Impact of Rac1 and Cdc42 Signaling during Early Herpes Simplex Virus Type 1 Infection of Keratinocytes[▽]

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Keratinocytes of the skin or mucosa are the primary entry portals for herpes simplex virus type 1 (HSV-1) in vivo. We hypothesized that dynamics of cell motility and adhesion contribute to the initial steps of HSV-1 infection of epithelial cells, and thus, we investigated the impact of Rac1 and Cdc42, which serve as key regulators of actin dynamics. Measurement of endogenous Rac1 and Cdc42 in the human keratinocyte cell line HaCaT indicated temporary changes in activity levels of Rac1/Cdc42 upon HSV-1 infection. Overexpression of Rac1/Cdc42 mutants in HaCaT cells demonstrated a decrease of infection efficiency with constitutively active Rac1 or Cdc42, while dominant-negative Rac1 had no effect. Accordingly, we addressed whether the absence of Rac1 and/or Cdc42 influenced infection, and we performed RNA interference studies. Both in HaCaT cells and in primary human keratinocytes, reduction of Rac1 and/or Cdc42 did not suppress infection. When mouse epidermis was infected ex vivo, we observed early HSV-1 infection in basal keratinocytes. Similar results were obtained upon infection of mouse epidermis with a keratinocyte-restricted deletion of the *rac1* gene, indicating no inhibitory effect on HSV-1 infection in the absence of Rac1. Our results suggest that HSV-1 infection of keratinocytes does not depend on pathways involving Rac1 and Cdc42 and that constitutively active Rac1 and Cdc42 have the potential to interfere with HSV-1 infectivity.

Mammalian Rho GTPases are well documented for their important roles in regulating the actin cytoskeleton. The Rho GTPases Rac1 and Cdc42 function as molecular switches and cycle between an active GTP-bound state and an inactive GDP-bound state (20). In cells, Rho GTPases exist mainly in their inactive form (34). Activation is mediated by guanine nucleotide exchange factors, while GTPase-activating proteins promote the hydrolysis of GTP to GDP. When bound to GTP, the Rho proteins can activate various downstream effectors, thereby stimulating diverse biological responses, such as actin dynamics, cell cycle progression, cell adhesion, and gene transcription (5). Viruses are very well adapted to host cell signaling and have evolved strategies to manipulate cellular responses to viral infection. Most importantly, viruses can not only counteract cellular signaling but also take advantage of signaling pathways to optimize their infection cycle (18). Recent observations indicate that herpesviruses can interact with actin and/or Rho GTPases during the three major phases of their replication cycle: entry, replication, and egress (15). During the viral entry phase, microtubule-based transport has been described for capsids traveling to the nucleus, while actin filaments may play a role in short-range movements (26). There is growing evidence that viruses induce Rho GTPase activity during the entry process, although the underlying mechanisms and the biological significance are often unclear. Studies of the entry of herpes simplex virus type 1 (HSV-1) into primary corneal fibroblasts and nectin-1-overexpressing CHO cells indicate an association of virions with actin-based cellular pro-

trusions (10). The Rho GTPase RhoA was activated during this process, while Cdc42 showed a brief activation at 1 min postinfection (p.i.). These features may be essential parts of a phagocytosis-like uptake of HSV-1 (10). Activation of Rac1 also occurs during entry of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) into fibroblasts, accompanied by increased RhoA activity levels. Inactivation of the Rho GTPases results in reduced delivery of viral DNA to the nucleus. Naranatt et al. suggest that human herpesvirus 8 induces Rho GTPases, thereby modulating microtubules and promoting trafficking of viral capsids (28). A role for Cdc42 signaling has been reported for pseudorabies virus during infection of sensory neurons (11). During entry, pseudorabies virus induces the formation of synaptic boutons, which are known sites for virus egress from axons (8, 35). Inhibition of Cdc42 suppressed formation of synaptic boutons, while inhibitors of Rac1 and RhoA had no effect. Thus, De Regge et al. conclude that Cdc42-induced formation of synaptic boutons during virus entry may serve as axon exit sites during virus egress (11). We previously addressed the putative functions of Rac1 and Cdc42 during the HSV-1 entry process in the well-studied epithelial cell line MDCKII (22). Endogenous Rac1 and Cdc42 were temporally activated upon infection. Interestingly, we found decreased infectivity when constitutively active Rac1 or Cdc42 was overexpressed, while no effect was observed upon overexpression of dominant-negative Rac1. These results suggest a mechanism involving virus-induced regulation of Rac1/Cdc42 activities in MDCKII cells (22).

HSV-1 enters its human host via the epithelia of mucosa, skin, or cornea. Epithelia are thought to be infected following apparent or inapparent injury. Mucosal, epidermal, and corneal keratinocytes therefore represent the primary entry portal for HSV-1. Cellular entry relies initially on the interaction of

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several viral glycoproteins with various cell surface receptors (21). The intercellular adhesion molecule nectin-1 can mediate HSV-1 entry into human keratinocytes (23). *Trans* interaction of nectins in turn leads to activation of Cdc42 and Rac1, representing one step of the actin reorganization that is involved in formation of adherens junctions (38). It is still unclear whether virus interaction with nectin-1 in epithelial cells induces similar Cdc42/Rac1-mediated cellular responses that play a role during the viral entry process. Further alternative HSV-1 receptors, particularly for entry into human keratinocytes *in vivo*, have not been excluded (23). Depending on the cell line investigated, HSV-1 can enter cells either by fusion of the viral envelope with the plasma membrane or by endocytic pathways (1, 10, 17, 27, 29, 30, 31). Furthermore, expression of the recently identified coreceptor PILR α (paired immunoglobulin type 2 receptor α) in CHO cells indicates HSV-1 uptake via fusion, while expression of nectin-1 in CHO cells leads to endocytic uptake, suggesting that the entry pathway into the same cell line depends on the cellular entry coreceptor used (2). For human keratinocytes, there is evidence that HSV-1 enters via endocytic pathways (30). Thus, the impact of actin dynamics on the entry process could vary depending on the preferred internalization pathway in the respective cell type.

