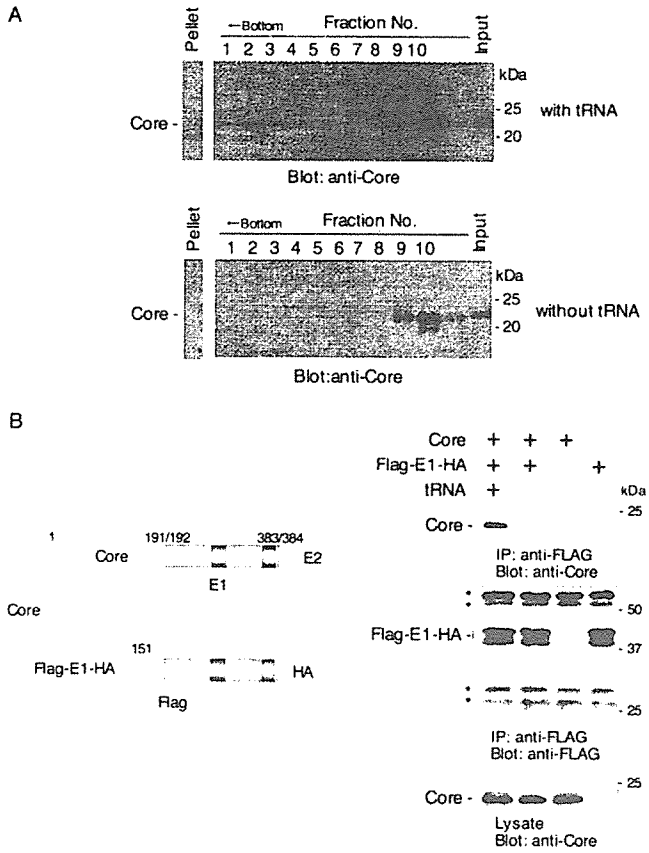

FIG. 3. HCV core protein binds to E1 protein in the presence of tRNA. (A) Cell lysates of 293T cells expressing HCV core protein were subjected to velocity sedimentation with a sucrose gradient in the presence or absence of tRNA. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA. (B, left) cDNAs used for expression. FLAG-E1-HA encodes FLAG tag after the signal peptide and HA tag after the transmembrane region. (Right) Immunoprecipitation analyses. Cell lysates of 293T cells expressing core and FLAG-E1-HA proteins were immunoprecipitated by anti-FLAG antibody in the presence or absence of tRNA. The asterisks indicate nonspecific bands.

was immunoprecipitated by using an anti-FLAG antibody. Coprecipitation of core protein with E1 was assessed by Western blot analysis using a core-specific monoclonal antibody. Although HCV core protein was clearly coprecipitated with FLAG-E1-HA in the presence of tRNA, little association was seen in the absence of tRNA (Fig. 3B, right). Nonspecific precipitation of the core protein with tRNA was not observed (data not shown). Although a small amount of the intracellular core protein may already associate with viral RNA under the intracellular conditions, a large amount of RNA may be required for oligomerization that is detectable by the sedimentation assay. Together, our results suggest that tRNA facilitates oligomerization of the HCV core protein and potentiates the interaction between the core protein and E1.

The region spanning amino acid residues 72 to 91 in the HCV core protein is crucial for binding to the E1 protein in yeast. The interaction between the HCV core and E1 proteins likely occurs on the cytosolic side of the cell membrane and, thus, presumably involves the posited cytoplasmic loop region in the polytopic form of the E1 glycoprotein. To investigate the possibility for this specific interaction in cells, core protein lacking the transmembrane region (Core1-173) was examined for interaction with the putative E1 cytoplasmic loop region in a yeast two-hybrid system (Table 1). When Core1-173 was expressed with the E1 cytoplasmic region (residues 288 to 346), the yeast was able to grow on the dropout plate lacking Trp, Leu, His, and Ade, suggesting that the core protein associates with the cytoplasmic loop of the E1 protein in yeast. To determine the region of the HCV core protein responsible for the interaction with the cytoplasmic domain of E1, deletion mutants of the core were tested. Association in the yeast two-hybrid system was seen with Core24-173, Core38-173, Core58-173, Core72-173, and Core1-151 mutants, but not with Core92-173 and Core1-25. Nonspecific interaction of the GAL4 activation domain with these core mutants was not observed. These results suggest that the region spanning from amino acid residues 72 to 91 in the HCV core protein is important for interaction with the cytoplasmic domain of the E1 protein in yeast.

Amino acid residues 72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein in mammalian cells. To examine the involvement of amino acid residues 72 to 91 of the HCV core protein in the interaction with the E1 protein in mammalian cells, FLAG-E1-HA was coexpressed with either a wild-type core or a deletion mutant lacking amino acid residues 72 to 91 (CoreΔ72-91) in 293T cells (Fig. 4A). Cell lysates were incubated with yeast tRNA, and FLAG-E1-HA was immunoprecipitated with anti-FLAG antibody. As shown in Fig. 4B (left), only the wild-type core protein, but not CoreΔ72-91, coprecipitated with E1. Self-oligomerization was also prevented by the deletion in CoreΔ72-91 (Fig. 4B, right). These results suggest that amino acid residues 72 to 91 in the HCV core protein play a crucial role in the interaction with the E1 protein and oligomerization of the core protein.

The E1 cytoplasmic domain interacts with the core protein in mammalian cells and inhibits the interaction with intact E1 protein in *trans*. To assess the involvement of the E1 cytoplasmic region in the interaction with core protein in mammalian

TABLE 1. Interaction between the core and the E1 cytoplasmic region in yeast

Bait	Growth with prey[a]			
	E1 cytoplasmic loop		No insert	
	Dropout	Control	Dropout	Control
Core1-173	+	+	−	+
Core24-173	+	+	−	+
Core38-173	+	+	−	+
Core58-173	+	+	−	+
Core72-173	+	+	−	+
Core92-173	−	+	−	+
Core1-151	+	+	−	+
Core1-25	−	+	−	+
No insert	−	+	−	+

[a] HCV core mutants were expressed as fusion proteins with the DNA binding region by using a bait plasmid. The HCV E1 cytoplasm region was expressed as a fusion protein with an activation domain by using a prey plasmid. Yeast growth was observed in dropout plates lacking Trp, Leu, Ade, and His (dropout) or plates lacking Trp and Leu (control). +, growth; −, no growth.

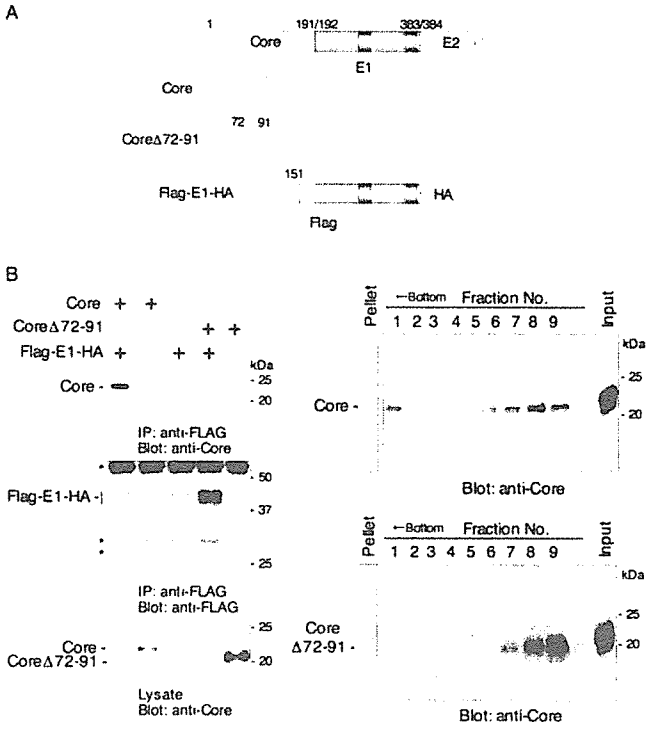

FIG. 4. Amino acid residues72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein. (A) cDNAs used for expression. CoreΔ72-91 is an HCV core protein carrying a deletion of amino acid residues 72 to 91. (B, left) FLAG-E1-HA was coexpressed in 293T cells with either a wild-type core or CoreΔ72-91, and the interaction was analyzed by immunoprecipitation in the presence of tRNA. The asterisks indicate nonspecific bands. (Right) Oligomerization of a wild-type core or CoreΔ72-91 in the presence of tRNA. Wild-type core protein was self-oligomerized, but CoreΔ72-91 was not.

