tag. As summarized in Fig. 3(b), the reference mAb specific for the EIAV gp90 linear epitope B was able to detect surface expression of 38 of the 41 substituted gp90GFP proteins; only three substituted constructs (E7, E8 and E23) were negative for detectable surface expression. The observed lack of mAb reactivity with the E23 construct was expected due to the substitution of the target epitope B by the VSV-G tag in this particular gp90 construct (Ball *et al.*, 1992). For the majority of the surface-expressed gp90GFP constructs, the level of surface expression was similar (±25%) to that observed with the unmodified gp90GFP; however, three constructs (E15, E18 and E22) were substantially reduced in terms of surface expression relative to the unmodified gp90GFP, despite the relatively similar levels of protein production in transfected cells (see Fig. 3a). As summarized in Fig. 3(c), the pattern of surface expression of the various gp90GFP constructs detected with the EIAV gp90 conformational epitope F-specific mAb was remarkably similar to that observed with the linear epitope B-specific mAb, demonstrating efficient flow cytometry analysis of the intact cells. As summarized in Fig. 3(a), all 41 of the substituted gp90GFP constructs were successfully produced in transfected 293T cells at expression levels that ranged from 50 to 100% of the expression levels detected for the parental gp90GFP transfection. As a measure of functional structure of the substituted gp90GFP mutants, we next assayed the level of surface expression of each construct in intact transfected 293T cells after labelling either with mAbs specific for EIAV gp90 or with a mAb specific for the VSV-G tag. As summarized in Fig. 3(b), the reference mAb specific for the EIAV gp90 linear epitope B was able to detect surface expression of 38 of the 41 substituted gp90GFP proteins; only three substituted constructs (E7, E8 and E23) were negative for detectable surface expression. The observed lack of mAb reactivity with the E23 construct was expected due to the substitution of the target epitope B by the VSV-G tag in this particular gp90 construct (Ball *et al.*, 1992). For the majority of the surface-expressed gp90GFP constructs, the level of surface expression was similar (±25%) to that observed with the unmodified gp90GFP; however, three constructs (E15, E18 and E22) were substantially reduced in terms of surface expression relative to the unmodified gp90GFP, despite the relatively similar levels of protein production in transfected cells (see Fig. 3a). As summarized in Fig. 3(c), the pattern of surface expression of the various gp90GFP constructs detected with the EIAV gp90 conformational epitope F-specific mAb was remarkably similar to that observed with the linear epitope B-specific mAb, demonstrating efficient

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**Fig. 2.** Summary of serial segment substitution and variable-domain deletions introduced into EIAV gp90 and used for mapping of receptor-binding domains. Schematic representation of EIAV gp90 fused at the C terminus to GFP and showing the EIAV gp90 sequence with serial segment substitutions with the VSV-G peptide indicated as boxed areas (E1–E41) above the corresponding amino acid sequences and individual variable-domain deletions indicated by bold and underlining. Shaded boxes indicate the gp90 signal sequence (SS) and cysteine residues predicted to form disulfide loops. The corresponding regions of epitope B and epitope E_N are indicated in italic.
surface expression of all but a small number of gp90GFP constructs. Finally, surface labelling of the transfected cells with the mAb specific for the VSV-G tag (Fig. 3d) revealed a greater range of surface reactivity, presumably reflecting the differences in accessibility of the VSV-G peptide tag incorporated into different parts of the gp90 sequence. Importantly, however, surface labelling with the VSV-G tag-specific mAb did effectively detect surface expression of the E19, E21, E22 and E23 constructs, which were non-reactive or poorly reactive with the mAbs to gp90.

Combining the data in Fig 3(b–d), it could be concluded that all of the substituted gp90GFP constructs were expressed at similar levels in transfected 293T cells and that all were expressed on the cell surface, except for the E7 and E8 constructs, which were not detected using any of the reference antibodies.

Using techniques similar to those described above, the total and surface expression levels of the single variable-domain-deleted gp90GFP protein mutants were also measured by...
flow cytometry using selected reference antibodies. As summarized in Fig. 4(a), all eight of the deleted gp90GFP constructs were produced successfully in transfected 293T cells at expression levels similar to the expression levels detected in cells transfected with the parental gp90GFP construct. As shown in Fig. 4(b), the reference epitope B-specific mAb was able to detect surface expression of seven of the eight deleted gp90GFP constructs; only the V4 mutant lacking gp90 epitope B failed to react to the reference mAb, as expected (Fig. 2). The levels of surface expression of the various gp90GFP constructs containing variable-domain deletions were similar to the surface expression levels observed with the parental gp90GFP. As summarized in Fig. 4(c), the pattern of surface expression of the deleted gp90GFP constructs detected with the epitope F-specific mAb was remarkably similar to that observed with the epitope B-specific mAb, demonstrating efficient surface expression of all of the deletion constructs, except the V3 and V4 constructs in which the target F-epitope is affected. Combining the data in Fig 4(b and c), it could be concluded that all of the deleted gp90GFP constructs were expressed on the cell surface.

