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Induction of IL-8 Release in Lung Cells via Activator Protein-1 by Recombinant Baculovirus Displaying Severe Acute Respiratory Syndrome-Coronavirus Spike Proteins: Identification of Two Functional Regions[1]

Ya-Jen Chang,[2]* Catherine Y.-Y. Liu,[2‡] Bor-Luen Chiang,[†] Yu-Chan Chao,[2,3‡] and Ching-Chow Chen[3]*

The inflammatory response and the intracellular signaling pathway induced by severe acute respiratory syndrome (SARS)-coronavirus (CoV) were studied in lung epithelial cells and fibroblasts. SARS-CoV spike (S) protein-encoding plasmid induced activations of IL-8 promoter and AP-1, but not NF-κB in these cells. Mutation of the AP-1, not the κB site, abolished the SARS-CoV S protein-induced IL-8 promoter activity. IL-8 release was effectively induced by vAtEpGS688, a baculovirus exhibiting the aa 17–688 fragment of S protein, and this induction was attenuated by the angiotensin-converting enzyme 2 Ab. Recombinant baculovirus expressing different deletion and insertion fragments identified the functional region of S protein from aa 324–688 (particularly the N-terminal aa 324–488 and the C-terminal aa 609–688), which is responsible for IL-8 production. Activations of AP-1 DNA-protein binding and MAPKs after vAtEpGS688 transduction were demonstrated, and SARS-CoV S protein-induced IL-8 promoter activity was inhibited by the specific inhibitors of MAPK cascades. These results suggested that the S protein of SARS-CoV could induce release of IL-8 in the lung cells via activations of MAPKs and AP-1. The identification of the functional domain for IL-8 release will provide for the drug design on targeting specific sequence domains of S protein responsible for initiating the inflammatory response. *The Journal of Immunology,* 2004, 173: 7602–7614.

C oronaviruses (CoV)[4] are a family of enveloped viruses with positive-stranded, capped, and polyadenylated RNA genomes ranging in size from 28 to 32 kb (1). Two-thirds of the viral genome starting from the 5′ end encode replicase proteins, Rep1a and Rep1b, for the amplification of CoV RNA. Structural proteins, including spike (S), envelope (E), membrane (M), nucleocapsid (N), and several others with unknown functions, are also expressed in the CoV (1). In March 2003, a new member of CoV, the severe acute respiratory syndrome (SARS)-CoV, was discovered as the causative agent of SARS (2–5). Ultimately, the virus infected >8000 people and killed >700, mostly in Asia. SARS-CoV does not belong to any of the previously defined genetic and serological CoV groups. SARS-CoV S protein is essential for the process of viral infection, and SARS-CoV N protein is important for the transcription of viral RNA. Both proteins can serve as targets for the early diagnosis of infection.

The S proteins of CoV are major targets for neutralizing Abs and form the characteristic corona of large and distinctive spikes on the viral envelopes (1, 6). Similar 20~40-nm complex surface projections constituted of S proteins also surround the SARS-CoV particles (2). The amino acid sequence homology between SARS-CoV S protein and other CoV is low (20–27% identity), except for some conserved regions in the S2 domain (3). The S1 domain of all characterized CoV, including SARS-CoV, mediates the initial high affinity interaction with the cellular receptor (7–9).

Receptor identification is important for the understanding of viral tropism, pathogenicity, and mechanism of entry, which may help in the development of therapeutics and vaccines. The angiotensin-converting enzyme 2 (ACE2) has been identified as the receptor on Vero E6 and 293T cells (10) for SARS-CoV. The receptor-binding domain (RBD) of SARS-CoV S protein was first characterized to be located in the N-terminal (NT) region of 318~510 aa (11). Afterward, two other groups identified the RBD to be situated between 303~537 and 270~510 aa of the NT region, respectively (12, 13), which were approximately the same region suggested by the first group. Although the entrance of SARS-CoV into the host cell has been demonstrated to be through the binding of S protein to ACE2, the immune response induced by SARS-CoV remains undercharacterized.

High serum levels of IL-8 and IL-6 in the acute stage (cytokine storm) associated with lung lesions were found in SARS patients (14). The elevations of the plasma chemokine IL-8 and Th1-related cytokine can induce the hyperinnate inflammatory response due to the SARS-CoV invasion of the respiratory tract. Corticosteroid can suppress the elevated IL-8, and subsequently alleviate the chemokine-associated pulmonary inflammation in SARS (15). In the present study, SARS-CoV S protein-induced IL-8 release in lung

Departments of *Pharmacology and †Pediatrics, College of Medicine, National Taiwan University and National Taiwan University Hospital, Taipei, Taiwan; and ‡Institute of Molecular Biology, Academia Sinica, Nankang, Taipei, Taiwan

epithelial cells (A549 and NCI-H520) and lung fibroblasts (HFL-1 and MRC-5), and the transcription factor involved are studied. Effects of transient transfections of the SARS-CoV S protein-encoding plasmid on the IL-8 promoter, AP-1, and NF-κB were examined. A recombinant baculovirus containing 688-aa length of SARS-CoV S protein (vAtEpGS688), which could transduce the lung cells via expressing the S protein on its surface, was used as a tool for measuring IL-8 release. Our results showed that SARS-CoV S protein induced AP-1 activation and up-regulated IL-8 release. A functional region of the S protein, aa 324–688, was identified as being essential for the induction of IL-8 release. The intracellular signaling pathway was closely examined, and it was found that MAPKs and AP-1, but not NF-κB, were activated.

Materials and Methods

Reagents and plasmids

Phosphospecific Abs to ERK, p38, and JNK, and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Abs specific to ERK, p38, and JNK were from Santa Cruz Biotechnology (Santa Cruz, CA), and Ab specific to ACE2 was from Alpha Diagnostic International (San Antonio, TX). RPMI 1640, FCS, penicillin, and streptomycin were from Invitrogen Life Technologies (Gaithersburg, MD). PD98059, SB203580, and SP600125 were from Calbiochem (San Diego, CA). Reagents for SDS-PAGE were from Bio-Rad (Hercules, CA). Poly(dI-dC) was from Amersham Biosciences (Piscataway, NJ). [γ-^{32}P]ATP (3000 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA), the SuperFect reagent was from Qiagen (Valencia, CA), and the luciferase assay kit was from Promega (Madison, WI). The full-length cDNA of the S protein of SARS-CoV (TW1 clone) characterized by the National Taiwan University SARS Research Team (16) was subcloned into the pcDNA3.1. The AP-1 and NF-κB luciferase reporters were from Stratagene (La Jolla, CA). The IL-8 promoter construct IL-8 wild-type (wt) (−162/+44), AP-1 site mutation (IL-8ΔAP-1), and κB site mutation (IL-8ΔκB) were gifts from A. Brasier (University of Texas Medical Brach, Galveston, TX). The dominant-negative (DN) mutant of ERK2 was from M. Cobb (South-Western Medical Center, Dallas, TX), p38 (T180A/T182F) was from J. Han (Scripps Research Institute, San Diego, CA), and JNK (T183A/Y185F) was from M. Karin (University of California, San Diego, CA). pSVfos was a gift from W. Chang (National Cheng Kung University, Tainan, Taiwan).

Transient transfection and luciferase activity assay

Cells grown to 60% confluence in 12-well plates were transfected with either the human IL-8 wt, AP-1-luc, or NF-κB-luc using SuperFect (Qiagen), according to the manufacturer's recommendations and our previous description (17).

In experiments using DN mutants, cells were cotransfected with reporter (0.3 μg) and β-galactosidase (0.15 μg), 0.45 μg of the pcDNA3.1-S or the empty vector, and either the DN ERK, p38, and JNK mutants, or the empty vector (0.6 μg).

Construction of recombinant baculoviruses

The coding sequence of enhanced GFP (EGFP) with a poly(A) termination signal was inserted into the pTriEx-3 (Novagen, Madison, WI) transfer vector at the NcoI and HindIII sites. The resulting pAtE plasmid contained the EGFP gene under the control of both p10 and cmv promoters, and expressed egfp in both insect and mammalian cells. The signal peptide (SP) of Autographa californica nucleopolyhedrovirus (AcMNPV) gp64 gene was amplified from purified wt AcMNPV genomic DNA using oligonucleotide primers GPSF and GPSR2 (for all primer sequences, see Table I). The NT of gp64 gene was amplified using GPSF and GPNT227R. The CT of gp64 gene was amplified using GPCF227 and GPCR. The SP/NT and CT fragments were cloned into the PstI/KpnI and KpnI/SmaI sites of the pBacPAK8 transfer vector (BD Clontech, Palo Alto, CA) under the control of a polyhedrin promoter. The polyhedrin promoter plus the partial gp64 gene and the poly(A) termination signal were cleaved out using the EcoRV and HindIII restriction sites and ligated into the PvuII and HindIII sites on the pAtE plasmid, creating the pAtEpG/pAtEpGf (f = full length gp64) plasmids.

