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Short Communication

Identification of a conserved linear epitope at the N terminus of the rabies virus glycoprotein

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A novel, linear B-cell epitope has been identified at the N terminus of the rabies virus (RABV) glycoprotein. Screening of a phage-display library demonstrated that two glycoprotein-specific mAbs recognized a conserved sequence, WxxxDI, which aligned between aa 14 and 19 of the mature glycoprotein. Screening of truncated glycoprotein fragments with both mAbs confirmed the location of the epitope in the N-terminal region. Alignment of amino acid sequences from a range of RABV isolates indicated that the site was conserved in most viruses. Alignment with representatives of other lyssaviruses suggested that it is conserved within phylogroup I, which includes the European bat lyssaviruses, but not phylogroup II. A 12 aa synthetic peptide of this epitope was recognized by both mAbs and sera from a subset of rabies-vaccinated dogs. In a multimeric form, the peptide could induce an epitope-specific response following immunization in rabbits and mice.

The rabies virus (RABV) glycoprotein is one of five proteins that are encoded by the virus's single-stranded, negative-sense RNA genome and is the only surface-exposed protein. It is postulated to form trimeric structures in the viral membrane (Gaudin *et al.*, 1992), although the crystal structure of the protein has not been elucidated. In immune responses following vaccination or infection, neutralizing antibodies are directed primarily against the glycoprotein (Cox *et al.*, 1977). Previous studies of the glycoprotein have identified both conformational and linear, non-conformational antigenic sites. All have relied upon the binding of neutralizing mAbs and subsequent sequencing of mutants that escape neutralization. There are two major conformational sites; the immunodominant site is antigenic site II, which is formed from two regions, aa 34–42 and 198–200 (Prehaud *et al.*, 1988). The second site, antigenic site III, is located between aa 330 and 338 with an arginine residue at position 333 (Seif *et al.*, 1985), a residue that is associated with neuroinvasion (Dietzschold *et al.*, 1983; Seif *et al.*, 1985). It has been shown that 97 % of mAbs raised against the glycoprotein will bind to these immunodominant sites (Coulon *et al.*, 1993). However, as these epitopes are conformational, many antibodies fail to recognize them in Western blotting assays and the presence of antibodies against both sites has not been demonstrated in polyclonal sera raised against current vaccines. A non-conformational, neutralizing epitope has been identified by Dietzschold *et al.* (1990), who identified a linear epitope between aa 244 and 281, and a second site was identified by Ni *et al.* (1995) between aa 249 and 268. The latter authors concluded that the glycoprotein has many linear epitopes, most of which are not recognized as strongly as conformational epitopes. Furthermore, Luo *et al.* (1997) have demonstrated a neutralizing linear epitope in this region, located around the tryptophan residue at position 251.

In order to identify linear epitope sites on the glycoprotein of the rabies Pasteur virus (PV), we have used phage display (Smith & Scott, 1993), a technique that has successfully identified linear epitopes on a number of viral glycoproteins (Grabowska *et al.*, 1999). Two mAbs that recognized the virus glycoprotein, MnAb1 and MnAb2 (a gift from Merial, France), were studied by Western blot. The PhD12 peptide library (New England Biolabs) was used to characterize the mAb-binding site and was performed by following the manufacturer's protocols. *Escherichia coli* strain ER2738 was used for amplification of eluted phage, which provided input phage for the next round of biopanning. After four rounds of biopanning for each mAb, ten clones were amplified and the region encoding the peptide insert was sequenced. Synthetic peptides were manufactured (Alta Bioscience) in a linear form or as a multiple antigenic peptide (MAP) and purified to >95 %. Peptide inoculations were carried out in rabbits by using 2 mg MAP ml^{-1}, injected subcutaneously with Freund's complete adjuvant, with subsequent inoculations given with Freund's incomplete adjuvant. Groups of five mice were inoculated with saline, monomeric peptide or MAP. The peptides were all diluted to 2 mg ml^{-1} in saline and the inocula were combined with equal quantities of MPL + TDM adjuvant (Sigma). The mice were inoculated and given boosters on days 21 and 42. Each mouse was tail-bled prior to the first inoculation and then on days 14 and 28. On day 59, they

