

Preparation and bioactivity of anti-Newcastle disease virus-phosphoprotein cytoplasmic transduction peptide antibody

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Abstract

On the basis of cell-penetrating peptide's character that it can penetrate cytomembrane and transfer macromolecular protein to cytoplasm so to play biological function, we took the experiments. The fuse penetrating peptide our experiment adopted is HIV-TAT derived fragment-CTP512, with good transmembrane effect and distinct cytoplasm-position. In this chapter, the research of transmembrane character was processed first. According to the tests on trans-membrane protein with different concentrations, the best trans-membrane concentration is 3 μ M. Afterwards, we found that the location of trans-membrane antibody is overlapping with phosphoprotein using indirect immunofluorescence test analysis. According to MTT test, there is no significant difference between CTP fusion protein and control on cell proliferation and viability. TCID₅₀ test was used to detect the protective effect of trans-membrane antibody on cell. Result showed that trans-membrane antibody has significant cell protection effect compared to the control in the order: ZL.103>ZL.17>Control. Fluorogenic quantitative PCR result showed that trans-membrane antibody can disturb the duplication and transcription of Newcastle disease virus. This results not only paved a good way to research the transport of disease related protein, but also provide a splendid tool on protein function research.

Background

Current research shows that, whether in the medical or veterinary field, an efficient system for transporting antibodies to cells or animal tissues is lacking, which restricts the research and development of antibodies as antiviral drugs[1, 2]. Previously, most carriers delivering antibodies into cells were viral carriers; however, issues related to safety and immunity have not been sufficiently addressed. At present, most research has mainly used antibody coding genes in commonly expressed eukaryotic carrier. However, these carriers do not possess the ability to infect cells, and they need an supporting method for inoculation into cells, such as Lipofectamine carrying an antibody-coding plasmid to infect cells, or the use of a particular injection device to guide antibodies into cells[3, 4]. However, these methods all involve infection in vivo, they are difficult to utilize in living animal tissues. Therefore, an appropriate delivery system is one of the key technologies in antiviral antibody research.

Recently, in the medical field, the discovery of cell-penetrating peptides, a new type of transbody, has shed light on antibody research[5, 6]. The mechanism underlying the entry of cell-penetrating peptides into cells is unclear; however, this mechanism could be used for biotherapy and scientific research. Cell-penetrating peptides can carry external material into cells[7]. To date, TAT, VP22, MPG, Pep-1, etc. have been used as cell-penetrating peptides[8]. Among them, HIV-TAT and its derived fragment are the most commonly used cell-penetrating peptides[9, 10]. However, because of the nuclear localization of TAT, its function with protein fusion is limited[11, 12]. By transforming the order and structure of HIV-TAT's amino acids, Kim developed a new type of cytoplasmic peptide-CTP-with good cell penetrating characteristics and notable cytoplasmic localization[13]. The Newcastle disease virus phosphoprotein is located in the cytoplasm, and thus, the interaction of transmembrane antibodies and this phosphoprotein should be investigated.

In this study, we generated chicken-derived cytoplasm-expressed genes fused to CTP sequences, which might show transmembrane transport of antibodies in cells, which might show transmembrane transport of antibodies in cells. After crossing the cytomembrane, the antibody will be localized in the cytoplasm in accordance with localization sequence.

Methods

Construction of a cytoplasmic transduction peptide-single chain antibody fusion protein

Anti-phosphoprotein scFvs have been cloned from the spleen of immunized chickens in our laboratory, as described in our previous study[14]. The CTP was added to the 3' terminus. The forward primer was 5'-CGGAATTCGCCGTGACGTTGGAC-3' with *EcoR* I; and the reverse primer was 5'-GAGTCATTCTGCGGCCGCACGGCGACGCTGGCGACGTTTCTTACGACCGTATAGGACGGTCAGGGTTGTCCC-3' with *Not* I and CTP. The anti-P CTP-scFv gene was amplified by PCR. After digestion with *EcoR*I and *Not*I, the CTP-scFv gene was subcloned into pET28a(+). Thus, the recombinant expression plasmid pET28a-CTP-scFv was obtained.

