Early herpes simplex virus type 1 infection is dependent on regulated Rac1/Cdc42 signalling in epithelial MDCKII cells

Sven Hoppe,1†† Mario Schelhaas,1†§ Verena Jaeger,1¶ Timo Liebig,1‖ Philipp Petermann2 and Dagmar Knebel-Mörsdorf1,2

The aim of this study was to understand how molecular determinants of epithelial cells influence initial infection by herpes simplex virus type 1 (HSV-1). Upon infection of the epithelial MDCKII cell line, enhanced association of virus particles with cells forming actin protrusions was observed, suggesting a putative role of actin dynamics in HSV-1 infection. Thus, the impact of the small Rho-like GTPases Rac1, Cdc42 and RhoA acting as key regulators of actin dynamics was addressed. Endogenous Rac1 and Cdc42 were temporarily activated at 15 and 30 min after HSV-1 infection. When constitutively active Cdc42 or Rac1 mutants were expressed transiently, a significant decrease in infectivity was observed, whereas expression of RhoA mutants had no influence. Furthermore, dominant-negative Cdc42 led to decreased infectivity, whereas dominant-negative Rac1 had no effect. So far, the study of potential effectors indicated that Rac1/Cdc42 mutants inhibited infectivity independently of p21-activated kinase (Pak1). The inhibitory effect of Rac1/Cdc42 mutant expression on HSV-1 infection was characterized further and it was found that binding, internalization and transport of HSV-1 were not affected by expression of Rac1/Cdc42 mutants. Thus, these results provide the first evidence for a role of Rac1/Cdc42 signalling during early HSV-1 infection and suggest a mechanism relying on virus-induced regulation of Rac1/Cdc42 activities.

INTRODUCTION

Herpes simplex virus (HSV) can cause a range of diseases, from mild, uncomplicated mucocutaneous infections to those that are life-threatening. The virus enters the host via mucosal epithelia, skin or cornea, followed by the establishment of local infection in epithelial cells. Upon primary infection, HSV gains access to the nervous system and becomes latent in neurons. The initial challenge for HSV is therefore to overcome the barrier function of skin or mucosa and enter the epidermis for productive infection.

HSV infection is initiated by attachment of viral envelope glycoproteins to cell-surface heparan sulphate proteoglycans (WuDunn & Spear, 1989; Herold et al., 1994). This initial contact facilitates subsequent binding to a co-receptor, which is required for virus internalization. Co-receptors known so far include a member of the tumour necrosis factor receptor family; others are related to members of the immunoglobulin superfamily, such as nectin-1 and nectin-2. A third class of co-receptors belongs to the protein family of sulphotransferases (Campadelli-Fiume et al., 2000; Spear et al., 2000; Spear, 2004). Recently, a new class of co-receptor or co-receptor has been added, which includes B5, a cell-surface membrane protein (Perez et al., 2005). HSV was initially thought to enter cells exclusively via fusion of the viral envelope with the plasma membrane. It has now been shown that HSV uptake can occur via direct penetration of the plasma membrane or via endocytic pathways, depending on the cell line (Nicola et al., 2003, 2005; Gianni et al., 2004; Nicola & Straus, 2004; Milne et al., 2005).

The knowledge of initial events during infection is based on infection studies in non-polarized cells. How HSV enters polarized epithelia, such as skin or mucosa, however, remains to be shown. Recently, we reported on the polar entry of HSV type 1 (HSV-1) into the epithelial MDCKII cell line, primary human keratinocytes and human foreskin.
epithelia. When viruses have access to basolateral membranes either in subconfluent cells or in wounded monolayer cultures, efficient entry is observed. In contrast, infected cells are rarely detectable in confluent cell monolayers, which support an HSV-1 entry mechanism via basolateral membranes (Schelhaas et al., 2003). These observations are in line with the general assumption that HSV-1 entry in vivo is facilitated at sites of lesions in skin or mucosa. Hence, our goal is to explore the characteristics of cells next to a wound that lead to preferential infection of HSV-1. Cellular characteristics such as migration, proliferation, cell adhesion and/or formation of cell–cell contacts may contribute to the initial steps of HSV-1 infection. The dynamics of cell motility rely on regulated recruitment of molecular scaffolds and are coupled to the coordinated organization of actin filaments (Small et al., 2002). Key regulators of actin dynamics are the small Rho-like GTPases RhoA, Rac1 and Cdc42 (Hall, 1998; Ridley, 2001). Rho GTPases function as molecular switches that cycle between an active, GTP-bound state and an inactive, GDP-bound state. They interact with a variety of downstream effectors, thereby controlling diverse biological effects such as actin dynamics, cell-cycle progression, cell adhesion and gene transcription (Bishop & Hall, 2000). The role of Rac1/Cdc42 signalling during HSV infection is still unknown. It has been reported that the US3 protein kinase of HSV may affect a signalling pathway involving Rac1/Cdc42 (Murata et al., 2000).

