FEBS Letters 567 (2004) 230–238 FEBS 28435

Rac1 and PAK1 are upstream of IKK-ε and TBK-1 in the viral activation of interferon regulatory factor-3

Christina Ehrhardt[a,b,1], Christian Kardinal[c,2], Walter J. Wurzer[a,b,1], Thorsten Wolff[d,3], Christoph von Eichel-Streiber[e], Stephan Pleschka[f,4], Oliver Planz[g,5], Stephan Ludwig[a,b,*]

[a]*Institute of Molecular Medicine (IMM), Heinrich-Heine-Universität, Universitätsstrasse 1, D-40225 Düsseldorf, Germany*
[b]*Institut für Medizinische Strahlenkunde und Zellforschung (MSZ), Julius-Maximilians Universität, Versbacher Strasse 1, D-97078 Würzburg, Germany*
[c]*Department of Pediatric Hematology and Oncology, Medizinische Hochschule, Carl-Neuberg-Strasse 1, D-30626 Hannover, Germany*
[d]*Robert-Koch Institut, Nordufer, D-13353 Berlin, Germany*
[e]*Institute of Medical Microbiology, Johannes Gutenberg-Universität, Obere Zahlbacher Strasse 63, D-55101 Mainz, Germany*
[f]*Institute für Medical Virologie, Justus-Liebig-Universität, Frankfurter Str. 107, D-35392 Giessen, Germany*
[g]*Institut für Immunologie, Bundesforschungsanstalt für Viruskrankheiten der Tiere (BFAV), Paul-Ehrlich Strasse, D-72076 Tübingen, Germany*

Received 22 January 2004; revised 13 April 2004; accepted 26 April 2004

Available online 8 May 2004

Edited by Hans-Dieter Klenk

Abstract The anti-viral type I interferon (IFN) response is initiated by the immediate induction of IFNβ, which is mainly controlled by the IFN-regulatory factor-3 (IRF-3). The signaling pathways mediating viral IRF-3 activation are only poorly defined. We show that the Rho GTPase Rac1 is activated upon virus infection and controls IRF-3 phosphorylation and activity. Inhibition of Rac1 leads to reduced IFNβ promoter activity and to enhanced virus production. As a downstream mediator of Rac signaling towards IRF-3, we have identified the kinase p21-activated kinase (PAK1). Furthermore, both Rac1 and PAK1 regulate the recently described IRF-3 activators, IκB kinase-ε and TANK-binding kinase-1, establishing a first canonical virus-induced IRF-3 activating pathway.

Keywords: Viral infection; Transcription factor; Signal transduction; Rho GTPase; Anti-viral response

* Corresponding author. Fax: +49-211-811-5892.
E-mail addresses: christina.ehrhardt@uni-duesseldorf.de (C. Ehrhardt), Kardinal.Christian@MH-Hannover.de (C. Kardinal), walter.wurzer@uibk.ac.at (W.J. Wurzer), wolfft@rki.de (T. Wolff), Chris.veichel@t-online.de (C. von Eichel-Streiber), stephan.pleschka@mikro.bio.uni-giessen.de (S. Pleschka), oliver.planz@tue.bfav.de (O. Planz), stephan.ludwig@uni-duesseldorf.de (S. Ludwig).

[1] Fax: +49-211-811-5892.
[2] Fax: +49-511-532-8119.
[3] Fax: +49-304-547-2328.
[4] Fax: +49-641-994-1209.
[5] Fax: +49-707-196-7150.

Abbreviations: MDCK, Madin–Darby canine kidney; IFN, interferon; dsRNA, double-stranded RNA; IRF-3, interferon-regualtory-factor-3; PRD, positive regulatory domain; PAK, p21-activated kinase; IKK, inhibitor of kappaB kinase; TBK-1, TANK-binding kinase; IL, interleukin; TLR, toll-like receptor; MAPK, mitogen-activated protein kinase; mab, monoclonal antibody; NAK, NF-κB activating kinase; JNK, Jun-N-terminal kinase; FPV, fowl plague virus; PR8, influenza strain A/Puerto-Rico/8/34; PKR, dsRNA-dependent protein kinase; wt, wild-type

1. Introduction

Interferon (IFN)β is one of the first anti-viral cytokines to be expressed upon virus infection, initiating an auto-amplification loop to cause an efficient and strong type I IFN response [1]. The IFNβ enhanceosome, which mediates the inducible expression of IFNβ, carries binding sites for transcription factors of three families, namely the AP-1 family members c-Jun and ATF-2, the NF-κB factors p50 and p65, and the interferon-regulatory factor (IRF-3) [2,3]. While AP-1 and NF-κB transcription factors are activated by a variety of stimuli, a strong IRF-3 activation is selectively induced upon virus infection, specifically by the double-stranded RNA (dsRNA) that accumulates during replication [4,5]. Thus, IRF-3 is a main determinant of a strong virus- and dsRNA-induced IFNβ response. Surprisingly, there is only very limited information with regard to the intracellular signaling chains that mediate virus-induced IRF-3 activation, especially those which lead to the virus specific C-terminal phosphorylation of the factor [6]. So far, most attempts have failed to identify the crucial mediators but rather helped to exclude a variety of signaling components and pathways [7–9]. Only until recently two non- canonical homologs of IκB kinases, namely inhibitor of κB kinase epsilon (IKK-ε), also known as IKK-i [10], and TANK-binding kinase (TBK-1), also known as NF-κB activating kinase (NAK) [11], have been identified as virus-induced IRF-3 activators [12,13]. However, the signaling chain upstream of these kinases has not been defined yet. Using an IRF-3-binding promoter element specifically responding to dsRNA or virus infections in a screening approach, we have identified Rac1 as a critical mediator of IRF-3 activation. This is a first demonstration that Rac1 is activated by a viral stimulus. Furthermore, the observation that the Rac1 effector p21-activated kinase (PAK1) also plays a role in IRF-3 activation and both Rac1 and PAK1 are upstream of IKK-ε establish a first virus-induced signaling cascade towards IRF-3.

0014-5793/$22.00
doi:10.1016/j.febslet.2004.04.069

2. Materials and methods

2.1. Viruses, cells and viral infections

Avian influenza virus A/Bratislava/79 (H7N7; fowl plague virus (FPV)) and human influenza virus A/Puerto-Rico/8/34 (H1N1) (PR8) were taken from the strain collection of the Institute of Virology in Giessen, Germany, and were used for infection of different cell lines. Sendai virus strain "Z" was taken from the strain collection of the Institute of Virology, Marburg, Germany, and has been passaged three times in embryonated chicken eggs with high multiplicity. Madin–Darby canine kidney (MDCK) cells or HEK293 cells were grown, respectively, in MEM or DMEM, both containing 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and cultured as described [14]. The A549 lung epithelial cell line was grown in Ham's F12 supplemented with 10% FBS and antibiotics. Infections of cells and titrations of progeny virus in the supernatants were essentially performed as described [15]. A receptor-neutralizing monoclonal antibody (mab) against the type I IFN receptor was purchased from PBL Biomedical Laboratories.

