Enterovirus 71 triggering of neuronal apoptosis through activation of Abl-Cdk5 signalling

Tsan-Chi Chen,1,2 Yiu-Kay Lai,2,3 Chun-Keung Yu4 and Jyh-Lyh Juang1,5*
1Division of Molecular and Genomic Medicine, National Health Research Institutes, Miaoli, Taiwan.
2Department of Life Science and Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan.
3Department of Bioresources, Da-Yeh University, Changhua, Taiwan.
4Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan.
5Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan.

Summary

The molecular mechanism behind what causes an infection of Enterovirus 71 (EV71) in young children to result in severe neurological diseases is unclear. Herein, we show that Cdk5, a critical signalling effector of various neurotoxic insults in the brain, is activated by EV71 infection of neuronal cells. EV71-induced neuronal apoptosis could be effectively repressed by blocking either Cdk5 kinase activity or its protein expression. Moreover, EV71-induced Cdk5 activation was modulated by c-Abl. The suppression of c-Abl kinase activity by STI571 notably repressed both the Cdk5 activation and neuronal apoptosis in cells infected with EV71. Although EV71 also induces apoptosis in non-neuronal cells, it did not affect Abl and Cdk5 activities in several non-neuronal cell lines. Intriguingly, coxsackievirus A16 (CA16), a genetically closely related serotype to EV71 that usually does not induce severe neurological disorders, could only weakly stimulate Abl, but not Cdk5 kinase activity. Taken together, our data suggest a serotype- and cell type-specific mechanism, by which EV71 induces Abl kinase activity, which in turn triggers Cdk5-signalling for neuronal apoptosis.

Introduction

Enterovirus 71 (EV71), a ss-(+)-RNA virus of the Picornaviridae family, was first identified in California in 1969 (Schmidt et al., 1974). Since then, many sporadic outbreaks of hand-food-and-mouth diseases (HFMD) associated with EV71 have occurred worldwide, particularly in many Asia-Pacific countries and in some non-Asian countries such as Brazil (Castro et al., 2005), Bulgaria (Melnick et al., 1980) and Germany (Kehle et al., 2003). Infection by EV71 in young children may cause severe neurologic diseases, including acute flaccid paralysis, aseptic meningitis, encephalitis and encephalomyelitis (Chang et al., 1999a; Huang et al., 1999; Chang et al., 2004a). Although some early molecular diagnostic methods of detecting EV71 have recently been developed (Singh et al., 2002; Chen et al., 2006b), no specific therapy has been developed to treat neurological diseases induced by EV71, in part due to the fact that the molecular mechanism by which EV71 induces neuronal apoptosis remains elusive.

Cdk5 is a serine/threonine protein of cyclin-dependent kinase (Cdk) (Lew et al., 1994), which expresses mainly in terminally differentiated neurons (Nguyen et al., 2002). In addition to the pleiotropic roles it plays in promoting and regulating normal neuronal functions and nervous system development (Philpott et al., 1997; Zhang et al., 1997; Gilmore et al., 1998), Cdk5 also functions as an important cellular effector of various neurotoxic insults when regulating neuronal apoptosis, such as under oxidative stress and amyloid accumulation (Butterfield et al., 2001; Gong et al., 2003). In these cases, Cdk5 kinase activity is elevated, so the pharmacological inhibition of Cdk5 kinase can attenuate neuronal apoptosis. Because of its potentially deleterious effects, Cdk5 kinase activity is tightly regulated in neuronal cells. Like other Cdk family proteins, Cdk5 forms a complex with a neuron-enriched regulator, p35, to regulate its kinase activity. Thus, either elevation of p35 expression level (Tsai et al., 1994; Chen et al., 2004) or the formation of p25, a cleaved form of p35, results in a more stable Cdk5 complex (Patrick et al., 1999; Kusakawa et al., 2000; Lee et al., 2000), promoting Cdk5 kinase activity in neuronal cells.

Although the association of either p35 or p25 with Cdk5 is needed to activate Cdk5 kinase, the association alone cannot fully stimulate Cdk5 activity. The phosphorylation of Cdk5 at tyrosine-15 (Cdk5-Y15) is also a critical...
regulatory event that further triggers Cdk5/p35 kinase activity during neuronal development or neuronal pathogenesis. In neuronal development, phosphorylation of Cdk5-Y15 in the Abl/Cables/Cdk5 trimolecular complex enhances Cdk5 kinase activity (Cowan and Henkemeyer, 2000; Zukerberg et al., 2000). In amyloid-triggered neuronal apoptosis, Abl phosphorylation of Cdk5-Y15 also plays a vital role in deregulating Cdk5 kinase (Lin et al., 2007). Similar to the deregulation of Cdk5, aberrant Abl activity during neuronal development or neuronal pathogenesis can stimulate Cdk5 kinase activity and protein subcellular localization in blood cells lead to leukaemia (Wetzler et al., 1993). Although both Abl and Cdk5 function in response to environmental stress, it remains unclear whether they are involved in virus-induced neuronal apoptosis.

This study tested the hypothesis that the infection of EV71, by analogy of neurotoxic insult, might deregulate Cdk5 activity and consequently result in neuronal apoptosis. We demonstrated that EV71 could stimulate c-Abl activity, which then led to the phosphorylation and activation of Cdk5 in neuronal cells. More importantly, we found that blockade of kinase activation of either protein could effectively ameliorate neuronal apoptosis. Our data provide a coherent picture of the regulation of Abl and Cdk5 activities in neuronal cells during EV71 infection. These findings may eventually lead to the development of targeted pharmacological strategies that can prevent the neuronal complications sometimes associated with severe HFMD.

