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MEK inhibition impairs influenza B virus propagation without emergence of resistant variants

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Abstract **Influenza A and B viruses are still a major worldwide threat. We demonstrate that influenza B virus infection induces signaling via the Raf/MEK/ERK cascade, a process required for efficient virus production. Expression of dominant-negative Raf and ERK mutants or treatment with a MEK inhibitor (U0126) strongly impaired viral propagation, while selective activation of the pathway resulted in increased virus titers. MEK inhibition appears to interfere with a distinct viral nuclear export process. Most importantly, no resistant virus variants emerged in the presence of U0126 demonstrating that influenza viruses cannot easily adapt to the missing cellular function.**

Key words: Influenza virus; Mitogen-activated protein kinase cascade; Mitogen-activated protein kinase/extracellular signal-regulated kinase inhibition; Viral resistance

1. Introduction

It has long been known that a variety of DNA viruses and retroviruses induce cellular signaling through the classical mitogen-activated protein kinase (MAPK) cascade [1–4]. However, only little is known about functional signaling via the Raf/MEK/ERK pathway induced by negative-strand RNA viruses [5–12].

Influenza A and B viruses, which represent related but distinct virus species, are highly contagious worldwide pathogens for humans. Important characteristics that distinguish influenza A and B viruses include the restricted host range of type B viruses to humans, a higher variability and antigenic diversity of the influenza A virus surface proteins and expression of at least one unique protein by type A or B viruses that has no obvious counterpart in the other type. Most importantly, influenza B viruses are not sensitive to the action of amantadine, a compound that efficiently blocks influenza A virus replication [13].

Both viruses possess a negative-strand RNA genome of eight segments that is replicated and transcribed in the nucleus of the infected cell. In the late stage of the replication cycle the viral genome forms ribonucleoprotein complexes (RNPs) that are exported to the cytoplasm to be packaged into progeny virions [14–16]. Thus, nuclear RNP export is an essential step during influenza virus replication. Due to the relatively small coding capacity influenza viruses extensively exploit and manipulate host cell functions to support viral replication [17]. Uncovering these dependencies could help to develop new strategies for antiviral therapy. We have recently shown that the Raf/MEK/ERK cascade is activated by type A influenza viruses in a biphasic manner to support viral propagation [8]. Here we investigated whether the related but distinct type B influenza viruses also regulate ERK signaling and whether such a control is equally required for virus propagation. Furthermore we examined whether resistant variants of influenza A and B viruses will emerge upon inhibition of the Raf/MEK/ERK signaling cascade.

2. Materials and methods

2.1. Viruses, cells and viral infections

Human influenza virus strains B/Lee/40 (B/Lee), B/Maryland/59 (B/Md), B/Massachusetts/6/93 (B/Mass) and avian influenza A/FPV/Bratislava/79 (H7N7) (FPV) viruses were used for infection of Madin–Darby canine kidney (MDCK) cells that were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) and antibiotics. Cells were washed with phosphate-buffered saline (PBS) and infected at the indicated multiplicity of infection (MOI) in PBS/BA (PBS containing 0.2% bovine serum albumin, 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 100 U/ml penicillin, 0.1 mg/ml streptomycin) for 1 h at room temperature. The inoculum was aspirated and cells were incubated with DMEM/BA (medium containing 0.2% bovine serum albumin (BSA), antibiotics and 2 μg/ml trypsin for

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Abbreviations: MDCK, Madin–Darby canine kidney; RNP, ribonucleoprotein complex; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK kinase/ERK kinase; JNK, c-Jun NH_2-terminal kinase; DMSO, dimethylsulfoxide

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B/Lee, B/Md and B/Mass). The MEK inhibitor U0126 (Promega) in dimethylsulfoxide (DMSO) or solvent alone were added 30 min prior to infections and the final concentrations indicated were kept constant throughout the experiments. Mitogenic activation of the Raf/MEK/ERK cascade was achieved by stimulation of cells with 100 ng/ml of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA). A standard plaque assay was used to assess the number of infectious progeny virus particles (PFU = plaque-forming units).

2.2. Mammalian expression vectors and transfections

The empty expression vector (pKRSPA, empty vector) and plasmids expressing dominant-negative mutants of ERK and Raf (pKRSPA-ERK2B3, pKRSPA-ERK2C3 and pKRSPA-RafC4B) were described previously [18]. A constitutively active mutant of Raf (pcDNA-Raf BxB-Cx/DD) was created by fusion of the kinase domain of c-Raf-1 to the membrane targeting signal of Ki-Ras and by exchange of two tyrosines at positions 340/341 to aspartic acid to mimic phosphorylation [19]. A constitutively active mutant of MEK1 (pBABE ΔStu1 MEK1 LIDEMANE) was generated by deletion of an autoinhibitory α-helix and by exchange of two activating phosphorylation sites at positions 218/222 to glutamic acid [18,20]. MDCK cells were transfected with Lipofectamine 2000 (Invitrogen).

