Arch Virol (2010) 155:491–498
DOI 10.1007/s00705-010-0608-1

ORIGINAL ARTICLE

Recombinant mouse beta-defensin 2 inhibits infection by influenza A virus by blocking its entry

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Received: 7 October 2009 / Accepted: 22 December 2009 / Published online: 2 March 2010

Abstract Human influenza A virus (IAV) is a major cause of life-threatening respiratory tract disease worldwide. Defensins are small cationic peptides of about 2–6 kDa that are known for their broad-spectrum antimicrobial activity. Here, we focused on the anti-influenza A activity of mouse β-defensin 2 (mBD2). The prokaryotic expression plasmid pET32a-mBD2 was constructed and introduced into *Escherichia coli* Rosseta gami (2) to produce recombinant mBD2 (rmBD2). Purified rmBD2 showed strong antiviral activity against IAV in vitro. The protective rate for Madin–Darby canine kidney cells was 93.86% at an rmBD2 concentration of 100 μg/ml. Further studies demonstrated that rmBD2 prevents IAV infection by inhibiting viral entry. In addition, both pretreatment and postinfection treatment with rmBD2 provided protection against lethal virus challenge with IAV in experimental mice, with protection rates of 70 and 30%, respectively. These results suggest that the mBD2 might have important effects on influenza A virus invasion.

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Introduction

Influenza caused by influenza A virus (IAV) has been a serious threat to human health. Up to now, more than 29,000 people have been infected, and nearly 3,000 have died in the new H1N1 influenza global pandemic (http://www.who.int/csr/disease/swineflu/updates/en/index.html). The emergence of novel variants of IAV and the increasing appearance of resistant strains of influenza virus highlights our need to search for new antiviral strategies.

The first line of defense against invading pathogens is the innate immune system. Key elements of this system are defensins, which are well known for their broad-spectrum antimicrobial activity against viruses, bacteria and fungi [1, 2]. Structurally, defensins are small (2–6 kDa), cysteine-rich cationic peptides. Based on the spatial distribution of their six cysteine residues and the connectivity of their disulfide bonds, they can be classified into three categories: α-, β-, and θ-defensins [2]. β-defensins differ from α-defensins (Cys1-Cys6, Cys2-Cys4, Cys3-Cys5) by having up to 45 residues and a different cysteine pairing Cys1-Cys5, Cys2-Cys4, Cys3-Cys6 and spacing [3]. β-defensins are predominantly produced by barrier epithelial cells, and they therefore play an important role when attacking microbes invade the epithelium. Additionally, β-defensins modulate the host's immune response and cell-mediated immunity via cytokine expression, providing an interface between the innate and adaptive immune responses [4]. In recent years, defensins have attracted increasing interest as potential antiviral peptides.

The human β-defensins are of particular significance in airway and lung host defense [5]. Similarly, mouse β-defensins have been shown to play a role in resistance to infection of the airway and lung [6, 7]. In our previous work, MDCK cells transfected with pcDNA 3.1(+)/mBD3

demonstrated clearly inhibited IAV replication, and mBD3 protected mice from IAV challenge through intramuscular injection of pcDNA 3.1(+)/mBD3 [8]. Mouse β-defensin 2 (mBD2), an inducible expression peptide in the airways, was first reported by Morrison et al. [9] in 1999. Mature mBD2 is a cationic peptide with 41 amino acids and a gene sequence similar to that of other mouse and human beta defensins. However, information on the antimicrobial activity of mBD2 has been scarce over the past decade; it has only been shown to possess activity against *Staphylococcus aureus* in vivo [10]. The effects of mBD2 on influenza virus have been poorly studied. The main site of influenza virus infection is the respiratory mucosa; thus, mBD2 may have important effects on influenza virus invasion. To study the anti-influenza activity of mBD2, in the present study, we have established an expression system for mBD2 in *Escherichia coli* (*E. coli*) using standard procedures of recombinant gene techniques. The conditions of cultivation and induction were optimized systematically to further improve the efficiency of mBD2 production. Purified rmBD2 showed antiviral activity against influenza virus by inhibiting viral entry. In addition, rmBD2 protected mice from lethal IAV challenge.