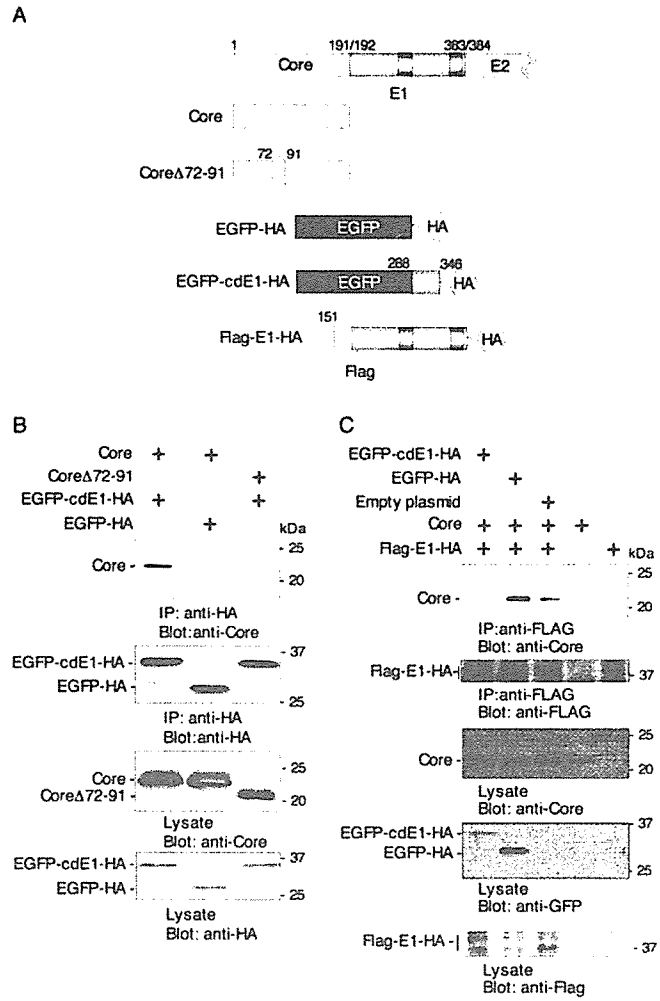

FIG. 5. Interaction of the E1 cytoplasmic loop with the core protein. (A) cDNAs used for expression. EGFP-cdE1-HA is an EGFP fusion protein carrying the E1 cytoplasmic region of amino acid residues 288 to 346 followed by an HA tag. (B) Wild-type core or CoreΔ72-91 was coexpressed with EGFP-cdE1-HA in 293T cells, and their interaction was analyzed by immunoprecipitation. EGFP-cdE1-HA coprecipitated with wild-type core protein, but not with CoreΔ72-91. (C) Inhibition of the interaction of the core protein with FLAG-E1-HA by expression of EGFP-cdE1-HA. Expression of EGFP-cdE1-HA but not of EGFP disrupted the interaction between core and E1 proteins.

cells, we constructed an enhanced green fluorescent protein (EGFP) fusion protein carrying the E1 cytoplasmic domain followed by an HA tag (EGFP-cdE1-HA) (Fig. 5A). Upon coexpression of EGFP-cdE1-HA with the wild-type core protein in 293T cells, the two proteins could be coprecipitated using anti-HA antibody (Fig. 5B). The mutant CoreΔ72-91 protein was unable to associate with EGFP-cdE1-HA (Fig. 5B). Together, these studies demonstrate that the cytoplasmic loop region of E1 is able to interact with the core protein and that core residues 72 to 91 are required for this association.

To further confirm the specificity of the interaction of the E1 cytoplasmic region with the core protein, we examined the ability of the EGFP-cdE1-HA protein to inhibit the association of the intact E1 protein (in Flag-E1-HA) with the wild-type core protein (Fig. 5C). Expression of EGFP-cdE1-HA but not EGFP-HA competed strongly with the interaction between core and the FLAG-tagged Flag-E1-HA protein. These results suggest that the cytoplasmic loop in the intact E1 glycoprotein can directly bind to HCV core protein. Interestingly, the EGFP-cdE1-HA protein was unable to inhibit this interaction in the context of the intact core and E1 and E2 polyproteins (data not shown), suggesting that expression of the core and E1 proteins in *cis* may prevent subsequent interaction with E1 expressed in *trans*.

Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes revealed that the region from Gln^{302} to Pro^{328} is highly conserved (Fig. 6A). To determine residues in the E1 cytoplasmic region that are critical for interaction with the core protein, blocks of four residues each in the conserved region were replaced with Ala in the polyprotein (core, E1, E2, and p7) (Fig. 6A). These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody; coprecipitated E1 protein was detected by immunoblotting using an anti-E1 monoclonal antibody (Fig. 6B). The replacement of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1

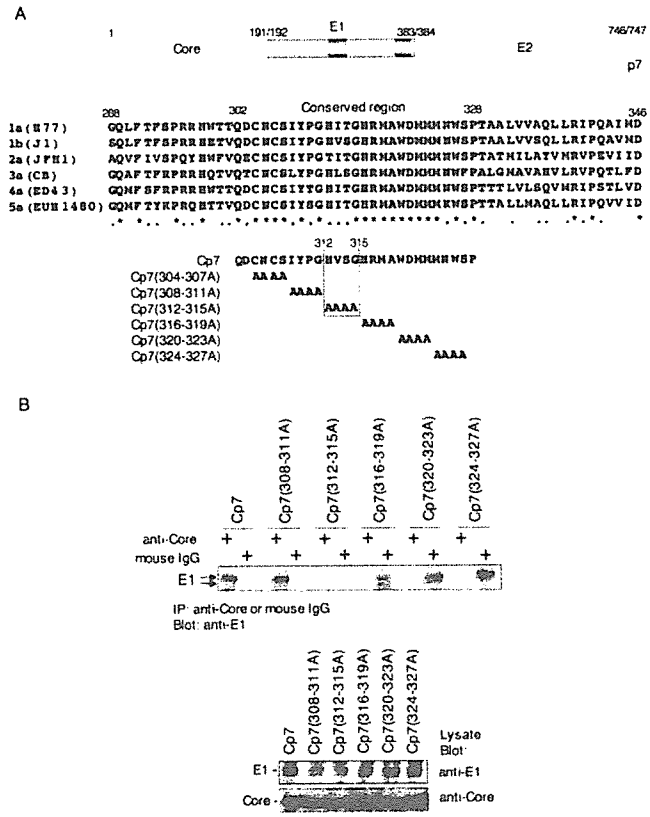

FIG. 6. Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. (A) Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes (1a, H77 [AF009606]; 1b, J1 [D89815]; 2a, JFH1 [AB047639]; 3a, CB [AF046866]; 4a, ED43 [Y11604]; 5a, EUH1480 [Y13184]). A conserved region from Gln^{302} to Pro^{328} is shown by gray shading. Mutant polyproteins consisting of the core, E1, E2, and p7 proteins with four residues each replaced by Ala in the conserved E1 region were constructed. Four amino acid residues, His^{312}, Val^{313}, Ser^{314}, and Gly^{315}, in the E1 cytoplasmic region of strain J1 and substitution of the amino acids with Ala in Cp7 (312-315A) are indicated by the box. (B) These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody or nonspecific mouse IgG in the presence of $MgCl_2$ and tRNA. The E1 protein that coprecipitated with the core protein was detected by immunoblotting. The substitution of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1 protein, Cp7 (304-307A), could not be examined due to the low level of expression.

protein could not be examined due to a low level of expression (data not shown). Among the mutant constructs examined, only the substitution at residues 312 to 315, Cp7 (312-315A), markedly diminished association with the core protein (Fig. 6B). These results suggest that this region in the E1 cytoplasmic domain of the J1 strain of HCV (His^{312}, Val^{313}, Ser^{314}, and Gly^{315}) is important for interaction with the core protein.

