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Expression cloning of functional receptor used by SARS coronavirus

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Abstract

We have expressed a series of truncated spike (S) glycoproteins of SARS-CoV and found that the N-terminus 14–502 residuals were sufficient to bind to SARS-CoV susceptible Vero E6 cells. With this soluble S protein fragment as an affinity ligand, we screened HeLa cells transduced with retroviral cDNA library from Vero E6 cells and obtained a HeLa cell clone which could bind with the S protein. This cell clone was susceptible to HIV/SARS pseudovirus infection and the presence of a functional receptor for S protein in this cell clone was confirmed by the cell–cell fusion assay. Further studies showed the susceptibility of this cell was due to the expression of endogenous angiotensin-converting enzyme 2 (ACE2) which was activated by inserted LTR from retroviral vector used for expression cloning. When human ACE2 cDNA was transduced into NIH3T3 cells, the ACE2 expressing NIH3T3 cells could be infected with HIV/SARS pseudovirus. These data clearly demonstrated that ACE2 was the functional receptor for SARS-CoV.

Keywords: Expression cloning; SARS-CoV; ACE2; Receptor; FACS sorting; TAIL-PCR; S glycoprotein; Insertion activation; LTR

Expression cloning is a powerful method used to isolate cellular receptors for viruses and was successfully used in the identification of receptors for HIV, Ebola virus, and HCV [1–3]. Using the method to isolate viral receptors, the cDNA library from susceptible cells was delivered into non-infectable cells and the receptor function gaining cells was then selected—either by challenging with a pseudovirus carrying a selection marker [1,2] or by FACS sorting with the virus' envelope protein containing the receptor binding domain as an affinity ligand [3]. The causative agent of SARS was identified to be a novel coronavirus [4–8]. Because the spike (S) glycoprotein of coronavirus was responsible for the binding to the specific receptor [9–12], we designed an expression cloning strategy to isolate the cellular receptor for SARS-CoV. Here we reported the expression cloning of ACE2 as the functional receptor for SARS-CoV. Our cloning strategy also suggested a novel approach for functional gene cloning through insertion activation of the endogenous genes.

Materials and methods

Soluble S protein expression and binding assay. For the construction of a series of truncated S1 proteins, the coding sequences for N-terminal 14–640, 14–502, and 14–440 residues of S protein were amplified from the full length S gene with primers: forward 5′-ACTGGATCCAGTGACC TTGACCGGT-3′; reverse for S640 5′-CATTCTAGAACTCAGCTCC TATAAGACAGC-3′; reverse for S500 5′-CATTCTAGAATTCAAAA GAAAGTACTAC-3′; and reverse for S440 5′-CATTCTAGAAATAT TTATAATTATAATTAC-3′. The PCR products were digested with *Bam*H I and *Xba*I and then ligated to a modified pFastBacI vector (Life Technologies, Rockville, MD) which contained the signal sequence of Tpa and a 9× His-tag. These proteins were expressed using a Baculovirus System (Life Technologies, Rockville, MD). For the binding assay, S1 protein conditioned mediums were mixed with the cells and incubated at 4 °C for 1 h. After washing, the cells were incubated for 30 min at 4 °C with anti-6× His mAb (R&D systems, Minneapolis) or sera from SARS patients. Normal mouse or human sera were used as control. The cells were then washed and incubated for 30 min with diluted fluorescein isothiocyanate-labeled antiserum. Cells were analyzed with a Moflo cytometer (Dako Cytomation, Denmark). For neutralization tests, S500

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was mixed with 1:50 diluted SARS patient's sera before used for binding assay.

cDNA library construction. The mRNA was isolated from Vero E6 cells using an mRNA isolating kit (Life Technologies, Rockville, MD). Double strand cDNAs were synthesized by using the SuperScript plasmid system (Life Technologies, Rockville, MD). *Bst*xI adaptor (Life Technologies, Rockville, MD) was ligated to the cDNA by T4 DNA ligase (Life Technologies, Rockville, MD) and then size-fractionated through a 0.8% agarose gel. cDNA ranging from 0.75 to 5 kb was cloned into *Bst*xI site of pMX vector which was provided by T. Kitamura (Tokyo University). The ligated DNA was transformed into *Escherichia coli* XL1-blue by electroporation.