Results

Activation of Cdk5 is essential for EV71-induced neuronal apoptosis

Previous studies have found EV71 infection induced cell apoptosis in non-neuronal cell culture systems, including in T lymphocyte cells (Chen et al., 2006a), lung cells (Wong et al., 2005) and muscle cells (Leong and Chow, 2006). To determine whether EV71 induced apoptosis in neuronal cell cultures, we infected SH-SY5Y cells (human neuroblastoma) with 1 moi (multiplicity of infection) of EV71. By using TUNEL staining, apoptotic cells were detected at 6 and 24 h post infection (hpi) (Fig. 1A, middle and right panels). Uninfected cells showed no clear staining pattern (Fig. 1A, left panel).

To test whether Cdk5 was involved in EV71-induced neuronal apoptosis, we first investigated whether EV71 affected the kinase activity of Cdk5. Using histone H1 as a substrate of Cdk5 kinase for in vitro kinase assay, we measured Cdk5 kinase activity in SH-SY5Y cells. Ten minutes post infection (infection time = 1 h) with 1 moi of EV71 resulted in a rapid induction of Cdk5 kinase activity (Fig. 1B). Within 3 hpi, the Cdk5 activity levelled off to an activity level about five times greater than that found in the control. To confirm the activation of Cdk5 was triggered by the infection of EV71, we compared Cdk5 activity in live viruses with that in heat-inactivated viruses, and found that the heat-inactivated virus did not stimulate the Cdk5 kinase activity (Fig. 1B, lane 2 of the upper panel). These results suggest that the EV71 infection elicited Cdk5 kinase activity in SH-SY5Y neuronal cells.

We used three different Cdk5 kinase inhibitors (rosco-vitine, Ros; indirubin 3'-oxime, IO; and olomoucine, Olo) to investigate whether Cdk5 needed to be activated before EV71 could trigger neuronal apoptosis. The Cdk5 kinase activity was suppressed by these kinase inhibitors at 6 hpi when the inhibitors were added before infection of EV71 (moi = 1) (Fig. 1C). Moreover, all three kinase inhibitors significantly prevented the apoptosis triggered by 1 moi of EV71 as assessed by WST-1 viability assay and TUNEL staining. Specifically, the cell viability of inhibitor-treated cells was approximately 80% compared with 8.4% in cells not treated with the inhibitors at 48 hpi (Ros, P-value 0.013; IO, P-value 0.005; Olo, P-value 0.007) (Fig. 1D). TUNEL staining at 24 hpi also revealed the similar results (Fig. 1E). These data clearly indicate that activated Cdk5 played an important role in promoting EV71-triggered neuronal apoptosis.

Enterovirus 71 triggers neuronal apoptosis via Abl-dependent Cdk5 Tyr15 phosphorylation

Because phosphorylation of Cdk5-Y15 (Cdk5-pY15) by Abl can stimulate Cdk5 kinase activity (Zukerberg et al., 2000), we investigated whether EV71 triggered Cdk5 activation via this process. Western blotting with an antiphospho antibody specific for Cdk5-pY15 showed that Cdk5 in EV71-infected cells produced 3.8 times the Cdk5-pY15 signal at 3 hpi than that in the uninfected cells (Fig. 2A). Heat-inactivated EV71 showed no such effect. Furthermore, we investigated whether or not EV71 would stimulate the formation of p25 and found that there was no detectable increase in p25 and no obvious decrease in p35 in cells infected with EV71 (Fig. 2B). These results suggest that EV71 infection causes an elevation in Cdk5-pY15 concurrent with the activation of Cdk5, but not with the formation of p25.

To test whether the elevation of Cdk5-pY15 was a result of the activation of Abl kinase activity, we examined the c-Abl kinase activity in EV71-infected SH-SY5Y cells. Using GST-CrkII as a substrate of c-Abl kinase for in vitro kinase assay, we found c-Abl kinase activity was notably increased in infected SH-SY5Y cells, where it levelled off around 3 hpi (Fig. 2C). c-Abl kinase activity did not increase in cells treated with heat-inactivated EV71. These results suggest that EV71 infection of SH-SY5Y cells induced kinase activity in both Abl and Cdk5. Moreover, similar to the results obtained using SH-SY5Y cells,
Fig. 1. EV71 triggers Cdk5 kinase activity and the blockade of Cdk5 activation suppresses the neuronal apoptosis.

A. EV71 caused neuronal apoptosis in SH-SY5Y cells. Fluorescent TUNEL staining indicated a dramatic increase of apoptotic cells induced by 1 moi of EV71 at 6 and 24 hpi. No obvious TUNEL signal was detected in the uninfected cells. Lower panels are phase images.

B. Assay of Cdk5 kinase activity over the post infection time-course. Immunocomplex using an anti-Cdk5 antibody was subjected to in vitro kinase assay using histone H1 as a substrate.

C. Several Cdk5 kinase inhibitors could effectively suppress Cdk5 kinase activity in SH-SY5Y cells. Cdk5 activity was analysed at 6 hpi by in vitro kinase assay in EV71-infected cells treated with or without kinase inhibitors. The concentration of the inhibitors used in this assay were 15 μM roscovitine (Ros), 100 nM indirubin 3'-oxime (IO) and 50 μM olomoucine (Olo).

D. WST-1 assay revealed a suppression of EV71-induced neuronal apoptosis by Cdk5 kinase inhibitors. Relative viability of infected cells was measured at 48 hpi.

E. Fluorescent TUNEL staining showed a marked reduction of EV71-induced neuronal apoptosis by Cdk5 kinase inhibitors (the first row) at 24 hpi. No noticeable TUNEL signal was detected in cells treated with inhibitor alone (the third row). The second and the last rows are phase images. Shown is the mean of three independent experiments ± SEM, and P-values are indicated above bars versus infected cells: *P < 0.05; **P < 0.01.
EV71 infection in two other neuronal cells, SVG p12 and IMR32, also induced kinase activities for c-Abl and Cdk5 at 6 hpi (Fig. 2D), suggesting the activated Abl might cause the increase of Cdk5-pY15 signal during the EV71 infection.