**Binding of gp90GFP mutants to ELR1**

We initially used the cell–cell binding assay to evaluate the ability of the panel of substituted gp90GFP proteins expressed on the surface of transfected 293T cells to bind target Cf2Th/ELR1 monolayer cells. The data in Fig. 3(e) indicated a clear distinction in the ability of the segment substitutions to affect the mutant gp90GFP binding to ELR1. Segment substitutions E1–E6 (aa 7–70) and E9–E14 (aa 82–147) in the N-terminal end and segment substitution E41 at the C-terminal end of the gp90 protein were all able to bind ELR1 with efficiencies similar to those observed for the unmodified gp90GFP, except that E11 and E13 reduced the ability to binding ELR1 and E10 increased it in the cell–cell binding assay. In marked contrast, all of the segment substitutions from E15 to E40 (aa 148–429) completely eliminated or greatly reduced the ability to bind ELR1 in the cell–cell binding assay. E20 substituted within the V3 region displayed reduced receptor-binding levels that were similar to those observed with the E11 construct. For E7 and E8 (aa 71–81), the lack of receptor binding was apparently due to their lack of surface expression.

As the preceding mapping studies all involved segment substitutions in the gp90 sequences, we next sought to map the determinants of receptor binding by gp90 by evaluating the effect of deletions of each of the defined variable domains (V1–V8) on gp90–ELR1 binding, using the cell–cell binding assays described above. The results in Fig. 4(d) summarize the ability of the individual variant-domain deletion mutant gp90 constructs to bind the ELR1 receptor in the cell–cell binding assay. The assay clearly indicated that deletions of V1, V2 or V3 sequences had no significant effect on receptor binding, whilst deletion of V4, V5, V6, V7 or V8 sequences completely eliminated receptor binding of gp90GFP constructs to ELR1. Combining the data in Fig 4(b and c), it could be concluded that all of the deleted gp90GFP constructs were expressed on the cell surface.

![Fig. 4. Characterization of expression and receptor-binding properties of variable-domain deletion gp90GFP constructs. (a) Total expression levels of fluorescent protein in 293T cells transiently transfected with the indicated variable-domain deletion gp90GFP constructs (V1–V8) or the parental gp90GFP construct. (b, c) Surface expression of variable-domain deletion gp90GFP constructs in transfected 293T cells as detected by surface staining with the reference mAb directed to the linear epitope B (b) and the conformational epitope F of EIAV gp90 (c). (d) Analysis of the receptor-binding properties of variable-domain deletion gp90GFP constructs in the cell–cell binding assay to measure binding of ligand 293T cells transfected with individual gp90 constructs to target Cf2Th/ELR1 cells. The expression and receptor-binding levels of the variable-domain deletion constructs are shown as the means (±SD) of at least three independent experiments.](image-url)
binding. Thus, cell–cell binding assays of variable-domain deletion mutants were in general consistent with the segment substitution mutant assays in mapping the critical determinants for ELR1 binding to the complex of discontinuous sequences in the C-terminal two-thirds of the gp90 protein sequences.

**DISCUSSION**

Studies of receptor usage by HIV-1, SIV and FIV have revealed a common theme of co-receptors in which sequential binding of viral SU proteins to two distinct surface proteins is required for infection of target cells. It has been shown that the sequential binding of the respective gp120 proteins to co-receptor proteins is required to induce the series of conformational changes that fully activate the fusogenicity of gp41 and achieve fusion of the viral and cellular membranes. In contrast, the simple retroviruses, such as avian and murine oncoviruses, employ a single cell receptor to infect target cells. This difference in receptor specificity between oncoviruses and lentiviruses also appears to be related to differences in the nature of the respective receptor-binding domains of HIV-1 gp120 and murine gp70 (Cordonnier et al., 1989; Fass et al., 1997; Battini et al., 1998; Kwong et al., 1998; Wyatt et al., 1998). For example, the CD4-binding site of gp120 is a depression formed at the interface of the outer domain with the inner domain and the bridging sheet of gp120 spanning a complex of discontinuous sequences, mainly in the C-terminal half of gp120. In contrast, the receptor-binding domain of gp70 has been localized to the VRA and VRB regions in the N-terminal third of the MuLV gp70 protein. Thus, the specificity and binding interactions of SU proteins with their respective receptor protein(s) appeared to differentiate oncoviruses from lentiviruses as definitively as the differences in their respective genomic structures.