The Myc-tagged expression plasmids, pRK5 Rac1wild-type (wt) and pRK5 Rac1N17, as well as the empty expression vector, pRK5, were provided by K.-D. Fischer, University of Ulm, Germany. Expression plasmids for FLAG-tagged IKK-i/IKK-ε wt and K38A mutant were kindly provided by S. Akira, Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Japan [10]. PCDNA3 FLAG-NAK/TBK-1 was a kind gift of Makato Nakanishi, Department of Biochemistry, Nagoya City University Medical School, Japan [11]. The plasmids pCMV PAK1 and pCMV PAK1 K299R were a kind gift of I. Haase, Department of Dermatology and Center for Molecular Medicine, University of Cologne (CMMC), Germany. The 4 × IRF3 construct contains four copies of the IRF3-binding positive regulatory domain (PRD)I/III motif of the IFNβ promoter in front of a luciferase reporter gene. The IFNβ promoter construct and plasmids expressing dominant-negative IRF-35A or GFP-IRF-3 were a kind gift of J. Hiscott, Lady Davis Institute for Medical Research, McGill University, Montreal, Canada. A plasmid expressing HA-tagged IRF-3 was kindly provided by S. Jennings, Institute of Virology, Freiburg, Germany. MDCK and HEK293 cells were transfected with Lipofectamine 2000 (Life Technologies) according to a protocol by Basler et al. [16]. The dsRNA analog poly(IC) was purchased from Sigma and was directly added to the cell supernatant for stimulation. The specific Rho GTPase inhibitor toxin B of the *Clostridium difficile* strain VPI 10463 (TcdB-10463) was purified at the Institute of Medical Microbiology, University of Mainz, Germany, and was used at the indicated concentrations.

2.2. Rac1 activity assay

The Rac1-binding domain of PAK3 (aa 65–136) fused to GST (plasmid kindly provided by Dr. Wolfgang Kranewitter, Salzburg, Austria) was expressed in *E. coli* induced with 1 mM IPTG. The washed bacteria were run through the FrenchPress and supernatants were cleared by centrifugation.

Host-cells were stimulated or infected for the indicated times, washed 2× with PBS and harvested in Lysis buffer (50 mM Tris, pH 7.4; 500 mM NaCl; 1% Triton X-100; 0.5% DOC; 0.1% SDS; 10 mM $MgCl_2$; and 10 μg/ml each of leupeptin, aprotinin and pefablock). 500 μg total cell lysate was mixed with 20 μg GST-RBD and rotated in the presence of 25 μl washed glutathione–Sepharose beads for 45 min at 4 °C. The beads were washed 3× with buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% Triton X-100; 10 mM $MgCl_2$; and 10 μg/ml each of leupeptin, aprotinin and pefablock), sample buffer was added to the beads and boiled. The protein samples were separated on an 12.5% SDS–PAGE gel, proteins were blotted onto nitrocellulose membranes and deteced by the anti-Rac1 antibody (clone 23A8; Biomol).

2.3. Luciferase assays, immunoprecipitations and Western blotting

For luciferase reporter gene assays cells were infected or stimulated 24 h after transfection and harvested at the indicated times in 150 μl of lysis buffer (50 mM Na-MES, pH 7.8; 50 mM Tris–HCl, pH 7.8; 10 mM DTT and 2% Triton X-100). Luciferase activities were determined as described elsewhere [17] and are given as 2.5-fold activation ±S.E.M. from three independent transfections.

For immunoprecipitations and Western blotting, cells were lysed in Triton lysis buffer (TLB; 20 mM Tris–HCl, pH 7.4; 137 mM NaCl; 10% glycerol; 1% Triton X-100; 2 mM EDTA; 50 mM sodium glycerophosphate; 20 mM sodium pyrophosphate; 5 μg/ml aprotinin; 5 μg/ml leupeptin; 1 mM sodium vanadate and 5 mM benzamidine) on ice for 10–20 min. Cell lysates were cleared by centrifugation and were either used for immunoprecipitation with the 12CA5 monoclonal anti-HA antibody (purified at the MSZ, Würzburg, Germany) and protein A agarose (Roche) or directly subjected to SDS–PAGE and subsequent blotting. Endogenous IRF-3 protein in crude lysates was detected with an anti-rabbit IRF-3 anti-serum (Zytomed). HA-IRF-3, GFP-IRF-3 or CREB-binding protein (CBP) were detected with the 12CA5 mab, an anti-GFP mab or an anti-CBP anti-serum (both from Santa Cruz Biotechnology), respectively. Protein bands were visualized in a standard enhanced chemiluminescence reaction (Amersham). In some of the assays, loading controls were performed with an ERK2 anti-serum or different Jun N-terminal kinase (JNK)1 anti-sera (Santa Cruz Biotechnology).

2.4. Immunecomplex kinase assays

MDCK cells expressing FLAG-tagged forms of IKK-i/IKK-ε or NAK/TBK-1 were lysed in TLB and lysates were used for immunoprecipitations using the anti-FLAG M2 mab (Sigma). Immunecomplexes were washed and then incubated with recombinant GST-IκBα as a substrate in the presence of 100 μM unlabeled adenosine triphosphate (ATP), 5 μCi [γ^{32}P]ATP and kinase buffer for 30 min at 30 °C, essentially as previously described for IKK2 kinase assays [14]. Samples were then subjected to SDS–PAGE, blotted onto nitrocellulose membranes and visualized by autoradiography. Western blot analysis with the anti-FLAG antibody was performed to monitor equal loading of kinases.

3. Results

In order to screen for signaling mediators of IRF-3 activation, we have constructed a specific promoter reporter gene plasmid carrying four copies of the IRF-3 responsive PRDI/III region of the IFNβ enhanceosome in front of a luciferase gene (4 × IRF-3). In the initial phase of a virus infection, this promoter element has been demonstrated to specifically bind to constitutively expressed IRF-3 as a dimer, while other inducible factors, such as IRF-1 or IRF-7 are only bound during the late amplification phase [1]. Accordingly, the 4 × IRF-3 promoter is rapidly and specifically activated in response to dsRNA stimulation or infection with a RNA virus, such as the influenza A virus which is a known activator of IRF-3 [18] (Fig. 1A). A significant luciferase activity is already detected 2 h post-virus infection (data not shown) which greatly rules out the involvement of inducible IRF-factors, such as IRF-1 or IRF-7. Activation of the factor required productive virus replication, since partially UV-inactivated influenza virus is only poorly inducing transcriptional activity (Fig. 1B). The same pattern of transcriptional activation is also observed with an IFNβ promoter/enhancer element, which is mainly controlled by IRF-3 in response to virus infection (Fig. 1C). Other signaling activators, such as TNF-α, IL-1β or TPA factors, which readily activate NF-κB or AP-1-dependent transcription failed to activate the 4 × IRF-3 promoter (Fig. 1D). Finally, the specificity of the construct to respond to IRF-3 in our experimental setting was confirmed by expression of a dominant negative IRF-3 mutant (IRF-3 5A) which efficiently impaired virus-induced transcriptional activity (Fig. 1E). With this specific tool, we screened for involvement of a variety of signaling proteins in the cell by co-expressing corresponding dominant-negative or constitutively active mutants. The screen included several components of the ERK, JNK, p38 and ERK5 mitogen-activated protein kinase (MAPK) signaling cascades and components of the IKK/NF-κB module.

