Antiviral Research 93 (2012) 110–117

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

MEK1–ERKs signal cascade is required for the replication of Enterovirus 71 (EV71)

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ARTICLE INFO

Article history:
Received 30 September 2011
Revised 4 November 2011
Accepted 6 November 2011
Available online 13 November 2011

Keywords:
EV71
Viral replication cycle
MEK1
MEK2
ERK
siRNAs

ABSTRACT

The role of the MEK1–ERK signaling cascade in the replication cycle of Enterovirus 71 (EV71), the primary cause of hand, foot and mouth disease (HFMD), has been analyzed. In vitro infection with EV71 induced a biphasic activation of ERK. The two phases of activation appeared to be triggered by different mechanisms, with the first phase being activated by the binding of viral particles to the membrane receptor of host cells and the second probably being in response to the production of new virus particles. Inhibition of ERK activation by U0126 was found to severely impair virus production. A similar reduction in EV71 replication was also observed when MEK1 expression was subject to knockdown using specific siRNAs. By contrast knockdown of MEK2 expression showed that it was dispensable for virus replication cycle, despite both MEK isoforms being activated and translocated to the nucleus equally well in response to virus infection. Overall, this study suggests distinct functions of the two isoforms of MEK in EV71 replication cycle, with an essential role for MEK1 in stimulating the ERK signaling cascade to promote virus replication. Taken together with our previous work on herpes simplex virus type 2 (HSV2) this study highlights MEK1 as a potential broad antiviral molecular target.

1. Introduction

Enterovirus 71 (EV71) is a single-positive-stranded RNA virus belonging to the Enterovirus genus of the *Picornaviridae* (Nasri et al., 2007). It has become increasingly recognized as the major etiological agent of the large outbreaks of hand, foot and mouth disease (HFMD) currently occurring in the Asia-Pacific region, including mainland China (Solomon et al., 2010; Yang et al., 2009; Yi et al., 2011). EV71-caused HFMD has been associated with a higher risk of developing severe neurological complications and fatalities (Chang et al., 2002; Solomon et al., 2010; Yi et al., 2011). This contrasts with disease caused by Coxsackievirus A16 (CAV16) and Coxsackievirus B3 (CVB3), which usually does not result in life-threatening manifestations. Consequently EV71 has become recognized as the most important neurotropic Enterovirus (EV) after the virtual eradication of poliovirus (Solomon et al., 2010; Yi et al., 2011).

The extracellular regulated kinase (ERK) is one of three major mitogen-activated protein kinases (MAPK) that are linked in a tiered cascade of serine/threonine kinases composed of Raf, MAPK/ERK (MEK) and ERK (Avruch, 2007; Chambard et al., 2007; McCubrey et al., 2007; Zhang and Liu, 2002). MEK1 and MEK2 are two Thr/Tyr dual isoforms of Raf-MEK1/2–ERK1/2 axis, ERK1 and ERK2, i.e., ERK1/2, are the only MEK1/2 subtracts that have been accepted to date (Belanger et al., 2003; Chambard et al., 2007; Giroux et al., 1999; Jacque et al., 1998). Thus, MEK1 and MEK2, play a key role in connecting the Raf and ERK. The inhibition of MEK has been studied as the most effective way of investigating the importance of the Raf/MEK/ERK signaling cascade for various cellular physiological and pathological processes (Kelemen et al., 2002; Kohno and Pouyssegur, 2006; Kolch, 2005; Ludwig, 2011; Luo et al., 2002). In many scenarios, the isoforms of MEK identified (MEK1 and MEK2) are functionally interchangeable, they share 80% sequence homology and are ubiquitously expressed in cells and tissues (Avruch, 2007; Chambard et al., 2007; Shaul and Seger, 2007). More recent studies have however added complexity to the signaling functions of MEK1 and MEK2 and suggest that each may perform non-redundant functions (Belanger et al., 2003; Brandt et al., 2010; Skarpen et al., 2008; Ussar and Voss, 2004). Thus, it is now believed that MEK1 but not MEK2 forms a signaling complex with Raf1 which suggests that in some cells the Raf1 signaling pathway preferentially activates ERKs via MEK1 (Jelinek et al., 1994). Furthermore, MEK1 and MEK2 have distinct ways to regulate ERK activity and cell cycle progression (Ussar and Voss, 2004) and have distinct effects on the regulation of IFN-β signaling (Brandt et al., 2010). Finally, MEK1 and MEK2 knockout mice have different phenotypes (Belanger et al., 2003; Giroux et al., 1999). Taken together, these observations indicate functional differences between MEK1 and MEK2.

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doi:10.1016/j.antiviral.2011.11.001

Virus amplification in host cells is a prerequisite for all the pathogenic consequences caused by virus infection and is reliant on many functioning components of cellular metabolism. One of these is the host cell's Raf-MEK1/2-ERK1/2 (ERK) signal cascade, necessary for cellular proliferation, migration, division, and survival. It has been demonstrated to be utilized by many DNA and RNA viruses to amplify themselves in host cells (Hunter et al., 1995; King et al., 1986; Moser and Schultz-Cherry, 2008; Perkins et al., 2002; Pleschka, 2008; Smith et al., 2000). These include a number of Enteroviruses, such as CVB3 (Luo et al., 2002), Echovirus 1 (Huttunen et al., 1998) and EV71 (Tung et al., 2011; Wang et al., 2010). More specifically our previous work with herpes simplex virus types 2 (HSV2) showed that only MEK1 is essential for HSV2 replication cycle in vitro (Zhang et al., 2010), highlighting that MEK1 and MEK2 can exert distinct, non-redundant effects on the replication cycle of viruses. Against this background, the objective of the present study was to determine the specific role(s) of the MEK isoforms in the replication cycle of EV71.