However, the recent finding that EIAV can utilize a single receptor protein, designated ELR1, to infect target cells revealed an unexpected variation in the monothematic model of lentivirus receptor specificity. Interestingly, ELR1 is a member of the TNFR protein family, and TNFR-like proteins have been identified as receptors for certain avian oncoviruses and as a co-receptor component (CD134) for FIV (Brojatsch et al., 1996; Adkins et al., 1997, 2000; de Parseval et al., 2004; Shimojima et al., 2004; Zhang et al., 2005; Barnard et al., 2006). Taken together, these observations suggest that EIAV may represent a critical transitional link between the simple oncoviruses and the more complex immunodeficiency lentiviruses in terms of genetic composition and receptor usage. The results of current mapping studies clearly reveal for the first time that the ELR1-binding domain of EIAV gp90 is composed of a complex of discontinuous segments located in the C-terminal two-thirds of the Env protein, whereas the sequences in the N-terminal third of gp90 appear unnecessary for receptor binding. Thus, these data indicate that EIAV gp90 resembles HIV-1 gp120 in the structural organization of its receptor-binding domain, whilst it resembles MuLV in the use of a single receptor for infection.

Whilst a number of different strategies have been employed to map the receptor-binding domain of HIV-1, we selected the combination of serial segment substitution and selected segment deletions to map the ELR1-binding domain of EIAV gp90. A potential limitation of these protein modifications is that the segment substitutions or deletions may alter the expression, processing or trafficking of the modified gp90 protein. Taking advantage of the GFP tag on the gp90 protein, we were able to demonstrate that all 41 segment-substituted (Fig. 3a) and all eight segment-deleted (Fig. 4a) constructs were expressed in transfected cells to levels that were similar to the parental gp90GFP construct. In addition, surface staining with a panel of reference mAbs (Fig. 3b–d and Fig. 4b,c) demonstrated that 47 of the 49 modified constructs could be detected at the surface of transfected cells at levels similar to the parental gp90GFP construct; only two segment-substituted constructs (E7 and E8) were not detected at the cell surface. It has been demonstrated that, when membrane proteins fail to fold properly or to associate into the necessary oligomeric complexes, they tend to be trapped in the endoplasmic reticulum (Doms et al., 1993). Thus, cell-surface expression has been used as one indication that the modified viral Env glycoproteins assume functional conformations that can interact properly with the complex of host cellular trafficking protein factors (Chiang et al., 1994; Connolly et al., 2002, 2003). We interpret the surface localization and the conformational antibody reactivity of the modified gp90GFP constructs as indicators of their ability to assume a functional structure, including the potential to bind its cognate receptor, ELR1.

We have suggested previously that lentivirus Env proteins share similar structural features, despite a lack of amino acid sequence homology (Gallaher et al., 1989, 1995). Subsequent studies have indicated further key similarities between EIAV gp90 and HIV-1 gp120 with respect to variable-domain structure and functional properties, especially in the respective V3 regions (Modrow et al., 1987; Leonard et al., 1990; Leroux et al., 1997, 2001; Zheng et al., 1997; Kwong et al., 1998). The current studies further demonstrate this similarity in lentivirus Env protein structure with respect to V3 structure and function. For example, the current studies clearly demonstrate that substitution or deletion in the V3 domain of EIAV gp90 retains receptor binding. Similarly, mutations in the V3 region of gp120 retained CD4-binding activity, although the V3 domain is important for co-receptor binding (Kwong et al., 1998; Suphaphiphat et al., 2003, 2007). Interestingly, we did observe that mAb directed to the E epitope contained in the V3 region of gp90 blocked binding of ELR1 and gp90-expressing cells in the standard cell–cell binding assay (Fig. 1b), in contrast to the sustained receptor binding observed with V3 substitutions or
deletions (Figs 3 and 4, respectively). Whilst the reason for this difference is not certain, it is possible that binding of mAb to the V3 segment may induce conformational changes or produce steric hindrance that inhibits binding to ELR1. This observation may imply some role for the gp90 V3 sequences in binding to the ELR1 protein. With the availability of purified gp90 and ELR1 proteins, it should be possible to produce complexes that can be analysed by X-ray crystallography to obtain a high-resolution characterization of EIAV Env–receptor interactions to elucidate the mechanisms by which receptor binding alters Env conformation and activates membrane fusion and infection.

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