In vivo HSV-1 entry may be enabled at sites of lesions in mucosa or skin. Upon injury, the epithelial barrier is broken and viruses come into contact with keratinocytes that would normally be protected by more differentiated, suprabasal layers of the epithelium. The wound healing reaction initiated in basal keratinocytes after injury is characterized by profound changes in cellular actin dynamics that are associated with changes in keratinocyte adhesion and motility. We speculate that this actin reorganization as part of the wound healing response could contribute to preferential viral infection at sites of injury. In epithelial cells, Rac1 and Cdc42 are known regulators of adhesion and migration (25).

Our recent results suggest a role of regulated Rac1/Cdc42 signaling for HSV-1 infection in the epithelial MDCKII cells. The rationale of the present study is to address the role of Rac1/Cdc42 in keratinocytes and epidermal sheets and to extend our previous analysis by choosing “knockdown” and “knockout” approaches. In addition to overexpression of Rac1 and Cdc42 mutants, we reduced Rac1 and/or Cdc42 in human keratinocytes by RNA interference. Furthermore, we used a mouse model to study the consequences of Rac1 deficiency in murine epidermis for HSV-1 infection. Interestingly, our findings suggest that initiation of HSV-1 infection in keratinocytes was independent of Rac1/Cdc42 signaling.

MATERIALS AND METHODS

Cells, viruses, and plasmids. HaCaT cells (6) were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 10% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 μ g/ml). Under starvation conditions, FCS was reduced to 0.5%. Primary human foreskin keratinocytes were prepared and cultured on feeder layers as described previously (40). In brief, primary human keratinocytes were maintained in keratinocyte culture medium (Ham's F12-DMEM (1:3); Invitrogen) containing 1.8 mM calcium ions and 10% FCS, penicillin (100 IU/ml), streptomycin (100 μ g/ml), adenine (1.8×10^{-4} M), glutamine (2 mM), hydrocortisone (0.5 μ g/ml), epidermal growth factor (EGF) (10 ng/ml), cholera enterotoxin (10^{-5} M), and insulin (5 μ g/ml) in the presence of mitomycin C-treated 3T3 fibroblasts, strain J2. Murine epidermal

keratinocytes were isolated from skin of wild-type newborn mice (C57BL/6) as described previously (39).

Murine epidermal sheets were taken from back skin of wild-type (C57BL/6) and mutant newborn mice. At 3 days after birth, mice were decapitated and skin pieces of about 15 mm in diameter were taken. In addition, skin was peeled from the tails of adult mice, and pieces of about 8 mm were used to prepare epidermal whole mounts as described previously (7). After incubation for 30 min at 37°C with 5 mg/ml dispase II (Roche) in phosphate-buffered saline (PBS), the epidermis was washed three times in PBS, gently removed from the underlying dermis as an intact sheet using forceps, and used immediately for infection studies.

Cells and epidermis were infected with purified preparations of HSV-1 wild-type strain 17 as described previously (36). Time zero was defined as the time when the virus inoculum was added to the cells/sheets at 37°C. Only primary murine keratinocytes were infected at 32°C. Virus titers were determined with Vero cells. Virus released into the cell medium (CRV) was obtained from supernatant of infected cells upon clarification. Cell-associated virus was harvested from infected cell pellets; upon three freeze/thaw cycles and subsequent centrifugation of the cell debris, CRV in the supernatant was taken for titration.

The time course of ICP0 expression in HaCaT cells was determined by Western blot analyses. Total protein extracts were prepared from mock-infected cells or from cells at 1, 2, 3, and 4 h p.i., and proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (10%). ICP0 was detected with rabbit anti-ICP0 antiserum (r191) (33) diluted 1:4,000. Staining with rabbit anti-PDI antiserum (SPA-980) diluted 1:4,000 was used as a loading control.

pRK5 expression vectors, encoding Myc-tagged L61Rac1, N17Rac1, L61Cdc42, and N17Cdc42, were obtained from V. Braga (Imperial College London, United Kingdom) and A. Hall (Sloan-Kettering Institute, New York). Plasmid EGFP-C1 (Clontech) was used as a control.

Rac1 mutant mice. Female mice with two alleles containing floxed exon 3 of the *rac1* gene (9) were intercrossed with mice expressing Cre recombinase under the control of the keratin 14 promoter (19). Mice used for experiments were genotyped as described previously (9). Efficient deletion of Rac1 within the epidermis was controlled by Western blot analysis. In brief, total protein extracts were prepared from epidermis of 3.5-day-old Rac1 E-KO and wild-type mice and from tail epidermis of adult mice. Proteins were resolved by SDS-PAGE gels (15%), and Rac1 was detected with mouse anti-Rac1 (monoclonal antibody 23A8; Upstate) diluted 1:500, followed by enhanced chemiluminescence (ECL; Pierce).