Upon HSV-1 infection of MDCKII cells, we observed activation of endogenous Rac1 and Cdc42. In order to address the impact of Rac1 and Cdc42 activities on the efficiency of HSV-1 infection, we expressed dominant-negative and constitutively active mutants of the GTPases prior to infection. Our results indicate that early HSV-1 infection is Rac1/Cdc42 signalling-dependent.

METHODS

Cells, viruses and plasmids. MDCKII cells (Hansson et al., 1986) were infected with HSV-1 wild-type strain 17 or with the HSV-1 recombinant VP26–green fluorescent protein (GFP), which were purified as described previously (Schelhaas et al., 2003). The VP26–GFP recombinant (Desai & Person, 1998) was reconstituted in HSV-1 strain 17 and was provided by Dr G. Elliott (Marie Curie Research Institute, Ox ted, UK).

For internalization studies, purified preparations of HSV-1/VP26–GFP (5 × 10⁶ particles) were incubated with 1 μg of either mAb DL11 (mouse anti-gD) (Muggeridge et al., 1988) or mAb 3-4 (mouse anti-gD) (Kühn et al., 1990) for 1 h at 37 °C prior to addition to cells. In control experiments, various amounts of mAb DL11 were incubated with virus particles to test the efficiency of blocking viral internalization (data not shown).

Expression vector pRK5 encoding myc-tagged wild-type (wt) Cdc42, wtRac1, wtRhoA, L61Cdc42, N17Cdc42, L61Rac1, N17Rac1, L63RhoA, N19RhoA, L61Rac1 37A, L61Rac1 40C or Pak1 L107F were obtained from Dr V. Braga (Imperial College London, UK) and Dr A. Hall (Sloan–Kettering Institute, New York, USA). Expression plasmid pCMV6, using the cytomegalovirus promoter to express the myc-tagged dominant-negative Pak1 K299R mutant, has been described by Sells et al. (1999). Prior to transient-expression assays, all cloned inserts were sequenced. Plasmid EGFP-C1 (Clontech) was used as a control.

Transient expression. For transfection, MDCKII cells were trypsinized, pelleted, washed with PBS and resuspended in Nucleofector solution T (Amaxa). Cells (4 × 10⁶) were transfected with 2 μg plasmid in a cuvette, utilizing program P29 of an Amaxa Nucleofector I. Cells were seeded on coverslips and infected at 6 h post-transfection at an m.o.i. of 50 p.f.u. per cell.

Immunocytochemistry and antibodies. Cells grown on coverslips, fixed in 2 % paraformaldehyde, permeabilized with 0-1 % Triton X-100 and stained as described previously (Schelhaas et al., 2003). At 2 h post-infection (p.i.), infected cells were visualized by ICP0 staining either with rabbit anti-ICP0 antiserum (r191) (Parkinson & Everett, 2000), diluted 1:500, or with mAb 11060 (mouse anti-ICP0) (Everett et al., 1993), diluted 1:2000. Capsid protein VP5 was visualized with mAb DM165 (McClelland et al., 2002), diluted 1:200. The myc-tagged RhoA, Rac1, Cdc42 and Pak1 mutant and wt forms were detected with mAb 9E10 (mouse anti-myc) (Santa Cruz), diluted 1:2000. Primary antibodies were visualized with fluoresochrome-conjugated anti-rabbit and anti-mouse IgG. Staining of F-actin was performed with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma). Specimens were mounted and viewed under a Zeiss Axiomert 135 and under a Leica DM RE microscope linked to a Leica SP/2 confocal unit as described previously (Schelhaas et al., 2003). Images were assembled by using Adobe Illustrator version 10 and Adobe Photoshop version 7.0.

The effects of Rac1, Cdc42 and RhoA mutant expression were quantified by counting about 200 transfected cells visualized with anti-myc antibodies in at least three independent experiments and calculating the number of infected cells visualized by ICP0 staining.

Rac1/Cdc42 activity assay. Cells (1 × 10⁶) seeded at low density were infected about 14 h after seeding. At various times p.i., cells were washed twice in ice-cold PBS. Activated Rac1 or Cdc42 was identified by binding specifically to the GST-fused p21-binding domain of human Pak1 by using an EZ-detect Cdc42 activation kit (Pierce). Cell lysates were treated as described by the manufacturer, except for an increased NaCl concentration of 500 mM in the lysis/binding/wash buffer (Pierce), to which a Proteinase Inhibitor Cocktail Tablet Complete (Roche) was added. The protein concentration of each lysate fraction was determined to confirm equal protein amounts per sample. Full blots were resolved by 15 % SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences) by blotting for 2 h at 40 V and 4 °C. Bound GTP–Rac1 or GTP–Cdc42 was detected with mAb 23A8 (mouse anti-Rac) or mAb 23D5 (mouse anti-Cdc42) (Sigma) at a dilution of 1:500 or anti-Cdc42 antibodies (EZ-detect; Pierce) at a dilution of 1:250, followed by enhanced chemiluminescence (ECL Plus; Amersham Biosciences).