2. Material and methods

2.1. Cell culture

Rhabdomyosarcoma (RD) cells and Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 2% or 10% heat-inactivated fetal bovine serum (FBS, GIBCO) at 37 °C in a 5% CO_2 humidified incubator.

2.2. Virus infection

EV71 (a clinical strain denoted as EV71-BC08, GU475127) was isolated from the vesicular fluid of a pediatric patient diagnosed with HFMD as described previously (Wang et al., 2010).

Monolayers of RD or HEK293 cells at 70–80% confluency were starved and infected with the wild type EV71 (EV71-BC08 stain) at various multiplicities of infection (MOI) as indicated in the text and figure legends. Virus adsorption was done for 1 h (designated as 0 h in the growth curve) at 37 °C before the inoculum was removed and incubation continued in DMEM containing 2% FBS at 37 °C. Supernatants collected at different time points post-infections (p.i.) were clarified by centrifugation (2000*g*), virus titers were determined by plaque assays using RD cells and carried out in triplicate.

Virus was inactivated by being UV irradiated for 30 min using a Phillips TUV 30 W bulb at a distance of 17 cm with occasional agitation, and then blind passaged for three generations in RD or HEK293 cells to confirm loss of replicative capacity (Beck et al., 1990).

2.3. Antibodies

Antibodies used for Western blot analysis were purchased from Cell Signaling Technology (anti-phospho-ERK1/2, anti-ERK1/2, anti-MEK1 and anti-MEK2), Santa Cruz Biotechnology (β-actin) and Abcam (polyclonal anti-EV71 VP1). In addition, rabbit MEK1 and MEK2 polyclonal antibodies (Bioworld Inc.), mouse EV71 polyclonal antibody (Abcam) and goat anti-rabbit Alexa Fluor 488 or goat anti-mouse Alexa Fluor 594 secondary antibodies (Invitrogen) were used for the immunofluorescence assay in this study.

2.4. MEK inhibitor

The MEK1/2 specific inhibitor U0126 (Pierce) was freshly dissolved at 10 mM in DMSO, and added to culture medium at final concentrations of 20 or 30 μM for 1 h prior to virus infection and kept in the medium throughout the experiment, or added for different intervals as described in the text or figure legends.

2.5. siRNAs and transient transfection

As described previously (Zhang et al., 2010), specific short interfering RNAs (siRNAs) targeting sequences for human MEK1, MEK2 mRNAs (named siMEK1 and siMEK2, respectively), and siRNA controls, designated as siMut1 for siMEK1 and siMut2 for siMEK2, where two base pairs different from the derived siRNAs, were used served as negative controls for nonspecific effects of siRNA and/or transfection reagents on cell viability and virus infection. All siRNAs were commercially synthesized by Gene Chem, Ltd.

For siRNA transfections, the INTERFERin reagent (Polyplus-transfection) was used according to the manufacturer's instructions. RD cells or HEK293 cells were grown in 12-well plates to 40% confluency. Cells were then transfected with 10 or 30 nM specific siRNAs and incubated for 36 h, the medium was then replaced with DMEM containing with 1% FBS and cells starved for 8 h. Then, cells were infected with EV71 either at an MOI of 1.5 or at the MOI indicated in the figure legends. The virus titers in supernatants harvested at 24 h p.i. were measured by plaque assay and cell lysates collected were analysed for viral proteins and genomic RNAs as well as cellular proteins.

2.6. Western blot analysis

Western blots were processed as previously described (Wang et al., 2010). Briefly, cells were lysed in a buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, 5 mM EDTA (pH 7.4), 1 mM Na_3VO_4, 30 mM NaF, 5% glycerol, 0.1% SDS, 1% Triton X-100, 10 mM *p*-nitrophenylphosphate, 1 mM glycerophosphate, supplemented with complete protease inhibitors (Roche). Cell lysates were obtained by centrifugation at 13000 rpm and 4 °C, total protein concentration determined by the Bicinchoninic Acid Protein Assay Kit (Pierce). Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to PVDF membranes (Millipore). Membranes were blocked for 2 h with 5% nonfat dry milk solution in Tris-buffered saline containing 0.1% Tween 20. Then membranes were blotted with specific primary antibodies, followed by incubation with secondary antibodies conjugated with horseradish peroxidase. Blots were developed with an enhanced chemiluminescent substrate (ECL) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

2.7. Real-time quantitative PCR (Q-PCR)

Viral RNAs from supernatants were prepared using Trizol reagent (Invitrogen). Then, 1 μg of total RNA was reverse transcribed into cDNA with the ReverAid First strand cDNA synthesis kit (Thermo Science). A quantitation standard curve was achieved using seven 10-fold serial dilutions of the recombinant plasmid standard DNA, which contained a 226 bp sequence derived from the VP1 gene of EV71, varying from 1×10^3 to 1×10^9 copies. Real-time quantitative (Q-PCR) was performed by targeting the VP1 gene in 96-well plates with the Roche Light Cycler 480 system. Each 20 μl of reaction contained 10 μl of 2× SYBR Green Supermix, 20 nM of target gene primer mix, and 20–50 ng of a cDNA template. The sequence of the primers used was as follows: Forward: 5′-GCA GCC CAA AAG AAC TTC AC-3′ and Reverse: 5′-ATT TCA GCA GCT TGG AGT GC-3′. The PCR reaction was set up as follows: initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 30 s at 94 °C, at 55 °C for 30 s and at 72 °C for 30 s. Quantified results for the experimental samples were extrapolated from the

standard curve, with all experimental samples being run in triplicate.