The various CT truncations of the SARS-CoV spike gene minus the signal sequence were obtained by PCR from a full-length TW1 spike clone (kindly provided by P. J. Chen of National Taiwan University). Six PCR fragments coding for spike gene fragments S280, S324, S488, S688, S763, and S966 were amplified (see Fig. 2B) and used to make the spike CT deletion expression vectors. The number here represents the most CT amino acid residue of the particular Spike fragment. The SpikeF2 primer was the universal 5′ primer. The reverse primers SpikeRX, in which X corresponded to a Spike amino acid residue number, are shown in Table I. All six PCR products were first cloned into pZeroBlunt (Invitrogen Life Technologies), then cut with SfiI restriction enzyme and inserted into SfiI-cut pAtEpG vector, resulting in pAtEpGSX, in which X represents the most CT spike amino acid residue (e.g., pAtEpGS280). To construct the spike insertion vectors, three internal fragments of the spike gene, S489–534, S489–609, and S489–688, were amplified using SpikeF489 and SpikeRX (X = 534, 609, or 688). These fragments were inserted into the SfiI site of the pAtEpGf vector, resulting in pAtEpGS489–534, pAtEpGS489–609, and pAtEpGS489–688.

All these spike-expressing plasmids plus the pAtE control plasmid were cotransfected with vAcRP23.Laz (BD Pharmingen, San Diego, CA), a linearized viral DNA of AcMNPV, into Spodoptera frugiperda (Sf21) cells using Lipofectin (Invitrogen Life Technologies). Ten recombinant baculoviruses were produced in total.

Baculovirus production in insect cells

The IPLB-Sf21 (Sf21) cell line was cultured at 26°C in TC100 insect medium, supplemented with 10% heat-inactivated FBS. It was used for the generation and propagation of wt and recombinant AcMNPV. All viral stocks were prepared according to the standard protocols described by O'Reilly et al. (18). The virus titers were determined by quantitative PCR (18, 19).

Viral DNA analysis

Recombinant baculoviruses were amplified at a multiplicity of infection (MOI) of 0.1 for 4 days in Sf21 cells before harvesting. The medium

Table I.   *PCR primers used for recombinant baculovirus construction*

Primer Name	Sequence (SfiI site underlined)
GPSF	5′-AGGCCTCAATGCTACTAGTAAATC-3′
GPSR2	5′-GGCCGCAAAGGCCGAATGCGCCGC-3′
GPNT22R	5′-GGCCGCAAAGGCCACGCCGTCTCGATGAAGCAC-3′
GPCF227	5′-GGCCACGGTGGCCATGATTCTCAAACAAAAGTC-3′
GPCR	5′-CCCGGGTTAATATTGTCTATTACG-3′
SpikeF2	5′-GGCCTTTGCGGCCGACCGGTGCACCACTTTTG-3′
Spikef489	5′-GGCCTTTGCGGCCATTGGCTACCAACCTTAC-3′
SpikeR280	5′-GGCCACCGTGGCCTGAGAACAATCAACAGCATC-3′
SpikeR324	5′-GGCCACCGTGGCCGGACACAAGTTTGTAATATTAGG-3′
SpikeR488	5′-GGCCACCGTGGCCCCAGTAGTGGTGTAAAAACC-3′
SpikeR534	5′-GGCCACCGTGGCCCCGAGTCCATTAAAATTAAAATTGAC-3′
SpikeR609	5′-GGCCACCGTGGCCGTAGAAACATCAGTGCAG-3′
SpikeR688	5′-GGCCACCGTGGCCATTGAACTATCAGCACCTAAAG-3′
SpikeR763	5′-GGCCACCGTGGCCACTTCACGTGTGTTGCGATC-3′
SpikeR966	5′-GGCCACCGTGGCCAGTCGCGAAAGGATGTCATTTAGCAC-3′

containing the viruses was centrifuged first at 800 rpm for 5 min to get rid of cellular debris. Viral DNA was then extracted from the supernatant using the High Pure Viral Nucleic Acid kit (Roche, Basel, Switzerland). PCR primer pairs SpikeF2 and GPCR (for deletion clones) or SpikeF489 and GPCR (for insertion clones) were used to amplify the fusion protein gene fragments.

Western blot analysis

Total proteins from Sf21 cells infected with the recombinant baculoviruses with an MOI of 5 were harvested 4 days postinfection and separated on 8% SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA) and probed with either rabbit anti-Spike Ab at 1/2000 dilutions, or rabbit anti-GP64 Ab (1/5000 dilution). The membranes were then probed with goat anti-rabbit IgG-HRP (Novagen) secondary Ab at 1/3000 dilutions.

For mammalian cells, after the recombinant virus transduction, cells were rapidly washed with PBS to remove medium and then lysed with the ice-cold lysis buffer, as described previously (17). The proteins were transferred to the nitrocellulose paper, and the Western blot was performed (20). Control experiments consisted of the cells transduced by the control vAtE virus. The immunoreactive proteins were detected using ECL.

Mammalian cell culture and virus transduction experiments

The human lung epithelial and fibroblast cell lines, A549, NCI-H520, HFL-1, and MRC-5, were obtained from the American Type Culture Collection (Manassas, VA) and cultured in F12K, RPMI 1640, F12K, and DMEM, respectively. All of the medium was supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded into six-well plate with the number of 10^5 overnight. In the next day, cells were incubated with the recombinant baculovirus for 2 h, and then the virus mixture was removed and replaced with fresh cell culture medium. Cells were then further incubated for 24 h posttransduction, and the medium was collected to analyze the release of IL-8.

IL-8 ELISA

Quantification of IL-8 was used kit from Amersham Biosciences and performed according to the manufacturer's recommendation.

RT-PCR

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen Life Technologies). The reverse-transcription reaction was performed using 2 μg of total RNA, which was transcribed into cDNA using the oligo(dT) primer, and then the cDNA was amplified for 30 cycles using two oligonucleotide primers derived from a published IL-8 sequence (5'-ATGACTTC CAAGCTGGCCGTGGCT-3' and 5'-TCTCAGCCCTCTTCAAAAACT TCT-3'), c-fos sequence (5'-GAATAACATGGCTGTGCAGCCAAATGCC GCAA-3' and 5'-CGTCAGATCAAGGGAAGCCACAGACATCT), and β-actin sequence (5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' and 5'-CTAGAAGCATTTGCGGGGACGATGGAGGG-3'). For IL-8 and c-fos, a PCR cycle consisted of a denaturation step (94ºC, 1 min), an annealing step (60°C for IL-8; 58°C for c-fos, 1 min), and an elongation step (72°C, 1.5 min). There was a total of 35 cycles, followed by an additional extension step (72°C, 7 min). For β-actin, PCR cycle was conducted for 30 s at 94°C, 30 s at 65°C, and 1 min at 70°C. The PCR products were subjected to electrophoresis on a 1.5% agarose gel. Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Preparation of nuclear extracts and the EMSA

After the recombinant virus infection and transduction for the indicated time, cells were washed with PBS for several times, and nuclear extracts were prepared, as described previously (17). Oligonucleotides corresponding to the consensus sequences of AP-1 site on the human IL-8 promoter (5'-AGAAACAGTCATTTC-3') were synthesized, annealed, and end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and EMSA was performed (17).

When supershift assays were performed, polyclonal Ab specific for anti-c-fos or anti-p65 was added to the nuclear extracts 30 min before the binding reaction, and the DNA/nuclear protein complexes were separated on a 4.5% polyacrylamide gel.

Statistical analysis

Data were analyzed using Student's t test. Values of $p < 0.05$ were considered significant.

Results

SARS-CoV S protein up-regulated IL-8 promoter and induced activation of AP-1, but not NF-κB, in the lung cells

Effects of transient transfections of the SARS-CoV structure protein-encoding pcDNA3.1-S on the IL-8 promoter of the lung cells were examined. The human wt IL-8 promoter-luciferase construct that contains the IL-8 promoter ($-162/+44$) was used to measure the promoter activity. Overexpressions of SARS-CoV structure protein (S, M, E, or N)-encoding plasmid induced different degrees of increase in the promoter activity (data not shown). SARS-CoV S protein activated IL-8 promoter of all lung cells (Fig. 1*A*). Its effect on the activities of the transcription factors, AP-1 and NF-κB, was also studied and showed the activation of AP-1 (Fig. 1*B*), but not NF-κB (Fig. 1*C*). To identify which *cis*-acting element was involved in the S protein-induced IL-8 promoter activity, the IL-8 promoter-luc constructs including $-162/+44$ (wt), AP-1 site ($-126/-120$) mutant (IL-8ΔAP-1), and κB site ($-82/-70$) mutant (IL-8ΔκB) were used. Our results revealed that the induction of IL-8 promoter activity was attenuated when using IL-8ΔAP-1, but not IL-8ΔκB in both A549 and HFL-1 cells (Fig. 1*D*), demonstrating the contribution of AP-1, but not κB element to the S protein-induced IL-8 transcription.