DISCUSSION

The biogenesis of the transmembrane glycoproteins involves a series of coordinated translation and membrane integration events that are directed by topogenic determinants within the nascent chains and that ultimately lead to the most favored topology for any given polypeptide (24). However, there is an increasing number of examples of glycoproteins that can assume multiple topological orientations. The large envelope protein of the hepatitis B virus, for instance, has been suggested to adopt distinct topologies that enable the protein to serve in virus assembly as a matrix-like protein and in virus entry as a receptor binding protein (22). An unglycosylated form of the HCV E2 protein has been identified and shown to interact with protein kinase R in the cytosol (45). In Newcastle disease virus, type I and polytopic forms of the fusion protein are present in the same cell, and the polytopic form is suggested to be involved in the membrane fusion event (31).

HCV glycoproteins E1 and E2 were shown to possess transmembrane domains and associate to form noncovalent heterodimers that are statically retained in the ER membrane upon recombinant expression (10, 29, 46). Previously, the E1 protein of genotype 1a was suggested to possess a single C-terminal transmembrane domain, based in part on its utilization of potential glycosylation sites (33) and on a model of the transmembrane domains of the E1 and E2 proteins, in which the C terminus reorients, upon signal peptidase cleavage, from the ER lumen to protrude slightly into the cytoplasm (7). In our study, we have suggested that the E1 protein can also adopt a polytopic topology in which the protein spans the ER membrane twice and includes an intervening cytoplasmic region. In this model, the membrane orientation of the C-terminal transmembrane region is inverted and translocation of the signal peptidase-cleaved C terminus is not required.

Our analysis revealed that the 305 mutant of the 1b genotype expressed by transfection exhibited a single band of 32 kDa, whereas that of genotype 1a expressed by recombinant vaccinia viruses has been reported to contain two bands (33). Although we do not know the reason for this discrepancy, it may relate to differences in the expression systems. HCV proteins expressed by vaccinia virus and Sindbis virus vectors formed disulfide-linked aggregates (9, 11, 34), and coexpression of a large amount of vaccinia viral proteins also may alter the proper processing of the expressed proteins, as suggested by Merola et al. (32). However, further work will be necessary to clarify the reasons for the differences in glycosylation patterns of E1 mutants obtained in the different expression systems.

Mottola et al. analyzed the determinants for ER localization of the E1 protein and showed that the juxtamembrane region of E1, between amino acid residues 290 and 333, was required for ER retention (41). This region lies within the ectodomain of the E1 protein in the type I topology and in the cytoplasmic region of the protein in the proposed polytopic form. ER localization determinants of transmembrane proteins have in general been located either in the cytosolic or in the transmembrane domain, not in the luminal ectodomain, except for the yeast Sec20 protein (41). Therefore, assignment of the ER localization signal to the cytoplasmic region of the E1 protein might further support the possibility of the polytopic topology model. Affinity purification and membrane reconstitution of the E1 protein carrying an affinity tag (S-peptide) in the putative cytoplasmic region are also consistent with this model (35). Together, these findings provide indirect support that the E1 glycoprotein can adopt a polytopic form.

As previously reported (20), oligomerization of the HCV core protein to form nucleocapsid-like particles requires the presence of stem-loop RNA structures, such as those in tRNA.

Here, we have demonstrated that self-assembly of the core protein occurs without envelope protein in the presence of tRNA and that tRNA is required for the association of E1 glycoprotein with the core protein, suggesting that oligomerization of the core protein may be a prerequisite for this interaction during virus assembly. Based on hydrophobicity and the clustering of basic amino acids, the HCV core protein is proposed to possess three domains: the N-terminal basic and hydrophilic region (domain 1; residues 1 to 118), a central basic and hydrophobic domain (domain 2; residues 119 to 174), and the hydrophobic signal sequence for E1 (domain 3; residues 175 to 191) (14). Biophysical characterization of the core protein indicated that the C-terminal residues 125 to 179 were critical for the folding and oligomerization of the core protein (21). Although our mutant HCV polyprotein containing Ala substitutions at residues 312 to 315 in the cytoplasmic region of the E1 protein exhibited a clear reduction in its interaction with the core protein, a substantial amount of residual binding was retained. These results suggest that regions other than the residues from 312 to 315 in the E1 protein are also involved in the interaction with the core protein.

In Semliki Forest virus, the cytoplasmic domain of the E2 glycoprotein, which corresponds to the E1 protein in HCV, has been shown to interact with the capsid protein (26, 49). Assembly of alphaviruses has also been found to require the specific interaction between the C-terminal cytoplasmic domain of the E2 protein and the capsid protein (17). Although the functional significance of the two forms of the HCV E1 protein is still unclear, the E1 cytoplasmic region among different HCV genotypes is well conserved and four amino acid residues, His^{312}, Val^{313}, Ser^{314}, and Gly^{315} of strain J1, were shown to be important for interaction with the core protein. Although the four amino acid sequences identified in strain J1 of genotype 1b are not strictly conserved among the different HCV genotypes (Fig. 6A), a pattern of polar-hydrophobic-polar-glycine residues can be discerned in all of them. The interaction of the cytoplasmic E1 protein with the core protein may indicate that the polytopic form is a mature E1 protein that is incorporated into virions.

In conclusion, the polytopic topology model of the HCV E1 protein and the interaction of oligomerized core protein with the cytoplasmic region of the E1 protein may provide clues to aid in understanding the biosynthesis and assembly of the HCV structural proteins. HCV core protein is also involved in the development of liver steatosis, type II diabetes mellitus, and hepatocellular carcinoma in transgenic mice (39, 40, 48). A detailed knowledge of the assembly of HCV particles will provide the basis for the development of effective therapeutics for chronic hepatitis C.

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8 Vaccines for severe acute respiratory syndrome

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Abstract

The SARS-coronavirus (SARS-CoV), which affected over 8000 individuals worldwide and was responsible for over 700 deaths in the 2002–2003 outbreak, caused acute respiratory distress and was highly lethal when it first emerged in 2002. Although public health measures eventually controlled the spread of SARS, the global impact of the epidemic was severe. Given concerns about future outbreaks and a lack of effective treatments for the virus, global research efforts have focused on the development of vaccines against SARS. Treatment with convalescent plasma has achieved some success, suggesting that induction of passive immunity might be useful to combat SARS. The inactivated SARS-CoV vaccine will likely be the first available vaccine against SARS

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since it is relatively easy to produce. However, there are serious concerns about the safety of this vaccine. The spike protein of SARS-CoV has been identified as the major inducer of neutralizing antibodies, while a receptor-binding domain of the S1 subunit of SARS-CoV S protein contains multiple conformational neutralizing epitopes. This suggests that recombinant proteins containing fragments of the S protein might be used to develop safe and effective SARS vaccines. Viral-vector vaccines and DNA vaccines are also an attractive option for SARS vaccines.