Materials and methods

Preparation of mBD2 peptide

The mBD2 peptide was synthesized by recombinant techniques. Briefly, cDNA for mature mBD2 was amplified using polymerase chain reaction (PCR) from the pcDNA3.1(+)-mBD2 plasmid (constructed by Dr. De Yang). Primers were designed according to the coding sequence of mBD2 (GenBank accession No. AJ011800) and synthesized by Invitrogen (Shanghai, China). The forward primer (mBD2-F), 5′-GCGGGTACC**GACGACGACGAC AAG**GCAGAACTTGACCACTG-3′, contained a restriction site for *Kpn I* (underlined) and codons for an enterokinase (EK) cleavage site (black) at the 5′ end, and the reverse primer (mBD2-R), 5′-GCGCTCGAGTCATTTCATGTAC TTGCAACAGG-3′, contained a restriction site for *Xho I* (underlined) at the 5′ end. The conditions of PCR amplification were as follows: 94°C, 4 min; 30 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 30 s; 72°C, 5 min. The PCR product was cleaved by *Xho I* and *Kpn I* (Takara Biotech Co. Ltd.), and the mBD2 fragment was inserted into similarly digested pET32a(+) vector (Novagen) to construct the expression plasmid named pET32a-mBD2. The sequence of pET32a-mBD2 was confirmed by DNA sequencing at the laboratory of Takara Biotech Co. Ltd. Plasmid pET32a-mBD2 was introduced into competent *E. coli* Rosseta gami (2) (Novagen). The optimized conditions for target protein expression were as follows: induction at A_{600} 0.6 with 0.6 mM isopropylthiogalactoside (IPTG) at 34°C, and harvest at 6 h postinduction in 2 × YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, with 100 μg/ml ampicillin). The cells were harvested by centrifugation, and each gram of cell paste was suspended in 10 ml of binding buffer (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4) containing 1 mM phenylmethyl sulfonylfluoride (PMSF). The cells were then lysed by sonication and centrifuged. The supernatant including the soluble protein fraction was isolated. Purification was performed using a ÄKTA Purifer system (Amersham Pharmacia Biotech) with a nickel affinity chromatography column, His Trap™ FF crude (GE, Healthcare), which was prepacked with the affinity medium Ni Sepharose 6 Fast Flow. The purified fusion protein (TrxA-mBD2) was desalted using Amicon® Ultra-15 10K Centrifugal Filter Devices (Millipore, USA) and digested by recombinant EK with His-tag (rEK-His, Zhongda Nanhai Marine Biotech, China) at 25°C for 16 h in the recommended buffer (0.2 mM Tris–HCl, 100 mM NaCl, pH 8.0). The mixture was further purified using His Trap™ FF crude. The released mature rmBD2 peptide (theoretical molecular weight 4.5 kDa) was obtained from the effluent of the loading sample of the digestion mixture and further desalted using Amicon® Ultra-15 3K Centrifugal Filter Devices. In addition, the released mature rmBD2 was examined by Tricine-SDS-PAGE [11] and western blot with a goat anti-mouse β-defensin 2 antibody (Santa Cruz). Finally, the bioactivity of rmBD2 was analyzed.

Determination of virus titer

Madin–Darby canine kidney (MDCK) cells were grown in DMEM (Gibco, USA) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. The medium applied in vivo and vitro did not contain serum.

The titer of IAV A/PuertoRico/8/34 (PR-8, H1N1) was determined by 50% tissue culture infective dose ($TCID_{50}$) analysis in MDCK cells and evaluated by the method of Reed and Muench.

Since the antimicrobial activity of β-defensins is sensitive to high salt (described below) and serum concentration [12], we initially evaluated the activity of anti-IAV with mBD2 in a low-salt medium (10 mM phosphate buffer, PB; 8.2 mM Na_2HPO_4,1.8 mM KH_2PO_4, pH 7.4) and a serum-free environment [13].

Test for protection of host cells against IAV infection

An MTT assay was employed for evaluating the activity of rmBD2 against IAV. MDCK cells were seeded in 96-well tissue culture plates and incubated at 37°C until they

formed a confluent monolayer. PR-8 (10 $TCID_{50}$) was preincubated with serial twofold dilutions of rmBD2 or TrxA-mBD2 (100–3.125 μg/ml) in 10 mM PB for 1 h. To initiate infection, the regular medium was aspirated from the confluent MDCK cell monolayer and washed with PBS to remove FCS, followed by addition the 100 μl of the virus-peptide mixture to MDCK cells for 1 h at 37°C. After the infection mixture was discarded, cells were washed two times with PBS and overlaid with 100 μl fresh DMEM medium. One hundred microlitre of the cell controls (PBS only) and virus controls (virus only) was incubated in parallel. The tray was incubated at 37°C in 5% CO_2 for 48 h, and cytotoxicity was measured using an MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide] kit (GMS10039, Genmed Scientifics Inc) following the manufacturer's instructions. The percent antiviral activity of the rmBD2 was calculated as follows: protective rate = [(mean optical density of test − mean optical density of virus controls)/(optical density of cell controls − mean optical density of virus controls)] × 100% [14].