2.3. Immune complex kinase assays, in vivo labelling and Western blotting

Cells were lysed in Triton lysis buffer (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 50 mM Na-glycerolphosphate, 20 mM Na-pyrophosphate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM Na-vanadate, 5 mM benzamidine) on ice for 10–20 min. Cell lysates were then centrifuged at 13 000 × *g* for 10 min at 4°C and supernatants were used for Western blot analyses and immune complex kinase assays. To determine the amount of newly synthesized viral hemagglutinin and nucleoprotein (HA_0, NP), blots from total cell lysates were incubated with a specific antiserum against influenza B virions and a monoclonal antibody (mAb) to NP (DPC Biermann). Proteins were detected with peroxidase-coupled streptavidin or protein A or G, followed by a standard enhanced chemiluminescence reaction (Amersham). To determine activity of the three MAPKs cell lysates were incubated with antisera to ERK2, c-Jun NH_2-terminal kinase 1 (JNK1) or p38 (Santa Cruz Biotechnology) and protein A agarose (Roche Molecular Biochemicals) for 2 h at 4°C. Immune complexes were used for in vitro kinase assays with purified myelin basic protein (MBP), glutathione *S*-transferase-(GST) fused c-Jun (1–135) or dominant-negative 3pK (3pK K > M) as substrates for ERK, JNK or p38 as previously described [18]. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto polyvinylidene difluoride membranes. Phosphorylated proteins were detected by a BAS 2000 Bio Imaging Analyzer (Fuji) and by autoradiography. Loading of kinases in the assays was monitored in blots using the respective anti-kinase antiserum.

2.4. Confocal laser scanning microscopy

MDCK cells grown on glass coverslips were infected (MOI = 1), incubated with media containing solvent (DMSO, 0.6%) or U0126 (60 μM, in DMSO), washed twice with PBS 6 h or 8 h after infection, fixed for 20 min with 4% paraformaldehyde, 1% Triton X-100 (in PBS) at room temperature. After being blocked, fixed cells were incubated with NP-specific mAb (1:50, DPC Biermann, Germany) diluted in PBS/3% BSA for 1 h. After additional washes cells were incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (1:100, Sigma, Germany) in PBS/3% BSA containing 1 μg/ml propidium iodide (PI, Sigma) for 1 h, washed, and mounted with Mowiol (Aldrich) in glycerol/H_2O supplemented with 2.5% DABCO (Merck). Fluorescence was visualized with a DM IRBE confocal laser scanning microscope (Leitz).

2.5. Cell viability analysis

MDCK cells were grown to near confluence in six-well dishes and either left untreated or incubated with 60 μM U0126 or 0.6% DMSO for 9 h, 24 h, 48 h or 72 h. After morphological examination cells were washed twice with PBS, trypsinized, and after two additional washing steps stained with 1 μg/ml PI. The proportions of dead and viable cells were then analyzed in flow cytometry analysis using a FACScan (Becton Dickinson).

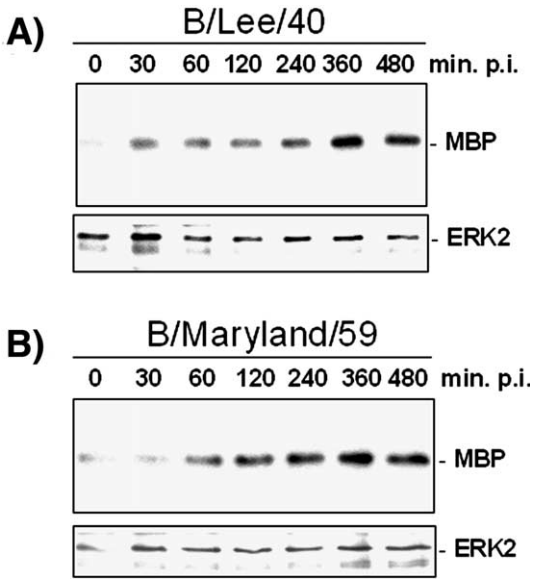

Fig. 1. Influenza B virus induces ERK activity. MDCK cells were infected with influenza virus B/Lee (A) and B/Md (B) in PBS/BA at MOI = 5. Cells were incubated in DMEM/BA and harvested at the indicated time points p.i. and ERK2 was immunoprecipitated from cell lysates as described previously [8]. Immune complexes were subjected to in vitro kinase assay using MBP as a substrate. Proteins were separated by SDS–PAGE, and labeled substrate bands were detected by autoradiography. Equal loading of ERK2 proteins was confirmed by Western blot.

