Membrane Accumulation of Influenza A Virus Hemagglutinin Triggers Nuclear Export of the Viral Genome via Protein Kinase Ca-mediated Activation of ERK Signaling*

Received for publication, September 16, 2005, and in revised form, March 29, 2006. Published, JBC Papers in Press, April 11, 2006, DOI 10.1074/jbc.M510233200

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† This work was supported by grants of the Deutsche Forschungsgemeinschaft (Grants IIKG-GRK370/3 and SFB 535 to S. P., SFB 593 to H.-D. K., and Lu 477/4-5 to S. L.). This work is part of the activities of the VIRGIL European Network of Excellence on Antiviral Drug Resistance supported by the Priority 1 Life Sciences, Genomics and Biotechnology for Health program in the 6th Framework Program of the EU (Grant LSHM-CT-2004-503359). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: MEK, MAPK/ERK kinase; ts, temperature-sensitive; wt, wild type; HA, hemagglutinin; MCD, methyl-β-cyclodextrin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PKC, calcium-dependent protein kinase; RNP, ribonucleoprotein; NP, nucleoprotein; MDCK, Madin-Darby canine kidney cells; PBS, phosphate-buffered saline; m.o.i., multiplicity of infection; TPA, 12-O-tetradecanoylphorbol-13-acetate; IFN, interferon; JNK, c-Jun NH2-terminal kinase; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; Cr-B, cholesterol B subunit; MITT, 3,4-5-dimethylthiazole-2-yl-2,5-diphenyltetrazolium bromide; ss, single strand; ds, double strand; wt, wild type; p.i., post infection; p.t., post transfection; dn, dominant negative; GM1, gangliotetraosylceramide.

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The JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 24, pp. 16707–16715, June 16, 2006

Membrane Accumulation of Influenza A Virus Hemagglutinin Triggers Nuclear Export of the Viral Genome via Protein Kinase Ca-mediated Activation of ERK Signaling*

Replication and transcription of the influenza virus genome takes place exclusively within the nucleus of the infected cells. The viral RNA genome, polymerase subunits, and nucleoprotein form ribonucleoprotein (RNP) complexes. Late in the infectious cycle RNPs have to be exported from the nucleus to be enwrapped into budding progeny virions at the cell membrane. This process requires viral activation of the cellular Raf/MEK/ERK (mitogen-activated protein kinase (MAPK)) signaling cascade that is activated late in the infection cycle. Accordingly, block of the cascade results in retardation of RNP export and reduced titers of progeny virus. In the present study we have analyzed the importance of cell-membrane association of the viral hemagglutinin glycoprotein for viral MAPK activation. We show that hemagglutinin membrane accumulation and its tight association with lipid-raft domains trigger activation of the MAPK cascade via protein kinase Ca activation and induces RNP export. This may represent an auto-regulative mechanism that coordinates timing of RNP export to a point when all viral components are ready for virus budding.

Influenza A and B viruses are important worldwide pathogens for humans and animals and cause devastating epidemic and pandemic outbreaks. During intracellular replication influenza viruses interact with many different cellular functions to promote their propagation (1, 2). Both influenza A and B viruses activate the cellular Raf/MEK/ERK3 signaling cascade. A block of this signaling pathway impairs RNP nuclear export and thereby virus replication without the emergence of resistant variants (3, 4).

Many DNA and retroviruses induce cellular signaling through mitogen-activated protein kinase (MAPK) cascades (5–8), and reports on functional MAPK signaling induced by negative strand RNA viruses are now accumulating (9–14). MAPK cascades are key regulators of cellular responses such as proliferation, differentiation, and apoptosis (15–19). The Raf/MEK/ERK cascade is the prototype of MAPK cascades. Growth factor-induced signals are transmitted by consecutive phosphorylation from the kinase Raf via MEK to ERK, which translocates to the nucleus to phosphorylate a variety of substrates (17, 19). Intracellular signal transduction is usually initiated by membrane proximal events, including complex protein-protein interactions between ligands, receptors, and kinases. Lipid rafts on the cell surface have been shown to play a crucial role in these processes. These membrane structures are cholesterol- and sphingolipid-rich dynamic assemblies, in the outer leaflet of the membrane bilayer. Such microenvironments form distinct areas within the lipid bilayer (21). They can change their size and protein composition in response to intra- or extracellular stimuli leading to specific protein-protein interactions. Lipid rafts can form platforms for receptors that are activated by ligand binding and other signaling components resulting in the activation of signaling cascades (22). Within lipid-raft domains kinases can alter the phosphorylation state of their substrates and membrane-anchored G-proteins can activate signaling, such as Ras that recruits Raf to the plasma membrane leading to the activation of MEK and ERK (22).

