BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **253,** 59–64 (1998)
ARTICLE NO. RC989724

Acute Infection of Sindbis Virus Induces Phosphorylation and Intracellular Translocation of Small Heat Shock Protein HSP27 and Activation of p38 MAP Kinase Signaling Pathway

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Received October 21, 1998

In general, viral infection is supposed to induce stress responses in the host cell. However, very few detailed observations about virus-induced stress responses have been reported. Here we investigated specific stress responses in Vero cells infected with Sindbis virus (SV), a single-stranded RNA virus, acute infection with which is known to cause apoptotic cell death in the host cells. Prior to the onset of apoptosis, p38 mitogen-activated protein kinase (MAPK) and c-Jun NH_2-terminal kinases (JNKs) were activated. Subsequently, a 27-kDa heat shock protein (HSP27) became phosphorylated, and intracellular distribution of HSP27 was changed from the cytoplasm to the perinuclear region. These results indicate that the cellular signaling cascades activated by pro-inflammatory cytokines and environmental stresses are also activated as a result of lytic infection with SV. These responses may contribute to the delayed onset of apoptosis in the host cells and the facilitation of viral replication. © 1998 Academic Press

Sindbis virus (SV), the prototype of alphavirus genus (Togavirus family) is a single stranded positive-sense RNA virus. Its characteristics, such as the virion structure, genomic organization, and replication machinary, have been well elucidated (1). During replication of the virus, protein synthesis in the host cells are dramatically shut-off (1, 2), and cytopathic morphological changes, such as membrane blebbing, and both cytoplasmic and nuclear condensation, can be observed, which suggests SV causes host cell death in an apoptotic fashion (3, 4, 5). In many cell lines, SV causes lytic infection as described above. On the other hand, in some cells such as post-mitotic neurons, persistent productive infection is achieved (4, 6). Although SV is usually considered avirulent for humans, SV-related Western equine encephalitis virus causes encephalitis in humans (1). In neonatal mice, infection with most strains of SV causes fatal encephalomyelitis (3, 7). SV can be a good model for investigating the viral and host determinants of the outcome of infection (3).

Extracellular stimuli, including proinflammatory cytokines (such as tumor necrosis factor (TNF)-α and interleukin-1), toxic reagents (such as anticancer drugs) and other environmental stresses (such as heat shock, ultraviolet light, x-ray, and hydrogen peroxide), activate signaling pathways mediated by c-Jun NH_2-terminal kinases (JNKs) and p38 mitogen-activated protein kinase (MAPK) (8, 9, 10). They also induce synthesis of heat shock proteins (HSPs) (11), and phosphorylation and intracellular translocation of small heat shock protein HSP27 (12, 13). Such responses are thought to play important roles for maintaining cellular homeostasis.

Acute viral infection is generally assumed to induce stress responses in host cells (11). Actually, SV-infection stimulate synthesis of HSP70 (14, 15), but other stress responses induced by viruses have never been described in detail, and the biological significance of the stress responses for viral replication and maintenance of homeostasis of the host cells is still unclear.

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Abbreviations used: SV, Sindbis virus; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase; MAPKAPK-2, MAPK-activated protein kinase-2; HSP, heat shock protein; DMEM, Dulbecco's minimum essential medium; MOI, multiplicity of infection; PFU, plaque forming units; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

