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Short Communication

Mapping epitopes in equine rhinitis A virus VP1 recognized by antibodies elicited in response to infection of the natural host

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Equine rhinitis A virus (ERAV) is an important respiratory pathogen of horses and is of additional interest because of its close relationship and common classification with foot-and-mouth disease virus (FMDV). As is the case with FMDV, the VP1 capsid protein of ERAV has been shown to be a target of neutralizing antibodies. In FMDV VP1, such antibodies commonly recognize linear epitopes present in the βG–βH loop region. To map linear B cell epitopes in ERAV VP1, overlapping fragments spanning its length were expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion proteins. These fusion proteins were tested for reactivity with sera from ERAV-infected horses and with polyclonal sera from ERAV-immunized rabbits and mice. Regions at the N- and C-termini as well as the βE–βF and the βG–βH loop regions contained B cell epitopes that elicited antibodies in the natural host. GST fusion proteins of these regions also elicited antibodies following immunization of rabbits and mice, which, in general, strongly recognized native ERAV VP1 but which were non-neutralizing. It is concluded that the N-terminal region of ERAV VP1, in particular, contains strong B cell epitopes.

Equine rhinitis A virus (ERAV) is an important respiratory pathogen of horses. Disease is characterized by fever (41 ± 0·5 °C) and clinical signs that include nasal discharge, coughing, anorexia, pharyngitis and lymphadenitis (Plummer, 1962). ERAV is classified with foot-and-mouth disease virus (FMDV), albeit as a separate cluster, in the genus *Aphthovirus*, family *Picornaviridae*. ERAV shares many physico-chemical and biological properties with FMDV and the genome structures and sequences of the two viruses are also similar (Li *et al.*, 1996; Newman *et al.*, 1973; Plummer, 1963; Studdert & Gleeson, 1978; Wutz *et al.*, 1996).

Picornavirus capsid proteins VP1, VP2 and VP3 share structural homology and are composed of wedge-shaped, eight-stranded, β-barrels, which differ in the size and conformation of the connecting loops between their strands and the extensions of their N- and C-termini (Rueckert, 2001). The amino acid sequences of the loops that connect the β-strands and the N- and C-terminal regions that extend from the β-barrel domain give each picornavirus its distinct morphology and antigenicity (Mateu, 1995; Rueckert, 2001). For example, the surface loops of VP1, VP2 and VP3 of poliovirus type 1 (PV-1) and human rhinovirus type 14 capsid structures protrude from the virion surface to form the receptor-binding canyon, as well as the major antigenic sites of these viruses. In comparison, the VP1 protein within the relatively smooth surface of FMDV particles contains a linear peptide, the βG–βH loop, which is both the dominant neutralization epitope and is also involved in receptor binding to various integrins via an RGD motif (Berinstein *et al.*, 1995; Danen *et al.*, 1995; Jackson *et al.*, 2000; Mateu *et al.*, 1995; Stanway, 1990; Strohmaier *et al.*, 1982).

In marked contrast to FMDV and human rhinoviruses, the predicted amino acid sequence of ERAV P1 and, in particular, VP1 has remained remarkably stable over time (Varrasso *et al.*, 2001). Amino acid variations that do occur among naturally occurring strains of ERAV locate mostly to the proposed βE–βF loop of VP1 and the βA2–αZ loop of VP2, although some variation also occurs at the N terminus and at the βC–βD and βG–βH loops of VP1 (Varrasso *et al.*, 2001). Amino acid sequence variation in these regions suggests that the regions may contain epitopes that elicit neutralizing antibodies. We have shown that the ERAV capsid protein VP1 contains B cell epitopes that elicit neutralizing antibodies and has receptor-binding activity (Warner *et al.*, 2001). It was anticipated, therefore, that the major antigenic sites for ERAV would be located within the surface structures of VP1. In particular, in the loop regions, which are not predicted to play an essential role in stabilizing the capsid structure and are, therefore, likely to be exposed to antibodies. In ERAV, the βG–βH loop of VP1 is smaller than that in FMDV and does not contain an identifiable integrin-binding motif. In this study, using panels of glutathione *S*-transferase (GST) fusion proteins comprising overlapping segments of VP1, we define further linear epitopes of ERAV VP1 that elicit antibodies in the natural host following infection and in rabbits and mice following immunization.