The influenza A virus genome consists of eight RNA segments of negative polarity coding for at least 10 viral proteins. The three subunits (PB1, PB2, and PA) of the viral RNA-dependent RNA-polymerase and the nucleoprotein (NP) form the biological active ribonucleoprotein (RNP) complexes. Because influenza viruses pursue a nuclear replication strategy the RNPs must undergo bidirectional nuclear transport. Upon infection the RNPs are released into the cytoplasm and transported into the nucleus. The nuclear residency of the RNPs must be well coordinated to assure efficient viral genome replication and production of viral mRNAs/proteins, whereas late in the replication cycle newly formed RNPs have to be exported from the nucleus and transported to the cell membrane for virus assembly (23, 24).

The HA serves two important functions early in the viral life cycle. It binds to the receptor determinant (25) and induces fusion between the viral and the cellular membrane (26). As shown for other viral glycoproteins (27) HA is post-translationally acylated by addition of palmitoyl acids at three highly conserved cysteine residues (28, 29). These modi-
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fications seem to be important for efficient fusion (30) as well as for virus assembly and interaction of HA with lipid rafts (31–33).

The virus-induced MAPK signaling is activated late in the viral replication cycle, and RNP nuclear export is dependent on this cellular function (3, 4). Our aim was to define viral factors/mechanisms that activate this MAPK signaling cascade. The facts that (i) Raf/MEK/ERK signaling is commonly activated by either membrane-resident or temporarily membrane-localized factors, (ii) HA associates with cholesterol and lipid rafts that can be involved in signaling (22, 30–32, 34, 35), and (iii) HA accumulates in the cellular membrane late in the viral replication cycle led us to investigate the role of HA as a possible viral inducer of MAPK signaling in more detail.

EXPERIMENTAL PROCEDURES

Viruses, Cells, and Infection—Avian influenza virus A/FPV/Rostock/34 (H7N1, Rostock), A/FPV/Bratislava/79 (H7N7, Bratislava), the Rostock-derived temperature-sensitive mutant ts227 characterized by a plaque forming units).

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Viruses, Cells, and Infection—Avian influenza virus A/FPV/Rostock/34 (H7N1, Rostock), A/FPV/Bratislava/79 (H7N7, Bratislava), the Rostock-derived temperature-sensitive mutant ts227 characterized by a cell-surface transport defect of HA at non-permissive temperature (40 °C), the recombinant viruses Ac1, Ac2, and Ac3 (30), the human influenza virus A/Puerto-Rico/8/34 (H1N1, PR/8), and the PR/8 mutant lacking the NS1 gene (ΔNS1) (36) were used for infection of Madin-Darby canine kidney (MDCK) cells grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum and antibiotics. Cells were washed with phosphate-buffered saline (PBS), infected at the indicated multiplicity of infection (m.o.i.), and further incubated as described previously (3). Activation of the Raf/MEK/ERK cascade was achieved by stimulation of cells with 100 ng ml−1 of the phorbol ester TPA. Standard plaque assays on MDCK cells were performed to assess the number of infectious progeny virus particles (in plaque forming units).

Mammalian Expression Vectors and Transfections—The expression vectors pCAGGS-MCS-C5, pCAGGS-Rostock-HA expressing the Rostock wtH7-HA, pCAGGS-Ac3-HA expressing the Ac3-HA, and the ERK-expression vector pKRS-PA-HA-MAPK2 (described previously (37, 38)) were used for transfection of MDCK cells. To monitor IFNβ promoter activity MDCK cells were transfected with an IFNβ promoter-dependent luciferase expression plasmid pIFNβ-Luc (39). Additionally, a constitutively active mutant of Raf (pcDNA-Raf BxB-Cx/DD, described in Ref. 40) was used. For transient expression of dominant negative mutants of PKCα and Ras, MDCK cells were transfected with pCMV-PKCα K-R and pKRS-PA-ΔN Ras (kindly provided by Ulf Rapp, MSZ Würzburg, Germany), respectively, and empty vectors as control. MDCK cells were transfected with Lipofectamine 2000 (Invitrogen).

Luciferase Assays—To determine IFNβ-dependent reporter gene activation MDCK cells were transfected with the IFNβ promoter-dependent luciferase expressing plasmid pIFNβ-Luc using Lipofectamine 2000 (Invitrogen). After 24 h, cells were either mock transfected or transfected with poly(L/C) (Sigma) at different concentrations. Cell extracts were prepared at 4 h.p.t. in cell lysis buffer (Promega) according to the manufacturer’s description and assayed for luciferase activity using a Microplate Luminometer (Molecular Devices). Luciferase activities were determined from two independent transfections analyzed in duplicates.