were killed humanely and a blood sample was retained. For rabbits, mice and rabies-vaccinated dogs, blood samples were tested by ELISA for reactivity to the peptide as follows. Each sample was tested in triplicate against the peptide at a concentration of 10 μg ml^{-1} in PBS or against a PBS-only background control in a Maxisorp plate (Nunc). The coated plate was incubated at 37 °C for 2 h. Plate contents were discarded, each well was filled with blocking buffer (PBS, 1 % Tween 20, 1 % non-fat milk) and the plate was incubated at 37 °C for 1 h. During the blocking incubation, samples were pre-diluted 1 : 250 in antibody diluent (PBS, 1 % Tween 80, 2 % fetal calf serum) and incubated at room temperature for 30 min. Blocking was followed by six washes with 1 % PBST (PBS, 1 % Tween 20; 1 min per wash, with agitation). On addition to the coated plate, samples were incubated for 1 h at 37 °C and then washed as above. Protein A–horseradish peroxidase (Sigma), diluted 1 : 6000 in antibody diluent, was incubated with the plate for 1 h at 37 °C, again followed by six washes with 1 % PBST. 3′,3′,5′,5′-Tetramethylbenzidine (Sigma) substrate was incubated with the plate for 25 min at room temperature in the dark. The reaction was stopped with 1 M sulphuric acid and A_{450} was measured. The fluorescent antibody virus neutralization (FAVN) test was used for the detection of rabies vaccine-specific antibodies in companion animal sera, as described by Cliquet *et al.* (1998).

Consensus sequences were obtained for both mAbs MnAb1 and MnAb2, panned against the peptide library [Fig. 1a (i and ii)]. For both target molecules, the amino acids tryptophan, aspartic acid and isoleucine were common to all sequences. In addition, four phage sequences for MnAb1 were identical to the consensus sequence of MnAb2, indicating that both antibodies probably bound to the same epitope site. Alignment of the consensus sequences with the mature PV glycoprotein (lacking the signal peptide) suggested that the proposed binding site was in a region near to the N terminus between aa 14 and 19 [Fig. 1a (iii)]. Further homology was observed at the terminal lysine for the consensus sequence for MnAb1 and at histidine and serine residues within the consensus sequence of MnAb2. In order to provide supporting evidence that the binding site of these antibodies was indeed the N-terminal domain, both were used to probe recombinant glutathione *S* transferase (GST) fragments of the RABV glycoprotein (Johnson *et al.*, 2002a). Identification of the N terminus as the site of the epitope is supported by the immunoblotting of recombinant glycoprotein fusion proteins with mAb MnAb2 (Fig. 1b). This antibody binds strongly to fragment RvG1-2, which encodes the first 128 aa of the mature glycoprotein, and supports the location of the epitope at the N-terminal region of the RABV glycoprotein. Confirmation that the region between aa 12 and 23 was the