Expression cloning. Briefly, 10 μg retroviral cDNA plasmid was transfected into 293GP cells (2×10^6 cells in a 10-cm culture dish) by calcium phosphate precipitation. Two days post-transfection, the culture supernatant was added with 8 mg/ml polybrene to ten 10-cm dishes, each containing 5×10^5 HeLa cells. Two days later FACS sorting screened the library-transduced cells. For FACS sorting, the library-transduced cells were prepared as binding assay and then subjected to FACS sorting. In the first two rounds of sorting, the 0.2–0.5% cells with the highest binding capacity were collected. After the third round, a cell population with high binding capacity emerged and were collected.

HIV/SARS pseudovirus infection assays. HIV/SARS pseudovirus was produced as described previously [1,13]. Briefly, the pHIV-luc (12 μg) and S expressing constructs (6 μg) were co-transfected into 2×10^6 293T cells. Supernatants were harvested 48 h later and used to infect cells in a 24-well plate (2×10^4 cells/well). The cells were lysed at 48 h post infection. Twenty microliters of lysate was tested for luciferase activity by the addition of 100 μl of luciferase substrate and measured for 10 s in a Wallac Multilabel 1450 Counter (Perkin–Elmer, Singapore).

Cell fusion assay. HeLa cells were transduced with S genes by retrovirus vector pBabe-puro and selected in culture medium containing 5 μg/ml puromycin for one week. The puromycin resistant cells were checked for S protein expression and named as S-HeLa. F5 cells or HeLa cells were mixed with S-HeLa at the ratio of 1:1 and co-cultured for 8 h, and the syncytium was examined under microscope.

Genomic PCR. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Germany). The retrovirus-transduced cDNAs were recovered from F5 genomic DNA by PCR using primers: forward 5′-CTGCCGGATCCCAGTGTGCTGGAAA-3′; and reverse 5′-ATC GTCGACCACTGTGCTGGAAA-3′. The resulting PCR fragment was purified and cloned into the T vector (Promega, Wisconsin) and sequenced. LTR specific and ACE2 specific primers we used are: 5′-CC GACTTGTGGTCTCGCTGTTCCTTGG-3′ and 5′-TTTGATAG AACAGGTCTTCGGCTTCGTGGT-3′.

TAIL-PCR. TAIL-PCR was performed as described [14] with the exception that the annealing temperature in the tertiary PCR was 42 °C. Long specific primer sets complementary to the LTR sequence were 5′-GTTCGCTTCTCGCTTCTGTTCGC-3′; 5′-CTCAATAAAA GAGCCCACAACCCCT-3′ and 5′-ACTTGTGGTCTCGCTGTTCC TTG-3′. Short arbitrary degenerate (AD) primer was 5′-CGTCGAGT GAGATGAA-3′.

RT-PCR. The total RNA was isolated with a Trizol reagent (Life Technologies, Rockville, MD). The expression of ACE2 was analyzed with primers: forward 5′-GCACTCACGATTGTTGGGACT-3′ reverse 5′-ATTAGCCACTCGCACATCCTC-3′. The expression of mouse ACE2 was analyzed with primers: forward 5′-GCATTG ACAATTGTTGGAACA-3′ reverse 5′-ATCACTCACTCGTACAT CTTC-3′.

Inhibition of HIV/SARS pseudovirus entry by anti-ACE2 antibody. F5 cells were pre-incubated with a medium containing 10 μg/ml goat anti-human ACE2 polyclonal antibody (R&D systems, Minneapolis); or 10 μg/ml normal goat sera for 15 min at 4 °C. Cells were challenged with HIV-luc/SARS pseudovirus in the presence of antisera. 48 h later the luciferase activity was tested.