2.8. Indirect immunofluorescence assay

RD cells seeded on coverslips were washed three times with ice-cold PBS at 5 h p.i., fixed for 20 min with 4% paraformaldehyde in PBS at room temperature and then washed a further three times with PBS. Then cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min, followed by three washes. Cells were then blocked with goat serum for 1 h, washed three times with PBS and incubated with diluted rabbit antibodies to MEK1, MEK2 or mouse anti-EV71 antibodies followed by either goat anti-rabbit Alexa Fluor 488 (green) or goat anti-mouse Alexa Fluor 594 secondary antibody (red). Then cells were washed extensively with PBS before being incubated for 10 min in PBS containing 1% BSA and 0.1% Hochst to stain nuclei. Images were obtained using an Olympus IX71 inverted fluorescence microscope with DP2-BSW imaging software. The images shown are representative of three independent experiments.

2.9. Cell viability assay

Cell viability following siRNA transfection and MEK1/2 inhibitor U0126 treatment was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) assay (Chemicon International), according to the manufacturer's recommendations. Briefly, RD and HEK293 cells were seeded in 96-well cell culture plates and treated with individual siRNAs and U0126 for 48 and 24 h prior to being incubated with AB solution for 4 h at 37 °C. Solution C containing isopropyl alcohol/HCl was then added, and absorbance read using an ELISA plate reader (Bio-Rad) at a test wavelength of 570 nm and reference wavelength of 630 nm. Cell viability was also assessed by visual inspection using phase-contrast microscopy.

2.10. Statistics

The relative amounts of the phospho-ERK1/2 (p-ERK1/2) were normalized by densitometric scanning of β-actin using band leader software (version 3.0). Statistical difference analysis was carried out using ANOVA or Student's *t*-test as indicated in the figures and legends.

3. Results

3.1. EV71 induced a biphasic activation of ERK1/2

Activation of the mitogenic Raf–MEK–ERK (ERK) kinase cascade has previously been shown to play an important role in EV71 replication cycle (Tung et al., 2011; Wang et al., 2010). To further elucidate the kinetics of EV71 induced-activation of the ERK pathway, growth arrested RD cells and HEK 293 cells were infected with EV71 at an MOI of 1.5 and cell extracts collected at different time points p.i. and blotted with the phospho-ERK1/2 (p-ERK1/2) antibody. Fig. 1A shows that infection of RD cells by EV71 induced ERK phosphorylation with a first transient peak at 15–30 min p.i., and a second peak from 8 to 12 h p.i. A similar biphasic activation of ERK was observed in EV71-infecetd HEK293 cells (Fig. 1B). The EV71 capsid protein VP1 began to be detected at 8 h in these infections and increased as the infection progressed (Fig. 1). Therefore EV71 infection initiated an early transient and late sustained biphasic activation of ERK1/2 in these two cell lines.

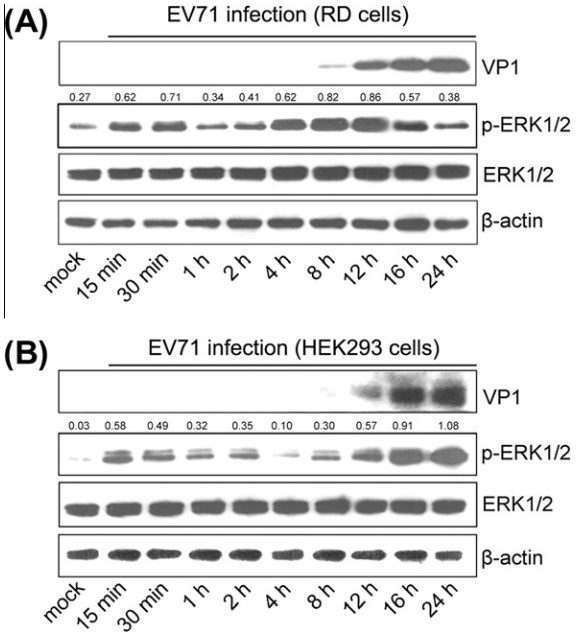

Fig. 1. Infection with EV71 resulted in a biphasic activation of ERK in RD and HEK293 cells. (A) RD cells were infected with wild type EV71 at an MOI of 1.5. At the indicated time points post-infection (p.i.), cells were collected and lysed for Western blot analysis to detect phospho-ERK1/2 (p-ERK1/2) and EV71 VP1 proteins. β-actin and total ERK1/2 (ERK1/2) were used as internal controls. (B) The experiment was performed as described in panel A except that HEK293 cells were used. The ratio of p-ERK1/2 to ERK1/2 signals at different time pairs shown in Arabic numeric in panel A and B was determined by densitometric scanning. The experiments were repeated three times.