Introduction

A new disease called severe acute respiratory syndrome (SARS) originated in China in late 2002 and spread rapidly throughout a number of countries (1). Clinically, SARS is characterized by high fevers, malaise, rigors, headache, dry cough, and progression to interstitial infiltration of the lungs, with an eventual mortality exceeding 10% in many countries. Laboratory findings include lymphopenia, and chest radiographs commonly exhibit unilateral or bilateral infiltrates (2). Following the WHO alert, a number of probable SARS cases were reported in other regions of China, as well as other Asian countries, including Singapore, Taiwan, Indonesia, Thailand, and the Philippines. International travel facilitated its spread to other continents. Before the initial epidemic ended, 8,098 probable cases of SARS, and 774 associated deaths, were reported to the WHO (www.cdc.gov/mmwr/mguide_sars.html). At the time of the outbreak, a global collaborative network to combat SARS was co-ordinated by the WHO. As a result of this international effort, a novel type of coronavirus (SARS-CoV) was identified as the etiologic agent of SARS (3)(4) in March 2003. The genomic sequence of SARS-CoV was completed and we now know that SARS-CoV has all the characteristic features of a coronavirus, but it is quite different from all previously known coronaviruses (group I~III), representing a new group of coronavirus (group IV) (5)(6). SARS-CoV is thought to be a mutant coronavirus transmitted from a wild animal, which has developed the ability to infect humans (5)(7). The SARS-CoV genome is a single-stranded positive-sense RNA sequence, approximately 30 kb in length, containing five major open reading frames encoding non-structural replicase polyproteins and structural proteins. These include spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, which are encoded in much the same order and are of similar size to the structural proteins of other coronaviruses (Fig.1). The S protein is involved in receptor binding, fusion, and entry, and is a major inducer of neutralizing antibodies, while the E protein plays a role in viral assembly, the M protein in virus budding, and the N protein in viral RNA packaging (for review, see reference (7)). In a model of MHV infection, S proteins are known to contain important virus-neutralizing epitopes that elicit neutralizing antibody responses in mice (8). The S protein

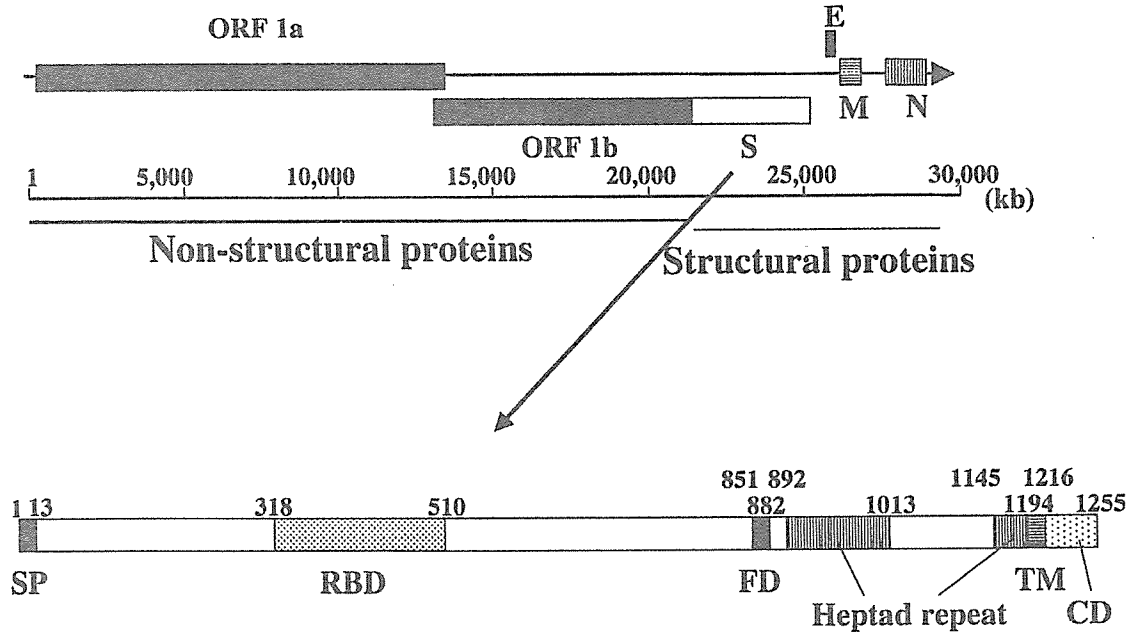

Figure 1. Genome organization and the structure of spike glycoprotein of SARS-CoV. Upper panel: S; spike glycoprotein, E; envelope protein, M, membrane glycoprotein; N, nucleocapsid protein. Lower panel: SP, signal peptide; RBD, receptor binding domain; FD, fusion domain; TM, transmembrane domain; CD, cytoplasm domain. The residue numbers of each region correspond to their amino acid positions in the S protein.

of SARS-CoV consists of a signal peptide and three domains: an extracellular domain, a transmembrane domain, and an intracellular domain. The extracellular domain consists of two subunits: S1 and S2 (7). The S1 subunit is responsible for binding of the virus to its receptor, angiotensin-converting enzyme 2 (ACE2) (9). A fragment located in the middle region of the S1 subunit is the receptor-binding domain (RBD) for ACE2 (10)(11)(12). The S2 subunit, which contains a putative fusion peptide and two heptad repeats (HR1 and HR2), is responsible for fusion between the virus and target cell membranes (Fig.1)(13). This indicates that the S protein may have potential use as a vaccine, resulting in the production of antibodies which prevent virus binding and fusion.

Since the initial outbreak, global research efforts have focused on developing effective vaccines against SARS. This review summarizes the progress made to date with regard to SARS vaccines.

Passive immunization

Treatment with convalescent plasma has been successfully used to treat SARS, suggesting that passive immunity might be a useful approach by which to combat SARS. (14) Subbarao *et al.* (15) have shown that passive transfer of murine neutralizing antibodies can prevent replication of SARS coronavirus in

the respiratory tract. Traggiai *et al.* (16) have isolated human monoclonal antibodies from immortalized B lymphocytes of people recovering from SARS. These antibodies have been shown to neutralize the virus *in vitro*, and to prevent viral replication in a mouse model of SARS-coronavirus infection. Since human serum containing antibodies to SARS-CoV is not available in sufficient amounts, neutralizing antibodies are thought to be a good alterative for passive immunization. Sui *et al.* have investigated the antiviral activity of a human monoclonal antibody to S1 protein that blocks receptor association (17), demonstrating the prophylactic effectiveness of this monoclonal antibody *in vivo* using a mouse model of SARS. (18) Alternatively, in China, pure concentrated human SARS hyperimmunoglobulins for intravenous injection have been prepared from pooled convalescent plasma samples, which are awaiting evaluation. ter Meulen *et al.* (19) have shown that prophylactic administration of a human IgG monoclonal antibody capable of reacting with whole inactivated SARS coronavirus is able to reduce SARS coronavirus replication within the lungs of infected ferrets, as well as prevent the development of SARS-coronavirus-induced macroscopic lung pathology and prevent viral shedding in pharyngeal secretions.

Active immunization

A number of groups are working on vaccines using inactivated SARS-CoV, recombinant SARS-CoV proteins and subunits, viral-vector vaccines and recombinant DNA vaccines.

