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Identification of a Novel B Cell Epitope on the Nucleocapsid Protein of Porcine Reproductive and Respiratory Syndrome Virus by Phage Display

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Abstract. A phage display peptide library targeting the nucleocapsid (N) protein of porcine reproductive and respiratory syndrome virus (PRRSV) strain CH-1a was generated and used for epitope mapping. After 3 rounds of biopanning with the monoclonal antibody (MAb) N3H2 directed against the N protein, 3 positive phages were screened and sequenced. These phages share a consensus sequence, IQTAFNQGA, which corresponds to the amino acid (AA) 79–87 segment of the CH-1a N protein. A small DNA fragment coding for IQTAFNQGA was expressed as a fusion product, and reacted to N3H2 in Western blots and indirect ELISA. Four truncated peptides (IQTAFNQG, IQTAFNQ, QTAFNQGA, and TAFNQGA) expressed as GST fusion products failed to react with N3H2. The sequences around the N3H2-binding site among the N proteins of 57 PRRSV strains were compared. Our results indicate that the IQTAFNQGA motif is highly conserved among North American and European isolates. We concluded that the precisely defined nona-peptide epitope is a novel conserved Linear B cell epitope on the N protein of PRRSV.

Key words: epitope mapping, phage display, porcine reproductive and respiratory syndrome virus

Introduction

Porcine reproductive and respiratory syndrome (PRRS) first emerged in North America in 1987. It is characterized by severe reproductive failure and respiratory distress [1]. The etiology of PRRS was originally described in The Netherlands [2], where the causative agent was identified as the Lelystad virus (LV), a RNA virus now commonly known as PRRS virus (PRRSV). In 1992, the first North American isolate responsible for PRRS was identified [3] and designated as VR-2332. Although North American and European isolates of PRRSV have similar morphology and genomic organization, they display significant genetic and antigenic variations, suggesting that they may represent two distinct genotypes [4,5]. In the last decade, panels of monoclonal antibodies (MAbs) have been raised against several PRRSV isolates and used to characterize the antigenic profile of the viral proteins. However, most of the identified epitopes were not defined precisely. Several domains of antigenic importance have been mapped on the nucleocapsid protein (N) of PRRSV [6–9].

Phage displayed peptide library technology provides a powerful tool for elucidating the structure and function of proteins [10,11], and has been used to locate a number of epitopes [12–15]. Fusion phages can be used in epitope mapping and vaccine design [16,17]. In the present study, we employed phage display methodology to map a conserved epitope on the N protein of the first Chinese isolate of PRRSV, CH-la [18].

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Materials and Methods

Virus Strains, MAb, Phagemid and Helper Phage

The PRRSV strain CH-la was isolated in China [18]. The N3H2 MAb directed against the N protein of CH-la was generated in our laboratory by immunization of BALB/c mice with the recombinant N protein expressed in *E. coli*. Phagemid pC89 and helper phage VCSM13 [19] were kindly provided by Dr Ying Wan at Third Military Medicine University, Chongqing, China.

Construction of PRRSV ORF7-Targeted Phage Library

Reverse transcription (RT)-PCR was performed to amplify the ORF7 gene which codes for the PRRSV N protein. The following primers based on the genomic sequence of CH-1a (GenBank accession no. AY0362626) were used for RT-PCR as described previously [20,21]: 5′-GTG GGA ATT CAG CGG AAC AAT GGG G TG-3′ and 5′-ATT CTA TCC TCG AGG ATC CCA AAG AAT ACC AG-3′. The PCR product was digested randomly by DNase I (TaKaRa) to generate a population of DNA fragments of different sizes and sequences, as described previously [13,22,23]. Small DNA fragments of 50–80 bp were isolated after size fractionation, and blunted with Klenow DNA polymerase (TaKaRa). The phagemid vector pC89 was digested with *Eco*RI and *Bam*HI, blunted and dephosphorylated. Approximately 10 ng of random DNA fragments were ligated to 40 ng of treated pC89 vector in the presence of T4 DNA ligase (Invitrogen) overnight at 16°C. Following phenol/chloroform extraction, ethanol precipitation, and a 70% ethanol washing, the ligation product was transformed into competent *E. coli* XL1-Blue cells (Stratagene). The transformed bacteria were incubated on a shaker at 200 rpm in 1 ml of SOC medium for 45 min at 37°C. A 2 μl aliquot of transformed bacteria was collected to determine the library size (number of transformed bacteria); the remainder was superinfected with VCSM13 helper phage [24]. Following overnight incubation, the phages were recovered from culture supernatants by two rounds of PEG-NaCl precipitation. Phage pellets were resuspended in 400 ml of 40% glycerol in phosphate-buffered saline (PBS, pH 7.4), and stored at −20°C.