Immune Complex Kinase Assays, in Vivo Labeling, and Western Blotting—Cell lysates were used for Western blot analysis and immune complex kinase assays as described before (3). Activated ERK and JNK were detected with phospho-specific monoclonal antibodies (mAb, Santa Cruz Biotechnology and BD Transduction Laboratories). After stripping of bound antibodies total ERK2 and JNK1 were detected using rabbit polyclonal antisera (Santa Cruz Biotechnology). Proteins recognized by primary Abs were further analyzed with peroxidase-coupled anti-species-specific Abs or streptavidin, followed by a standard enhanced chemiluminescence reaction (Amersham Biosciences). To determine HA-ERK activity cell lysates were incubated with the 12CA5 anti-HA mAb (purified at the Institute of Molecular Virology, Munster, Germany) and protein Agarose (Roche Applied Science) for 2 h at 4 °C. Immune complexes were used for in vitro kinase assays with purified myelin basic protein (Sigma) as substrate for ERK as previously described (38). After Western blot, proteins were detected by a BAS 2000 Bio Imaging Analyzer (Fuji) and autoradiography. Quantification of specific bands was done with the PC-BAS software package (Fuji). Loading in the assays was monitored in Western blots using anti-Erk2- and anti-JNK1-specific antisera (Santa Cruz Biotechnology) or the 12CA5 anti-HA mAb.

HA-Surface Expression—MDCK cells were infected with different strains of influenza A virus as indicated (m.o.i. = 1). Cells were incubated for 8 h. Then the cells were detached with trypsin, fixed in PBS/4% paraformaldehyde, and stepwise incubated with anti-H7-HA mAb and then with anti-mouse FITC-conjugated mAb (Calbiochem) for 30 min on ice each. Finally, HA-surface expression was determined by FACS analysis using an ELITE Analyzer (Beckman Coulter) or FACS Calibur (BD Biosciences).

Membrane Cholesterol Depletion and Quantification—To deplete membrane cholesterol MDCK cells grown in 10-cm dishes were washed with PBS and incubated with either fresh media or media containing 5 μM lovastatin to inhibit cholesterol synthesis (Sigma) for 7 h at 37 °C, 5% CO2. Then methyl-β-cyclodextrin (MCD, Sigma), solved in ddH2O, was added to deplete membrane cholesterol, was added (5 mM). Cells were incubated additionally for 1 h at 37 °C, 5% CO2.

In the cholesterol re-addition experiment the compound (20, 200, and 400 μg) was solved in ethanol, mixed with MCD (5 mM) in a total volume of 80 μl of media, and then added to the cell supernatant. Cells were further incubated for 1 h before lysis.

For cholesterol determination cell pellets were sonicated in 600 μl of chloroform/methanol (2:1, v/v) for 30 s. 120 μl of water was added, and after phase separation and centrifugation the chloroform layer was collected and dried in a stream of nitrogen (41). Acetylation was performed by adding 500 μl of acetic anhydride/pyridine (4:1, v/v) and incubation for 16 h at room temperature. The sample was redisolved in 500 μl of dichloromethane, and 2 μl was used for gas chromatographic analysis (Philips, Pye Unicam PU 4500). Cholesterol acetate was separated on a CP Sil 24cp column (0.25-mm inner diameter, Macherey and Nagel, Germany) by raising the temperature from 250 °C to 325 °C at 6 °C/min and detected by flame ionization. Quantification was performed with acetylated cholesterol standards. A total of two samples of untreated and three samples of cholesterol-depleted cells was measured, and the standard error was calculated.

Confocal Laser Scanning Microscopy and Immunofluorescence Assay (IFA)—MDCK cells grown on glass coverslips were infected as indicated, incubated in media with or without lovastatin (5 μM), MCD (5 mM), or TPA (100 ng ml−1, Sigma) as indicated, washed with PBS at the indicated time points post infection, and fixed with 4% paraformaldehyde with or without 1% Triton X-100 (in PBS) at room temperature. Then cells were incubated with the NP-specific mAb N7C4 (a kind gift from L. Stitz). Alternatively, infected cells were incubated with a combination of the NP-specific mAb and a phospho-specific anti-PKCα mAb (Santa Cruz Biotechnology) or the H7-HA-specific mAb. After additional washes permeabilized cells were incubated with FITC-labeled goat anti-mouse IgG (Sigma) in PBS/3% bovine serum albumin (containing 300 ng ml−1 propidium iodide, Sigma, when indicated) for
Lipid-Raft Co-patching Assay—MDCK cells grown on glass coverslips were washed once with PBS and then incubated with FITC-conjugated cholera toxin B subunit (Ct-B, *Vibrio cholerae*, Sigma, 50 μg/ml) in PBS/bovine serum albumin (3%) for 30 min at 4 °C, washed, and then incubated with anti-Ct-B antibody (Sigma) for 30 min at 4 °C followed by 15 min at 37 °C. After one additional washing step cells were fixed with 4% paraformaldehyde for 30 min at room temperature and mounted as above.

