Poliovirus entry into human brain microvascular cells requires receptor-induced activation of SHP-2

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Viruses use specific receptor molecules to bind selectively to target cells. Receptors have often been considered as mere docking sites, but they may also possess intrinsic signaling capacities that serve to prime the cell for entry and infection. Poliovirus (PV) initiates infection by binding to the PV receptor (PVR) and causes paralytic poliomyelitis by replicating within motor neurons of the brain and spinal cord. We have examined the process by which PV enters cultured human brain microvascular endothelial cells (HBMEC), an *in vitro* model of the blood–brain barrier. We found that PV enters HBMEC by dynamin-dependent caveolar endocytosis, and that entry depends on intracellular signals triggered by virus attachment to PVR. Tyrosine kinase and RhoA GTPase activation initiated by PVR ligation were both essential. Virus attachment also induced tyrosine phosphorylation of PVR; this permitted the association of PVR with SHP-2, a protein tyrosine phosphatase whose activation was required for entry and infection. The results indicate that receptor-induced signals promote virus entry and suggest a role for tyrosine phosphatases in viral pathogenesis.

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Introduction

The blood–brain barrier (BBB) prevents the free passage of leukocytes, toxins and microbial pathogens from the bloodstream into the central nervous system (CNS). Poliovirus (PV), a single-stranded RNA virus belonging to the Picornaviridae family, causes paralytic poliomyelitis by entering the CNS and replicating within motor neurons of the brain and spinal cord (Pallansch and Roos, 2001). PV infection begins within the mucosa of the oropharynx and intestine; virus then enters the bloodstream and spreads to

secondary sites of replication. Virus has been proposed to reach the CNS both by moving (in a retrograde manner) through peripheral neurons to the spinal cord, and by passing from the bloodstream through the BBB (Yang *et al*, 1997; Pallansch and Roos, 2001; Racaniello, 2006).

Despite many years of PV research (reviewed in Racaniello, 2006), the mechanism by which PV gains entry into host cells has not been defined. PV interaction with target cells depends on attachment to the PV receptor, PVR (also known as CD155 or Necl-5), an adhesion molecule that participates in cell adhesion, motility, and proliferation (reviewed in Ogita and Takai, 2006). Virus interaction with PVR is known to trigger conformational changes in the virion that are essential for disruption of the viral capsid and release of viral RNA into the cytoplasm (reviewed in Hogle, 2002). However, a number of studies, primarily conducted on HeLa cells, have left it unclear whether PV entry occurs by a specific endocytic route, and even whether PV virions must enter host cells in order to release their RNA (reviewed in Hogle, 2002).

We have examined the mechanism by which PV enters human brain microvascular endothelial cells (HBMEC) (Stins *et al*, 2001), which replicate many of the functional and morphologic characteristics of brain endothelium. HBMEC form intercellular tight junctions that prevent paracellular movement of ions and macromolecules, and they have provided a model for studying the mechanisms by which bacteria, fungi, and protozoan parasites interact with the endothelium to cross the BBB (reviewed in Kim, 2006). We find that virus enters HBMEC by a caveolin-dependent endocytic process, and that specific PVR-mediated signals, including the recruitment and activation of a protein tyrosine phosphatase (PTPase), SHP-2, are essential for virus entry and infection.

Result

PVR is required for infection of HBMEC

We first examined the role of PVR in PV infection of HBMEC. PVR was detected by immunofluorescence microscopy both on the apical and basolateral cell surfaces of HBMEC and within intercellular junctions (Figure 1A), and PV infected HBMEC equally from the apical or basolateral side (not shown). PVR expression and binding were required for PV infection, as PV attachment and infection were blocked by the anti-PVR monoclonal antibody (mAb) D171 (not shown) and inhibited by PVR-specific siRNA (Figure 1B).