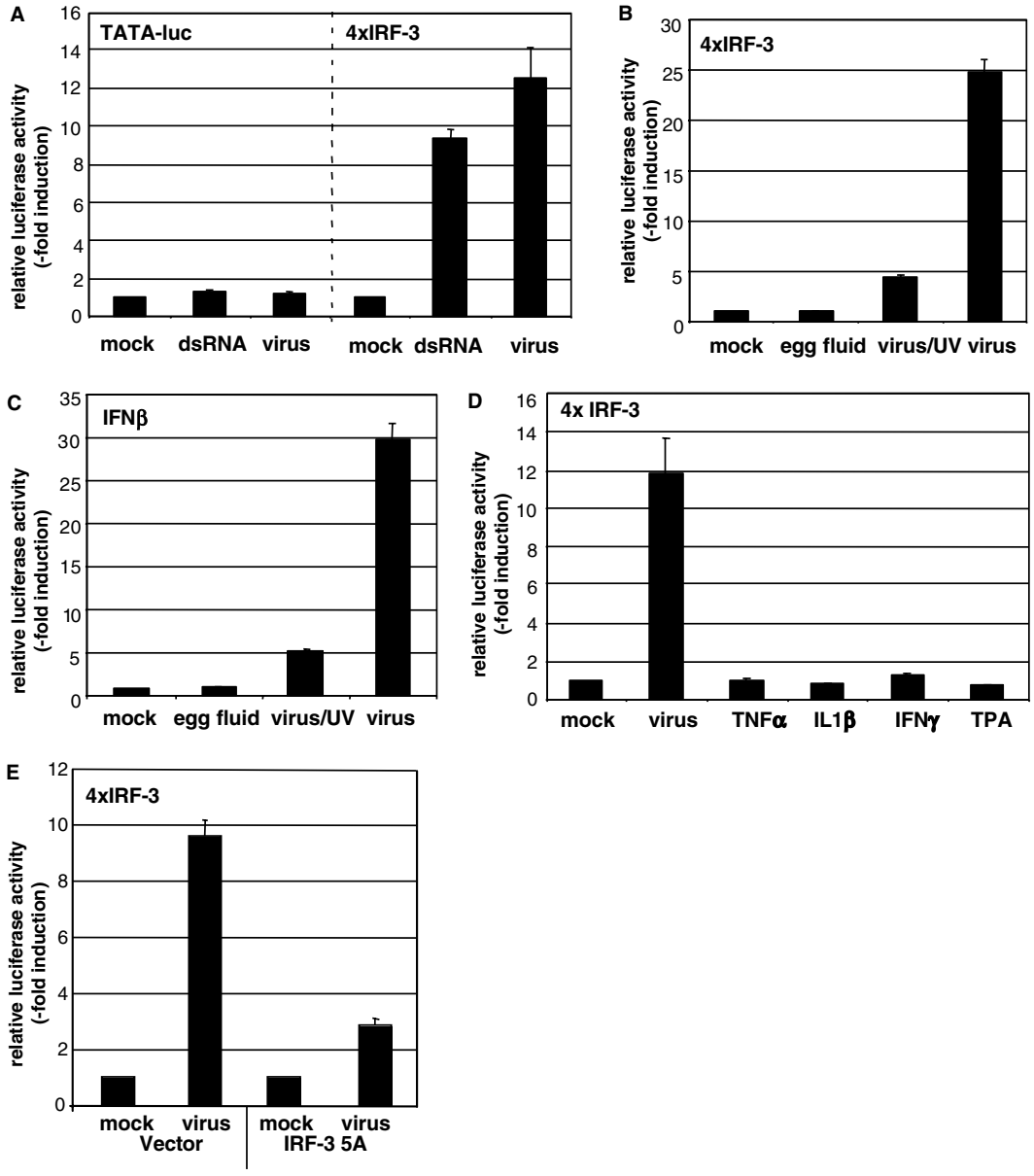

Fig. 1. The 4 × IRF-3 promoter element specifically responds to productive RNA-virus infection and dsRNA stimulation. MDCK cells (A–C,E) or 293 cells (D) were transfected with the empty TATA-luciferase vector (A, left side), with the 4 × IRF-3 luciferase plasmid (A right, B,D,E) or with the IFNβ promoter luciferase plasmid (C). In (E), cells were cotransfected with empty vector or a plasmid expressing IRF-3 5A. After transfection, cells were untreated (mock), infected with replication-competent (A,B,D,E) or with partially UV-inactivated influenza A virus (B,C) grown in embryonated chicken eggs (MOI = 5), treated with the equal amount of egg fluid from uninfected eggs (B,C), or with 50 μg/ml of the dsRNA analog poly(IC) (dsRNA) (A), 10 ng/ml TNF-α (C), 500 U/ml IL1β 50 ng/ml IFNγ or with 100 ng/ml TPA (C). After 4 h of infection or stimulation, cells were lysed and assayed for luciferase activity. Data represent means ± S.E.M. of three independent transfections and are given as the ratio of luciferase activity relative to the uninfected or unstimulated control.

However, none of these mutants, that had been shown to efficiently act dominant-negatively or positively on their cellular counterparts in other systems, showed any significant effect on virus- or dsRNA-induced 4 × IRF-3 promoter activity (data not shown). These results were in line with and partly supported the negative data of earlier studies mainly performed with more or less specific pharmacological inhibitors [7–9].

In the light of all these negative findings, it was quite significant that the activation of the 4 × IRF-3 promoter element was strongly affected by the Rho GTPase family member Rac1. Indeed, Rac1 was activated in response to virus infections or dsRNA stimulation as determined in a GTPase pull-down assay (Fig. 2A). Overexpression of wt Rac1 (Racwt) results in enhanced virus-induced 4 × IRF-3 promoter activity, while expression of the dominant-negative mutant of Rac1 (RacN17) leads to a strong inhibition (Fig. 2B). The same effects of wt and mutant Rac1 were observed upon dsRNA stimulation, suggesting that accumulation of viral RNA upon productive virus replication is the main inducing component (Fig. 2C). Rac1-dependent virus or dsRNA-induced promoter activity is observed as early as 1–2 h post-infection (data not shown) consistent with an early activation of Rac1 (Fig. 2A).