Flow-cytometric analysis for virus internalization. To remove virions attached to the cell surface, infected cells were treated with protease K. At 1 or 2 h p.i., cells were washed three times with ice-cold PBS and incubated with 0-5 mg protease K ml⁻¹ (catalogue no. P6556; Sigma) diluted in PBS for 45 min at 4 °C. Digestion was blocked with 3 % BSA in PBS, followed by three washing steps. Prior to infection with HSV-1/VP26–GFP (50 p.f.u. per cell), 4 × 10⁶ cells were transfected with 2 μg myc-tagged plasmids L61Rac1, N17Rac1 or N17Cdc42. At 6 h post-transfection, cells were infected and, at 2 h p.i., cells were incubated with protease K followed by fixation in 2 % paraformaldehyde, and permeabilized with 0-1 % saponin (Sigma) in 20 mM EDTA, 0-02 % NaN₃, 2 % fetal calf serum (FCS) (FACS permeabilization buffer). Subsequently, cells were incubated overnight at 4 °C with mAb 9E10.
(mouse anti-myc) (Santa Cruz), diluted 1:500 in FACS permeabilization buffer. The primary antibody was visualized by incubation with Alexa Fluor 660-conjugated anti-mouse IgG (Molecular Probes), diluted 1:500, for 2 h at room temperature. Cells were washed twice with FACS permeabilization buffer and once in FACS buffer (i.e. FACS permeabilization buffer without saponin) and analysed by using a FACS Calibur (Becton Dickinson). Fixation and size of cells led to high autofluorescence. Thus, we determined the gate upon analysis of mock-infected cells (Fig. 3). When cells were transfected and infected, we gated for transfected cells and set a further gate for transfected plus infected cells (Fig. 3c).

RESULTS

Experimental design

Infection studies were performed with highly purified stocks of HSV-1. The emphasis was on the initial steps of infection that were investigated in individual MDCKII cells. We chose MDCKII cells because these cells have been a long-standing model for epithelial polarity and the effects thereof. HSV-1 infection was visualized by staining infected cells with an antibody directed against the HSV-1 immediate-early protein ICP0. As early as 2 h p.i., ICP0 is detectable in the cell nucleus, indicating the successful delivery of the viral genome and initiation of viral gene expression (Schelhaas et al., 2003).

Preferred HSV-1 entry sites in subconfluent MDCKII cells

In subconfluent MDCKII cells, infection is detectable in peripheral cells of cell islets (Schelhaas et al., 2003). To characterize further the preferred entry sites, cells were infected with an HSV-1 recombinant that expressed a VP26–GFP fusion protein. VP26 is a capsid protein and decorates the outer surface of the capsid shell (Zhou et al., 1995). When subconfluent cells were analysed at 30 and 60 min p.i., VP26–GFP was observed preferentially in association with cells protruding their plasma membrane and forming lamellipodia to contact neighbouring cells (Fig. 1a, c). Quantification revealed a threefold increase in virus particles that were associated with protrusion-forming cells, compared with association with other peripheral cells characterized by a strong cortical actin bundle (Fig. 1b). Thus, we assume that lamellipodium-forming cells represent preferred targets for HSV-1 infection in MDCKII cells.

Influence of HSV-1 infection on Rac1 and Cdc42 activation

In general, Cdc42 and Rac1 stimulate the formation of protrusions at the leading edge of migrating cells and induce filopodia and membrane ruffles, respectively, whereas RhoA activation leads to the formation of stress fibres (Hall, 1998). In order to address whether HSV-1 entry influences Rac1/Cdc42 signalling, we investigated the level of activated Rac1 and Cdc42 upon infection of MDCKII cells by pull-down assays (Benard et al., 1999). It is worth mentioning that the level of activated Rac1 differs in subconfluent cultures versus

Fig. 1. HSV-1 entry into subconfluent MDCKII cells. Cells were infected with HSV-1/VP26–GFP (50 p.f.u. per cell) and fixed at 30 min (a) or 60 min (c) p.i. F-actin was visualized with TRITC-phalloidin (red). (b) Virus particles were counted in about 200 cells at 30 min p.i. Big dots (virus aggregates) were counted as one. Results are mean ± SD values from two independent experiments and P values are given above the diagram. Cells of cell islets were distinguished as lamellipodium-forming cells, other peripheral cells and central cells. A peripheral cell (left) and a lamellipodium-forming cell (right) are highlighted (a). Bars, 10 μm.
confluent cultures (Noren et al., 2001). Thus, we performed control experiments that demonstrated decreased Rac1 activity in subconfluent MDCKII cells compared with confluent cultures, confirming recent results (Noren et al., 2001) (data not shown).