The Spike-gp64 fusion protein can be expressed on the surface of recombinant baculoviruses

To assemble a functional SARS-CoV S protein and express it on the surface of baculovirus, a NT fragment of the S protein was fused to the CT half (aa 227–589) of the AcMNPV GP64 protein. Six CT deletion fragments of the S protein, S280, S324, S488, S688, S763, and S966, were chosen, as shown by the illustration in Fig. 2*A*. The SP of the S protein was also replaced by the GP64 SP, ensuring the positioning of the fusion protein onto the surface of baculoviral membrane. The CT deletion as well as three insertion clones in which three S internal fragments were inserted into aa position 227 of the full-length GP64 protein were made. They were used for further analysis of the functional domain of SARS-CoV S protein in inducing IL-8 release.

All the S-GP64 fusion proteins were driven by the baculoviral *polyhedrin* promoter. A selection marker, EGFP, was cloned after the *cmv* and *p10* promoters of the pTriEx vector (Invitrogen Life Technologies), so EGFP would be expressed both in insect and mammalian cell lines. A control EGFP-only construct without the fusion gene was also made, and served as a negative control in all experiments.

The recombinant baculoviruses were checked for the incorporation of the fusion constructs by PCR with Spike F2 or Spike F489 and GPCR primers (see Table I) using purified viral DNA as templates (Fig. 2*A*). The reaction should produce only the recombinant fusion construct, *spike-gp64*, but not the wt *gp64* gene. The expected product sizes for *spike-gp64* fusion constructs GS280, GS324, GS488, GS688, GS763, and GS966 were 1,729, 1,861, 2,353, 2,950, 3,178, and 3,787 bp, respectively. The expected sizes for insertion constructs GS489–534, GS489–609, and GS489–688 were 1,246, 11,471, and 1,708 bp, respectively (Fig. 2*B*). Sf-21 cell lysates infected with these recombinant baculoviruses were analyzed with 8% reducing SDS-PAGE. The expected sizes for the deletion clones S280, S324, S488, S688, S763, and S966 were 69, 74, 93, 114, 122, and 144 kDa without glycosylation. For the insertion clones S489–534, S489–609, and S489–688, their expected sizes without glycosylation were 66, 74, and 82 kDa, respectively. However, the observed sizes ranging from 80 to 160 kDa (Fig. 2, *C* and *D*) indicated the extensive glycosylation has taken place on these fusion proteins. Indeed, the ability to perform complex glycosylation has long been one of the advantages of the
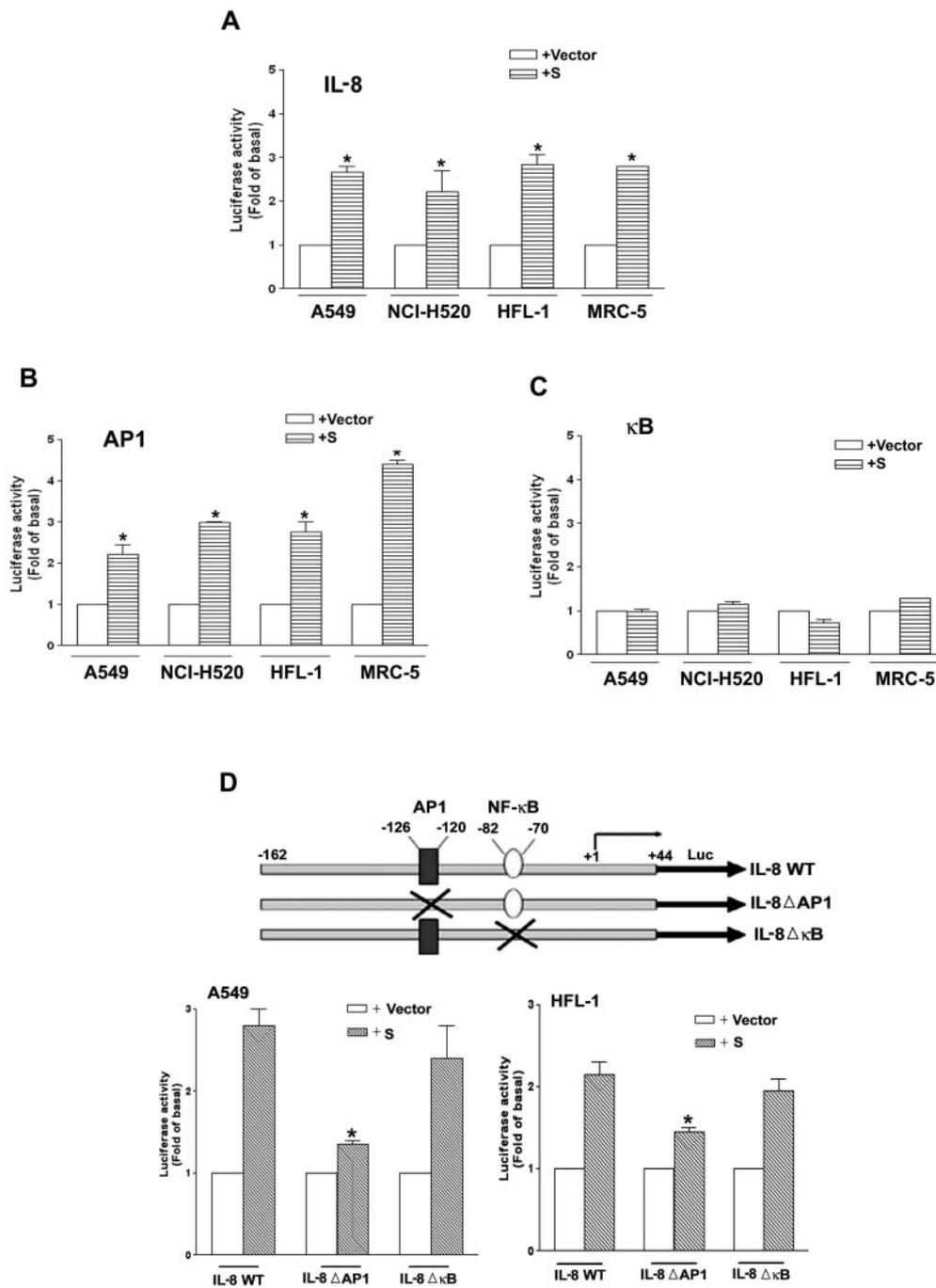
FIGURE 1. Effects of SARS-CoV S protein-encoding plasmid on IL-8 promoter, AP-1, and NF-κB activities in lung cells. A549, NCI-H520, HFL-1, or MRC-5 cells were cotransfected with IL-8 wt-Luc (*A*), AP-1-Luc (*B*), or NF-κB-Luc (*C*), as well as pcDNA3.1-S, or the empty vector. Luciferase activity was measured, as described under *Materials and Methods*. The results were normalized to the β-galactosidase activity and expressed as the mean ± SE of three independent experiments performed in triplicate. *, $p < 0.05$ compared with empty vector. *D*, *Upper diagram*, Schematic diagram of the 5′ regulatory region of the human IL-8 gene. A549 or HFL-1 cells were cotransfected with the IL-8 wt, IL-8ΔAP-1, or IL-8ΔκB luciferase expression vector as well as pcDNA3.1-S or the empty vector, and then the cell extracts were prepared and assayed for luciferase and β-galactosidase activity. The luciferase activity was normalized using the β-galactosidase activity and expressed as the mean ± SEM of three independent experiments performed in triplicate. *, $p < 0.05$ as compared with IL-8 wt.

baculovirus expression system. As the S protein belongs to the type I glycoprotein family, the increases in protein sizes were almost certainly resulting from glycosylation.

Using recombinant baculovirus vAtEpGS688 to study the effect of SARS-CoV S protein on the IL-8 release