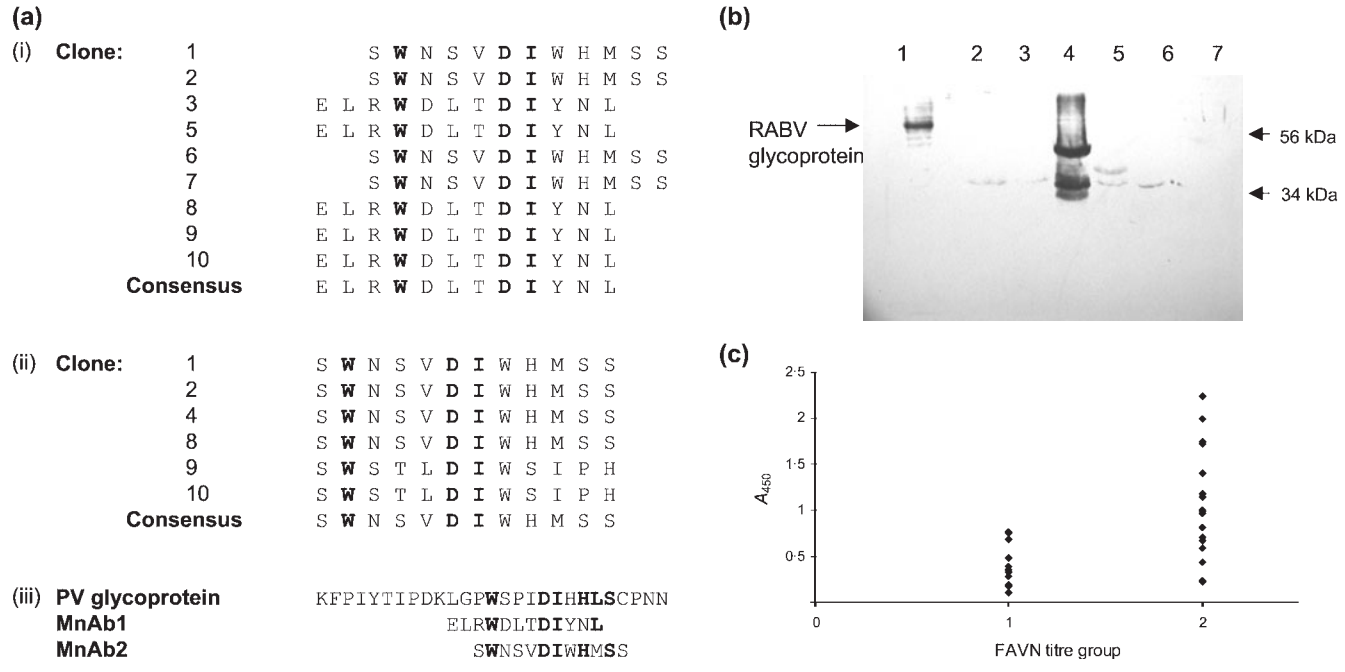

Fig. 1. (a) Coding sequences derived from phage clones for mAbs MnAb1 (i) and MnAb2 (ii), with consensus sequences shown below. Consensus sequences for both epitopes are aligned with the PV glycoprotein aa 1–27 (iii). Numbering refers to the mature glycoprotein. (b) Reactivity of mAb MnAb2 with the RABV glycoprotein (lane 1), GST supernatant without IPTG (lane 2), GST supernatant with IPTG (lane 3) and recombinant fusion proteins RVG1-2 (lane 4), RVG3-4 (lane 5), RVG5-6 (lane 6) and RVG 7-8 (lane 7). For further details on the antigens used, see Johnson *et al.* (2002a). (c) Detection of antibody in dog sera post anti-rabies vaccination against the 12 aa peptide. Sera shown were grouped into (1) FAVN-naïve and (2) vaccinated (FAVN > 0·5 IU ml^{-1}).

location of the epitope site was obtained by synthesis of a 12-mer peptide that was identical to this region. This peptide was detected by both mAbs by ELISA (data not shown). To establish immunogenicity of the epitope, a MAP of the epitope was synthesized and used to inoculate both rabbits and mice. Sera obtained from rabbits detected both the MAP and monomeric conformations of the peptide by ELISA (data not shown); both forms of the peptide were detectable after the first test bleed. Similar results were obtained following inoculation of mice with the MAP (Table 1). An antibody response was detected after inoculation with the multimeric peptide, which was not observed following inoculation with either saline or the monomeric form of the peptide. However, when mouse serum raised against the MAP was tested by FAVN, it was shown to have no neutralizing activity against the challenge virus standard at neutral pH and reflected the failure of both original mAbs to neutralize virus. The prototype ELISA was modified for screening two panels of dog sera, one of which consisted of naïve animals with no vaccination history and the second of sera from recently vaccinated dogs with a detectable response, as measured by FAVN. The results from this screening (Fig. 1c) suggest that the synthetic peptide mimic was recognized ($A_{450} > 1$) by sera from a subset of rabies-vaccinated dogs. This subset was characterized by elevated levels of neutralizing antibody (>10 IU ml^{-1}).