0006-291X/98 $25.00

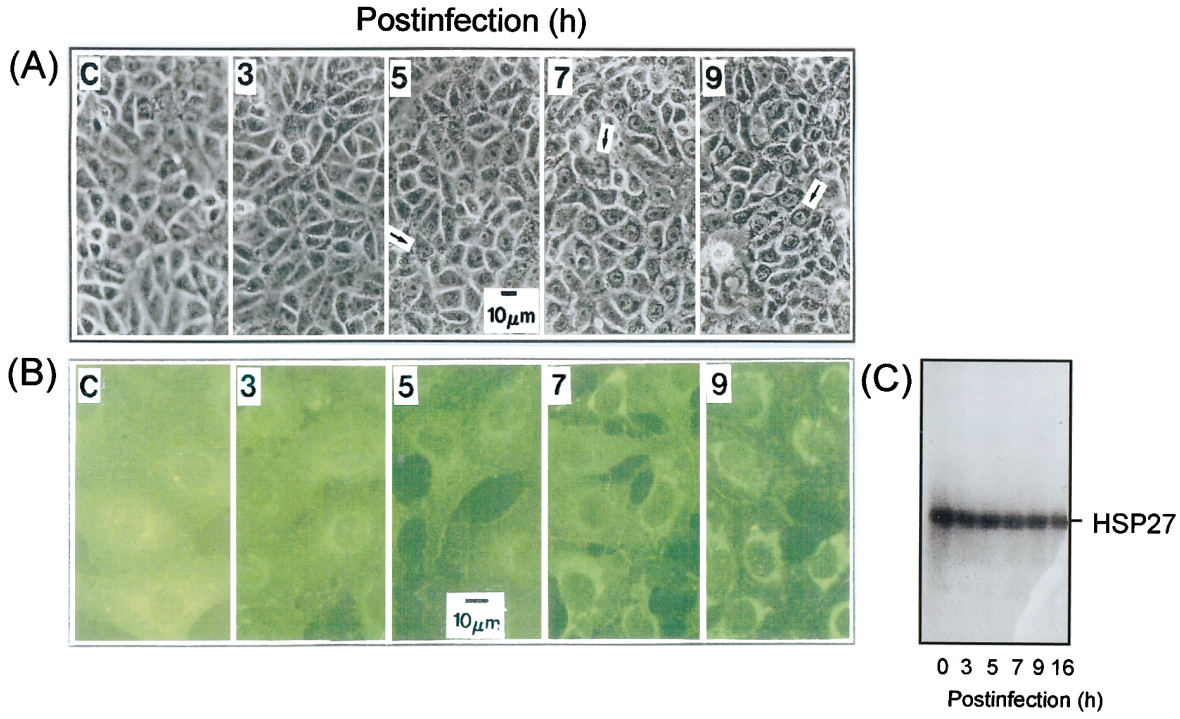

FIG. 1. Subcellular localization of HSP27 in SV-infected Vero cells. (A) Photomicrographs of SV-infected Vero cells at indicated times (hours after infection). Note that dense granular structures are seen in perinuclear region(arrowheads). C, uninfected control cells. (B) Perinuclear accumulation of HSP27 in SV-infected cells. Cells were fixed at indicated times (hours after infection) and immunostained with anti-HSP27. C, uninfected control cells. (C) Immunoblot detection of HSP27.

Here we report that infection of SV induced (i) intracellular translocation of HSP27 from cytoplasm to perinuclear region, (ii) phosphorylation of HSP27, (iii) activation of signaling pathways via stress-activated protein kinases such as JNKs and p38 MAPK, which resulted in phosphorylation of HSP27. Our results suggest viral infection induce stress responses in host cells, and these responses may modify the replication of viruses and host cell death.

MATERIALS AND METHODS

Cell culture. Vero cells at passage 120 were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum at 37°C and 5% CO_2 (16). In some experiments, actinomycin D (Sigma; St Louis, MO) or SB 203580 (Calbiochem; San Diego, CA) was added to the culture at 2 μg/ml or 10 μM, respectively. Labeling of cells with ^{32}P was performed by incubation for the appropriate time in phosphate-free DMEM with [^{32}P]H_3PO_4 (37MBq/ml in culture; NEN, 315 to 337 TBq/mmol), 10% of normal DMEM, and 10% dialyzed fetal bovine serum (GIBCO, Life Technologies Inc.; Gaithersburg, MD).