Stable expression of pET28a-scFv-CTP

Trans1-Blue cells transformed with the recombinant plasmid pET28a-scFv-CTP were grown at 37°C in Luria Bertani (LB) medium supplemented with 100 mg/ml kanamycin until the bacteria reached the logarithmic growth phase (at OD₆₀₀=0.7), and the cells were then induced by the addition of 1 mM isopropyl b-d-thiogalactopyranoside. Purification of the His-tagged phosphoprotein was carried out in accordance with the manufacturer's procedures for Ni²⁺-NTA resin-packed columns. The final protein concentration was determined by the BCA kit.

Optimal transfection of the pET28a-scFv-CTP fusion protein

BHK21 cells were passaged in 6 well plates. Cells in 100 ml cell culture bottles were washed twice using DMEM. After 1 min of digestion using trypsin, the cells were suspended in 4 mL DMEM(10% FBS with penicillin-streptomycin), added to 6 well plates(0.6 ml of cell suspension per well), and cultured in an incubator overnight. When the cell confluence reached 80%, the cells were treated with protein at different concentrations as following: 1) 1 mL of DMEM (without FBS); 2) 1 mL of DMEM (without FBS) with scFv at a final concentration of 3 µM; 3) 1 mL of DMEM (without FBS) with CTP-scFv at a final concentration of 1 µM; 4) 1 mL of DMEM(without FBS)with CTP- scFv at a final concentration of 3 µM; 5) 1 mL of DMEM(without FBS) with CTP-scFv at a final concentration of 4 µM. After 2 h of culture, the cells were washed twice in DMEM (or: after 3 h of incubation) and washed twice in PBS, and the medium was replaced with new medium for another 24 h of culture. The cells were washed three times in PBS, and then, the expression was observed. After 1 min of digestion using trypsin, the cells were blown slightly and suspended in 1 mL DMEM(10% FBS with penicillin-streptomycin). The suspension were collected in EP tubes and Western blotting performed to analyse the transmembrane effect.

Cytoplasmic localization of CTP-scFv

The pET28a-scFv-CTP fusion protein was transduced into BHK21 cells as described above. After 24 h, the cells were fixed with 4 % paraformaldehyde for 30 min and incubated with 0.1 % TritonX-100 for 10 min. The cells were incubated with the primary antibody mouse anti-His tag mAb at a dilution of 1:50 for 2 h, followed by incubation with the secondary antibody FITC- conjugated goat anti-mouse IgG at 1:25 for 1 h at room temperature. The cells were analysed and photographed with confocallaser scanning microscope(Leica TCS-SP).

MTT assy of the effect of CTP on cell viability

Cells were collected in the logarithmic phase, used to generate were made into 10^5 cell/ml suspensions using DMEM containing 10% FBS. Then, the cells were inoculated in 96 culture plate. After 24 h, pET28a-scFv-CTP and scFv cells were treated with 0.5 mg/ml DMEM. Every sample had 4 wells in parallel. The cells were cultured at 37°C and 5 % CO₂. After 24 h, the cells were treated with 20 µl of 5 mg/ml MTT(in PBS). After incubation for 4 h, the supernatant was removed, and the culture plate was inverted on several layers of filter paper to wipe away the supernatant. DMSO (150 µl per well) was added, and the cells were shaken in a shaker for 30 min. The OD values were detected 570nm/490nm using a microplate reader.

Virus infection

To assess the effect of the anti-P CTP-scFv antibody on NDV production, we transduced BHK21 cells with the pET28a-scFv-CTP fusion protein. Then, the cells were infected with the virus at a multiplicity of infection of 0.01. The supernatants of the cell cultures were harvested at 12, 24, 36 and 48 h. The samples were serially diluted, and assayed for virus titer by TCID₅₀ assays[15].

