Chapter 19
Signaling Pathways of SARS-CoV In Vitro and In Vivo

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Abstract Severe acute respiratory syndrome (SARS) is a respiratory illness with variable symptoms that was recognized as the first near-pandemic infectious disease of the twenty-first century. A novel human coronavirus, named SARS coronavirus (SARS-CoV), derived from SARS patients was reported as the etiologic agent of SARS. Studying the signaling pathways of SARS-infected cells is key to understanding the molecular mechanism of SARS viral infection. Cell death is observed in cultured Vero E6 cells after SARS-CoV infection. From SARS-CoV infection to cell death, p38 mitogen-activated protein kinase (MAPK) is a key participant in the determination of cell death and survival. Two signaling pathways comprising signal transducer and activator of transcription 3 (STAT3) and p90 ribosomal S6 kinase (p90RSK) are downstream of p38 MAPK. AKT and JNK (Jun NH_2-terminal kinase) signaling pathways are important to establish persistent infection of SARS-CoV in Vero E6 cells. Expression studies of SARS-CoV proteins indicate that the viral proteins are able to activate signaling pathways of host cells. The study of signaling pathways in SARS-CoV patients is difficult to perform compared with in vitro studies due to the effects of the human immune system. This review highlights recent progress in characterizing signal transduction pathways in SARS-CoV-infected cells in vitro and in vivo.

19.1 Introduction

Severe acute respiratory syndrome (SARS) is a respiratory illness with variable flu-like symptoms and pneumonia, which is caused by the SARS coronavirus (SARS-CoV) (Drosten et al. 2003; Ksiazek et al. 2003; Peiris et al. 2003a, 2003b; Poutanen

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et al. 2003; Tsang et al. 2003). SARS was first recognized in China in November 2002 and subsequently spread to 29 other countries, thus emerging as the first near-pandemic infectious disease of the twenty-first century. A worldwide total of 8,096 cases of SARS, of which 774 (9.6%) resulted in death, was reported by the World Health Organization (WHO) (http://www.who.int/csr/sars/country/table2004_04_21/en/index.html).

SARS-CoV belongs to the *Coronaviridae* family (order *Nidovirales*) of enveloped single-stranded positive RNA viruses (Marra et al. 2003; Rota et al. 2003; Thiel et al. 2003). The SARS-CoV genome is approximately 30 kb in length and is the longest known amongst the RNA virus genomes. The SARS-CoV genomic RNA has a cap structure and a poly-A tail at the 5′ and 3′ ends, respectively. SARS-CoV genome replication occurs in the cytoplasm. During viral replication, a full-length genomic negative-stranded RNA is transcribed from genomic positive-stranded RNA by the viral RNA polymerase that is initially translated from genomic RNA. Approximately 60% of SARS-CoV genomic RNA encodes viral polymerase and its related proteins. The mRNA transcription of coronavirus is unique, because all mRNAs have a nested set structure. The mRNAs have a 5′ leader sequence of approximately 70 nucleotides and poly-A tails at the 3′ end. Mouse hepatitis virus (MHV), which is a prototype of coronavirus, has seven mRNAs, whereas SARS-CoV has at least nine mRNAs. The leader RNA is transcribed from the 3′ end of full-length genomic negative-stranded RNA. There is strong evidence that the leader RNA is transcribed as small sized-RNA, which is approximately 70 bases in length. The leader RNA binds to intragenic initiation sites on negative-stranded RNA, and then viral RNA polymerase starts to transcribe mRNA at the site. The SARS-CoV viral genomic RNA comprises 14 open reading frames (ORFs), and eight of the encoded proteins are unique compared with other coronaviruses. These unique proteins are thought to be involved in the pathogenetic mechanism of SARS-CoV.

Large overlapping polyproteins (1a and 1b) encoded by approximately 60% of the SARS-CoV viral genome are processed into 16 nonstructural proteins including polymerase and proteases (chymotrypsin-like cysteine protease and papain-like protease). These proteins are thought to be essential in viral replication and transcription. The viral particle of SARS-CoV mainly consists of four structural proteins, spike (S), membrane (M), envelope (E), and nucleocapsid (N) (Fig. 19.1). The viral particle may also comprise viral accessory proteins that bind to the structural proteins. The S protein binds to the viral receptor of host cells and enables the virus to enter the cytoplasm by endocytosis.

