An efficient system to generate truncated human angiotensin converting enzyme 2 (hACE2) capable of binding RBD and spike protein of SARS-CoV2

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ABSTRACT

Human angiotensin converting enzyme 2 (hACE2) mediates the cell entry of both SARS-CoV and SARS-CoV2 and can be used as a drug target. The DNA encoding the truncated hACE2 (30-356aa) was cloned into pET-28a (+) and expressed in *Escherichia coli* Rosetta (DE3). The recombinant hACE2 (rhACE2) was purified by affinity chromatography on a Ni-NTA column and characterized with SDS-PAGE and Western blot. The binding activity of rhACE2 to Spike protein of SARS-CoV2 was evaluated in S protein-overexpressed HEK293A cells (HEK293A-SP cells) through flow cytometry. The prokaryotic expression system is able to produce approximately 75 mg protein per liter, which would be useful for infection mechanism study, and drug screening and development of SARS-CoV2.

1. Introduction

The global outbreak of COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), has posed a serious threat to human health. The scientists around the world have been racing to fight with this coronavirus. Many studies have been done for elucidating the mechanism of infection. It has been found that the spike (S) protein of SARS-CoV2 binds to angiotensin converting enzyme 2 (ACE2) on cell membrane to initiate the cell entry [1].

ACE2 is a metalloprotease located on the surface of endothelial and other cells with a full length of 805 amino acids (aa) which includes an extracellular domain (18-740aa), a relatively short transmembrane region (741-761aa) and an intracellular region (762-805aa) in which the extracellular region is the main structural region that exerts enzyme activity. Its extracellular domain includes N-terminal peptidase domain (PD, 18-605aa) and C-terminal collectrin-like domain (CLD, 606-740aa). ACE2 was initially identified as a homolog (or a variant) of angiotensin converting enzyme (ACE) which is a key protease that cleaves angiotensin I to form angiotensin II. Angiotensin II (Ang II) is a key regulator of renin-angiotensin system (RAS) to mediate biological functions through two G protein-coupled receptors, angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). Subsequent studies indicated that ACE2 negatively regulates the activated RAS by degrading Ang II into heptapeptide angiotensin 1-7 [2]. Moreover, it has

demonstrated that ACE2 plays a critical role in mediating the entry of SARS-CoV and SARS-CoV2 to human cells [3].

It's been reported that SARS-CoV/SARS-CoV2 bind to the peptidase domain of ACE2 through its receptor binding domain (RBD) of spike protein and participate in the viral infection process. The strategies of blocking SARS-CoV2 Spike protein and ACE2 interaction has been widely used for anti-virus drug development. The amino acid substitution analysis indicated that K353, K31, E35, M82, N53, N90, and N322 in ACE2 would influence the binding activity of SARS-CoV/SARS-CoV2 [4]. In order to keep the functional integrity, we designed to generate the fragment (30-356aa) of hACE2 for further study. In this study, we have demonstrated that recombinant hACE2 (30-356aa) can be effectively expressed in *E. coli* Rosetta (DE3). The functional analysis indicated that hACE2 (30-356aa) can bind to specific antibodies and S protein of SARS-CoV2. The recombinant protein would provide a useful tool for SARS-CoV2 infection study, anti-viral drug screening and development.

2. Materials and methods

2.1. Bacteria strains and vectors

Escherichia coli Rosetta (DE3) strain was purchased from Novagen and used for cloning and expression. Plasmids pcDNA3.1-ACE2-Flag and

pcDNA3.1-Spike-Myc were purchased from Beyotine biotechnology (Shanghai, China). Of which, pcDNA3.1-hACE2-Flag was used as a template to obtain hACE2 gene through polymerase chain reaction (PCR) and pcDNA3.1-Spike-Myc was used to construct the SARS-CoV2 S protein-overexpressed HEK293A (HEK293A-SP) cells. The plasmid pcDNA3.1 was obtained from Dr. Sun Qiming's Lab, Dept of Biochemistry, School of Medicine, Zhejiang University. The expression plasmid pET-28a (+)/RBD was constructed previously in our lab. The hACE2 gene was inserted to pET-28a (+) vector with a C-terminal His tag (6 × His).

2.2. Reagents and antibodies

DNA gel extraction kits (Omega Bio-tek, USA), AxyPrep plasmid miniprep kit (Axygen Scientific Inc, USA) were used for DNA fragments purification and plasmid extraction according to the manufacturer's instructions. The BCA protein quantification kit and T4 DNA ligase were from Vazyme Biotech (Nanjing, China). The following reagents were purchased from Sangon Biotech (Shanghai, China): urea, imidazole, Ni-NTA sefinose resin, dialysis membrane (cutoff MW, 14 KD), polyethylene glycol-20000 (PEG20000), 50 × TAE buffer, two-color pre-stained protein marker. The 2 × Hieff PCR Master mixture, nucleic acid gel stain (10000 × in water), and DNA marker were obtained from Yeasen Biotech (Shanghai, China). NcmECL Ultra Kit was obtained from NCM Biotech (Suzhou, China) and used for detection of Western blot.