Reverse transcription and real-time PCR

The transfected BHK21 cells grew exponentially in 96-well plates on glass cover-slips, and 24 h after transfection, they were infected with NDV at a multiplicity of infection of 0.1. Four hours after infection, the culture medium was removed and the cells were collected. Total RNA was isolated from the lysed cells using TRIzol reagent according to the procedures described by the manufacturer. Reverse transcription was carried out by using reverse transcriptase in a 20 µl reaction mixture, containing 200 ng of total RNA and specific primers for P-mRNA (5'-TTTTTTTTTTTTTTTTTTT-3'), P-cRNA (5'-TGGTGATCAGCCATTCAGCGCAAGGC-3'), and P-vRNA (5'-TACCCAGCAGACCAGGGCGAATATG-3'), at 42 °C for 1 h. The RT reaction mix was then used for real-time PCR using specific primers for P (sense 5'- CCTTTACAGACGCGGAGATTG-3' and antisense 5'-GTTTTGCCTTGTGGGATTGC-3') and β-actin (sense 5'-GCATCCACGAACTACATTCAACTC-3' and antisense 5'-CACTGTGTTGGCATAGAGGTCTTTG-3'), and SYBR Green I dsDNA binding dye. Real-time PCR was performed at 95 °C for 3 min for 1 cycle and then 94 °C for 45 s, 55 °C for 30 s and 72 °C for 1 min for 40 cycles. The PCR products were assessed with a Rotor-Gene 2000 Real-time Cyclor and analysed with Rotor-gene software. Cycle times (Ct) were analyzed with a reader of 0.2 fluorescence units. The duplicate cycle times were averaged and normalized to the cycle time of β-actin. All reactions were performed in duplicate.

Results

Construction of pET28a-scFv-CTP

The RT-PCR products had the expected sizes. A desired PCR product of 783 bp was obtained after agarose gel electrophoresis. Then, the phosphoprotein gene was digested by a double restriction enzyme digest and further cloned into the pET28a(+) vector. The recombinant plasmid pET28a-P was successfully constructed, and the enzyme digestion analysis showed the expected results(Fig 1. A).

Expression of anti-P pET28a-scFv-CTP

To obtain the purified pET28a-scFv-CTP fusion protein, we transformed the constructed prokaryotic expression plasmid pET28a-scFv-CTP into Trans1-Blue cells and induced the cells with 1 mM IPTG. A high level of protein expression was obtained. A fusion protein with a molecular weight of approximately 33 kDa was detected by SDS-

PAGE (Fig 1. B). The recombinant protein was purified through Ni-chelating affinity chromatography, and the purity was above 85%, as shown by SDS-PAGE analysis (Fig 1. C).

Transmembrane activity of the pET28a-scFv-CTP fusion protein

Immunofluorescence studies were carried out to identify transduced pET28a-scFv-CTP fusion proteins that could penetrate cells. The results showed that the purified pET28a-scFv-CTP fusion proteins had efficient transmembrane activity, and the optimal concentration of the pET28a-scFv-CTP fusion protein was 3 μM .

Subcellular localization of the transduced ZL.17 and ZL.103

The cellular localization of the fusion proteins ZL.17 and ZL.103 was investigated. BHK21 cells were transduced with the purified fusion proteins ZL.17 and ZL.103 carrying a His tag. The results showed that the anti-P fusion proteins ZL.17 and ZL.103 were distributed uniformly throughout the cell (in both the cytoplasm and nucleus)(Fig 1.D).

The pET28a -scFv-CTP fusion protein influenced the cell viability

MTT assays were performed to determine whether the fusion protein was harmful to BHK21 cells. The results indicated that there was no cytotoxic effect of the pET28a-scFv-CTP fusion protein on the BHK21 cells (Fig 2).

The anti-P pET28a-scFv-CTP fusion protein affected NDV F48E9 virus production

Stably transduced cell lines were then infected with the virus at a multiplicity infection of 0.01. At 24 h, 36 h, and 48 h after infection, the supernatants of the cell cultures were harvested, serially diluted, and assayed for viral titre using the TCID₅₀ method. The results revealed that the viral titres were reduced in the cell lines stably expressing all three anti-P CTP intrabodies, in the order of ZL.17> ZL.103>control, compared to that of empty vector transfected cells. These results suggested that virus production was potently inhibited by ZL.17 and ZL.103 (Fig 3).