3. Results

3.1. Influenza B virus infection results in the activation of the Raf/MEK/ERK cascade

MDCK cells were infected with three influenza B virus strains (B/Lee, B/Md, B/Mass). At the indicated time points cell lysates were prepared and subjected to an immune complex kinase assay to analyze ERK activity as a measure for induction of the Raf/MEK/ERK cascade [8]. Infection of MDCK cells with B/Lee or B/Md leads to activation of ERK that steadily increases to reach a plateau around 6 h post infection (p.i.) (Fig. 1). Essentially the same results were obtained for B/Mass (data not shown). This indicates that the activation kinetics induced by influenza B viruses differs from that of influenza A viruses that show a biphasic rather than a linear activation of ERK [8].

3.2. Influenza B virus-induced Raf/MEK/ERK activity is critical for viral propagation

To determine the role of virus-induced ERK activity for influenza B virus propagation MDCK cells were infected with B/Mass at low MOI to allow multi-cycle replication. The infected cells were incubated for 96 h in the presence or absence of 60 μM U0126 in DMSO or the solvent alone (0.6%). To analyze the efficacy of U0126 the inhibitor was given either once (directly after infection) or twice (directly and 24 h p.i.) while the other samples received the solvent DMSO. The production of infectious progeny virions was determined in supernatants taken every 12 h. After 60 h p.i. virus titers of U0126-treated cells were reduced by more than 1 log compared to the solvent control (Fig. 2A). Furthermore, in MDCK cells transfected with plasmids transiently expressing dominant-negative forms of ERK and Raf, virus titers

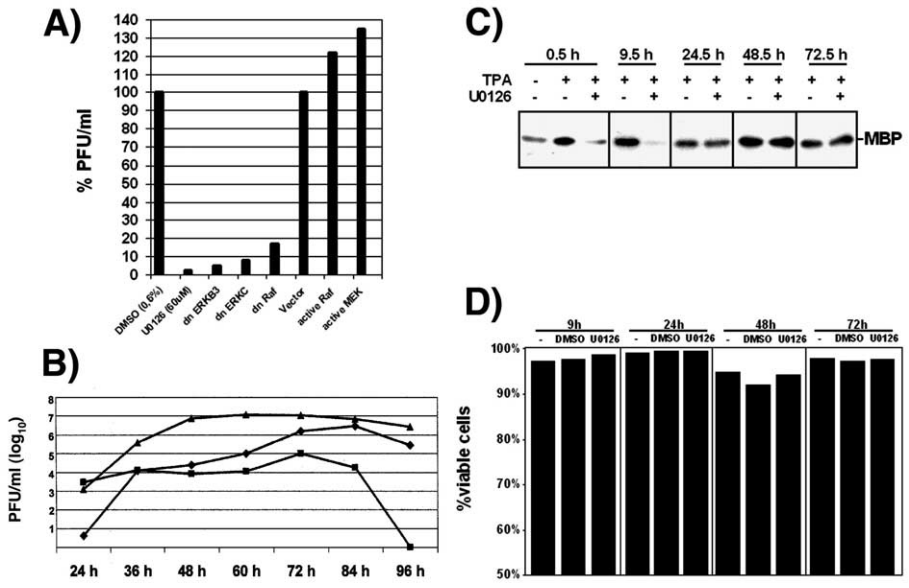

Fig. 2. Blockade of Raf signaling impairs influenza B virus propagation. A: MDCK cells grown in DMEM, 10% FCS and antibiotics were either infected with B/Mass at MOI = 1 and incubated for 60 h in DMEM/BA in the presence of U0126 (60 μM) or the solvent DMSO (0.6%) or were first transfected with plasmids expressing dominant-negative (dn) forms of ERK (ERK2B3, ERK2C3 (dn ERKB3, dn ERKC3)), or Raf RafC4B (dn Raf)) as well as constitutively active forms of Raf (pcDNA-Raf BxB-Cx/DD (active Raf)) and MEK (pBABE ΔStu1 MEK1 LIDEMANE, (active MEK)) or empty vector. Virus titer (PFU/ml) was determined 48 h p.i. by standard plaque assay on MDCK cells shown as the mean of duplicates titered with different dilutions. PFU titers of U0126-treated or plasmid-transfected samples were calculated as percentage of DMSO- or vector-transfected controls respectively, set at 100%. B: MDCK cells were infected with B/Mass (MOI = 0.01) and incubated in the presence of U0126 (applied once p.i. (♦) and twice 24 h p.i. (■)) or DMSO (▲) (see A) for 96 h. Medium samples collected every 12 h p.i. were analyzed for infectious virions by plaque assay. C: MDCK cells were either left untreated or treated with 60 μM U0126 for the times indicated. Cells were then stimulated for 15 min with 100 ng/ml of TPA as a strong activator of the Raf/MEK/ERK cascade. Cell lysates were subjected to ERK2 immune complex kinase assays using MBP as a substrate. Induction of the Raf/MEK/ERK cascade by TPA is effectively inhibited by U0126 up to 9.5 h but increases after 24.5 h of U0126 incubation and is unaffected after 48.5 h. D: MDCK cells in duplicate were left untreated or were treated with U0126 (60 μM) or the solvent DMSO (0.6%) for 9 h, 24 h, 48 h or 72 h. Cells were stained with PI (1 μg/ml) and analyzed by flow cytometry using FACS analysis. The amount of surviving cells is shown as percentage ± S.E.M. of the total cell number, which was arbitrarily set at 100%.

