

Signal Transduction in SARS-CoV-Infected Cells

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ABSTRACT: Severe acute respiratory syndrome (SARS) is a newly found infectious disease that is caused by a novel human coronavirus, SARS coronavirus (SARS-CoV). Because the mortality rate of SARS patients is very high, understanding the pathological mechanisms of SARS not only *in vivo* but *in vitro* is important for the prevention of SARS. Activation of signaling pathways caused by SARS-CoV infection leads to the phosphorylation and activation of downstream molecules. Two conflicting cellular programs, apoptosis to eliminate virus-infected cells and survival to delay apoptosis by producing antiviral cytokines, occur in SARS patients. Recent studies regarding SARS and SARS-CoV have clarified that activation of mitogen-activated protein kinases (MAPKs) plays important roles in upregulation of cytokine expression and apoptosis both *in vitro* and *in vivo*. Both Akt and p38 MAPK are keys for determination of cell survival or death in SARS-CoV-infected cells *in vitro*. Agents being developed to target these signaling cascades may be important for the design of anti-SARS-CoV drugs. This review highlights recent progress regarding SARS-CoV biology, especially signal transduction in SARS-CoV-infected cells.

KEYWORDS: SARS; apoptosis; MAPK

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a newly found infectious disease that is caused by a novel coronavirus, SARS coronavirus (SARS-CoV).^{26,34} In late 2002, SARS spread from China to more than 30 countries, causing severe outbreaks of atypical pneumonia. Although the mechanism of SARS pathogenesis *in vivo* may involve both the effects of viral replication in the target cells and immune responses to viral antigens, there is a lack of molecular pathological data, including data regarding the signaling pathways of SARS-CoV infection. As viral virulence and the mortality rate of SARS

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patients are very high, understanding the pathological mechanisms of SARS is important for the prevention of SARS.

Generally, both proapoptotic and prosurvival signaling pathways are activated during viral replication. Many pro- and antiapoptotic proteins are involved in these pathways in cells. Several reports indicated that activation of physiological intracellular signaling cascades caused by SARS-CoV infection leads to the phosphorylation and activation of downstream molecules. For example, mitogen-activated protein kinases (MAPKs) are well-known signal transducers that respond to extracellular stimulation by cytokines, growth factors, viral infection, and stress, and in turn regulate cell differentiation, proliferation, survival, and apoptosis.^{15,23,40} Recent studies regarding SARS and SARS-CoV have clarified that activation of MAPKs plays important roles in upregulation of cytokine expression and apoptosis both *in vitro* and *in vivo*. This review highlights progress regarding SARS-CoV biology, especially signal transduction in SARS-CoV-infected cells (FIG. 1).

p38 MAPK

Apoptosis, which is fundamentally different from necrosis, is an active and physiological type of cell death, which can be induced by viral infection, viral replication, and production of viral proteins. The monkey kidney cell line, Vero E6, is widely used in SARS-CoV research due to its high sensitivity to infection with the virus. Several studies have shown that SARS-CoV infection of Vero E6 cells induces apoptosis, detected by DNA fragmentation and caspase activation.^{28,42} p38 MAPK is strongly activated by stress and inflammatory cytokines. Mouse hepatitis virus (MHV), a prototype coronavirus, was able to induce activation of p38 MAPK into virus-infected cells.¹ p38 MAPK activation in CD14 monocytes was also observed in SARS patients.²⁴ Phosphorylation of p38 MAPK was significantly upregulated at 18 h postinfection (h.p.i.) in SARS-CoV-infected Vero E6 cells.²⁸ Cytopathic effects of SARS-CoV-infection were partially prevented by adding the p38 MAPK-specific inhibitor, SB203580, to the cells. Although p38 MAPK can promote both cell death and survival,¹⁰ the results of inhibitor studies indicated that the p38 MAPK signaling pathway may be involved mainly in cell death in SARS-CoV-infected Vero E6 cells. Several downstream targets of p38 MAPK were phosphorylated in virus-infected cells, and SB203580 effectively inhibited phosphorylation of these proteins in SARS-CoV-infected cells. MAPK-activated protein kinase (MAPKAPK)-2, which is activated in response to stress and growth factors,^{12,13} was phosphorylated in virus-infected cells. Hsp-27, which is a substrate of MAPKAPK-2 and is known to show antiapoptotic activity by inhibiting apoptosome formation,¹⁴ survival factors,²⁰ cAMP response element-binding protein (CREB), and activation of transcription factor (ATF)-1, was also phosphorylated in virus-infected cells. Phosphorylation of Hsp-27, CREB, and ATF-1 may induce an antiapoptotic environment in