Anti-P pET28a-scFv-CTP inhibited viral transcription and replication

To determine the effect of anti-P intrabodies on viral transcription and replication, we measured the production of three different forms of NDV RNA (mRNA, cRNA and vRNA), by using a previously described approach[16]. Total RNA was isolated at 4 h postinfection when sufficient viral RNA transcription and replication had occurred. Viral mRNA, cRNA, and vRNA were reverse transcribed using the appropriate primers, and the cDNA products were measured by real-time PCR. Data from the real-time PCR analysis showed that the levels of P-specific mRNA were significantly decreased in the presence of ZL.17 and ZL.103. The levels of P-specific cRNA were significantly inhibited by the two anti-P CTP intrabodies. In addition, P-specific vRNA production was inhibited by the two anti-P intrabodies in the following order: ZL.17>ZL.103>Control(Fig 4). These results suggested that anti-P CTP intrabodies have inhibitory effects on the production of NDV mRNA, cRNA and vRNA.

Discussion

CTP is an antibody protein fused to a transport region[17, 18]. Through its transmembrane function, CTP can conduct the transmembrane transport of antibodies (expressed protein), and then localize in the cytoplasm[19, 20]. As this antibody is the protein itself, it does not need to be expressed in the host cell, which ensures the safety

of this system. There are multiple cell-penetrating peptides, that are composed of 7-16 amino acids[8]. Generally, there is no or little homology in the primary structure and secondary structure of these cell-penetrating peptides; however, the common feature is that cell-penetrating peptides are rich in amino acid residues, especially arginine, that interact with the negative pole on the cell surface[21]. Cell-penetrating peptides have low toxicity, and in addition to biomacromolecules such as polypeptides, proteins and nucleic acids, they can carry molecular drugs, eikonogen and so on; hence, cell-penetrating peptides are commonly used in cell research.

Our report is the first showing that through protein prokaryotic expression and purification technology, combined with a peptide fusion strategy for cytoplasmic translocation, we could transport an extrinsic peptide into the cytoplasm of BHK21 cells and specifically inhibit the replication and transcription of Newcastle disease virus via a phosphoprotein. This system is a good tool for researching the transport of disease-related proteins and studying protein function.

In the past 20 years, research on cell-penetrating peptides has suggested a promising method to surmount the biological membrane barrier. In 1988, the HIV-1-Tat protein was first reported to pass through the cytomembrane; the antennapedia transcription protein in *Drosophila* was reported to possess similar transcription characteristics[22, 23]. The transduction ability of small peptide sequences for these transcription factors identified after that of other macromolecules, such as cell-penetrating proteins[24]. Many cell-penetrating peptides have been identified thus far[25, 26]. These peptides initially became Trojan horses or protein-transducing regions and then act as cell-penetrating peptides through internalization, showing endocytosis and promoting transport, which is a primary internalization pathway[27]. Among them, the previously identified protein-HIV-TAT and its derived fragment are well studied cell-penetrating peptides. However, cell-penetrating peptides show no specificity for tissues and cells; hence, an specific method is needed in drug delivery.

Because the phosphoprotein in Newcastle disease virus participates in the transcription and replication of RNA and is located in the cytoplasm after infecting cells, the fused cell-penetrating peptide our experiment adopted was the HIV-TAT-derived fragment CTP512, which has good transmembrane effects and distinct cytoplasmic localization[10, 28, 29].

At present, an unsolved problem is how to transport therapeutic biomacromolecules across the cytomembrane. One issue is the low bioavailability caused by biomacromolecule size and fluid behaviour and the subsequent restriction on passage across the membrane. This problem has become increasingly important; instead of biomacromolecules, new drugs have to penetrate one or more lipid bilayers to reach the target location. Many studies have demonstrated the high efficiency of cell-penetrating peptides in carrying polypeptides in vitro. Schwarze has proven the transmembrane effect of cell-penetrating peptides in vitro via the fusion of penetrating peptides to b-galactosidase; protein transport was realized by intraperitoneal injection in mice[30]. Hence, cell-penetrating peptides are a promising tool in macromolecule delivery.