To further confirm this hypothesis, we studied whether suppressing Abl kinase activity with STI571 (also known as Gleevec or Imatinib Mesylate) would suppress the EV71-induced Cdk5 kinase activity. This was determined by measuring Cdk5-pY15 level and the in vitro kinase activity of Cdk5. We found that STI571 markedly suppressed EV71-induced Cdk5 kinase activity and Cdk5-pY15 signal at 6 hpi (Fig. 3A), suggesting that EV71-triggered Cdk5 kinase activity could be regulated via the Abl phosphorylation of Cdk5-pY15 in neuronal cells. Because EV71-induced neuronal apoptosis could be suppressed by blocking the activation of Cdk5, we wanted to test whether the apoptosis could be also rescued by inhibiting the activation of Abl. After pretreating the infected SH-SY5Y cells with 15 μM of STI571, we compared the...
Fig. 3. Abl is essential for initiation of Cdk5 kinase activity and EV71-induced neuronal apoptosis.
A. Suppression of Abl kinase activation with STI571 prohibited the EV71-induced Cdk5 activation. SH-SYSY cells were treated with 15 μM of STI571 and harvested at 6 hpi for the in vitro kinase assay of Abl and Cdk5, using GST-CrkII and histone-H1 as the substrates respectively.
B. STI571 significantly rescued the cell viability upon EV71 infection at 24 hpi as determined by WST-1 assay.
C. TUNEL staining of neuronal cells treated with STI571. EV71-induced neuronal apoptosis was marked abrogated by STI571 at 24 hpi. No evident TUNEL signal was detected in the STI571-treated cells. Lower panels are phase images.
D. Disruption of c-Abl expression effectively diminished the EV71-induced kinase activity of c-Abl and Cdk5 and phosphorylation of Cdk5-Y15. Cells transfected with shabl or shcdk5 at 6 hpi were subjected to in vitro kinase assays with the indicated substrates.
E. Using shRNA to knockdown protein expression of c-Abl or Cdk5 suppressed EV71-induced neuronal apoptosis. Relative cell survival rate was determined by WST-1 assay for EV71-infected cells transfected with or without shRNA plasmids at 48 hpi.
F. TUNEL staining of neuronal cells treated with shabl or shcdk5 at 24 hpi. Lower panels are phase images. Shown is the mean of three independent experiments ± SEM, and P-values are indicated above bars versus infected cells: *P < 0.05.
cell viability of the treated and untreated infected cells at 24 hpi by WST-1 assay, and found the STI571-treated cells to have significantly higher cell viability than the untreated ones (75.3% vs. 34.3%; P-value 0.032) (Fig. 3B). Moreover, the TUNEL staining at 24 hpi also showed the similar results (Fig. 3C). This finding suggests that the activation of Abl is linked to the induction of Cdk5 kinase activity and neuronal apoptosis.

To exclude the possibility that these compounds had a non-specific drug effect, we transfected the lentiviral short hairpin RNAi (shRNA) constructs to repress the expression of Abl and Cdk5. Immunoblotting showed that the shRNA-abl (shabl) and shRNA-cdk5 (shcdk5) efficiently reduced the expression of endogenous c-Abl and Cdk5 by approximately 90% and 80%, respectively (data not shown). As was found in our experiments with STI571, the reduction in c-Abl expression resulted in the suppression of both c-Abl and Cdk5 kinase activities as well as Cdk5-pY15 level (Fig. 3D, lane 4). In contrast, a reduction of Cdk5 expression only repressed the Cdk5 activation but did not noticeably affect c-Abl kinase activity (Fig. 3D, lane 6), further supporting the idea that Abl is the upstream regulator of Cdk5 kinase during EV71 infection. Moreover, shRNA knockdown of c-Abl or Cdk5 effectively rescued the cell viability of the EV71-infected cells at 48 hpi from 9.1% back to 69.7% (P-value 0.033) and 82.2% (P-value 0.012), respectively (Fig. 3E). In accord with the WST-1 assay, the TUNEL staining also revealed the similar results (Fig. 3F). Taken together, these results suggest that Abl-dependent phosphorylation of Cdk5 is required for the initiation of EV71-induced neuronal apoptosis.

**Enterovirus 71-induced Abl-Cdk5 activation is independent of viral transcription and replication**

To provide some insight into the mechanism that underlies the EV71-induced Abl-Cdk5 activation, we UV irradiated the EV71 before the infection of target cells because UV irradiation can eliminate the virus transcription and replication while maintaining the capability to bind to the cell surface receptor molecules and the function of endocytosis (Beck et al., 1990). As shown in Fig. 4, the UV-inactivated EV71 still effectively initiated both the c-Abl and Cdk5 kinase activities in neuronal cells at 6 hpi as determined by in vitro kinase assay. Because Abl kinase activity has been demonstrated to be stimulated by the interaction of cellular ligands with the receptors, including PDGF receptor or adhesion receptors (Hernandez et al., 2004), our result raises a possibility that the EV71 interaction with the host surface protein or receptor might involve in the initiation of Abl and/or Cdk5 kinase activity. In accord with this idea, a recent study has shown that the interaction of coxsackievirus B3 (CVB3) with a cell surface protein, DAF (GPI-anchored protein decay-accelerating factor) can activate Abl kinase, resulting in the alteration of intercellular junctional structures between epithelial cells and enhancement of virus entry (Coyne and Bergelson, 2006). In addition to the initiation of Abl kinase, the UV-inactivated CVB3 can also activate ERK1/2 kinase (Luo et al., 2002) or induce production of chemokines (Shen et al., 2004) and cytokines (Henke et al., 1992) in different cell types. Taken together, these and our data suggest that some enterovirus-mediated cellular responses, including apoptotic and cytotoxic responses might be initiated at the early phase of infection, when virus just attaches onto the host cell surface.