Results and discussion

The expression of soluble S protein containing the receptor binding domain

The S glycoproteins of coronaviruses carry out essential virus entry functions [11,12]. In some coronaviruses, proteolytic cleavage of the S protein yields the amino-terminal S1 and carboxyl-terminal S2 subunits, which function in receptor binding and membrane fusion, respectively [9,10]. Although there is not yet direct evidence that the S protein of SARS-CoV is cleaved into subunits, the S1 and S2 domains of the SARS-CoV S protein can be identified through their homology with the S1 and S2 subunits of other coronaviruses.

To determine whether the S1 domain of SARS-CoV S protein can interact with SARS-CoV permissive Vero E6 cells, we expressed a series of truncated S1 proteins tagged with 9× His in the baculovirus expression system: S640 (14–640), S500 (14–502), and S440 (14–440). These fragments would be secreted into the culture medium because of the signal peptide ligated to their N terminal, and their existence in conditioned medium was tested by ELISA. The binding of these proteins to Vero E6 cells was analyzed by FACS using sera from SARS patients or anti-6× His mAb. We found that both S640 and S500 were able to bind with Vero E6 cells while S440 was unable to do so (Fig. 1A). Further study with S500 showed that it did not bind to non-infectable cells such as HeLa cells (Fig. 1B). Moreover, this S500 binding could be blocked if S500 was pre-incubated with neutralizing sera from convalescent SARS patients (Fig. 1C). These studies indicate that S500 could specifically interact with a putative receptor expressed on the surface of Vero E6 cells. Similar results were recently reported that the receptor binding domain (RBD) of the S protein located in the N-terminal 318–510 residuals [15,16]. Our results further indicated that 503–510 residuals were unnecessary for binding with the receptor.

The screen of retroviral cDNA library transduced cells by FACS sorting

Based on the binding affinity of S500 to the putative receptor on permissive cells, we designed an expression cloning strategy to isolate the cellular receptor for SARS-CoV. We transfected the cDNA library of highly susceptible Vero E6 cells into non-infectable HeLa cells by pMX retrovirus expression vector; we then screened the retroviral cDNA library transduced cells by FACS using the soluble S500 as an affinity ligand. In the first two rounds of FACS sorting, only 0.2–0.5% of positive cells were collected. After 5 rounds, a HeLa cell clone (F5) was enriched and characterized. The clone was isolated because its high affinity to S500, which was even higher than that of Vero E6 cells (Fig. 2A).

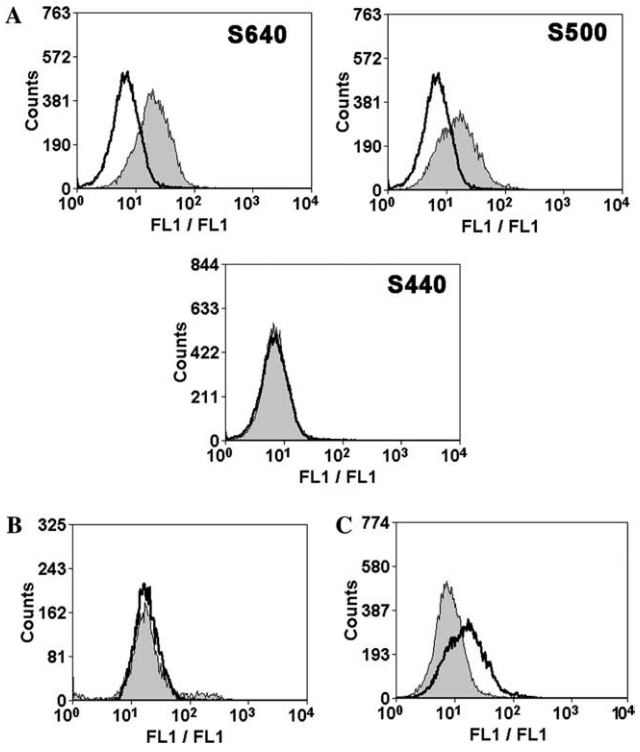

Fig. 1. Soluble S1 proteins could bind with SARS-CoV susceptible Vero E6 cells. (A) S500 was sufficient to bind with Vero E6 cells. Binding of variously truncated S1 proteins to Vero E6 cells was measured by FACS using sera from SARS patient and a FITC-labeled anti-human IgG secondary antibody. Vero E6 cells were incubated with S1 conditioned mediums (shaded area) or culture medium (white). (B) Binding of S500 to HeLa cells was measured by FACS. HeLa cells were incubated with S500 conditioned medium (shaded area) or culture medium (white). (C) Pre-incubation of S500 with sera from SARS patient (shaded area) blocked the binding of S500 to Vero E6 cells.