To mimic the real situation in SARS-CoV infection, the recombinant baculoviruses were used for further study. The baculovirus

only infects insect, but not mammalian cells in normal condition. vAtE is the control baculovirus with *cmv* promoter to drive EGFP without S protein. This virus goes into the host cells with lower efficiency than those carrying the S protein. A recombinant baculovirus containing 688 aa of SARS-CoV S protein (vAtEpGS688) was used to examine the effect on the IL-8 release. vAtEpGS688 could enter the lung cells via the expression of S protein on viral surface. The entry of virus into the host cells was detected by
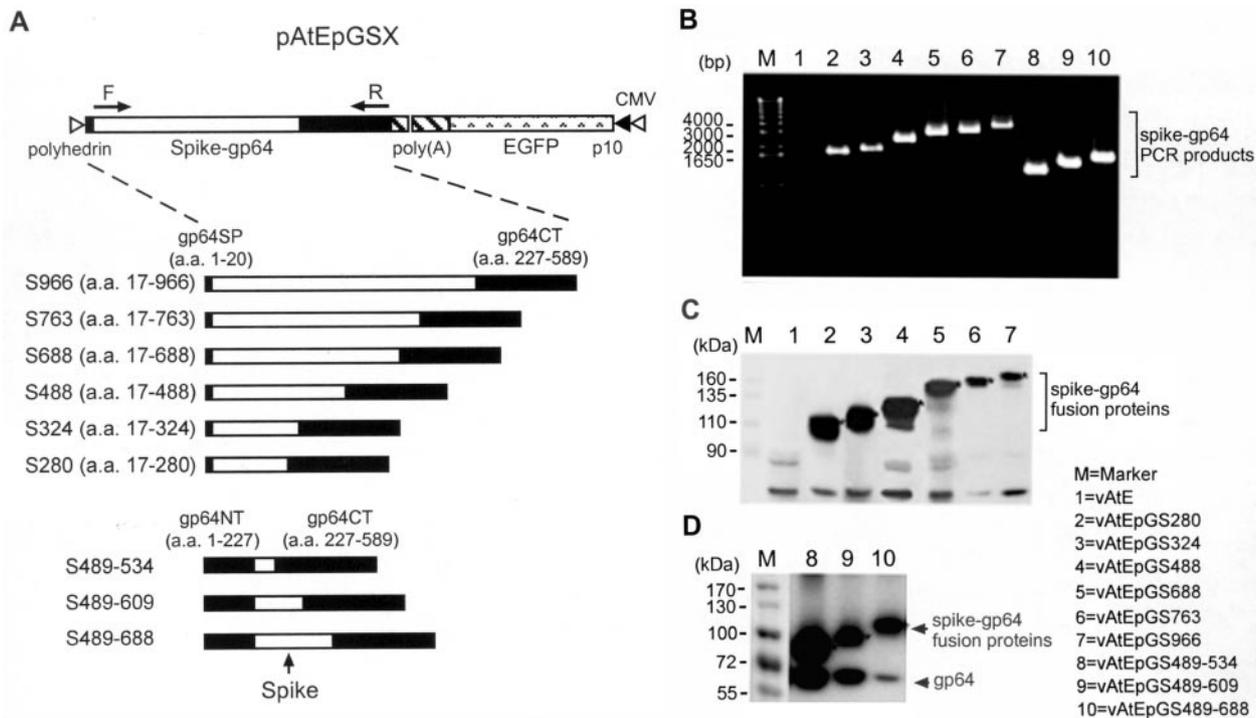
FIGURE 2. An illustration of the genetic organization of the pAtEpGSX plasmid and all the S-GP64 fusion protein fragments (*A*). The EGFP marker was driven by both *cmv* and *p10* promoters, and the S-GP64 fusion protein was produced under the *polyhedrin* promoter. The X in pAtEpGSX represents the length of the S insertion (e.g., pAtEpGS966 for the construct containing the S966 fragment). Two arrows, F and R, indicate the positions of the PCR primers used in *B*. *B*, Viral DNA samples were purified from the 10 recombinant baculoviruses and used as template in PCR using Spike F2 (deletion clones) or Spike F489 (insertion clones) and GPCR as the primers. *C* and *D*, Cell lysates from Sf-21 cells infected with these recombinant baculoviruses were analyzed by Western blotting using either anti-S or anti-GP64 Ab. A star indicates the position of the S-GP64 fusion protein in each lane.

EGFP fluorescence. As shown in Fig. 3, the successful entry of virus carrying the S688 fragment into the lung cells was demonstrated.

Up-regulation of IL-8 release by SARS-CoV S protein was demonstrated by ELISA. Either full-length S protein treatment, vAtEpGS688 transduction, or pcDNA3.1-S transfection can induce the IL-8 release in A549 cells. The expression of S protein after pcDNA3.1-S transfection was demonstrated by Western blot (Fig. 4*A*). A stronger capability of IL-8 release was induced by vAtEpGS688 transduction compared with S protein treatment and pcDNA3.1-S transfection (Fig. 4*A*). Therefore, vAtEpGS688 was most effective in inducing IL-8 release and used for further investigation of the intracellular signaling pathway. vAtEpGS688-induced IL-8 release in the lung cells was found at 24 h transduction and declined after 48 h (Fig. 4*B*). To further explore the time-course effect, vAtEpGS688 was found to increase the IL-8 release at 12 h transduction in A549 cells and at 8 h in HFL-1 cells, and

to reach maximum at 24 h (Fig. 4*C*). IL-8 mRNA expression was also increased at 3 h transduction (Fig. 4*D*), suggesting that IL-8 release was induced at the transcriptional level.

Role of ACE2 in vAtEpGS688-induced IL-8 release and localization of the functional domain of S protein

ACE2 was identified as a functional receptor for the SARS-CoV (10). VeroE6 cells showed abundant expression of ACE2, and all the lung cells also expressed ACE2 protein (Fig. 5*A*). The blocking effect of anti-ACE2 Ab on vAtEpGS688-induced IL-8 release was observed (Fig. 5*B*). To examine which domain on the S protein mediated IL-8 release in the lung cells, series of recombinant baculovirus clones with various deletions, including vAtEpGS280, vAtEpGS324, vAtEpGS488, vAtEpGS688, vAtEpGS763, and vAtEpGS966, were designed. The induction of IL-8 release was significant using vAtEpGS488, vAtEpGS688, vAtEpGS763, and vAtEpGS966. Dramatic drops of IL-8 release in A549 and HFL-1
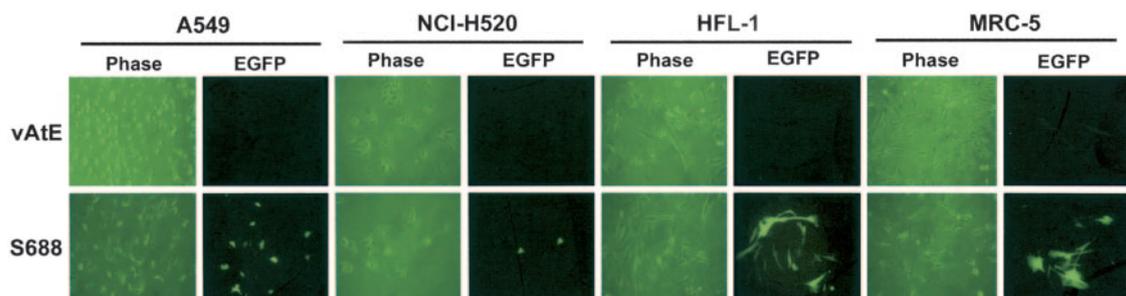