Cell Viability Analysis—MDCK cells were grown in 96-well dishes and either left untreated or incubated in media containing 5 μM lovastatin for 7 h at 37 °C, 5% CO₂. Then MCD was added to a 5 mM final concentration. Cells were incubated for one additional hour. Medium was replaced by fresh medium (Dulbecco’s modified Eagle’s medium/10% fetal calf serum, antibiotics). After further incubation for 1 h the medium was replaced by 200 μl of MTT-Mix (Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, antibiotics, containing tetrazolium bromide (175 μg/ml, Sigma)). After 90 min incubation media were replaced by PBS/4% paraformaldehyde, and cells were fixed for 30 min at room temperature. Cells were dried and, after addition of isopropanol plates, vigorously agitated for 10 min at room temperature. Photometrical analysis was done at 550 nm excitation in an enzyme-linked immunosorbent assay reader (Diagnostic Pasteur Type LP 400). 16 sam-

**FIGURE 1.** Viral replication intermediates do not induce ERK activation. A, MDCK cells were transfected with an IFN β1 promoter-dependent luciferase expression plasmid. 24 h later cells were transfected with poly(I/C) at the indicated amounts. Luciferase expression indicating IFN β1 promoter activation was analyzed 4 h.p.t. The results are based on two independent experiments with luciferase activities determined in duplicates. B, MDCK cells infected with either ΔNS1 or wild type PR/8 virus (PR/8, m.o.i. = 1) were incubated for 6 and 9 h. After Western blot ERK and JNK activation was analyzed with phospho-specific mAbs. Subsequently, loading was controlled with a mAb against ERK2 and JNK on the according blot.

**FIGURE 2.** HA-surface expression induces ERK activation and nuclear RNP export. A, MDCK cells uninfected (black line) or infected (gray areas) with either wild type virus (WT) or the ts mutant (ts227) (m.o.i. = 1) were incubated at 33 °C or 40 °C for 8 h. The amounts of cells with HA-surface expression at 33 °C and 40 °C (wt: 60%, ts227: 4%) were detected by FACS using H7HA-specific mAb. The results are based on two independent experiments with titer determined at different dilutions. B, MDCK cells uninfected (--) or infected either with wild type virus (WT) or ts227 (m.o.i. = 1) were incubated at 33 °C or 40 °C for 6 and 8 h. After Western blot ERK activation was analyzed with a phospho-specific mAb. Subsequently, loading was controlled with an mAb against ERK2 on the according blot. Respective bands of three independent experiments were quantified, and relative ERK activation was calculated and normalized to the loading control. Virus types as well as the time of analysis post infection (p.i.) are indicated. C, MDCK cells were infected with wild type virus (WT) or ts227 (m.o.i. = 1) and incubated at 33 °C or 40 °C for 10 h. RNPs were stained with anti-NP mAb and FITC-conjugated secondary Abs (green). Nuclei were counterstained with propidium iodide (red). Intracellular RNP localization was analyzed by confocal microscopy. The merger of both channels is shown.
RESULTS

Viral Replication Intermediates Do Not Induce the Raf/MEK/ERK Signal Cascade—Activation of most of the influenza virus-induced signaling processes, such as JNK, p38, or NF-kB activation are primarily due to the accumulation of viral RNA (ssRNA or dsRNA) (42). In clear contrast to that, ERK is not activated in response to the dsRNA analog (poly(I/C)), although the compound in these concentrations readily activated the dsRNA-responsive IFN-β promoter. This strongly suggests a completely different mode of ERK activation (Fig. 1A). A further argument against viral dsRNA as an inducer of ERK activity comes from experiments using the ΔNS1 virus. Because this virus mutant lacks the viral dsRNA-binding protein NS1, infection with ΔNS1 resulted in a boost of dsRNA-induced signaling events compared with the isogenic wild type (wt) control. Although this was true for JNK1 activity, a known dsRNA effector, there was no change in ERK activation 9 h p.i. and even a reduced rather than an enhanced ERK activity 6 h p.i. if the mutant is compared with the wt-virus (Fig. 1B). This again strongly indicates that virus-induced ERK activation occurs by a dsRNA-independent mechanism. It might still be argued that virus-induced secretion of cellular factors, such as cytokines and chemokines, might lead to activation of the MAPK cascade in an autocrine or paracrine fashion. But when MDCK cells were analyzed for ERK activity after they were treated with UV-inactivated supernatants from either mock infected or virus-infected cells collected at different time points p.i., no effect on ERK activation was detected (data not shown).