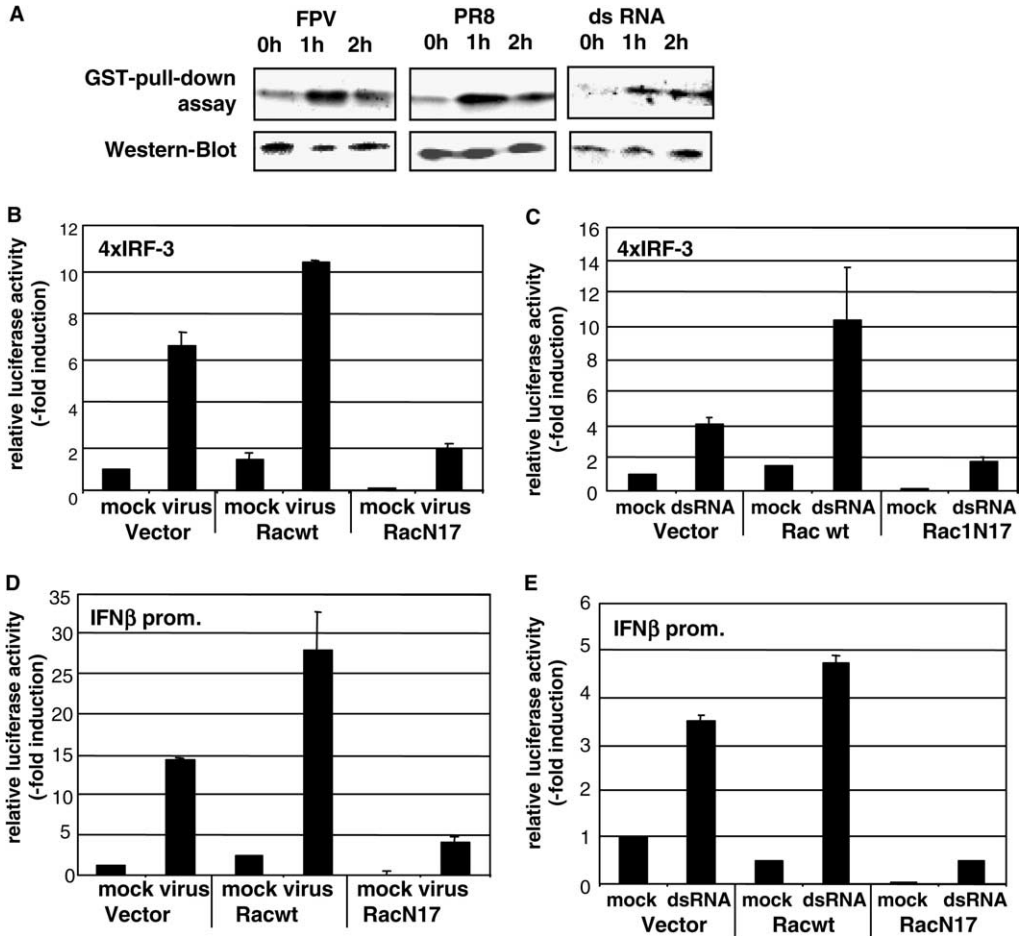

Fig. 2. Rac1 is activated and critical for virus and dsRNA-induced transcription from the 4 × IRF-3 and the IFNβ promoter. (A) 293 cells or A549 cells were infected with different influenza A viruses (FPV or PR8, MOI = 10) or stimulated with dsRNA (100 μg/ml) for the times indicated. Cell lysates were subjected to GST-Rac1BD pull-down assays that allow to selectively precipitate the active GTP loaded form of Rac1. Control blots were performed in full cell lysates. (B–E) MDCK cells were transfected with the 4 × IRF-3 (B,C) or IFNβ (D,E) reporter gene plasmid and co-transfected with empty vector, a Rac1 (Racwt) expression construct, or a plasmid expressing dominant-negative Rac1 (RacN17). Subsequently, cells were either left unstimulated (*mock*), infected with influenza A virus (MOI = 5) for 4 h (B,D) or treated for 4 h with 50 μg/ml poly(IC) (dsRNA) (C, D). Lysates were then prepared and assayed for luciferase activities.

In accordance with the finding that early transcription from the IFNβ promoter/enhanceosome is mainly controlled by IRF-3, the same results were obtained when using an IFNβ promoter reporter gene plasmid (Fig. 2D and E). Other Rho GTPases such as wt and mutant RhoA showed a much weaker effect, while no influence was seen with wt or mutant Cdc42 (data not shown). This suggests a predominant Rac1 specific function in IRF-3-dependent transcriptional activation.

IRF-3 is strongly phosphorylated at the C-terminus in response to virus infection or dsRNA stimulation, and this regulates its nuclear translocation and transactivation potential [2,4,19]. IRF-3 phosphorylation can be monitored by a retardation of the protein in SDS–PAGE [6] and was detectable as early as 1–2 h after dsRNA stimulation or infection with influenza A virus (Fig. 3A and B). If Rac1N17 was transiently overexpressed in infected cells, the amounts of the slower migrating form of IRF-3 protein are strongly reduced (Fig. 3C).

Another way of specifically inhibiting the activity of Rac1 and other Rho GTPases, such as Cdc42 and RhoA, is treatment with the *C. difficile* toxin B. This leads to a reduced intrinsic GTPase activity and impaired membrane-cytosol cycling due to chemical modification [20,21]. In the presence of the toxin TcdB-10463, virus- or dsRNA-induced IRF-3 phosphorylation was also efficiently blocked (Fig. 3D and E). The effect of the toxin most likely has to be attributed to its action on Rac1, since in transfection assays dominant-negative mutants of RhoA or Cdc42 showed no significant activity (data not shown).

Upon virus-induced phosphorylation IRF-3 forms dimers that migrate to the nucleus [6]. Thus, dimerization is another hallmark of IRF-3 activation. To analyze whether Rac affects IRF-3 dimerization, we performed co-immunoprecipitation experiments with transfected IRF-3 fused to two different protein-tags. GFP-IRF-3 specifically immunoprecipitates with HA-IRF-3 from lysates of infected cells (Fig. 3F), indicating virus-induced dimerization. The dimerization is enhanced in cells expressing Rac1wt but nearly abolished in Rac1N17 expressing cells (Fig. 3F).

After translocation to the nucleus, IRF-3 dimers bind to transcriptional coactivators such as the CBP, which finally results in a strong transcriptional activity of the IRF-3