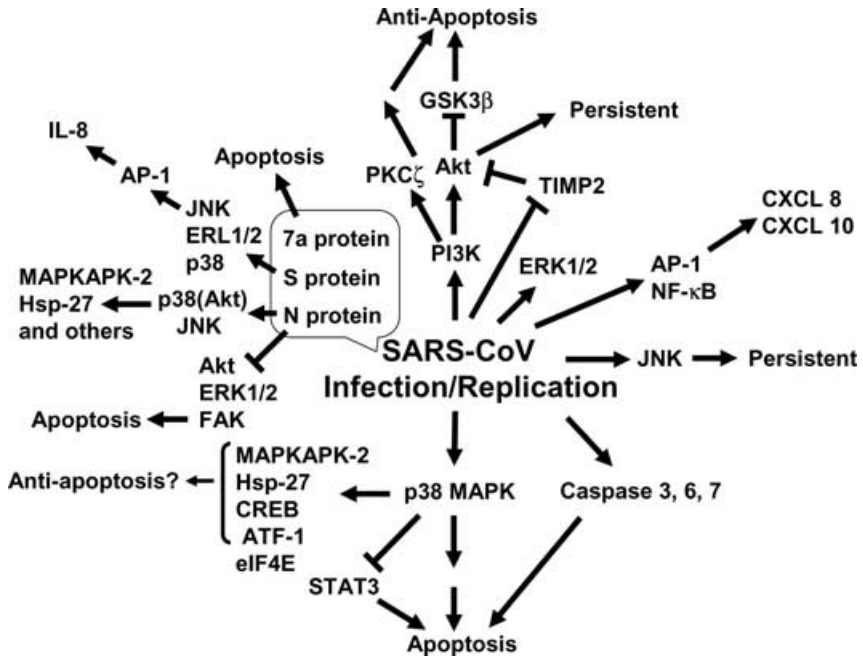


FIGURE 1. Schematic representation of signal transduction on SARS-CoV infection.

SARS-CoV-infected cells. Nucleocapsid (N) protein was able to induce phosphorylation of Hsp-27 and CREB in transfected cells as described below.³⁶ The translation initiation factor, eukaryotic initiation factor 4E (eIF4E), is known to enhance translation rates of cap-containing mRNAs.¹⁶ The phosphorylation of eIF4E was utilized to promote virus-specific protein synthesis in the case of MHV.¹ Although the levels of phosphorylated eIF4E were increased by SARS-CoV infection, the activated eIF4E was not advantageous for viral protein synthesis as demonstrated by the similar kinetics of viral protein accumulation in infected Vero E6 cells in the presence and absence of SB 203580. There may be other substrates of p38 MAPK that are inducible on apoptosis of Vero E6 cells caused by SARS-CoV infection, determination of the apoptosis-inducible target molecules of p38 MAPK is important for the development of anti-SARS-CoV drugs.

ERK1/2

Extracellular signal-regulated kinase (ERK) 1/2 was phosphorylated in SARS-CoV-infected Vero E6 cells,²⁷ whereas ERK1/2 was downregulated in N protein-expressing COS-1 cells as described below.³⁶

JNK

c-Jun N-terminal kinase (JNK) was phosphorylated in SARS-CoV-infected Vero E6 cells.²⁷ A recent study indicated that persistent infection was established after most of the SARS-CoV-infected Vero E6 cells had died by apoptosis.³⁰ SP600125, an inhibitor of JNK, and LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K), inhibited the establishment of persistence, whereas PD98059, an inhibitor of MEK1/2, and SB203580, an inhibitor of p38 MAPK, did not. Thus, two signaling pathways of JNK and PI3K are important for the establishment of persistence in Vero E6 cells.

STAT3

In Vero E6 cells, signal transducer and activator of transcription (STAT) 3 is constitutively phosphorylated at tyrosine (Tyr)-705 and is slightly phosphorylated at serine (Ser)-727.²⁷ However, SARS-CoV replication induced dephosphorylation of STAT3 at Tyr-705. As STAT3 is a major transcription factor activated in response to cytokines, such as interleukin-6 (IL-6) and IL-10, inhibition of STAT3 signaling by dominant negative and antisense oligonucleotides against STAT3 resulted in decreases in cell viability and apoptosis.^{17,31} Thus, STAT3 is thought to act as an antiapoptotic transcription factor. Although inhibitors of MEK and JNK had no effect on the phosphorylation status of STAT3 in virus-infected cells, two inhibitors of p38 MAPK (SB203580 and SB202190) partially inhibited dephosphorylation of STAT3 at Tyr-705, suggesting that the p38 MAPK signaling pathway is upstream of Tyr-705 dephosphorylation of STAT3 in Vero E6 cells. The level of STAT3 phosphorylation at Ser-727 was increased in virus-infected cells. Although the effect of Ser-727 phosphorylation on the function of STAT3 remains unresolved, it was reported that phosphorylation of Ser-727 of STAT3 negatively modulated its Tyr phosphorylation.⁶ Interestingly, Tyr-705 dephosphorylation and Ser-727 phosphorylation showed almost the same timing in SARS-CoV-infected cells. STAT3 phosphorylated at Tyr-705 was localized mainly in the nucleus in mock-infected cells, whereas STAT3 disappeared from the nucleus in virus-infected cells. As STAT3 acts as an activator of transcription in the nucleus, STAT3 may lack its transcriptional activity in SARS-CoV-infected Vero E6 cells, and thus result in a decrease in antiapoptotic activity in the cells.