HA-Surface Expression Induces ERK Signaling and Nuclear RNP Export—Type A influenza virus-induced signaling displays a biphasic ERK activation both early and late in the viral replication cycle (3). Interestingly the late phase of MAPK activation is absent upon non-productive infection of HeLa cells (data not shown). These cells have been reported to show defects (i) in the maturation and membrane insertion of the HA, (ii) in M1 production, and (iii) in the budding process (43–45). Influenza B virus infection only shows late ERK activity (4).

We investigated whether HA-membrane accumulation could be a possible viral inducer of the Raf/MEK/ERK cascade, which is normally activated by membrane resident cellular factors (17, 46, 47). MDCK cells were infected with either wild type virus (WT) Ac1, Ac2, or Ac3 (m.o.i. = 1), and ERK activation was analyzed at the time points indicated. After Western blot ERK activation was analyzed with a phospho-specific mAb. Subsequently, loading was controlled with an mAb against ERK2 on the according blot. Respective bands of three independent experiments were quantified and relative ERK activation was calculated and normalized to the loading control. Virus types as well as the time of analysis p.i. are indicated. Δ, MDCK cells were infected with wild type virus (WT) or Ac3 (m.o.i. = 1) and incubated for the time indicated at 37 °C. RNPs were stained (green). Nuclei were counterstained with propidium iodide (red). Intracellular RNP localization was analyzed by confocal microscopy. The merger of both channels is shown.
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temperatures showed that at 40 °C ERK activation was not affected in wt-infected cells but reduced in ts227-infected cells at late time points of infection, where ERK activation normally increases (3, 4) (Fig. 2B). To investigate MAPK signaling-dependent nuclear RNP export, intracellular RNP localization was investigated with cells infected with either virus incubated at 33 °C or 40 °C (Fig. 2C). The RNPs in wt-infected cells incubated at 33 °C or 40 °C were found in the cytoplasm at 10 h p.i. indicating efficient nuclear RNP export. In contrast only a small fraction of RNPs in ts227-infected cells incubated at 40 °C were found in the cytoplasm. It is thus tempting to speculate that HA-surface expression is functionally linked to the activation of MAPK signaling and concomitant viral RNP export.

Stability of HA/Lipid-Raft Association Affects HA-induced MAPK Signaling—In wt-HA fatty acid side chains are attached to three highly conserved cysteine residues in the cytoplasmic tail at amino acid positions 551, 559, and 562 (numbering according to HA of A/FPV/Rostock/34). By employing recombinant Rostock virus mutants expressing HAs with alterations in these cysteine residues (mutants: Ac1, C551A; Ac2, C559A; and Ac3, C562A), it was demonstrated that loss of palmitoylation from the distally located site significantly reduced the strength of HA/lipid-raft association (30). Interestingly, the tightness of the HA/lipid-raft interaction closely correlated with the infectivity in that weakening of raft association led to a clear reduction in virus particle infectivity, which was shown to be due to impaired HA fusion activity. Because lipid rafts are involved in signal transduction (22), we investigated the importance of the HA/lipid-raft association for ERK activation. MDCK cells were infected at m.o.i. = 1 with either wt or the mutant viruses. ERK activation and intracellular RNP localization (Fig. 3 A and B) were analyzed at different time points p.i. The results show that ERK activation by wt, Ac1, and Ac2 did not significantly differ at 6, 8, and 10 h p.i., whereas obviously Ac3 infection always led to a reduced ERK activation compared with the wt (Fig. 3A). When the nuclear RNP export of wt- and Ac3-infected cells was compared, the onset of RNP export in wt-infected cells was observed to be around 6 h p.i., while at the same time point export in Ac3-infected cells was significantly retarded. Only as late as 8 h p.i. was the amount of cytoplasmic RNPs increased (Fig. 3B). The results indicate that tight association of HA with lipid rafts correlates with efficient induction of MAPK signaling.