SARS-CoV has the potential to cause respiratory illness in human patients. Cytokine storm occurs in SARS-CoV-infected patients and is one of the observed pathologic mechanisms of SARS-CoV infection. On the other hand, apoptotic cell death is observed in vitro when SARS-CoV-sensitive cultured cells such as Vero E6 cells are used (Mizutani et al. 2004c). Various signaling pathways are activated during the entire process of viral infection, from S protein–ACE2 (Angiotensin-converting enzyme-2) binding for internalization into the host cells to apoptotic cell death (Mizutani 2007). The most common signaling pathways are mitogen-activated

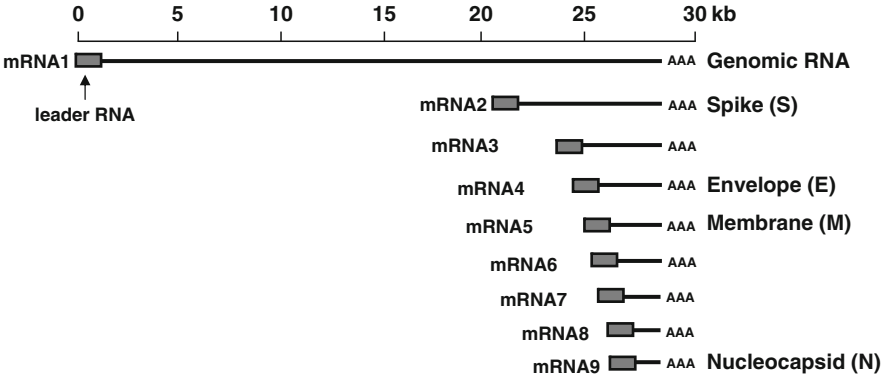

Fig. 19.1 SARS-CoV mRNAs in infected cells. Structural proteins are indicated

protein kinase (MAPK) pathways, which include Jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPK. These three major MAPKs are highly conserved in a wide range of species from yeast to mammals and are regulatory proteins of cell death and cell survival in living cells. Thus, MAPKs are key to the process of apoptosis (Garrington and Johnson 1999). MAPKK kinase (MAPKKK) activates MAPK kinase (MAPKK), and then MAPKK activates MAPK. Generally, the ERK signaling pathway promotes cell survival and proliferation, and JNK and p38 MAPK induce apoptosis. However, the role of each MAPK varies depending on cell type and stimulation. Many signaling pathways are activated in virus-infected cells, and cross-talk activation between signaling pathways occurs. Thus, signaling pathways regulating cell death and survival in virus-infected cells is highly complex.

Analysis of activated signaling pathways in SARS-CoV-infected cells and patients is required for understanding the pathogenesis of SARS. This review highlights recent progress in characterizing signal transduction pathways induced by SARS-CoV infection in vivo and in vitro.

19.2 p38 MAPK Signaling Pathway in Viral Infection

The p38 MAP kinase is expressed in response to stressors, and viral infection generally induces activation of p38 MAPK. The roles of p38 MAPK in viral infection/replication have been researched recently as described below.

Environmental stresses, such as UV irradiation, oxidative stimuli and pro-inflammatory cytokines, are able to induce activation of p38 MAPK. There are at least four isoforms of p38 MAPK: p38α, p38β, p38γ, and p38δ (1999; Platanias 2003; Lee et al. 2004), but these isoforms are generally not distinguished in the field of virology. However, these four isoforms exhibit different properties and have

different cellular functions. The p38α and p38β MAPKs have more than 70% similarity at the amino acid sequence level, and their functions are inhibited by the pyridinyl imidazole inhibitor SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole]. Conversely, p38γ and p38δ MAPKs, which have 60% similarity to p38α, are not inhibited by SB203580. Furthermore, p38α and p38β MAPKs are widely expressed in tissues, whereas the expression of p38γ and p38δ MAPKs is tissue-specific. In the field of virology, because SB203580 is generally used as an inhibitor of the p38 MAPK signaling pathway, it can be used in studying the role of p38α and/or p38β MAPKs in SARS-CoV infection. The kinases upstream of p38 MAPK are MKK3 (MAPK kinase 3) and MKK6 (MAPK kinase 6), which are known to phosphorylate and activate p38 MAPK. Interestingly, MKK6 has affinity to PKR in the presence of double-stranded RNA, poly(rI;rC) and PKR is able to activate MKK6, but not MKK3. This result indicated that interaction of MKK6 and PKR provides a mechanism to regulate activation of p38 MAPK (Silva et al. 2004). In hepatitis C virus-core-expressing cells, PKR has an important role in cell cycle arrest and was shown to interact strongly with p38 MAPK (Spaziani et al. 2006). As upstream of p38MAPK, transforming growth factor (TGF)-β-activating kinase (TAK1), apoptosis signal-regulatory kinase (ASK1), and MAPKKK4 are known as MAPKKKs.