The mouse anti-6 × His tag monoclonal antibodies was from Proteintech Group (USA). The rabbit polyclonal antibody against SARS-CoV2 Spike RBD was from ABclonal Technology (Wuhan, China). Antibodies against the following agents were obtained from BBI Life Sciences (Shanghai, China): rabbit anti-ACE2 polyclonal antibody, rabbit anti-Myc tag polyclonal antibody, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG. Goat anti-mouse IgG-Alexa Fluor® 647 antibody was purchased from HuaBio (Hangzhou, China).

2.3. Plasmid constructions

According to the prediction and structural analysis of ACE2 in vertebrates [4], we tried to obtain the hACE2 (30-356aa) which is able to bind with RBD of SARS-CoV2. The DNA encoding hACE2 (30-356aa) was amplified by PCR using specific primers. The pcDNA3.1-hACE2-Flag (Beyotine biotech, Shanghai) containing whole gene of ACE2 was used as a template. The sequences of designed primers including the *Nco* I or *Xho* I restriction enzyme site were listed here: forward primer 5′ CATGCCATGGACAAGTTTAACCACGAAGCCG 3′ and reverse primer 5′ CCGCTCGAGGAAGTCGCCCTTCCCCAGGTCCCAAG 3′. PCR amplification was performed in mixture containing 2 × Hieff PCR Master Mix (DNA polymerase, dNTPs, Mg+, dye), template, primers, sterile H_2O. PCR was carried out for initial pre-denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 45 s and final extension at 72 °C for 10 min. The PCR products were directly cloned into vector pET-28a (+) to obtain expression plasmid pET-28a (+)/hACE2. After transformation into *Escherichia coli* Rosetta (DE3) competent cells, each clone was subjected to DNA sequencing to verify the desired sequence.

2.4. Expression and purification of recombinant hACE2

The expression plasmid was introduced into *Escherichia coli* Rosetta (DE3) by $CaCl_2$-mediated method. The cells were grown with shaking until an optical density at 600 nm reached 0.4–0.6. The expression was induced at 37 °C for 6 h in the presence of 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were then harvested by centrifugation at 4000 rpm for 10 min and the pellet was re-suspended in phosphate-buffered saline (PBS) for sonication. After sonication, the supernatant and pellet were separated by centrifuging at 1,2000 rpm for 10 min, and analyzed by SDS-PAGE. The pellet (inclusive body) was

washed with PBST (PBS, 0.1% Triton X-100) for three times. After washed with distilled water, the pellet was finally dissolved in NTA buffer containing 8 M urea and purified by affinity chromatography with a Ni-NTA sefinose column (1 × 1 cm). The bound rhACE2 was eluted with 1 × NTA buffer containing 20 mM–250 mM imidazole and 8 M urea respectively. The fractions containing rhACE2 were collected and dialyzed with urea gradient including 4, 2 and 0 M in NTA buffer, and PBS respectively. The recombinant protein was concentrated and filter sterilized. The SDS-PAGE (12%) was used to analyze the purity of the recombinant protein and the protein concentration was measured by the BCA method.

2.5. Western blot analysis

The purified rhACE2 proteins were subjected to SDS-PAGE (12%) and transferred to a solid PVDF membrane (Millipore, USA) at 100 V for 60 min. Membranes were blocked with blocking buffer (5% no-fat milk dissolved in TBST (TBS, 0.1% Tween-20) and incubated separately with mouse anti-6 × His tag monoclonal antibodies (1:1500) and rabbit anti-ACE2 polyclonal antibody (1:2000) for 1 h at room temperature. The membranes were washed with TBST for three times, then the HRP-conjugated goat anti-rabbit IgG or mouse IgG antibodies (1:10,000) was incubated with membranes for 1 h at room temperature followed by three times washing with TBST.

For detection of S protein expressed in HEK293A-SP cells, the cells were washed with ice-cold PBS and then lysed in RIPA lysis buffer (Beyotime Biotech) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF). An equal amount of each protein sample was subjected to SDS-PAGE separation, and then transferred onto PVDF membranes. Membranes were blocked for 30 min with the blocking buffer and followed by incubation with the primary antibody and secondary antibodies. The anti-Myc tag and anti-SARS-CoV2 Spike-RBD rabbit polyclonal antibodies (1:2000) were used as primary antibodies. The HRP-conjugated goat anti-rabbit IgG antibody (1:10,000) was used as secondary antibody. The membranes were detected and visualized by using NcmECL Ultra detection reagents under the ChemiDoc™ Touch Imaging system (Bio-Rad, USA) and analyzed by Image Lab software.

