Rhinoviral Infections Activate p38MAP-Kinases Via Membrane Rafts and RhoA

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Key Words

Rhinovirus • Infection • p38MAP-Kinase • Membrane Rafts • G-proteins

from membrane rafts via the small G-protein RhoA to p38-K to infect human cells.

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Abstract

Rhinoviral infections belong to the most frequent human infections characterized by common cold, chronic bronchitis, exacerbations of asthma, otitis media and sinusitis. Here, we define molecular mechanisms that mediate infections of human epithelial cells with human rhinovirus strain 14 (RV14). We demonstrate that RV14 activates p38-MAP-Kinase (p38-K) in a biphasic time course. Early stimulation of p38-K by RV14 was observed a few minutes after initiation of the infection, while the late increase of p38-K activity occurred 7-12 hrs upon infection. The stimulation of p38-K was mediated by the small G-protein RhoA, which was activated by RV14. Transfection of a genetic construct preventing RhoA activation blocked RV14-induced p38-K activation. Further, integrity of cholesterol and sphingolipid-enriched membrane domains was required for RV14-mediated p38-K activation, which was inhibited by destruction of membrane rafts. The data indicate that RV employs a signaling cascade

Introduction

Human Rhinovirus (RV), a member of the Picornaviridae family, is the most frequent cause of common cold, but it has been also associated with more serious sequelae such as chronic bronchitis, exacerbations of asthma, otitis media and sinusitis. There are over 100 serotypes of RV that are divided into two groups. The major group, which includes 90% of all RVs, uses intracellular adhesion molecule-1 (ICAM-1) as a cellular receptor [1, 2], while the minor group receptor is represented by members of the low density lipoprotein receptor family (LDL) [3]. RV infections are initiated by binding of the virions to their receptors on the cell surface. Although some of the molecular details of the interaction of LDL with rhinoviruses still require definition, it has

been shown that the ICAM-1 receptor binds to the canyon regions of the viral capsid resulting in a conformational change that is important for the later phases of the infection including translocation of the viral particles into the cell and release of genomic material into the cytoplasm [4, 5].

RV, as well as other respiratory pathogens, have been shown to induce production of many cytokines/chemokines leading to an inflammatory response, which is considered to cause the above-mentioned symptoms [6-8]. It has been also demonstrated that RV infection increases ICAM-1 expression on the cell surface [9, 10]. However, little is known about the signaling pathways involved in RV-infection, and the characterization of these pathways may provide new therapeutic strategies for the treatment of inflammation associated with respiratory virus infection.

In an attempt to identify signaling events involved in rhinoviral infections, we tested whether RV activates mitogen-activated protein kinases (MAPKs) that are central components of signal transduction pathways in the regulation of multiple cell functions such as proliferation and differentiation, cytokine production and apoptosis. In mammalian cells, three distinct subgroups of MAPK-families have been identified: extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK) and p38-MAP-Kinase (p38-K). MAPKs are activated by phosphorylation on threonine and tyrosine-residues by a signaling cascade which includes MKKK (MAPKKK or MEKK) and MKK (MAPKK or MEK) (for a detailed review see Kyriakis and Avruch) [11]. P38-Ks are activated by different stimuli ranging from inflammatory cytokines to environmental stresses and viral infection [12-15]. Upstream activators of p38-K include members of the RhoGTP-binding protein family, Ras-proteins, PI-3-K and Src-like tyrosine kinases [16-20]. P38-Ks are known to regulate several downstream molecules, in particular MAPKAPK2 and ATF2 [16, 21] that are involved in transcriptional control and gene regulation.

In the present study we investigated the regulation and function of the p38-K pathway upon RV infection, using the major group rhinovirus type 14 (RV14). We present evidence that RV14 induces phosphorylation and activation of p38-K in HeLa cells, with a biphasic accumulation of activated p38-K. We also report that lipid rafts in the plasma membrane and the small GTP-ase RhoA function upstream of p38-K upon rhinoviral infection of epithelial cells.