were also reduced about 10-fold (Fig. 2A). In contrast, expression of constitutively active forms of Raf and MEK led to increased virus titers. It should be stressed that transfection efficiencies in these assays were around 60%, thus the effects are 'diluted' by 40% of untransfected wild-type cells. Nevertheless a significant reduction or enhancement of viral replication could be achieved, demonstrating a critical role of Raf, MEK and ERK for influenza B virus propagation. The viral growth curve in the presence of a single dose of U0126 differed significantly from the solvent control. Influenza B virus titers were already detectable at 36 h p.i. in the control with increasing titers up to 60 h p.i. Thereafter virus titers declined. In contrast, in U0126-treated cultures virus growth was severely delayed and was only detectable as late as 60 h p.i. with reduced peak titers at 84 h p.i. (Fig. 2B). Since the activity of the single dose of inhibitor declined gradually in cell culture and was only barely detectable 24 h p.i. and later (Fig. 2C), recovery of virus growth correlated well with the decrease in MEK inhibition. This becomes evident when U0126 was applied twice resulting in a titer reduction of more than 10-fold throughout the full time course of the experiment (Fig. 2B). In conclusion, inhibition of ERK signaling not only delayed virus growth, but also resulted in a reduced peak activity, even with a 'one-shot' treatment.

3.3. MEK inhibition by U0126 is not toxic for MDCK cells

Neither U0126 nor the solvent DMSO was toxic for the host cells in the concentrations used (Fig. 2D) as determined by PI staining and flow cytometry analysis of MDCK cells. Furthermore, the inhibitor had no adverse effect on vesicular stomatitis virus (Rhabdoviridae) propagation indicating that cells remain principally viable and permissive for virus replication (data not shown). This has also been confirmed for U0126 concentrations that effectively act against Borna disease virus (Bornaviridae) in several cell types [9].

3.4. U0126 neither affects production of viral HA and NP nor reduces activity of other virus-induced MAPK cascades

Next, we investigated whether the accumulation of viral proteins was affected by U0126, thereby leading to a reduction of progeny virions. The accumulation of both an early viral protein, the nucleocapsid protein (NP), and a late viral protein, the hemagglutinin (in the uncleaved form, HA_0), was analyzed. Even though the anti-NP mAb does not detect the B/Md-NP very well, only slight differences in NP amounts of U0126- or DMSO-treated cells were detectable (Fig. 3A). Equally, the amounts of HA_0 detected in infected inhibitor- or solvent-treated cells at 10–14 h p.i. did not differ significantly. Partial differences in the protein amounts of HA and NP may also in part correspond to minor loading differences as can be seen in the loading control. Taken together these results indicate that as for influenza A viruses [8] the decrease in virus titers by MEK inhibition cannot be attributed to severe effects on the viral protein production.

Influenza A virus infection results in activation of JNK and p38 MAPK [11,21]. Therefore we analyzed whether type B

virus infection would equally induce activation of these kinases and whether U0126 would influence their activity. The results obtained from immune complex kinase assays of ERK2, JNK and p38 show that all three kinases are activated by influenza B virus infection, but as compared to virus-activated ERK, there is no cross-inhibition of JNK or p38 pathways by U0126 (Fig. 3B). This demonstrates the specificity of the inhibitor under our experimental conditions.

3.5. MEK inhibition impairs nuclear RNP export

The viral RNPs that are composed of the viral RNA segments, the NP and the polymerase proteins undergo bidirectional transport across the nuclear membrane during the viral replication cycle. First, RNPs are transported into the nucleus where transcription and replication occurs. At a late stage of the infection cycle, newly formed RNPs are exported to the cytoplasm and are assembled into progeny virions at the cellular membrane.