2.6. Enzyme-linked immunosorbent assay (ELISA)

First, 96-well microplate (CoStar, USA) was coated with 0.625, 1.25, 2.5, 5, 10, 20 μg/mL of rhACE2 and placed at 4 °C overnight. After washing with PBST (PBS, 0.05% Tween-20), the plate was blocked with 3% non-fat milk in PBST for 1 h at 37 °C. Recombinant RBD of SARS-CoV2 (OkayBio, Nanjing, China) (1:2000) in TBST was added to each well followed by incubation at 37 °C for 1 h. Then the plate was washed for three times with PBST, the anti-RBD rabbit polyclonal antibody was added at the dilution of 1:5000 in PBST and incubated at 37 °C for 1 h. After washing with PBST three times, HRP-conjugated goat anti-rabbit IgG antibody (1:5000) was added and incubated at 37 °C for 0.5 h. Wash with PBST for six times to remove the nonspecific binding proteins. Finally, the tetramethylbenzidine (TMB) solution (Solarbio Science Technology, Beijing) was added into the wells of microplate and incubated at 37 °C for 15–20 min, followed by adding 2 M H_2SO_4 to terminate this reaction. Optical density at 450 nm (OD450) was measured by Synergy HT Microplate Reader (Bio-Tek, USA). The data were analyzed by GraphPad Prism 7.0.

2.7. Cell lines, cell culture and transfection

HEK293A cell line was a kindly gift from Dr. Qiming Sun's lab (School of Medicine, Zhejiang University). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, Israel) with 10% fetal bovine serum (FBS, ExCell Bio) and 1% penicillin–streptomycin (Sangon Biotech, China) in a 5% CO_2 incubator at 37 °C. Transfection with plasmid DNA was performed with Hieff *trans-*
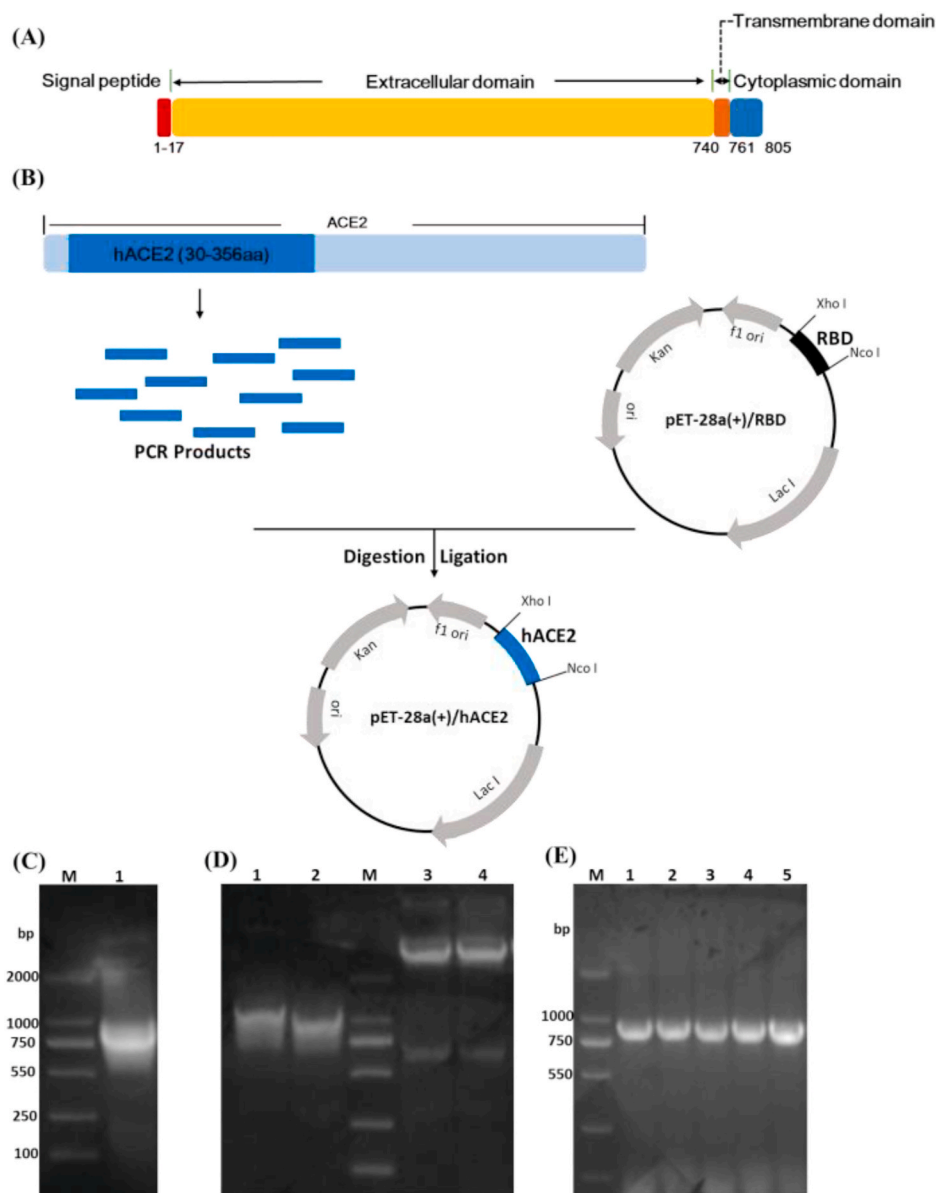
Fig. 1. Cloning of hACE2 gene and construction of recombinant plasmid pET-28a (+)/hACE2. (A) Domains of the full-length hACE2. (B) Construction map of expression plasmid pET-28a (+)/hACE2. (C) PCR products of hACE2. (D) Preparation of target gene and plasmid. Lane 1–2, PCR products digested with *Nco* I and *Xho* I; lane 3–4, pET-28a (+)/RBD digested with *Nco* I and *Xho* I. (E) The recombinant clones were confirmed by PCR. All the DNA fragments were subjected to agarose 1% gel electrophoresis and visilized under UV light.