Materials and Methods

Cells and Viruses

HeLa cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and were maintained in MEM (EAGLE) containing 10% fetal calf serum (FCS), 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μ M non-essential amino acids, 100 units/ml penicillin, 100 μ g/ml streptomycin (all from Invitrogen). Rhinovirus serotype 14 (ATCC; Manassas, VA) was amplified in HeLa cells and titered by Tissue Culture Infectious Dose 50 (TCID₅₀; amount of virus required to infect 50% of monolayers) according to the Spearman-Kärber method. The virus was stored at -80°C until use. In order to prevent replication, viruses were exposed to UV-light at 1200 mJ/cm² for 30 min as previously described [22].

Drugs and antibodies

Methyl- β -Cyclodextrin (M β -CD) and α -Cyclodextrin (α -CD) were purchased from Sigma (Deisenhofen, Germany). The p38-K inhibitor SB203580 was from Calbiochem (La Jolla, CA, USA). Rabbit-anti-phospho-p38 MAPK, rabbit-anti-phospho-ATF2 antibodies and rabbit-anti-ATF2 antibody were obtained from Cell Signaling Technology (MA, USA) and used at a dilution of 1:1000. Rabbit-anti-p38-K, rabbit-anti-RhoA and AP-coupled goat-anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Inc., CA, USA) and were also diluted 1:1000 for use.

Viral infection, inhibition of p38-K and disruption of rafts

HeLa cells were grown in 6-well plates (5 $\times$ 10⁵ cells per well). MEM medium containing 2% FCS was added to the cell culture prior to drug treatment. Rafts were disrupted by treatment of the cells with methyl- β -cyclodextrin (1 mM) for 45 min prior to infection or 4 hrs after infection. The inactive isomer α -cyclodextrin (1 mM) served as control. Inhibition of p38-K was achieved by treatment of the cells with SB203580 (25 μ M). RV14 was added at a multiplicity of infection (MOI) of 20 or 50 as indicated. After 1 h incubation at 37°C, the virus inoculum was removed, and fresh medium containing 10% FCS was added to the culture.

Protein preparation and Western blot analysis

At various time points after infection, culture supernatant was removed and the cells were lysed in 25 mM HEPES (pH 7.3), 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM EDTA, 10 mM sodiumpyrophosphate, 10 mM NaF, 125 mM NaCl, 1 mM Na₂VO₄, and 10 μ g/ml each of aprotinin/leupeptin. The cellular debris was removed by centrifugation at 14,000 rpm for 15 min. Samples were analysed by SDS-PAGE followed by transfer to a nitrocellulose membrane (Amersham Biosciences, NJ, USA). The membranes were blocked for 45 min with 4% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20, washed and incubated for 60 min at room temperature with the primary antibodies. The membranes were washed, incubated with alkaline phosphatase-conjugated goat-anti-rabbit immunoglobulin G (1:15,000, Santa Cruz

Fig. 1. RV14 induces an activation of p38-K. (A) RV14 induces a biphasic phosphorylation of p38-K (pp-p38), which serves as a measurement for activation of the kinase. P38-K phosphorylation shows an early peak 30-60 min and a second peak 7-12 hrs after infection. (B) Titration of viral MOIs indicates that phosphorylation of p38-K occurs at a minimum of 5 virus particles/cell and reaches an optimum at 20 virus particles/cell. HeLa cells were infected for the indicated time with RV14 at an MOI of 1:50 (A) or for 30 min with the indicated MOIs of RV14 (B). Cells were lysed, proteins were separated by SDS-PAGE, blotted and phosphorylation of p38-K was determined by the use of a phospho-specific anti-p38-K antibody that detects phosphorylation of p38-K at Thr180/Tyr182. Aliquots of the samples were blotted with anti-p38-K antibodies to control the amount of protein in the samples (lower blots). Panel A and B show representative results of 5 or 2 independent experiments, respectively.

Biotechnology Inc.) and developed with the TROPIX chemoluminescence detection system.