Membrane Cholesterol Depletion Affects MAPK Signaling in Influenza A Virus-infected Cells—The results obtained so far suggest that both, HA-membrane accumulation as well as strong HA/lipid-raft interaction, are mandatory for efficient viral activation of MAPK signaling. We therefore analyzed the impact of lipid-raft disruption by cholesterol depletion with MCD (22) on influenza virus-induced MAPK signaling. Successful lipid-raft disruption by MCD treatment was demonstrated in co-patching experiments (49). Lipid rafts can be concentrated and visualized through specific binding of FITC-conjugated cholera toxin (Ct-B) to the lipid-raft component GM1 (50) and cross-linking untreated or were treated with MCD for different time points as indicated. ERK activation was analyzed at time points indicated. After Western blot ERK activation was analyzed with a phospho-specific mAb. Subsequently, loading was controlled with an mAb against ERK2 on the according blot. Respective bands of three independent experiments were quantified, and relative ERK activation was calculated and normalized to the loading control. Infection, MCD treatment, and the time of analysis post infection (p.i.) are indicated. C, intracellular RNP localization (green) of infected (m.o.i. = 1) MDCK cells that were either treated with MCD or left untreated, was determined by confocal laser scanning microscopy 6 h p.i. Nuclei were counterstained with propidium iodide (red). The merger of both channels is shown. D, influenza virus-infected (m.o.i. = 1) MDCK cells were left untreated or were treated with MCD and cholesterol for different time points and concentrations as indicated. ERK activation was analyzed at 9 h p.i. by Western blot. Respective bands of three independent experiments were quantified, and relative ERK activation was calculated and normalized to the loading control. Infection, MCD treatment, and cholesterol treatment are indicated.
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with an anti-Ct-B mAb resulting in bright membrane-resident spots in untreated cells. In contrast MCD treatment leads to a diffuse staining of the total cell surface indicating the dissipation of GM1 and disruption of the lipid rafts (49) (Fig. 4A). Taken together, at non-toxic MCD concentrations (verified in MTT assays), the amount of membrane-expressed HA (analyzed by FACS) was not significantly affected, whereas a large portion (31%, as measured by gas chromatography) of membrane cholesterol was depleted (data not shown), and lipid rafts were disintegrated.

After these initial control studies MDCK cells were infected and treated with MCD as indicated (Fig. 4B). Disruption of the lipid rafts evidently impaired ERK activation. Whereas virus infection leads to continuously increasing ERK activation from 4 to 9 h p.i., MCD treatment impaired further increase of ERK activation after 6 h p.i. To elucidate whether cholesterol depletion by itself would affect MAPK signaling we stimulated cells with TPA. These cells were either treated further with MCD for 4, 6, and 8 h or left untreated. Here we could not detect an impact of cholesterol depletion on TPA-stimulated ERK activation (data not shown). Not only did lipid-raft disruption of virus infected cells lead to reduced ERK activation and impaired nuclear RNP export (Fig. 4C), but also infectious virus titers declined (Table 1A). Even though the decline in virus titers might also reflect additional effects of MCD on virus integrity, these results overall further support the idea that a tight HA/lipid-raft association is important for viral induction of the MAPK cascade.

To test the specificity of the MCD treatment infected cells were treated with MCD and increasing concentrations of cholesterol (Fig. 4D). The results show that ERK activation achieved upon MCD treatment could be strongly increased by extra cholesterol in a dose-dependent response indicating that indeed membrane cholesterol depletion and thereby lipid-raft disintegration affects influenza virus-induced ERK activity.

Transient Expression of HA Induces MAPK Signaling—The results suggest that HA by itself might lead to ERK activation. To test this MDCK cells were transfected with a combination of plasmids expressing HA-tagged ERK and either (i) empty vector, (ii) vector expressing the wtH7-HA, or (iii) the Ac3-HA. FACS analysis indicated that cell-surface expression of H7-HA and Ac3-HA was similar (~35–40%, Fig. 5A). Analysis of ERK activity revealed strong kinase activation in cells expressing the H7-HA, whereas activity in Ac3-HA-expressing cells was significantly weaker (Fig. 5B). An induction of ERK could also be achieved by transfecting vectors expressing H3–HA (data not shown). It can therefore be concluded that HA expression alone is sufficient to induce signaling via the Raf/MEK/ERK cascade, and, like in virus-infected cells, inefficient lipid-raft association of the Ac3-HA results in reduced ERK activity.