**Enterovirus 71 does not trigger Abl-Cdk5 signalling in non-neuronal cells**

In addition to neuronal cells, EV71 also triggers apoptosis in non-neuronal cells. Therefore, it was important to investigate whether EV71 would also activate Abl-Cdk5 signalling to trigger apoptosis in non-neuronal cells. Using TUNEL staining (Fig. 5A) and WST-1 assay (Fig. 5B), we demonstrated that EV71 did induce apoptosis in the non-neuronal cells tested, including Hs 840.T (pharynx cells), MRC5 (lung cells) and RD (muscle cells). However, the in vitro kinase assays did not show the infection of EV71 (moi = 1) to these cells affected the kinase activity of c-Abl and Cdk5 at 6 hpi (Fig. 5C), suggesting that EV71 did not trigger the Abl-Cdk5 signalling in non-neuronal cells.
Coxsackievirus A16 is less potent in stimulating Abl kinase activity and does not trigger Cdk5 kinase

Coxsackievirus A16 (CA16) is genetically close to EV71 and is also a major aetiological agent for HFMD in the Asia-Pacific region, but the virus shows much less propensity to cause neurological disease during acute infection (Chang et al., 1999b). Thus, it is of interest to investigate whether CA16 could stimulate the activation of Abl and Cdk5 in neuronal cells. To compare the effect of these two serotypes on kinase activities, we first had to measure the virus titre. Using 100-fold serial dilutions of control plasmid as the normalization standard, we were able to use quantitative real-time polymerase chain reaction (PCR) (with a set of pan-enterovirus-specific primers) to define the absolute virus copy number for each serotype (Fig. 6A). With this information, we infected SH-SYSY cells with low (moi = 1) or high (moi = 10) titres of CA16 and used them in the in vitro kinase assay. Time-course analysis showed that the low virus titre of CA16 could only temporarily elevate the c-Abl kinase activity at some late post infection time point (between 18 and 24 hpi). A low virus titre of EV71 or a high virus titre of CA16, however, stimulated the c-Abl kinase activity at 6 hpi (Fig. 6B, upper panel). Unlike EV71, CA16-induced c-Abl kinase activity did not appear to trigger subsequent Cdk5 kinase activity. Nor did we observe any alteration of Cdk5-pY15 signal or Cdk5 kinase activity during the extended post infection time periods (Fig. 6B, third and fourth panels).

To investigate whether Abl activation itself by CA16 triggers apoptosis in neuronal cells, we suppressed Abl protein expression or kinase activity before infecting cells with CA16 (moi = 1). However, we found that neither treat-
ment noticeably rescued the CA16-induced neuronal apoptosis (Fig. 6C), suggesting that the CA16-induced Abl kinase, unlike the EV71-induced one, was not essential for the neuronal apoptosis. This finding further supports our idea that the Abl-Cdk5 signalling pathway is a serotype-specific mechanism for the EV71-induced apoptosis in neuronal cells.

Although CA16 is considered less virulent than EV71 to neuronal cells, its diminished ability to induce Abl and Cdk5 activities might be also due to the lower viral load or reduced replication capacity. To test this, we analysed the copy number of intracellular viral transcript over the post infection period. Using real-time PCR analysis, we found the initial viral load of these two serotypes of virus to be

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd, Cellular Microbiology, 9, 2676–2688
fairly equivalent. After 12 hpi, EV71 had slightly higher replication ability than CA16 (Fig. 6D). This finding suggests that the diminished ability of CA16 to induce Abl and Cdk5 activities was not primarily due to actual viral load in cells.

Because CA16 infection did not vigorously regulate Cdk5, such an infection might not cause neuronal cell death. To test this possibility, we compared the cell viability of EV71- and CA16-infected SH-SY5Y cells over the post infection period. Using WST-1 assay, we found that neuronal cells possessed significantly greater resistance to CA16-induced apoptosis (76.3%) than they did to EV71-induced apoptosis (38.6%) at 24 hpi (Fig. 6E). However, at a prolonged stage of post infection (48 hpi), both serotypes of virus caused severe neuronal apoptosis. Although the molecular mechanism underlying the CA16-induced apoptosis has not been investigated, our results suggest that the activation of Abl-Cdk5 signalling does not seem to be essential to CA16-induced neuronal apoptosis. Moreover, this observation also suggests the possibility that there are two different signalling pathways involved in the regulation of the neuronal apoptosis triggered by these two serotypes.

**Discussion**

Several lines of evidence indicate that neurotoxicity enhances Cdk5 activity, leading to pathological neuronal death (Smith and Tsai, 2002). One famous example is the neurotoxic insult of amyloid β (Aβ) which results in the degeneration of neurons found in Alzheimer’s disease (AD) (Butterfield et al., 2001). By analogy, the neurotoxic insult of EV71 might deregulate Cdk5 activity, leading to neuronal apoptosis. This study tests this hypothesis. Our findings were very similar to those found in previous studies of neurodegenerative diseases. We found that in neuronal cells EV71 infection induced Abl activity, which subsequently triggered phosphorylation and activation of Cdk5. This was confirmed when we blockaded c-Abl activation. Activation of Cdk5 was prevented and apoptosis was rescued. Conversely, repression of Cdk5 activity or protein expression only repressed the neuronal apoptosis but did not affect c-Abl kinase activity. These findings support the idea that Abl's modulation of Cdk5 kinase activity might play an important role in the EV71 pathogenesis in nervous system. Therefore, the targeting of Abl and Cdk5 by EV71 may be required for neuronal apoptosis, although the pathway by which EV71 activates Abl kinase requires further investigation.