Transfection of wildtype and transdominant N19Rho A

HeLa cells were seeded onto a 12-well plate and transfected by the Lipofectamine method (Invitrogen Inc., Karlsruhe, Germany) or by use of the Superfect Transfection Kit from Qiagen (Hilden, Germany) following the protocol provided by the manufacturer. Cells were transfected with Myc-tagged wild-type RhoA or Myc-tagged mutant N19RhoA (kind gifts from Dr. Christoph Hauck, University of Wuerzburg, Germany). Transfection efficiency was controlled by western blotting employing anti-c-Myc-antibodies (not shown). RV14 infection of the cells was initiated 24 hrs after transfection. The samples were prepared and analysed by Western-blotting for p38-K phosphorylation as described above. For FACS-analysis of p38-K phosphorylation in wild-type RhoA- and N19RhoA-transfected cells, the cells were infected 24 hrs after transfection with RV14 (MOI 20) for the indicated time, fixed and permeabilized employing the Cytotfix/Cytoperm Kit (BD Pharmingen, Heidelberg, Germany). Cells were then stained consecutively with anti-phospho-p38 antibodies and anti-c-Myc antibodies clone 9E10 (Roche, Mannheim, Germany) (both diluted 1:100) for 45 min at room temperature followed by each 20 min consecutive staining with FITC-donkey anti-rabbit IgG (1:200) and Cy5-coupled donkey anti-mouse IgG (1:500). Cells were analyzed by FACS. Cells that express wild-type RhoA- or N19RhoA were identified by staining with anti-c-Myc antibodies clone 9E10. This population was gated and the activity of p38-K was determined by measuring the FITC signal representing phospho-p38-K.

RhoA activity assays

To determine the activity of RhoA, we employed the finding that only active RhoA binds to Rhotekin. We, therefore, incubated cell lysates from un-infected and infected cells with a GST-fusion protein of Rhotekin and determined the amount

of RhoA binding to this GST-fusion protein, which reflects the status of RhoA activity. GST-Rhotekin was purified from bacterial cultures 2 hrs after induction with 0.1 mM isopropylthiogalactoside (IPTG). Bacteria were lysed with 20% sucrose, 10% glycerol, 50 mM Tris (pH 8.0), 2 mM $MgCl_2$, 2 mM DTT and 10 μ g/ml each of aprotinin/leupeptin. The samples were cleared by centrifugation and GST-proteins were purified from the supernatants employing glutathione agarose beads (Amersham). To assay active RhoA, HeLa cells were grown on 25 cm^2 tissue culture flasks, treated with methyl- β -cyclodextrin or left untreated and infected with virus for the indicated time. The cells were scraped into 500 μ l of 50 mM Tris (pH 7.4) 1% NP-40, 100 mM NaCl, 2 mM $MgCl_2$, 10 μ g/ml each of aprotinin/leupeptin and centrifuged to remove cellular debris. Aliquots of the supernatants were mixed with 50 μ l of a 50% slurry of glutathione agarose beads and rotated at 4°C for 30 min. The beads were then centrifuged, washed, resuspended in SDS-sample buffer and eluted proteins were separated on a 15% SDS-PAGE gel. The samples were analysed by Western blotting employing anti-RhoA antibodies and secondary AP-coupled anti-rabbit antibodies. The blots were developed using the TROPIX chemoluminescence system.

Results

Activation of p38-K by RV14

Initially, we examined whether RV14-infection induced p38-K phosphorylation in HeLa cells. The cells were inoculated with RV14 at an MOI of 50, and cell lysates were prepared at various times after infection. Non-infected cells served as negative control. Western blot analysis indicates a biphasic phosphorylation of p38-K upon infection. A first peak of p38-K phosphorylation was detected already 30 min after infection followed by

a decline of p38-K phosphorylation within the next hours (Fig. 1A). Phosphorylation of p38-K peaked again 7-8 hrs after infection (Fig. 1A) and the levels remained elevated up to 12 hrs after infection and declined to background at 24 hrs after infection (Fig. 1A). Expression levels of p38-K were similar in all samples (Fig. 1A) suggesting that RV-infection caused phosphorylation of p38-K without *de novo* synthesis of protein.