To verify whether the F5 cells indeed expressed a functional receptor for the entry of SARS-CoV, F5 cells were infected with HIV-luc/SARS pseudovirus that was demonstrated to be able to mimic the entry of wild-type SARS-CoV [13]. We found that the F5 cells were highly susceptible to HIV/SARS pseudovirus while non-transduced control HeLa cells were non-infectable (Fig. 2B). The presence of a functional receptor for S protein in the F5 cells was further confirmed by the cell–cell fusion assay. For this purpose, we established S-expressing HeLa cells (S-HeLa) by transducing the S gene into HeLa cells. When the F5 cells were co-cultured with S-HeLa cells, many syncytiums could be observed (Fig. 2C). These results demonstrated that F5 cells expressed a functional receptor required for HIV/SARS pseudovirus or SARS-CoV infection, which not only had a high binding affinity with the S protein but also was sufficient to mediate the entry of the virus.

The isolation of SARS-CoV receptor gene

Two strategies were used to recover the cDNA sequence encoding the receptor protein for HIV/SARS pseudovirus in the F5 cells. Recombinant retroviruses could ectopically express the encoded cDNA library proteins to confer the infectivity of the HIV/SARS pseudovirus. Alternatively, the retroviral vector used for cloning cDNA libraries had two long terminal repeat sequences (LTRs) on both sides of the cDNA insert. The LTRs could function as a promoter to initiate the transcription of their downstream sequences [17]. Integration of recombinant retroviruses containing two LTR promoter sequences into the upstream region of an endogenous gene might thus transcriptionally activate the expression of the downstream endogenous gene by the second LTR promoter element in the virus. We analyzed both possibilities by extracting the genomic DNAs from F5 cells. The virus-encoded cDNA was amplified by PCR using the primers flanking inserts in the retroviral vectors. A 1.3 k PCR product was cloned. DNA sequencing revealed the cDNA encoded triosephosphate isomerase (TPI). Analysis of the expression of TPI in non-permissive and permissive cells to infection suggested that there was no correlation between the susceptibility of the HIV/SARS pseudovirus and TPI expression in various cells (data not shown). Therefore, we tested the possibility that the LTR–TPI–LTR fragment might accidentally integrate into the upstream region of the endogenous receptor gene for S protein so that the second LTR activated the transcription of the endogenous receptor gene in the F5 cells. The expression of this receptor protein conferred the infectivity of F5 cells to HIV/SARS pseudovirus. To identify the genome DNA sequences downstream of the retroviral LTR element, we used the thermal asymmetric interlaced (TAIL) PCR, a method for recovery of DNA fragment adjacent to known sequences [14,18]. After three rounds of TAIL-PCR, a 300 basepair (bp) band and a 900 bp band were detected (Fig. 3A). The PCR products were cloned and sequenced. Sequence alignment indicated that one of them was part of the retrovirus vector sequence and the other was the 5′ region of the human ACE2 gene. The retrovirus integrated into the 5′ end of the endogenous ACE2 gene so that there was only 169 bp from the end of retroviral LTR to the start codon (ATG) of endogenous ACE2. The integration of the retroviral LTR into the upstream region of the endogenous ACE2 gene was further confirmed by genomic DNA PCR using primers specific to LTR and ACE2 (Figs. 3B and D).