To map the location of ERAV VP1 B cell epitopes, two sets of GST fusion proteins were prepared. The first set comprised four overlapping fragments (GST–NT, GST–VP1.2, GST–VP1.3 and GST–VP1.4) designed to encompass the complete VP1 protein, where each fragment contained one or more of the predicted surface loops (Fig. 1). The second set of GST fusion proteins (GST–CA, GST–DE, GST–EF and GST–CT) was designed to contain the individual loop regions between the predicted β-sheet and α-helical structural elements of VP1 (Fig. 1).

The antigenicity of both sets of fusion proteins was investigated by Western blotting and probing with polyclonal ERAV antisera prepared either by infection of horses or by immunization of rabbits with the ERAV.393/76 strain (Hartley *et al.*, 2001) (Fig. 2). When the large VP1 fragments were probed with horse ERAV antisera (horses C and G; Hartley *et al.*, 2001), GST–NT and GST–VP1.4 showed some reactivity, while GST–VP1.2 and GST–VP1.3 showed reactivity only marginally above background (Fig. 2a). When the same VP1 fragments were probed with rabbit ERAV antiserum made to whole inactivated virus, the N-terminal (GST–NT) and full-length GST–VP1 reacted strongly, while the fragment VP1.2 reacted to a lesser degree (Fig. 2a) and fragments VP1.3 and VP1.4 showed no reactivity. The pattern of reactivity seen with both the horse and rabbit ERAV antisera maps a strong B cell epitope(s) to the N-terminal region of VP1. The reactivity observed when the proteins were probed with sera from the natural host also indicates that there are additional linear epitopes along the length of VP1, in particular, in the region contained within fragment GST–VP1.4 and possibly also in a more central region.

To define more precisely the antigenic regions of VP1, a second set of fusion proteins, comprising the individual loop regions of VP1, was investigated for reactivity to the ERAV antisera. The fusion proteins GST–NT, GST–EF, GST–GH and GST–CT reacted strongly with the horse