3.2. Inhibition of ERK1/2 activation resulted in a significant reduction in virus replication

To demonstrate the importance of ERK activation to EV71 replication cycle, U0126, an inhibitor specific for both MEK1 and MEK2, was used for investigating the effect on cellular ERK activation and EV71 capsid protein VP1 expression. As shown in Fig. 2A and B, treatment of cells with U0126 (30 μM) resulted in a severe suppression of both EV71-induced early- and late-phase ERK phosphorylation and a sharp reduction of EV71 VP1 protein at 12, 16 and 24 h p.i. Next the effect of U0126 treatment on EV71 progeny titers was examined. Fig. 2C shows that the progressive increase in virus titer was reduced by 46%, 77%, and 96% in RD cells, or 40%, 76%, and 90% in HEK293 cells treated with U0126 (30 μM),respectively, when compared with control cells without drug treatment. The data also revealed that the inhibitory effect of U0126 was dose-dependent (Fig. 2D). It should be noted that, at all concentrations of U0126 used, there was no significant difference in viability between U0126-treated cells and mock-cells, as detected by the MTT assays (data not shown).

3.3. UV-irradiated EV71 induced early activation of ERK, but failed to stimulate the second phase of activation

UV-irradiated (inactivation) virus retains the capability for receptor binding and endocytosis into host cells, but due to the thymidine dimers generated by UV treatment, it fails to transcribe viral genes and express viral proteins (Luo et al., 2002; Perkins et al., 2002). To examine the correlation between EV71 replication and the kinetics of virus-mediated ERK1/2 activation, UV-irradiated EV71 was used to 'infect' RD and 293 cells. Fig.3A and B showed that in this case, the early increase in ERK1/2 phosphorylation remained, whereas the level ERK1/2 phosphorylation seen

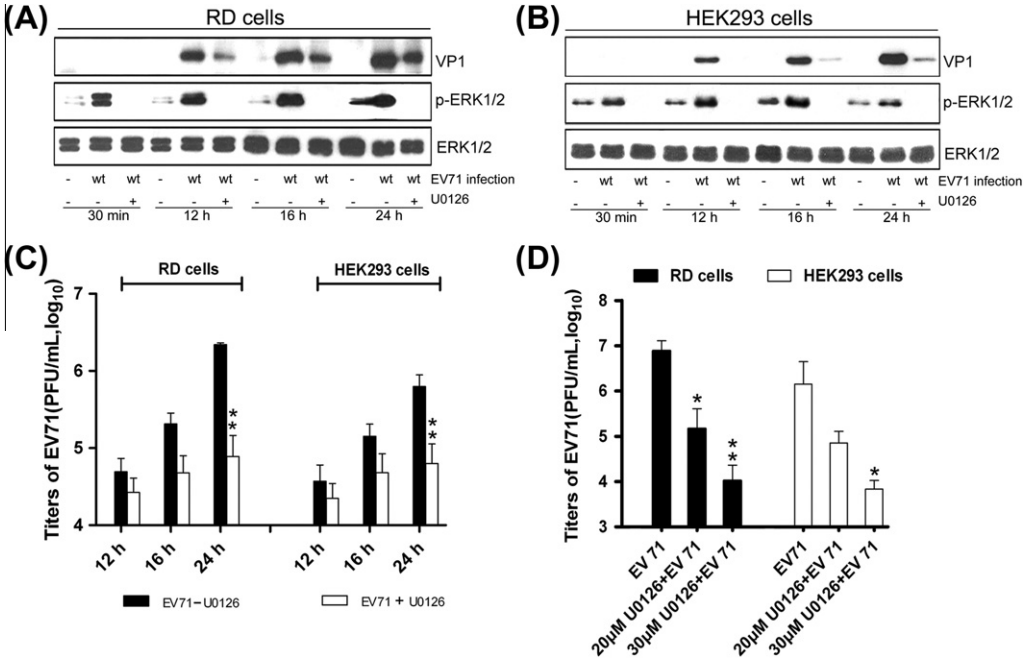

Fig. 2. Suppression of ERK activation and EV71 replication by treating RD and HEK293 cells with U0126. (A) RD cells were pre-incubated without or with U0126 (30 μM) for 1 h, and then followed by infection with wild type EV71 at an MOI of 1.5, U0126 was present throughout the experiment. The cells and supernatants were collected separately at different time points as indicated. Cell lysates were blotted with anti-p-ERK1/2, anti-ERK1/2 and anti-EV71 VP1 antibodies. (B) The experiment was performed as described in panel A except that HEK293 cells were used. The experiments were repeated three times. (C) Virus titers in the supernatants collected in panel A and B were measured by plaque forming assay. (D) RD and HEK293 cells were treated with U0126 as described in the panel A and B, but with two different concentrations. Virus titers in culture supernatants collected at 24 h p.i. were measured by plaque forming assay. Data shown were the means ± standard deviations ($n = 3$). The titers on y-axis of panel (C) and (D) represented logarithmic values. The values obtained with U0126 that were significantly different from those in its absence are indicated by the asterisk ($^{*}P < 0.05$, $^{**}P < 0.01$ by ANOVA).