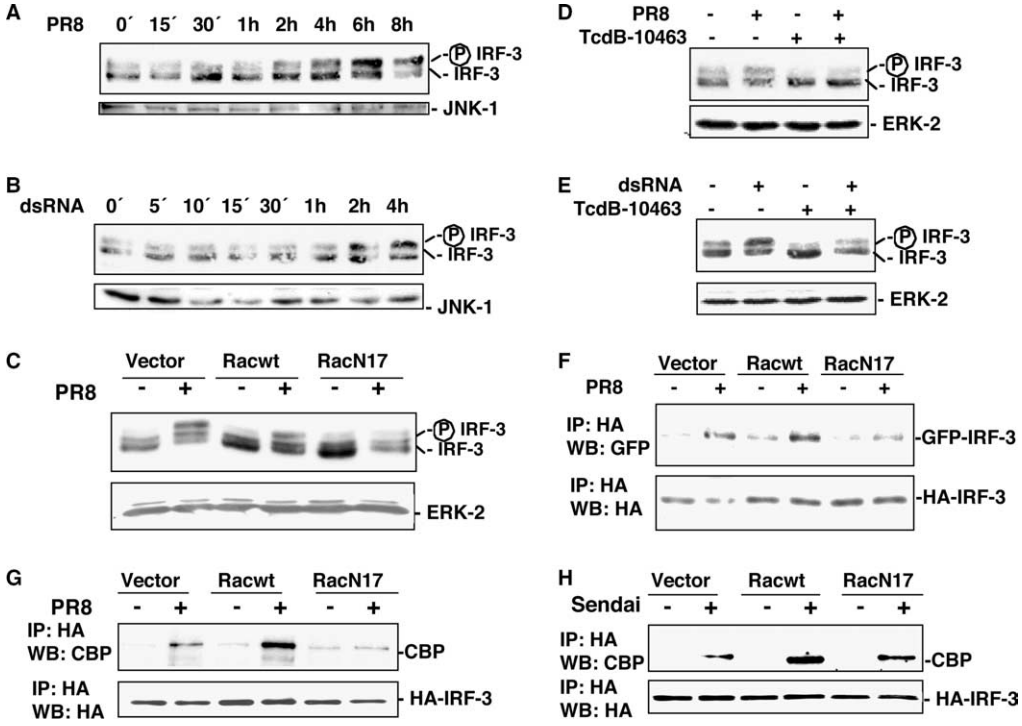

Fig. 3. Virus- or dsRNA-induced IRF-3 phosphorylation, dimerization and association with CBP is regulated by Rac1. MDCK cells were infected with either influenza A virus (MOI = 5) (A,C,D,F,G) or Sendai virus (MOI = 10) (H) or stimulated with 50 μg/ml poly(IC) dsRNA (B,E). Phosphorylated and unphosphorylated IRF-3 were detected by Western blotting as described in Section 2. (D,E) Cells were preincubated for 2 h with 10 ng/ml of the *C. difficile* toxin B-10463 prior to infection with influenza A virus (MOI = 5) or stimulation with dsRNA (50 μg/ml) for 3 h. Proteins were detected in lysates as described. (C,F,G,H) Cells were transfected with empty vector, Rac1wt or Rac1N17 24 h prior to infection with influenza A virus for 3 h (C) or 4 h (D,F,G) (MOI = 5) or Sendai virus for 6 h (H) (MOI = 10). In some of the assays, cells were co-transfected with HA-tagged IRF-3 alone (G,H) or in combination with a GFP-tagged IRF-3 (F). IRF-3, GFP-IRF-3 and CBP proteins were either directly detected in the crude protein lysate (A–E) or upon co-immunoprecipitation with HA-IRF-3 (F–H). Equal protein loads were verified with JNK1-(A,B), ERK2- (C–E), or an anti-HA-tag blots (F–H).

harboring complex [6]. CBP association upon influenza virus infection was also detected in HA-IRF-3 immunoprecipitates and again was enhanced in Rac1wt expressing cells but abolished in cells expressing Rac1N17 (Fig. 3G). Interestingly, the same effects of Rac1wt and mutant expression were observed if cells were infected with Sendai virus (Fig. 3H), indicating that Rac1 involvement may be a general phenomenon in RNA virus-induced IRF-3 activation.

Since Rac1 is obviously not a kinase, the remaining task was to identify the Rac effector(s) that mediate the signal leading to phosphorylation and transcriptional activity of IRF-3 in virus-infected cells. Among the variety of Rac effectors, the kinase PAK1 has been previously demonstrated to be involved in signaling leading to gene expression changes [22]. Thus, we tested wt and various mutants of PAK1 in the IRF-3 promoter–reporter gene assay. Although wt PAK1 did not influence IRF-3 promoter activity, we observed a pronounced effect of dominant negative PAK1 (PAK K299R) (Fig. 4A). This indicates that PAK1 activity is not sufficient yet required for full virus-induced IRF-3 activation. Expression of dominant negative PAK resulted in impaired dimerization (Fig. 4B and C) and CBP association (Fig. 4D and E), both in influenza and Sendai virus-infected cells. Thus, PAK1 is at least one of the signaling components downstream of Rac1 that is involved in RNA virus-induced IRF-3 activation.

Recently, two independent publications introduced two non-canonical homologs of the IκB kinase family, namely IKK-ε and TBK-1 as novel activators of IRF-3 [12,13]. Thus, the question arose whether Rac1 and PAK1 are involved in the pathway leading to viral IKK-ε/TBK-1 and subsequent IRF-3 activation. IRF-3 promoter reporter gene assays revealed that overexpression of Rac1 did not result in enhanced transcriptional activity caused by expression of wt IKK-ε in uninfected cells, however, once cells were infected an additive effect was observed (Fig. 5A). This is in full accordance with the observations shown in Fig. 2 where Rac1wt overexpression only results in enhanced IRF-3 activity in the presence of a viral or dsRNA stimulus. Conversely, expression of dominant negative Rac1 efficiently impaired IRF-3-mediated transcriptional activation induced by IKK-ε (Fig. 5A). Similar results were obtained in parallel experiments using wt and dominant-negative PAK1 (Fig. 5B). This is an indication that Rac1 and IKK-ε act in an additive manner, however, it does not necessarily mean that Rac1 is upstream of IKK-ε. To address this point, we expressed both FLAG-tagged versions of IKK-ε and TBK-1 in mock- and virus-infected cells and co-expressed wt or dominant-negative forms of Rac1 or PAK1. Both kinases were subsequently immunoprecipitated from cell lysates and subjected to in vitro kinase assays to test for IKK-ε and TBK-1 activity. Influenza virus infection resulted in a significant activation of IKK-ε and TBK-1 in vector transfected cells (Fig. 5C and D). Virus-induced activities of both kinases were enhanced in cells overexpressing Rac1wt but strongly impaired in cells expressing the dominant negative mutant of the