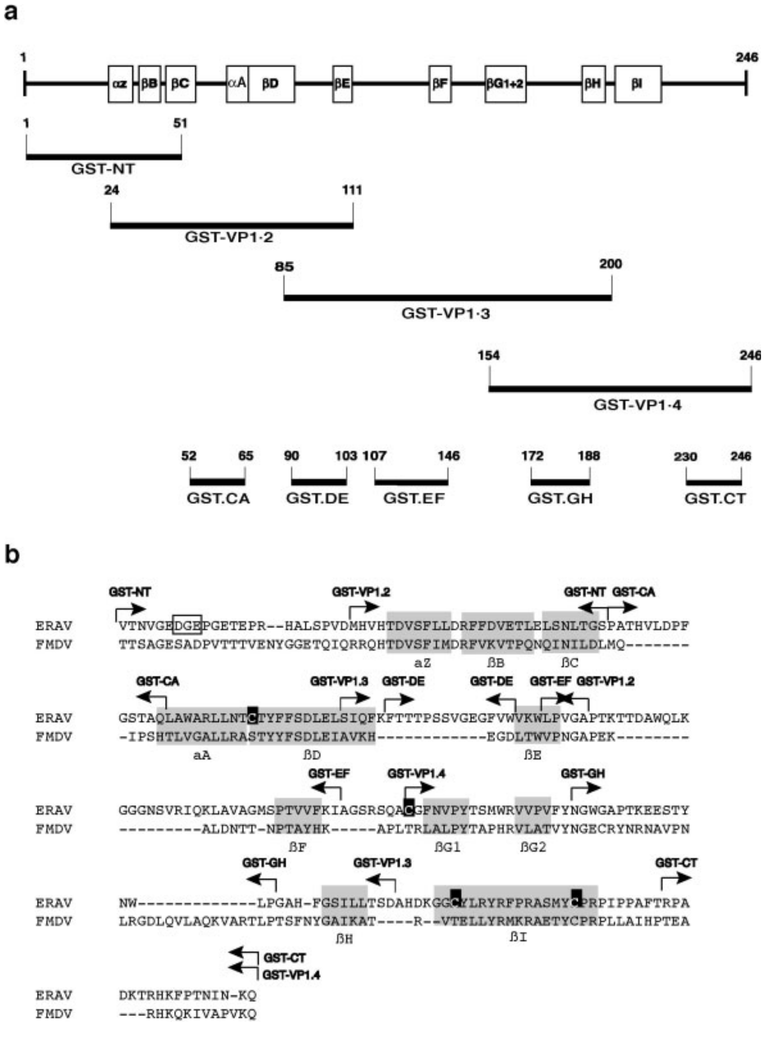

Fig. 1. (a) Schematic representation of the VP1 protein of ERAV.393/76. Locations of each of the VP1 fragments and peptides used as GST fusion proteins in this study are indicated. Amino acid numbering is from the first residue of VP1. (b) Amino acid alignment of ERAV VP1 as compared to FMDV. The fusion proteins used in this study are indicated by arrows.

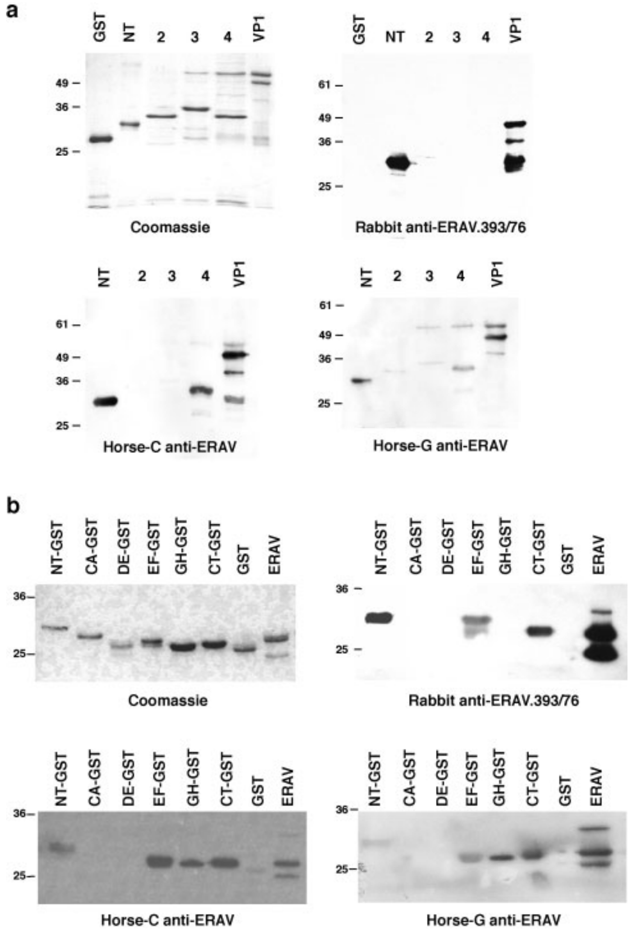

Fig. 2. The proposed loop regions of ERAV.393/76 VP1 contain B cell epitopes. SDS-PAGE and Western blot analysis of GST, GST–VP1 fragments (a) and GST–VP1 loop regions (b). Purified fusion proteins were separated by 12 % SDS-PAGE under reducing conditions and stained with Coomassie blue or transferred to PVDF membranes (Immobilon, Millipore) and probed with ERAV.393/76 rabbit antisera diluted 1 : 1000 and horse ERAV antisera diluted 1 : 250 (horses C and G). Blots were probed with HRP-conjugated swine anti-rabbit IgG (1 : 1000, Dako) or HRP-conjugated goat anti-horse IgG (1 : 20 000, Sigma) and developed using ECL substrate (Amersham). Autoradiographs were scanned with a Umax Astra 3450 scanner and published using Adobe Photoshop 8.0.