liposomal transfection reagent (Yeasen Biotech, China) according to the manufacturer's instructions.

To obtain stably-expressing Spike protein, HEK293A cells were transiently transfected with plasmid pcDNA3.1-Spike-Myc harboring full-length SARS-CoV2 Spike protein. After 24 h of transfection, selection reagent G418 (700 μg/ml) was added to the cells in the fresh complete medium. After 14 days, cells were digested with 0.25% Trypsin-EDTA (NCM Biotech, China) and harvested with complete DMEM medium supplied with 700 μg/ml G418. After 7 days of incubation, single clones were seeded and picked up from 96 well-plates through gradient dilution, then each single clone was transferred to a new dish with fresh complete DMEM medium. To keep the resistant phenotype of established transfected cells, the stable cell lines were maintained in medium with 400 μg/ml G418.

2.8. Flow cytometry

Flow cytometry analysis was performed to evaluate the binding ability of rhACE2 to HEK293A cells stably expressing of SARS-CoV2-Spike protein. Briefly, 2×10^5 cells were incubated with rhACE2 or hACE2 (Sangon Biotech, China) for 30 min at 2–8 °C, after three times

washing with PBS containing 2% FBS, then anti-His-tag mouse monoclonal antibody was added (1:200) and incubated for 30 min at 2–8 °C, followed by three times washing mentioned above. Then Goat anti-mouse IgG-Alexa Fluor® 647 antibody (1:200) was added to incubate for 30 min in the dark at 2–8 °C. Wash the cells for three times, and re-suspend cells in an appropriate volume of PBS buffer. The samples were processed by ACEA NovoCyteTM (USA) and further analyzed by FlowJo V10 software.