Transient expression. For transfection of plasmids expressing Rac1 and Cdc42 mutants, HaCaT cells were trypsinized, pelleted, washed with PBS, and resuspended in Nucleofector solution V (Amaxa). Cells (1×10^6) were transfected with 2 μ g plasmid in a cuvette, utilizing program U-20 of an Amaxa Nucleofector I device. Cells were seeded on coverslips and infected at 22 h posttransfection at a multiplicity of infection (MOI) of 50 PFU/cell for 2 h. Alternatively, HaCaT cells were seeded on coverslips (6×10^5) in DMEM with 10% FCS and transfected with 2 μ g plasmid mixed with Lipofectamine LTX and Plus reagent (Invitrogen) in Opti-MEM I (Invitrogen) according to the manufacturer's instructions. At 22 h posttransfection, cells were infected at an MOI of 50 PFU/cell for 2 h.

siRNA-mediated knockdown. For the knockdown of human Rac1, the validated small interfering RNA (siRNA) HS RAC1 6HP (Qiagen) was used. In addition, Cdc42-siRNA (GAUAAACUACCACUGUCCATT) (Eurogentec) directed against human Cdc42 and RhoA-siRNA (GAAGUCAAGCAUUUCU GUC) (Eurogentec) directed against human RhoA, as well as nonspecific control siRNAs (Qiagen, Eurogentec), were used. HaCaT cells (1.5×10^4) and human primary keratinocytes (1.5×10^4) seeded on coverslips were transfected with the siRNAs for Rac1 (40 nM), Cdc42 (20 nM), RhoA (20 nM), or the nonspecific controls (20 or 40 nM) mixed with HiPerFect transfection reagent (Qiagen) diluted in Opti-MEM I (Invitrogen). At 70 h posttransfection, cells were infected at an MOI of 20 PFU/cell. At 2 h p.i., infected cells visualized by ICP0 staining were counted. The knockdown of each experiment was controlled by Western blot analyses using mouse anti-Rac1 (monoclonal antibody 23A8; Upstate) diluted 1:500, mouse anti-Cdc42 (monoclonal antibody 610929; BD Biosciences) diluted 1:250, mouse anti-RhoA (26C4; Santa Cruz) diluted 1:200, and rabbit polyclonal anti-VDAC (600-401-882; Rockland) diluted 1:1,000 for normalization.

Histology, immunocytochemistry, and antibodies. HaCaT cells and primary keratinocytes grown on coverslips were fixed with 2% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and stained as described previously (36). At 2 h p.i., infected cells were visualized by ICP0 staining either with rabbit anti-ICP0 antiserum (r191) (33), diluted 1:500, or with mouse anti-ICP0 (mono-

FIG. 1. ICP0 expression in HaCaT cells and primary human and mouse keratinocytes. Subconfluent HaCaT cells (A and B), primary human keratinocytes (D), or primary mouse keratinocytes (E) were infected with HSV-1 (20 PFU/cell) and fixed at 1, 2, 3, and 4 h p.i., respectively. F-actin was visualized with TRITC-phalloidin (red). Cells were stained with mouse anti-ICP0 (green), visualized with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (Molecular Probes). Total protein extracts were prepared from uninfected HaCaT cells (mock) and from cells at 1, 2, 3, and 4 h p.i. Proteins were resolved on SDS-PAGE gels (10%) and stained with rabbit anti-ICP0 antiserum. Staining with rabbit anti-PDI (protein disulfide isomerase) antiserum was used as a loading control. Single stainings and merged images of confocal projections are shown. Bar, 50 μ m (A and B) or 80 μ m (D and E).

FIG. 2. Endogenous Rac1 and Cdc42 activities upon HSV-1 infection. HaCaT cells kept under normal serum conditions (nonstarved) (A and B) or starved for 7 days (C and D) were infected with HSV-1 (20 PFU/cell). The levels of GTP-bound Rac1 and Cdc42 were determined in mock-infected (mock) cells or in cells at 15, 30, or 60 min p.i. Pull-down experiments were performed, and equal amounts of bead fractions and lysate fractions were resolved on SDS-PAGE gels (15%). The GTP-bound forms of Rac1 and Cdc42 in bead fractions and total Rac1 in lysate fractions were stained with mouse anti-Rac1 or mouse anti-Cdc42 antibodies.

clonal antibody 11060) (14), diluted 1:2,000. The Myc-tagged Rac1 and Cdc42 mutants were detected with mouse anti-Myc (monoclonal antibody 9E10; Santa Cruz), diluted 1:2,000.