ERAV antisera (horses C and G), whereas GST–CA and GST–DE showed no reactivity (Fig. 2b). A similar pattern of reactivity was found when the same antigens were probed with rabbit ERAV antisera, with the exception that the rabbit antisera did not react against the fusion protein encompassing the βG–βH loop (GST–GH) (Fig. 2b). The pattern of reactivity obtained with both the horse and rabbit antisera indicates the presence of B cell epitopes at the N- and C-termini and within the βE–βF and βG–βH loop regions of VP1. This is generally consistent with the pattern of reactivity seen in the larger VP1 fusion protein fragments, although some additional reactivities were seen with the smaller fragments. While the βE–βF loop region is contained within the larger fragment GST–VP1.3, GST–EF but not GST–VP1.3 was reactive to the rabbit ERAV antisera, which may indicate that the βE–βF loop region may present differently in the GST–VP1.3 fusion protein. The antibodies in horse sera to each of the fusion proteins were specific for each independent loop region, since antibodies that were affinity purified to GST–NT, GST–EF, GST–GH and GST–CT, using a method described by Crabb *et al.* (1992), reacted only with the homologous antigen and showed no cross reactivity with the other fusion proteins by Western blot (data not shown). Together, these results confirm the presence of antibodies in post-infection horse sera that are specific for the ERAV residues present in the βE–βF and βG–βH loop regions and the N-terminal 50 and C-terminal 16 residues of VP1.

To determine if the individual antigenic fusion proteins (GST–NT, GST–EF, GST–GH and GST–CT) elicit ERAV antibodies in rabbits, two rabbits were immunized with 75 μg fusion protein in Freund's complete adjuvant (FCA) per rabbit, and sera collected after twice boosting with 25 μg of the same fusion protein in Freund's incomplete adjuvant (FIA). Antisera from these rabbits were used to probe purified ERAV virion proteins in Western blot. VP1-specific antibodies were detected in sera from rabbits immunized with each of the fusion proteins (Fig. 3a), confirming the presence of authentic VP1 epitopes within the fusion proteins. As described by Warner *et al.* (2001), rabbits immunized with the full-length recombinant VP1 (GST–VP1) produced neutralizing antibodies at a level comparable to rabbit ERAV antisera. Neutralizing antibodies were not detected in sera from rabbits immunized with any of the fusion proteins GST–NT, GST–EF, GST–GH or GST–CT (SN titres <10).

Mice were immunized in a similar manner: by injection with 20 μg of each of the fusion proteins GST–NT, GST–GH and GST–CT emulsified in FCA. Sera were collected after twice boosting with 20 μg of the same fusion protein in FIA. Sera were then used to probe purified ERAV.393/76 virion proteins in Western blots (Fig. 3a). Sera from mice immunized with GST–NT and GST–CT reacted strongly with VP1 of purified ERAV.393/76 virions, showing that VP1-specific antibodies were produced in response to immunization with each of these fusion proteins. Surprisingly, mice immunized with GST–GH did not produce antibodies that react against ERAV VP1. Again, none of these antisera contained neutralizing antibodies (SN titres <10). Mice were not immunized with GST–EF.