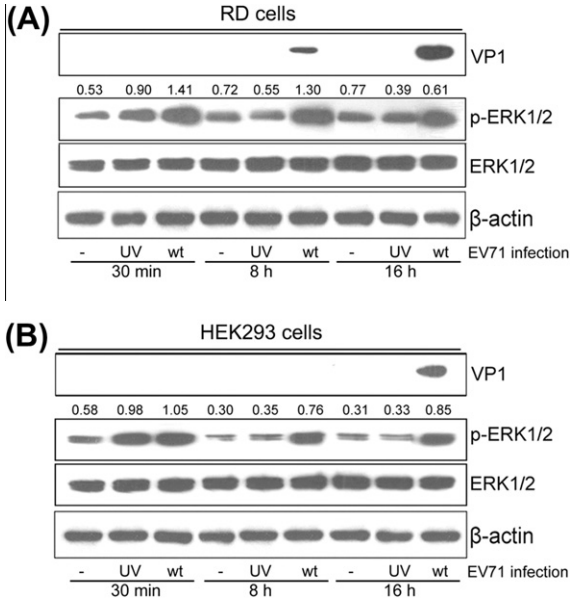

Fig. 3. Ultraviolet-irradiated EV71 (UV-EV71) induced early-phase phosphorylation of EKR1/2 in RD and HEK293 cells. RD cells (A) and HEK293 cells (B) were infected with wild type EV71 (wt) or UV-EV71 (UV) at an MOI of 1.5. At the indicated time point p.i., cells were collected for Western blot analyses to examine p-ERK1/2, ERK1/2 and EV71 VP1. β-actin was used as an internal control. The ratio of p-ERK1/2 to ERK1/2 signals at different time pairs shown in Arabic numeric in panel A and B was determined by densitometric scanning. The experiments were repeated three times.

at later event was comparable to that of uninfected mock control. Together, these data demonstrate that both phases of ERK activation can be correlated with the EV71 replication cycle, with only EV71-receptor interaction and internalization being required for early ERK1/2 activation whereas the second phase of activation is linked to viral gene transcription and protein synthesis.

3.4. Individual depletion of the MEK isoforms acted differentially in EV71 replication

To investigate if the two MEK isoforms have differential effects on EV71 replication cycle, siRNA depletion of the individual isoforms was carried out using siMEK1 and siMEK2. Fig.4A and B shows that transfection of siMEK1 and siMEK2 achieved marked and very specific knockdown effects, with no cross-silencing by each other's siRNA or by respective mutated siRNAs (mu) being observed. This result was confirmed by immunofluorescence staining and microscopic imaging of treated cells (Fig.4C and D). It is also worth noting that no evidence of cytotoxicity was seen in these experiments even at 30 nM siRNA which achieved the maximal knockdown effect in a titration experiment (data not shown). Next, knockdown of MEK1 was seen to result in a dose dependent reduction of EV71 VP1 protein expression (Fig. 4A), analogous to that seen in U0126 treated cells (Fig. 2A and B). By contrast, silencing of MEK2 showed no significant change in the expression of VP1 protein as compared to that of siMut2 control (Fig. 4B). Moreover, ERK activation in virus-infected cells was reduced by siRNAs for the two respective MEKs, as compared with their cognate mutated siRNAs. In addition, EV71 titers were decreased by about 80% and 71% (10 nM), and 98% and 97% (30 nM) in siMEK1-transfected RD cells and 293 cells, respectively, as compared to that of cells treated with siMut ($^{*}P < 0.05$, $^{**}P < 0.01$ by Student's t-test, Fig. 4E). No significant reduction of viral progeny titers was achieved in siMEK2-transfected cells (Fig. 4E). Finally, a real time PCR assay was applied to quantify the EV71 VP1 gene. The data revealed that VP1 RNA at 8 and 16 h p.i. were significant reduced in EV71-in-