HSV-1 infection studies were performed in cells at low density, forming nearly no cell islets to achieve a high infection rate. Upon HSV-1 infection, endogenous Rac1 activity increased at 15–30 min p.i., followed by a decrease to the initial activity level at 60 min p.i. (Fig. 2a). The same pattern of temporary activation was observed for Cdc42 (Fig. 2a). Quantification of four independent experiments showed at least a fourfold increase at 30 min p.i. compared with uninfected cells (Fig. 2c). In addition, Rac1 activity increased again at 120 min p.i., although the level of increase varied (Fig. 2a, c). As soon as we observed a significant increase of Rac1 activity at 120 min, Cdc42 activity also increased (Fig. 2c). These observations provide evidence for a mechanism of early HSV-1 infection that involves a temporary activation of endogenous Rac1 and Cdc42.

Impact of transient expression of Cdc42 and Rac1 mutants on HSV-1 infection

In order to address the impact of virus-induced temporary activation of Rac1 and Cdc42 on successful HSV-1 infection, we expressed Rac1 and Cdc42 mutants prior to infection. MDCKII cells were transfected with myc-tagged versions of constitutively active or dominant-negative Rac1 and Cdc42 mutants, followed by infection with HSV-1, which was visualized by ICP0 staining at 2 h p.i. A transient-expression system based on electroporation (Amaxa) was set up, which allowed us to study the effects of overexpressed GTPase mutants that potentially interfere with cell viability upon prolonged expression. The aim was to achieve high transfection efficiency with minimal time of gene expression. At 8 h post-transfection, cell survival was not affected.

When GFP was expressed as control, at least 80% of the transfected cells were infected (Fig. 3). In contrast, Cdc42 and Rac1 mutants affected the efficiency of HSV-1 infection significantly (Fig. 3a, b). Interestingly, both the constitutively active L61Cdc42 and L61Rac1 mutants reduced the number of infected cells to 22%, indicating a significant drop in HSV-1 infectivity (Fig. 3b). In contrast, inactive Rac1 had no influence on the number of HSV-1-infected cells, whilst inactive Cdc42 expression led to inhibitory effects (Fig. 3a). When the wt versions of Cdc42 and Rac1 were overexpressed, no significant loss of infectivity was observed (Fig. 3c). As a control, we performed transfection experiments with RhoA mutants and wt RhoA. Upon overexpression of constitutively active, dominant-negative or wt RhoA, only minor effects on infectivity were observed (Fig. 3). In summary, our results indicate that perturbations of Rac1 and Cdc42 signalling can interfere with HSV-1 infectivity of MDCKII cells.

In order to demonstrate the specificity of the Rac1 and Cdc42 mutants upon transient expression in MDCKII cells, we visualized the actin cytoskeleton. Whilst GFP expression did not influence the actin cytoskeleton, we observed characteristic changes of the F-actin organization upon overexpression of wt GTPases, such as long filopodia and short stress fibres in wt Cdc42-expressing cells, and membrane protrusions, short filopodia and actin accumulation along cell–cell contacts in wt Rac1-expressing cells (data not shown). Upon overexpression of the dominant-active mutants, enhanced effects on the formation of characteristic F-actin structures were detectable (Fig. 4c.
Fig. 3. HSV-1 infection of MDCKII cells transfected with wild-type or mutant forms of Rac1, Cdc42 and RhoA. Cells were transfected with either constitutively active Rac1 (L61Rac1) or Cdc42 (L61Cdc42), dominant-negative Rac1 (N17Rac1) or Cdc42 (N17Cdc42) or wt forms followed by HSV-1 infection (50 p.f.u. per cell) at 6 h post-transfection. At 2 h p.i., cells were fixed and co-stained with rabbit anti-ICP0 antibodies (red) and mouse anti-myc (green), visualized with Cy3-conjugated anti-rabbit IgG (Jackson) and Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes), respectively. The number of ICP0-expressing cells per mutant- or wt-expressing cell was determined in at least three independent experiments. As a control, the percentage of ICP0-expressing cells that were transfected with a GFP-expressing plasmid is shown (a–c). The percentage of infected cells that were transfected with the indicated plasmids is given. Results are mean ± SD values. Merges of confocal projections demonstrate expression of ICP0 and of dominant-negative mutants (a), constitutively active mutants (b) or wt forms (c). Bar, 10 μm.
d), demonstrating the dominant effect on the actin cytoskeleton. When inactive N17Rac1 was expressed, cells rounded up and showed no characteristic F-actin structures (Fig. 4b), which confirmed a dominant-negative effect of the mutant. In contrast, N17Cdc42 expression led to the formation of filopodia and strong actin fibres similar to the phenotype of L61Cdc42-expressing cells (Fig. 4a), which did not necessarily reflect the expected phenotype of dominant-negative Cdc42. Taken together, the visualization of the actin cytoskeleton confirmed the specific effects of the overexpressed mutants, except for N17Cdc42, which showed a phenotype similar to that of the constitutively active Cdc42.