We then tested the expression of ACE2 in non-infectable HeLa, NIH/3T3 cells and in susceptible F5, Vero E6, and Huh7 [13]. By RT-PCR, we found that ACE2 were strongly expressed in F5, Vero E6, and Huh7 cells; however, it could not be detected in HeLa and NIH/3T3 cells (Fig. 3C). To further confirm the receptor function of ACE2, we pre-incubated the F5 cells with anti-ACE2 polyclonal antibody before challenging the cells with the

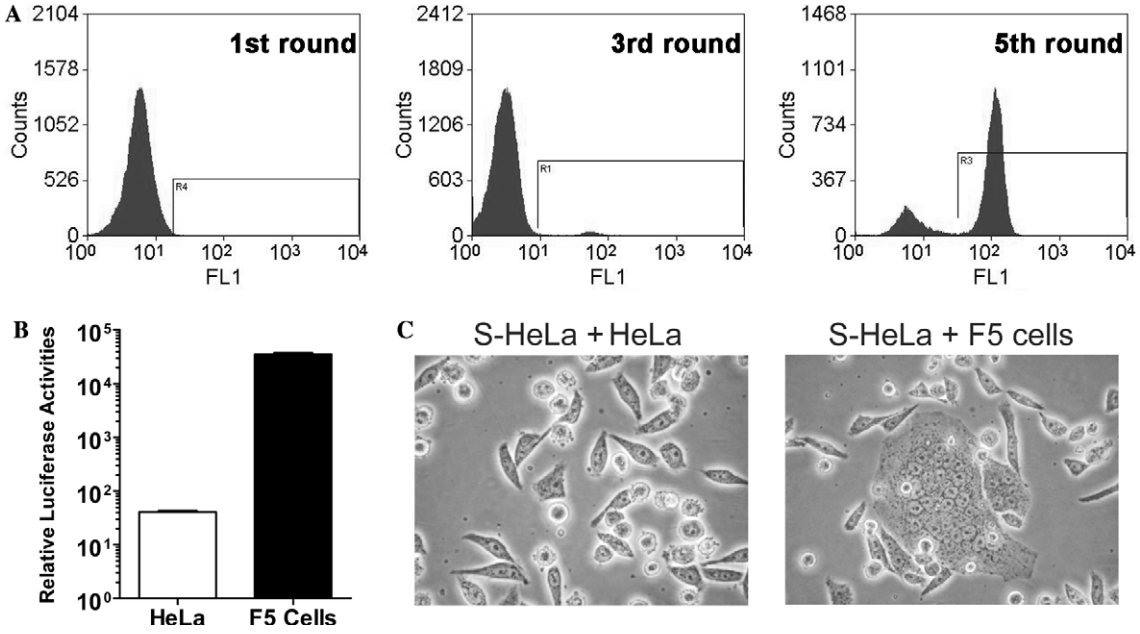

Fig. 2. HIV/SARS susceptible clone F5 enriched from cDNA library transduced HeLa cells by FACS sorting with S500. (A) A HeLa cell clone with high binding affinity to S500 was enriched after five rounds of FACS sorting. In each round, the cells shown in gate were collected and cultured. (B) F5 cells and non-transduced HeLa cells were infected with HIV-luc/SARS pseudovirus and luciferase activity was measured after two days. The experiment was repeated for three times. (C) Syncytiums could be observed when F5 cells were co-cultured with S protein expressing HeLa cells (S-HeLa). Non-transduced HeLa cells were used as control and co-cultured with S-HeLa.