Alignment of the N terminus of the RABV glycoprotein has suggested that within genotype 1 (classical RABV), this region is strongly conserved (Johnson *et al.*, 2002b). Comparison of the extreme N terminus, which encodes the first 40 aa of the glycoprotein, of isolates from diverse locations throughout the world demonstrates that, of 18 sequences, 15 were identical to that of the PV vaccine strain (Fig. 2a). Of those that were not, one had a substitution outside the epitope region (RV56) and two, from the USA (RV53) and Zimbabwe (RV122), had single substitutions within the epitope. Both were also at critical tryptophan or aspartic acid residues. Alignment of the N-terminal sequence of the RABV glycoprotein of each of the seven lyssavirus genotypes (Fig. 2b) demonstrated that sequences from genotypes 2 (Lagos bat virus) and 3 (Mokola virus) contained three and five substitutions within the epitope site, respectively, whereas genotypes 4 (Duvenhage virus), 6 (European bat lyssavirus type 2) and 7 (Australian bat lyssavirus) were identical and genotype 5 (European bat lyssavirus type 1) had a single substitution. The epitope appears to be conserved within the proposed phylogroup 1 (genotypes 1, 4, 5, 6 and 7) (Badrane *et al.*, 2001).

Table 1. Detection of antibody by peptide ELISA and FAVN in mice inoculated with saline, monomeric peptide and multimeric peptide (pre-inoculation bleed and bleed 3)

Controls for ELISA were MnAb2 (+) and naïve dog sera (−); FAVN controls were OIE (Office International des Epizooties) positive sera at 0·5 IU ml^{-1} and negative dog sera. Titres are calculated in relation to the results of the OIE positive control at 0·5 IU ml^{-1}. ND, Not determined.

Inoculum	ELISA A_{450}	FAVN (IU ml^{-1})
Saline		
Pre	0·064 (±0·047)	ND
Bleed 3	0·311 (±0·14)	0·15 (±0·028)
Monomer		
Pre	0·130 (±0·086)	ND
Bleed 3	0·235 (±0·088)	0·17 (±0·00)
MAP		
Pre	0·046 (±0·009)	ND
Bleed 3	0·890 (±0·522)	0·17 (±0·00)
Controls		
+	4·640 (±0·321)	0·5
−	0·141 (±0·018)	0·17

Antibodies play a pivotal role in protecting vaccinated individuals against the development of rabies (Hooper *et al.*, 1998) and provide a useful tool for measuring the response to anti-rabies vaccination in animals (Fooks *et al.*, 2002). mAb studies by Coulon *et al.* (1993) suggested that very few antibodies that recognize linear epitopes are capable of showing neutralizing activity, although some neutralizing mAbs that bind linear sites have been described (Dietzschold *et al.*, 1990; Luo *et al.*, 1997). Identification and characterization of B-cell epitopes on the RABV glycoprotein to which antibodies bind have assisted in the understanding of the immunology of vaccination against this disease. By using phage-display and immunoblotting techniques, we have identified a linear epitope near to the N terminus of the RABV glycoprotein and defined the residues critical for antibody binding. A peptide mimic of this site is detected by the original mAbs that were used to identify the epitope and is immunogenic in both rabbits and mice. Previous studies have suggested that the N-terminal region of the RABV glycoprotein contains antigenic sites that are detected by the sera of rabbits (Dietzschold *et al.*, 1982) and dogs (Johnson *et al.*, 2002a) that have received anti-rabies vaccine. Attempts to locate epitope sites by using neutralization-escape mutants have identified a subset of antibodies that neutralize the rabies virus at acidic pH (Raux *et al.*, 1995; Gaudin *et al.*, 1996). These studies suggested that residues at the N terminus of the glycoprotein (lysine 10, proline 13 and serine 16) were critical to antibody binding. This partially overlaps the epitope site that has been identified by this study. However, this subset of antibodies bound to conformational epitopes, as evidenced by a failure to detect the glycoprotein by immunoblotting (a property of MnAb1 and MnAb2), and could neutralize the virus, a property that was not shown by either MnAb1 or MnAb2 at neutral (Table 1) or acidic (data not shown) pH. Therefore, we postulate that the epitope identified is a novel, linear site that has not been described or characterized previously. Furthermore, we have demonstrated that a peptide with identical sequence

(a)

Isolate	Country	Glycoprotein sequence (aa 1–40)
PV	Vaccine	KFPIYTIPDKL**GPWSPIDIHHLS**CPNNLVVEDEGCTNLSG
Rv53	USA	--------S----**C**------------------------
Rv56	USA	----------------------------------N----
Rv63	Poland	--
Rv73	Belize	--
Rv103	Morocco	--
Rv122	Zimbabwe	----------------**Y**------------------S---
Rv128	Zimbabwe	--
Rv202	Turkey	--
Rv245	Russia	--
Rv277	Pakistan	--
Rv304	Russia	--
Rv313	Germany	--
Rv425	South Africa	--
Rv440	Russia	--
Rv464	Mozambique	--
Rv484	Nigeria	--
Rv661	France	--
Rv677	France	--