To define a dose-response curve, we infected epithelial cells with different MOIs of RV14, i.e. MOIs

of 0.5, 1, 5, 10 and 20, and determined the phosphorylation of p38-K. Non-infected cells were used as control. Samples were prepared 12 hrs after infection and the level of p38-K activation was determined by immunoblotting. As shown in Fig. 1B, a slight phosphorylation of p38-K was already induced by an MOI of 5 and increased in a dose-dependent manner, with the highest level of activation being obtained with an MOI of 20 (Fig. 1B). Therefore, the further experiments with RV14 infections were conducted with an MOI of 20.

Fig. 3. Late activation of p38-K seems to be involved in replication of the virus. (A) RV14 induces phosphorylation of ATF2 via p38-K. Pretreatment of the cells with the p38-K inhibitor SB203580 prevents phosphorylation of ATF2 upon cellular infection with RV14. Cells were infected for 12 hrs with RV14 at an MOI 20, lysed and activity of ATF2 was determined by measuring the phosphorylation of ATF2 employing phospho-specific antibodies (upper panel). The lower panel displays aliquots blotted with anti-ATF2-antibodies to control for similar amounts of ATF2 in all samples. The data are representative for 2 experiments. (B) The late activation of p38-K is prevented by irradiation of RV14 prior to infection, while the early peak of p38-K phosphorylation is not affected by inactivation of the virus. Cells were infected with UV-irradiated or viable virus for the indicated time and phosphorylation of p38-K was determined as above. The lower blots show that expression of p38-K is similar in all samples. The blots are representative for 3 independent studies. (C) Treatment of HeLa cells 4 hrs after the initial infection with methyl- β -cyclodextrin does not prevent late phosphorylation of p38-K, while addition of methyl- β -cyclodextrin prior to infection prevents the late phosphorylation of p38-K. This suggests that the late activation of p38-K is not caused by uptake of a second wave of released viruses but rather to replication and/or intracellular processing of the virus, while the early phosphorylation is involved in the uptake of the virus. The isomer α -cyclodextrin was without effect. The upper blot shows phosphorylation of p38-K employing phospho-specific antibodies, the lower blot reveals the analysis of total p38-K in aliquots of the samples. The data are representative of 3 independent experiments.

RV14 triggers p38-K activation via cholesterol and sphingolipid-enriched membrane rafts and small G - proteins of the Rho-family

In an attempt to identify upstream events of p38-K activation by RV14, we investigated a role of detergent-insoluble, cholesterol and sphingolipid-enriched membrane rafts in rhinovirus infection and, in particular, rhinovirus-induced p38-K activation. To this end we pre-incubated HeLa cells with the drug methyl- β -cyclodextrin that disrupts rafts by interference with cholesterol. Methyl- β -cyclodextrin was added 45 min prior to infection with RV14 (MOI 20). Methyl- β -cyclodextrin almost completely inhibited RV14-induced p38-K phosphorylation (Fig. 2A), while the structurally related, but inactive isomer α -cyclodextrin was without effect. Inhibition of RV14-induced p38-K-activation by pre-treatment of the cells with methyl- β -cyclodextrin was not restricted to an early infection time, but also observed 8 hrs after initiation of the infection (Fig. 2A). This suggests that intact rafts are required for activation of p38-K upon infection with

rhinovirus.

In order to identify further upstream regulators of p38-K, we tested for an involvement of small G proteins of the Rho-family in p38-K activation by RV14. In particular, we assessed the role of RhoA for activation of the p38-K cascade upon infection with RV14. To this end, HeLa cells were transfected with a plasmid encoding the dominant negative mutant N19RhoA or the wild type form of RhoA that served as a control. Cells were infected with RV14 and the activity of p38-K was assessed by immunoblotting using antibodies selective for phospho-p38-K (Fig. 2B). The data reveal that RV14-induced activation of p38-K was markedly reduced by transfection with N19RhoA 30 min, but also 2, 4 and 8 hrs after infection (Fig. 2B upper and lower panel). This result is consistent with the finding that RV14-infections resulted in a stimulation of RhoA (Fig. 2C) suggesting a regulation of p38-K by RhoA upon RV14-infection.