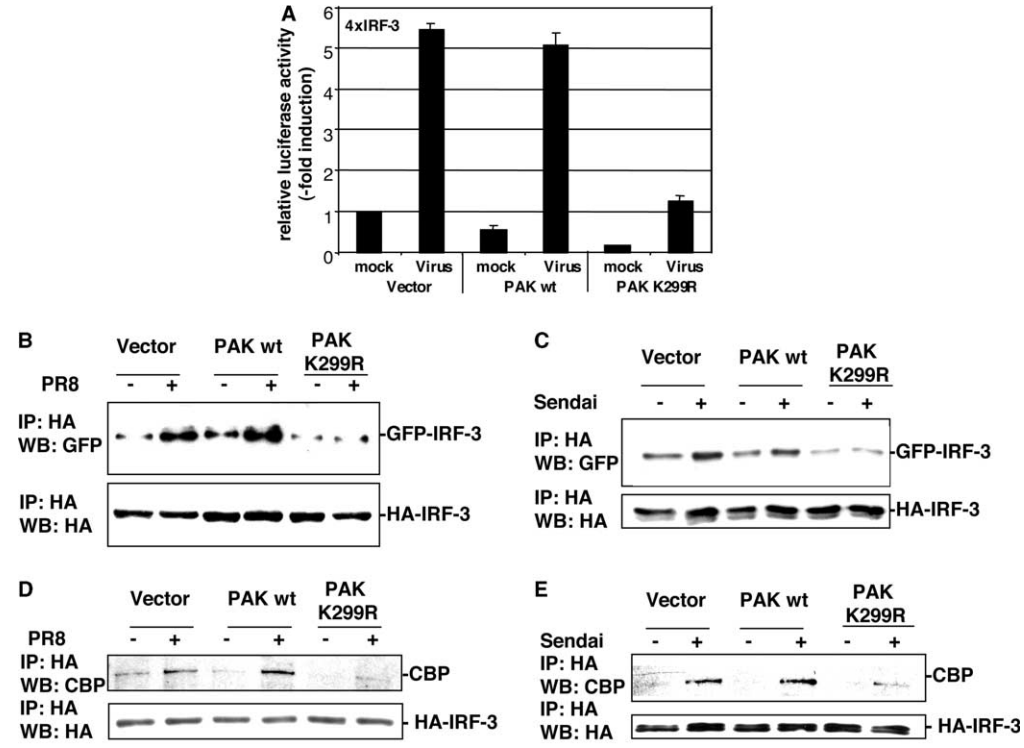

Fig. 4. Inhibition of PAK1 activity impairs virus-induced IRF-3 phosphorylation, dimerization, CBP association and transcriptional activity. MDCK cells were transfected with empty vector, a wt PAK1 (PAK wt) expression construct, or a plasmid expressing dominant-negative PAK1 (PAK K299R). (A) Cells were co-transfected with the 4 × IRF-3 reporter gene plasmid and subsequently left unstimulated (mock) or infected with influenza A virus (MOI = 5) for 4 h followed by determination of luciferase activity in the cell lysates. (B–E) Cells were co-transfected with a plasmid expressing HA-IRF-3 (D,E) or with two plasmids expressing either HA- or GFP-tagged IRF-3 (B,C). Cells were subsequently infected for 4 h with PR8 (MOI = 5) (B,D) or for 6 h with Sendai virus (MOI = 10) (C,E). Cells were subjected to immunoprecipitation of HA-IRF-3 (B–E) and co-immunoprecipitated GFP-IRF-3 (B,C) or CBP (D,E) was detected by Western blot. Equal protein loads of HA-IRF-3 in the immunoprecipitates were verified by an anti-HA-tag Western blot.

GTPase (Fig. 5C). Similar results were obtained in a parallel assay expressing wt and dominant negative PAK1 (Fig. 5D). This clearly indicates that both Rac1 and its effector PAK1 are upstream of IKK-ε and TBK-1 during influenza virus-induced activation of the kinases.

IRF-3 is a central mediator of anti-viral gene expression by regulating type I IFNs and other anti-viral genes. If Rac1 is involved in IRF-3 activation and the subsequent onset of the IFN response, then inhibition of Rac1 in infected cells should result in an enhanced virus production. This was indeed the case, both in cells pre-treated with the inhibitor toxin TcdB-10463 and in cells expressing dominant-negative Rac1 (Fig. 6A–D). We observed virus-titers of up to 2.5-fold the level in controls, using two different viruses and a variety of different cell types (Fig. 6A–D). Although the effects are relatively weak due to a strong counter-regulation of the IFN induction by influenza virus, they are consistently observed in several cell types and with different virus isolates. Moreover, a similar degree of enhanced virus replication is observed in infected cells treated with an anti-type I IFN receptor blocking antibodies (Fig. 6G) and in cells expressing a dominant-negative mutant of the bona fide IRF-3 activator IKK-ε (Fig. 6F). Finally, as expected we observed a stronger effect of Rac1wt and mutant on Sendai virus replication (Fig. 6E). Thus, our results clearly demonstrate that in cells with impaired Rho GTPase activity, similar to cells expressing dominant-negative IKK-ε, important components of the innate anti-viral activity are missing.

4. Discussion

Rho GTPases play a role in a variety of cellular responses such as reorganization of the cytoskeleton, transcriptional activation and apoptosis regulation [23,24]. Here, we have discovered a novel function of the Rho GTPase Rac1 and its effector PAK1 in the innate immune response to virus infections. Rac1 represents the first signaling GTPase shown to be activated in response to virus infections and to be involved in activation of IRF-3. Furthermore, we were able to track the Rac-mediated signal to be transmitted via PAK1 to IKK-ε and TBK-1, which have been just recently identified as IRF-3 activating kinases [12,13].

The finding that overexpression of Rac1wt (Fig. 2) or an active form of Rac (Rac1L61) (data not shown) did not significantly induce IRF-3 transcriptional activity on its own indicates that Rac1 activity is required, yet not sufficient, to induce IRF-3 activation. The same holds true for PAK1 indicating that most likely more than one pathway targets IKK-ε and/or IRF-3 during virus infection. A variety of intracellular signaling mediators have been tested by us and others for involvement in virus-induced IRF-3 activation [7–9]. Among these were also several Rac1 effectors such as the IKK/NF-κB pathway and the p38 and JNK MAPK pathway. Since dominant-negative mutants or specific inhibitors of these Rac1 effectors did not block IRF-3-dependent-promoter activation, we can at least rule out their involvement. IRF-3 is