To investigate further the production of antibodies to ERAV, rabbit fusion protein antisera were also tested for their ability to bind to purified whole virions in ELISA. ELISA was carried out as described previously (Crabb *et al.*, 1995), with the exception that wells were coated with 1 μg ml^{-1} purified ERAV.393/76 and that bound rabbit antibodies were detected using a 1 : 1000 dilution of

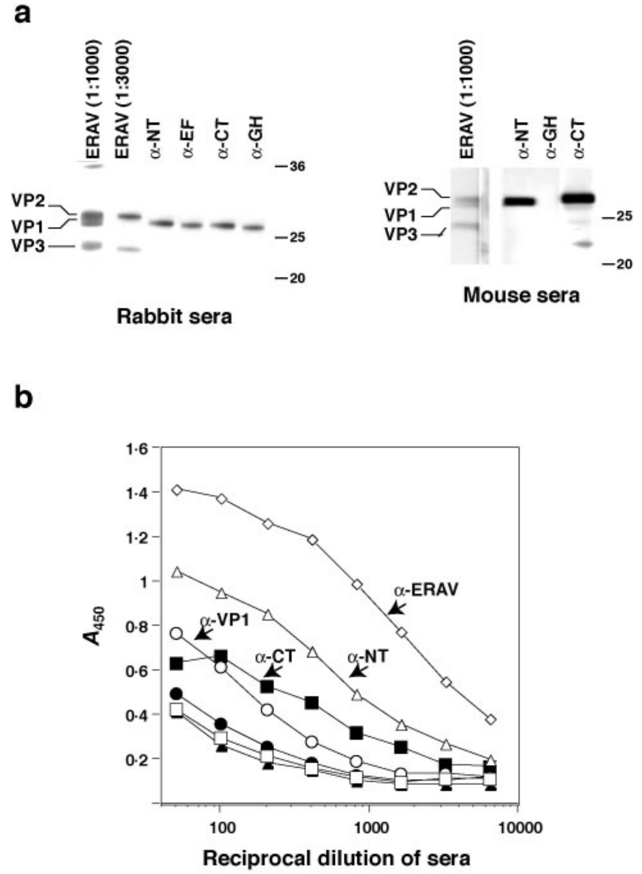

Fig. 3. (a) The VP1 loop region fusion proteins elicit antiviral antibodies. Purified ERAV.393/76 virions were separated by 12·5 % SDS-PAGE and transferred to a PVDF membrane. Membrane strips were probed with rabbit antisera (α) prepared against each of the VP1 loop region fusion proteins GST–NT, GST–EF, GST–GH and GST–CT or mouse antisera (α) prepared against the fusion proteins GST–NT, GST–GH and GST–CT. The positions of molecular mass standards (kDa) are shown on the left. (b) ELISA was used to detect binding of rabbit fusion protein antisera to purified ERAV. Pooled rabbit pre-immunization serum (□), rabbit ERAV antisera (α, ◇), rabbit GST–VP1 antisera (α, ○), rabbit GST–NT antisera (α, △), rabbit GST–EF antisera (●), rabbit GST–GH antisera (▲) and rabbit GST–CT antisera (α, ■) were titrated on purified ERAV.393/76 virus-coated wells. Bound antibody was detected with HRP-conjugated swine anti-rabbit IgG (Dako, 1 : 1000) and developed with TMB substrate (Sigma).

horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (Dako). Despite the finding that antibodies elicited to each of these fusion proteins bound to VP1 in Western blot, only antibodies to GST–NT reacted strongly to whole ERAV.393/76 in ELISA, although full-length VP1 (GST–VP1) and the C-terminal fragment (GST–CT) elicited somewhat lower antibody titres to ERAV (Fig. 3b). Neither the rabbit GST–EF antisera nor the rabbit GST–GH antisera showed any significant reactivity.