Viral infection and titration. SV (strain AR339) were obtained from the National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo, Japan. For propagation of viruses, Vero cells were infected with SV at multiplicity of infection (MOI) of 0.3 to 0.5 plaque forming units (PFU). For viral infections, viral suspension was incubated with 1×10^6 cells for 60 min at a MOI of 50 PFU (17). Viral titration was performed by plaque assay with methyl cellulose overlay.

Antisera and antibodies. Rabbit antisera specific for the phosphorylated and both phosphorylated and non-phosphorylated forms of ERK1 or ERK2, JNK1 or JNK2, and p38 MAPK were purchased from New England Biolabs (Beverly, MA). Goat polyclonal antibody to HSP27 were purchased from Santa Cruz Biotech (Santa Cruz, CA). Alkaline phosphatase-conjugated secondary antibodies were from New England Biolabs and Santa Cruz Biotech.

Protein analysis. For sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), cells were lysed with SDS sample buffer after several wash with Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS(−)). Approximately 100μg of total cell protein was loaded to each lane. Gels were stained with Coomassie brilliant blue R250.

For two-dimensional (2D-) PAGE, cells were lysed with sample buffer for the first-dimensional separation [8M urea, 2% Nonidet P40 (NP40), 5% β-mercaptoethanol, 10% glycerol, 0.4% ampholines (pH 5 to 7), 0.1% ampholines (pH 3 to 10)], and protein equivalent of 3×10^5 cells was analyzed as described previously (18).

For immunoblot analysis, the separated protein was transferred to polyvinylidene difluoride membranes (19), which were then incubated with 5% non fat dried milk in blocking buffer [50mM Tris-HCl (pH 7.4), 0.15M NaCl, 0.1% Tween 20] for 1 h at room temperature, with primary antibody at a dilution of 1:1500 in blocking buffer for 16 h at 4°C, and with alkaline phosphatase-conjugated secondary antibody at a dilution of 1:1500 for 2 h at room temperature. The membranes were then subjected to chemiluminescence reactions with CDP-star (New England Biolabs) and exposed to X-ray film (Kodak; Rochester, NY), or subjected to coloring reactions with NBT/BCIP (Boehringer Mannheim; Mannheim, Germany).

Immunofluorescence staining. Cells are seeded on 18mm diameter coverslips in 12-well dishes. At serial time points after infection, coverslips were removed, treated with 2% formalin for 20 min, and permealized with 1% NP40 in PBS(−). The staining procedures have been