Since MEK inhibition affects export of influenza A virus RNPs [8] we investigated the intracellular RNP localization during influenza B virus infection in the presence or absence of U0126 by immunofluorescence at different time points (Fig. 4). NP is the main component of the RNPs and serves as an indicator for RNP localization [22]. To identify cell nuclei the cellular DNA was counterstained with PI. The RNPs of DMSO-treated cells started to appear in the cytoplasm at 6 h p.i. indicating the onset of nuclear export of newly formed RNPs. In contrast RNPs were still retained in the nucleus of U0126-treated cells at the same time point. This picture changed only slightly at 8 h p.i. as only a small fraction of RNPs was located in the cytoplasm of U0126-treated cells. These results demonstrate that MEK inhibition negatively affects nuclear export of influenza B virus RNPs and implies that RNP packaging into progeny virions is impaired.

3.6. Influenza B and A viruses cannot adapt to the selective pressure of MEK inhibition

The influenza virus polymerase does not possess a 'proofreading' function resulting in a high error rate. This allows the virus to rapidly adapt to certain selection pressures by mutation, thereby generating resistant variants as shown for the antiviral agent amantadine [23]. Amantadine has a strain-specific inhibitory effect on type A but not on type B influenza viruses [24]. The agent blocks the ion channel activity of the viral M2 protein. It is a common observation that amantadine-sensitive influenza A virus strains rapidly develop resistance in vitro and in vivo [23,25–27].

To assess whether continued MEK inhibition may also lead to the emergence of resistant influenza B and A virus variants we performed multi-passage experiments (Fig. 5). MDCK cells were infected with B/Mass or FPV [28] at low MOI in the presence or absence of U0126 (50 μM) or the solvent