FIGURE 3. Induction of EGFP fluorescence by vAtEpGS688 transduction in lung cells. The cells transduced by vAtE or vAtEpGS688 (S688) and the induction of the EGFP fluorescence were shown at 24 h posttransduction in A549, NCI-H520, HFL-1, or MRC-5 cells. The control vAtE virus showed no EGFP fluorescence.
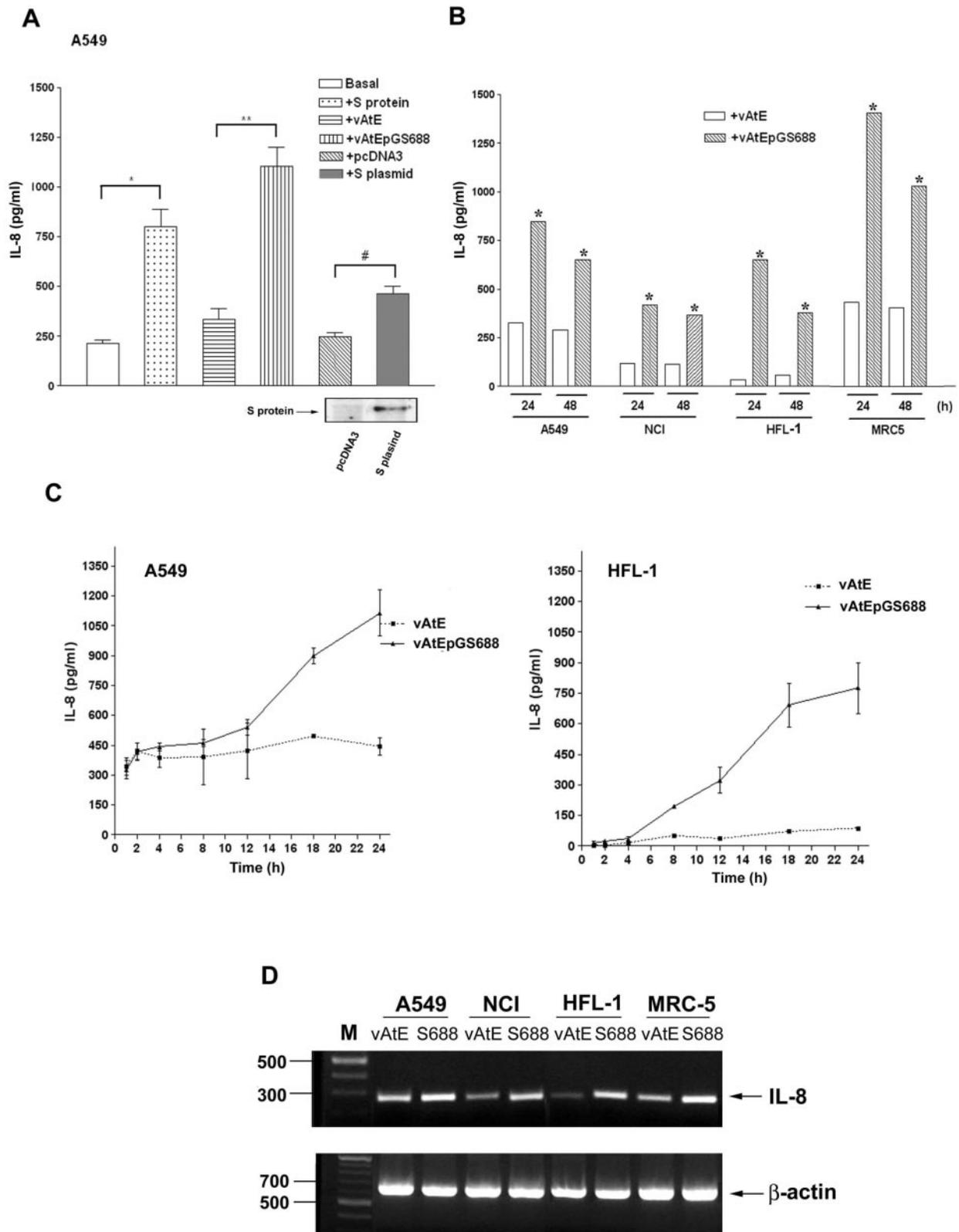
FIGURE 4. Comparison of IL-8 release by S protein, vAtEpGS688 transduction, and pcDNA3.1-S transfection, and the time course of vAtEpGS688-induced IL-8 release. *A*, A549 cells were treated with S protein (5 μg/ml), transduced by EG or vAtEpGS688 at an MOI of 50, or transfected with 2 μg of pcDNA3.1 or pcDNA3.1-S. The medium was collected from the cells following 24-h treatment, transduction, or transfection, and IL-8 levels were detected using ELISA kit. The results were expressed as the mean ± SE for three independent experiments performed in triplicate. Immunoblot of cell extracts from transfected A549 cells to detect the S protein level by Western blot using anti-S protein Ab. *, $p < 0.05$ compared with Basal. **, $p < 0.05$ compared with control vAtE. #, $p < 0.05$ compared with the empty vector. *B*, A549, NCI-H520, MRC-5, or HFL-1 cells were transduced with vAtE or vAtEpGS688 at an MOI of 50, and then the medium was collected from the cells at 24 or 48 h, and the IL-8 levels were detected. *C*, A549 or HFL-1 cells were transduced with vAtE or vAtEpGS688 at an MOI of 50, and then the medium was collected from the cells at the indicating time and the IL-8 levels were detected. *D*, A549, NCI-H520, MRC-5, or HFL-1 cells were transduced by vAtE or vAtEpGS688 (S688) at MOI of 50, and RNA was isolated at 3 h posttransduction. Total RNA (2 μg) was used for RT-PCR, as described under *Materials and Methods*.

FIGURE 5. Identification of ACE2 expression in lung cells and location of functional regions in SARS-CoV S protein mediating IL-8 release. *A*, The VeroE6 and lung cell lysates were prepared, and 50 μg of total proteins was subjected to Western blot analyses using anti-ACE2 Ab. *B*, Cells were preincubated with control IgG or anti-ACE2 Ab (8 μg/ml), and then transduced with vAtE or vAtEpGS688 (S688) at MOI of 50. The medium was collected at 24 h, and IL-8 levels were detected. The results were expressed as the mean ± SE for three independent experiments performed in triplicate. *, $p < 0.05$ compared with vAtE in the absence of anti-ACE2 Ab. **, $p < 0.05$ compared with vAtEpGS688. *C*, *Upper diagram*, Schematic diagram of the recombinant baculovirus-encoding series of deletion clones of SARS-CoV S protein, including vAtEpGS280, vAtEpGS324, vAtEpGS488, vAtEpGS688, vAtEpGS763, and vAtEpGS966. A549 or HFL-1 cells were transduced with vAtE or series of deletion clone at an MOI of 50, and then the medium was collected at 24 h and IL-8 levels were detected. The results were expressed as the mean ± SE for three independent experiments performed in triplicate. #, $p < 0.05$ compared with vAtE. *, $p < 0.05$ compared with vAtEpGS324, and **, $p < 0.05$ compared with vAtEGS488. *D*, *Upper diagram*, Schematic diagram of the recombinant baculovirus encoding various insert clones of SARS-CoV S protein, including vAtEpGS489–534, vAtEpGS489–609, and vAtEpGS489–688. A549 cells were transduced with vAtE at an MOI of 50, 100, or 150, or with vAtEpGS488, vAtEpGS688, vAtEpGS489–534, vAtEpGS489–609, or vAtEpGS489–688 at an MOI of 50, and then the medium was collected at 24 h and IL-8 levels were detected. The results were expressed as the mean ± SE of three independent experiments performed in triplicate. *, $p < 0.05$ compared with the basal.
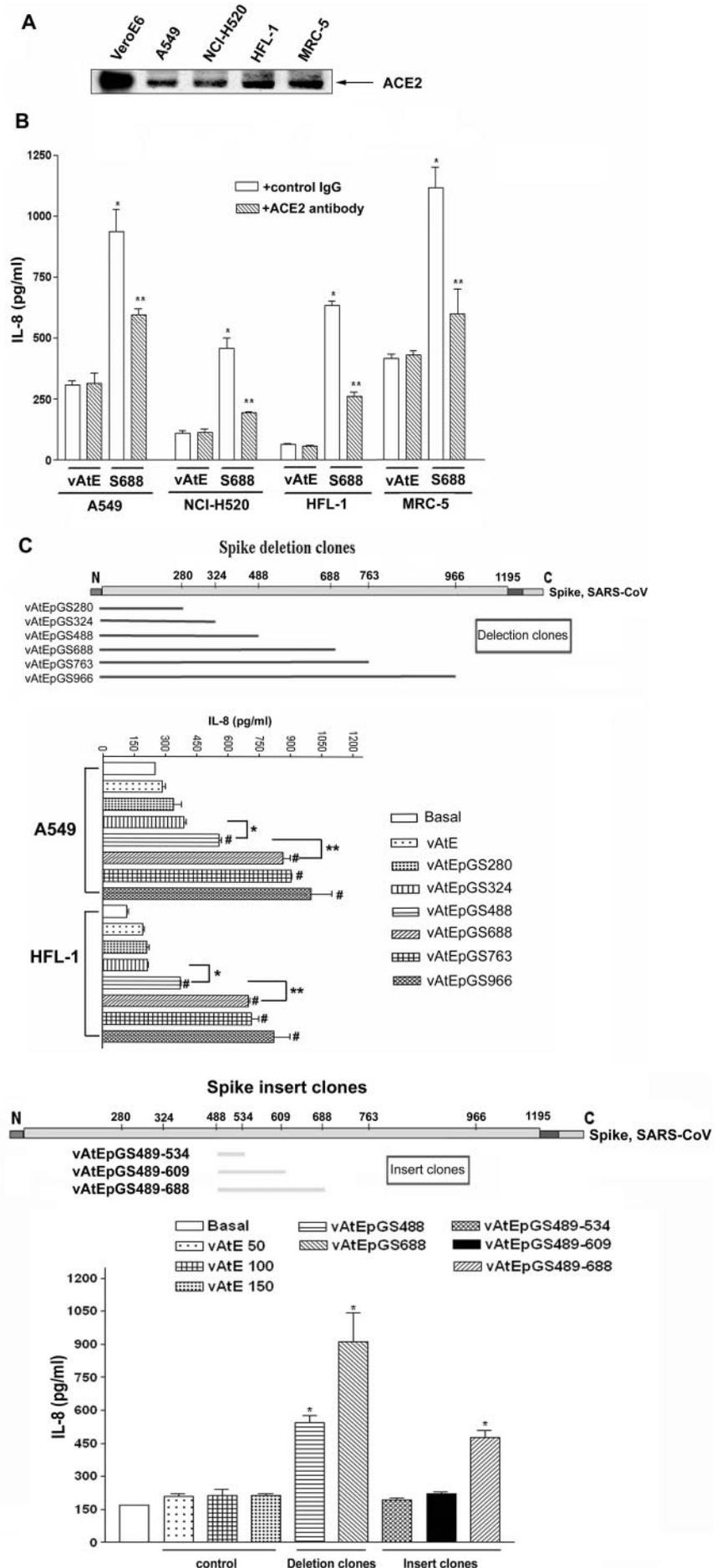
cells were seen by vAtEpGS488 transduction compared with vAtEpGS688, and by vAtEpGS324 transduction compared with vAtEpGS488 (Fig. 5C). These findings suggested that two regions of S protein, aa 324~488 and aa 489~688, mediated the induction of IL-8 release in the lung cells. Further examined by insert clones, the IL-8 release was induced by vAtEpGS489–688, but not vAtEpGS489–534 and vAtEpGS489–609 (Fig. 5D), indicating that aa 609–688 may be critical for the induction of IL-8 release.