Confluency of cell monolayer assay

A confluent monolayer of MDCK cells in a 24-well tissue culture plate was washed with PBS to remove FCS and incubated with 10^4 $TCID_{50}$ PR-8 per well in 10 mM PB at 37°C for 1 h. Non-internalized PR-8 was removed by briefly washing the cells with PBS, and the medium was replaced with fresh DMEM medium. rmBD2 (100 μg/ml) was applied (a) at the onset of the entry period (cells were incubated with PR-8 for 1 h, rmBD2 on entry) or (b) 1 h after onset of entry (non-internalized PR-8 was removed, mBD2 after entry), and incubation with the cells was then continued for 1 h. Peptide-free controls (virus only) and negative controls (PBS only) were incubated in parallel. The plate was incubated at 37°C in 5% CO_2, and confluency of the cell monolayer was observed by light microscopy 20 h after infection. Loss of cell monolayer confluency due to cytopathic effects (CPE) of viral infection was quantified as a decrease in the percentage of the area occupied by the attached cells.

Exploring the mechanism of anti-IAV with rmBD2

The procedure was performed according to the methods reported by Sun and Sinha [15, 16]. Nearly confluent of MDCK cells in a 48-well tissue culture plate were washed with PBS to remove FCS and incubated with 10 $TCID_{50}$ PR-8 per well in 10 mM PB on ice for 90 min. Unbound PR-8 was removed by thoroughly washing the cells three times, and the culture was transferred to 37°C for 1 h to permit internalization. Non-internalized PR-8 was removed by briefly washing the cells with 0.1 M glycine–0.1 M NaCl buffer, pH 3.0, and fresh medium was added. The plate was incubated at 37°C in 5% CO_2. rmBD2 (100 μg/ml) was added (a) during the 4°C binding period, (b) when cells were shifted to 37°C, or (c) immediately after glycine treatment for 1 h to determine whether mBD2 inhibited infection if present during these time windows. The virus-free controls (peptide only) and peptide-free controls (virus only) were incubated in parallel. At 6 h after infection, cells were fixed in 4% paraformaldehyde-PBS and permeabilized with 0.5% Triton X-100. The cells were incubated with a fluorescein-tagged influenza monoclonal antibody against nuclear protein (NP) (ab20921, Abcam, UK) at 37°C for 30 min and then washed with PBS and analyzed for infection by immunofluorescence microscopy (DMI 6000B, Leica, Germany). Viral infection of MDCK cells was measured as the percentage of cells expressing influenza NP [15]. At least 100 cells were counted for each sample to determine the percent infected.

Animal test

Five-week-old female BALB/c mice were obtained from the Animal Laboratory Centre of Sichuan University. The procedure was performed according to the methods reported by Jiang et al., Hazrati et al. and Jones et al. [8, 17, 18] with modifications. Briefly, mice (12 mice per group) were lightly anesthetized by ether inhalation and inoculated intranasally with rmBD2-treated (100 μg/ml) PR-8 (20 LD_{50}), PBS-treated or untreated PR-8. The infected mice that received untreated virus were anesthetized and administered 2 mg/kg rmBD2 or PBS intranasally 6 h postinfection, with readministration daily for 5 days. Mice were observed for 14 days, and the living and dead mice were counted. On the third day postinfection, two mice from each group were sacrificed, and the lungs were collected. The tissues were homogenized in 1 ml of cold PBS and clarified by centrifugation, and the supernatants were stored at −70°C. Viral titers ($TCID_{50}$) of tissues were evaluated by serial titration on MDCK cells.

Statistical analysis

Data were obtained from three independent experiments, each performed in triplicate and reported as mean ± standard deviation. Comparison between groups in the antiviral assays was performed using the one-way ANOVA q-test with significance defined at $P < 0.05$.