Although our data support the idea that the molecular mechanism of EV71-induced pathological apoptosis in neuronal cells is similar to that induced by amyloid accumulation, it remains unclear whether it holds true in other tissue cell types. It is reasonable that different cell types might use different signalling pathways in response to the same virus, and that the outcomes might be different. To test this idea, we used three different non-neuronal cell lines to investigate whether an EV71 infection would affect the kinase activity of c-Abl or Cdk5. Neither the kinase activity of c-Abl nor that of Cdk5 was actively stimulated, indicating that the activation of Abl-Cdk5 signalling is more related to apoptotic signalling in neuronal cells than in other types of cells.

However, from this conclusion it cannot be inferred that the activation of Abl alone is enough to initiate the apoptotic signalling nor can it be inferred that activated Abl will always trigger Cdk5 kinase activity during an enterovirus infection. Our study of the kinase response to CA16 infection addressed these issues. CA16 is not a highly virulent serotype and it usually only causes mild, self-limited case of HFMD. Therefore, we wanted to investigate whether or not CA16 would trigger Abl-Cdk5 signalling for neuronal apoptosis. We found that CA16 also caused neuronal apoptosis and eventually stimulated c-Abl kinase activity after prolonged post infection period. Nevertheless, the kinase activity of Cdk5, the downstream target for Abl, did not increase. Moreover, the blockade of Abl activation by inhibitor or RNAi did not seem to suppress the CA16-induced apoptosis in neuronal cells. It appeared that the enterovirus infection in neuronal cells does not necessarily trigger Abl-Cdk5 kinase activity for apoptosis, or the delayed activation of Abl kinase during the CA16 infection is actually an indirect response to the secondary intracellular stress. Accumulated evidence indicates that Abl functions as a stress sensor for oxidative and genotoxic stimuli, particularly the reactive oxygen species (ROS). It has been observed that, during the virus infection, cells are induced to produce intracellular ROS (Schwarz, 1996). In response to ROS stress, c-Abl is activated and trans-localized to mitochondria to modulate cell death (Ito et al., 2001). However, multiple components or downstream signalling pathways may be involved in determining the final outcome of the activation of Abl kinase (Shaul, 2000). Signals downstream of c-Abl activation include many critical cellular effectors, such as stress-activated protein kinase (Kharbanda et al., 1995), PKD (Storz et al., 2004), Rac/JNK (Boureux et al., 2005) and p38 mitogen-activated protein kinase (Pandey et al., 1996). Although additional studies are needed to further elucidate the comprehensive cellular response programs induced by CA16, we believe that the above findings indicate that the CA16-induced apoptotic signalling in neuronal cells probably comes about through a mechanism that is independent of Abl-Cdk5 pathway.

In general, infection with less virulent serotypes of enterovirus typically results in a milder disease without severe neurological implications (Muir and van Loon, 2001). By analogy, the neurotoxicity caused by these two serotypes.
EV71-induced Abl-Cdk5 signalling for neuronal apoptosis

Given the fact that CA16 is not as neurotropic as EV71 in neuronal cells (Fig. 6E), we wanted to test whether this difference was because it was less virulent or productive than EV71 in neuronal cells. We found the replication of these two viruses was comparable during the post infection period, but EV71 triggered an earlier apoptotic response than that by CA16 (Figs 6B and 6E). Therefore, the severity of apoptosis might not rely on virus load as much as on host–virus interaction. If so, apoptosis-inducing kinases may be important in determining viral resistance and severity of infection in vivo. Therefore, this study provides clues for the future exploration of differences that may be found in the molecular pathological mechanisms behind infection by EV71 compared with that of CA16.

Two cellular proteins, namely FasL and caspase 9, have been shown to be activated in the EV71-triggered apoptotic cascade in Jurkat T cells (Chen et al., 2006a) and SK-N-MC cells (human neuroblastoma) (Chang et al., 2004b), respectively. However, these two proteins are involved in the modulation of most downstream effector phase of the apoptotic cascade that might be common in most apoptotic events. Moreover, these studies did not address whether inhibition of these proteins could prevent EV71-induced apoptosis.

Host-virus interplay may be the base for many of multifaceted outcomes of virus infection. Thus, understanding how and why viruses cause disease in specific host cells is essential to obtaining a deeper understanding of EV71-induced neuronal apoptosis. While the impact of variation in serotypes or cell types on the severity of virus infection may be expected, we have focused on the importance of Abl-Cdk5 signalling pathway for EV71-induced neuronal apoptosis. Because our study found that pharmaceutical suppression of Abl or Cdk5 kinase activity could effectively inhibit the EV71-induced neurotoxicity or Cdk5-mediated neuronal loss, it may provide new potential therapeutic targets for development of therapeutic agents. Our findings also emphasize the importance of host-virus interplay in the manifestation of enterovirus infections.

Experimental procedures

Viruses and cell cultures

Enterovirus 71 strain TW2272/98 (GenBank Accession no. AF119795) and CA16 (Shih et al., 2003) were propagated in RD cells (human embryonal rhabdomyosarcoma, CCL-136), which were maintained in MEM supplemented with 4.5 g l⁻¹ glucose and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco) without antibiotics.

Six cell lines, obtained from American Type Culture Collection (ATCC), including SH-SY5Y cells (human neuroblastoma, CRL-2266), SVG p12 cells (human astroglia, CRL-8621), IMR32 cells (human neuroblastoma, CCL-127), Hs 840.T cells (human pharynx papilloma, CRL-7573), MRC-5 cells (human embryonal lung, CCL-171) and RD cells, were grown as a monolayer in a proper media as described in ATCC, supplemented with 10% FBS, 20 U ml⁻¹ penicillin and 20 µg ml⁻¹ streptomycin (Gibco) and maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO₂ until infected with a virus.