Inactivated vaccines

Several reports have shown that inactivation of SARS-CoV with formaldehyde, UV light, and β-propiolactone can induce virus-neutralizing antibodies in immunized animals (20)(21)(22)(23)(24). In fact, the first inactivated SARS-CoV vaccine is being tested in clinical trials in China. However, there are serious concerns about the safety of this inactivated vaccine, and there is concern that production workers are at risk of infection during handling of the concentrated live SARS-CoV. Incomplete virus inactivation may cause SARS outbreaks among vaccinated populations (25). Takasuka *et al.* (26) have immunized mice with UV-inactivated SARS-CoV with or without adjuvant, and have shown that UV-inactivated SARS-CoV elicits marked humoral immunity, producing sustained antibody secretion and the production of memory B cells. They have also shown that UV-inactivated virions induce regional lymph node T cell-proliferation and marked cytokine production (IL-2, IL-4, IL-5, IFN-γ and TNF-α) following re-stimulation *in vitro*. He *et al.* (21) have demonstrated that SARS-CoV inactivated by beta-propiolactone elicits high titers of antibodies capable of recognizing the S

protein in immunized mice and rabbits. They have also shown that mouse and rabbit antiserum significantly inhibits S protein-mediated virus entry, suggesting that inactivated SARS vaccines might induce potent neutralizing antibodies capable of preventing SARS-CoV entry. Several other groups have also suggested that inactivated SARS-CoV virions might be useful. Kong *et al.* (27) have evaluated immune responses following exposure to different combinations of priming and boosting with DNA, adenoviruses, as well as inactivated viral vaccines. The ability to boost gene-based vaccines with adjuvanted inactivated virus shows clear enhancement of the CD4 and antibody responses. CD8 responses are not similarly enhanced by boosting.

Protein and/or subunit vaccines

At the present time, SARS-CoV S glycoprotein and related fragments are thought to be good candidates for the development of recombinant vaccines (Fig.1)(17)(22)(28)(29)(30)(31)(32). The N-terminal region of SARS-CoV S protein (S1) contains a putative RBD that is responsible for cell attachment (11)(31). He *et al.* (21) have shown that a recombinant fusion protein containing a 193-amino acid RBD (residues 318-510) and a human IgG1 Fc fragment induces potent antibody responses in immunized rabbits. These antibodies recognize the RBD of the S1 domain and completely inhibit SARS-CoV infection at a serum dilution of 1:10,240. Rabbit antiserum has been shown to effectively block binding of S1 to ACE2. van den Brink *et al.* have shown that a human monoclonal antibody specific for the RBD of SARS-CoV strain FM1 can effectively bind to the RBDs of most SARS-CoV strains (33). This suggests that antibodies directed against the RBD of a SARS-CoV isolate might neutralize infection by a broad spectrum of SARS-CoV strains. Therefore, recombinant proteins containing RBD or vectors encoding RBD might be used as vaccines for preventing infection by SARS-CoV with distinct genotypes. Recently, Pogrebnyak *et al.* (34) have demonstrated successful expression of the N-terminal region of the SARS-CoV S1 protein in transgenic plants, sufficient to induce an antibody response in mice fed the transgenic material. Mice parenterally primed with this plant-derived antigen have been observed to develop an immune response following booster immunization. This method has a number of practical, economic, and safety advantages over conventional systems for the production and delivery of subunit vaccines.

Ho *et al.* (35), Mortola and Roy (36), and Huang *et al.* (37), have all reported the formation of virus-like particles (VLPs) following the expression of several structural proteins of SARS-CoV using a baculovirus expression system or plasmid transfection of mammalian cells. These findings further advance our understanding of the morphogenesis of SARS-CoV and enable the generation of safe, conformational mimetics of the SARS-CoV that may facilitate the development of vaccines.

Viral-vector vaccines

Gao *et al.* (38) have constructed recombinant forms of adenovirus expressing the SARS-CoV S protein S1 fragment, M protein, and N protein. Intramuscular vaccination with all three recombinant adenoviruses on days 0 and 28 induces broad, virus-specific immunity in rhesus macaques. In their research, six vaccinated macaques demonstrated antibody responses against S protein, as well as T-cell responses against N protein. SARS-CoV specific neutralizing antibodies are detected in blood samples from all vaccinated animals *in vitro*. Bisht *et al.* (29) have constructed recombinant forms of a highly attenuated modified vaccinia virus Ankara (MVA) containing a gene encoding full-length SARS-CoV S protein. Expression of S protein alone after intranasal or intramuscular injection of recombinant MVA raises neutralizing antibody titers. Furthermore, four weeks after a second immunization, challenge of vaccinated and control animals with SARS-CoV shows reduced titers of SARS-CoV in the respiratory tracts of vaccinated animals. SARS-CoV replication in naive mice is reduced upon transfer of serum from mice immunized with recombinant MVA to naive mice. These findings suggest that MVA-based vaccines are potentially useful in the fight against SARS. Our group has constructed a series of recombinant DIs, a highly attenuated vaccinia strain (39), expressing a gene encoding four structural proteins (E, M, N and S), expressed individually or simultaneously. These recombinant DIs elicit the production of SARS-CoV-specific serum IgG antibodies and T-cell responses in vaccinated mice following intranasal or subcutaneous administration. Mice that have been subcutaneously vaccinated with recombinant DIs expressing E, M, N and S proteins combined demonstrate the greatest titers of serum neutralizing IgG antibodies and marked protective immunity against SARS-CoV challenge in the absence of a mucosal IgA response. This indicates that the potent immune response elicited by subcutaneous injection of recombinant DIs expressing S in combination with other membrane components (E, M, and N), may be capable of preventing mucosal infection by SARS-CoV (40). On the other hand, Weingartl's group have found that ferrets (*Mustela putorius furo*) immunized with recombinant rMVA expressing SARS-CoV S protein develop more rapid and vigorous neutralizing antibody responses, compared to control animals following challenge with SARS-CoV. However, marked inflammatory responses in the liver are also observed, suggesting that vaccination with recombinant MVA expressing SARS-CoV S protein may induce hepatitis (41)(42). This information is extremely important for development of safe SARS vaccines. Irregardless, extra caution should be taken when human trials of SARS vaccines are initiated given the potential for liver damage as a result of immunization, as well as the potential for inadvertent viral infection. Bukreyev *et al.* have constructed a recombinant attenuated parainfluenza virus (PIV) expressing SARS-CoV S protein, and

have immunized monkeys with a single dose of this recombinant PIV administered via the respiratory tract. Neutralizing serum antibodies can be detected in all vaccinated animals. Following SARS-CoV challenge, viral shedding is observed in control animals but not vaccinated monkeys (30). These results suggest that a vector mucosal vaccine expressing SARS-CoV S protein alone might be highly effective as a single-dose vaccine against SARS. This same group of researchers have further investigated the contribution of each structural protein to protective immunity by expressing them both individually and in combination using a recombinant PIV vector (32). Immunization with recombinant PIV expressing S provides complete protection against SARS-CoV challenge in the lower respiratory tract and partial protection in the upper respiratory tract. This suggests that S protein is the only antigen capable of inducing the production of neutralizing antibodies and subsequent protection against SARS-CoV infection. Faber *et al.* (43) have constructed recombinant rabies viruses (RVs) expressing SARS-CoV N or S proteins, and have studied their immunogenicity in mice. A single inoculation with recombinant RV expressing SARS-CoV S protein induces a marked SARS-CoV-neutralizing antibody response. On the other hand, antibodies against SARS-CoV N protein are not observed following a single inoculation with SARS-CoV N protein-expressing recombinant RV, probably because a single inoculation with RV primarily induces antibodies against a surface G protein, and not against internal RV proteins.

DNA vaccines

Yang *et al.* (28) have shown that a DNA vaccine encoding the S glycoprotein of SARS-CoV induces T cell and neutralizing antibody responses, as well as protective immunity, in a mouse model. Alternative forms of S protein have been analyzed by DNA immunization. DNA expression vectors induce a robust immune response mediated by CD4 and CD8 cells. Furthermore, neutralizing antibodies are observed in mice vaccinated with DNA expression vectors encoding a form of S with a preserved transmembrane domain. Viral replication is reduced by more than six orders of magnitude in the lungs of mice vaccinated with S plasmid DNA expression vectors, and protection is mediated by a humoral, rather than a T-cell-dependent, immune mechanism. Wang *et al.* (44) have produced marked titers of neutralizing antibodies using DNA vaccines encoding full-length S protein or S protein segments in rabbits. They have demonstrated major and minor neutralizing epitopes within the S1 and S2 subunits, respectively. These findings suggest that DNA vaccines based on the S glycoprotein effectively induce immune responses leading to protective immunity against SARS in animal models.