Results

Preparation of rmBD2 peptide

The expression vector pET32a-mBD2 (Fig. 1) was constructed using standard procedures of recombinant techniques. Fused with the TrxA·Tag, the mBD2 gene was under the control of a T7 promoter. Between the TrxA·Tag and mBD2 coding sequence, there was a His·Tag, which served as the detection and purification tag in later steps. This expression plamid was introduced into *E. coli* Rosseta-gami (2) to obtain the recombinant strain Rosseta-gami (2)/pET32a–mBD2, to produce the fusion target protein. Under the optimized fermentation parameters, the fusion protein TrxA-mBD2 constituted a high percentage (≥95%) of the soluble fraction (Fig. 2a). The rEK-His-cleaved fusion protein was further purified using His Trap™ FF crude, and the purified mature rmBD2 was analyzed by Tricine-SDS-PAGE and western blot (Fig. 2b). The expression of soluble mature rmBD2 was achieved in *E. coli* Rosseta-gami (2) with a volumetric productivity of 6 mg/L. After purification, the recombinant mature mBD2 was obtained. The results of bioactivity assays showed that rmBD2 inhibits the growth of *Staphylococcus aureus* (ATCC 25923), and the peptide was almost completely inactive when the concentration of NaCl was higher than 150 mM (data not shown).

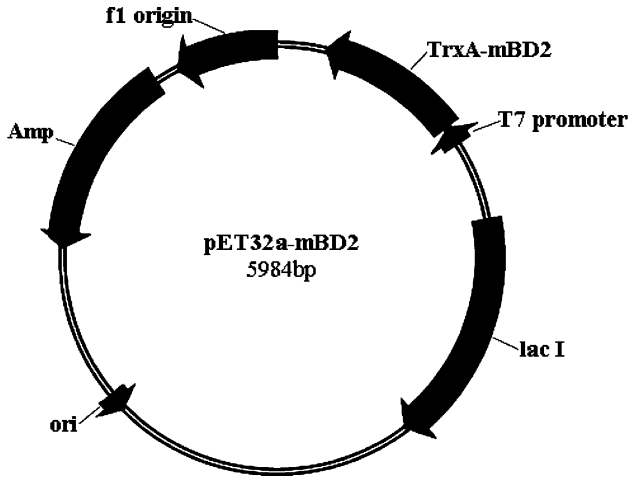

Fig. 1 Schematic representation of the expression vector pET32(a)-mBD2

Antiviral activity of rmBD2 against IAV

A cytotoxicity study (MTT assay) demonstrated that the rmBD2 did not induce significant cytotoxicity in MDCK cells until a concentration 150 μg/ml (data not shown). The activity of rmBD2 against IAV was analyzed by MTT assay. Briefly, MDCK cells were incubated with rmBD2-treated PR-8, and the cytotoxicity was evaluated at 48 h