**Influence of potential effectors of Rac1/Cdc42 signal-transduction pathways**

To gain insights into the Rac1/Cdc42 mutant-induced responses that trigger the interference with HSV-1 infectivity, we performed transfection studies with constitutively active L61Rac1 containing additional effector-site amino acid substitutions. Rac1 and Cdc42 with a Y40C effector-site substitution no longer interact with a variety of CRIB (Cdc42–Rac interactive binding) motif-containing target proteins and are unable to activate the JNK MAP kinase pathway. However, they can still induce changes of the actin cytoskeleton. In contrast, a further mutant containing an F37A effector-site substitution is unable to induce lamellipodia and filopodia and still interacts with CRIB-containing proteins (Lamarche et al., 1996). When we transfected the constitutively active L61Rac1 37A mutant into MDCKII cells, we observed rounded cells with no specific actin structures (Fig. 5b). Upon expression of the L61Rac1-40C mutant, we detected actin accumulation at cell–cell contacts and filopodium formation (Fig. 5b). These results demonstrate effects in MDCKII cells similar to those described previously for fibroblasts (Lamarche et al., 1996). Interestingly, HSV-1 infection of mutant-expressing MDCKII cells indicated an inhibitory effect on infectivity when the L61Rac1 37A mutant was expressed, whilst expression of L61Rac1 40C only resulted in a minor reduction in the number of infected cells (Fig. 5a). Thus, we suggest that the actin cytoskeleton participates in the Rac1-induced inhibitory effect on HSV-1 infectivity and that activation of the JNK MAP kinase pathway by Rac1 does not play a significant role in infection. The inhibitory effect of the L61Rac1 37A mutant still interacting with CRIB-containing proteins implies that binding to effectors with a CRIB motif may play a role for inhibition. Among the best-established Rac effectors are the CRIB-containing p21-activated kinases (Paks). Paks are highly conserved serine/threonine kinases that are activated by GTP-bound forms of Rac1 and Cdc42 and are implicated in many Rac-mediated responses, including cell migration (Kiosses et al., 1999; Sells et al., 1999). The interaction of Pak with Rac1/Cdc42 is blocked by the Y40C effector-site substitution, but not by the F37A mutation (Lamarche et al., 1996). As the F37A mutation led to inhibition of HSV-1 infectivity, Pak might be a potential effector. We tested this hypothesis by transiently expressing constitutively active and dominant-negative Pak1 mutants prior to infection. Neither of the mutants, however, showed an effect on the number of infected cells (Fig. 6). In addition, staining of the actin cytoskeleton revealed no significant changes upon mutant expression (data not shown). Thus, the inhibitory effects of Rac1/Cdc42 mutant expression on HSV-1 infectivity seem to be independent of Pak1 activation.

**Fig. 4.** F-actin changes upon transient expression of Rac1 or Cdc42 mutants. (a–d) MDCKII cells were transfected with either Rac1 or Cdc42 mutant, fixed at 6 h post-transfection and stained with TRITC-conjugated phalloidin (red). Transfected cells were stained with mouse anti-myc (green) visualized with Alexa Fluor 488-conjugated anti-mouse IgG (a–d). Single stainings and merges of confocal projections are presented. Bar, 10 μm.
Influence of Rac1/Cdc42 mutants on internalization of HSV-1 particles

To explore whether the inhibitory effects of Rac1/Cdc42 mutant expression on HSV-1 infection affected the internalization of viral particles, we performed further infection studies with the recombinant HSV-1/VP26–GFP. MDCKII cells were transfected with each of the mutants L61Rac1, N17Rac1 or N17Cdc42, followed by infection, and virions internalized into transfected cells were determined by flow-cytometric analyses at 2 h p.i. To quantify only internalized viruses, we removed particles attached to the cell surface by incubation with proteinase K. The efficiency of this treatment was shown after infection with HSV-1/VP26–GFP for 1 h at 4°C. Whilst washing steps were inefficient, proteinase K treatment resulted in complete removal of attached virions (Fig. 7a). As a control, we blocked virus internalization by incubation of HSV-1/VP26–GFP with the neutralizing mAb DL11 (anti-gD), which has been suggested to block both receptor binding and HSV-1 infection (Whitbeck et al., 1999). In addition, we treated another batch of HSV-1/VP26–GFP with mAb 3-4, a non-neutralizing antibody. At 1 h p.i. with DL11-treated virions, we observed 14% of cells with internalized viruses, whilst infection with the non-neutralizing mAb 3-4-treated virions led to 39% of cells with internalized viruses, confirming the inhibitory effect of mAb DL11 (Fig. 7b).