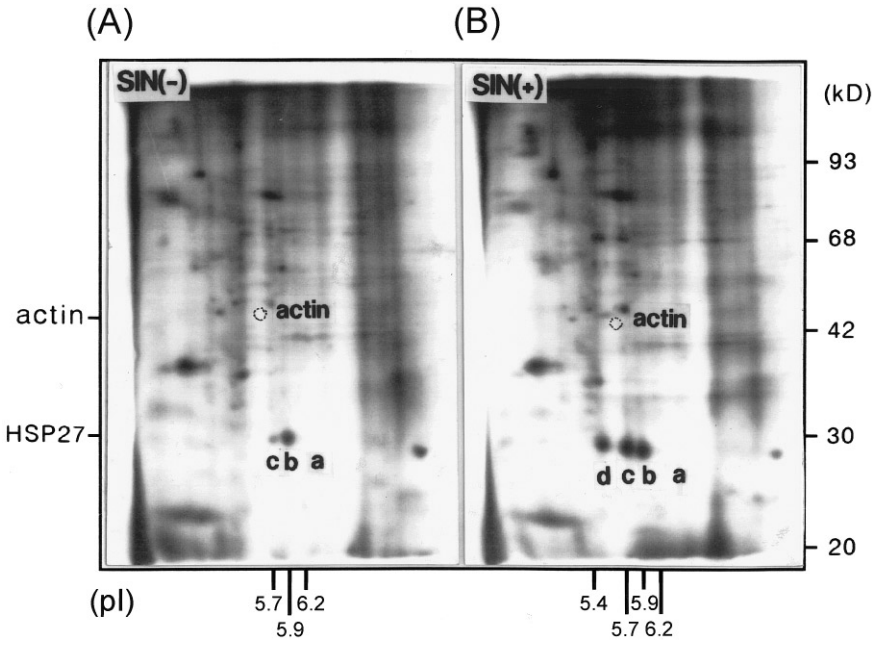

FIG. 2. Analysis of HSP27 phosphorylation in SV-infected cells by 2D-PAGE. Mock-infected (A) or SV-infected (B) cells were labeled for 2 h, between 3 and 5 h after infection, with $[^{32}P]H_3PO_4$. The cells were then solubilized with the sample buffer and analyzed by 2D-PAGE. Immunoblot analysis showed dots, denoted as a, b, c, and d, that were isoforms of HSP27 (data not shown). The position that corresponds to actin is indicated.

described (16). Fluorescein isothiocyanate-conjugated secondary antibody was purchased from Organon Teknika-Cappel (Durham, NC). The coverslips were mounted in Crystal Mount (Biomedia; Northfield, IL) and observed under a fluorescence microscope GB200 (Olympus; Melville, NY) with a laser-scanning confocal immunofluorescence microscopy system (Meridian Instrument; Kent, WA).

Protein kinase assay. Virus-infected or uninfected cells (1×10^7) were homogenizied with 15 strokes of a Teflon-glass homogenizer in lysis buffer [50mM β-glycerophosphate (pH 7.2), 5mM EGTA, 2mM EDTA, 1mM dithiothreitol, 2mM sodium orthovanadate, 10mM leupeptin, 10 μ g/ml aprotinin, 1mM phenylmethyl sulfonyl fluoride]. After centrifugation at 30,000g for 20min at 4°C, supernatant was applied to a MONO Q Column HR5/5 (Amersham Pharmacia Biotech; Uppsala, Sweden) equilibrated with lysis buffer without protease inhibitors. Proteins were eluted at a flow rate of 0.5ml/min with a linear gradient of NaCl (0 to 1M) in the same buffer. 30 fractions were collected and assayed for protein kinase activity with rn-HSP27 (Stress Gen; Victoria, British Columbia) as the substrate (20).

RESULTS

Time course of viral growth and induction of apoptosis. We first examined the relationship between time course of viral growth and of the onset of cell death induced by SV-infection. When Vero cells were infected with SV at a MOI of 50 PFU, production of progeny virions first became apparent at 3 h after infection. The viruses replicated rapidly and achieved maximum titers of 10^9PFU/ml at 9 h after infection in a single step multiplication cycle. The infected cells became rounded and shrunken by 5 h (Fig. 1A). These appearances represent early signs of cytopathic effects. Chromosomal DNA fragmentation and formation of blebs, characteristics of apoptotic changes (3), became apparent by the time viral growth was almost completed. Thus, there was a period of several hours between initiation of viral replication and the onset of apoptosis, during which maximum viral production was achieved. To reveal the mechanisms by which SV-infection induces delayed onset of apoptosis, we made the following studies.

SV-infection induced intracellular redistribution of HSP27. Light microscopy of SV-infected Vero cells revealed accumulation of dense granular structure in the periphery of the nucleus concomitant with the appearance of cytopathic effect (Fig.1A, arrowheads). Immunofluorescence staining of SV-infected Vero cells with antibody to HSP27 (anti-HSP27) showed that the anti-HSP27 staining became concentrated in the perinuclear region between 5 and 9 h after SV-infection (Fig.1B), while its distribution in non-infected cells was throughout the cytoplasm (Fig.1B). The staining pattern with anti-HSP27 in the SV-infected cells corresponded to the light-microscopic dense granular structure in the perinuclear region. Similar changes have been observed during heat shock and other non-viral cellular stresses (12, 13). Different from the responses induced by heat shock (12), however, the amount of HSP27 protein remained almost constant during SV-infection (Fig. 1C). These results indicate that stress responses, involving the redistribution of HSP27, were induced in the host cells by SV-infection.