2.9. Statistical analysis

Statistical analysis was carried out by using GraphPadPrism7.0 statistical software. Statistical significance for OD450nm of ELISA between groups was determined by grouped three replicates, and statistical significance for mean fluorescence intensity between groups was determined by grouped two replicates value in side-by-side sub-columns analysis, and p value of less than 0.05 was considered to be statistically significant. Asterisks indicate statistical compared to negative group (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).
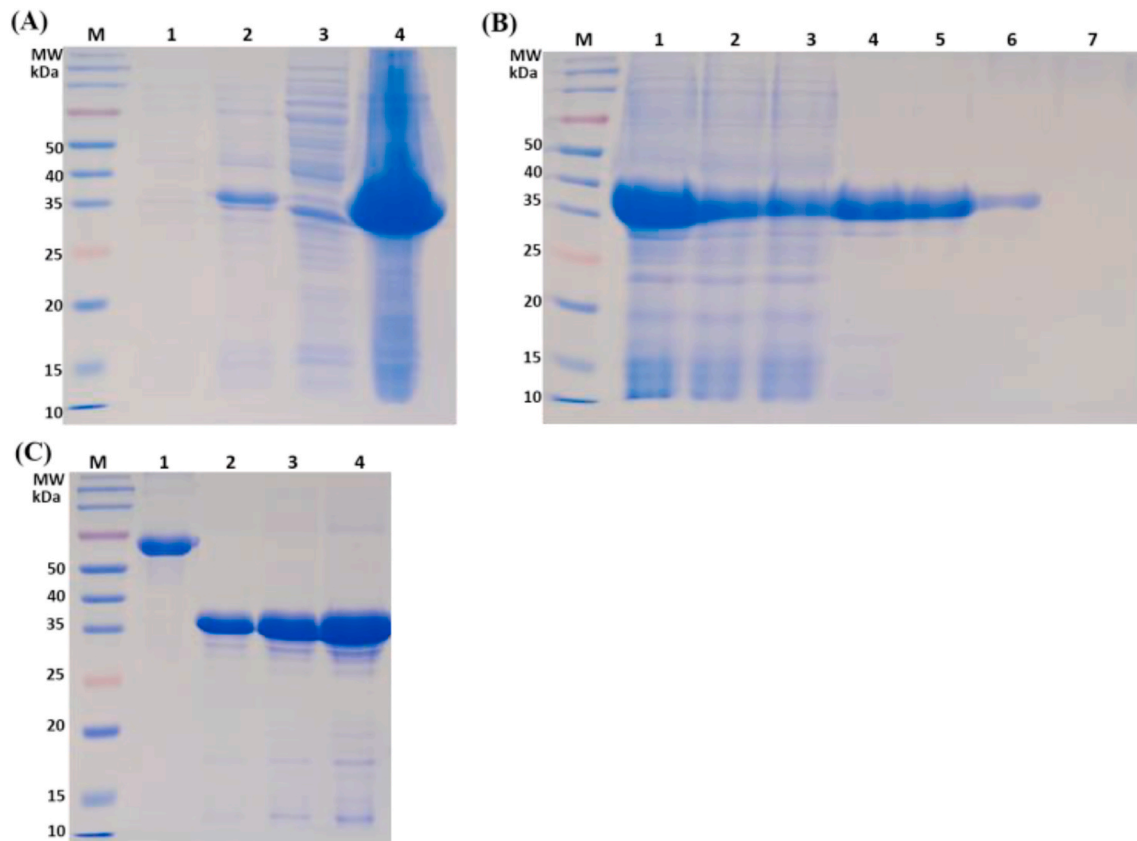
Fig. 2. Expression, purification and refolding of the recombinant hACE2. (A) Expression of recombinant hACE2. Lane 1, non-induced bacteria lysate; lane 2, bacteria lysate induced with 1 mM IPTG for 6–7 h; lane 3, soluble fraction after sonication; lane 4, pellet after sonication. (B) Purification of recombinant hACE2 through Ni-NTA sefinose column. Lane 1, crude sample before loading (denatured protein by 8 M urea); lane 2–7, eluted fractions of recombinant protein by using NTA buffer containing 20 mM, 50 mM, 100 mM, 150 mM, 200 mM and 250 mM imidazole and 8 M urea. (C) Refolding recombinant protein after dialysis. Lane 1, 0.5 mg/ml BSA (control); lane 2–4, 0.5 mg/ml, 1 mg/ml, 2 mg/ml rhACE2 protein.

3. Results

3.1. Construction of recombinant plasmid pET-28a (+)/hACE2

The full-length human ACE2 contains 805aa, which consists of a signal peptide (1-17aa), an extracellular domain (18-740aa), a transmembrane domain (741-761aa) and the cytoplasmic domain (762-805aa) (Fig. 1A). Of which, the DNA coding hACE2 (30-356aa) was amplified by PCR using the specific primers and the template of pcDNA3.1-hACE2-Flag vector. The entire strategy of recombinant vector construction was shown by a schematic diagram (Fig. 1B). The size of desired DNA fragment is 981bp identified by 1% agarose gel electrophoresis (Fig. 1C). The DNA fragment was digested with *Nco* I and *Xho* I and subcloned into the plasmid pET-28a (+) (Fig. 1D) to generate the recombinant expression plasmid pET-28a (+)/hACE2. The products of ligation were transformed into *Escherichia coli* Rosetta (DE3) by CaCl$_2$ method. Single clones were subjected to DNA extraction and identified through PCR reaction. The band of each PCR product examined by 1% agarose gel electrophoresis showed all test samples were positive (Fig. 1E). These positive clones were further confirmed by DNA sequencing from Sangon Biotech (Shanghai, China). Sequencing results showed that the recombinant expression vector pET-28a (+)/hACE2 was constructed successfully.