Downstream targets of p38 MAPK are well-known as mitogen and stress-activated protein kinase 1 (MSK1), MAP kinase-interacting kinase 1 (MNK1), and MAPK-activated protein kinase 2 and 3 (MAPKAPK 2 and 3) (Freshney et al. 1994). These signaling pathway proteins have further downstream targets. For example, MNK1 activates the eukaryotic translation initiation factor 4E (eIF4E) (Gingras et al. 1999). MAPKAPK2 activates heat shock protein 27 (HSP27), cAMP response element-binding protein (CREB), and transcription factor-1 (ATF-1) (Garrido et al. 2003; Tan et al. 1996). Mouse hepatitis virus (MHV) A59 strain induces interleukin-6 (IL-6) production via eIF4E phosphorylation under p38 MAPK activation (Banerjee et al. 2002). Inhibitors of p38 MAPK inhibit transcription of viral mRNA and production of viral protein, indicating that p38 MAPK is utilized to promote viral protein synthesis. Conversely, p38 MAPK enhances transcription of CHOP (C/EBP homologous protein) encoded by the growth arrest- and DNA damage-inducible gene 153 (GADD153) (Wang et al. 1996). The p38 MAPK-induced apoptosis via activation of the CHOP pathway occurs in cells infected with Japanese encephalitis virus (JEV) (Su et al. 2002). The H5N1 subtype of influenza virus induces tumor necrosis factor alpha (TNF-α) expression via activation of p38 MAPK (Lee et al. 2005). The p38 MAPK signaling pathway is thought to primarily induce apoptosis in virus-infected cells. As described in HCV infection, p38 MAPK is able to promote cell survival. Although p38 MAPK is activated in many cases of viral infection, viral proteins sometimes negatively regulate p38 MAPK. The ORF 61 protein of varicella-zoster virus (VZV) is known to repress phosphorylation of p38 MAPK for negative regulation of cellular proinflammatory responses (Rahaus et al. 2005). Thus, activation or inactivation of p38 MAPK occurs in the pathogenesis of disease caused by viral infection.

19.3 p38 MAPK Signaling Pathway in SARS-CoV-Infected Cells

The p38 MAPK signaling pathway takes part in cell death, as previously described. Apoptosis is an active and physiologic type of cell death and is a host cell's protective mechanism for preventing the spread of viral particles before production of viral particles. Vero and Vero E6 cells, which are monkey kidney cells, are widely used in SARS-CoV research because of their high susceptibilities to infection due to lack of interferon genes. Apoptosis has been shown to be inducible by infection with SARS-CoV (Mizutani et al. 2004c; Yan et al. 2004). Cytopathic effects (CPEs), defined as focal cell rounding and DNA fragmentation typical of apoptosis, are observed in SARS-CoV-infected Vero E6 cells at 24 h post-infection (h.p.i.) (Mizutani et al. 2004c). Activated caspase 3, which has an essential role in apoptosis, was detected at peak levels at 24 h.p.i. On the other hand, the phosphorylation level of p38 MAPK reached a maximum at 18 h.p.i. in virus-infected cells. The phosphorylated p38 MAPK was active, as shown by using an in vitro kinase assay. The CPE observed in SARS-CoV-infected cells is slightly inhibited by SB203580, and therefore p38 MAPK activation is thought to induce CPE of virus-infected cells. However, DNA fragmentation is not inhibited by the inhibitor. Apoptosis and CPE are thought to be linked, and activation of p38 MAPK is a promoter of cell death in Vero E6 cells infected by SARS-CoV. However, SB203580 treatment of Vero E6 cells indicates that there is no requirement for p38 MAPK activation in SARS-CoV replication. The p38 MAPK signaling pathway perhaps has other roles in SARS-CoV-infected cells.