Finally, we tested whether membrane rafts and RhoA are organized in a sequential signalling pathway triggered

by rhinovirus. The results demonstrate that pre-incubation with methyl- β -Cyclodextrin prevented activation of RhoA (Fig. 2C) suggesting a signalling cascade from rafts via RhoA to p38-K upon infection of human epithelial cells with RV14.

The late activation of p38-K is related to viral replication

To define functional consequences of RV14-induced p38-K phosphorylation, we tested whether activation of p38-K in RV14-infected cells results in phosphorylation of its downstream substrate ATF2. The cells were lysed 12 hrs after infection, proteins separated on a 7.5% SDS-PAGE and subjected to Western blot analysis using an ATF2-phospho-specific antibody. The results demonstrate that infection of epithelial cells with RV14 triggered a phosphorylation of ATF2, which was almost completely inhibited by pre-incubation of the cells with the specific p38-K inhibitor SB203580 (25 μ M) (Fig. 3A). These data suggest that p38-K activation by RV14 is related to regulation of gene expression.

To gain some insight into this hypothesis, we infected HeLa cells with UV-irradiated viruses, which are replication-defective, but are still able to be internalised by the cells. Cells were also infected with non-irradiated virus as a control. Samples were prepared at 30 min, 4 hrs and 8 hrs after infection and tested for p38-K phosphorylation by Western blotting. The results reveal that both irradiated virus and intact virus induce a very similar phosphorylation of p38-K 30 min after infection (Fig. 3B), whereas only non-irradiated, intact virus triggered phosphorylation of p38-K 8 hrs after infection (Fig. 3B). In contrast, UV-irradiated RV was without a significant effect on p38-K phosphorylation 8 hrs after application (Fig. 3B). These results suggest that the early peak of phosphorylated p38-K is involved in viral adhesion/uptake and/or cytokine induction, while the late peak requires viral integrity.

To further test the hypothesis that the late phosphorylation of p38-K is related or caused by replication of the virus, we treated cells with methyl- β -cyclodextrin 4 hrs after infection with RV14. We assumed that methyl- β -cyclodextrin added 4 hrs after infection prevents re-infection of the cells by a new generation of virus resulting in a new cycle of p38-K stimulation. The cells were lysed 8 hrs after infection and analysed for p38-K phosphorylation. The results (Fig. 3C) reveal that methyl- β -cyclodextrin added 4 hrs after infection did not inhibit the late peak of phosphorylated p38-K, while the control sample treated with methyl- β -cyclodextrin prior

to infection showed a strong inhibition of p38-K phosphorylation. The inactive isomer α -cyclodextrin had no effect on p38-K activation in either case. This suggests that the late peak of p38-K phosphorylation is caused by intracellular processing of the virus, viral replication and or related events, while a re-infection with a wave of newly assembled viruses seems to be unlikely.

Discussion

The present study indicates an activation of p38-K upon infection of human epithelial cells with human rhinovirus 14. The data further suggest a signaling cascade from membrane rafts via the small G-protein RhoA to p38-K initiated by rhinoviral infection.

Our studies reveal a biphasic activation of p38-K upon infection with RV14. However, in the present study we focused on the later peak of p38-K activity, which was reached approximately 8 hrs after initiation of the infection. The early peak of p38-K activity was also observed upon incubation with UV-irradiated RV14 and, thus, is very likely related to adhesion and/or uptake of the virus or signaling events triggered by adhesion and/or internalization. This notion is also consistent with findings of Griego et al. [6] and Hall et al. [23], who demonstrated an early activation of p38-K upon infection of BEAS 2B cells with RV39 or epithelial cells with RV16, respectively. These authors concluded that early activation of p38K is involved in the release of pro-inflammatory cytokines, e.g. MCP, IL-1, G-CSF and GM-CSF [6, 23].