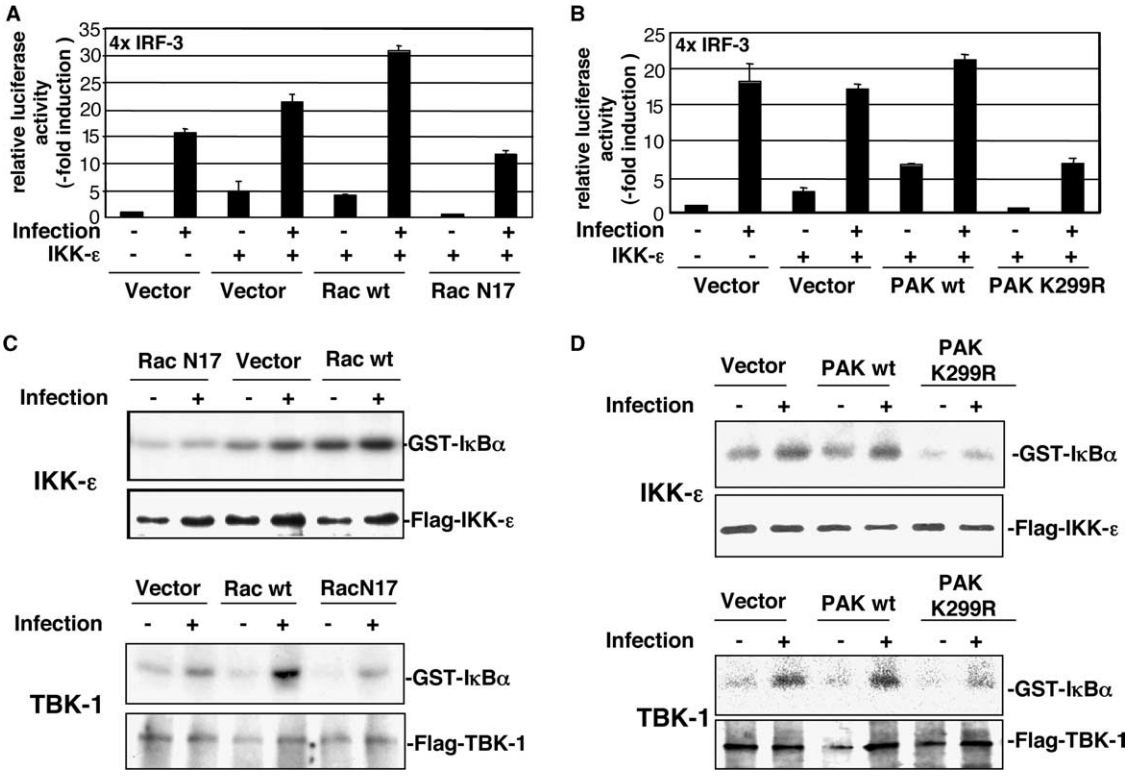

Fig. 5. Rac1 and PAK1 are upstream mediators of virus-induced IKK-ε and TBK-1 activation. (A,B) MDCK cells were transfected with the 4 × IRF-3 reporter gene plasmid and cotransfected with empty vector or wt IKK-ε. Cells were cotransfected with either wt or dominant negative Rac1 (A) or PAK1 (B). 24 h later, cells were either left unstimulated (mock) or infected with influenza A virus (MOI = 5) for 4 h followed by determination of luciferase activity in the cell lysates. (C,D) MDCK cells were transfected with constructs expressing either FLAG-IKK-ε or FLAG-TBK-1 and cotransfected with wt or dominant negative Rac1 (C) or PAK1 (D). 24 h post transfection cells were infected for 4 h and subsequently harvested. Kinase activities of FLAG-IKK-ε or FLAG-TBK-1 in the lysates were determined as described in Section 2. Equal loadings of FLAG-tagged kinases in the assays were controlled by anti-FLAG Western blot.

phosphorylated on multiple sites at the C-terminus without any apparent sequence consensus [2]. Thus, it is most likely that more than one kinase mediates phosphorylation for full viral activation of the factor. Recently, serine 369 of IRF-3 has been identified as the minimal phosphoacceptor site required for in vivo activation of IRF-3 in response to virus infection and dsRNA stimulation [19]. Since the same site appears to be targeted by IKK-ε [12], and IKK-ε is downstream of Rac1 (Fig. 6), it is most likely that the GTPase also acts via this site following virus infection.

Another open question is the nature of the virus or dsRNA sensor in infected cells, which appears to be located upstream of Rac1. At least two dsRNA responsive signaling mediators have been identified so far, the dsRNA-dependent protein kinase (PKR) [25] and the toll-like receptor-3 (TLR3) [26]. While a link between PKR and Rac1 is yet to be established, at least for one member of the TLR family, TLR2, signaling via Rac1 was demonstrated [27]. Interestingly, in these studies TLR2-Rac1 signaling targeted NF-κB activity directly on the p65 level via a pathway parallel to the IKK/IκB module [27]. This supports a report that prominent Rac1 effector functions may well be independent of common effectors such as MAPK pathways or IKK/NF-κB [28]. Furthermore, Rac1 recruitment to the TLR required tyrosine phosphorylation [27], while in another study geldanamycin, an inhibitor of Src-family tyrosine kinases, was shown to affect virus-induced IRF-3 activation. Finally, similar to our findings, downstream signaling from the TLR was blocked by dominant-negative Rac1 but not by dominant negative-Cdc42 [7]. Taking these findings together, it appears likely that TLR3 also signals via Rac1 in a virus- or dsRNA-specific manner. It remains elusive whether Rac1 acts downstream or parallel to the recently identified adaptor proteins TICAM-1, TRIF and TRAM that have been shown to mediate TLR3 generated signals into IFNβ production [29–31].

Another alternative mechanism of Rac1 involvement in viral IRF-3 activation may work via endosomal sensing of viral ssRNA through TLR7 [32–34], a process that would explain the very early onset of the IRF-3 response upon virus infection.

In a final set of experiments, we have demonstrated that inhibition of Rac1 indeed leads to enhanced virus production to a same degree as observed with dominant negative IKK-ε or upon inhibition of type I IFN signaling in influenza virus-infected cells. This fully supports our finding that Rac1 controls activation of a transcription factor critical for the onset and amplification of the anti-viral IFN response. One might argue that the effects on replication are not too impressive. However, it should be kept in mind that although influenza viruses activate IRF-3 [18], these pathogens also express an efficient IFN antagonist, the viral NS1 protein, to keep the consequences of IRF-3 activation and anti-viral gene expression limited [35]. In this light, one would not expect much stronger differences. Furthermore, the same effects were also observed with Sendai

