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Generation of a mouse scFv library specific for porcine aminopeptidase N using the T7 phage display system

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Porcine aminopeptidase N (pAPN) is a common cellular receptor for swine transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV). To investigate single-chain fragment variable (scFv) repertoire against pAPN, the genes encoding the immunoglobulin light chain variable region (VL) and heavy chain variable region (VH) were amplified by reverse transcript polymerase chain reaction (RT-PCR) using a series of degenerate primers from the spleen of BABL/c mice immunized with native pAPN. The VL and VH amplicons were combined randomly by a 12 amino acid flexible linker by splicing by overlap extension PCR (SOE-PCR), which produced the scFv gene repertoire. After ligation of the scFv gene repertoire into the T7Select10-3b vector, a mouse scFv phage library specific for pAPN was produced through in vitro packaging. The primary scFv library against pAPN contained 2.0×10^7 recombinant phage clones, and the titer of the amplified library was 3.6×10^9 pfu/mL. *Bst*NI restriction analysis and DNA sequencing revealed that 28 phage clones from the primary pAPN scFv library showed excellent diversity. The effectiveness of the scFv library against pAPN was verified further by phage ELISA using the recombinant protein of the pAPN C subunit as coating antigen. The construction and evaluation of a murine scFv library against the common receptor pAPN of porcine coronaviruses TGEV and PEDV using the T7 phage display system are described.

1. Introduction

Porcine aminopeptidase N (pAPN) is a type II transmembrane glycoprotein belonging to the membrane-bound metallopeptidase family; it is composed of 963 amino acids and has a molecular weight of 150–160 kDa (Delmas et al., 1994; Hooper, 1994). The pAPN is distributed mainly on the surface of porcine intestinal epithelial cells, where some pAPN proteins are cleaved into a B subunit (aa 1–572, 95 kDa) and a C subunit (aa 573–963, 50 kDa) by trypsin (Kenny and Maroux, 1982; Semenza, 1986; Jongeneel et al., 1989). pAPN has been revealed to be a common cellular receptor for swine transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) to allow their entry into cells (Delmas et al., 1992; Li et al., 2007).

TGEV and PEDV belong to group I of the genus Coronavirus, family Coronaviridae, order Nidovirales, and cause severe diarrhea and high mortality rates of up to 100% in piglets (Pensaert and Debouck, 1978; Reynolds and Garwes, 1979). In the past 30 years, disease caused by TGEV and PEDV has broken out frequently in many swine-raising countries, and has led to heavy economic losses (Jung and Chae, 2005; Kim et al., 2007; Vemulapalli et al., 2009; Chen et al., 2010). Many studies of these viruses have been reported over the past decades, but TGEV and PEDV still exist and cause frequent occurrences of piglet diarrhea in various countries, notably in China (Sun et al., 2007, 2008; Elia et al., 2010; Ribes et al., 2011; Nogales et al., 2011). Most researchers have focused mainly on how to kill or control the pathogens TGEV and PEDV. Only a little information has been reported regarding anti-viral measures against TGEV and PEDV that are based on the cellular receptor pAPN (Liu et al., 2009; Ren et al., 2010).

The single-chain fragment variable (scFv) is a small engineered antibody in which the variable light chain (VL) and heavy chain (VH) of the antibody are connected by a short polypeptide linker. The scFv holds important potential for receptor blockade, pathogen neutralization and therapeutic antigen targeting in vivo (Haidaris et al., 2001; Pansri et al., 2009). At present, isolation and

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Table 1
Primers for PCR amplification of the immunized mouse VH and VL genes.