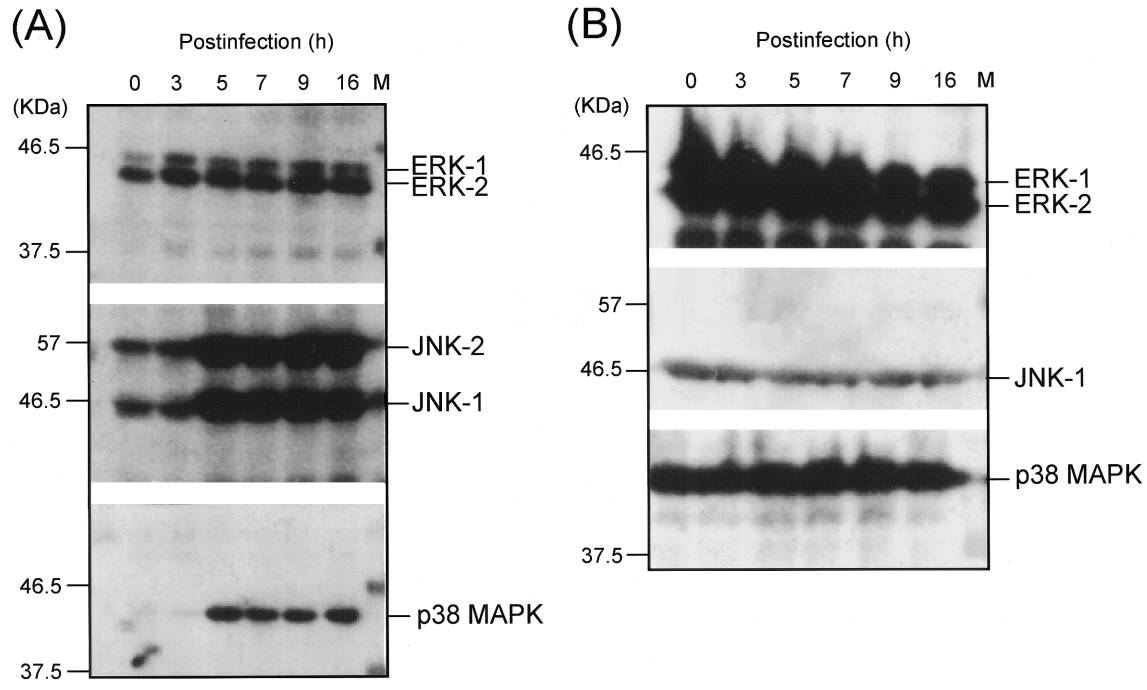

FIG. 3. Phosphorylation of MAP kinases in SV-infected Vero cells. At the indicated time after infection, cell lysates were subjected to SDS–PAGE and immunoblot analysis with antibodies specific to the phosphorylated (A) or both the phosphorylated and the nonphosphorylated (B) forms of MAPKs. Rabbit antisera specific to the phosphorylated form of MAPKs were reactive with ERK1 and ERK2 phosphorylated on Tyr204, JNK1 and JNK2 on Thr183 and Tyr185, and p38 MAPK on Tyr182, respectively. Lane M, size markers.