3.2. Expression and purification of the recombinant hACE2

Escherichia coli Rosetta (DE3) host strain is designed to enhance the expression of eukaryotic proteins that contain codons rarely possessed in *Escherichia coli*. This strain provides tRNAs for 7 rare codons (AGA, AGG,

AUA, CUA, GGA, CCC, and CGG) on a compatible chloramphenicol-resistant plasmid. The tRNA genes are driven by their native promoters [5]. The correct clone was subjected to protein expression by induced with 1 mM IPTG. SDS-PAGE analysis of the induced bacteria sample showed that there was a distinct band with the molecular weight about 39 kDa compared with the un-induced one. There was a large amount of insoluble protein existed in the pellet (inclusion body) but not in the supernatant after sonication (Fig. 2A). It's been reported that expression system of pET-28a (+) usually causes insoluble inclusion body. In order to acquire enough rhACE2 similar to natural structure with correct spatial structure which is essential for the functional activity. We used 8 M urea to dissolve and denature the inclusion bodies followed by affinity purification through Ni-NTA sefinose resin system. The results of SDS-PAGE showed that the fractions eluted with NTA buffer containing 100 mM, 150 mM, 200 mM imidazole and 8 M urea contained relatively pure recombinant proteins with high yield (Fig. 2B). The rhACE2 denatured proteins were collected and refolded by serial dialysis. Two times 1 × PBS buffer were carried to the dialysis procedure finally, then the protein was concentrated with PEG20000. Here we processed the expression system with 200 ml LB medium and finally obtained 15 mg of rhACE2. The purity of rhACE2 is approximately 90% with few undesired faint bands analyzed by SDS-PAGE at the concentration of 0.5 mg/ml (Fig. 2C). The yield of rhACE2 reached about 75 mg with 1 L of LB medium expression system, which represents an efficient way to produce recombinant hACE2.

3.3. Antigenic assay and RBD binding activity of recombinant hACE2

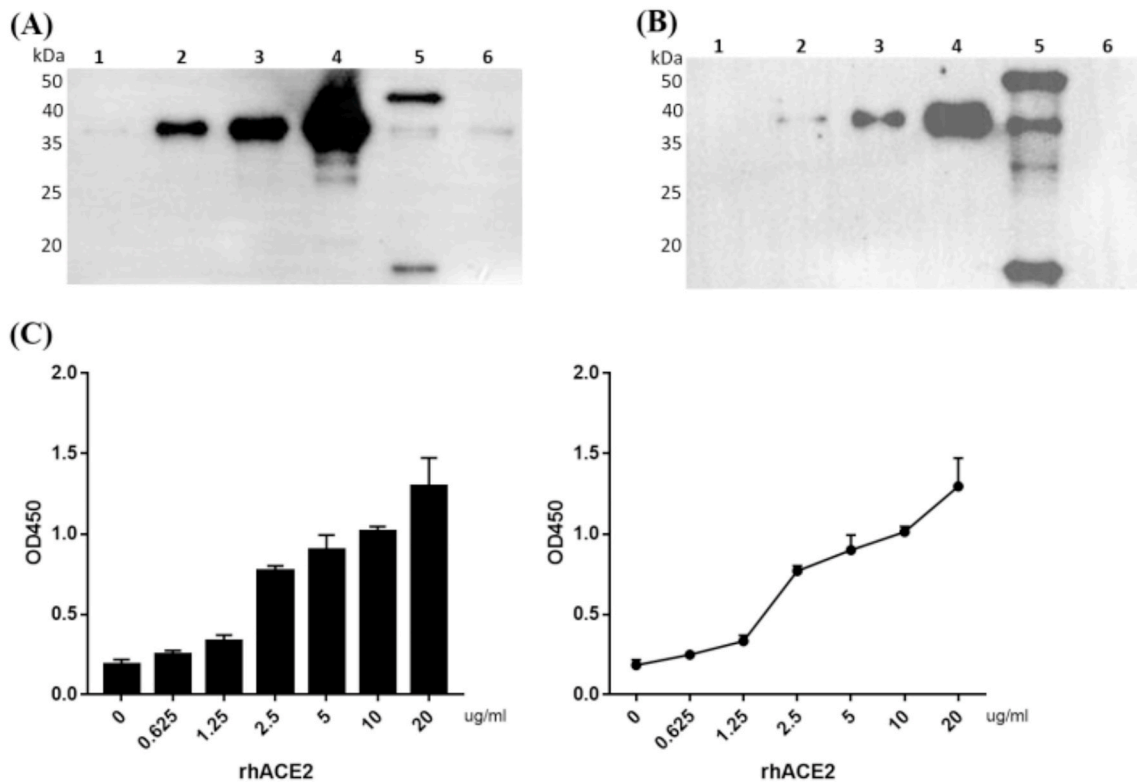To identify the function of the purified recombinant hACE2, the