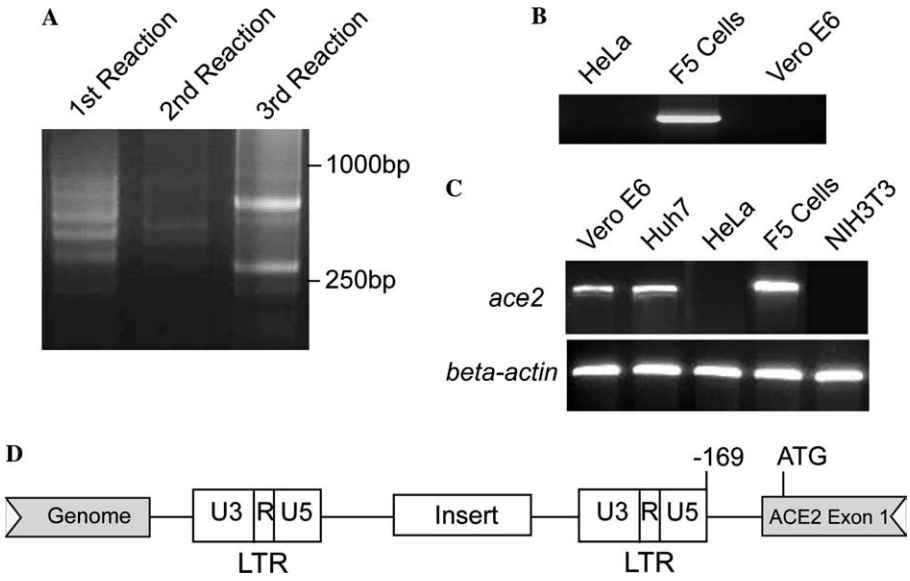

Fig. 3. The insertion of LTR in F5 cells activated the expression of endogenous ACE2 gene. (A) After three TAIL-PCRs, a 900 bp band (top) and a 300 bp band (bottom) were detected by agarose gel. The PCR products were sequenced. The 900 bp PCR products consisted of a partial sequence of LTR and a partial sequence of the 5′ end of ACE2 gene. (B) A 300 bp band was detected in genomic DNA from F5 cells by PCR using primers specific to LTR and the ACE2 gene. Genomic DNAs from non-transduced HeLa cells and Vero E6 cells were used as control. (C) By RT-PCR, the expression of ACE2 was detected in Vero E6, Huh7, and F5 cells but not in non-transduced HeLa and NIH/3T3 cells. (D) Schematic representation of integration of LTR into the 5′ end of endogenous ACE2 gene. The start codon of ACE2 gene was 169 bp downstream to the 3′ LTR so that the ACE2 gene was under the transcriptional control of the 3′ LTR.

pseudovirus and found that the infection could be abrogated (Fig. 4A). We then cloned the ACE2 cDNA from F5 cells by RT-PCR and transfected it into NIH3T3 cells. The ACE2-expressing NIH3T3 cells exhibited similar infectivity to that of F5 cells to HIV/SARS pseudovirus (Fig. 4B). These results clearly demonstrated that ACE2 was the functional receptor for SARS-CoV. When we were preparing this manuscript, Li et al. [19] reported on

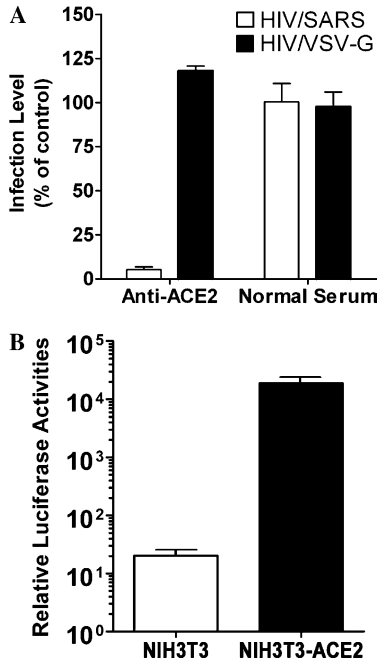

Fig. 4. ACE2 mediated the entry of HIV/SARS pseudovirus. (A) The goat anti-human ACE2 polyclonal antibody could abrogate the entry of HIV-luc/SARS pseudovirus into F5 cells. Normal goat sera were used as control. (B) The ACE2 expressing NIH3T3 cells could be infected with HIV/SARS pseudovirus. NIH3T3 cells transduced with pMX vector were used as control.

identifying the SARS-CoV receptor ACE2 by a biochemical approach, which was consistent with our finding described here using an independent expression cloning approach.

In this study, we showed that the RBD of S protein was located in the N-terminal 14–502 residuals and we identified ACE2 as the functional receptor by an expression cloning strategy. By the insertion of a retroviral LTR into the upstream region of endogenous ACE2, the expression of ACE2 is activated in the normally non-permissive HeLa cells for the infection of SARS-CoV. Therefore, our research suggested a novel approach for functional gene cloning through activation of the endogenous genes by random insertions of retrovirus into mammalian genomes.

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