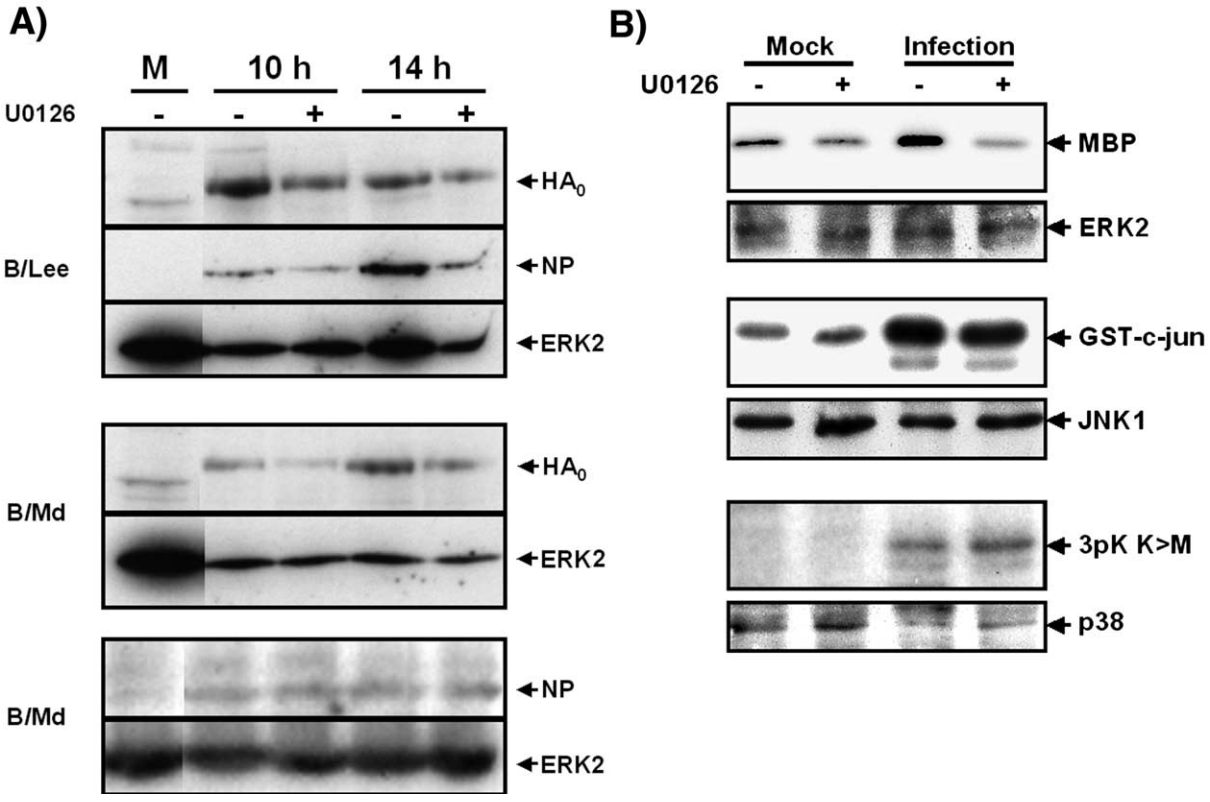

Fig. 3. MEK inhibition neither affects viral protein accumulation nor impairs other MAPK cascades. A: MDCK cells were infected with B/Lee and B/Md at MOI = 5 in the presence (+) or absence (−) of U0126 (50 μM) and were lysed at the indicated times p.i. Cell lysates were subjected to SDS–PAGE and blotted on nitrocellulose membranes. Blots were incubated with an antiserum against influenza B virions (HA) and nucleoprotein (NP). Loading of protein in the lysates was monitored with an antiserum against ERK2. B: MDCK cells were infected with B/Md at MOI = 5 in the presence (+) or absence (−) of U0126 (50 μM) and incubated overnight. Cell lysates were subjected to immunoprecipitation with antisera to ERK2, JNK1 or p38. Subsequently immune complex kinase assays were performed as described in Section 2 using purified MBP, GST-c-Jun (1–135) or 3pK K > M as substrates for ERK, JNK or p38 respectively. Loading of the kinases in these assays was monitored in blots using the respective anti-kinase antibodies.

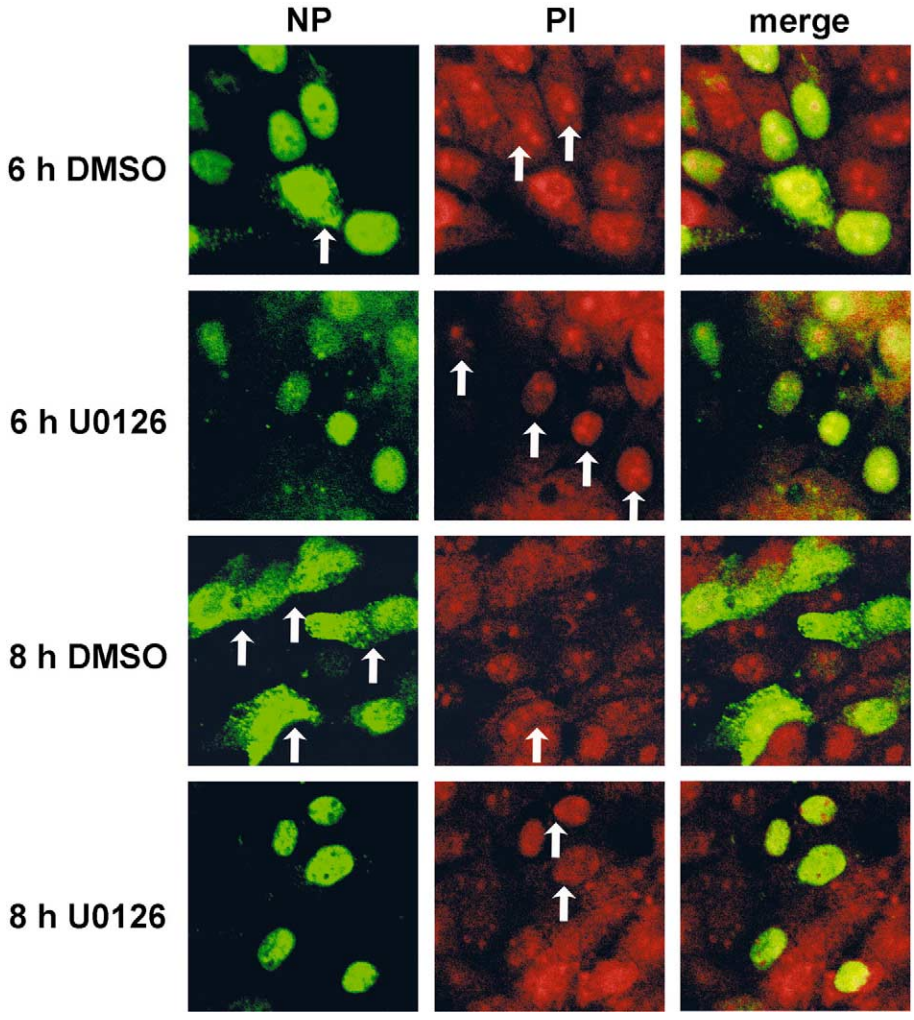

Fig. 4. Intracellular localization of viral RNPs. MDCK cells were infected with B/Mass (MOI = 1) and incubated with media containing U0126 (50 μM) or the solvent DMSO (0.5%). At the indicated time points (6, 8 h p.i.) cells were fixed, permeabilized and incubated with an anti-influenza B virus NP mAb and subsequently with a FITC-coupled anti-mouse Ab and PI. The intracellular distribution of the viral RNPs and the cellular DNA was determined by confocal laser scanning microscopy. Cytoplasmic localization of viral RNPs (NP, green) and of selected nuclei (PI, red) is indicated (white arrows). The merger shows that the intracellular DNA and the RNPs co-localize within the nucleus.