At MOI of 50, the control baculovirus showed no entry into the lung cells (Fig. 3). When the MOI was increased to 100 and 150, successful entry of vAtE baculovirus was seen and no toxicity caused (data not shown). However, no significant increase in IL-8 release was induced by vAtE at MOI of 150. Previous report has shown that baculovirus treatment on hepatocytes at MOI of 1500 did not cause any toxic effect (21). Therefore, no toxic effect on the human lung cells is expected at MOI of 150. These results excluded the entry of baculovirus itself, inducing the IL-8 release.

vAtEpGS688 induced AP-1 DNA-protein complex formation

Because S protein-encoding plasmid induced IL-8 promoter activity and AP-1 activation, vAtEpGS688 transduction-induced AP-1 activation was examined by EMSA (Fig. 6). vAtEpGS688 increased AP-1 DNA-protein binding at 30 min posttransduction, and the activation was sustained to 120 min and declined at 240 min in A549 cells (Fig. 6Ab, lanes 2–5). No AP-1 DNA-protein binding was seen by vAtE transduction (Fig. 6Aa, lanes 2–5). To identify the specific subunits involved in the formation of the AP-1 complex, supershift assays were performed using Abs specific for anti-c-fos or anti-p65. Incubation of nuclear extracts with anti-c-fos Ab attenuated AP-1 DNA-protein binding (Fig. 6Ab, lane 7), but no attenuation occurred in the presence of anti-p65 Ab (Fig. 6Ab, lane 6). These results indicated the component of c-fos in AP-1 complex. Excess cold AP-1 probe blocked the AP-1 DNA-protein binding, and confirmed the specificity of this probe (Fig. 6Ab, lane 8). In HFL-1 cells, little AP-1 DNA-protein binding was induced by vAtE (Fig. 6Ba); however, vAtEpG688 significantly induced AP-1 DNA-protein binding at 30 min, and sustained to 240 min posttransduction (Fig. 6Bb, lanes 2–5).

Because AP-1 complex was demonstrated to contain c-fos, the effect of vAtEpGS688 transduction on c-fos mRNA expression in the lung cells was examined by RT-PCR. As shown in Fig. 6C, vAtEpGS688 induced increases in c-fos mRNA expression at 1 and 3 h transduction. Overexpression of c-fos increased AP-1 and IL-8 promoter activity in A549 cells, and S protein-induced activities were also augmented by the overexpression of c-fos (Fig. 6D).

vAtEpGS688 activates MAPKs and the inhibitors and DN mutants of MAPKs inhibit IL-8 promoter activity

The transcriptional activity of AP-1 has been reported to be regulated by MAPKs (22–27). Therefore, vAtEpGS688-induced MAPK activation was examined. In A549 cells, phosphorylations of ERK1/2 were evident at 30 min of vAtEpGS688 transduction. Both p38 and JNK were activated at 30 min, and maximal effects were seen at 60 min (Fig. 7A). Activations of ERK1/2 and p38 in HFL-1 cells by vAtEpGS688 were seen at 60 min and sustained to 240 min; however, JNK was not activated (Fig. 7B).

To assess the role of various MAPKs in S protein-induced IL-8 promoter activity, cells were transfected with pcDNA3.1-S following pretreatment with various MAPK pathway inhibitors. The MEK inhibitor PD98059, the p38 inhibitor SB308520, and the JNK inhibitor SP600125 all attenuated IL-8 promoter activity in A549 cells (Fig. 7C), which was also attenuated by cotransfection with the DN mutants of ERK2 (DN), p38 (DN), or JNK (DN) (Fig. 7D), suggesting the involvement of all three MAPKs in the SARS-

CoV S protein-induced IL-8 promoter activity in the lung epithelial cells. PD98059 and SB308520, but not SP600125, inhibited IL-8 promoter activity in HFL-1 cells (Fig. 7C), which was also attenuated by cotransfection with the DN mutants of ERK2 (DN) and p38 (DN), but not JNK (DN) (Fig. 7D), suggesting the involvement of ERK and p38, but not JNK, in the SARS-CoV S protein-induced IL-8 promoter activity in the lung fibroblasts.

Because pcDNA3.1-S could not induce NF-κB activation (Fig. 1C) and IL-8 promoter activity was not affected by the mutation of κB site (Fig. 1D), the role of NF-κB was further examined by measuring the degradation of IκBα. vAtEpGS688 transduction did not induce IκBα degradation (Fig. 7E), confirming that vAtEpGS688-induced IL-8 release was not dependent on NF-κB.

Discussion

SARS is a recently emerged infectious disease characterized by persistent fever, respiratory symptoms with lung consolidation, lymphopenia, and respiratory failure in life-threatening cases (28). It has been shown that overproductions of specific inflammatory cytokines and CC chemokine IL-8 are the hallmark of viral infection. SARS sequelae such as transendothelial migration of polymorphonuclear cells into the lung tissues, multiple organ dysfunction, and acute respiratory distress syndrome have been postulated to associate with cytokine and chemokine dysregulation (29). IL-8 has been shown to be elevated in blood and alveolar spaces (30), and exhibits a positive correlation with the number of polymorphonuclear cells in bronchoalveolar fluid of patients with pneumonia and acute respiratory distress syndrome (31). The elevations of IL-8 and cytokines have been found in the plasma of SARS-CoV-infected patients (14) and can induce the hyperinnate inflammatory response due to the SARS-CoV invasion of the respiratory tract. Accordingly, it is worthwhile to investigate the induction of chemokine, such as IL-8 in the lung cells, which may be the cause or consequence of pulmonary inflammation and immune hyperreactivity in SARS. Of all the SARS coat protein-encoding plasmids (S, E, and M) studied, only S protein induced activation of IL-8 promoter in the lung cells (data not shown, and Fig. 1).

Based on the studies of other CoV, the S glycoprotein is thought to be particularly important in the infectious process. First, it is the site for the virus to interact with the cognate receptor (32). Second, it has fusion activities (33). Third, it contains sites in which major neutralizing Abs are directed (34). This glycoprotein is therefore relevant to the ability of the virus to evade the host's immune system (35). Therefore, we aim to produce a recombinant baculovirus that mimics SARS-CoV in its host range and infection mechanism. As it was essential to produce a functional S protein on the viral surface, the strategy involved replacing just the receptor-binding region of its own envelope protein, GP64, with the receptor-binding region of SARS-CoV S protein. The remaining GP64 C terminus part of the fusion protein provided the fusion, oligomerization, and the transmembrane domains, which ensured the fusion proteins its proper timeric organization and surface localization on the recombinant baculoviruses. The SP of the S protein was also replaced by the GP64 equivalent, enabling the fusion protein to be directed onto the baculoviral membrane. At the end, a series of S protein fragments from S280 to S966 cloned on a baculovirus vector were used as a tool to dissect its functional domain in inducing IL-8 release. The effectiveness of the recombinant S-coated baculoviruses in inducing IL-8 response is a testimony to the successful conservation of the S protein structural architecture.