In this paper, we performed a bioactivity analysis of the transmembrane fusion protein we generated. First, we investigated whether the pET28a-scFv-CTP protein can enter BHK21 cells. The results showed that the pET28a-scFv-CTP fusion protein successfully entered the cell and was expressed in a concentration-dependent manner, with 3 μ M being the optimum concentration. Cellular localization under fluorescence microscopy revealed that transmembrane antibodies were mainly distributed in the cytoplasm and were colocalized with phosphoproteins of the Newcastle disease virus. TCID50 analysis demonstrated that the two transmembrane antibodies could protect against Newcastle disease virus infection. Fluorogenic quantitative PCR analysis demonstrated that the

transmembrane antibodies can disturb the production of cRNA, vRNA and mRNA, which are related to the transcription and replication of viruses, to inhibit viral transcription and replication.

Conclusion

Our experiment verified that, the transmembrane fusion antibodies we generated not only have efficient transmembrane characteristics, but also transport the protein of interest to target locations to exert bioactivity. According to the current report, cell penetrating peptides do not possess tissue and cell targeting properties, and pharmacokinetics and body distribution studies have not been carried out, so more studies must be performed before these molecular can be used in clinical applications[7, 31]. However, the results identified a new method for the prevention and treatment of Newcastle disease, and provide a basis for screening drugs targeting internal structural proteins of Newcastle disease viruses.

Abbreviations

NDV Newcastle disease virus P phosphoprotein CTP cytoplasmic transduction peptide

Declarations

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Authors' contributions

Jianguo Zhu conceived and designed the experiments. Benqiang Li performed the experiments. Man Wang and Fanqing Zhang contributed data analysis. All authors have read and approved the final version of manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and use committee of Shanghai Academy of Agricultural Science.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

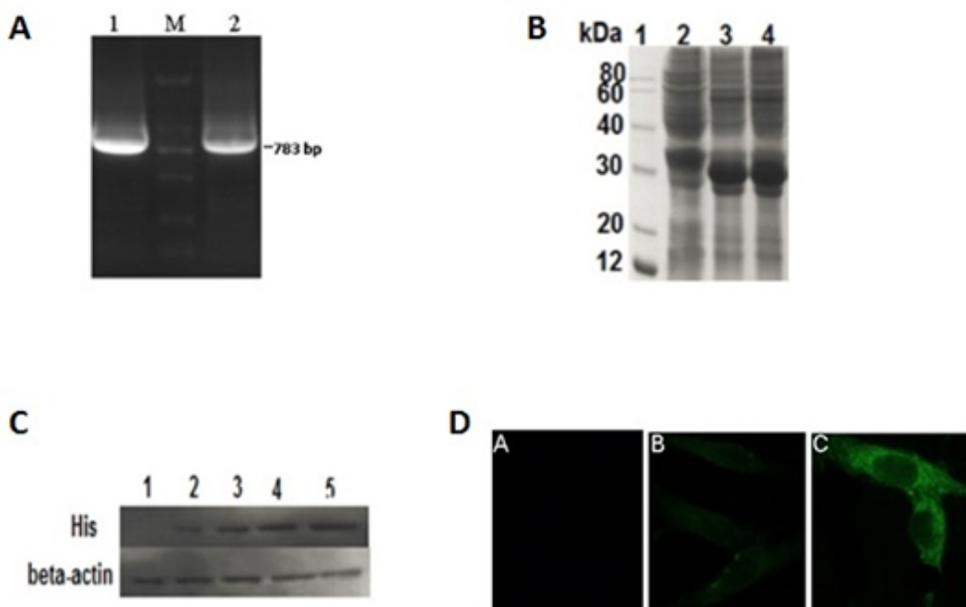


Figure 1

A. Gel electrophoresis of the pET28a-scFv-CTP genes. M. DNA Marker 1.2, pET28a-scFv-CTP ZL.17 and ZL.103 gene; B. Expression of the recombinant plasmid pET28a-scFv-CTP in E.coli. 1. Protein Marker 2. Induced pET28a-scFv-CTP vector control; 3-4. Expression of pET28a-scFv-CTP ZL.17 and ZL.103; C. Western blot analysis of the CTP-scFv fusion protein transduction. Lane 1.negative control; Lane 2. 1 μM CTP-scFv; Lane 3. 2 μM CTP-scFv; Lane 4. 3 μM CTP-scFv; Lane 5. 4 μM CTP-scFv; D. Subcellular localization of CTP-scFv in BHK21 cells.