Library sizes were estimated from an aliquot of transformed bacteria by plating of serial dilutions. To characterize the randomicity of the library, 10 colonies were selected randomly from the titrating plate and phagemid DNA was prepared. PCR was then done to determine the lengths of the inserted fragments. The following primers were used for the amplification: forward primer 5′-TGA GCG GAT AAC AAT TTC AC-3′ and reverse primer 5′-CGC CGA CAA TGA CAA CAA CC-3′. Three of the 10 selected colonies were subjected to sequencing of the inserted fragments.

Biopanning of the Gene-Targeted Peptide Library

Three rounds of biopanning were carried out according to the New England Biolabs (NEB) phage display manual, as previously described [13,22,23]. The gene-targeted peptide library was added to a plate previously coated with the N3H2 MAb and incubated for 1 h. The unbound phages were washed away and the bound phages were eluted with low pH buffer. The eluted phages were amplified by inoculating early-log XL1-Blue *E. coli*.

Phage ELISA

After 3 rounds of biopanning, 40 individual clones were cultured and tested for their ability to bind N3H2 in a sandwich ELISA as described by NEB's Ph.D.C7C manual. Briefly, ELISA plates were coated overnight with 100 μg/ml of N3H2. After 2 h of blocking with 4% skim milk, 40 phage clones were added to the wells (10^{11} pfu/100 μl per well) and incubated for 1 h at room temperature. Bound phages were detected with horseradish peroxidase (HRP)-conjugated anti-phage pVIII MAb (Pharmacia), and visualized with OPD. Each sample was subjected to three independent ELISAs. Wild-type phage VCSM13 (10^{11} pfu/100 μl) and tris-buffered saline (TBS; 100 μl) served as negative controls.

Phage Sequencing

The positive samples yielded by the sandwich ELISA were sequenced with the universal primer 5′-TGA GCG GAT AAC AAT TTC AC-3′.

Construction of Recombinant Expression Vector

Oligonucleotides coding for amino acid (AA) 79–87, 79–86, 79–85, 80–87 and 81–87 of the N protein were codon optimized [25] and cloned into the pGEX-6p-1 prokaryotic expression vector (Pharmacia), generating pEp703, and pEp703-1 to 4. The expressed fusion proteins were analyzed by SDS-PAGE and Western blot using standard procedures [26].

Western Blot

The *E. coli* BL21 (DE3) cells transformed by pEp703 and 4 deleted mutants were subjected to induction with 1 mM isopropyl-D-thiogalactopyranoside (IPTG). Cells were harvested and suspended in PBS (0.1 M, pH7.4). The expressed fusion proteins (GST-ep, GST-ep-1 to 4) were analyzed by SDS-PAGE and Western blot using the MAb N3H2 as described by Sambrook et al. [26].

Results

Generation of a PRRSV ORF7-Targeted Peptide Library Displayed by Phage

Serial dilution plating of transformed bacteria indicated that the library size was approximately 2×10^4 clones/ml in size, which is enough for epitope mapping with the methods described by Oleksiewicz et al. [22]. The lengths of the inserted fragments showed diversity in the 10 clones picked randomly from the library, and the displayed sequences of the 3 sequenced clones were dispersive.

Identification of Epitopes with MAb Labeling

Three of 40 phage clones reacted with MAb N3H2 in the phage ELISA, while the wild-type phage VCSM13 control did not (Fig. 1). The positive phage clones (PL7, PL27, and PL33) showed similar affinities to N3H2. The 3 sequenced displayed phages shared a consensus sequence, IQTAFNQGA, which corresponds to AA 79–87 of the N protein of the PRRSV CH-la strain (Fig. 2). To further characterize the epitope recognized by N3H2, a DNA fragment coding for IQTAFNQGA was expressed as a GST fusion protein in *E. coli*. Western blot analysis revealed that the GST fusion protein product reacted with N3H2 (Fig. 3).