Mouse VL forward primers	
MVL-F1	5′-cggcatttcgtc*GAATTC*cGAYATTGTDHTVWCHCAGTC-3′
MVL-F2	5′-cggcatttcgtc*GAATTC*cGAYATTNWKMTVAHDCAGTC-3′
MVL-F3	5′-cggcatttcgtc*GAATTC*cGAYRTYBWRMTSACMCARWC-3′
MVL-F4	5′-cggcatttcgtc*GAATTC*cGAYATYSWGMTGACNCARBC-3′
MVL-F5	5′-cggcatttcgtc*GAATTC*c GAYRYTGTKRTRMYYMRGDW-3′
Mouse VL reverse primers	
MVL-R1	5′-tcctcctgagccgccgccgccagaaccaccaccaccCCGTTYNAKYTCCARCTTDG-3′
MVL-R1	5′-tcctcctgagccgccgccgccagaaccaccaccaccMCSTWBNABHKYCAVYYTDG-3′
Mouse VH forward primers	
MVH-F1	5′-ggtggtggtggttctggcggcggcggctcaggaggaSAKGTBMAGCTBMAGSASTC-3′
MVH-F2	5′-ggtggtggtggttctggcggcggcggctcaggaggaSAGGTYCARCTBCARCARTC-3′
MVH-F3	5′-ggtggtggtggttctggcggcggcggctcaggaggaSAVGTSMWSBTGRWGSARTC-3′
MVH-F4	5′-ggtggtggtggttctggcggcggcggctcaggaggaGAKGTGMAVSKGRTGGARTC-3′
MVH-F5	5′-ggtggtggtggttctggcggcggcggctcaggaggaGARGTRMARSTTSWBGAGTC-3′
MVH-F6	5′-ggtggtggtggttctggcggcggcggctcaggaggaSAKGTBMMNYTVVWVSWRYS-3′
Mouse VH reverse primers	
MVH-R1	5′-ttctatgcgc*AAGCTT*ttaYGARGARACDSTGASMRKRGT-3′
MVH-R2	5′-ttctatgcgc*AAGCTT*ttaYGARGARRMSKKKASWGWGRT-3′
MVH-R3	5′-ttctatgcgc*AAGCTT*ttaYGAGGAGACKGTGASHGDGGH-3′
Primers for generation of scFv genes in SOE-PCR	
MscFv-F	5′-cggcatttcgtc*GAATTC*c-3′
MscFv-R	5′-ttctatgcgc*AAGCTT*tta-3′

S = G/C, R = G/A, K = G/T, M = A/C, Y = C/T, W = A/T, H = A/C/T, B = C/G/T, V = A/C/G, D = A/G/T, and N = A/T/G/C.

identification of interesting scFvs are carried out through a powerful phage display library. In the current study, the T7 phage display system was use to construct a mouse scFv library specific for pAPN, the common receptor for TGEV and PEDV. The scFv library against pAPN was generated, and the potential utility of the scFv library was confirmed by restriction analysis, sequencing, and specific antigen binding. The pAPN scFv library and its preparation will provide useful information and a basis for further research into anti-viral agents against TGEV and PEDV, and other coronaviruses that use APN as a cellular receptor.

2. Materials and methods

2.1. Primers for mouse scFv gene repertoire

Primers for PCR amplification of the immunized mouse VL and VH genes were designed according to the degenerate primers described by Okamoto et al. (2004). The 5′ ends of the mouse VL forward primers and VH reverse primers were modified to include *Eco*RI and *Hin*dIII sites, respectively. In order to splice the VL and VH amplicons, a complementary overlapping sequence encoding a flexible linker of 12 amino acids (GGGGSGGGGSGG) was added to the 5′ ends of the mouse VL reverse primers and VH forward primers, respectively. The primers MscFv-F and MscFv-R were designed for generation of the scFv genes by splicing by overlap extension PCR (SOE-PCR). All primers are shown in Table 1.

2.2. Immunization of mice with pAPN

Female BALB/c mice, 6–8 weeks old (Harbin Veterinary Research Institute Experimental Animal Center, Harbin, China), were injected subcutaneously with 50 μg of native pAPN from porcine kidney (Sigma–Aldrich, St. Louis, MO, USA) emulsified in an equal amount of complete Freund's adjuvant (Sigma, St. Louis, MO, USA). At 3-week intervals two boosters of 50 μg of the native pAPN emulsified in an equal amount of incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) were administered. The spleens of the immunized mice were isolated 3 days after the last booster inoculation, and stored at −80 °C.

2.3. RNA extraction and cDNA synthesis

Total RNA was prepared from spleen tissue of the immunized mice using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The mRNA was purified from extracted total RNA using a Poly AT tract mRNA isolation kit (Promega, Madison, WI, USA). The first strand cDNA was generated using an OrientExpress Oligo (dT) cDNA Synthesis Kit (Novagen, San Diego, CA, USA) according to the manufacturer's instructions.