The exact function of the late p38-K activation is presently unknown, but the late appearance suggests that intracellular events initiated by viral infection trigger p38-K activation. Since replication-deficient RV14 did not trigger late p38-K-stimulation, it might be involved in the replication cycle, although this has to be proven. The notion that p38-K might be involved in RV-replication is consistent with previous findings on Herpes simplex type 1 [24], Rabies virus [25], encephalomyocarditis virus [13], HIV [26], varicella-zoster virus [27] or Hepatitis C [28] to name a few examples. Collectively, these studies indicate a stimulation of p38-K by these viruses that is directly or indirectly involved in and critical for viral replication.

Our data further suggest a signaling cascade from sphingolipid- and cholesterol-enriched membrane domains via the small G-protein RhoA to p38-K. Inhibition of RhoA was achieved by transfection of a transdominant inhibitory RhoA mutant, which permits specific inhibition of this small

G-protein without inhibition of other small G-proteins [29]. The integrity of small membrane rafts was disrupted by extraction of cholesterol employing methyl- β -cyclodextrin. This drug interferes with the cholesterol metabolism and, thus, destroys rafts [30, 31].

Rafts are dynamic assemblies of membrane lipids and are constituted by a preferential interaction of sphingolipids and cholesterol resulting in a separation from other phospholipids in the cell membrane [32]. We have recently shown that rhinoviral infections result in an activation of the acid sphingomyelinase that releases ceramide from sphingomyelin in the cell membrane [33]. Ceramide molecules associate and form ceramide-enriched membrane microdomains that spontaneously fuse to one or a few large macrodomains, also named membrane platforms [34-36]. Ceramide-enriched membrane platforms have been shown to serve the clustering of receptor molecules, for instance CD95 [36], CD40 [37] or Fc γ RII [38]. In addition, studies by Zhang et al. demonstrated that the release of ceramide within rafts is intimately linked to the assembly of multi-protein complexes, for instance the NADPH-oxidase [39]. At present, it is unknown whether the modification of rafts mediated by ceramide is required for activation of RhoA upon rhinoviral infection or whether the integrity of distinct domains in the cell membrane is necessary for coupling rhinoviral receptors to RhoA. Further, it remains to be determined whether rafts and/or ceramide-enriched domains are involved in the assembly of intracellular signaling molecules leading to RhoA- and finally p38K-activation or whether the predominant role of these domains consists in clustering of receptor molecules involved in rhinoviral infection.

At present it is unknown how rafts are coupled to

RhoA and p38-K. Since many G-proteins are present in the cytosolic face of rafts and seem to interact with rafts via their farnesyl- or prenyl-chains, it is conceivable that the integrity of rafts is required for the function of RhoA in the infection with rhinoviruses. G-protein-coupled receptor molecules and the small G-proteins Cdc42 and Rac have been shown to regulate p38-K upon stimulation (for a recent review see [40]). However, preliminary data from our group suggest that Rac does not play a role in RV14-induced p38-K stimulation. It should be noted that our data indicate a marked reduction of p38-K activation upon transfection of transdominant inhibitory RhoA, although no complete inhibition was achieved. This might be explained by some low residual activity of endogenous RhoA even after transfection of N19RhoA, an incomplete transfection efficiency or may indicate the presence of alternative pathways leading to activation of p38-K by rhinoviral infection.

In conclusion, our studies demonstrate that infection of HeLa cells with RV14-stimulates p38-K phosphorylation in a biphasic manner. Activation of p38-K triggers phosphorylation of its downstream substrate ATF2, and blockade of p38-K activity by its specific inhibitor SB203580 resulted in a decrease of ATF2 phosphorylation. We identified two components acting upstream of the RV14-induced p38-K stimulation, i.e. the small G protein RhoA and lipid rafts of the plasma membrane.

Acknowledgements

The study was supported by the Graduiertenkolleg GRK 1045 and the DFG grant Gu 335/10-3 to E.G.

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