Fig. 3. Antigenic and binding activity analysis of recombinant hACE2 in protein level. (A) Detection of rhACE2 by Western blotting with anti-6 × His Tag monoclonal antibody. (B) Detection of rhACE2 by Western blotting with rabbit anti-ACE2 polyclonal antibody. Line 1–4, rhACE2 with the concentration of 0, 0.5, 2.5, 12.5 ng/ul; line 5, 12.5 ng/ul hACE2 (purchased from Sangon Biotech) (positive control); line 6, 12.5 ng/ul BSA (negative control). (C) ELISA assay. The rhACE2 binds to SARS-CoV2 RBD protein in a concentration-dependent way. Values are mean ± SD. Experiments were performed in triplicate.
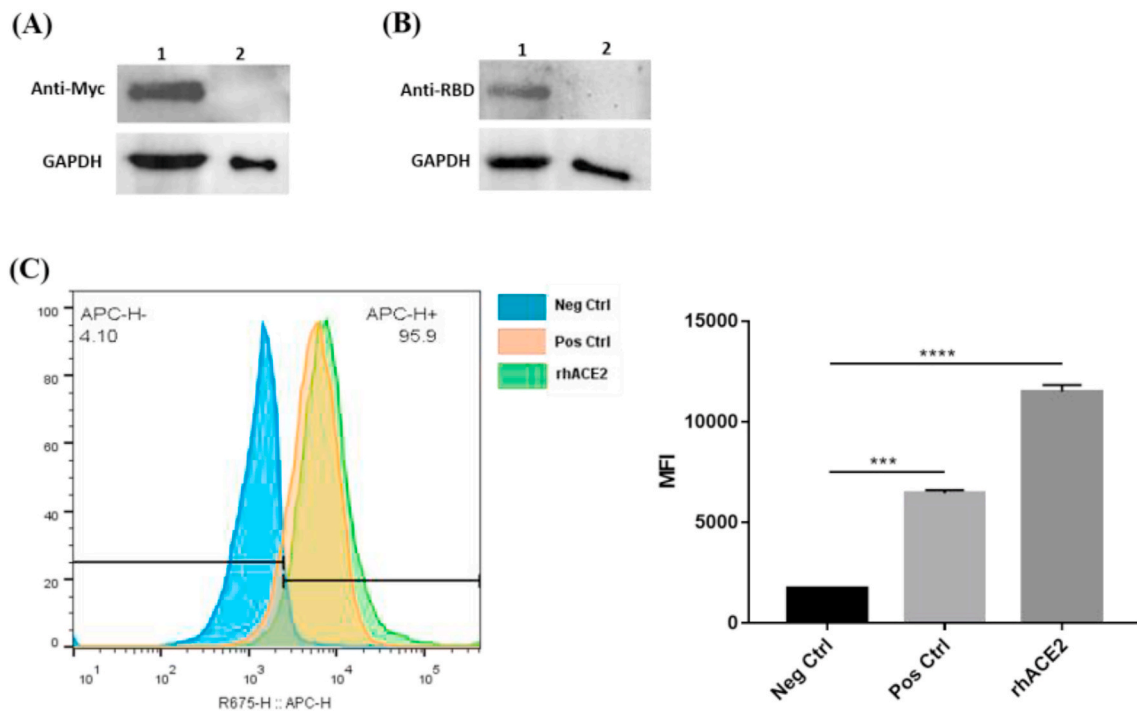

Fig. 4. Recombinant hACE2 is able to bind to the HEK293A-SP cells. (A) (B) WB analysis of the overexpressed Spike protein on HEK293A-SP cells detected with rabbit anti-Myc and rabbit anti-RBD polyclonal antibody respectively. Lane 1, cell lysate of HEK293A cell transfected with plasmid pcDNA3.1-Spike-Myc; lane 2, cell lysate of HEK293A cell transfected with empty pcDNA3.1 vector (control). (C) Binding analysis of rhACE2 to HEK293A-SP cells by flow cytometry. The mean fluorescence intensity (MFI) are mean ± SD. Experiments were performed in double. HEK293A-SP cells transfected with pcDNA3.1-Spike-Myc showed more fluorescence intensity than HEK293A, indicating rhACE2 is able to bind the S protein-overexpressed cells. The BSA and commercial hACE2 (purchased from Sangon Biotech) were used as negative control (Neg Ctrl) and positive control (Pos Ctrl) respectively.

antigenic bioactivity experiment was examined by Western blot. Two tests were carried out by using the anti-His tag and anti-ACE2 antibodies respectively. The specific bands with 39 kDa were detected with both antibodies and also showed in a concentration-dependent manner (Fig. 3A and B). Most importantly, whether the rhACE2 possesses the binding activity with SARS-CoV2 receptor bind domain (RBD) need to be determined. Therefore, we did the enzyme-linked immunosorbent assay (ELISA) to prove this point. Briefly, the rhACE2 proteins were coated to 96-well microplates at several increased concentrations followed by addition with RBD protein (OkayBio, China), the anti-RBD antibodies was used for final detection. The significant differences can be observed among the tested groups with different concentrations of rhACE2 (Fig. 3C). Taken together, our data demonstrated that the rhACE2 can bind to SARS-CoV2 RBD.