(b)

Virus (genotype)	Genbank acc. no.	Glycoprotein sequence (aa 1– 40)
PV (1)	M13215	KFPIYTIPDKL**GPWSPIDIHHLS**CPNNLVVEDEGCTNLSG
LBV (2)	AF298148	D--L----E-I---**T**---**LI**--------QS-----GTS-V
MOKV (3)	Y09762	E--L----E-I**EK-T**---**MI**--------LS-E---NTE-P
DUVV (4)	AF298146	---F----------T-T-
EBLV-1 (5)	AF298143	----------I--------**N**--------I-------T-TP
EBLV-2 (6)	AF298144	----------T-M--------T-TV
ABLV (7)	AF006497	---L----------I-------S---

Fig. 2. Alignment of the first 40 aa of the PV glycoprotein and 18 other RABV isolates (a) or each of the seven currently recognized lyssavirus genotypes (b). The sequence encoding the proposed epitope is highlighted. The procedures used have been described previously (Johnson *et al.*, 2002b).

to that of the RABV glycoprotein is recognized by mAbs MnAb1 and MnAb2 and is therefore an accurate mimic of this novel epitope. This is an invaluable step in confirming the significance of this epitope in sera raised in recipients of rabies vaccines. However, the failure of sera raised against this peptide to neutralize RABV (Table 1) suggests that a peptide-vaccine approach using the epitope sequence identified in this study would not protect against challenge. Structural prediction analysis suggests that the epitope is antigenic whilst also indicating that it is not surface-exposed, providing a possible explanation for the failure of antibodies raised against it to neutralize virus (Table 1). However, the contribution of the N-terminal domain to the postulated properties of the RABV glycoprotein (receptor binding, membrane translocation etc.) is not known. Certain amino acids that are known for their involvement in active sites include serine, aspartic acid and histidine, which are all present in this epitope. Histidines, in particular, are associated with structural transitions (Roche & Gaudin, 2002). The critical residues of this epitope appear to be tryptophan (position 14), aspartic acid (position 18) and isoleucine (position 19), with further residues at positions 21, 22 and 23 that also contribute to the recognition of both mAbs. Tryptophan residues are predicted to form binding sites in protein–protein interactions (Ma *et al.*, 2003) and replacement of residues with tryptophan has been shown to increase ligand binding within murine major histocompatibility complex class I binding proteins (Smith *et al.*, 2002). This appears to be borne out in the detection of B-cell epitopes on the RABV glycoprotein. In a screen of six mAbs by using phage display, the most commonly observed residue was tryptophan (Veterinary Laboratories Agency, unpublished data) and previous studies that have detected linear epitopes on the glycoprotein have identified a site at tryptophan 251 (Ni *et al.*, 1995; Luo *et al.*, 1997). The epitope site is conserved between RABV isolates and lyssaviruses (phylogroup 1), but is probably hidden under native conditions. Roche & Gaudin (2002) have suggested that an increase in acidity can cause conformational changes in the native glycoprotein conformation, resulting in exposure of previously hidden hydrophobic residues. Such conformational changes could occur during the inactivation process of vaccine production, which might explain the detection of antibodies against a hidden site in vaccinated animals.

Screening of post-rabies vaccination sera by using techniques such as ELISA is of increasing importance in the control of animal movements, when attempting to minimize the risk of rabies reintroduction. The aim of such screening is to identify those animals that have developed a measurable antibody response to vaccination, and are therefore protected against the disease, prior to movement of an animal into a new country. Screening assays use whole, inactivated virus (Cliquet *et al.*, 2004) or recombinant viral proteins (Inoue *et al.*, 2003) as the antigenic target. Peptides offer a cost-effective alternative in the production of the appropriate antigenic component of such assays. From this

study, we conclude that a single peptide mimic of this epitope when used alone is a poor discriminator of vaccination status of sera, compared with current methods. However, it is possible that a panel of peptides or larger peptide fragments may offer a viable alternative for future assay development.

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