The downstream targets of p38 MAPK are phosphorylated in SARS-CoV-infected cells. The level of phosphorylated eIF4E is increased in SARS-CoV-infected cells (Mizutani et al. 2004c). However, the activated eIF4E does not regulate viral protein synthesis, as demonstrated by the similar kinetics of viral protein accumulation in infected Vero E6 cells in the presence and absence of SB203580. Both MAPKAPK-2 and its substrate HSP-27 are phosphorylated in virus-infected cells. HSP-27 is known as an anti-apoptotic protein as it inhibits apoptosome formation (Garrido et al. 2003). CREB is also known to mediate a survival signal under various conditions (Tan et al. 1996; Ginty et al 1994; von Knethen et al. 1998), and CREB is also phosphorylated in SARS-CoV-infected cells. The expression of SARS-N protein in transfected COS-1 cells induces phosphorylation of p38 MAPK, HSP-27, and CREB (Surjit et al. 2004), whereas the viral-N protein expression system of vaccinia virus (DIs-N) does not induce phosphorylation of p38 MAPK (Mizutani et al. 2006d). Activation of the p38 MAPK pathway induces actin reorganization in COS-1 cells devoid of growth factors (Surjit et al. 2004). Furthermore, the 7a protein of SARS-CoV induces apoptotic cell death and phosphorylation of p38 MAPK in 293 T cells (Kopecky-Bromberg et al. 2006). However, SB203580 does not prevent cell rounding, apoptosis, and chromatin condensation induced by the 7a protein. The differences in the results are most likely due to the use of different cell cultures and expression

systems. Overall, phosphorylated proteins downstream of p38 MAPK have the potential to induce an anti-apoptotic environment in SARS-CoV-infected cells. However, activated p38 MAPK in SARS-CoV-infected cells is thought to be able to promote both cell death and survival. Perhaps there are other substrates of p38 MAPK that are inducible on cell death of Vero E6 cells caused by SARS-CoV infection, or perhaps there is cross-talk between the p38 MAPK signaling pathway and other signaling pathways inducing cell death.

19.4 Downstream Signaling Pathways of p38 MAPK

In Vero E6 cells, signal transducer and activator of transcription (STAT) 3 protein is constitutively phosphorylated at Tyr-705 and is slightly phosphorylated at Ser-727 (Mizutani et al. 2004a). SARS-CoV infection is able to induce dephosphorylation of STAT3 Tyr-705 after 18 h.p.i. On the other hand, Ser-727-phosphorylated STAT3 is slightly increased at the same point in time. The activity of STAT transcription factors is induced by phosphorylation of a single tyrosine residue, leading to dimerization via an intermolecular SH2 phosphotyrosine interaction (Shuai et al. 1993, 1994; Schindler et al. 1992a, 1992b). STAT3 is known to be activated in response to interleukin-6 (IL-6) and IL-10, and is thought to act as an anti-apoptotic transcription factor (Rajan and Mckay 1998; Grandis et al. 2000; Mora et al. 2002). Tyr-705 phosphorylation of STAT is necessary for its activation (Shuai et al. 1993, 1994; Schindler et al. 1992b), suggesting that SARS-CoV infection leads to a decrease in STAT3 activation. Furthermore, STAT3 does not act as a transcriptional enhancer in SARS-CoV-infected Vero E6 cells, as shown by the disappearance of Tyr-705-phosphorylated STAT3 from the nuclear fraction post SARS-CoV infection. The proteins upstream of STAT3 in the signaling pathway are Janus kinases (JAK1 and 2) and Tyk2, which are phosphorylated at low levels in mock-infected Vero E6 cells, even after virus infection. The signal transducing adaptor molecule 1 (STAM1), which is known to be associated with Jak2 and Jak3 via the immunoreceptor tyrosine-based activation motif, is upregulated in SARS-CoV-infected Vero E6 cells (Leong et al. 2005). Therefore, Tyr-705 dephosphorylation of STAT3 in virus-infected cells is independent of its upstream kinases, and there may be other signaling pathways regulating STAT3 phosphorylation and dephosphorylation. Two inhibitors of p38 MAPK (SB203580 and SB202190) partially inhibit dephosphorylation of STAT3 at Tyr-705, indicating that the p38 MAPK signaling pathway is upstream of Tyr-705 dephosphorylation of STAT3 in SARS-CoV-infected Vero E6 cells. Inactivation of STAT3 via p38 MAPK activation may induce cell death in SARS-CoV-infected cells. However, the kinetics of STAT3 after SARS-CoV infection varies for different cell types. The suppressors of cytokine signaling-3 (SOCS3) mRNA are suppressed in SARS-CoV-infected Caco-2 cells (Okabayashi et al. 2006), leading to continuous activation of STAT3.