SV-infection induced phosphorylation of HSP27. HSP27 are known to be phosphorylated in response to various stimuli, including heat shock, growth factor, toxic reagents, and proinflammatory cytokines (12, 21). To elucidate whether SV-infection induces phosphorylation of HSP27, we carried out 2D-PAGE analysis. When SV-infected cells were labeled with [^{32}P]-orthophosphate for 2 h, between 3 to 5 h after infection, we detected three phosphorylated isoforms of approximately 27-KDa protein at pI value of 5.9, 5.7 and 5.4 (Fig. 2B, denoted as b, c and d, respectively). Immunoblot analysis showed these were isoforms of HSP27 (data not shown). The phosphorylated form at pI 5.9 was also detected in uninfected cells (Fig.2A). A non-phosphorylated form of HSP27 (pI 6.2, denoted as a) was detected by immunoblotting with anti-HSP27 (data not shown). These results indicate SV-infection induce phosphorylated isoforms of HSP27 at pI 5.7 and 5.4, which were shown to correspond to those phosphorylated at two and three sites, respectively (22).

SV-infection activated p38 MAPK and JNKs pathways. The signaling pathways activated by stress signals such as heat shock and cytokines have been well characterized (8, 9), and most stress signals induce activation of so-called stress-activated MAPKs such as p38 MAPK, which can result in phosphorylation of HSP27 (23-26). The phosphorylated isoforms of MAPKs induced by their own dual specific kinases (what we call MAP kinase kinases) correspond to the conformations in their active state (27, 28). We therefore examined the phosphorylation state of MAPKs with antibodies specific for the phosphorylated forms of those proteins. After SV-infection, the phosphorylation of p38 MAPK and JNKs were increased, while that of extracellular signal-regulated kinases (ERKs) remained almost constant during infection (Fig.3A). Especially, the phosphorylation of p38 MAPK started to be increased gradually at 3 h after infection, and at 9 h after infection, it reached a value 10 times of that in noninfected control cells (Fig.3A). Immunoblot analysis with antibodies recognizing both phosphorylated and non-phosphorylated forms of MAPKs showed that the amounts of p38 MAPK, JNKs and ERKs remained constant during SV-infection (Fig.3B).

We next examined the activity of MAP kinase-activated protein kinase (MAPKAPK)-2, because MAPKAPK-2 is one of the kinases directly activated by p38 MAPK and is capable of phosphorylating HSP27 (25, 26). The activity of MAPKAPK-2 in SV-infected cells was assayed with purified HSP27 as substrate, as previously described (20). At 5 h after infection, the activity of MAPKAPK-2 was increased fourfold (data not shown). These results indicate SV-infection induce activation of signaling pathways of p38MAPK and JNKs, but not that of ERKs, as observed in other stresses (8, 9).

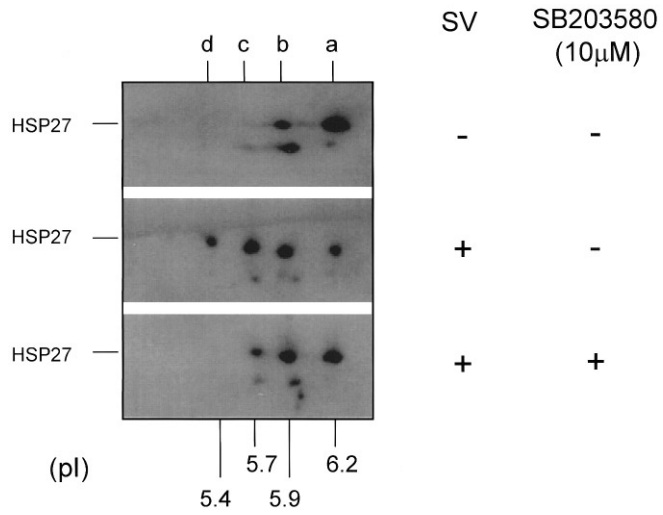

FIG. 4. Phosphorylation of HSP27 induced by SV infection in the presence of p38 MAPK-specific inhibitor SB 203580. Vero cells were incubated for 3 h with(+) or without(−) 10μM SB 203580 and were then infected with SV in the continued presence of the inhibitor. At 9 h after infection, cells were lysed and subjected to 2D-PAGE and immunoblotting with anti-HSP27.

