Virology 410 (2011) 299–306

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Phage displayed peptides recognizing porcine aminopeptidase N inhibit transmissible gastroenteritis coronavirus infection *in vitro*

Xiaofeng Ren [a,*], Boqi Liu [a], Jiechao Yin [b], Heng Zhang [a], Guangxing Li [a,*]

[a] College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, China
[b] College of Life Sciences, Northeast Agricultural University, Harbin 150030, China

ARTICLE INFO

Article history:
Received 26 May 2010
Returned to author for revision 22 June 2010
Accepted 16 November 2010
Available online 21 December 2010

Keywords:
Coronavirus
TGEV
Peptides
Phage display
Vaccine

ABSTRACT

Porcine aminopeptidase N (pAPN) is a cellular receptor of transmissible gastroenteritis virus (TGEV), a porcine coronavirus. Interaction between the spike (S) protein of TGEV and pAPN initiates cell infection. Small molecules, especially peptides are an expanding area for therapy or diagnostic assays for viral diseases. Here, the peptides capable of binding the pAPN were, for the first time, identified by biopanning using a random 12-mer peptide library to the immobilized protein. Three chemically synthesized peptides recognizing the pAPN showed effective inhibition ability to TGEV infection *in vitro*. A putative TxxF motif was identified in the S protein of TGEV. Phages bearing the specific peptides interacted with the pAPN in ELISA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays confirmed the protective effect of the peptides on cell infection by TGEV. Moreover, the excellent immune responses in mice induced by the identified phages provided the possibility to develop novel phage-based vaccines.

Introduction

Transmissible gastroenteritis virus (TGEV) is the causative agent of transmissible gastroenteritis (TGE), a highly contagious enteric disease in swine. The typical clinical symptoms of TGE consist of vomiting, acute diarrhea, and dehydration. Older animals usually recover, but in newborn piglets mortality rates may reach 100% (Saif and Wesley, 1992; Laude et al., 1993; Schwegmann-Wessels et al., 2002; Ren et al., 2008). TGE prevalence causes enormous economic losses in pig industry. TGEV is an enveloped, single-stranded, positive-sense RNA virus that belongs to the family *Coronaviridae* and order Nidovirales (Enjuanes et al., 2000; Yin et al., 2010). They own the largest RNA viral genome, about 30 kb. TGEV produces at least eight subgenomic mRNAs during viral replication and each mRNA consists of 3′ co-terminal nested sets (Laude et al., 1993; Vaughn et al., 1995).

Three major structural proteins of coronavirus: the spike (S), the integral membrane (M) glycoprotein, and the nucleocapsid (N) protein are translated from mRNAs 2, 5 and 6, respectively (Spaan et al., 1988; Laude et al., 1993; Almazán et al., 2000). The M protein is an abundant component of coronaviruses (Rottier, 1995; Ren et al., 2010b). The M protein as the major interferon inducing component has been proposed to play a role in innate immune response to coronaviruses (Charley and Laude, 1988; Laude et al., 1992). Roughly one-third of TGEV M protein assumes a topology in which part of the endodomain constitutes a fourth transmembrane segment, thereby positioning the carboxy terminus of the molecule on the exterior of the virion (Masters, 2006; Risco et al., 1995). TGEV N phosphoprotein complexes with the genomic RNA in a beads-on-a-string fashion to form the nucleocapsid (Suñé et al., 1990; Cavanagh et al., 1994). The S protein of coronaviruses is a large transmembrane protein with the amino terminus exposed to the virus surface and the carboxy terminus inside the virus particle and it assembles into trimers to form the distinctive viral surface spikes (Delmas and Laude, 1990). Coronavirus S protein plays an important role in inducing neutralizing antibodies (Garwes et al., 1978; Jiménez et al., 1986; Laude et al., 1987; Suñé et al., 1990) and it is also related to host cell tropism (Jacobs et al., 1986; Schwegmann-Wessels et al., 2003, 2009; Ren et al., 2006), pathogenicity (Siddell, 1995; Krempl et al., 1997), fusion (Collins et al., 1982; Spaan et al., 1988), hemagglutination activity (Krempl et al., 2000; Krempl and Herrler, 2001) and interaction with its cellular receptors such as porcine aminopeptidase N (pAPN) (Delmas et al., 1992; Liu et al., 2009; Ren et al., 2010a).

APN, also called CD13 in human is a type II transmembrane ectopeptidase of 150 kDa that contains a zinc-binding motif (HEIAH) and forms a noncovalently bound homodimer on the cellular membrane (Liu et al., 2009). APN was extensively expressed on various cell lines such as hematopoietic cells of myeloid origin, fibroblasts, brain cells, and epithelial cells of the liver, kidney, and intestine, etc. (Tsukamoto et al., 2008; Zhang et al., 2008). It is reported that aminopeptidase N (pAPN) is a cellular receptor for most of group 1 coronaviruses including human coronavirus 229E (HCoV-229E), TGEV, feline coronavirus (FCoV) and canine coronavirus

* Corresponding authors. Fax: +86 451 55103336.
E-mail addresses: rxfemail@yahoo.com.cn, renxf@neau.edu.cn (X. Ren), lgxemail@yahoo.com.cn (G. Li).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.11.014

(CCoV) (Delmas et al., 1992; Tresnan et al., 1996; Yeager et al., 1992). Generally, APN as receptors for many coronaviruses are species-specific (Delmas et al., 1994; Kolb et al., 1997), although the feline APN (fAPN) can also serve as a receptor for canine coronavirus, TGEV and human coronavirus 229E in addition to feline infectious peritonitis virus (Tresnan et al., 1996). The pAPN consists of several identified regions, which include the initiator methionine, cytoplasmic topological domain, transmembrane region, cytosolic Ser/Thr-rich junction region, metalloprotease region, and TGEV spike glycoprotein-interacting region, respectively (Fig. 1).