2.4. Generation of mouse scFv gene repertoire

The PCR amplification of the mouse VL and VH genes was carried out in a 50 μL mixture containing 10 nmol of each forward and reverse primer, 4 μL cDNA extracted from spleen tissue of the immunized mice, 0.25 mmol each dNTP mixture (Takara, Dalian, China), 10× buffer, and 0.5 U ExTaq DNA polymerase (Takara, Dalian, China). The amplification conditions comprised an initial denaturation at 95 °C for 5 min, followed by 35 PCR cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. The amplified products of the mouse VL and VH genes were purified with a Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany). A SOE-PCR was performed for generation of the mouse scFv gene. Briefly, the SOE-PCR reaction contained 100 ng of purified VL products and 100 ng of purified VH products, 10 nmol of MscFv-F and MscFv-R primers, 0.25 mmol each dNTP mixture (Takara, Dalian, China), 10× buffer, and 0.5 U ExTaq DNA polymerase (Takara, Dalian, China). The cycling conditions for the SOE-PCR were the same as mentioned previously.

2.5. Cloning of mouse scFv gene repertoire into T7Select10-3b vector

The mouse scFv gene products were digested with restriction enzymes *Eco*RI and *Hin*dIII. After purification of the digested products, cohesive ligation of the scFv gene products (0.12 pmol) with T7Select 10-3b *Eco*RI/*Hin*dIII Vector Arms (0.04 pmol) was carried out in a working volume of 5 μL using T4 DNA Ligase (Novagen, San Diego, CA, USA) at 16 °C for 12 h. The ligation products were added directly to 25 μL T7 Packaging Extracts (Novagen, San Diego, CA, USA), and the mixtures were incubated for 2 h at 22 °C for in vitro packaging. After the reaction had been stopped by the addition of

270 μL sterile LB medium, the mouse scFv library against pAPN was obtained from the packaged phages. The primary pAPN scFv library was amplified by liquid lysate amplification according to the T7Select System Manual. The titers of the primary and amplified library were determined by plaque assay.

2.6. BstNI restriction analysis and DNA sequencing

Thirty phage clones were selected randomly from the primary scFv library against pAPN, and they were cultured by liquid lysate amplification. After purification by the PEG/NaCl method, the genomic DNA of each phage clone was extracted by simple heat treatment. The inserted scFv gene of each recombinant phage was amplified by PCR using the primers T7SelectUP (5′-GGAGCTGTCGTATTCCAGTC-3′) and T7SelectDOWN (5′-ACCCCTCAAGACCCGTTTA-3′), which were specific for amplification of the region surrounding the multiple cloning site of the vector T7Select 10-3b. The amplified product of each phage clone was digested with the frequent cutting restriction enzyme *Bst*NI (New England Biolabs Inc., Beverly, MA, USA). Furthermore, the nucleotide sequences of three scFv clones with different restriction patterns were obtained. The framework region (FR) and complementarity determining region (CDR) of each sequenced mouse VL and VH gene were analyzed using the IMGT/V-QUEST program version 3.2.20 of the international immunogenetics information system (http://www.imgt.org./IMGT_Vquest/share/textes/).

2.7. scFv library validation

To determine the presence of antigen-specific scFv that bound to the recombinant C subunit of pAPN (pAPN-C) expressed in *Escherichia coli* in a previous study, a biopanning procedure was run for enrichment of the specific scFv phages according to the T7Select System Manual. Twenty-five single phage clones were selected from output phages of the third round of biopanning, and their reactivity with pAPN-C was analyzed by phage ELISA. Briefly, the ELISA plate wells were coated using the purified pAPN-C protein at 4 °C for 12 h. After blocking with skimmed milk, 1.0×10^8 pfu of selected phages were added to the well, and incubated at 37 °C for 1 h. Phages that bound to the immobilized pAPN-C protein were detected by incubation with HRP-conjugated anti-T7 tag antibody (EMD Chemicals Inc., San Diego, CA, USA), followed by a coloration reaction using the substrate TMB solution (Amresco, USA). The absorbance at 450 nm was measured. The phages generated by the T7Select Control Insert were used as negative controls. The sample OD_{450} value/negative control OD_{450} value (S/N) > 2 was determined as the positive standard.