Virus infection

Cells were grown to 70–80% confluence before infection. For virus absorption, the cells were infected for 1 h with virus at an moi of 1, or equal volume of inactive virus (by heat inactivation at 65°C for 30 min or by UV irradiation at 200 mJ cm⁻²) in serum-free medium. After 1 h infection, the infected cells were washed with phosphate-buffered saline (PBS) and cultured in proper medium before harvesting at the indicated time points. For kinase inhibition assay, SH-SY5Y cells preincubated with kinase inhibitors for 24 h before infection were coincubated with the same concentration of inhibitors during and after the infection before harvesting at the indicated time points.

Western blotting

For Western blotting, cell lysates were prepared with lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 137 mM NaCl, 50 µM EDTA, protease inhibitor mixture and 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 14 000 g for 10 min at 4°C, supernatants were collected and mixed with an equal volume of protein sample buffer (2x, 120 mM Tris-HCl, pH 8.0, 20% Glycerol, 4% SDS, 2.5% β-mercaptoethanol and 0.05% bromophenol blue). Lysates were separated by SDS-PAGE gel and transferred to nitrocellulose membrane (Millipore). The membrane was blocked with blocking buffer containing 5% BSA in TBST (20 mM Tris-HCl, pH 7.5, 500 mM NaCl and 0.1% Tween 20) for 1 h at room temperature and then incubated overnight at 4°C with the appropriate primary antibody diluted in the blocking buffer. The primary antibodies used in this study were purchased from CHEMICON [EV71], and Santa Cruz Biotechnology [β-actin, c-Abl (K-12), Cdk5 (DC17), p-Cdk5 (Tyr15), p35 (C-19 and N-20) and p53 (DO-1)]. After hybridization with primary antibody, the membrane was washed three times with TBST before hybridization with anti-goat HRP, anti-mouse HRP, or anti-rabbit HRP secondary antibodies diluted in TBST, according to the primary antibody used. The blots were then washed three times with TBST and developed by chemiluminescence (NEN).

Immunoprecipitation and kinase assay

Immunoprecipitations were performed as previously described with minor modification (Lin et al., 2004). The supernatant from lystate was incubated with anti-c-Abl (K-12) or anti-Cdk5 (DC17) (Santa Cruz) at 4°C for 12 h and subsequently with protein A- (for c-Abl) or G- (for Cdk5) agarose beads (Roche) at 4°C for 1 h. The bead-bound proteins were released by boiling in protein sample buffer after being washed twice with lysis buffer.

For assay of Cdk5 kinase activity, the immunoprecipitates were placed in kinase buffer (20 µl; 50 mM HEPES, pH 7.0, 10 mM MgCl₂ and 1 mM dithiothreitol), and then mixed with 10 µCi of [γ-³²P]-ATP and 2 µg of histone-H1 (Calbiochem). The mixtures
were incubated at 30°C for 30 min. The isotope-labelled samples were separated by SDS-PAGE before being transferred to nitrocellulose membrane and analysed by exposure on film. For assay of Abl kinase activity, GST-CrkII proteins were used as the substrate following the same protocol.

**TUNEL staining**

To detect cell apoptosis, cells were stained with *In Vitro* Cell Death Detection Kit, Fluorescein (Roche), according to manufacturer’s instructions with minor modification. Briefly, cells were grown to 50–60% confluence on the 12-mm cover glass placed in 24-well culture plate before infection. After virus infection, cells were fixed with 4% paraformaldehyde solution in PBS, pH 7.4 for 1 h. The fixed cells were washed twice with PBS and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) on ice for 10 min. Cells were then washed twice with PBS and incubated with TUNEL reaction mixture at 37°C for 1 h in a humidified atmosphere in the dark. After that, stained cells were washed once with PBS, mounted with aqueous mounting medium (Serotec), and directly analysed under fluorescence microscope.

**Cell viability assay**

Cell viability was evaluated with the formazan-based WST-1 assay (Roche), according to manufacturer’s instructions. The absorbance of samples was measured at 450 nm against a reference at 690 nm with a spectrophotometer, SpectroMAX plus (Molecular Devices). The percentage of cell viability was calculated as the ratio of the optical density values relative to the control cells.

**shRNA knockdown**

To knock down Abl and Cdk5 expression, the lentiviral short hairpin RNAi (shRNA) constructs, which were obtained from National RNAi Core Facility in Taiwan, were transfected into SH-SY5Y cells according to previously published procedures (Moffat *et al.*, 2006). In brief, 2 days before EV71 infection, cells seeded in a 6-well plate were transfected with 5 μg Abl shRNA (clone ID: TRCN0000039899; NCBI no. NM_005157) or 5 μg Cdk5 shRNA (clone ID: TRCN0000021467; NCBI no. NM_004935) using a 3 μl Lipofectamine 2000 Transfection Reagent (Invitrogen).

**Reverse transcription and real-time PCR**

To determine the relative virus copy number, 5.0 × 10^5 cells was isolated by the RNeasy Mini kit (QIAGEN) and eluted to a final concentration of 1 μg μl^-1^. For reverse transcription of viral RNA to cDNA, 1 μg total RNA was mixed with the antisense strand (10 pmol) of a pan-enterovirus-specific primer set (sense, 5′-GTGTGAAAGAGTTCTATTGAGC-3′; antisense, 5′-ATTGTCACCATAACGAGCCA-3′), and then adjusted to a final solution of 20 μl with reverse transcription mixture, containing Moloney murine leukaemia virus (M-MLV) reverse transcriptase reaction buffer (5×; Promega), dNTP (10 pmol each) and M-MLV reverse transcriptase (100 U; Promega). The mixture was incubated at 42°C for 1 h. To determine relative transcript level of each target, cDNA was amplified using a LightCycler FastStart DNA Master SYBR Green I (Roche) with the pan-enterovirus-specific primer set. The real-time PCR reactions were carried out as suggested by the manufacturer with 20 μl of the reaction mixture (2 μl of cDNA, 2 μl of LightCycler FastStart Enzyme, 3 mM MgCl₂ and 10 pmol of specific primer set), and the results were analysed by LightCycler Software, version 3.5 (Roche).