Next, internalization of virus particles was determined upon transient expression of the Rac1/Cdc42 mutants. Although L61Rac1 or N17Cdc42 expression led to an inhibitory effect on infection compared with N17Rac1 expression (Fig. 3a, b), we observed no difference in internalization. Expression

![Fig. 5. HSV-1 infection upon overexpression of Rac1 effector-site mutants. (a) Cells were transfected with constitutively active effector-site mutants L61Rac1 40C or L61Rac1 37A and, for comparison, with L61Rac1 and N17Rac1, followed by HSV-1 infection (50 p.f.u. per cell) at 6 h post-transfection. At 2 h p.i., cells were fixed and co-stained with rabbit anti-ICP0 and mouse anti-myc antibodies. The number of ICP0-expressing cells per mutant-expressing cell was determined in three independent experiments. As a control, the percentage of ICP0-expressing cells that were transfected with a GFP-expressing plasmid is shown. Results are mean ± SD values. (b) Transfected cells were fixed at 6 h post-transfection and stained with TRITC-conjugated phalloidin (red) and mouse anti-myc (green) visualized with Alexa Fluor 488-conjugated anti-mouse IgG. Single stainings and merges of confocal projections are shown. Bar, 10 μm.](image)

![Fig. 6. HSV-1 infection upon overexpression of Pak1 mutants. Cells were transfected with either constitutively active Pak1 (L107F PAK) or dominant-negative Pak1 (K299R PAK) and, for comparison, with L61Rac1 and N17Rac1, followed by HSV-1 infection (50 p.f.u. per cell) at 6 h post-transfection. At 2 h p.i., cells were fixed and co-stained with rabbit anti-ICP0 and mouse anti-myc antibodies. The number of ICP0-expressing cells per mutant-expressing cell was determined in two independent experiments. As a control, the percentage of ICP0-expressing cells that were transfected with a GFP-expressing plasmid is shown. Results are mean ± SD values.](image)
Fig. 7. FACS analyses of HSV-1 particles upon transient expression of Rac1 and Cdc42 mutants. (a) MDCKII cells were incubated with HSV-1/VP26–GFP (100 p.f.u. per cell) for 1 h at 4 °C. Cells were treated with proteinase K for 45 min at 4 °C or washed with PBS followed by an incubation for 45 min at 4 °C. After fixation, VP26–GFP-positive cells were sorted from about 5000 cells. (b) HSV-1/VP26–GFP inocula were incubated with the neutralizing mAb DL11 or non-neutralizing mAb 3-4 for 1 h at 37 °C prior to infection of cells. At 1 h p.i., cells were treated with proteinase K followed by fixation and FACS analysis. (c) Cells were transfected with L61Rac1, N17Rac1 or N17Cdc42, followed by infection with HSV-1/VP26–GFP (50 p.f.u. per cell) at 6 h post-transfection. In addition, L61Rac1-transfected cells were infected with HSV-1/VP26–GFP treated with mAb DL11 prior to infection. At 2 h p.i., cells were treated with proteinase K, fixed and stained with mouse anti-myc (9E10) (Santa Cruz) visualized with Alexa Fluor 660-conjugated anti-mouse IgG (Molecular Probes). Filled curves represent cells transfected with L61Rac1, N17Rac1 or N17Cdc42 and infected with HSV-1/VP26–GFP; open curves represent cells transfected with L61Rac1 and infected with mAb DL11-treated HSV-1/VP26–GFP. Histograms show only transfected cells and the bar represents the gate for transfected plus infected cells (%). (d) Fold increase in internalization into cells expressing either of the mutants compared with internalization of mAb-treated virions was determined in three independent experiments. Results are mean ± SD values.
of L61Rac1, N17Cdc42 and N17Rac1 led to 38, 39 and 44% of cells with internalized virions, respectively (Fig. 7c). Only when L61Rac1-expressing cells were infected with mAb DL11-treated virions did we find a reduced number of cells with internalized viruses (Fig. 7c). Quantification revealed a four- to fivefold increase in internalization of HSV-1/VP26–GFP into cells expressing L61Rac1, N17Rac1 or N17Cdc42 compared with internalization upon treatment with the neutralizing mAb DL11 (Fig. 7d). In summary, our observations indicate that the inhibitory effects of Rac1/Cdc42 mutant expression did not result from a block of virus internalization.