A protein in which the S1 domain (residues 12–672) of the SARS-CoV S protein fused to the Fc region of human IgG1 has been shown to associate with the ACE2-overexpressed 293T and
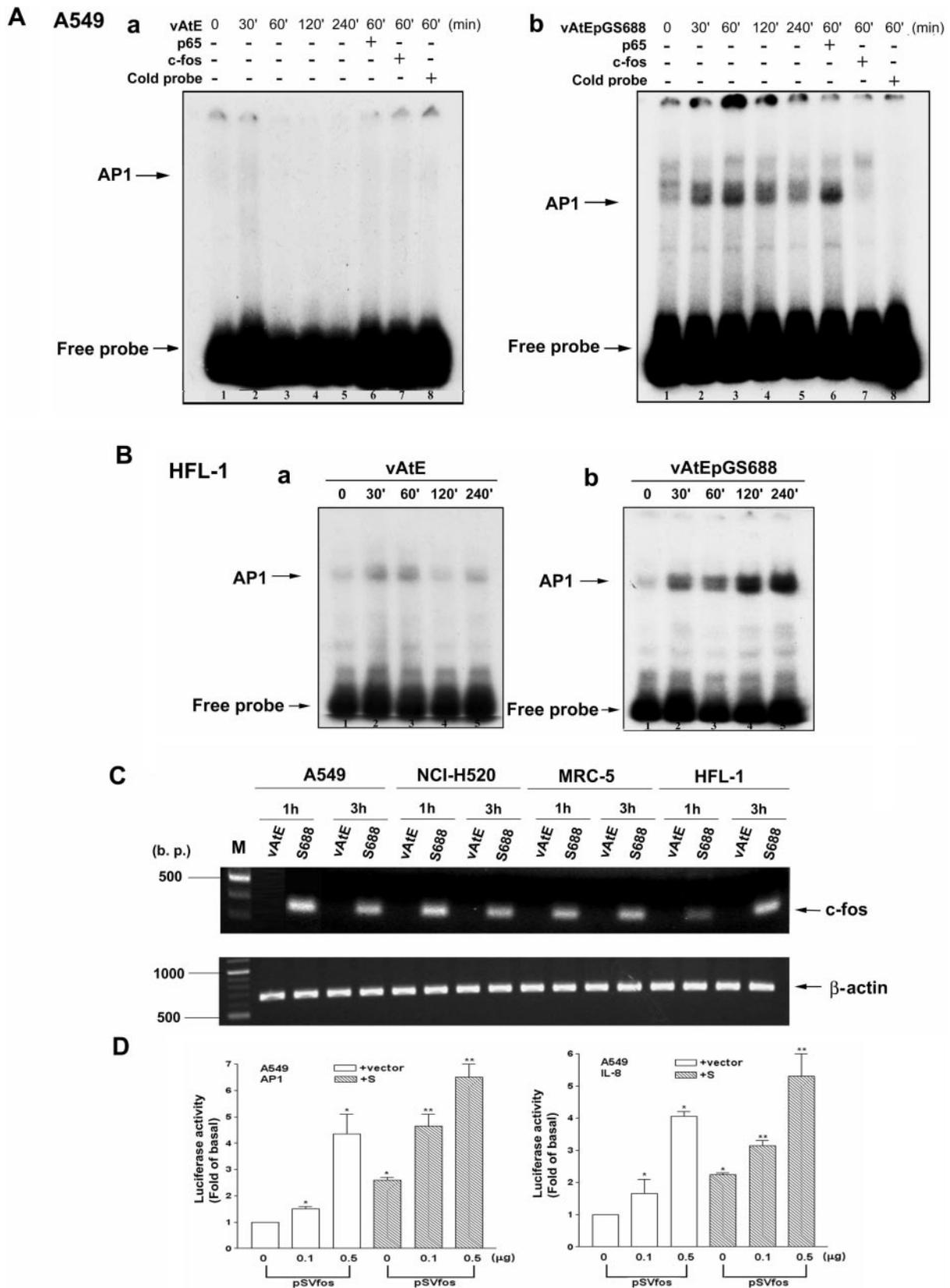
FIGURE 6. Kinetics of vAtEpGS688 transduction-induced AP-1 DNA-protein binding and c-*fos* mRNA expression in lung cells. *A* and *B*, A549 (*A*) or HFL-1 (*B*) cells were transduced with vAtE (*a*) or vAtEpGS688 (*b*) at MOI of 50 for the indicated time, and then nuclear extracts were prepared and AP-1 DNA-protein-binding activity was measured by EMSA. The supershift assays were performed using 2 μg of anti-p65 or anti-c-*fos* Ab, as described under *Materials and Methods*. *C*, A549, NCI-H520, MRC-5, or HFL-1 cells were transduced by vAtE or vAtEpGS688 (S688) at an MOI of 50, and RNA was isolated at 1 and 3 h posttransduction. Total RNA (2 μg) was used for RT-PCR, as described under *Materials and Methods*. *D*, A total of 0.1 or 0.5 μg of pSVfos plasmid was cotransfected with IL-8 wt-Luc or AP-1-Luc in the absence or presence of pcDNA3.1-S. Luciferase activity was measured, as described under *Materials and Methods*. The results were normalized to the β-galactosidase activity and expressed as the mean ± SE of three independent experiments performed in triplicate. ∗, $p < 0.05$ compared with the empty vector. ∗∗, $p < 0.05$ compared with equal amount of pcDNA3.1-S alone.
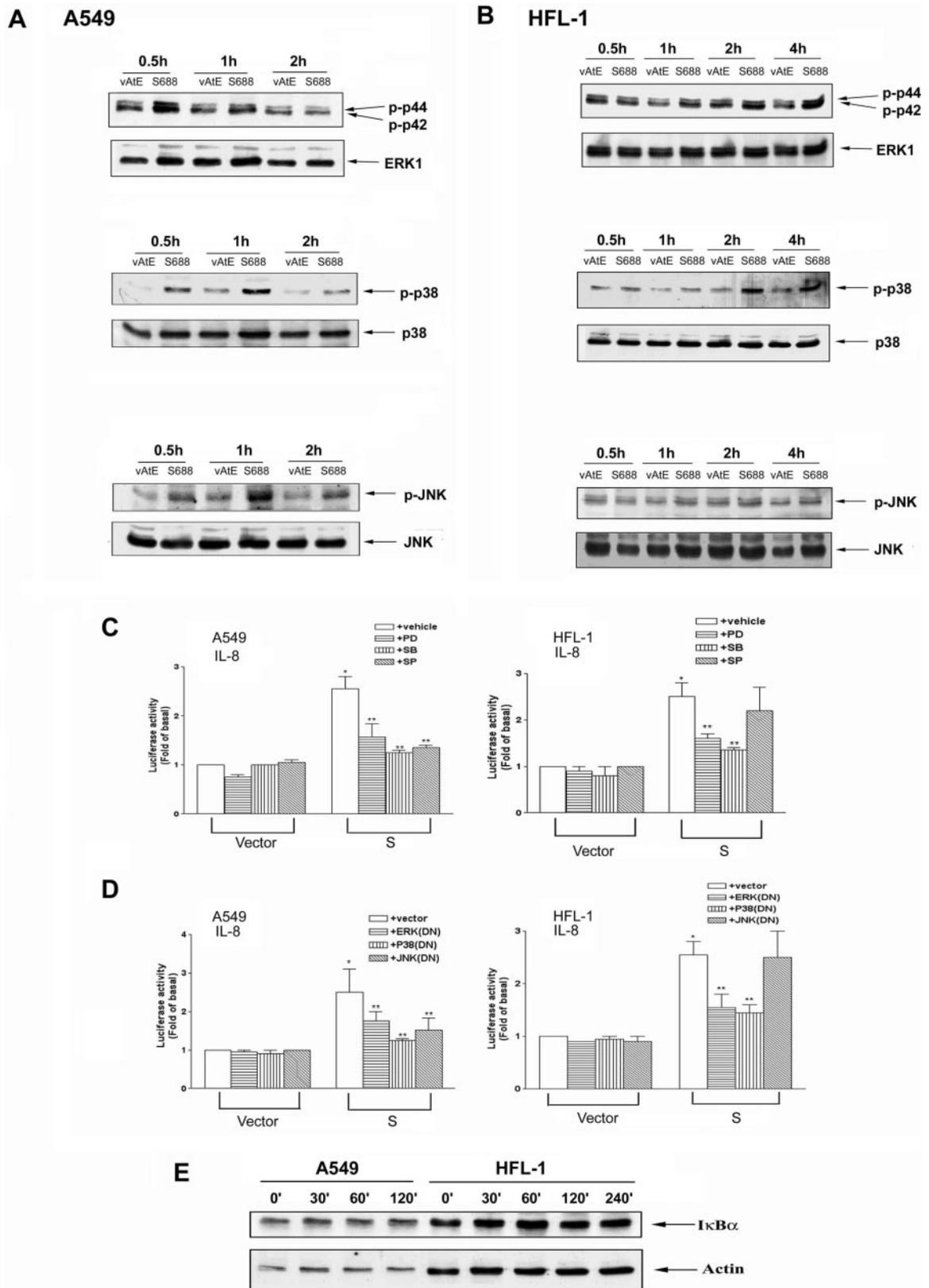
FIGURE 7. vAtEpGS688 transduction-induced MAPK activations in lung cells and effects of MAPK cascade inhibitors on pcDNA3.1-S-induced IL-8 promoter activity. A549 (*A*) or HFL-1 (*B*) cells were transduced with vAtE or vAtEpGS688 (S688) at an MOI of 50 for the indicated time. Cell lysates were prepared and subjected to Western blot analyses using phospho-specific MAPK Abs that recognize p-ERK, p-p38, or p-JNK. The expression levels of ERK, p38, or JNK were detected. *C* and *D*, A549 or HFL-1 cells transfected with IL-8 wt-Luc were pretreated with PD98059 (50 μM), SB203580 (30 μM), or SP600125 (30 μM) (*C*) or cotransfected with the DN mutants of ERK (DN), p38 (DN), or JNK (DN), or the respective empty vector (*D*). Luciferase activity was measured, as described under *Materials and Methods*. The results were normalized to the β-galactosidase activity and expressed as the mean $\pm$ SE of three independent experiments performed in triplicate. *, $p < 0.05$ compared with the empty vector. **, $p < 0.05$ compared with pcDNA3.1-S alone. *E*, A549 or HFL-1 cells were transduced with vAtEpGS688 (MOI:50) for the indicated time. Cell lysates were prepared and subjected to Western blot analyses using anti-IκBα or anti-actin Ab, as described under *Materials and Methods*.