A serine/threonine kinase, p90 ribosomal S6 kinase (RSK), belongs to another signaling pathway, which is regulated by p38 MAPK. Generally, p90RSK is phosphorylated at Thr-573 by ERK (Gavin and Nebreda 1999; Smith et al. 1999), and this phosphorylation induces autophosphorylation at Ser-380, and then PDK1 (phosphoinositide-dependent kinase 1) phosphorylates at Ser-221 (Frödin et al. 2000; Jensen et al. 1999; Richards et al. 1999). No significant differences are observed in phosphorylation levels of p90RSK at Ser-221 and Thr-573 in SARS-CoV-infected Vero E6 cells (Mizutani et al. 2006a). However, Ser-380 of p90RSK is phosphorylated in virus-infected confluent cells. Thus, phosphorylation of p90RSK Ser-380 is upregulated without upregulation of Thr-573 in SARS-CoV-infected cells. The phosphorylation of Ser-380 is decreased in SB203580-treated virus-infected cells, indicating that p38 MAPK can induce phosphorylation of Ser-380. Furthermore, p90RSK phosphorylates CREB (Frodin and Gammeltoft 1999). In SARS-CoV-infected Vero E6 cells, p38 MAPK activation induces phosphorylation of p90RSK Ser-380, and then CREB is thought to be phosphorylated by activated p90RSK. Thus, p90RSK may have anti-apoptotic activity in SARS-CoV-infected cells.

19.5 ERK1/2 Activation by SARS-CoV Infection

The SARS-CoV S protein is able to induce phosphorylation of ERK1/2 in HEK293T cells (Liu et al. 2007). The S-induced protein kinase C (PKC)/ERK signaling pathway promotes nuclear factor-kappa B (NF-κB) binding to the cyclooxygenase-2 (COX-2) promoter. Similar results have been reported using the N protein of SARS-CoV (Yan et al. 2006). SARS-CoV S protein expression induces release of interleukin-8 (IL-8) via ERK and p38 MAPK signaling pathways including activator protein 1 (AP-1) in A549 cells (Chang et al. 2004). On the contrary, phosphorylation of ERK1/2 is downregulated in N protein-expressing COS-1 cells in the absence of serum (Surjit et al. 2004). Thus, viral proteins can potentially up- or downregulate phosphorylation of ERK1/2. ERK1/2 is observed to be phosphorylated in SARS-CoV-infected Vero E6 cells (Mizutani et al. 2004a). After treatment with MAPK/ERK kinase 1 and 2 (MEK1/2)-specific inhibitor (PD98059), SARS-CoV-infected Vero E6 cells exhibit no significant changes in activated caspase-3 or caspase-7. Thus, activation of ERK1/2 is not sufficient to prevent cell death by SARS-CoV infection. Furthermore, activation of ERK1/2 is not necessary to establish persistent infection of SARS-CoV in Vero E6 cells (Mizutani et al. 2005).

19.6 JNK Activation by SARS-CoV

The SARS-CoV S protein induces CREB binding to COX-2 promoter mediated via the phosphatidylinositol 3-kinase (PI3K)/PKC/JNK pathway in HEK293T cells (Liu et al. 2007). Expression of the SARS-CoV N protein induces phosphorylation

of JNK in Vero E6 cells (Mizutani et al. 2006d) and in COS-1 cells in the absence of serum (Surjit et al. 2004). The phosphorylation level of Jun, which is dependent upon activation of JNK, also increases in the absence of serum. The SARS-CoV N protein can activate AP-1, which is composed of homodimers and heterodimers of Fos, Jun, CREB, and activating transcription factor (ATF) subunits, in Vero and Huh7 cells (He et al. 2003). The viral accessory proteins, 3a and 7a, phosphorylate JNK1 and JNK3 in HEK293T cells (Kanzawa et al. 2006). Overall, viral proteins are able to induce phosphorylation of JNK in several cell lines. SARS-CoV infection induces phosphorylation of JNK in Vero E6 cells after at least 12 h.p.i. (Mizutani et al. 2004a). The Vero E6 cells begin to show rounding at 24 h.p.i and persistently infected cells are observed after 48 h.p.i (Mizutani et al. 2005). At this time, JNK, Akt, and p38 MAPK are phosphorylated in virus-infected cells. Treatment with an inhibitor of JNK (SP600125), and PI3K (LY294002), inhibits the establishment of persistence, whereas treatment with an inhibitor of MEK1/2 (PD98059) and p38 MAPK (SB203580) does not inhibit persistence of infection (Mizutani et al. 2005). Thus, two different signaling pathways of JNK and PI3K/Akt are important for the establishment of persistently infected Vero E6 cells (Mizutani et al. 2006d, 2007).