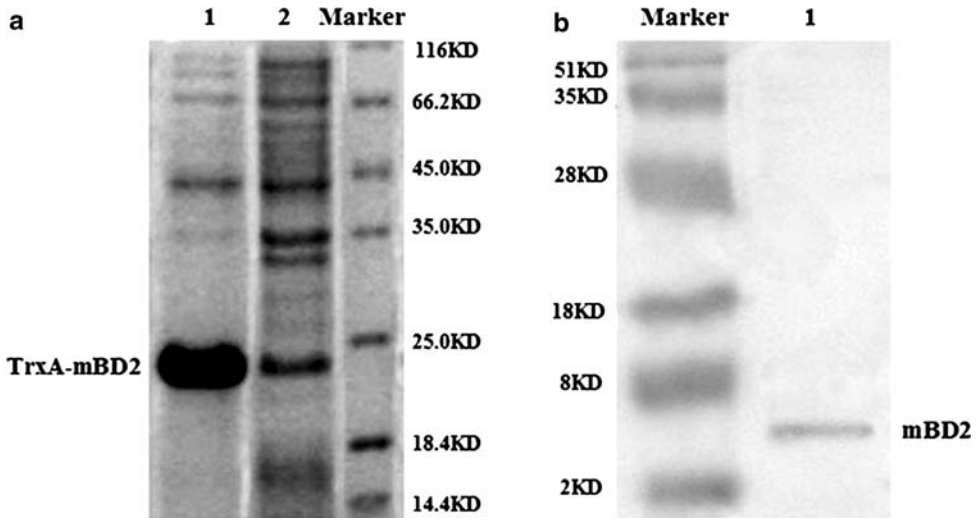

Fig. 2 Expression of mBD2 in *E. coli* Rosseta-gami (2)/pET32a-mBD2. **a** Expression of TrxA-mBD2 fusion protein in the *E. coli* Rosseta-gami (2)/pET32a-mBD2 cells monitored by 15% SDS-PAGE. Expression vector pET32a–mBD2 was introduced into *E. coli* Rosseta-gami (2) to obtain a recombinant strain for producing fusion target protein TrxA-mBD2. Under optimized fermentation parameters (induction at A_{600} 0.6 with 0.6 mM IPTG and at 34°C in 2xYT medium and harvest at 6 h postinduction), the fusion protein TrxA-mBD2 constituted a high percentage (≥95%) in the soluble fraction. *Lane 1* soluble protein of Rosseta-gami (2)/pET32a-mBD2, *Lane 2* insoluble protein of Rosseta-gami (2)/pET32a-mBD2. **b** Released mature rmBD2 peptide was detected by western blot analysis. The fusion protein was specifically digested with rEK-His, and the mixture was further purified by His Trap™ HP crude. Released mature rmBD2 peptide was obtained from the effluent of the loading sample of the digestion mixture and further desalted and condensed using Amicon® Ultra-15 3K Centrifugal Filter Devices. The mature rmBD2 peptide was detected by western blot analysis with goat anti-mouse *β*-defensin 2 antibodies. *Lane 1* eluent fraction from the nickel affinity column

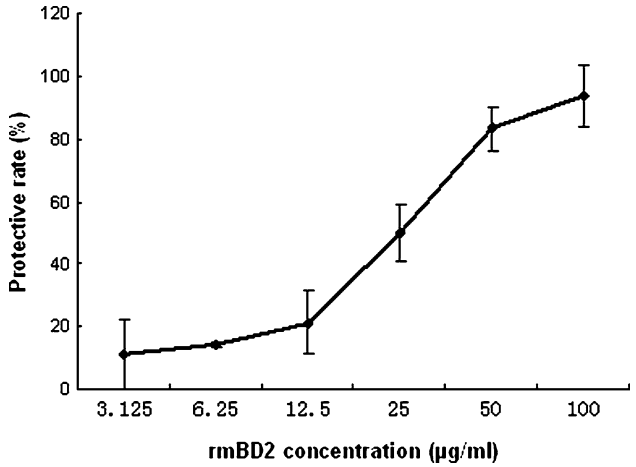

Fig. 3 Protection of MDCK cells from infection by influenza virus PR-8 (MTT assay). The viruses ($10TCID_{50}$) were preincubated with different concentrations (100–3.125 μg/ml) of rmBD2 for 1 h at 37°C, followed by addition of the virus-peptide mixture to a confluent monolayer of MDCK cells for 1 h at 37°C. After the infection mixture was discarded, cells were washed with PBS and overlaid with fresh DMEM. After incubation at 37°C for 48 h, the cytotoxicity was measured using an MTT kit. The percentage of antiviral activity of rmBD2 was calculated as follows: protective rate = [(mean optical density of test − mean optical density of virus controls)/(optical density of cell controls − mean optical density of virus controls)] × 100%. At the highest concentration (100 μg/ml), rmBD2 protected 93.86% cells from infection by influenza virus PR-8

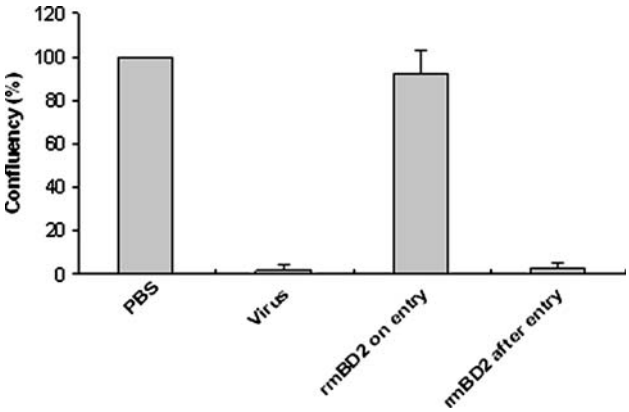

Fig. 4 rmBD2 inhibits influenza virus entry into MDCK cells. A confluent monolayer of MDCK cells was incubated with 10^4 $TCID_{50}$ viruses/well at 37°C for 1 h. Non-internalized viruses were removed briefly washing with low-pH buffer, and the medium was replaced with fresh DMEM medium. rmBD2 (100 μg/ml) was applied (1) during the entry period (cells were incubated with viruses) and (2) after the entry period (noninternalized viruses were removed). The plate was incubated at 37°C, and the confluency of the cell monolayer was observed under a light microscope 20 h after infection. Loss of cell monolayer confluency due to cytopathic effects of viral infection was quantified as a decrease in the percentage of the area occupied by the attached cells

postinfection using an MTT kit. Under these conditions, rmBD2 was effective at protecting MDCK from infection with PR-8 and decreased virus-induced cell death in a dose-dependent manner [Fig. 3]. The protective rate was 93.86% at an rmBD2 concentration of 100 μg/ml. However, the fusion protein TrxA-mBD2 had no anti-influenza activity (data not shown).