(0.5%). The U0126 concentration selected still allowed a low-level replication giving the residual progeny virus a chance to adapt to the selective pressure. 48 h p.i. the same amount of solvent or inhibitor was added again. Supernatant was harvested after an additional 24 h and 0.1 ml of a 10^{-2} dilution was used for the next passage. Five passages were performed and the virus titer was determined after each passage (Fig. 5A). As a reference, influenza A virus (FPV) was passaged in parallel in the presence of the drug amantadine (5 μM) and supernatants were harvested 24 h p.i., 0.1 ml of a 10^{-3} dilution was used for the next passage and then proceeded as described above (Fig. 5B). An initial reduction in B/Mass titer of 63% with U0126 treatment was enhanced in the following passages (99%, 96%, 97%, 94%). The virus titer did not rise again indicating that B/Mass cannot adapt to MEK inhibition. For the influenza A virus FPV the reduction of virus titers in the presence of U0126 remained constant throughout all five passages (82%, 92%, 77%, 95%, 89%) and is in the range of what was previously reported [8]. Again, emergence of resistant variants was not observed. In contrast, amantadine treatment resulted in a rapid selection of resistant variants, reflected by a steady decrease of inhibition (93%, 54%, 38%, 1%, 0%). Even though the initial titer reduction for B/Mass is not as strong as for FPV the results indicate that influenza A and B viruses do not adapt easily to MEK inhibition.

4. Discussion

Influenza A and B viruses are responsible for millions of infections and thousands of deaths in the human population worldwide each year. Therefore new information about their replication strategies could support new approaches in the fight against these foes.

Here we demonstrated that influenza B virus infection leads to a time-dependent increase of cellular ERK activity. Virus propagation is strongly dependent on the activity of the Raf/MEK/ERK signaling cascade since not only U0126 treatment but also expression of dominant-negative forms of Raf and ERK repressed virus propagation by at least 1 log step (Fig. 2A). Accordingly, constitutively active Raf and MEK supported an increase in virus titers (Fig. 2A).

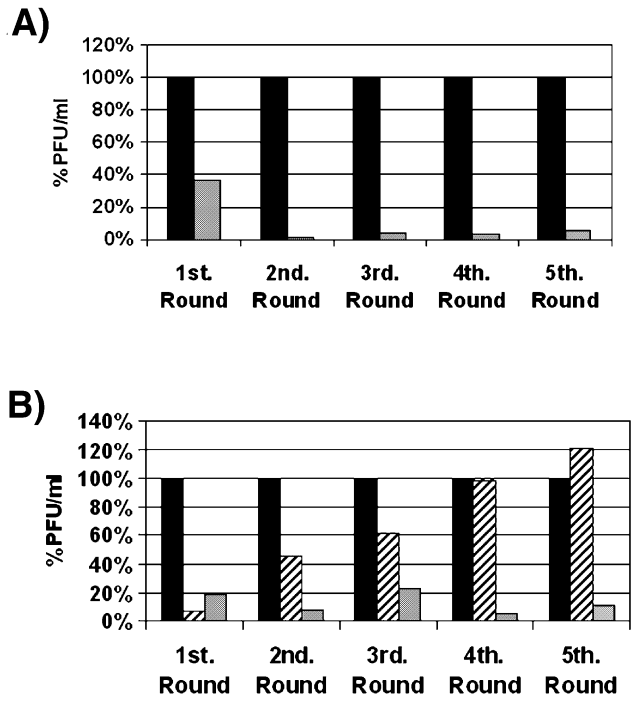

Fig. 5. U0126 treatment does not select for resistant influenza virus variants. A: MDCK cells were infected with B/Mass (MOI = 0.01) and incubated 48 h with media and the solvent DMSO (0.5%, black bars) or U0126 (50 μM, gray bars). The same amount of inhibitor and solvent was added to the media again and cells were incubated another 24 h. Diluted supernatant was used for a second round of infection of fresh MDCK cells. Five passages (1st, 2nd, 3rd, 4th, 5th round) were performed and after each the virus titer (PFU/ml) was determined by plaque assay. PFU titers of U126-treated samples were calculated as percentage of DMSO controls set at 100%. Shown is the mean of duplicate experiments titered in different dilutions. B: MDCK cells were infected with FPV (MOI = 0.01) and incubated 24 h with media containing the solvent DMSO (0.5%, black bars), amantadine (5 μM, hatched bars) or U0126 (50 μM, gray bars). Diluted supernatant was used for a second round of infection of fresh MDCK cells. Five passages (1st, 2nd, 3rd, 4th, 5th round) were performed and after each the virus titer (PFU/ml) was determined by plaque assay. PFU titers of amantadine- and U0126-treated samples were calculated as percentage of DMSO controls set at 100%. Shown is the mean of duplicate experiments titered in different dilutions.