Kim *et al.* (45) have shown that DNA vaccination with antigen linked to calreticulin dramatically enhances major histocompatibility complex class I presentation of linked antigen to CD8 T cells. Thus, they have employed this method to create effective DNA vaccines using SARS-CoV N protein as the target antigen. Vaccination with naked CRT/N DNA generates the most potent N-specific humoral and T-cell-mediated immune responses in vaccinated C57BL/6 mice, compared to all other DNA constructs tested. Mice vaccinated with calreticulin /N DNA have significantly reduced viral titers of N protein-expressing SARS-CoV following challenge with SARS-CoV. Zhu *et al.* (46) have shown that immunization of mice with N-based DNA vaccines leads to N-specific antibody production and CTL activity. Zhao *et al.* (47) have also performed DNA immunization with the full length N gene and have shown that the N protein of SARS-CoV is an important B cell immunogen and that it can elicit broad-based cellular immune responses. Jin *et al.* (48) have performed a DNA vaccine experiment using a chemical adjuvant to show that E, M, and N gene constructs can induce high levels of specific antibodies, T cell proliferation, and IFN-c and DTH responses, along with *in vivo* cytotoxic T cell activity specifically directed against SARS-CoV antigens. The most marked immune responses occur following immunization with constructs encoding the nucleocapsid protein. Okada *et al.* (49) have demonstrated that SARS (M) DNA and (N) DNA vaccines induce SARS-CoV-specific CTL activity and T cell proliferation in mouse models and humans *in vivo* using SCID-PBL/hu.

Conclusions

Convalescent serum can be used to prevent the spread of SARS, as demonstrated in Hong Kong in 2003 (14). However, the use of convalescent serum is limited to those with symptoms at present. Investigators have drawn on their experiences with animal coronavirus vaccines to examine a number of different strategies for vaccine generation, including the use of inactivated viruses, purified protein, DNA, viral vectors and virus-like particles. An inactivated vaccine has been developed over a very short time period, however, there are a number of concerns regarding the safety of this inactivated vaccine (25). S protein has been demonstrated to be essential for inducing neutralizing antibodies against SARS-CoV since vaccines based on S protein induce neutralizing antibody responses. The N protein of SARS-CoV is also a potential target antigen for vaccine development, in particular, to induce cellular immunity. To achieve maximal protective immunity, immunogens used to develop vaccines should resemble the pathogenic virus themselves and be non-pathogenic. Purified VLPs of SARS-CoV closely fit this criteria and thus, provide attractive candidates for the development of a SARS vaccine (35) (36)(37). Attenuated strains of SARS-CoV may also be used, however, there is

no report of such kind of studies until now. It is also important to develop means to test the efficacy of various vaccines, since animal models to do so are lacking at the moment. Although there have been no reports of SARS in 2004 and 2005, it is strongly felt that SARS-CoV persists in an unidentified animal reservoir, such that the resurgence of SARS is still a serious threat. Therefore, SARS vaccines capable of eliciting potent neutralizing antibody responses and subsequent protection against infection and transmission are still likely to be developed.

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Mechanisms of establishment of persistent SARS-CoV-infected cells

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Abstract

Previously, we reported the establishment of cells with persistent SARS-CoV infection after apoptotic events and showed that both JNK and PI3K/Akt signaling pathways are important for persistence by treatment with inhibitors at the early stages of SARS-CoV infection. However, the mechanisms of establishment of persistent infection are still unclear. In this study, we investigated which signaling pathways play important roles in escape from apoptosis in cells infected with SARS-CoV. In persistently infected cells at 50 h.p.i., PI3K/Akt, JNK, p38 MAPK and Bcl-2 were phosphorylated and the protein levels of Bcl-2 and Bcl-xL were increased. When surviving cells were treated with the JNK-specific inhibitor, SP600125, at 50 h.p.i., all cells died, suggesting that the JNK signaling pathway is necessary for maintenance of persistently infected cells. Among the signaling pathways in persistently infected cells, Akt and JNK were phosphorylated in SARS-CoV-nucleocapsid (N) protein-expressing Vero E6 cells using vaccinia viral vector (DIs), strongly suggesting that N protein-induced phosphorylation of Akt and JNK are necessary to establish persistence. These results indicated that at least four proteins, Akt, JNK, Bcl-2 and Bcl-xL, are necessary for survival of persistently SARS-CoV-infected cells.

Keywords: SARS; JNK; Bcl-2; Bcl-xL; Nucleocapsid protein

Severe acute respiratory syndrome (SARS) is a newly discovered infectious disease with atypical pneumonia caused by SARS coronavirus (SARS-CoV). SARS became a global health threat due to its rapid transmission and high fatality rate [1,2].

Vero E6 is a cell line derived from African green monkey kidney cells and is sensitive to SARS-CoV. Many laboratories use this cell line to study SARS-CoV. Infection of Vero E6 cells with SARS-CoV induces apoptosis *via* activation of caspase-3 [3]. Akt and mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal protein kinase (JNK), extracellular signal-related kinase (ERK) 1/2, and p38 MAPK, are phosphorylated in SARS-CoV-infected Vero E6 cells [3–5]. Especially, activation of p38 and inactivation of Akt by SARS-CoV infection induce cytopathic effects and apoptosis in virus-infected cells, respectively. Phosphorylation of p38 MAPK is known to regulate signal transducer and activator of transcription 3 and 90 kDa ribosomal S6 kinases [5,6]. Although the majority of virus-infected cells die by apoptosis, we found that a small population of virus-infected cells remained alive and these cells grew with production of virus [7]. Four groups, including ours, independently reported persistent infection of cultured cells by SARS-CoV [8–10]. We found that JNK and PI3K/Akt signaling pathways are important for the establishment of persistent SARS-CoV infection in Vero E6 cells when these specific inhibitors were added soon after viral adsorption.

In this study, we further analyzed the mechanisms of establishment of persistent SARS-CoV infection in Vero

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E6 cells. We found that anti-apoptotic proteins, Bcl-2 and Bcl-xL, are important for persistent infection. Nucleocapsid (N) protein of SARS-CoV was suggested to play important roles in phosphorylation of Akt and JNK.

Materials and methods

Cells and virus. Vero E6 cells were subcultured routinely in 75-cm^3 flasks in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 0.2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) fetal bovine serum (FBS), and maintained at 37 °C in an atmosphere of 5% CO_2. The medium was changed to 2% FBS DMEM before virus infection. SARS-CoV, which was isolated as Frankfurt 1 and kindly provided by Dr. J. Ziebuhr, was used in the present study. Infection was usually performed at a multiplicity of infection (m.o.i.) of 5. DIs-N expressing N RNA of SARS-CoV and DIs-GFP expressing GFP RNA were described in our previous study [11]. Confluent Vero E6 cells were infected with DIs-N and -GFP at 5 m.o.i.

Fixing and staining of cells. The cells in 24-well plates were fixed with 10% formaldehyde for at least 24 h, and stained with 0.1% naphthol blue-black for 30 min. This convenient method was described by Everitt and Wohlfart for determination of the actual or relative number of cells in anchorage culture [12]. After washing out with water, the plates were scanned with a GT-9400UF scanner (Epson, Tokyo, Japan). The dye–protein complexes were released hydrolytically with 0.1 M NaOH and measured spectrophotometrically at 660 nm. When cells and the supernatant contained infectious SARS-CoV, the cell number was counted using this method. The number of cells that did not contain SARS-CoV was counted using the WST-1 cell proliferation assay system (Takara, Shiga, Japan).