Precise Defining of the Epitope

To precisely define the epitope, 4 constructs with deletions at both termini of IQTAFNQGA were generated to express the polypeptides IQTAFNQG, IQTAFNQ, QTAFNQGA, and TAFNQGA as GST fusion products in *E. coli*. The 4 fusion proteins showed no reactivity with MAb N3H2 in Western blot analysis. This suggests that the nonamer peptide IQTAFNQGA is the minimal epitope recognized by MAb N3H2.

Sequence Alignment

The N protein sequences from 41 North American-type and 16 European-type PRRSV isolates were aligned using DNAstar software. Sequence comparison revealed that IQTAFNQGA (located between AA 79 and 87) was well-conserved among the European and North American genotypes of PRRSV. There was only one mutation found in this region. Q^{80} in CH-1a is mutated to H^{80} in the Kitasato 93-1 strain, which was isolated in Japan in 1993 (Fig. 4).

Discussion

In this study, a linear epitope IQTAFNQGA (named Ep703) was identified between AA 79 and 87 on the N protein of PRRSV. The sequence of Ep703 is identical to that of AA 80–88 of the N protein of the well-defined, European-type PRRSV LV strain. The homologous LV strain sequence is a component of a conformational epitope consisting of AA 51–67 and AA 80–90 [7]. The CH-1a N protein had 2 mutations in AA 50–66 compared to AA 51–67 of the LV strain N protein (LV A^{60} to CH-1a T^{59}, and LV L^{65} to

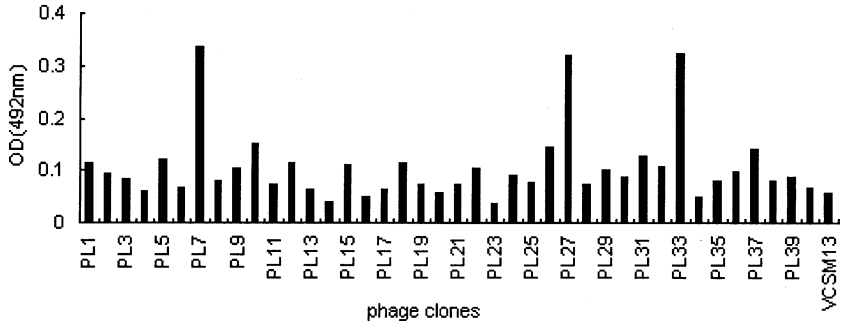

Fig. 1. The phage displayed peptides of the PRRSV N protein detected by sandwich ELISA. Forty phage clones picked after 3 rounds of biopanning were used as antigens, and wild-type phage VCSM13 was used as a control in the ELISA. The ELISA revealed 3 positive phage clones: PL7, PL27, and PL33.

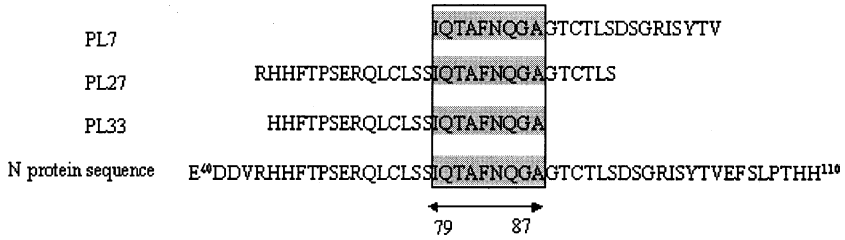

Fig. 2. Comparison of the peptides displayed on the 3 phages and N protein of the PRRSV CH-1a strain. The 3 phage clones share a consensus sequence with AA 79–87 of the PRRSV N protein.