HA-induced Signal Leading to ERK Activation Is Transmitted via PKCa—Extracellular stimuli leading to activation of the Raf/MEK/ERK cascade are normally transmitted via G-protein-coupled receptors or receptor-tyrosine kinases. This involves activation of Ca2+-dependent PKCs and Ras, respectively (47). It was shown before that influenza virus infection results in increased intracellular Ca2+ concentration (51) and an increase in PKC activity in human polymorphonuclear leukocytes (52). Reduction of intracellular Ca2+ concentration by EDTA was followed by decreased PKC activity, supporting the hypothesis that isoforms of Ca2+-dependent, conventional PKCs are activated during influenza virus infection (52). Furthermore, influenza virus entry seems to be dependent on PKCβII but not on PKCα activity, indicating a role for PKCs early in infection (53).

To this end we analyzed the activation/activity of PKCa and ERK over time in influenza virus-infected cells, either left untreated or treated with Verapamil (Fig. 6A). Activation was analyzed by phospho-specific mAbs in Western blots, whereas activity was measured in immune complex kinase assays. As expected ERK activation/activity increased with the time course of the viral replication cycle. Interestingly we could also detect an increase in PKCa activity. Verapamil significantly reduced both parameters for ERK as well as for PKCa. These results argue for a specific link between PKCa and ERK activity in influenza virus-infected cells.

To compare the impact of the Raf-activating signal transmitters PKCa and Ras for influenza virus replication, we further analyzed their role in MDCK cells transiently expressing a dominant negative (dn) version of PKCa and Ras versus cells transfected with empty vector (Fig. 6B). dnPKCa expression demonstrated a strong titer reduction at 6 h p.i., which was still significant at 8 h p.i. For both time points the
decrease in virus titer was less prominent when dnRas was expressed. In an additional attempt to elucidate whether PKCα/H9251 activation correlates with influenza virus replication, virus-infected cells were analyzed by immuno-fluorescence studies using phospho-specific antibodies directed against phosphorylated PKCα (P-PKCα, Fig. 6C). We could detect activated PKCα specifically in virus-infected cells. The signal strength correlated with RNP export and was clearly reduced in Ac3-infected cells and almost absent in Verapamil-treated cells. Accordingly, nuclear RNP export was strongly impaired by the inhibitor. This result was supported by analysis of cells transiently expressing wtH7-HA or Ac3-HA (Fig. 6C). Again we found a reduced P-PKCα signal in cells expressing the Ac3-HA. Taken together it seems likely that the signal for virus-induced ERK activation
is triggered by HA-membrane accumulation, and lipid-raft interaction is mainly transmitted via PKCα.

**DISCUSSION**

For a successful propagation all viruses strongly depend on cellular functions, including intracellular signaling events, and influenza virus is no exception from this rule. We have recently shown that activation of the Raf/MEK/ERK cascade (2–4) as well as the activity of caspases controlled in a NF-κB-dependent manner (55–57) are essential prerequisites for an effective nuclear RNP export. Inhibition of either one of these cellular pathways strongly impairs nuclear RNP export and the amount of infectious progeny virions formed. These findings are of importance when considering that RNP export must be well coordinated to allow sufficient genome replication and viral protein production to form functional virions.

Nevertheless the viral inducer of the MAPK cascade has remained elusive so far. Even though most of the influenza virus-induced signaling processes arise from the accumulation of viral RNA, results from poly(I/C) transfection, infection with ΔNS1 virus, and passing of supernatants from infected cells argue against dsRNA and/or secreted cellular factors as inducers of MAPK activity (Fig. 1). In contrast, the reduced ERK activation observed upon infection with ts227, a virus mutant that exhibits an HA transport defect at restrictive temperature (Fig. 2A), indicates that proper membrane accumulation of HA is an important requirement for viral ERK activation (Fig. 2B) leading to enhanced nuclear RNP export at late time points of infection (Fig. 2C). Considering that Raf is activated by membrane-resident factors, the correlation between cascade activation and membrane accumulation of HA late in the viral replication cycle supports the model that HA could trigger this event.

Membrane proteins implicated in signal transduction (22) can interact with lipid rafts via fatty acid side chains (58) and so does HA (32) as well as glycoproteins of many other enveloped viruses (30). A tight HA/lipid-raft association within the cellular membrane seems to be important for influenza virus entry (59, 60) and effective production of infectious virions (31, 61). The recombinant virus Ac3 was found to show the weakest lipid-raft association and the lowest infectivity of three virus mutants (Ac1, Ac2, and Ac3) (30). Even though no change in the amount of viral proteins as well as lipid composition of the viral membrane was observed for these virus mutants, less infectious Ac3 particles were produced. This could be attributed to impaired fusion pore formation (30). Here we show that reduced ERK activation (Fig. 3A) and as a consequence marked retardation of nuclear RNP export (Fig. 3B), which is most likely related to the weakened HA/lipid-raft interaction, are additional causes for the impaired Ac3 propagation. An equally striking RNP export defect that negatively affected virus titers was only detected by MEK inhibition (3, 4). In agreement with recently published data demonstrating that lipid-raft anchoring of the Ac1- and Ac2-HA is not or only mildly affected (30), a less significant effect on ERK activation for the mutant viruses Ac1 and Ac2 was found compared with wt-virus. In view of these results we suppose that, besides membrane accumulation of HA, the strength of HA/lipid-raft association is directly correlated with viral ERK activation.