As other STAT signal transduction pathways are involved in SARS-CoV infection, there have been many reports that treatment with interferons (IFNs) can inhibit viral replication *in vivo* and *in vitro*. However, there have been no detailed studies of IFN signal transduction in SARS-CoV-infected cells. IFN-alpha receptor recognizes STAT1 and STAT2 via Jak1 and Tyk2, whereas the IFN-gamma receptor recognizes STAT1 via Jak1 and Jak2. Signal-transducing adaptor molecule 1 (STAM1) was upregulated in SARS-CoV-infected Vero E6

cells.²⁵ As STAM1 is known to associate with Jak2 and 3 via the immunoreceptor Tyr-based activation motif, it may play an important role in signal transduction of cytokine receptors by SARS-CoV infection.

AKT

As mentioned above, the PI3K signaling pathway (including Akt) plays important roles in establishing persistent infection by SARS-CoV. Akt is phosphorylated at both Ser-473 and threonine (Thr)-308 residues via a PI3K-dependent mechanism on stimulation by growth factors, insulin, and hormones.³⁹ One of the most important functions of activated Akt in cells is the prevention of apoptosis. Akt has many downstream targets and it induces cell survival via phosphorylation of the forkhead transcription factor (FKHR) family, glycogen synthase kinase 3 β (GSK-3 β), caspase-9, and Bad.^{2,8,9} In SARS-CoV-infected confluent Vero E6 cells, Ser-473 of Akt was phosphorylated at 8 h.p.i. and maximal phosphorylation was observed at 18 h.p.i., whereas phosphorylation of Thr-308 was not observed. As Akt is thought to show its full activity when phosphorylated at both Ser-473 and Thr-308, the total activity of Akt may be low in Vero E6 cells. Therefore, the phosphorylated Akt in SARS-CoV-infected cells cannot prevent apoptosis.

Interestingly, tissue inhibitor of metalloproteinase 2 (TIMP2), which activates Ras and leads to Ras/PI3K complex formation, was shown to be downregulated in virus-infected Vero E6 cells at 12 h.p.i. by microarray analysis.²⁵ The acute Ser dephosphorylation of Akt at 24 h.p.i. after tentative phosphorylation in virus-infected cells may be due to downregulation of TIMP2.

PKC

Protein kinase C (PKC) is one of the major cellular mediators of biological functions. The PKC superfamily is classified as subsuperfamilies based on activation profiles: conventional PKC (cPKC α , β I, β II, γ novel PKC (nPKC δ , ϵ , η , θ), atypical PKC (aPKC ζ , ι / λ) PKC μ , PKD and PKC ν . PKC ζ , which was discovered as a unique PKC isotype,³² is thought to be one of the most important PKC, because PI3K-dependent activation of PKC ζ mediates B-cell survival by nerve growth factor.²² Akt has been reported to interact with PKC ζ .²¹ PKC ζ was phosphorylated in SARS-CoV-infected Vero E6 cells,²⁷ suggesting that this molecule is activated as an antiapoptotic response to SARS-CoV infection.

TRANSCRIPTION FACTORS

Human intestinal epithelial Caco-2 cells, which are highly permissive to SARS-CoV, have been used for analysis of cellular gene expression

by microarray analysis. The proinflammatory chemokines, interleukin 8 (CXCL8), and interferon- γ -inducible protein 10 (CXCL10), were upregulated in SARS-CoV-infected Caco-2 cells.⁷ These two chemokines are regulated by AP-1 and NF- κ B, which were also activated by viral infection. CXCL10 levels were significantly elevated in the blood of SARS patients^{38,41} and in macrophages *in vitro*.⁵ As described above and below, CREB was phosphorylated in virus-infected Vero E6 cells and N protein-expressing COS-1 cells.