**Acknowledgements**

This work was supported by grants from National Science Council (94WIC500055) and National Health Research Institutes. We are grateful to Drs Shin-Ru Shih and Luan-Yin Chang for providing enteroviruses and critical suggestions. We also thank the National RNAi Core Facility for providing lentiviral sh RNAi constructs, and Dr Hsiu-Ming Shih, Dr Tzu-Yang Lin and Ms. Chiu-Hui Huang for their constructive opinions.

**References**

Beck, M.A., Chapman, N.M., McManus, B.M., Mullican, J.C., and Tracy, S. (1990) Secondary enterovirus infection in the murine model of myocardiitis. Pathologic and immunologic aspects. *Am J Pathol* 136: 669–681.

Boureux, A., Furstoss, O., Simon, V., and Roche, S. (2005) Abl tyrosine kinase regulates a Rac/JNK and a Rac/Nox pathway for DNA synthesis and Myc expression induced by growth factors. *J Cell Sci* 118: 3717–3726.

Butterfield, D.A., Drake, J., Pocernich, C., and Castegna, A. (2001) Evidence of oxidative damage in Alzheimer’s disease brain: central role for amyloid beta-peptide. *Trends Mol Med* 7: 548–554.

Castro, C.M., Cruz, A.C., Silva, E.E., and Gomes Mde, L. (2005) Molecular and seroepidemiologic studies of Enterovirus 71 infection in the State of Para, Brazil. *Rev Inst Med Trop Sao Paulo* 47: 65–71.

Chang, L.Y., Lin, T.Y., Hsu, K.H., Huang, Y.C., Lin, K.L., Hseueh, C., et al. (1999a) Clinical features and risk factors of pulmonary oedema after enterovirus-71-related hand, foot, and mouth disease. *Lancet* 354: 1682–1686.

Chang, L.Y., Lin, T.Y., Huang, Y.C., Tsao, K.C., Shih, S.R., Kuo, M.L., et al. (1999b) Comparison of enterovirus 71 and coxsackievirus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. *Pediatr Infect Dis J* 18: 1092–1096.

Chang, L.Y., Tsao, K.C., Hsia, S.H., Shih, S.R., Huang, C.G., Chan, W.K., et al. (2004a) Transmission and clinical features of enterovirus 71 infections in household contacts in Taiwan. *JAMA* 291: 222–227.

Chang, S.C., Lin, J.Y., Lo, L.Y., Li, M.L., and Shih, S.R. (2004b) Diverse apopotic pathways in enterovirus 71-infected cells. *J Neurovirol* 10: 338–349.

Chen, F., Wang, Q., Wang, X., and Studzinski, G.P. (2004) Upregulation of Egr1 by 1,25-dihydroxyvitamin D3 contributes to increased expression of p35 activator of cyclin-dependent kinase 5 and consequent onset of the terminal phase of HL60 cell differentiation. *Cancer Res* 64: 5425–5433.
Chen, L.C., Shyu, H.W., Chen, S.H., Lei, H.Y., Yu, C.K., and Yeh, T.M. (2006a) Enterovirus 71 infection induces Fas ligand expression and apoptosis of Jurkat cells. J Med Virol 78: 780–786.

Chen, T.C., Chen, G.W., Hsiung, C.A., Yang, J.Y., Shih, S.R., Lai, Y.K., and Juang, J.L. (2006b) Combining multiplex reverse transcription-PCR and a diagnostic microarray to detect and differentiate enterovirus 71 and coxsackievirus A16. J Clin Microbiol 44: 2212–2219.

Cowan, C.A., and Henkemeier, M. (2000) More cables to Abl. Neuron 26: 543–544.

Coyne, C.B., and Bergelson, J.M. (2006) Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. Cell 124: 119–131.

Gilmore, E.C., Ohshima, T., Goffinet, A.M., Kulkarni, A.B., and Herrup, K. (1998) Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. J Neurosci 18: 6370–6377.

Gong, X., Tang, X., Wiedmann, M., Wang, X., Peng, J., Zheng, D., et al. (2003) Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuroreport 14: 33–46.

Henke, A., Mohr, C., Sprenger, H., Graebner, C., Stelzner, A., Nain, M., and Gemsa, D. (1992) Cox sackievirus B3-induced production of tumor necrosis factor-alpha, IL-1 beta, and IL-6 in human monocytes. J Immunol 148: 2270–2277.

Hernandez, S.E., Krishnaswami, M., Miller, A.L., and Koleske, A.J. (2004) How do Abl family kinases regulate cell shape and movement? Trends Cell Biol 14: 36–44.

Huang, C.C., Liu, C.C., Chang, Y.C., Chen, C.Y., Wang, S.T., Hernandez, S.E., Krishnaswami, M., Miller, A.L., and Koleske, A.J. (2000) More cables to Abl. Neuron 26: 543–544.

Ito, Y., Pandey, P., Mishra, N., Kumar, S., Narula, N., Kharbanda, S., et al. (2001) Targeting of the c-Abl tyrosine kinase to mitochondria in endoplasmic reticulum stress-induced apoptosis. Mol Cell Biol 21: 6233–6242.

Kehle, J., Roth, B., Metzger, C., Pfiztnzer, A., and Enders, G. (2003) Molecular characterization of an Enterovirus 71 causing neurological disease in Germany. J Neurovirol 9: 126–128.

Kharbanda, S., Pandey, P., Ren, R., Mayer, B., Zon, L., and Kufe, D. (1995) c-Abl activation regulates induction of the SEK1/stress-activated protein kinase pathway in the cellular response to 1-beta-D-arabinofuranosylcytosine. J Biol Chem 270: 30278–30281.