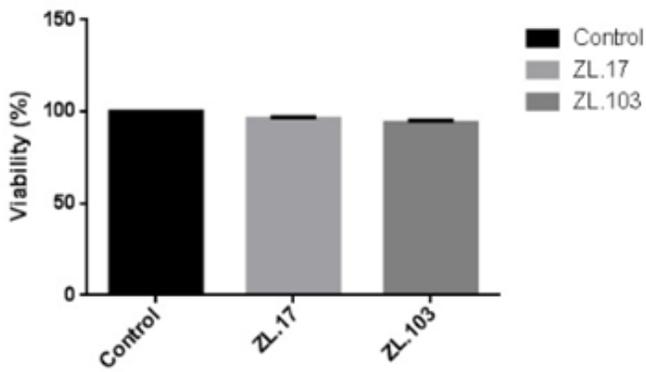


Figure 2

The optical absorbance of the reaction was measured spectrophotometrically at 490 nm with an ELISA plate reader. The results shown are the average of three different experiments performed in triplicate.

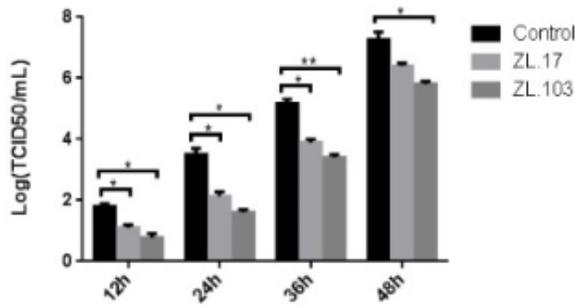


Figure 3

Determination of the effects of anti-P intrabodies on virus production. BHK21 cell lines expressing different intrabodies were infected with Newcastle disease virus. Viral titres in the culture supernatants were measured at different time points by TCID50 assays. The data shown are from one of the three experiments. ** $P \leq 0.01$, * $P \leq 0.05$

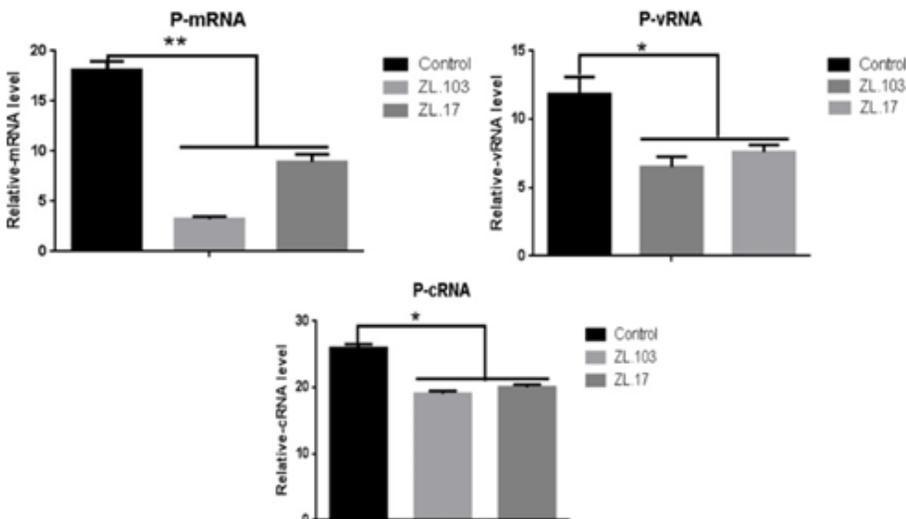


Figure 4

Determination of the role of anti-P intrabodies in Newcastle disease viral RNA production. Data from real-time PCR showed that the levels of P-specific mRNA were significantly decreased in the presence of ZL.17 and ZL.103.

*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$