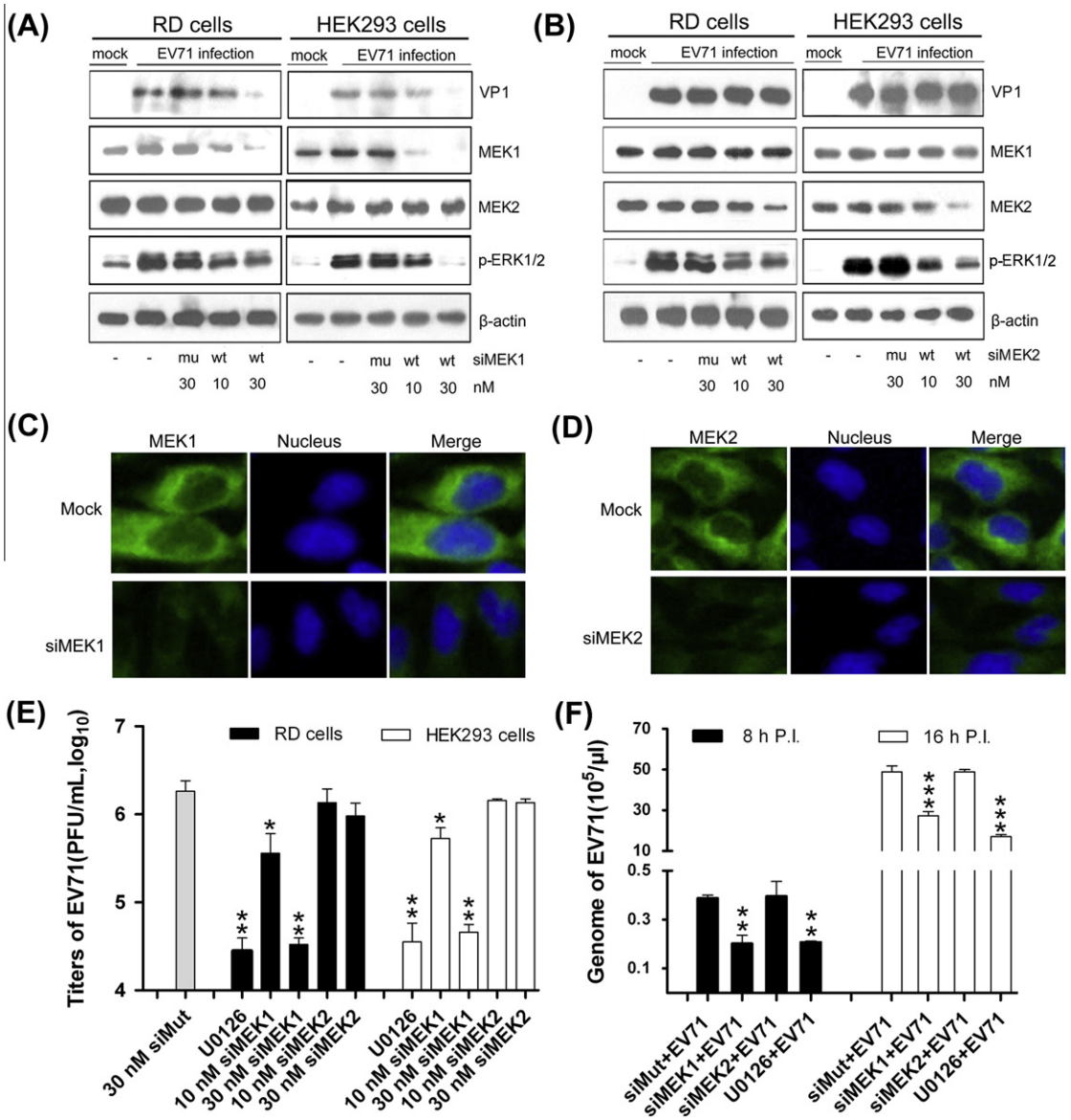

Fig. 4. Knockdown of MEK1 and MEK2 differentially impacted on EV71 replication in RD and HEK293 cells. (A) RD and HEK293 cells were transiently transfected with the siMEK1 (wt) or siMut1 (mu) at the indicated doses. Cells treated with U0126 were used as a control. The cells were infected with wild type EV71 (MOI = 1.5) at 36 h post-transfection. Culture supernatant and cells were collected at 24 h p.i. Cell lysates were prepared to detect MEK1, EV71 VP1 by Western blots with specific antibodies. (B) The experiment was performed as panel A except that siMEK2 or siMut2 was used to examine the effect of siMEK2 on EV71 replication. β-actin was used for a loading control. (C) RD cells were transiently transfected with 30 nM siMEK1 for 48 h, and then fixed, permeabilized, stained with anti-human MEK1 to determine the level of MEK1 knockdown (green). Cellular nuclei were stained with Hochst (blue). (D) The experiment was performed as in panel (C) except that siMEK2 and anti-human MEK2 were used to examine the effect of specific knockdown MEK2 in cells (green). Digital images were captured using an Olympus IX71 Inverted fluorescence microscope with DP2-BSW imaging software (400× magnification). Results included are representative of at least three independent experiments. (E) Supernatants collected in panels A and B were assayed for virus titers (PFU/ml). The 30 nM siMut plot represented the average viral progeny titers in 30 nM siMut1 and siMut2 treated cells. The data represent the average of three independent experiments shown as the means ± standard deviations. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with 30 nM siMut by Student's *t*-test. (F) Cells were transfected with 30 nM siMEK1 or siMEK2, and infected at 36 h post-transfection with 1.5 MOI of EV71, cells treated with U0126 as a control. Supernatants were collected at 8 or 16 h p.i. for Q-PCR detection. The data represent the average of three independent experiments and are shown as the means ± standard deviations ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with respective siMut by Student's *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fected RD cells pre-transfected with siMEK1, to a level similar to that seen in infected cells treated with U0126 (Fig. 4F). Taken together, these data suggest that MEK1 but not MEK2 activity is required for amplification of EV71.

3.5. The proliferation of EV71 altered localization of the MEK isoforms in RD cells

To probe the cellular localization of the site EV71 replication and the concomitant effect on the MEK isoforms, a double-label immunofluorescence assay was carried out. The results showed that MEK1 and MEK2 were predominantly located in the cytoplasm prior to virus infection (Fig. 5A, the first panel). However, 5 h after infection with EV71 redistribution of MEK had occurred in EV71-antigen positive cells, with some of it appearing in the nucleus, even in the presence of U0126. By contrast, EV71 replication was confined exclusively to the cytoplasm at 5 h p.i. (Fig. 5A, middle and bottom panels). The immunofluorescence imaging data also revealed that EV71-antigen positive cells were reduced by 68% and 79%, respectively in MEK1-knockdown and U0126-treated cells, whereas there was only a 17% reduction of EV71-antigen positive cells in siMEK2-deleted cells (Fig. 5B, $^{**}P < 0.01$ by ANOVA).