3.4. Recombinant hACE2 is able to bind the S protein expressed on the surface of HEK293A-SP cells

We attempted to study the binding activity of rhACE2 to the HEK293A-SP cells which are stably expressed S protein of SARS-COV2. First, the HEK293A was transfected with plasmid pcDNA3.1-SARS-CoV2 Spike-Myc in order to obtain a cell line stably expressing SARS-CoV2 Spike protein. We found that there is a remarkable expression of Spike protein in the transfected HEK293A cells comparing to the control group. The anti-Myc tag (Fig. 4A) and anti-RBD (Fig. 4B) antibodies were used for Western blot analysis, indicating the S protein was expressed in the HEK293A-SP cells. Moreover, we found that rhACE2 is able to bind to HEK293A-SP cells with higher affinity than commercial hACE2 protein (positive control) (Fig. 4C). Thus, data from this part of our study demonstrate that the rhACE2 expressed in *E. coli* is able to bind SARS-CoV2 Spike protein in the cell surface of HEK293A-SP cells, which indicates that recombinant protein possesses biological activity.

4. Discussion

Mechanism studies demonstrate that coronavirus invade host cells by fusing its membrane with a host cell membrane, then releasing its genome into the cytoplasm and initiating the viral replication cycle. During the infection process, a small set of viral surface transmembrane glycoproteins bind to host cell surface receptor and mediates fusion.

Previous studies indicated that ACE2 is associated with hypertension and cardiovascular disease. However, since ACE2 was identified as an essential receptor for the infection of coronavirus SARS-CoV in 2003, many studies have been completed [6]. It's reported that SARS-CoV invaded cells through the cell surface receptor ACE2 from the assay performed in vitro [7], which also was identified in vivo study [8]. Since the outbreak of the new coronavirus SARS-CoV2, it was found that the genome sequences of SARS-CoV2 obtained from patients of COVID-19 share 79.6% sequence identity to SARS-CoV [3]. Thus, it's speculated that the pathogenic mechanism of SARS-CoV-2 is similar to SARS-CoV. Later, it was reported that the SARS-CoV2 also enters human cells by hijacking their surface receptor ACE2 through its outer membrane spike [9]. This process will release both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), resulting in toxic damage to different target cells of the host [10]. The target tissues of human body include the lung, the main target organ of viral infection, multiple organ systems such as the intestine, liver, kidney and nervous system with varying degrees of toxic damage [11].

The S glycoprotein of the coronavirus SARS-CoV/SARS-CoV2 forms a characteristic coronal spinous processed on the viral envelope and the ACE2-Spike interaction leads to the endocytosis of virus particles through ACE2 internalization, induces the fusion of the virus and the host cell which promote the infection of coronavirus. From the above studies on the pathogenic mechanism of SARS-CoV/SARS-CoV2, it can be inferred that the targeted therapy strategy for blocking the interaction of between ACE2 and S protein in the treatment of new coronavirus

infection is the specific human anti-virus drugs [12] or soluble ACE2 fragments [13]. Therefore, rhACE2 is necessary for the preclinical research of anti-virus drugs development.

In this study, we have established an efficient prokaryotic expression and identification system of rhACE2. This purified rhACE2 possesses efficient antigenicity and binding ability to SARS-CoV2 RBD and S protein in HEK293A-SP cells.

5. Conclusion

In summary, we provide a high-level expression and identification system of human angiotensin converting enzyme 2 (hACE2). The rhACE2 (30-356aa) has biological activity and is able to bind both RBD and S protein of SARS-CoV2 in vitro. The rhACE2 obtained from the methods described in the study is useful for basic mechanism research, and use as a target for anti-viral drug screening and development. Our finding will contribute to generate valuable antibodies or fusion protein against SARS-CoV2.

Author Statement

Xiangzheng Gao: Methodology; Investigation; Validation; Formal analysis; Data Curation; Writing - Original Draft; Visualization.
Keying Liang: Methodology; Investigation.
Shengsheng Mei: Methodology; Investigation.
Shanshan Peng: Investigation.
Eu Gene Vong: Investigation.
Jinbiao Zhan: Conceptualization; Resources; Term; Writing - Review & Editing; Visualization; Supervision; Project administration; Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

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