This assumption is further supported by the results obtained from lipid-raft disruption in the wt-virus-infected cells. This treatment led to (i) a drastic reduction of virus-induced ERK activation at late time points p.i. (Fig. 4B), (ii) reduced virus titers (Table 1A), and (iii) impaired RNP export (Fig. 4E). Interestingly lipid-raft disruption by itself had no effect on TPA-induced ERK activation (data not shown). The fact that re-addition of cholesterol quenched the MCD effect on ERK activation demonstrates the specificity of the MCD action (Fig. 4C).

Artificial TPA stimulation of the MAPK cascade in wt-virus- and Ac3-infected cells (Table 1B) confirmed the hypothesis that impaired ability to activate ERK results in reduced RNP export and therefore in reduced virus titers. The relative increase in infectious Ac3-virions is stronger than for wt-virus, demonstrating suboptimal RNP export in Ac3-infected cells. As the Ac3 mutant was shown to be affected in fusion pore formation leading to reduced plaque titers (30), the detectable TPA-mediated titer increase could even be stronger. Nevertheless, improved RNP export (data not shown) and increased Ac3 titers upon TPA treatment indicates that reduced ERK activation and hence impaired nuclear RNP export should, at least in part, account for the reduced Ac3 titers. The TPA effect could also be mimicked by expression of a constitutively active form of Raf in Ac3-infected cells (Table 1C), supporting the idea that TPA-induced ERK activation can compensate the defect of the Ac3 mutant. Our finding, that transient expression of wtH7-HA results in ERK activation, clearly shows the role of HA as a viral trigger of MAPK signaling. It seems to be an HA type-independent effect, because transient expressed H3—HA also induced ERK activity (data not shown). In several experiments the activation by wtH7-HA was repeatedly stronger than by the mutated Ac3–HA (Fig. 5B). Although HA seems to be sufficient to trigger ERK activation we cannot exclude the involvement of other viral proteins or processes in the context of the virus-infected cell. In an attempt to elucidate which cellular factors are involved in signal transmission upstream of Raf we investigate the role of PKCα. The weak effect of dnRas on virus titer (Fig. 6B) and the low amount of phospho-tyrosine detected by immunofluorescence assay in virus-infected cells (data not shown) argue against a prominent role of Ras. In contrast dnPKCα (Fig. 6B) and treatment with the calcium channel blocker Verapamil significantly reduced virus titers (Table 1D). Furthermore the immunofluorescence assay signal for pPKCα evidently correlated with virus infection and RNP export and was diminished by Verapamil (Fig. 6C). The fact that pPKCα could also be detected in cells transiently expressing viral wtH7–HA demonstrates a link between HA expression and signal transmission via PKCα. Considering that transient expression of Ac3–HA resulted only in weak PKCα activation, HA/lipid-raft association seems to be important for this pathway.

The picture emerging represents an auto-regulative circuit that initiates nuclear RNP export by membrane accumulation of the HA. These feedback mechanisms appear to be beneficial for influenza virus replication. Likewise accumulation of PB1, PB2, PA, and NP seems to stabilize viral cRNA, synthesized by the viral polymerase, which otherwise is degraded, thus allowing its replication into viral RNA (62). Triggering RNP export by accumulation of a major structural component expressed late in the viral replication cycle assures sufficient replication and transcription of the viral genome and thereby production and accumulation of viral proteins necessary for virion formation. Continuously accumulated HA interacts with lipid rafts and by a yet unknown mechanism finally leads to the activation of the Raf/MEK/ERK signal cascade via PKCα, which in turn regulates RNP export. This ensures that exported RNPs can be efficiently packaged into progeny virions. Interference with such a mechanism important for influenza virus replication might have interesting therapeutic implications, such as the blockade of the MAPK pathway by MEK inhibitors.

**Acknowledgments**—The influenza A viruses were taken from the strain collections in Giessen and Marburg. We thank S. Becker, E. Mühlberger, and L. Stitz for providing reagents and thankfully acknowledge critical reading of the manuscript by W. H. Gerlich. Furthermore, we thank C. Menge for assistance in FACS analysis and J. Lampe and W. Mink for excellent technical assistance.