To prepare epidermal sections, murine epidermis was embedded in Tissue Tek (Sakura) and frozen in liquid nitrogen, and 8- μ m frozen cross sections were cut using a CM3050 (Leica) cryomicrotome. Hematoxylin-and-eosin (H-E) staining was performed according to standard histological procedures. For immunostaining, tissue sections were washed once with PBS, fixed with 0.5% paraformaldehyde for 10 min, washed two times with PBS, blocked with 5% normal goat serum for 30 min, and then incubated for 60 min with mouse anti-ICP0 (monoclonal antibody 11060) (14) diluted 1:2,000 and rabbit polyclonal anti-mouse keratin 14 (AF64; Covance) diluted 1:2,000. Primary antibodies were visualized with fluorochrome-conjugated anti-rabbit or anti-mouse immunoglobulin G (AF488 or AF555) (Molecular Probes) for 45 min at room temperature. Staining of F-actin was performed with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) for 15 min. For immunostaining of epidermal whole mounts, murine epidermis was fixed with 3.4% formaldehyde for 2 h, washed two times with PBS, blocked with 0.5% milk powder, 0.25% gelatin from cold water fish skin, and 0.5% Triton X-100 in 0.2% PBS-Tween 20 for 1 h, and then incubated overnight with primary antibodies, followed by overnight incubation with secondary antibodies at room temperatures. Microscopy was performed using a Zeiss Axiophot microscope, a Leica DM RE microscope linked to a Leica SP/2 confocal unit as described previously (36), and an Olympus IX81 confocal microscope with Olympus FluoView software, version 1.7b. Images were acquired using the Adobe Photoshop software program, version 9.0.

The effects of Rac1 and Cdc42 mutant expression were quantified by counting about 300 transfected cells visualized with anti-Myc antibodies in at least 3 independent experiments and calculating the number of infected cells visualized by ICP0 staining.

Upon transfection of siRNAs, infected cells were visualized with anti-mouse ICP0 (monoclonal antibody 11060) (14) and about 500 cells per siRNA were counted.

Rac1/Cdc42 activity assay. HaCaT cells (3×10^6) were seeded (in 10-cm dishes), followed by infection at about 24 h after seeding. In addition, HaCaT cells (3.5×10^5) seeded in 10-cm dishes were starved for 7 days (0.5% FCS) prior to infection. Control experiments were performed to demonstrate that Rac1 and Cdc42 activation was still possible in starved HaCaT cells; at 15 min after EGF addition, we observed an increase in activated Rac1 compared to results under mock conditions, where only medium was replaced (data not shown). Further controls excluded that medium replacement alone could increase activated Rac1 or Cdc42 at 15 and 30 min postreplacement (data not shown). Starved and

nonstarved cells were infected at an MOI of 20 PFU/cell or mock infected (replacement of medium). At various times p.i., activated Rac1 or Cdc42 was identified by specific binding to the glutathione-S-transferase-fused p21-binding domain of human Pak-1 using the EZ-detect Cdc42 activation kit (Pierce) as described previously (22). Proteins in bead fractions were resolved on SDS-PAGE gels (15%) and transferred to polyvinylidene difluoride membranes (GE Healthcare) by blotting for 2 h at 40 V and 4°C. The bound GTP-Rac1 or GTP-Cdc42 were detected with mouse anti-Rac1 (monoclonal antibody 23A8; Upstate) diluted 1:500 or anti-Cdc42 antibodies (EZ-detect; Pierce) diluted 1:250 followed by enhanced chemiluminescence (for Rac1, ECL [Pierce]; for Cdc42, ECLplus [GE Healthcare]).

RESULTS

Infection of keratinocytes. Infection studies were performed with HaCaT cells, representing undifferentiated human keratinocytes (6). In addition, undifferentiated primary human or murine epidermal keratinocytes were infected. To visualize HSV-1 infection in individual cells, cells were stained with an antibody directed against the viral immediate-early protein ICP0 (13). The cellular localization of ICP0 went through distinct phases during early infection. At 1 h p.i., ICP0 was detectable in distinct nuclear foci, followed by more diffuse nuclear ICP0 staining at 2 h p.i. and a cytoplasmic localization of ICP0 at 3 h p.i. (Fig. 1A and B). The amount of ICP0 increased during this time period (Fig. 1C). When subconfluent HaCaT cells were infected, we first observed infected cells at the rim of cell islets, suggesting preferred viral entry at the periphery of cell islets (Fig. 1A). At later time points, however, and upon infection at a high MOI, cells in the inner part of the cell islets were also infected. Infection of primary human keratinocytes was detected in monolayers with poor cell-cell contacts and in peripheral basal cells of islets as described earlier (Fig. 1D) (36). Similar observations were made for primary murine keratinocytes (Fig. 1E).

Rac1 and Cdc42 activities upon HSV-1 infection. We initially investigated the putative effect of HSV-1 infection on endogenous Rac1 and Cdc42 activation. In subconfluent HaCaT cells, the level of activated Cdc42 was rather high whereas the level of activated Rac1 was variable, ranging from high to low levels. When cells with a low Rac1 activity level were infected, we observed an increase at 15 and 30 min p.i. and a decrease at 60 min p.i. (Fig. 2A). In contrast, the high Cdc42 activity level in mock-infected cells did not increase after infection (Fig. 2B). Therefore, cells were serum starved for at least 7 days prior to infection, which resulted in reproducibly lower activation levels of Rac1 and Cdc42 (data not shown). The numbers of infected cells were comparable in starved and nonstarved cells (data not shown). Upon infection of starved cells, we observed an increase of endogenous Rac1 activity at 15 and 30 min p.i., followed by a decrease (Fig. 2C). Similar results were observed for the endogenous Cdc42 activity level (Fig. 2D). Thus, we conclude that infection can lead to fluctuation of Rac1 and Cdc42 activity levels.