Peptide ligands that target a specific protein surface own broad applications as therapeutics by interfering protein–protein interactions. Phage display libraries provide a powerful and inexpensive way to identify such peptides. Phage random peptide library consists of a pool of billions of peptides that can be produced by the fusion of random nucleic acid sequences to the N terminus of one of the capsid protein genes (pVIII or pIII) of a filamentous bacteriophage (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990). By optimal biopanning, a single clone of phages with a desired binding specificity from a random phage library can be identified. This approach has been applied successfully in numerous aspects, including antibody engineering (Hayden et al., 1997), peptide and protein drug discovery and manufacture (Kay et al., 1998), diagnostic analysis (Ren et al., 2010c), and vaccine development (Lesinski and Westerink, 2001).

At present, the precise localization of receptor-binding domains (RBD) in TGEV S protein remains unclear. At the same time, the RBD-containing peptides may be useful small molecules for diagnosis and therapy in viral infection. In this study, we identified, for the first time, the specific peptides recognizing pAPN that blocks the binding to TGEV, based on phage display technology. The peptides competitively blocked TGEV infection *in vitro*. They, therefore, can be used as specific antiviral inhibitors. A putative TxxF motif was identified in the S protein. Moreover, the peptide-containing phages elicited effective immune response *in vivo*, demonstrating a potential perspective in development of phage-based vaccines.

Results

Expression of pET-apn

The gene encoding a truncated pAPN with the deletion of cytoplasmic topological domain and transmembrane region (Fig. 1) was amplified using PCR and then cloned into pET30a vector. The authenticity of the recombinant plasmid pET-apn was confirmed by DNA sequencing. The pAPN-bearing bacteria were induced with IPTG to express the protein of interest, after pET-apn was transformed into host bacteria. SDS-PAGE indicated that the fusion protein was about 112 kDa and the immunoreactivity of this protein was confirmed by Western blot and immunofluorescence (data not shown).

Enrichment efficacy and binding activity of phages after biopanning

After four rounds of biopanning using the pAPN-protein as an immobilized target, the enrichment efficacy of phages for each round was analyzed. The titer of phages in elution buffer and the amplified phages was increased with the increased panning times (Table 1). Ten phage clones were numbered 1 to 10 and picked from the last round panning and amplified in host cells. The binding activity of the selected phages to the target protein was assayed using ELISA. Our results showed that they had a specific binding activity to the pAPN (Fig. 2).

Analysis of the peptide sequences

The DNA of the phages was extracted and then the heterologous genes encoding the peptides on the surface of the recombinant phages were amplified by primer-specific PCR. The DNA sequencing reports showed that three identical peptides were identified among the ten phages (Table 2). Phage numbers 1, 4 and 6 had a consensus peptide sequence SVVPSKATWGFA named S. Phages 2, 7, 8 and 10 had a consensus sequence HVTTTFAPPPPR named H. The consensus sequence derived from Phages 3, 5 and 9 was FKPSSPPSITLW named F. The results showed that that the three peptides have sequence homologies and that a TxxF(AK)PxxP overlapping motif could be identified. These identified motifs were compared with TGEV S protein sequence. Several TxxF motifs were identified in the S protein (Supplementary Fig. 1).

Binding activity of peptides to pAPN and its inhibitory effect on cell infection

ELISA results showed that the three peptides were capable of binding the pAPN. TGEV coated plates have a higher binding activity than these peptides. There was no significant binding between the IBV S protein and the anti-pAPN antibody (Fig. 3). Virus infection inhibition assay showed that ST cells infected by TGEV produced significant cytopathic effect (CPE) at 48 h post infection. In contrast, the CPE number of peptide-treated ST cells infected by TGEV decreased in a dose-dependent manner, and the maximum inhibitory activity of TGEV infection *in vitro* was achieved at a concentration of 20 μg/ml of each peptides (Fig. 4).

Conventional MTT assays were used to analyze the effect of the peptides on the proliferation of ST cells infected by TGEV. Our results showed that OD_{490} value of peptide-treated ST cells infected by TGEV was higher than virus infection control, indicating that the proliferation of cells infected by TGEV was enhanced by the peptides (Fig. 5). The pre-treatment of the peptides led to increasing number of viable cells, also confirming the antiviral ability of the biologically active peptides.

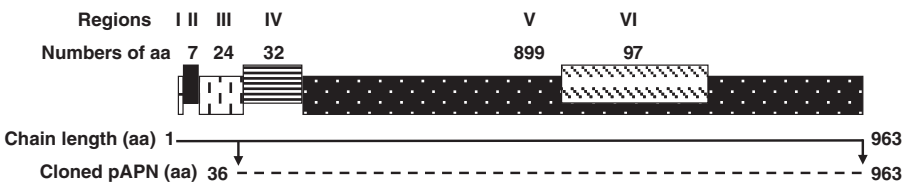

Fig. 1. Schematic drawing of porcine aminopeptidase N. The linear structure of porcine aminopeptidase N (pAPN) was classified into six regions. The feature key of regions I to VI consists of initiator methionine, cytoplasmic topological domain, transmembrane region, cytosolic Ser/Thr-rich junction region, metalloprotease region, and TGEV spike glycoprotein-interacting region, respectively. The number of amino acids in each region is indicated. Region VI (from 717–813 aa) is overlaid within region V (from 65–963 aa). The bold line shows the chain of pAPN and the broken line shows the length of the cloned pAPN used for expression. It should be noted that the sizes of the boxes or the lines are not proportional to the length of the amino acid chain.