APOPTOSIS-INDUCIBLE VIRAL GENES

The genome of SARS-CoV is approximately 30 kb in length and contains 14 potential open-reading frames (ORFs), including nine viral proteins with no homologues in other coronaviruses. The N protein, which is a 423 amino acid predicted phospho-protein of 46 kDa with a short Ser-rich stretch and a putative bipartite nuclear localization signal, is known to show little homology with molecules in other coronaviruses. The N protein has been shown to undergo self-association through the C-terminal 209 amino acid region³⁵ and was able to induce apoptosis of COS-1 cells in the absence of growth factors.³⁶ Both JNK and p38 MAPK were upregulated in the transfected cells, whereas ERK and Akt were downregulated. Activated p38 MAPK by N protein induced actin reorganization into cells in the absence of growth factors. The downstream targets of p38 MAPK, MAPKAPK-2, and Hsp-27, were phosphorylated in the cells as well as in the virus-infected Vero E6 cells. Downregulation of focal adhesion kinase (FAK) activity and fibronectin expression was observed due to N protein expression, supporting the hypothesis that apoptosis induced by N protein is caused by interference with the integrin signaling pathway, and that serum factors are able to inhibit apoptosis by maintaining the activity of FAK through alternative pathways. Caspase-3 and -7 were activated only in the absence of growth factors in N-expressing cells. However, SB203580, an inhibitor of p38 MAPK, failed to inhibit caspase-3 activation. Thus, there are two independent functional properties of N protein, p38 MAPK-dependent actin reorganization, and caspase activation. Another group showed that the levels of transcription factors, c-Fos, ATF2, CREB, and FosB, are increased by expression of N in Vero and Huh-7 cells.¹⁸ Spike (S) protein of SARS-CoV also induced activation of AP-1 and IL-8 promoter, possibly via activation of MAPKs, in A549 and HFL-1 cells.⁴ As high serum levels of IL-8 were observed in patients in the acute stage of SARS,¹⁹ activation of the IL-8 promoter via AP-1 induced by S protein may explain these clinical observations. The binding of S protein to a viral receptor, angiotensin-converting enzyme (ACE)-2, may be a trigger for activation of these MAPKs.

Two unique proteins of SARS-CoV, 3a (U274, X1) and 7a (U122, X4), have been studied. The 3a protein is located mainly in the Golgi apparatus and contains three transmembrane regions,⁴³ whereas 7a encodes a protein of 122

amino acids containing a probable cleaved signal sequence and a C-terminal transmembrane helix. As the C-terminal tail also contains a typical endoplasmic reticulum (ER) retrieval motif, 7a is localized to the perinuclear region in both SARS-CoV-infected and -transfected cells.¹¹ The overexpression of 7a, but not of 3a, induced apoptosis via a caspase-dependent pathway.³⁷

CONCLUSIONS

In SARS patients, two conflicting cellular programs occur: “apoptosis” to eliminate virus-infected cells and “survival” to delay apoptosis by producing antiviral cytokines. The control mechanisms balancing cell survival against cell death *in vitro* are important for understanding the pathology *in vivo*. As mentioned above, activation of p38 MAPK is involved in the regulation of IL-8 *in vivo*. Both Akt and p38 MAPK are keys for determination of cell survival or death in SARS-CoV-infected cells *in vitro*. Activation of the p38 MAPK signaling pathway and dephosphorylation of STAT3 via p38 MAPK induced by SARS-CoV infection have partially proapoptotic roles in Vero E6 cells. On the other hand, the levels of Akt, which inactivates proapoptotic pathways, are too low to block apoptosis signaling pathways. As Akt activation is necessary to establish persistently infected cells that escape from apoptotic cell death on viral infection, Akt plays an important role in delaying apoptosis in SARS-CoV-infected cells. Agents being developed to target p38 MAPK and its downstream molecules (for downregulation) and Akt (for upregulation) may be important for the design of anti-SARS-CoV drugs.

ADDENDUM

In recent papers, Surjit *et al.* reported that phosphorylated N protein shuttled from nucleus to cytoplasm by binding to 14-3-3 (J. Virol. 2005. 79: 11476–11486) and N protein blocked S phase progression (J. Biol. Chem. 2006. 281: 10669–10681). Yuan *et al.* reported that 7a protein blocked cell cycle progression at G0/G1 phase (Virology 2006. 346. 74–85). Inhibition of cell proliferation was caused by dephosphorylation of Akt (by us; FEMS Immunol. Med. Microbiol. 2006. 46: 236–243). Okabayashi *et al.* reported that production and response of IFN were not suppressed by SARS-CoV-infection (J. Med. Virol. 2006. 78: 417–424). We reported regulation 90 kDa ribosomal S6 kinase phosphorylation (FEBS Lett. 2006. 580: 1417–1424), importance of Bcl-2 and Bcl-xL for the establishment of persistent infection (Biochem.Biophys.Res.Comm. 2006. 347: 261–265), and enhancement of cytotoxicity against Vero E6 cells persistently infected with SARS-CoV by *Mycoplasma fermentans* (Arch. Virol. In press).

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