In this study, ERAV VP1 B cell epitopes were mapped to regions at the N- and C-termini and to the predicted βE–βF and βG–βH loop regions. Loop regions are not predicted to play an essential role in the formation of the capsid structure and are predicted to project from the capsid surface. They are, therefore, also likely to elicit antibodies. Direct comparison of the known structure of FMDV VP1 with that predicted for ERAV VP1 (Wutz *et al.*, 1996) shows that most of the predicted loops of ERAV VP1 are larger than those of FMDV. In particular, the βE–βF loop of ERAV, which contains a strong B cell epitope, is more than double the size of that in FMDV (32 compared to 14 aa in FMDV) and in the case of FMDV is not reported to contain any antigenic sites. Antigenicity of the βE–βF loop of VP1 is not described commonly amongst picornaviruses but has been demonstrated for hepatitis A viruses and PV-2 and -3 (Luo *et al.*, 1988; Mateu, 1995; Page *et al.*, 1988; Ping & Lemon, 1992). The βG–βH loop of ERAV VP1 is considerably shorter than that of FMDV (23 compared to 37 aa) and does not contain a recognized integrin-binding motif. We have shown that the βG–βH loop contains B cell epitopes that elicit antibodies in horses following infection but not in rabbits following immunization. The presence of the strongest B cell epitope of ERAV VP1 within the N-terminal peptide and the strong binding of rabbit GST–NT antisera to whole virus particles in ELISA suggests that at least part of this region may be oriented more towards the virus surface. In support of this is the fact that, in comparison to FMDV, the N terminus of VP1 in ERAV is highly hydrophilic (Varrasso *et al.*, 2001). In FMDV, the N terminus of VP1 is confined to the interior of the capsid, within a deep and predominantly hydrophobic cleft formed by the interface between VP2 and VP3 (Acharya *et al.*, 1989; Curry *et al.*, 1997; Lea *et al.*, 1994), although N-terminal residues of PV-1, which are known also to be located internally in the capsid, have been shown to elicit virus-neutralizing antibodies (Fricks & Hogle, 1990; Roivainen *et al.*, 1994).

Immunization of rabbits and mice with each of the fusion proteins (GST–NT, GST–EF, GST–GH and GST–CT) resulted in antibodies that reacted specifically with VP1, in addition to being recognized by sera from ERAV-infected and -immunized animals. Taken together, these results confirm the presence of authentic viral VP1 epitopes within the fusion proteins. However, while each of these fusion proteins elicited the production of antibodies that bound to reduced and denatured viral VP1, only GST–NT and GST–CT (and full-length GST–VP1) induced antibodies that bound to whole virus particles in ELISA and none of the polyclonal sera were neutralizing. Neutralization epitopes may exist as a linear sequence within VP1, as has been shown for FMDV, or as conformational epitopes comprising a combination of different surface loops (Xie *et al.*, 1987). The induction of virus-neutralizing antibodies would require the presentation of authentic, conformationally intact epitopes, which may not be present when these individual loop fragments are expressed in *Escherichia coli* as GST fusion proteins. The correct conformation must exist on the surface of intact virus or when the complete VP1 is expressed as a GST fusion protein, since both these immunogens have been shown to induce the production of neutralizing antibodies (Hartley *et al.*, 2001; Warner *et al.*, 2001).

An enhanced response could result from combining the epitopes from the small fusion proteins into a single peptide or fusion protein to represent conformational epitopes. Proteins in whole virions would be folded in such a way that various regions of the capsid would lie in close proximity to one another, despite appearing distant in a linear representation of the sequence. In FMDV, the VP1 C terminus is reported to lie close to the βG–βH loop region (Acharya *et al.*, 1989). This provides structural evidence for the enhanced immunogenicity that has been reported for a FMDV VP1 hybrid peptide containing βG–βH loop residues and C-terminal residues 200–213 (Brown, 1992; DiMarchi *et al.*, 1986). Peptides containing both regions induced antibodies that provided complete protection against infection with approximately one-hundredth the dose compared to that of a βG–βH loop peptide that did not contain any C-terminal residues (DiMarchi *et al.*, 1986).

In summary, this work shows that the N-terminal region of ERAV VP1 contains strong B cell epitopes. Other small regions of ERAV VP1, namely the βE–βF and βG–βH loops and the C-terminal region, also contain epitopes recognized by sera from ERAV-infected horses.

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