Effects of transient expression of Rac1 and Cdc42 mutants on HSV-1 infection. In MDCKII cells, we observed an inhibitory effect on HSV-1 infectivity upon overexpression of constitutively active L61Rac1 or L61Cdc42 mutants (22). Here we investigated the effect of Rac1/Cdc42 mutants in HaCaT cells and obtained comparable results. When constitutively active L61Rac1 and L61Cdc42 were expressed prior to infection, we observed a reduced number of infected cells compared to results in control experiments. Whereas overexpression of green fluorescent protein led to infection of at least 80% of the transfected cells, active Rac1 and Cdc42 reduced the number of infected cells to 17% and 16%, respectively (Fig. 3A). In contrast, dominant-negative Rac1 had no influence on the number of infected cells, while dominant-negative Cdc42 reduced the number of infected cells to 35% (Fig. 3A). Thus, the inhibitory effect of the active Rac1 and Cdc42 mutants was more severe than the inhibitory effect of the dominant-negative Cdc42 mutant. Overexpression of wild-type Rac1 or Cdc42 had no effect on the number of infected cells (data not shown).

The results were based on two different transfection methods; transient expression was achieved either by electroporation (Amaxa) or by a liposome-mediated transfer (Invitrogen).

FIG. 3. HSV-1 infection of HaCaT cells transfected with mutant forms of Rac1 and Cdc42. Cells were transfected with either constitutively active Rac1 (L61Rac1) or Cdc42 (L61Cdc42) or dominant-negative Rac1 (N17Rac1) or Cdc42 (N17Cdc42), followed by HSV-1 infection (50 PFU/cell) at 22 h posttransfection. Transfection was performed by electroporation (Amaxa) (A) or by liposome-mediated transfer (Invitrogen) (B and C). At 2 h p.i., cells were fixed and costained with rabbit anti-ICP0 antibodies (red) and mouse anti-Myc (green), visualized by AF555-conjugated antirabbit (Molecular Probes) and AF488-conjugated antimouse (Molecular Probes), respectively. (A) The number of ICP0-expressing cells per mutant-expressing cell was determined in at least three independent experiments. As a control, the number of infected cells that were transfected with a green fluorescent protein-expressing plasmid (GFP) is shown. The percentages of infected cells that were transfected with the indicated plasmids are given. Results are mean $\pm$ standard deviation values. (B) Single stainings and merged images of confocal projections are shown. (C) Single immunofluorescence analyses and overlays of double stainings are shown. Bar, 50 μ m.

FIG. 4. HSV-1 infection of HaCaT cells upon silencing of Rac1, Cdc42, or RhoA. HaCaT cells were transfected with Rac1-siRNA, Cdc42-siRNA, RhoA-siRNA, or nonspecific control siRNAs, followed by infection with HSV-1 (20 PFU/cell) at 72 h posttransfection. (A) Total protein extracts were prepared, and proteins were resolved on SDS-PAGE gels (15%). Expression of the Rho GTPases was analyzed with mouse

In both cases, the minimal time of mutant gene expression prior to infection was about 22 h to minimize potential interference with cell viability upon prolonged expression. Upon electroporation, cells attached; however, neither spreading nor formation of cell-cell contacts was observed. Therefore, putative effects of overexpressed Rac1 and Cdc42 mutants on cell morphology were detectable only upon liposome-mediated transfection. While we observed characteristic changes of the F-actin organization in MDCKII cells upon overexpression of Rac1 and Cdc42 mutants (22), only minor effects were visible in HaCaT cells (data not shown). The most obvious effect was the increased size of active Rac1-expressing cells compared to that of untransfected or inactive Rac1-expressing cells (Fig. 3B). The phenotype of either active or inactive Cdc42-expressing cells showed no difference from that of untransfected cells (Fig. 3C).

Effects of reduced Rac1, Cdc42, and RhoA on HSV-1 infection of human keratinocytes. The observation that inactive Cdc42 but not inactive Rac1 reduced the number of infected cells suggested that only Cdc42-dependent pathways play a role during initiation of HSV-1 infection. We analyzed the putative role of Cdc42 and Rac1 during infection by siRNA-mediated knockdown of Rac1 and Cdc42 in HaCaT cells and primary human keratinocytes. As a control, the effect of RhoA silencing was investigated. Previous results indicate that expression of RhoA mutants does not interfere with HSV-1 infectivity at least in MDCKII cells (22).

At 72 h posttransfection, efficient silencing of both Rac1 and Cdc42 was observed when either of the siRNAs was transfected or both siRNAs were transfected at the same time. Furthermore, transfection of RhoA siRNAs resulted in efficient knockdown of RhoA. According to Western blot analyses, expression of Rac1, Cdc42, or RhoA was reduced to 4 to 10% compared to that for samples transfected with negative-control siRNAs (Fig. 4A). At 70 h posttransfection, HaCaT cells were infected for 2 h at an MOI of 20 PFU/cell. When infected cells were visualized by ICP0 staining, nearly no effect on the number of infected cells was observed (Fig. 4C). Upon silencing of RhoA, a slight increase in the number of infected cells was visible, which might be related to an easier access of virus to inner cells of cell islets (Fig. 4B and C). To test whether silencing of both Rac1 and Cdc42 had an effect on the efficiency of infection, we performed Western blot analyses. Interestingly, similar levels of ICP0 expression were observed when only Rac1 or Cdc42 and both Rho GTPases were silenced (data not shown).