Inhibitors. The JNK inhibitor, SP600125, and PI3K/Akt inhibitor, LY294002, were purchased from Calbiochem (San Diego, CA, USA) and Cell Signaling Technology Inc. (Beverly, MA, USA), respectively. These inhibitors were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. The same volume of DMSO alone was used as a control.

Western blotting. The whole-cell extracts were electrophoresed on 5–20% gradient polyacrylamide gels, and transferred electrophoretically onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA). In the present study, we applied two sets of samples to polyacrylamide gels, and the membranes were divided into two halves after blotting, or membranes were examined once using a LumiGLO Elite chemiluminescent system (Kirkegaard and Perry Laboratories, Gaithersburg, ML, USA), and then stripped using Restore Western blot stripping buffer (Pierce, Rockford, IL, USA) for the second detection. The following antibodies, obtained from Cell Signaling Technology Inc., were used in the present study at a dilution of 1:1000: rabbit anti-phospho Akt (Ser473) antibody, rabbit anti-Akt antibody, rabbit anti-phospho ERK (Thr202/Tyr204) antibody, rabbit anti-ERK antibody, rabbit anti-p38 MAPK (Thr180/Tyr182) antibody, rabbit anti-p38 MAPK antibody, rabbit anti-phospho SAPK/JNK (Thr183/Tyr185) antibody, rabbit anti-SAPK/JNK antibody, rabbit anti-phospho Bcl-2 (Ser 70) antibody, and anti-Bcl-xL antibody. Mouse anti-Bcl-2 antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA) and used at a dilution of 1:500. Mouse anti-β-actin antibody was purchased from Sigma and used at a dilution of 1:5000. Rabbit anti-SARS Nucleocapsid protein antibody was described previously [3].

Results

Phosphorylation of signaling pathways in cells persistently infected by SARS-CoV

As indicated in our previous studies, apoptotic signals, cleaved caspase-3 and DNA fragmentation, are detected at 18 and 24 h post-infection (h.p.i.) in SARS-CoV-infected Vero E6 cells [3]. At 24 h.p.i., cells begin to show rounding and persistently infected cells are observed after 48 h.p.i. To investigate which signaling pathways are phosphorylated in persistently SARS-CoV-infected cells, protein samples were obtained from these cells at 50 h.p.i. Vero E6 cells were prepared at confluency in T-25 flasks with 2% fetal bovine serum (FBS) containing Dulbecco's modified Eagle's medium (DMEM), and infected with SARS-CoV at 5 m.o.i. On the other hand, Vero E6 cells were prepared in T-25 flasks at several concentrations with 2% FBS containing DMEM as controls because surviving cell number is different (less than 5% of total cells) in each experiment. At 50 h.p.i., surviving cells and controls were washed with 2% FBS containing DMEM 5 times (with pipetting 25 times). Although most dead cells were washed out, a fraction of dead cells were attached to surviving cells and could not be removed completely by washing. As phosphorylation status of signaling pathways sometimes changes following trypsinization and centrifugation, sample buffer for Western blotting analysis was added directly to the washed cells. We obtained a protein sample from mock-infected cells, with a similar cell number to persistently infected cells. The protein samples seemed to contain a maximum of 50% proteins from surviving cells. Western blotting analysis was performed using antibodies to phosphorylated proteins of signaling pathways. As shown in Fig. 1, Akt, JNK, and p38 MAPK were phosphorylated in surviving cells. On the other hand, Akt was phosphorylated in control cells, while JNK and p38 MAPK were not. As Akt was dephosphorylated in both confluent and subconfluent cells after 18 h.p.i. as shown in our previous studies [4,13], detection of strongly phosphorylated Akt was

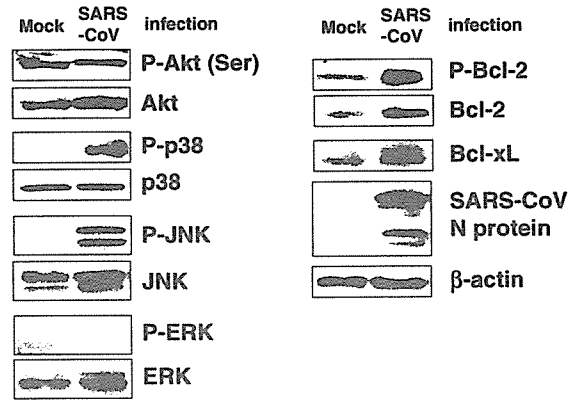

Fig. 1. Phosphorylation status of signaling pathways in persistently SARS-CoV-infected cells. Vero E6 cells were prepared at confluency in T-25 flasks with 2% fetal bovine serum (FBS) containing Dulbecco's modified Eagle's medium (DMEM), and infected with SARS-CoV at 5 m.o.i. At 50 h.p.i., surviving cells and controls were washed 5 times with 2% FCS containing DMEM (pipetting a total of 25 times). Mock-infected subconfluent cells, similar in number to surviving cells that had escaped from apoptosis by SARS-CoV infection, were also washed in the same manner. Western blotting analysis was performed using these protein samples.

suggested to reflect a feature of surviving cells that had escaped from cell death. In addition, the phosphorylated Akt in surviving cells indicated anti-apoptotic activity. The levels of the anti-apoptotic proteins, Bcl-2 and Bcl-xL, and phosphorylated Bcl-2, were also increased in surviving cells. The anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-xL, play important roles in inhibiting mitochondria-dependent cell death pathways [14]. This result suggested that Akt and JNK are important to establish persistent infection as indicated in our previous study, and that Bcl-xL and Bcl-2 are important for survival. Interestingly, p38 MAPK was strongly phosphorylated in surviving cells, suggesting that persistently infected cells consist of a balance between cell death and survival.

Importance of PI3K/Akt and JNK for establishment of persistently virus-infected cells

In our previous study, we showed that treatment of Vero E6 cells with the JNK inhibitor, SP600125, and PI3K/Akt inhibitor, LY294002, 1 h after inoculation with SARS-CoV prevented persistent SARS-CoV infection [7]. Therefore, we concluded that activation of JNK and PI3K/Akt by SARS-CoV infection is important for the establishment of persistence. To investigate whether these inhibitors affect the establishment of viral persistence in cells at the late stage of SARS-CoV infection, cells were treated with inhibitors at 50 h.p.i. As shown in Fig. 2A and B, SP600125 killed the cells completely, while LY294002 did not. As LY294002 has an inhibitory effect on cell proliferation, as indicated in Fig. 2C and in our previous study [13], the growth rate of persistently infected cells was slow. On the other hand, treatment with SP600125 in the absence of SARS-CoV infection did not affect cell proliferation. As Akt in virus-infected cells was dephosphorylated after 18 h.p.i., as shown in our previous studies [4,13], phosphorylation of Akt at the early stage of infection may be important for preventing apoptosis. However, this result strongly suggested that once persistence is established, phosphorylation of Akt is not necessary for survival. Therefore, we concluded that activation of PI3K/Akt is essential for the establishment of persistent infection with SARS-CoV at time points before cell death, whereas activation of JNK is required at the time of establishment of persistence.