19.7 PI3K/Akt Activation by SARS-CoV

Akt, which is also known as protein kinase B (PKB), is phosphorylated at both Ser-473 and Thr-308 residues via the PI3K signaling pathway upon stimulation by growth factors, insulin, and hormones (Toker 2000; Brazil and Hemmings 2001; Scheid and Woodgett 2003; Welch et al. 1998). The main role of Akt is inhibition of apoptosis via phosphorylation of the forkhead transcription factor (FKHR) family, glycogen synthase kinase-3β (GSK-3β), caspase-9, and Bcl-associated death protein (Bad) (Cardone et al. 1998; Cross et al. 1995; Datta et al. 1997). Interestingly, GSK-3 regulates phosphorylation of N protein (Wu et al. 2009). The M protein of SARS-CoV induces apoptosis in both HEK293T cells and transgenic *Drosophila* (Chan et al. 2007). The M protein-induced apoptosis involves mitochondrial release of cytochrome *c* protein. In SARS-CoV-infected Vero E6 cells, Ser-473 of Akt is phosphorylated at 8 h.p.i. and maximal phosphorylation is observed at 18 h.p.i. (Mizutani et al. 2004b), after which Akt is dephosphorylated. Thr-308 phosphorylation has not been detected in Vero E6 cells. The phosphorylation of Ser-473 of Akt by viral infection is inhibited by LY294002, which is an inhibitor of the PI3K signaling pathway. An in vitro kinase activity assay of Akt in SARS-CoV-infected cells indicated that Akt is highly phosphorylated only at serine residues, but Akt activity is low. Therefore, weak activation of Akt cannot prevent apoptosis induced by SARS-CoV infection in Vero E6 cells. The phosphorylation of Akt in virus-infected cells is necessary to establish persistence, but Akt is not phosphorylated after establishing persistent cell lines (Mizutani et al. 2005, 2006d), suggesting that activation of PI3K/Akt is essential for the establishment of persistent infection with

SARS-CoV at points in time before cell death. The above characterizations of Akt in SARS-CoV-infected Vero E6 cells are mainly derived from experiments using confluent cells. When subconfluent Vero E6 cells are infected by SARS-CoV, cell proliferation is inhibited (Mizutani et al. 2006c). SARS-CoV infection induces dephosphorylation of a serine residue of Akt without phosphorylation in subconfluent cultures. Thus, downregulation of Akt activity in SARS-CoV-infected cells prevents cell proliferation.

19.8 NF-κB Activation and Inhibition by SARS-CoV Proteins

The SARS-CoV N protein is able to activate NF-κB in Vero E6 cells (Liao et al. 2005). As described above, the S- and N-induced PKC/ERK signaling pathway promotes NF-κB binding to the COX-2 promoter (Liu et al. 2007; Yan et al. 2006). SARS-CoV S and N proteins may cause inflammation of the lungs by activating *COX-2* gene expression. The 3a and 7a viral accessory proteins enhance NF-κB mediated transcription in HEK293T cells (Kanzawa et al. 2006). In contrast, the N protein inhibits interferon production in 293 T cells via inhibition of NF-κB (Kopecky-Bromberg et al. 2007). The M protein also suppresses NF-κB activity (Fang et al. 2007). Growth arrest and apoptosis via caspase-3 and caspase-9 activities are induced in SARS-CoV 3C-like protease ($3CL^{pro}$)-expressing human promonocyte HL-CZ cell line (Lin et al. 2006). The SARS-CoV $3CL^{pro}$ may increase activation of NF-κB and upregulate cytochrome *c* oxidase and downregulate Hsp-70, inducing mitochondrial-mediated apoptosis (Lai et al. 2007). Viral papain-like protease (PLP) regulates antagonism of IRF3 and NF-κB signaling pathways (Frieman et al. 2009).

19.9 Inhibitory Effects of Viral Proteins on the Cell Cycle

The 3a protein of SARS-CoV has the potential to inhibit cell cycle progression at the G_1 phase in HEK293, COS-7, and Vero cells (Yuan et al. 2005, 2007). The C-terminal region of the 3a protein, which includes a potential ATPase motif, is essential to inhibit the cell cycle. The 3a protein expression reduces cyclin D3 level and inhibits retinoblastoma (Rb) phosphorylation. The p53 phosphorylation is increased by 3a expression. The 7a protein expression also blocks cell cycle progression at the G_0/G_1 phase in HEK293, COS-7, and Vero cells by mechanisms similar to those of the 3a protein (Yuan et al. 2006). The N protein is a substrate of cyclin-dependent kinase (CDK) as well as GSK, MAPK, and casein kinase II (Surjit et al. 2005). The N protein directly binds to cyclin D and inhibits activity of the cyclin D–CDK4 complex. The N protein also inhibits CDK2 activity by direct binding to the CDK2–cyclin complex, resulting in blocking the S phase progression

in COS-7 and Huh7 cells (Surjit et al. 2006). Therefore, proteins of SARS-CoV may have the ability to inhibit the progression of the host cell cycle, but further detailed analysis is required in SARS-CoV-infected cells.