When we extended our studies to primary human keratinocytes, we observed an efficient reduction to 9% with Rac1 and to 3% with Cdc42 siRNAs at 72 h posttransfection (Fig. 5B). Visualization of infected cells by ICP0 staining revealed that

the knockdown of either Rac1 or Cdc42 did not reduce the number of infected cells (Fig. 5A and C). Furthermore, silencing of Rac1 and Cdc42 seemed to have no influence on the preferred infection of rim cells when cell islets of the same size were compared to samples transfected with nonspecific siRNAs (Fig. 5A). These observations suggest that reduced Rac1 and Cdc42 did not lead to changes in cell-cell contacts that may enhance virus accessibility to cells in the inner part of cell islets.

In summary, our results indicate that reduced Rac1, Cdc42, and RhoA expression did not interfere with infectivity, suggesting that initiation of HSV-1 infection is independent of both Rac1- and Cdc42-mediated signaling pathways in human keratinocytes. To exclude that further steps in the infection cycle were affected by Rac1 and Cdc42 silencing, we determined the virus titer at 36 h p.i. The titers of both cell-released and cell-associated virus were comparable in cells transfected either with nonspecific siRNAs or with Rac1 and Cdc42 siRNAs (Fig. 4D), further demonstrating that HSV-1 infection was not limited by reduced Rac1 and Cdc42.

HSV-1 infection of murine epidermis. To understand the putative role of Rac1 during initiation of HSV-1 infection in the intact epithelium, we performed infection studies of murine epidermis. Initially, we established a protocol for ex vivo infection studies of epidermal sheets. Skin from the backs of newborn mice was prepared, and the epidermis was separated from the dermis by dispase treatment. Subsequently, the epidermal sheets were allowed to float on virus-containing medium, thus giving HSV-1 access to the basal epidermal layer. Since the sheets were thin, the epidermis was submerged and only the cornified layer remained above the virus-containing medium. At 3 h p.i., infected cells were detected by ICP0 staining of tissue sections.

Histological staining of tissue sections visualized the back skin and isolated epidermis of newborn mice. The back skin can be roughly divided into the epidermis and the dermis with the developing hair follicles (Fig. 6A). Hair follicles resemble outgrowths of the epidermis and are still connected with the isolated epidermis after dispase treatment (Fig. 6B). The interfollicular epidermis between the hair follicles is composed of a basal layer of proliferative keratinocytes and suprabasal layers of differentiating progeny. The terminally differentiated cells form the cornified layer (Fig. 6A and B). Interestingly, infected cells were detected only in the basal layer of keratinocytes, as identified by positive staining with an antibody against keratin 14 (Fig. 6D). In addition to the basal interfollicular epidermis, outer root sheath cells of the developing hair follicles were infected (Fig. 6C and D). At 3 h p.i., we observed some disruption of the epidermal layers. Even though this could also be observed after floating on virus-free medium

anti-Rac1, mouse anti-Cdc42, or mouse anti-RhoA antibodies. Staining of PDI or VDAC was used as a loading control. (B) At 2 h p.i., cells were fixed and costained with TRITC-conjugated phalloidin (red) and mouse anti-ICP0 (green) visualized with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G. Single stainings and merged images of confocal projections are shown. (C) The number of infected cells upon transfection of either of the specific or nonspecific siRNAs was determined by counting about 1,000 cells in at least two independent experiments. Results are mean $\pm$ standard deviation values. (D) At 36 h p.i., the virus titer was determined in cells transfected with either of the specific or nonspecific siRNAs in two independent experiments. Open bars indicate the titer of CRV, and filled bars indicate the titer of cell-associated virus (CAV). Bar, 50 μ m.

FIG. 5. HSV-1 infection of primary human keratinocytes upon silencing of Rac1 or Cdc42. Primary human keratinocytes were transfected with Rac1-siRNA, Cdc42-siRNA, or nonspecific control siRNAs, followed by infection with HSV-1 (20 PFU/cell) at 72 h posttransfection. (A) At 2 h p.i., cells were fixed and costained with TRITC-conjugated phalloidin (red) and mouse anti-ICP0 (green) visualized with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G. Single stainings and merged images of confocal projections are shown. Bar, 80 μ m. (B) Total protein extracts were prepared, and proteins were resolved on SDS-PAGE gels (15%). Expression of Rac1 and Cdc42 was analyzed with mouse anti-Rac1 or mouse anti-Cdc42 antibody. (C) The number of infected cells upon transfection of either of the specific or nonspecific siRNAs was determined in at least two independent experiments.

(Fig. 6B), there might be additional virus-specific disruptions of the epidermis. In addition to the infection of epidermal sheets, we performed infection studies with samples of whole murine skin that were allowed to float on virus suspension. This, however, did not result in infection (data not shown). In summary, our results demonstrate efficient infection of basal keratinocytes of murine epidermis once the surface of the basal layer was exposed to virus-containing medium.