3. Results

3.1. Amplification of the VL and VH genes from immunized mice

The cDNA was prepared using the extracted total RNA from spleen tissue of the immunized mice by reverse transcription (RT). The VL and VH genes were amplified clearly at about 400 and 340 bp by PCR using the synthesized cDNA as a template. Furthermore the amplified products of the VL and VH genes were combined randomly by the 12 amino acid flexible linker, using splicing, by SOE-PCR, resulting in the scFv gene repertoire (Fig. 1).

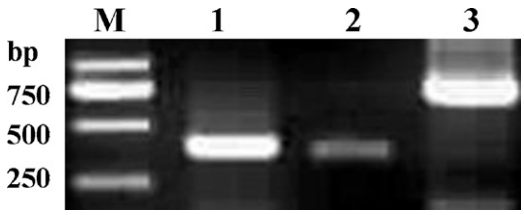

Fig. 1. Amplification of the VL and VH genes from immunized mice. Lane M: DNA Marker DL2000; Lane 1: PCR products of the VH gene; Lane 2: PCR products of the VL gene; Lane 3: Amplified products of the scFv gene by SOE-PCR.

3.2. Diversity analysis of the scFv library

The primary scFv library specific for pAPN was generated by cloning of the scFv gene repertoire into the T7Select10-3b vector and in vitro packaging; subsequently the primary library generated was amplified by the liquid lysate method. The titers of the primary and amplified library were 2.0×10^7 pfu/mL and 3.6×10^9 pfu/mL, respectively. Diversity analysis of the primary library was carried out by *Bst*NI fingerprinting of the 30 random phage clones. The *Bst*NI digestion pattern indicated that 28 phage clones had a different digestion pattern, with the exception of two unamplified scFv clones (Fig. 2). Sequence analysis revealed that the framework region (FR) and complementarity determining region (CDR) of the three random scFv clones showed the greatest difference in amino acid sequences (Table 2).

3.3. Validation of the scFv library by phage ELISA

After performing three rounds of biopanning for the primary scFv library, the reactivity of 25 scFv phage clones with the recombinant protein pAPN-C was evaluated by phage ELISA. Among the 25 phage clones, 10 phage clones showed positive reactions (S/N value > 2) with the recombinant protein pAPN-C in the phage ELISA (Fig. 3).

4. Discussion

As a basic functional unit of the antibody, the single-chain fragment variable (scFv) region maintains antigen specificity, and has a wide range of biomedical applications (Intorasoot et al., 2007; Bhatia et al., 2010). The phage library in which the scFv gene repertoires are expressed on the surface of phages becomes a powerful tool for isolation and identification of scFv molecules of interest (Cabezas et al., 2008; Tang et al., 2009). Here we describe a simple and rapid method for the generation and evaluation of a murine scFv library specific for porcine aminopeptidase N (pAPN), a common cellular receptor for TGEV and PEDV, using the T7Select Phage Display System. Compared with other similar technologies, the

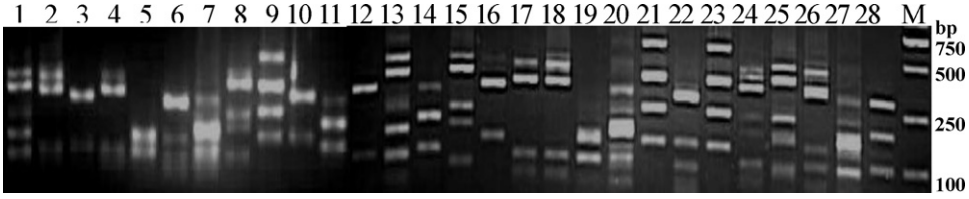

Fig. 2. Fingerprint analysis of the scFv phage clones by the frequent-cutting enzyme *Bst*NI. Lane 1–25: The restriction patterns of each scFv clone; Lane M: DNA Marker DL2000.

Table 2
The VL and VH amino acid sequences of scFv clones selected from primary library.