**Impact of the inhibitory effect of the Rac1 mutant on HSV-1 infection**

To investigate further the inhibitory effect of Rac1/Cdc42 mutant expression, we wanted to exclude an inhibitory effect specific for the ICP0 promoter. Thus, we determined expression of another viral gene encoding the major capsid protein VP5 in cells expressing Rac1 mutants. VP5 is a leaky-late (βl) gene that is transcribed prior to viral DNA replication. Subconfluent MDCKII cells infected at a high m.o.i. showed capsid staining in peripheral cells of cell islets at 1 and 2 h p.i., whereas VP5-expressing cells were only rarely detectable at these time points. At 3 and 4 h p.i., cells with nuclear VP5 staining increased (Fig. 8a). In comparison to ICP0, detection of VP5-expressing cells was delayed (Fig. 8a). Thus, the effects of either constitutively active L61Rac1 or dominant-negative N17Rac1 mutants were determined at 4 h p.i. ICP0 and VP5 expressions were observed in N17Rac1-expressing cells, whilst L61Rac1 expression resulted in neither ICP0 nor VP5 expression (Fig. 8b, c). The block of ICP0 and VP5 expression suggests that at least Rac1 mutant expression leads to a general inhibition of viral gene expression. Interestingly, L61Rac1-expressing cells showed no VP5 expression, but staining of viral capsids, which seemed to accumulate near the nucleus (Fig. 8c). These results are a first hint that HSV-1 particles cannot only cross the plasma membrane, but are also transported to the nuclear periphery when Rac1/Cdc42 signalling is perturbed.

**DISCUSSION**

Our results indicate that endogenous Rac1 and Cdc42 activities were activated upon HSV-1 infection of MDCKII cells. The increase of Rac1 and Cdc42 was observed at 15 and 30 min p.i., followed by a decrease at 1 h and a further increase at 2 h p.i. At 1 h p.i., gene expression of ICP0 was rarely detectable, suggesting that the time of temporary Rac1 and Cdc42 activation correlated with viral capsids on their way to the nucleus. The initial mediator of transient Rac1 and Cdc42 activation may be viral receptors in response to HSV-1 binding and/or internalization. Transfected human nectin-1 has been shown to act as HSV-1 receptor in MDCK cells (Yoon & Spear, 2002). Furthermore, trans-interactions of human nectins in nectin-1-MDCK cells induce the formation of filopodia and lamellipodia, which is based on activation of Cdc42 and Rac1 (Kawakatsu et al., 2002; Fukuhabara et al., 2004). Thus, it is conceivable that endogenous dog nectin, as a potential virus entry receptor in MDCKII cells, mediates transient Rac1 and Cdc42 activation upon HSV-1 entry. The further increase of Rac1/Cdc42 at 2 h p.i. may be related to expression of structural genes.

To address the impact of endogenous Rac1/Cdc42 activation, we expressed Rho GTPase mutants transiently to interfere with Rac1/Cdc42 signalling prior to infection. HSV-1 infectivity of MDCKII cells, as measured by ICP0 expression, decreased upon overexpression of Rac1 or Cdc42 mutants, whereas RhoA expression had no significant effect. These results indicate that Rac1/Cdc42 signalling plays a role during early HSV-1 infection. Interestingly, active Rac1 inhibited HSV-1 infection. Inactive Rac1 did not interfere with infectivity, implying that competition of inactive Rac1 for binding to cellular guanosine nucleotide-exchange factors plays no significant role during early infection. In contrast, inactive Cdc42 reduced infectivity as well as active Cdc42, suggesting that cycling between active and inactive forms is essential for successful infection. As the actin changes, however, are similar upon expression of both Cdc42 mutants, it remains unclear whether expression of inactive Cdc42 indeed led to a dominant-negative effect. Alternatively to N17Cdc42, a dominant-negative effect on Cdc42 signalling can be obtained by overexpression of the CRIB domain of the Wiskott–Aldrich syndrome protein (N-WASP), an effector of Cdc42 (Machesky & Insall, 1998). When we expressed the N-WASP fragment prior to infection, HSV-1 infection was not influenced (data not shown). Changes of the actin cytoskeleton differed from those observed upon N17Cdc42 expression, indicating different effects on Cdc42 signalling, which in turn may explain the opposing results obtained with N17Cdc42 and N-WASP. Altogether, our results indicate that interference with Rac1/Cdc42 signalling leads to a loss of HSV-1 infectivity.

As it will be of major interest to determine possible targets of Rac1 and Cdc42 that trigger the inhibitory effect on infectivity, we initially tested Pak1 as a putative effector molecule. Paks are key downstream effectors of Rac1 and Cdc42, conferring regulation of actin dynamics (Bokoch, 2003). As expression of constitutively active or dominant-negative Pak1 mutants had no influence on HSV-1 infection, we assume that the inhibitory effect of active Rac1/Cdc42 does not involve activation of Pak1. Participation of further Pak family members, however, cannot be excluded.