Phosphorylation of signaling pathways by SARS-CoV-nucleocapsid protein

Next, we investigated which signaling pathways are phosphorylated by nucleocapsid (N) protein of SARS-CoV because several reports indicated that expression of N protein induces phosphorylation of signaling pathways. Surjit et al. reported that ERK, phosphorylated Akt and Bcl-2 are down-regulated, whereas JNK, p38 MAPK activation, activated caspase-3 and -7 are up-regulated in COS-1 cells in the absence of growth factors [15]. They

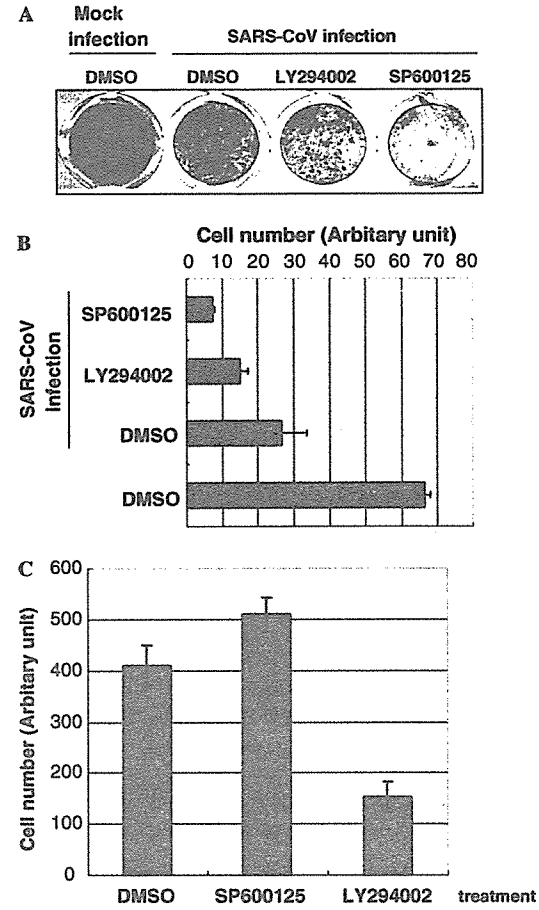

Fig. 2. Effects of JNK and PI3K/Akt inhibitors on cell viability of persistently SARS-CoV-infected cells. (A) Confluent Vero E6 cells in 24-well plates were infected with SARS-CoV for 50 h, and then LY294002 (10 μM) and SP600125 (20 μM) were added to the cells. All wells contained the same volume of DMSO. After incubation for 7 days, the cells were fixed with 10% formaldehyde and stained with 0.1% naphthol blue-black. (B) Stained cells were quantified by measuring the absorbance at OD_{660} with addition of NaOH. (C) Subconfluent cells were treated with inhibitors for 5 days, and then cells were counted using the WST-1 cell proliferation assay system [6].

suggested that N protein is able to induce apoptosis under stress conditions. To understand which signaling pathways are phosphorylated by N protein in Vero E6 cells persistently infected with SARS-CoV, we made an N expression plasmid. Transfection of the N expression plasmid was performed using transfection reagents, Magnetfection and VeroFect (OZ Biosciences, Marseille, France), which our screen of transfection reagents suggested to be the best transfection systems for Vero E6 cells, which have low transfection efficiency (data not shown). However, levels of expression of N protein by these two reagents were far lower than those in SARS-CoV-infected Vero E6 cells. Therefore, we next used the vaccinia virus expression system (DIs-N) [11]. Vero E6 cells were infected with DIs-N at 5 m.o.i. and protein samples were obtained at 18 h.p.i.

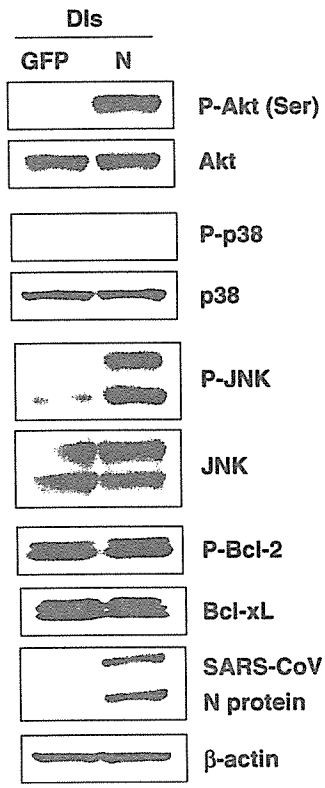

Fig. 3. Modulation of signaling pathways by N expression. Confluent Vero E6 cells in 24-well plates were infected with DIs-N and DIs-GFP at 5 m.o.i. Protein samples were obtained at 18 h.p.i. and Western blotting analysis was performed.

We used DIs-GFP, which expresses GFP protein in infected cells, as a control at the same m.o.i. As shown in Fig. 3, both Akt and JNK were phosphorylated in DIs-N-infected cells as compared with DIs-GFP-infected cells. There was no significant difference in the amount of Bcl-2, Bcl-xL, and phosphorylated p38 MAPK. This result suggested that phosphorylation of Akt and JNK induced by N protein in SARS-CoV-infected Vero E6 cells plays important roles for the establishment of persistence.

Discussion

In our previous study, the signaling pathways of JNK and Akt were shown to be important for establishment of persistent SARS-CoV infection, when these inhibitors were added soon after SARS-CoV infection [7]. Approximately 95% of confluent Vero E6 cells died 2 days after infection with SARS-CoV. The remaining 5% of cells that survived grew with persistent virus infection. When 24-well plates were used for experiments, the persistently infected cells reached confluence by 7 days. Interestingly, the PI3K/Akt inhibitor, LY294002, permitted cell survival when added after apoptotic events, but activation of JNK was also necessary for survival after apoptotic events. Our previous study demonstrated the importance of Akt activation for proliferation of SARS-CoV-infected cells [4]. Phosphorylation of Akt was down-regulated in subconfluent cells by SARS-CoV infection [13]. Nevertheless, LY294002-treated surviving cells that had escaped from SARS-CoV-induced apoptosis could still grow slowly. One of the reasons for this is that PI3K/Akt inhibitor needs 3 days after treatment to inhibit cell proliferation [13]. Therefore, persistent cell colonies may grow slightly in the presence of LY294002. SARS-CoV replicates in surviving cells, and these cells are still alive in the presence of LY294002, suggesting that the signaling pathway of PI3K/Akt is not necessary to prevent apoptosis in cells with persistent virus infection. The apoptotic signaling pathways may be blocked independent of PI3K/Akt in surviving cells. In this study, we demonstrated that the anti-apoptotic proteins Bcl-2 and Bcl-xL were present at elevated levels in persistently infected cells. Because Bcl-2 is slightly increased and phosphorylated at acute infection (24 h.p.i.), but not Bcl-xL (data not shown), Bcl-xL may be more important than Bcl-2 for survival. These results indicated that the PI3K/Akt signaling pathway is important for cell survival in the early stages of SARS-CoV-induced apoptosis, whereas the JNK, Bcl-2, and Bcl-xL pathways are important after apoptotic events. We found SP600125 that slightly prevented SARS-CoV-induced apoptosis (unpublished data). The JNK signaling pathway is one of the key factors for understanding persistence of SARS-CoV.

Interestingly, when we used the N expression system of vaccinia virus (DIs-N), Akt and JNK were phosphorylated in Vero E6. The differences in the results between our study and that reported by Surjit et al. using COS-1 are most likely due to the use of different cell cultures and different expression systems [15]. It is not yet clear whether N protein alone is able to induce phosphorylation of these signaling pathways in Vero E6 cells because we have no useful system for plasmid transfection of these cells. Both Akt and JNK were phosphorylated in our system due to additional stress by expression of vaccinia viral proteins. Because Bcl-2 and Bcl-xL were not increased by N protein expression, these anti-apoptotic proteins may not be downstream of Akt and JNK signaling pathways.

In this paper, we showed possible mechanisms of establishment of persistent SARS-CoV infection. Further investigations are necessary to determine signaling pathways, which are able to up-regulate Bcl-2 and Bcl-xL levels.

Acknowledgments

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