HSV-1 infection of epidermis from Rac1 conditional knock-out mice. Recently mice with a keratinocyte-restricted deletion of the *rac1* gene were generated (9). We used this model to

study the consequences of Rac1 deficiency in epidermal keratinocytes on the efficiency of HSV-1 infection. The mice were generated by intercrossing animals with floxed *rac1* alleles (9) with such expressing Cre recombinase under the control of the keratin 14 promoter. Mice with two floxed *rac1* alleles and the presence of the keratin 14-Cre transgene are referred to as Rac1 E-KO mice. Deletion of Rac1 from keratinocytes was shown by Western blot analysis of epidermal lysates from 3-day-old Rac1 E-KO mice (Fig. 7B). Comparison to control littermates heterozygous for the floxed *rac1* allele and expressing the Cre recombinase showed efficient deletion of Rac1 in

FIG. 6. HSV-1 infection of mouse epidermis. (A) H-E-stained skin section of 3-day-old mouse. (B) Epidermal sheets were separated from dermis by dispase II treatment, followed by incubation on medium or virus-containing medium. At 3 h p.i., infected and uninfected epidermal sections were fixed and H-E stained. (C) At 3 h p.i., epidermal sections were fixed and stained with mouse anti-ICP0 (green) visualized with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G. Merged image with phase contrast is shown. (D) At 3 h p.i., epidermal sections were fixed and costained with mouse anti-ICP0 (green) and rabbit anti-keratin 14 (red) visualized with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G and Alexa Fluor 555-conjugated anti-rabbit immunoglobulin G, respectively. Merged image of confocal projections is shown. The red dashed line (A) represents the basement membrane, and the white dashed line (D) represents the border of the cornified layer. Asterisks indicate the cornified layer, arrowheads point to developing hair follicles, arrows indicate infected cells in the outer root sheath, and the bracket denotes interfollicular epidermis. Bar, 50 μ m.

the Rac1 E-KO mouse epidermis in addition to similar levels of Cdc42 and RhoA (Fig. 7B). The complete phenotype of the Rac1 E-KO mice will be described elsewhere.

When epidermal sheets of newborn Rac1 E-KO mice were infected, we observed infection of basal keratinocytes of the

interfollicular epidermis and of cells of the outer root sheath of the developing hair follicles (Fig. 7D). The comparison to infected epidermis of control littermates revealed no significant difference, indicating that the absence of Rac1 had no effect on initiation of infection.

To further assess the spatial distribution of infected cells in the basal layer of the epidermis, we prepared epidermal whole mounts from the tail skin of adult mice. Epidermal whole mounts are preferentially prepared from tail skin, since mouse back skin contains a high density of hair follicles and thin interfollicular epidermis (7). Deletion of Rac1 in epidermal lysates of tail skin from 1-year-old Rac1 E-KO mice was demonstrated by Western blot analysis (data not shown). The epidermal sheets were separated from the dermis by dispase treatment, followed by infection. Since the surface of the basal keratinocytes with the overhanging hair follicles was visualized, the distribution of the infected cells became visible (Fig. 8A). In wild-type mice, the dispase treatment partially disrupted the hair follicles at the level of the sebaceous glands (Fig. 8B). Since hair development in Rac1 E-KO mice was severely impaired, only defective hair follicles with no sebaceous glands were visible (Fig. 8C). Costaining of keratin 14 and ICP0 revealed infection throughout the basal layer of keratinocytes from wild-type and Rac1 E-KO mice without any preferred region (Fig. 8B and C). In addition to the interfollicular epidermis, infection was found in the outer root sheath, the hair bulb, and undifferentiated keratinocytes at the periphery of sebaceous glands (Fig. 8B). Upon infection, ICP0 is initially visible in the nucleus followed by relocalization to the cytoplasm in the course of infection. From the presence of ICP0 in the cytoplasm, we conclude that basal keratinocytes of murine epidermis were very efficiently infected in both Rac1-deficient and wild-type epidermis (Fig. 8D).

DISCUSSION

Cytoskeletal modifications are thought to require the activation of Rho GTPases. Bacterial pathogens can modulate or mimic Rho GTPase signaling, leading to modifications of the actin cytoskeleton that help the bacteria to invade a host cell and/or gain motility in the cell (4). There is first evidence that viruses also take advantage of Rac1/Cdc42 signaling; the most prominent example is vaccinia virus (16). During herpesvirus infection, activation of Rho GTPases is often observed; however, the functional significance is not yet fully understood. We addressed the impact of Rac1 and Cdc42 during initiation of HSV-1 infection in keratinocytes and performed overexpression and RNA interference studies. In addition, we used a mouse model to study the effects in the epidermis, which serves as an entry portal for HSV-1 *in vivo*. Our findings provide the first evidence that Rac1/Cdc42 signaling pathways are not required for efficient initiation of HSV-1 infection in keratinocytes.

Initially we determined the endogenous levels of GTP-bound Rac1 and Cdc42 and found temporary changes in Rac1/Cdc42 activities upon infection. The most obvious explanation

is that viral receptors such as nectin-1 mediate these activity changes in response to HSV-1 binding and/or internalization.

Overexpression studies of HaCaT cells revealed inhibitory effects of constitutively active Rac1 and Cdc42 on HSV-1 infectivity, in line with our studies of MDCKII cells (22). Since the inhibitory effects were measured by ICP0 expression, multiple steps during the entry process could be effected. In MDCKII cells, virus internalization is not blocked by active Rac1 and Cdc42, and capsids seem to accumulate at the nucleus, suggesting that further steps of early infection are inhibited (22). In addition, we found no evidence for the involvement of Pak1, a key downstream effector of Rac1 and Cdc42 conferring regulation of actin dynamics (22). We assume that in keratinocytes as well, virus internalization is not blocked by active Rac1 or Cdc42 but transport and/or delivery of capsids is inhibited. This assumption is supported by the observations that microtubules can also be targets of Rho GTPase regulation, suggesting that Rac1/Cdc42 mutant expression interferes with microtubule-based transport of the capsids (32, 41). Since activated Rac1 and Cdc42 have been shown to inhibit the clathrin-dependent endocytosis of E-cadherin (24), one might envision a block of the endocytic pathway that is taken by HSV-1 in keratinocytes. Thus, the characterization of the viral entry pathway will be of major interest. Furthermore, we cannot rule out a block of viral transcription by overexpression of active Rac1 or Cdc42. One possible explanation is the induction of an antiviral response by Rac1 via interferon regulatory factor 3 (IRF3) (12). Our initial results with MDCKII cells, however, suggest that IRF3 is not activated after overexpression of active Rac1 (data not shown).