rmBD2 inhibits influenza virus infection by blocking entry into cells

An assay of the confluency of the cell monolayer showed that at a higher virus titer (10^4 $TCID_{50}$) of infection, rmBD2 applied during the entry period prevented a substantial loss of attached cells in each field of view caused by the cytopathic effects of PR-8; however, rmBD2 applied after the virus-internalization step had almost no effect on the confluency of the cell monolayer compared with the virus control (Fig. 4). To test further whether rmBD2 affects the entry of influenza virus into host cells, MDCK cells were exposed to PR-8 for 90 min on ice to allow viral attachment. Unbound PR-8 was removed by washing, and the culture was incubated at 37°C for 1 h to permit internalization. Then, the cells were washed at low pH to inactivate noninternalized PR-8, and fresh medium was added. rmBD2 or control buffer was added during the 4°C binding period (time A), at the time of the temperature shift to 37°C (time B, internalization) or immediately after glycine treatment (time C, postentry). At 6 h after infection, cells were fixed, and viral infection of MDCK cells was measured as the percentage of cells expressing influenza NP, using NP antibody. The results are shown in Fig. 5. At 100 μg/ml, rmBD2 inhibited IAV infection if added at time A. An antiviral activity persisted if rmBD2 was added at time B, but not if added at time C.

Protection of mice from lethal IAV challenge

To evaluate the efficacy of rmBD2 in vivo, BALB/c mice were inoculated intranasally with rmBD2-treated PR-8; untreated PR-8 (20 LD_{50}), mice from groups of untreated virus were given 2 mg/kg of mBD2 or PBS intranasally 6 h postinfection, with readministration daily for 5 days. Mice administered rmBD2 alone exhibited no toxicity in vivo, as measured by clinical signs and weight loss (data not shown). Pretreatment of PR-8 virus with rmBD2 resulted in 70% survival of mice (Fig. 6), and administration of rmBD2 after infection caused a delay in mortality and resulted in 30% survival. In contrast, none of the mice in the control groups survived. Protection was accompanied by a decrease in viral titers in the lungs of infected animals. On the third day postinfection, lungs were isolated and homogenized, and lungs tissue titers were determined on MDCK cells by $TCID_{50}$ analysis. Viral titers were significantly lower in the pretreated group by the third day