Phage clones	Chain type	FRI	CDRI	FR2	CDR2	FR3	CDR3	FR4
mouC-1	VL	DIVLTQTTLTLSVTIGQPASISCKSS	QSLLDSDGKTY	LNWLLQRPGQSPKRLIY	LVS	KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYC	WQGTHFPQT	FGGGTKLESNNS
mouC-15		DIVMTQSTSSLAMSVGQKVTMSC	KSSQSLLNSSNQKNYLA	WYQQKPGQSPKLLVY	FASTRES	GVPDRFIGSGSGTDFTLTISSVQAEDLADYFC	QQHYSTPWT	FGGGTKLEIK
mouC-21		DIVLTQTTAIMSASPGEKVTMTCSA	SSSVSY	MHWYQQKPGSSPKLWIY	STS	NLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYC	HQYHRSPYT	FGGGTKLEIK
mouC-1	VH	QVQLQESGPGLVAPSQSLSITCTVS	GFSLTGYG	VNWVRQPPGKGLEWLGM	IWGDGST	DYNSALKSRLSISKDNSKSQVFLKMNSLQTDDTARYYC	ARQGNYFDY	WGRAATLIV
mouC-15		QVQLPESGPGLVAPSQSLSITCTVS	GFSLTGYG	VNWVRQPPGKGLEWLGM	IWGDGST	DYNSALKSRLSISKDNSKSQVFLKMNSLQTDDTARYYC	ARGGNYFDY	WGQGTTLIV
mouC-21		RGEAAESGPGLVAPSQSLSITCTVS	GFSLTDYG	VSWIRQPPGKGLEWLGV	IWGGGST	YYNSALKSRLSISKDNSKSQVFLKMNSLQTDDTARYYC	ARDRGILRYFDY	WGQGTTLIVSS

Fig. 3. Phage ELISA of the phage clones generated from the third round of biopanning.

T7Select Phage Display System is easy to use and has the capacity to display peptides of up to about 50 amino acids or 1200 amino acids in size using the T7Select phage display vectors of different copy numbers.

In the current study the optimized degenerate primer sets reported by Okamoto et al. (2004) were chosen to amplify the immunoglobulin light chain variable region (VL) and heavy chain variable region (VH) genes. It has been revealed that these primer sets showed good coverage for amplification of the whole VL and VH gene repertoire by RT-PCR. The VL and VH amplicons were connected by a flexible linker of 12 amino acids by SOE-PCR, to generate the scFv gene repertoire. A number of studies have shown that the length of the linker region between the VH and VL genes affects the activity of scFv molecules, and a linker 12 amino acids in length is suitable to maintain the affinity of the scFv molecules (Wang et al., 2008). In this study the mid-copy number vector T7Select10-3b with *Eco*RI/*Hin*dIII arms for directional cloning of appropriately prepared inserts was used to construct the scFv phage library against pAPN. Generally, each recombinant T7 phage produced by the T7Select10-3b vector is capable of displaying 5–15 copy scFv molecules, and is suitable for the stable expression of the scFv gene and other genes up to about 750 bp in size. The size of the primary scFv library and the amplified scFv library specific for pAPN was 2.0×10^7 pfu/mL and 3.6×10^9 pfu/mL, respectively. Although this is considered to represent a sufficiently large scFv library, the size of both the primary library and the amplified library was slightly lower than the recommended size (6×10^8 pfu/mL and 10^{10} pfu/mL, respectively). It is suggested that the inserted scFv genes may affect the growth of the recombinant phages to a certain extent.

Using BstNI restriction analysis and DNA sequencing we have shown that the scFv phage library against pAPN has a sufficient diversity. In addition, among the 30 random phage clones from the primary scFv library against pAPN, the inserted scFv genes of two phages were not amplified. This demonstrates that there may have been some non-recombinant phages in the scFv phage library constructed. The phage ELISA indicated that, in the output phages of the third round of biopanning, forty percent of phage clones demonstrated a positive reaction with the recombinant protein pAPN-C. This result suggests that a large number of mouse spleen lymphocytes were activated and had proliferated as a result of the repeated immunization using the well-characterized native pAPN as immunogen. The pAPN scFv phage library will have a potential use for further receptor antagonist screening and analysis of the function of the receptors of TGEV and PEDV.

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