Phosphorylation of HSP27 induced by SV-infection were dependent on activation of p38 MAPK pathway. To confirm phosphorylation of HSP27 is dependent on p38 MAPK pathway in this system, we examined SV-induced phosphorylation of HSP27 in the presence of p38 specific inhibitor, SB 203580 (26). As shown in Fig. 4, treatment of SV-infected Vero cells with 10μM SB 203580 caused disappearance of the isoform at pI 5.4 and decrease of that at pI 5.7, compared with non-treated cells infected with SV. This result indicates phosphorylation of HSP27 induced by SV-infection is mostly dependent on activation of p38 MAPK pathway.

Effects of actinomycin D on viral replication and cellular responses. Although Vero cells are not known to produce interferon-α(29), other proinflammatory cytokines induced by viral infection may have triggered the responses observed above. To examine this possibility, Vero cells were infected with SV in the presence of transcriptional inhibitor actinomycin D. However, intracellular translocation of HSP27 and phosphorylation of p38 MAPKs and JNKs were still inducible by SV-infection (data not shown). Viral replication was often enhanced in the presence of actinomycin D, presumably due to the facilitation of the virus-induced shutdown of protein synthesis in the host cells. Actinomycin D by itself did not induce redistribution of HSP27 and phoshorylation of p38 MAPK and JNKs. (data not shown).

DISCUSSION

In the present study, we have shown that acute infection of SV directly induce phosphorylation and intracellular translocation of HSP27, and activation of JNKs and p38 MAPK. These phenomena are also inducible by proinflammatory cytokines, toxic reagent, and other environmental stresses (9, 12). In our observations, other single-stranded RNA viruses such as poliovirus and Japanese encephalitis virus also induced very similar stress responses (data not shown). So, these results described here enable us call these viruses as “stress-inducible agents”. Further studies with different viruses and host cells would be required to generalize this concept.

Intracellular physiological signaling pathway could modulate apoptotic changes induced by SV. For example, a dominant negative form of Ras protein delays the onset of apoptosis in neuronal PC12 cells (30). SV-induced p38 MAPK activation, and resulting HSP27 phosphorylation observed here may also play a role for delaying SV-induced cell death. In transfection studies, overexpression of wild-type HSP27 confer cellular resistance to cytotoxicity induced by TNF-α, anti-Fas antibody, oxidative stress, and hyperthermia (12, 21, 31-34). On the other hand, cells expressing non-phosphorylatable mutant of HSP27 were much less resistant to heat shock (34). These results indicate phosphorylation of HSP27 is essential for its protective function against environmental stresses. Actually, Vero cells infected with SV in the presence of SB 203580 tended to die faster than in the absence of the inhibitor (unpublished observation). Due to delaying onset of apoptosis by activating p38 MAPK pathway, viral production might be facilitated. Further analyses are required to evaluate this hypothesis. The use of inhibitors specific for p38 MAPK would be a helpful strategy for these studies.

If these stress responses, which induce HSP27 phosphorylation and translocation, indeed confer resistance against SV-induced cell death, modulation of these stress signaling pathway could have therapeutic potentiality for the virus-induced diseases. While wildtype neonatal mice are susceptive to SV-induced encephalomyelitis, neonatal mice expressing anti-apoptotic human Bcl-2 protein in neurons were resistant (3, 7). This observation suggests that protection from neural cell death can prevent mice from the fatal SV-induced encephalomyelitis. On the other hand, the nature of acquired immunodeficiency syndrome (AIDS) caused by infection of human immunodeficiency virus (HIV) is recently assumed to be the depletion of CD4-positive T-lymphocytes by the apoptotic changes induced by HIV infection (35-37). So anti-apoptotic medication could be a fascinating therapy for virus-induced diseases. In these regards, further studies would be necessary to reveal the mechanism of stress responses induced by viruses, which may result in the protection of cell death.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan and from Otsuka Pharmaceutical Co., Ltd.

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