19.10 Apoptotic Signaling Pathway

SARS-CoV infection induces apoptotic cell death in Vero E6 cells, via dephosphorylation of STAT3 by p38 MAPK activation, and inactivation of Akt, as previously described. Recent study suggest that SARS-CoV triggers apoptosis via protein kinase R (PKR) (Krähling et al. 2009). Overexpression of SARS-CoV proteins can induce apoptosis in variable cell lines. Induction of apoptosis by various viral proteins may occur at different stages of the infection cycle. SARS-CoV $3CL^{pro}$ expression in HL-CZ cells induces apoptosis via caspase-3 and caspase-9 (Lin et al. 2006). Furthermore, $3CL^{pro}$ expression in HL-CZ cells upregulates proteins located in the mitochondria, but downregulates Hsp-70, which antagonizes apoptosis-inducing factor (Lai et al. 2007). The SARS-CoV 8a protein, localized in the mitochondria of infected cells, increases mitochondrial transmembrane potential, reactive oxygen species production, and caspase-3 activation, resulting in inducing apoptosis in Vero, HEK293, and Huh7 cells (Chen et al. 2007). ORF 6 induces apoptosis via caspase-3 mediated, ER stress and JNK-dependent pathways (Ye et al. 2008). SARS-CoV N protein modulates the TGF-β signaling pathway to block apoptosis of SARS-CoV-infected host cells (Zhao et al. 2008). In the absence of serum, the SARS-CoV N protein can induce apoptosis by activating the mitochondrial pathway (Zhang et al. 2007), and/or by downregulating ERK and Akt signaling pathways (Surjit et al. 2004) in COS-1 cells, but not in Hep-G2 and Huh-7 cells (Zhang et al. 2007). The SARS-CoV S protein and its C-terminal domain (S2) induce apoptosis in Vero E6 cells, but the S1, E, M, and N proteins are not able to induce apoptosis in Vero E6 cells (Chow et al. 2005). In contrast, the SARS-CoV M and N proteins can induce apoptosis in human pulmonary fibroblast (HPF) cells (Zhao et al. 2006). The M protein induces apoptosis through modulation of the Akt pathway and mitochondrial cytochrome *c* release in HEK293T cells and transgenic *Drosophila* [85]. Overexpression of SARS-CoV 3a protein in Vero E6 cells induces apoptosis, mediated through a caspase-8-dependent pathway or p38 MAPK (Law et al. 2005; Waye et al. 2005; Padhan et al. 2008). The 3a protein expression in *Drosophila* induces apoptosis, which could be modulated by cellular cytochrome *c* levels and caspase activity (Wong et al. 2005). The SARS-CoV 3b protein induces both necrosis and apoptosis in Vero E6 cells (Khan et al. 2006). The SARS-CoV 7a protein interacts with pro-survival proteins, basal cell lymphoma-extra large (Bcl-xL), B cell lymphoma 2 (Bcl-2), Bcl-w, A1, and myeloid cell leukaemia sequence 1 (Mcl-1), at the endoplasmic reticulum and the mitochondria, resulting in triggering apoptosis in HEK293T and Vero E6 cells (Tan et al. 2007). Interestingly, the 7a protein does not interact with the pro-apoptotic members, Bcl-2 associated X protein (Bax), Bcl-2 homologous killer (Bak), Bad,

and Bcl-2 interacting domain (Bid). However, a mutant virus without the 7a/7b gene is able to induce extensive CPEs in the Vero cell line (Yount et al. 2005), suggesting that the 7a protein is not a dominant contributor to virus-induced cell death in this cell culture system. The SARS-CoV N protein downregulates the level of Bcl-2 in COS-1 cells (Surjit et al. 2004). The SARS-CoV E protein induces apoptosis in Jurkat T cells in the absence of growth factors, but apoptosis is inhibited by overexpression of Bcl-xL via interaction with the E protein (Yang et al. 2005). Apoptosis is also inhibited by overexpression of Bcl-2 in SARS-CoV-infected Vero cells (Bordi et al. 2006). In the virus-infected Vero cells, downregulation of Bcl-2 and upregulation of Bax are observed (Ren et al. 2005). Bcl-xL activation plays important roles in establishing persistent infection of SARS-CoV (Mizutani et al. 2006b). The N protein upregulates the Bcl-xL protein level (Mizutani et al. 2006d). These reports indicate that Bcl-xL activation is the key to preventing apoptosis due to SARS-CoV infection. The other viral proteins localized in the mitochondria of infected cells may also interact with Bcl-xL and other pro-survival proteins.