Overexpression of dominant-negative Rac1 did not interfere with infectivity in HaCaT cells, while dominant-negative Cdc42 showed inhibitory effects. Based on studies of MDCKII cells, it remained unclear whether overexpression of inactive Cdc42 indeed leads to a dominant-negative effect, since overexpression of N-WASP, an effector of Cdc42, had no inhibitory effect (22). We therefore performed silencing experiments to address the impact of Rac1/Cdc42 signaling. Interestingly, we found that reduced expression of both Rac1 and Cdc42 had no effect on the efficiency of HSV-1 infection in human keratinocytes. To exclude that this effect was restricted to keratinocytes, we also tested MDCKII cells. Upon reduction of Rac1, the number of infected cells was comparable (data not shown). Reduced Cdc42 expression interfered with the formation of cell-cell contacts and probably allowed even higher efficiency of infection, since nearly all MDCKII cells were accessible for the virus (data not shown). Apparently, reduced Rac1 and Cdc42 activities had no effect on initiation of HSV-1 infection in both MDCKII cells and keratinocytes. Minor changes in virus egress, however, cannot be excluded, although we observed no

FIG. 7. HSV-1 infection of epidermis from Rac1 E-KO mice. (A) Newborn wild-type control and Rac1 E-KO mice are shown. (B) Epidermal lysates from wild-type control and Rac1 E-KO mice were resolved on SDS-PAGE gels (15%) and stained with mouse anti-Rac1, mouse anti-Cdc42, or mouse anti-RhoA antibody. (C) Epidermal sheets of wild-type control and Rac1 E-KO mice were infected for 3 h. Epidermal sections were fixed and H-E stained. (D) Infected epidermal sections were costained for keratin 14 (red) and ICP0 (green). Confocal projections and merged images are shown. The arrow points to infected cells in the outer root sheath. Bar, 50 μ m.

FIG. 8. Localization of HSV-1-infected cells in epidermis from Rac1 E-KO mice. (A) Scheme illustrating the epidermal whole mounts with the visible surface of the basal keratinocyte layer. The star indicates the hair bulb, and the arrow points to the outer root sheath. (B and C) Epidermal sheets prepared from tail skin of adult wild-type control and Rac1 E-KO mice were costained for keratin 14 (red) and ICP0 (green). Confocal projections and merged images are shown. The arrowhead points to sebaceous glands, and the arrow indicates infected cells of the outer root sheath. Boxed areas of ICP0-stained cells are magnified. Bar, 80 μ m. (D) Single stainings of confocal projections are shown. Boxed areas are shown as magnified merged images of a confocal layer with 4',6'-diamidino-2-phenylindole (DAPI)-stained nuclei. One nucleus is circled for each merged image. Bar, 6.6 μ m.

significant drop in virus production in HaCaT cells with reduced Rac1 or Cdc42.

The recent development of Rac1 conditional knockout mice has provided a new tool for studying Rac1 function in vivo (20), in particular since a general Rac1 knockout causes lethality early in embryogenesis (37). Rac1 has been shown to be highly expressed in basal undifferentiated keratinocytes in both human and mouse epidermis (3). Thus, we used a mouse model with a keratinocyte-restricted deletion of the *rac1* gene to address the functional significance of Rac1 activities for infection of the epidermis. Initially we established an ex vivo infection assay. After preparation of epidermal sheets followed by infection, we observed ICP0 expression in basal keratinocytes at 3 h p.i. Visualization of ICP0 in tissue sections and epidermal whole mounts indicated no difference in the efficiency of infection when Rac1 was absent. We therefore conclude that HSV-1 infection is initiated in the basal layer of the epidermis independently of Rac1 signaling pathways. So far, changes in late phases of infection, such as cell-to-cell spreading, cannot be ruled out. It is worth mentioning that the characterization of skin from Rac1 E-KO mice showed no obvious defects in differentiation, proliferation, cell-cell contacts, and basement membrane deposition in Rac1-deficient epidermis. Instead, there is a defect in hair follicle formation and maintenance (9). Since the interfollicular Rac1-deficient epidermis is maintained, it is less surprising that HSV-1 infection was not affected. Physiological stress, such as wound healing, however, may be a situation that results in altered HSV-1 infection, since epidermal wound healing is impaired in the absence of Rac1 (39).

Our finding that Rac1/Cdc42 signaling is not required for initiation of HSV-1 infection in keratinocytes makes it unlikely that virus-induced activation of Rac1 and Cdc42 during the entry process has functional significance, at least in keratinocytes. Based on the inhibitory effects of the overexpressed active Rac1 and Cdc42 mutants, we hypothesize that efficient infection requires downregulation of virus-induced Rac1/Cdc42 activation. This, in turn, may reflect the need for dynamic changes in the host cell actin cytoskeleton during viral infection.

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