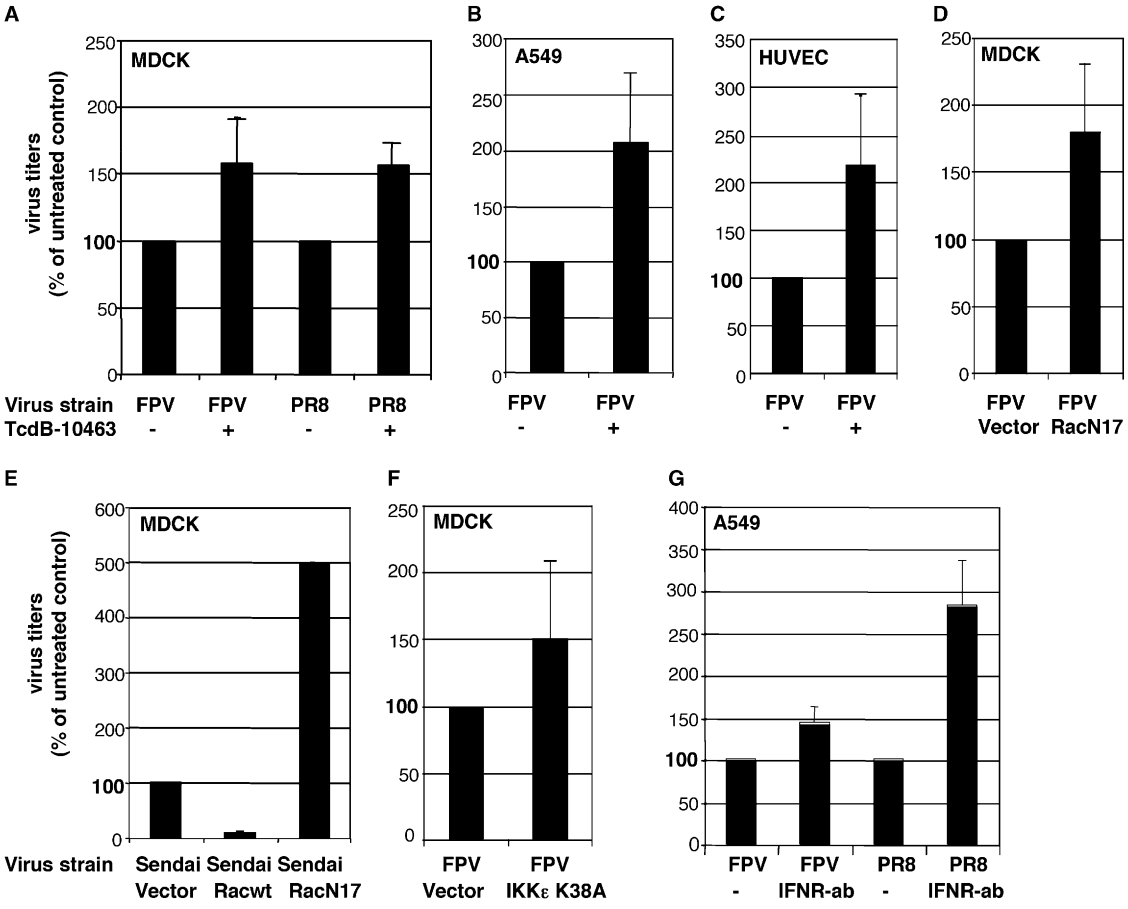

Fig. 6. Inhibition of Rac1 activity results in enhanced influenza A virus or Sendai virus propagation. MDCK cells (A), A549 (B) or HUVEC (C) were treated with toxin B-10436 (10 ng/ml) before and during infection with different influenza A viruses (MOI = 1). (D–F) Alternatively, MDCK cells were transfected with vector or plasmids expressing Rac1wt (E), Rac1N17 (D,E) or dominant negative IKK-ε (IKK-ε K38A) (E) prior to infection with influenza virus (MOI = 1) or Sendai virus (MOI = 0,1). (G) A549 cells were incubated with 20 μg/ml of a type I IFN receptor blocking antibody and infected with different influenza A viruses (MOI = 1). Supernatants were assayed for progeny virus yields 13 h (A–C), 8 h (D) or 24 h (E,G) post-infection in standard plaque titrations. Virus yields of mock-treated cells were arbitrarily set as 100%.

virus, suggesting that Rac1 involvement in the anti-viral response may be a general phenomenon for RNA viruses.

In summary, we have identified a novel function for the Rho GTPase Rac1 upstream of PAK1 and IKK-ε/TBK-1 in the innate immune response to virus infections targeting a major determinant of virus-induced type 1 IFN expression, IRF-3.

Acknowledgements: We are very thankful to S. Akira, K.D. Fischer, I,. Haase, J. Hiscott, S. Jennings and M. Nakanishi for providing plasmids. We also thank Martina Koch and Nicole Wittig for excellent technical assistance and Andris Avots and Randy Cassada for critical reading of the manuscript. The work was supported by grants Lu477/4-5 and GRK 1045 of the Deutsche Forschungsgemeinschaft (DFG), the Fonds der Chemischen Industrie (FdChI) and the Forschungskommission der Medizinischen Fakultät, University of Düsseldorf, Germany.

References

[1] Taniguchi, T. and Takaoka, A. (2002) Curr. Opin. Immunol. 14, 111–116.
[2] Hiscott, J., Pitha, P., Genin, P., Nguyen, H., Heylbroeck, C., Mamane, Y., Algarte, M. and Lin, R. (1999) J. Interferon. Cytokine Res. 19, 1–13.
[3] Thanos, D. and Maniatis, T. (1995) Cell 83, 1091–1100.
[4] Lin, R., Heylbroeck, C., Pitha, P.M. and Hiscott, J. (1998) Mol. Cell. Biol. 18, 2986–2996.
[5] Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E. and Fujita, T. (1998) EMBO J. 17, 1087–1095.
[6] Servant, M.J., Grandvaux, N. and Hiscott, J. (2002) Biochem. Pharmacol. 64, 985–992.
[7] Iwamura, T. et al. (2001) Genes. Cells 6, 375–388.
[8] Servant, M.J., ten Oever, B., LePage, C., Conti, L., Gessani, S., Julkunen, I., Lin, R. and Hiscott, J. (2001) J. Biol. Chem. 276, 355–363.
[9] Smith, E.J., Marie, I., Prakash, A., Garcia-Sastre, A. and Levy, D.E. (2001) J. Biol. Chem. 276, 8951–8957.
[10] Shimada, T., Kawai, T., Takeda, K., Matsumoto, M., Inoue, J., Tatsumi, Y., Kanamaru, A. and Akira, S. (1999) Int. Immunol. 11, 1357–1362.
[11] Tojima, Y. et al. (2000) Nature 404, 778–782.
[12] Sharma, S., ten Oever, B.R., Grandvaux, N., Zhou, G.P., Lin, R. and Hiscott, J. (2003) Science 300, 1148–1151.
[13] Fitzgerald, K.A. et al. (2003) Nat. Immunol. 4, 491–496.
[14] Goebeler, M., Gillitzer, R., Kilian, K., Utzel, K., Brocker, E.B., Rapp, U.R. and Ludwig, S. (2001) Blood 97, 46–55.
[15] Pleschka, S., Wolff, T., Ehrhardt, C., Hobom, G., Planz, O., Rapp, U.R. and Ludwig, S. (2001) Nat. Cell Biol. 3, 301–305.
[16] Basler, C.F., Wang, X., Muhlberger, E., Volchkov, V., Paragas, J., Klenk, H.D., Garcia-Sastre, A. and Palese, P. (2000) Proc. Natl. Acad. Sci. USA 97, 12289–12294.
[17] Ludwig, S., Ehrhardt, C., Neumeier, E.R., Kracht, M., Rapp, U.R. and Pleschka, S. (2001) J. Biol. Chem. 276, 10990–10998.

[18] Kim, M.J., Latham, A.G. and Krug, R.M. (2002) Proc. Natl. Acad. Sci. USA 99, 10096–10101.
[19] Servant, M.J., Grandvaux, N., ten Oever, B.R., Duguay, D., Lin, R. and Hiscott, J. (2003) J. Biol. Chem. 278, 9441–9447.
[20] Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995) Nature 375, 500–503.
[21] Genth, H., Aktories, K. and Just, I. (1999) J. Biol. Chem. 274, 29050–29056.
[22] Aspenstrom, P. (1999) Curr. Opin. Cell Biol. 11, 95–102.
[23] Bar-Sagi, D. and Hall, A. (2000) Cell 13, 227–238.
[24] Van Aelst, L. and D'Souza-Schorey, C. (1997) Genes Dev. 11, 2295–2322.
[25] Williams, B.R. (1999) Oncogene 18, 6112–6120.
[26] Alexopoulou, L., Holt, A.C., Medzhitov, R. and Flavell, R.A. (2001) Nature 413, 732–738.
[27] Arbibe, L. et al. (2000) Nat. Immunol. 1, 533–540.
[28] Murga, C., Zohar, M., Teramoto, H. and Gutkind, J.S. (2002) 0ncogene 21, 207–216.
[29] Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. and Seya, T. (2003) Nat. Immunol. 4, 161–167.
[30] Yamamoto, M. et al. (2003) Nat. Immunol. 4, 1144–1150.
[31] Fitzgerald, K.A. et al. (2003) J. Exp. Med. 198, 1043–1055.
[32] Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. and Reis, E.S.C. (2004) Science 303, 1529–1531.
[33] Heil, F. et al. (2004) Science 303, 1526–1529.
[34] Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Iwasaki, A. and Flavell, R.A. (2004) Proc. Natl. Acad. Sci. USA.
[35] Talon, J., Horvath, C.M., Polley, R., Basler, C.F., Muster, T., Palese, P. and Garcia-Sastre, A. (2000) J. Virol. 74, 7989–7996.