Kusakawa, G., Saito, T., Onuki, R., Ishiguro, K., Kishimoto, T., and Hisanaga, S. (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J Biol Chem 275: 17166–17172.

Lee, M.S., Kwon, Y.T., Li, M., Peng, J., Friedlander, R.M., and Tsai, L.H. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 405: 360–364.

Leong, W.F., and Chow, V.T. (2006) Transcriptomic and proteomic analyses of rhabdomyosarcoma cells reveal differential cellular gene expression in response to enterovirus 71 infection. Cell Microbiol 8: 565–580.

Lew, J., Huang, Q.Q., Qi, Z., Winklein, R.J., Aebersold, R., Hunt, T., and Wang, J.H. (1994) A brain-specific activator of cyclin-dependent kinase 5. Nature 371: 423–426.

Lin, H., Juang, J.L., and Wang, P.S. (2004) Involvement of Cdk5/p25 in digoxin-triggered prostate cancer cell apoptosis. J Biol Chem 279: 29302–29307.

Lin, H., Tin, T.Y., and Juang, J.L. (2007) Abl deregulates Cdk5 kinase activity and subcellular localization in Drosophila neurodegeneration. Cell Death Differ 14: 607–615.

Luo, H., Yanagawa, B., Zhang, J., Luo, Z., Zhang, M., Esfandiaei, M., et al. (2002) Coxsackievirus B3 replication is reduced by inhibition of the extracellular signal-regulated kinase (ERK) signaling pathway. J Virol 76: 3365–3373.

Melnick, J.L., Schmidt, N.J., Mirkovic, R.R., Chumakov, M.P., Lavrova, I.K., and Voroshilova, M.K. (1980) Identification of Bulgar strain 258 of enterovirus 71. Intervirology 12: 297–302.

Moffat, J., Grueneberg, D.A., Yang, X., Kim, S.Y., Kloepfer, A.M., Hinkle, G., et al. (2006) A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 124: 1283–1298.

Muir, P., and van Loon, A.M. (1997) Enterovirus infections of the central nervous system. Intervirology 40: 153–166.

Nguyen, M.D., Mushynski, W.E., and Julien, J.P. (2002) Cycling at the interface between neurodevelopment and neurodegeneration. Cell Death Differ 9: 1294–1306.

Pandey, P., Raingeaud, J., Kaneki, M., Weichselbaum, R., Davis, R.J., Kufe, D., and Kharbanda, S. (1996) Activation of p38 mitogen-activated protein kinase by c-Abl-dependent and -independent mechanisms. J Biol Chem 271: 23775–23779.

Patrick, G.N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L.H. (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 402: 615–622.

Philpott, A., Porro, E.B., Kirschner, M.W., and Tsai, L.H. (1997) The role of cyclin-dependent kinase 5 and a novel regulatory subunit in regulating muscle differentiation and patterning. Genes Dev 11: 1409–1421.

Schmidt, N.J., Lennette, E.H., and Ho, H.H. (1974) An apparently new enterovirus isolated from patients with disease of the central nervous system. J Infect Dis 129: 304–309.

Schwarz, K.B. (1996) Oxidative stress during viral infection: a review. Free Radic Biol Med 21: 641–649.

Shaull, Y. (2000) c-Abl: activation and nuclear targets. Cell Death Differ 7: 10–16.

Shen, Y., Xu, W., Chu, Y.W., Wang, Y., Liu, Q.S., and Xiong, S.D. (2004) Coxsackievirus group B type 3 infection upregulates expression of monocyte chemoattractant protein 1 in cardiac myocytes, which leads to enhanced migration of mononuclear cells in viral myocarditis. J Virol 78: 12548–12556.

Shih, S.R., Tsai, K.N., Li, Y.S., Chueh, C.C., and Chan, E.C. (2003) Inhibition of enterovirus 71-induced apoptosis by allphoycocyanin isolated from a blue-green alga Spirulina platensis. J Med Virol 70: 119–125.

Singh, S., Chow, V.T., Phoon, M.C., Chan, K.P., and Poh, C.L. (2002) Direct detection of enterovirus 71 (EV71) in clinical specimens from a hand, foot, and mouth disease outbreak in Singapore by reverse transcription-PCR with universal enterovirus and EV71-specific primers. J Clin Microbiol 40: 2823–2827.

Smith, D.S., and Tsai, L.H. (2002) Cdk5 behind the wheel: a role in trafficking and transport? Trends Cell Biol 12: 28–36.
Storz, P., Doppler, H., and Toker, A. (2004) Protein kinase Cdelta selectively regulates protein kinase D-dependent activation of NF-kappaB in oxidative stress signaling. *Mol Cell Biol* 24: 2614–2626.

Tsai, L.H., Delalle, I., Caviness, V.S. Jr, Chae, T., and Harlow, E. (1994) p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* 371: 419–423.

Wetzler, M., Talpaz, M., Van Etten, R.A., Hirsh-Ginsberg, C., Beran, M., and Kurzrock, R. (1993) Subcellular localization of Bcr, Abl, and Bcr-Abl proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. *J Clin Invest* 92: 1925–1939.

Wong, W.R., Chen, Y.Y., Yang, S.M., Chen, Y.L., and Horng, J.T. (2005) Phosphorylation of PI3K/Akt and MAPK/ERK in an early entry step of enterovirus 71. *Life Sci* 78: 82–90.

Zhang, Q., Ahuja, H.S., Zakeri, Z.F., and Wolgemuth, D.J. (1997) Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling. *Dev Biol* 183: 222–233.

Zukerberg, L.R., Patrick, G.N., Nikolic, M., Humbert, S., Wu, C.L., Lanier, L.M., *et al.* (2000) Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. *Neuron* 26: 633–646.