19.11 Signaling Pathways in SARS Patients

Western blots are used to analyze signaling pathway proteins of cultured cells infected with SARS-CoV or transfected with plasmids encoding viral proteins. Thus, the kinetics of phosphor-proteins regulating signaling pathways is important for understanding which signaling pathways are activated in virus-infected cells. However, in vivo analysis and amounts of mRNA from whole blood or tissues of SARS patients are primarily analyzed using DNA microarrays. Unfortunately, when the level of mRNA related to a signaling pathway increases in SARS patients, as measured by DNA microarray analysis, the results do not suggest activation of particular signaling pathways, due to the analysis being performed on a mixed population of cells. The roles of signaling pathways may be different amongst different cell types. Analyses of signaling pathways in virus-infected patients are still difficult to perform for these reasons. However, flow cytometric analysis of cell samples from virus-infected patients provides an improved method for the investigation of signaling pathways in vivo. Flow cytometric analysis of phospho-p38 indicated that augmented p38 MAPK phosphorylation of CD14 monocytes was associated with suppressed p38 MAPK phosphorylation of CD8 lymphocytes, suggesting that altered leukocyte p38 activation contributes to abnormal blood cytokine profiles in SARS patients (Lee et al. 2004).

Analysis of cell apoptosis in SARS patients is key to understanding the signaling pathways that regulate apoptosis. In SARS patients, lymphopenia caused by depletion of T lymphocytes by apoptosis is a common abnormality (Chen et al. 2006). Compared to healthy controls, SARS patients have significantly lower lymphocyte and platelet counts and have significantly higher vascular cell adhesion molecule-1 (sVCAM-1) levels and soluble Fas ligand (sFasL) levels, as determined using

ELISA (enzyme-linked immunosorbent assay). SARS patients also have intracellular activated caspase-3 fragment levels, as measured using flow cytometry (Peiris et al. 2003b). Liver impairment commonly occurs amongst patients with SARS, indicating that SARS-CoV may be localized in the liver (Chau et al. 2004). The pathologic features, perhaps due to apoptosis, are the presence of acidophilic bodies, ballooning of hepatocytes, and mild to moderate lobular activities. The thyroid glands of SARS-infected patients show extensive injury due to apoptosis of the follicular epithelial cells and the parafollicular cells, as measured using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (Wei et al. 2007). Necrosis is also observed in splenic lymphoid tissue and lymph nodes of SARS patients (Ding et al. 2003). MyD88-mediated innate immune signaling and inflammatory cell recruitment to the lung in BALB/c mice may be required for protection from lethal recombinant mouse-adapted SARS-CoV infection (Sheahan et al. 2008). Further detailed analysis of apoptosis in cells of SARS patients is required, but the initial reports indicate the activation of apoptotic signaling pathways in SARS patients.

19.12 Conclusion

Both pro-apoptotic and pro-survival signaling pathways are activated in SARS-CoV-infected cells (Fig. 19.2). The balance of activities of signaling pathways is important for determination of cell death or cell survival. In SARS patients, analysis of signaling pathways is further complicated because many cell types respond to viral infection. For example, immune cells infected by SARS-CoV produce and release cytokines, and the cytokines activate other cells. Thus, in SARS patients, many types of cells are infected by SARS-CoV, compared with one type of cell used for in vitro experiments. In addition, the viral proteins that interact with cellular proteins in signaling pathways must be further clarified to understand the

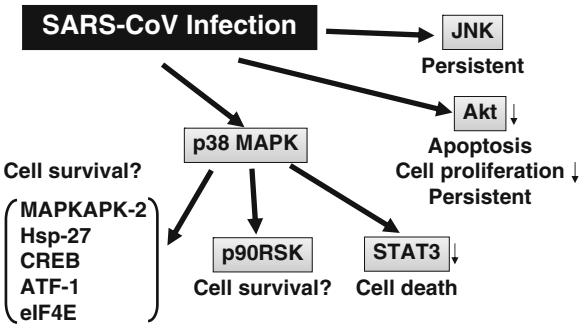

Fig. 19.2 Signaling pathways in cells infected with SARS-CoV. Because each report in this chapter used different cultured cells, this figure is shown based on our experiments using Vero E6 cells

molecular mechanisms of SARS-CoV infection. It is particularly important to determine the viral proteins that are necessary and sufficient to fully activate signaling pathways leading to apoptotic cell death. Determining the SARS-CoV-induced signaling pathways in SARS patients will enable the development of therapeutic reagents that can inhibit the pathways of apoptotic cell death and production of cytokines.

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