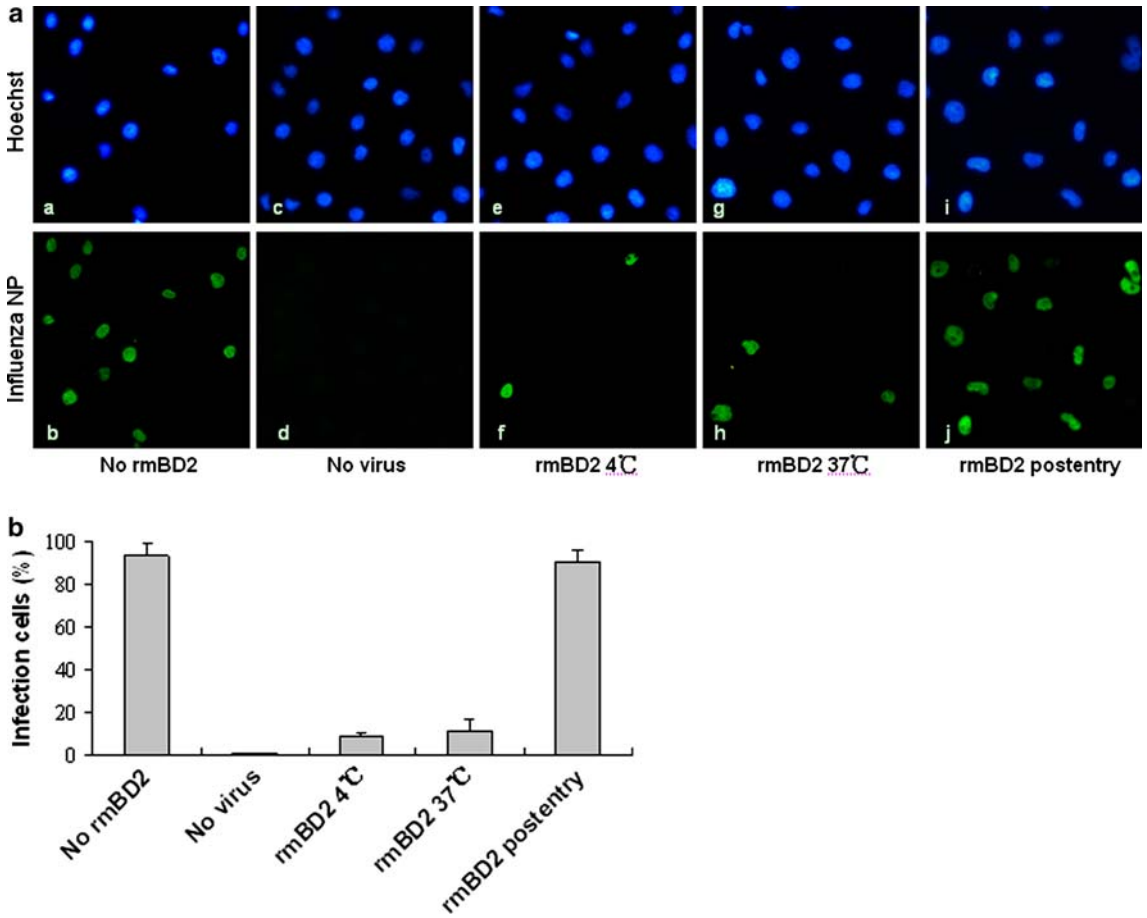

Fig. 5 Treatment of influenza virus with rmBD2 on the entry reduces virus infectivity. **a** MDCK cells were exposed to virus for 90 min on ice to allow viral attachment. Then, unbound viruses were removed, and the culture was incubated at 37°C for 1 h to permit internalization. Cells were briefly washed with a low-pH buffer to remove non-internalized viruses, and fresh medium was added. rmBD2 was added during the 4°C viral binding period, at the time of temperature shift to 37°C or immediately after glycine treatment (postentry). The cells were analyzed by direct immunofluorescence microscopy using fluorescein-tagged anti-NP antibodies (*b*, *d*, *f*, *h*, *j*) at 6 h after infection, or nuclei were stained with Hoechst 33258 (*a*, *c*, *e*, *g*, *i*). **b** Quantitation of data in panel A. Viral infection of MDCK cells was measured as the percentage of cells expressing influenza nuclear protein. At least 100 cells were counted for each sample to determine the percentage of cells infected

postinfection (Table 1). In contrast, the titers decreased only slightly when rmBD2 was administrated after infection. The $TCID_{50}$ values of the mBD2-pretreated and after-infection groups differed from those of the PBS groups ($P < 0.05$). These studies demonstrate that both pretreatment with rmBD2 and administration of rmBD2 postinfection protected mice from PR-8 infection.

Discussion

We have found that one of the mouse β-defensins, mBD2, was effective at protecting MDCK cells from being infected by IAV, and further studies showed that rmBD2 prevented influenza virus infection by blocking viral entry. There are three stages in influenza virus entry, including binding, internalization and fusion [15]. To define the step(s) in IAV invasion that was/were blocked by rmBD2, kinetic assays were performed as described previously. rmBD2 added before or at the time of internalization strongly inhibited infection, as assessed 6 h later by counting the percentage of cells expressing viral nuclear protein (Fig. 5). In contrast, when rmBD2 was added after the step of internalization (postentry), there was almost no effect on the expression of viral nuclear protein. These results are consistent with those of the cell monolayer confluency assay (Fig. 4), in which rmBD2 applied during the entry period inhibited the loss of attached cells caused by the cytopathic effects of influenza virus, but not if applied during the time of after entry. Thus, these data indicate that rmBD2 prevents influenza virus infection by blocking viral entry rather than by inhibiting the subsequent stages of an ongoing infection. These findings were supported by an animal test in which both pretreatment and