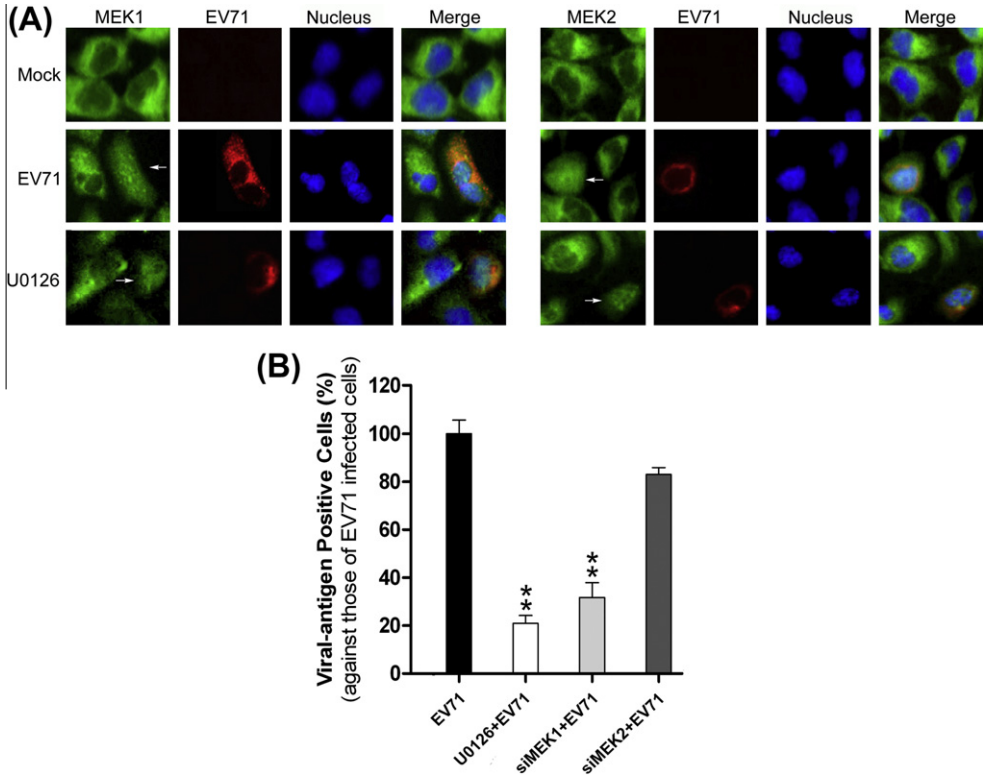

Fig. 5. The subcellular distribution of EV71 replication and the MEK isoforms determined by fluorescence microscopy (400× magnification). (A) RD cells pretreated with or without U0126 (30 μM) were infected with EV71 (0.5 MOI). At 5 h p.i., cells were fixed, permeabilized, and then stained with anti-human MEK1, or anti-human MEK2 and Alexa Fluor 488 secondary antibodies (green) to track the localization of respective MEK isoform, or with anti-EV71 antibodies and goat anti-mouse Alexa Fluor 594 secondary antibodies (red) to localize progeny EV71 distribution in infected cells in a double-labeled immunofluorescence imaging assay. The arrow indicates the redistribution of distinct MEK isoform in the EV71-antigen positive cell. (B) Cells were transfected with 30 nM siMEK1, or siMEK2, and then infected with EV71 (0.5 MOI) at 36 h post-transfection. Cells treated with U0126 were used as a control (5 h p.i.). The immunofluorescence staining was done as described in panel A. The histogram represents the inhibition of virus replication determined by the percentage of virus antigen-positive cells in distinct MEK isoform knockdown cells, or U0126 treated cells against those of EV71 infected control (100%). The plots shown are representative of three independent experiments with the means ± standard errors. The asterisks indicate $^{**}P$ values of <0.01 by Student's t test. Digital images were captured using an Olympus IX71 Inverted fluorescence microscope with DP2-BSW imaging software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The level of fluorescence in these experiments was however not quantified and hence only the subcellular distribution of EV71 replication and the MEK isoforms can be inferred and not their levels of expression. Overall this data demonstrated an isoform-specific effect of MEK1, but not MEK2 on promoting EV71 replication by enumerating the percentage of viral antigen-positive cells and that this leads by an as yet unknown mechanism to MEK redistribution in both cytoplasm and nucleus.

3.6. Inhibition of the activation of MEK–ERK cascade prevented EV71-induced CPE

To determine the effects of U0126, siMEK1 and siMEK2 on EV71-induced CPE, RD and HEK293 cells were pretreated with U0126 or siMEK1, or siMEK2, and infected with EV71 (Fig. 6). CPE was examined under phase-contrast microscopy every 12 h for 3 days p.i. At 24 h p.i., pretreatmemt with siMEK1 and U0126 markedly suppressed the morphological changes induced by EV71 infection, as compared to siMEK2 transfection and EV71 controls, consistent with the distinct functioning of the two MEK isoforms in EV71 replication cycle.

4. Discussion

Viruses utilize components of their host cell's metabolic machinery for productive infection. Activation of the Raf/MEK/MAPK signaling cascade has been shown to be one of the specific changes seen in response to infection with a variety of viruses including EV71 (Hunter et al., 1995; King et al., 1986; Moser and Schultz-Cherry, 2008; Perkins et al., 2002; Pleschka, 2008; Smith et al., 2000; Tung et al., 2011; Wang et al., 2010). In this study, infection of cells with live EV71 (wt) triggered both an early transient and a later sustained activation of ERK1/2, while infection with UV-inactivated EV71 only induced the early phase of activation suggesting that the two phases involve different control mechanisms, with viral protein and RNA synthesis only being necessary for the second phase, consistent with the results seen for CVB3 and HSV2 (Luo et al., 2002; Perkins et al., 2002).