The antiviral activity of the MEK inhibitor appears to correlate with its stability. At early time points when U0126 exerted its full activity virus production was efficiently suppressed (Fig. 2B) by a single inhibitor dose. Decline of U0126 activity in cell culture that was obvious 24 h post treatment and later (Fig. 2C) allowed the residual virus to further replicate. However, a delay in growth and a reduction in peak titers was still achieved. Recovery from MEK inhibition also demonstrated that the U0126 concentrations were not irreversibly toxic for the cell. This was independently confirmed as the viability of MDCK cells treated with U0126 was unaffected (Fig. 2D). A second dose of U0126 24 h p.i. led to more than 1 log reduction of the virus titer which persisted up to 96 h p.i. (Fig. 2B) demonstrating that MEK inhibition indeed impairs and does not just delay virus replication. Thus, as long as a MEK inhibitor is biologically active in cells an efficient reduction of influenza B virus replication could be achieved. The accumulation of viral HA and NP only showed very small differences in solvent- or U0126-treated cells (Fig. 3A) indicating that the significant reduction of virus titers does not result from altered expression of viral proteins. This is consistent with earlier studies using influenza A viruses [8].

Previously it was shown that influenza A virus infection activates the JNK and p38 MAPK pathways [11,21]. Here we demonstrate that influenza B virus infection likewise activates these MAPK cascades (Fig. 3B) in a U0126-unaffected manner. This lack of cross-inhibition and the results obtained with dominant-negative kinases indicate that U0126 exerts its effect on virus replication specifically through MEK inhibition.

As observed for influenza A viruses [8] U0126 treatment leads to impaired nuclear RNP export at late stages of the viral replication cycle (6–8 h p.i.) and even for prolonged times (10 h p.i., data not shown). Thus both virus types rely on virus-induced MEK activity for efficient nuclear export of their RNPs. The molecular mechanism by which ERK activity regulates RNP export is not clear at the moment. However, there is first evidence of distinct ERK-controlled export pathways. In lipopolysaccharide (LPS)-treated mouse macrophages MEK inhibition results in a specific retention of the tumor necrosis factor (TNF) mRNA in the nucleus [29]. This is also observed in cells deficient for Tpl-2, an activator of MEK and ERK. In these cells the failure to activate MEK and ERK by LPS again correlated with TNF mRNA retention while other cytokines are normally expressed [29]. Thus the ERK pathway may regulate a specific cellular export process presumably by regulating factors binding to the specific AU-rich regions in certain mRNAs [29] but leaves other export mechanisms unaffected. It is likely that such a specific export pathway is employed by influenza A and B viruses. Although activated upon influenza B virus infection, other MAPKs such as p38 appear not to be involved in control of such a pathway. When the p38-specific inhibitor SB203580 was used, the nuclear export of viral RNPs remained unaffected (data not shown), indicating that the virus-induced p38 signaling is not required for RNP export. This may also explain why U0126, in contrast to other more general export blockers such as leptomycin B that efficiently blocks influenza A [30,31] and B virus RNP export (data not shown), is not toxic for cells. How ERK signaling controls RNP export is not understood yet and is under investigation. It could well be directly by phosphorylation of a viral protein involved in the RNP export process as for example the M1 [32,33] or by regulating a cellular factor as mentioned above.

The obvious antiviral activity of MEK inhibitors and the fact that inhibition of the cascade does not severely affect cell viability ([8,9] and Fig. 2D) suggest that the signaling pathway may serve as a new target for an antiviral approach. Another prerequisite for a potent anti-influenza compound would be that the drug does not induce resistant variants, which usually occurs rapidly with influenza A viruses. Therefore we investigated whether repeated passaging of influenza type A and B viruses in the presence of the drug would lead to emergence of resistant mutants. In contrast to amantadine, a compound targeting a viral protein of some influenza A type viruses, U0126 shows no tendency to generate escape mutants of both A- and B-type influenza viruses (Fig. 5). Although this outcome could not be predicted for a new inhibitor, it is consistent with an earlier study reporting failure to obtain

drug-resistant influenza virus mutants to inhibitors of cellular factors, such as methyltransferase inhibitors or a broad-band kinase inhibitor (H7) [27]. Thus, the virus cannot easily adapt to a missing cellular function. Therefore, transient blockade of the MAPK cascade, a cellular pathway that supports influenza A and B virus propagation, may be a novel strategy for antiviral control of these pathogens.

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