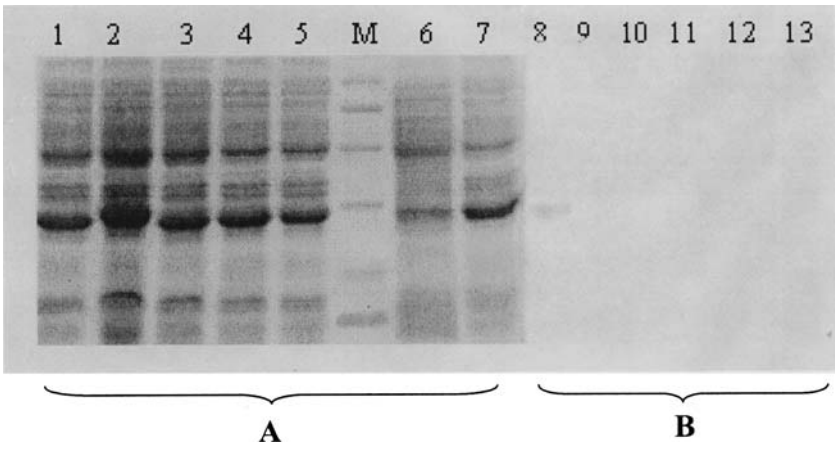

Fig. 3. Detection of the fusion protein, GST-ep, by SDS-PAGE and Western blot analysis. (a). lanes 1–5, the expressed protein of recombinant plasmids, GST-ep and 4 shorter peptides (~26 kDa); M, the protein marker standards (97.4, 66.2, 43.0, 31.0, 20.1, 14.4 kDa, respectively); Lane 6, the whole cellular protein of *E. coli* BL21 transformed with recombinant plasmid pEp703, without IPTG induction; lane 7, the whole protein of *E. coli* BL21 transformed with plasmid pGEX-6p-1, with IPTG induction. (b). Western blot analysis of the GST-ep (lane 8), GST-ep-1 to 4 (lanes 9–12) and GST (lane 13).

CH-1a R^{64}). In addition, there were three cysteine residues (C^{27}, C^{76}, C^{90}) in the N proteins of CH-1a and other North American-type isolates, but only two cysteine residues (C^{27}, C^{76}) in the N proteins of the LV and other European-type isolates.

It is possible that S–S bond formation differed between the two genotypes. Disulfide linkage plays a critical role in maintaining protein structure [27,28]. Furthermore, comparative analysis indicated that the N proteins showed less than 60% AA