The inhibition of ERK activation by U0126 was found to severely impair virus production in two different cells. A similar reduction in EV71 growth was seen following the knockdown of MEK1 expression using specific siRNAs, whereas silencing of MEK2 had little effect. Overall these data support activation of the ERK pathway as being a necessary cellular modification to support EV71 proliferation, with changes in MEK1 being the essential step. The mechanism underpinning these changes in the cellular environment following virus infection remains elusive. It is possible that, analogous to the activation event in CVB3, upstream components in the ERK pathway such as Ras and Raf1 are turned on by EV71, leading to the formation of a signaling complex with MEK1, and that this then induces non-discriminative activation of ERK1/2 (Luo et al., 2002). This hypothesis is in line with the observed mobilization of some MEK into the nucleus, although it remains

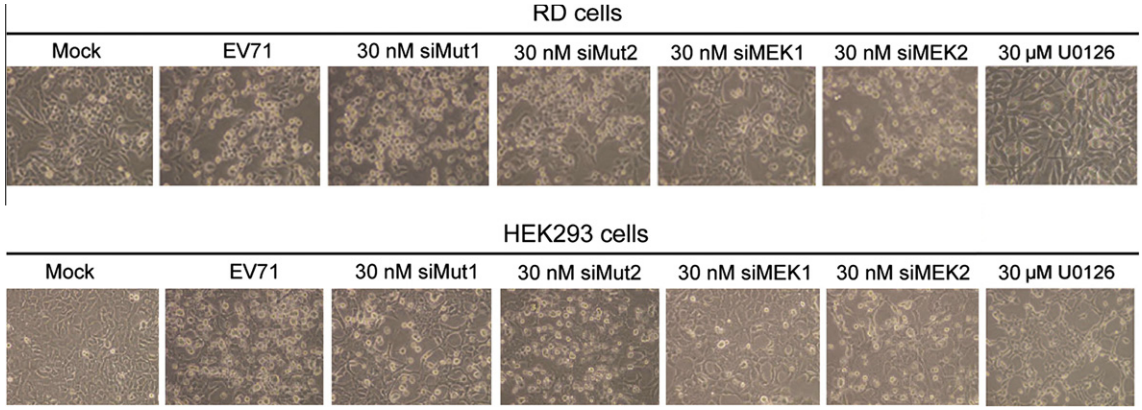

Fig. 6. CPE induced by EV71 in RD or HEK293 cells. RD cells (A) and HEK293 cells (B) were pre-incubated without or with U0126 at the indicated doses for 1 h, followed by infection with EV71 at an MOI of 1.5. EV71 infection, siMut1 and siMut2 were used as controls. The effects of U0126, siMEk1 and siMEk2 on CPE were examined from 8 h p.i., and images were taken at 24 h p.i.

unclear why both isoforms of MEK appear in the nucleus simultaneously following EV71 infection. It is possible that both MEK isoforms share the same adaptor to transport them to the nucleus (Kolch, 2005; Seger et al., 1994; Yao and Seger, 2009), and that only MEK1 implements functions necessary for virus replication cycle. However, at present, the possibility that the nuclear translocation is only a byproduct of MEK activation and has nothing to do with the virus replication amplification cannot be excluded.

The findings in this study are consistent with our recent report showing that MEK1 but not MEK2 plays a critical role in HSV2 propagation (Zhang et al., 2010). The fact that these two viruses have different types of genomes and pursue distinct subcellular replication strategies (Brooks et al., 2004; Yi et al., 2011), suggests that MEK1 is more generally important for virus replication cycle, which provides a basis for considering isoform–specific drug design, although the fact that the two isoforms share 80% identity, would make such efforts challenging.

Finally, in related recent studies, cellular ERK levels have been shown to be the major causal factor for upregulation of COX-2 expression induced by EV71 infection, which may participate in EV71-induced CNS damage (Tung et al., 2010, 2011). This additional evidence together with our study strongly supports the notion that MEK1–ERK1/2 signalling cascade as a central "hub" in EV71 replication cycle and pathogenesis. EV71 has been documented as the most important neurotropic EV after the eradication of the poliovirus (Solomon et al., 2010; Yi et al., 2011), and currently, no effective drug is available clinically for use against EV71 infection.

Consequently, components of the ERK pathway, such as MEK1/2, represent excellent targets for the development of antiviral drugs (Arita et al., 2009; Hunter et al., 1995; King et al., 1986; Moser and Schultz-Cherry, 2008; Perkins et al., 2002; Pleschka, 2008; Smith et al., 2000; Tung et al., 2011). In fact several promising small molecule MEK inhibitors, such as ARRY-142886 and CI-1040, have already entered clinical trials for cancer therapy (Kohno and Pouyssegur, 2006; Pleschka, 2008). Therefore, blockage of this cellular pathway, including isoform specific inhibition demonstrated in this study, may finally result in completely novel concepts of antiviral therapy.

Acknowledgments

This work was supported by Grants from National High-Tech Research and Development Program of China (863 Program, No. 2007AA02Z317) and the National Natural Science Foundation of China (No. 30470084). We would like to thank Dr. Malcolm A. McCrae for critical reading and revision of the manuscript.

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