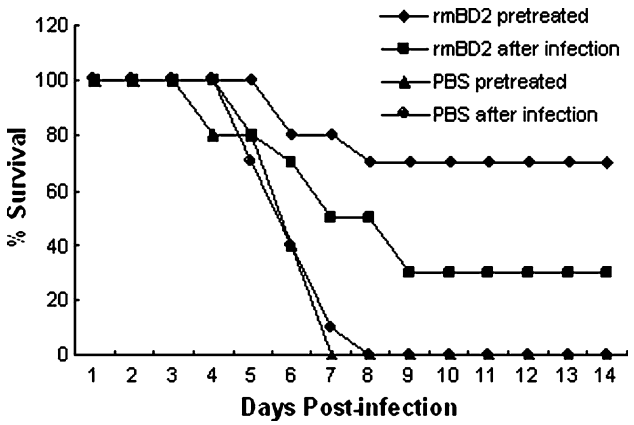

Fig. 6 rmBD2 protects mice from lethal influenza A virus infection. Mice were lightly anesthetized by ether inhalation and intranasally inoculated with rmBD2-treated (100 μg/ml) PR-8 ($20LD_{50}$), PBS-treated or untreated PR-8. Mice infected with untreated virus were anesthetized and 2 mg/kg rmBD2 or PBS was administered intranasally 6 h postinfection, with readministration daily for 5 days. Mice were observed for 14 days, and the living mice were counted. Both pretreatment and postinfection treatment with rmBD2 provided protection against lethal virus challenge with IAV in experimental mice, with protective rates of 70 and 30%, respectively

Table 1 Protection against lethal virus infection in experimental mice

Group	Lung tissues titer[a]
mBD2 pretreated	0.778 ± 0.127[b]
mBD2 after infection	2.139 ± 0.048
PBS pretreated	3.053 ± 0.316
PBS after infection	3.101 ± 0.327

[a] The titer numbers of are mean $-\log10$ of $TCID_{50} \pm$ SD. For example, 0.778 means the $TCID_{50}$ was $10^{-0.778}$

[b] The $TCID_{50}$ of the mBD2-pretreated and after-infection groups differed from that of the PBS groups ($P < 0.05$)

postinfection treatment with rmBD2 provided protection against lethal virus challenge with IAV in experimental mice as well as a decrease in viral titers in the lungs of infected animals.

Natural antiviral defenses are mediated by the innate and adaptive immune systems. Adaptive immune responses to one strain of IAV do not afford lasting protection against other strains, such as epidemic IAV, because its effector molecules are typically highly pathogenic. In contrast, those of innate immunity, such as defensins have a broader spectrum and appear to provide some protection against IAV. Recently, several defensins were shown to inhibit IAV in vitro via a variety of mechanisms. For example, the synthetic primate θ-defensin retrocyclin 2 (RC2) and human β-defensin 3 (HBD3) have been showed to block viral infection by a mechanism directly related to their ability to cross-link cell membrane glycoproteins into a fusion-resisting barricade [19], whereas the α-defensins human neutrophil peptides (HNPs) induce impairment of cellular pathways and increase neutrophil activity [20]. In addition, these defensins are also inhibitory to several other enveloped viruses, such as human immunodeficiency viruses [13, 21] and herpes simplex viruses [16, 18] by different mechanisms. Together, these findings indicate that the mechanisms of antiviral activity of defensins are complex and vary depending on the specific peptide and virus. Understanding the mechanisms of the wide-ranging antiviral activity of defensins is essential for developing them into effective antiviral agents.

As for any biological system, understanding the biological role of defensins requires having access to the target peptides, preferably in recombinant form. Because of its well-established expression systems, fast growth rate and low cost, *E. coli* is commonly used as a host cell to express peptides. In our present study, the employed expression system is *E. coli* pET/Origami, in which the *E. coli* Origami host strain has mutations in both the thioredoxin reductase and the glutathione reductase genes to enhance cytoplasmic disulfide bond formation greatly [22]. Meanwhile, the Rosseta strains supply rare tRNAs to enhance the expression of eukaryotic proteins. Fusion expression with a partner of TrxA [23] in the pET32a(+) vector alleviates the obstacles usually associated with using *E. coli* as a host cell for expression of antibiotic peptides, including host-killing activity and the susceptibility of the product to degradation [24]. Between the TrxA·Tag and mBD2 coding sequence, there is a His·Tag, which serves as the detection and purification tag in later steps. After precise cleavage of TrxA-mBD2 and purification with a nickel affinity chromatography column, functional rmBD2 was obtained.

In summary, we have identified a novel antiviral peptide, mBD2, which inhibits IAV by blocking its entry into cells. Both pretreatment and postinfection treatment with mBD2 provide protection against lethal virus infection with IAV in experimental mice. Additionally, we have established an efficient prokaryotic expression system for mBD2, and this may be a good way to produce large amounts of functional defensins at low cost and rich amount. As we continue to cope with yearly influenza epidemics, and as the H5N1 and new H1N1 pandemic continue to spread, understanding how these viruses interact with cells and developing tools to block this earliest step in viral infection are of enormous importance for public health. Further exploration the mechanism of the anti-influenza activity of rmBD2 in vivo could lead to the development of new strategies to prevent IAV infection.

Acknowledgments This work was financially supported by National Natural Science Foundation of China (No. 30671964, People's Republic of China).

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