ACE2-expressing Vero E6 cells, and to precipitate ACE2. These results demonstrated that ACE2 serves as a functional receptor for SARS-CoV (10). Our data showed that vAtEpGS688 induced IL-8 releases in the lung cells and these effects were blocked by the anti-ACE2 Ab, implying the existence of functional ACE2 receptors for SARS-CoV in the lung cells. Removal of residues 966–688 had no effect on IL-8 release. However, removal of residues 688–489 or 488–325 resulted in dramatic drops in IL-8 release. These results suggested that two separate regions, one aa 324–488, and the other aa 489–688, are essential for the induction of IL-8 release in the lung cells. To identify the functional domain mediating IL-8 release, the region S489–688 was inserted into the GP64 protein and expressed on the surface of baculovirus. This insert clone was sufficient for inducing the IL-8 response. Neither S489–534 nor S489–609 insert clone induced IL-8 release, suggesting that the critical region may be limited to a small area in CT aa 609–688. The possibility that baculovirus itself may induce IL-8 release was excluded by the facts that control vAtE at MOI of 150 that enters A549 cell successfully did not induce IL-8 release. This is the first report to find the increase of IL-8 release in lung cells by SARS-CoV S protein, although elevated plasma level of IL-8 in the early stage of SARS patients has been reported (14, 15, 36). Histological examination of lung biopsy from a patient of probable SARS by anti-IL-8 Ab hybridization revealed plenty of IL-8 in pulmonary tissue (National Taiwan University Hospital SARS research group, unpublished data).

A small fragment containing residues 318–510 retained ACE2 association and bounded ACE2 more efficiently than did the full-length S1 domain (11). This 193-aa peptide also more efficiently blocked S protein-mediated infection of ACE2-overexpressed 293T cells than did the full S1 domain. The ACE2 RBD located between residues 303 and 537 or 270 and 510 is also reported recently (12, 13). The functional region from residues 324 to 488 for IL-8 release in lung cells is within the reported ACE2 RBD; however, another region from residues 609 to 688 is not covered by this reported ACE2 RBD identified in Vero E6 and ACE2-overexpressed 293T cells (11, 12). This may be a functional region in S protein unidentified before. The results from our study and others (11, 12) suggested that ACE2 is also the receptor for S protein in the lung cells. Our study demonstrated that full-length S protein and recombinant baculovirus displaying S protein induced

much stronger IL-8 release than did S-expressing plasmid (Fig. 4A), suggesting that extracellular S protein may activate a cell surface receptor, possibly ACE2, which in turn switches on a downstream cascade of signal pathway to induce IL-8 release. Whether ACE2 is the receptor in lung cells to trigger IL-8 release remains to be further investigated. As presented in Fig. 8, we propose that the function region of S protein, located from residues 324 to 688, might act through ACE2 or unknown coreceptor(s) to induce IL-8 release in lung cells.

A sequence spanning nt −1 to −133 within the 5′ flanking region of the IL-8 gene is essential and sufficient for transcriptional regulation of the gene (37). Mutation and deletion analyses demonstrated that the promoter elements contain NF-κB, AP-1, and C/EBP binding sites (37–39). The promoter region is regulated in a cell type-specific fashion, requiring a NF-κB element plus either an AP-1 or a C/EBP element (40). For example, the AP-1 element along with the NF-κB site are sufficient for the full expression of this promoter in gastric cell lines (41), whereas in a fibrosarcoma cell line, only the C/EBP and NF-κB sites are required (42). The AP-1, but not NF-κB, is demonstrated to be required for the SARS-CoV S protein-stimulated IL-8 expression in lung cells. Supershift assays identified the component of c-*fos* in the AP-1 complex, and vAtEpGS688 transduction really induced c-*fos* mRNA expression in lung cells. Furthermore, overexpression of c-*fos* itself increased AP-1 and IL-8 promoter activity and augmented S protein-induced activity, indicating the important role of c-*fos* and AP-1 in the S protein-induced IL-8 release in lung cells.

A lot of studies reported that AP-1 activation leading to IL-8 release was regulated by the MAPKs. For example, both adenovirus type 7 and respiratory syncytial virus induced IL-8 production through the activation of ERK in A549 cells (22, 23), and the rhinovirus or TNF-α induced IL-8 production through the activation of p38 in BEAS-2B or rheumatoid fibroblast cells (24, 25). IL-1 induced IL-8 production through the activation of JNK in KB epithelial cells (26). In this study, our data demonstrated the activations of MAPKs as well as AP-1 by SARS-CoV S protein and their involvements in IL-8 production. This signaling pathway is probably related to ACE2 because the inhibition of IL-8 release by anti-ACE2 Ab was seen. MAPK is activated via phosphorylation of threonine and tyrosine residues by MAPK kinase (MKK) (43). The observation of protein interaction between MKK7 and ACE

FIGURE 8. Schematic representation of the signaling pathways involved in the SARS-CoV S protein-induced IL-8 release in lung cells. S protein acts through ACE2 or unknown coreceptor(s) to activate ERK1/2, p38, or JNK, leading to the activation of AP-1 on the IL-8 promoter, and initiation of IL-8 mRNA expression and protein release. Two functional domains aa 324–488 and 609–688 are essential for the induction of IL-8 release.
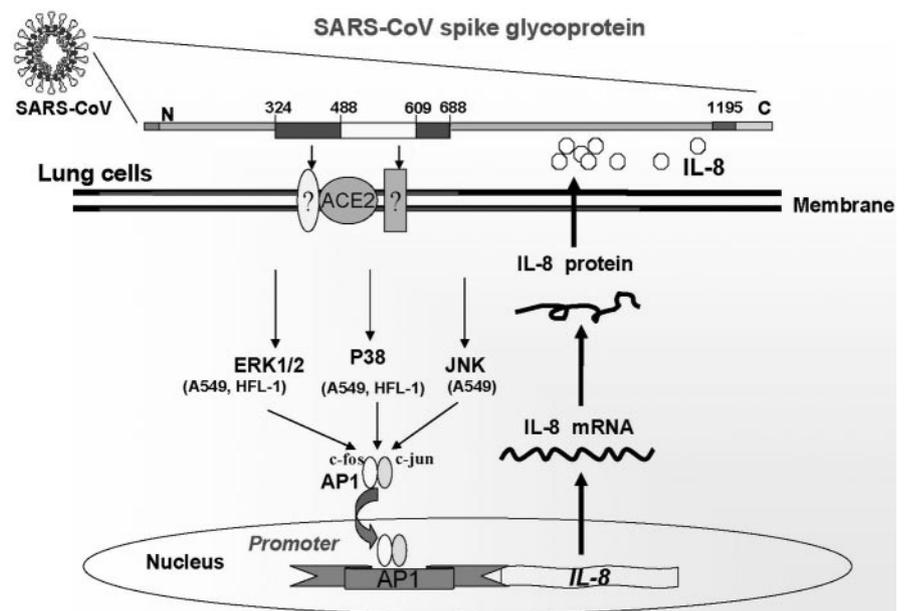
(another member in ACE family) suggested that activation of MKK7 is involved in ACE signaling. How ACE activates MAPKs remains unknown. ACE2 served as a functional receptor in cell surface, which was only reported recently (10, 11). However, its role in the intracellular signaling transduction has never been reported. The possibility that SARS-CoV S protein binds ACE2 and results in conformational changes leading to initiation of the intracellular signaling is not excluded. Although IL-8 promoter contains element for the binding of NF-κB, our results showed that neither NF-κB activation nor IκBα degradation was affected by S protein, indicating that the induction of IL-8 by the SARS-CoV S protein is not dependent on NF-κB.

In addition to S protein, we also found the activation of IL-8 promoter in lung cells by another structural N protein (data not shown). The biological function of CoV N protein is thought to participate in the replication and transcription of viral RNA and to interfere with the cell cycle of host cells (44, 45). The SARS-CoV nucleocapsid gene encodes a 50-kDa protein harboring a putative nuclear localization signal (KKDKKKK, aa 370–376). The N protein in many CoV is highly immunogenic and abundantly expressed during infection (46, 47). However, its intracellular effects on host gene regulation remain unknown. The cytoplasmic localization of SARS-CoV N protein is reported recently (48), suggesting that N protein may interact with unknown cytoplasmic and nuclear proteins to regulate host gene expression.

In summary, the present study is the first to provide the evidence that SARS-CoV could induce chemokine release in lung epithelial cells and fibroblasts via its S protein. We also identified the functional region of S protein from aa 324–688 (particularly the NT aa 324–488 and the CT aa 609–688) is responsible for IL-8 production in the lung cells. The NT region overlapped with the RBD of S protein-binding ACE2 reported recently, while CT region might be a new functional site. S protein triggers the activations of MAPKs and AP-1, and then initiates IL-8 release in lung cells. A schematic representation of the involvements of these molecules in the SARS-CoV S protein-induced IL-8 release is shown in Fig. 8. These may partly explain the clinical observation of dramatic cytokine storm and inflammation responses in SARS patients. These findings not only offer new tools to study the entry of the SARS virus into lung cells and localize the receptor binding or functional domain, but also could help in the development of novel vaccine immunogens and therapeutics for prevention and treatment of SARS.

Acknowledgment

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