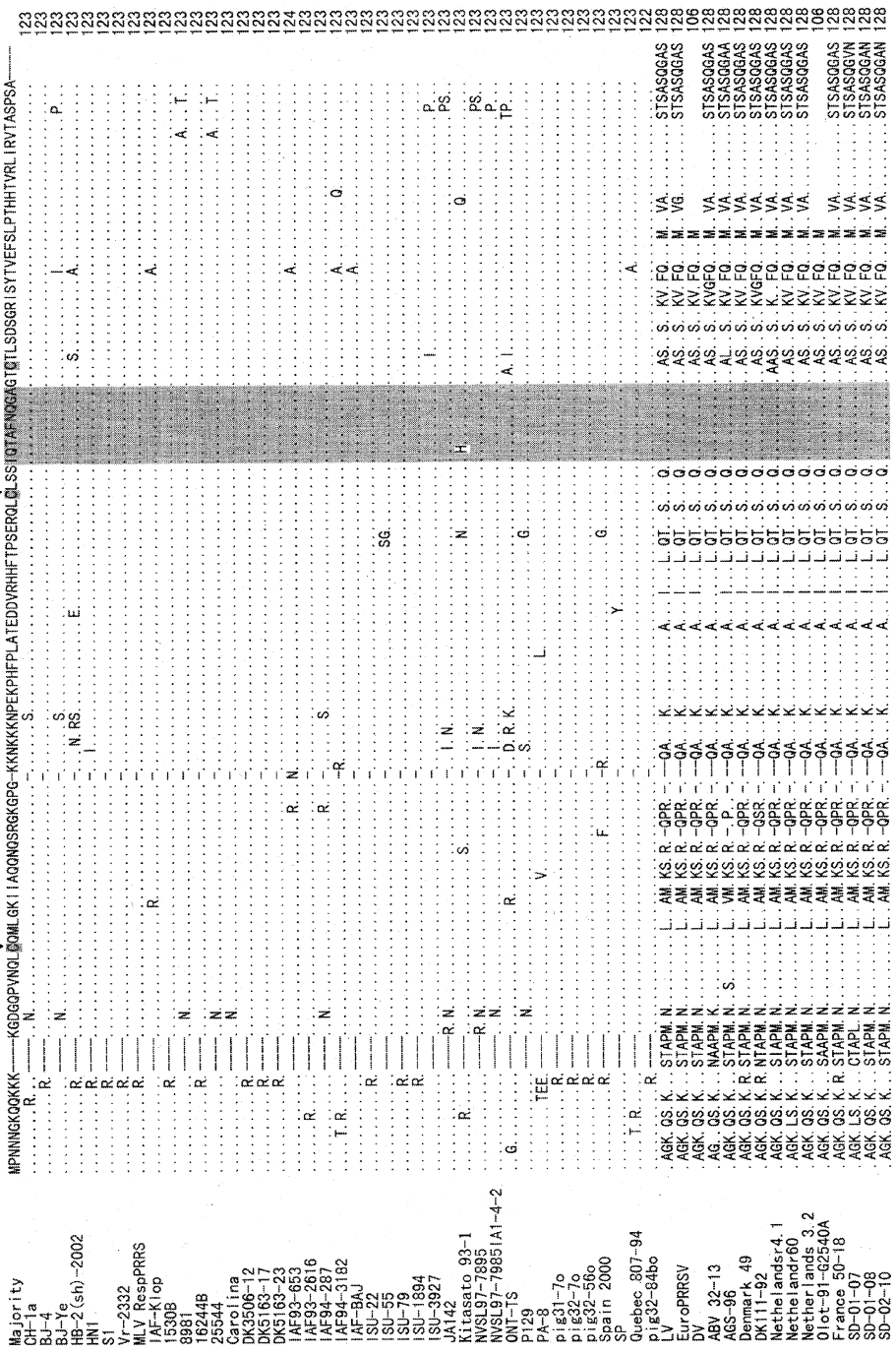

Fig. 4. Alignment of PRRSV N protein sequences among North American- and European-type isolates. A total of 57 isolates, including 41 North American and 16 European, were included. The motif IQTAFNQGA (shadowed) is highly conserved among all the isolates, excepting one amino acid mutation (^{80}Q → H) in the Kitasato 93-1 strain.

identity between CH-1a and LV, suggesting that their different antigenicity is due to a difference in the conformational structure of the proteins. Ep703's secondary structure is predicted to be composed mainly of beta-sheets. The three-dimensional structure of the N protein of the North

American-type SP strain has been illustrated [28] and provides a useful basis for virus type comparison. There exists 96% AA identity between the N proteins of the CH-1a and SP isolates. According to the three-dimensional antigenic structure of the N protein of the SP strain [28], the AA 79–87 region is located at a turn from an alpha-helix to a beta-sheet, suggesting that this epitope is exposed on the surface of the N protein and readily available for binding. This highly conserved linear B-cell epitope on the N protein may constitute a valuable diagnostic tool.

Previous researchers have mapped epitopes of the N protein. A panel of 12 MAbs raised against the PRRSV PA-8 was used to describe nine overlapping protein fragments ranging in length from 25 to 112 AA [6]. The domains 30–52, 69–123, and 37–52, were each independently identified by 3 separate MAbs from this panel. Seven of the MAbs tested specifically recognized a conformational epitope formed in part by AA 52–69 [6]. The Ep703 (AA 79–87) is encompassed by the antigenic domain AA 69–123. Therefore, the present study identifies a more precise epitope.

Yang and colleagues described 5 continuous epitopes (designated EpORF7-A through E) in the N protein of the PRRSV with a panel of 24 MAbs raised against the American isolate ISU-P [8]. But the sequences for the five epitopes were not clearly identified. Therefore, it is not clear whether the Ep703 epitope overlaps with any of the 5 epitopes. Casal and others reported two discontinuous epitopes localized in the first 78 residues of the N protein, and a linear common epitope (AA 50–66), but the linear epitope is not recognized by a pool of pig sera, and it was not finely defined [29].

Oleksiewicz and colleagues [22] screened phage display libraries of PRRSV protein fragments with the sera from experimentally infected pigs and identified 10 linear epitope sites (ES) of 11–53 AA in length. Of the 10 ES identified, eight localized to the replicase polyprotein, one to GP3, and one to GP4. The GP4 ES was shown to be an immunodominant epitope, in that six experimentally PRRSV-infected pigs consistently had very high antibody titers against it. This immunodominance likely prevented the authors from identifying more ES in the structural proteins of PRRSV, including the Ep703 epitope defined in our study.

In summary, we identified a hitherto novel B cell epitope between AA 79 and 87 on the PRRSV N protein by gene-targeted phage display peptide library, and this epitope is highly conserved among PRRSV isolates.

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