We initiated studies to determine the early step in HSV-1 infection that was blocked by disturbed Rac1/Cdc42 signalling. Our results indicate that the inhibitory effect of Rac1/Cdc42 mutants, as measured by ICP0 expression, does not correlate with a block of virus internalization. As viruses can still cross the plasma membrane, Rac1/Cdc42 signalling...
may play a role during further steps of early infection. ICP0 as well as VP5 expression was inhibited, thus we assume a general block of viral gene expression by Rac1/Cdc42 mutants. This block may be due to inhibitory effects on viral gene transcription or lack of successful delivery of the viral genome into the nucleus. This may, in turn, be related to perturbed trafficking through the cytoplasm and positioning at the nuclear pore. Visualization of capsids in transfected cells without viral gene expression suggests no general block of transport through the cytoplasm, as capsids

**Fig. 8.** VP5 and ICP0 expression during HSV-1 infection and upon overexpression of Rac1 mutants. (a) MDCKII cells were infected with HSV-1 (50 p.f.u. per cell) and fixed at 1, 2, 3 and 4 h p.i. Co-staining was performed with rabbit anti-ICP0 antibodies (red) and mouse anti-VP5 (DM165) (green), visualized with Alexa Fluor 555-conjugated anti-rabbit IgG (Jackson) and Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes), respectively. (b) Cells were transfected with either N17Rac1 or L61Rac1 followed by infection with HSV-1 (50 p.f.u. per cell) at 6 h post-transfection. At 4 h p.i., cells were fixed and co-stained with rabbit anti-ICP0 antibodies (red) and mouse anti-myc (green) visualized with Alexa Fluor 555-conjugated anti-rabbit IgG (Jackson) and Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes), respectively (c), or with mouse anti-VP5 (green) visualized with Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) and TRITC-conjugated anti-myc (red). Confocal images are shown as single stainings and merges. Bar, 10 μm.
were found in the nuclear periphery. Thus, delivery of the capsids upon transport, positioning at the pore complex and/or genome import into the nucleus may be blocked. It is still open whether HSV-1 enters MDCKII cells by fusion of the plasma membrane or by endocytosis. If HSV-1 enters MDCKII cells via an endocytotic pathway, the inhibitory effect of Rac1/Cdc42 mutants may be related to escape from endosomes. Recently, it has been reported that HSV-1 enters epithelial cells, such as human keratinocytes, via an endocytic pathway (Nicola et al., 2005). As we cannot rule out a block of viral gene expression upon Rac1/Cdc42 mutant expression, one might envision a Rac1-induced antiviral response via the interferon-regulatory factor 3 (IRF3; Ehrhardt et al., 2004). Induction of interferon-stimulated genes through IRF3 can occur upon HSV-1 entry and is counteracted by ICP0 (Eidson et al., 2002).

Given the virus-induced activation of Rac1/Cdc42 at 15 and 30 min p.i., the inhibitory effect of constitutively active Rac1/Cdc42 strengthens the impact of the virus-induced temporary activation and decrease at 1 h p.i. In addition, these results suggest a virus-induced regulation of Rac1/Cdc42 activities, which may be essential for successful infection. Our observation of enhanced association of virions with cell protrusions further suggests that HSV-1 prefers entry sites of highly dynamic F-actin reorganization and turnover. As Rac1/Cdc42 mutants, however, inhibited an infection step after internalization and cytoplasmic transport, the putative participation of dynamic F-actin during internalization remains open. So far, our results provide the first evidence that early HSV-1 infection relies on regulated Rac1/Cdc42 signalling. It will be of great interest to unravel further the Rac1/Cdc42-induced changes that play a role during HSV-1 infection.

ACKNOWLEDGEMENTS

We are grateful to Gill Elliott for kindly providing HSV-1 recombinant VP26–GFP. We thank Vania Braga, Alan Hall and Stephan Ludwig for plasmids, Claude Krummenacher, Roselyn Eisenberg and Gary Cohen for the anti-gD mAb DL11, Joachim Kühn for anti-gD mAb 3-4, Roger Everett for antibodies against ICPO and Frazer Rixon for antibodies against HSV capsid. Amaza is acknowledged for supporting the transfection experiments and Bertram Huth for help with the figures. Additional thanks go to Vania Braga and Carien Niessen for advice, many discussions and helpful comments. Finally, we acknowledge Christoph Göttlinger and Ari Waisman for their help with the flow-cytometric analyses.

REFERENCES


Viana Braga and Carien Niessen for advice, many discussions and helpful comments. Finally, we acknowledge Christoph Göttlinger and Ari Waisman for their help with the flow-cytometric analyses.