Kinetics of PV internalization

To determine the kinetics of PV internalization into polarized HBMEC, we used an immunofluorescence-based confocal microscopy assay that permitted us to distinguish virus on the cell surface from virus that had already penetrated the plasma membrane. PV was permitted to attach to HBMEC

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Figure 1 PVR localization and involvement in PV binding and infection in HBMEC. (A) Monolayers grown on collagen-coated slides were fixed and stained with antibodies against PVR or the junctional proteins ZO-1 (top) or E-cadherin (bottom). (B) PVR siRNA blocks PV binding and infection: monolayers transfected with control (CON) or PVR siRNA were exposed to S^{35} -labeled virus for binding (25 000 c.p.m.s) or unlabeled virus (1 PFU/cell) for infection. Cells were lysed following binding at 16°C for 1 h for S^{35} -PV binding assay or washed and incubated for 14 h and stained for infection with anti-VP1 antibody. (C) Immunofluorescence-based assay to determine kinetics of PV internalization (as described in Materials and methods). Cells exposed to PV (or no virus, bottom row) for 1 h at 16°C (0 min), or incubated at 37°C for the indicated times, were fixed (but not permeabilized) and stained with anti-VP1 antibody followed by anti-mouse-AF-594 secondary antibody (red, VP1_{unperm}). Cells were then refixed, permeabilized, and re-stained with anti-VP1 antibody, followed by incubation with anti-mouse-AF-488 secondary antibody (green, VP1_{perm}). Red fluorescence (or overlapping red and green fluorescence) indicates virus on the cell surface; green fluorescence in the absence of red indicates internalized virus. (D) Relative fluorescence signal intensities of VP1_{unperm} and VP1_{perm} (determined as described in Materials and methods) at the indicated times. (E) Quantification of virus internalization. Red and green fluorescence were measured, and the index of internalization was calculated as described in Materials and methods. The data shown in (B, D, E) are shown as mean $\pm$ standard deviation. Statistical analysis was performed using the Student's *t*-test as described in Materials and methods. Asterisks indicate $P < 0.01$ for panel B. In merged confocal images, the white line indicates the position of the XZ or YZ cross-section.

monolayers at 16°C. The temperature was raised to 37°C and, at various times, monolayers were fixed and stained (without permeabilization) with antibody specific for the viral capsid protein VP1, followed by secondary antibody conjugated to Alexa Fluor (AF)-594. The resulting red staining (termed

VP1_{unperm}) is a measure of virus accessible to antibody on the extracellular surface of intact cells. To detect internalized virus, monolayers were refixed after AF-594 incubation, permeabilized, and stained again with anti-VP1 antibody, followed by secondary antibody conjugated to AF-488. The

green staining (termed VP1_{perm}) identifies virus accessible to antibody after permeabilization.

At 16°C (0 min), virus was detected along the apical surface and at intercellular junctions (Figure 1C). Between 60 min and 2 h, virus remained concentrated at the junction, with no divergence of VP1_{unperm} and VP1_{perm} signals, indicating that virus had not yet internalized. By 2.5–3 h post-infection (p.i.), there was a dramatic decrease in VP1_{unperm} signal, which corresponded with the appearance of VP1_{perm} within what appeared to be cytoplasmic vesicles (Figure 1C). At 4 h p.i., both VP1_{unperm} and VP1_{perm} signals had decreased significantly and coincided with the disappearance of cytoplasmic vesicles containing VP1_{perm} (Figure 1D). Thus, by 4 h p.i., virus had entered the cytoplasm and the capsid had presumably undergone some level of degradation so that it was no longer detectable with anti-VP1 antibody. By quantifying the relative signal intensities of VP1_{unperm} and VP1_{perm} (as described in Materials and methods), we found that virus remained on the surface for up to 2 h p.i., and did not enter the cytoplasm until 2.5–3 h p.i. (Figure 1E).

Kinetics of PV uncoating

Interaction of PV with PVR induces conformational changes in the capsid, made evident by the virion's reduced sedimentation velocity (135S rather than 160S); the resulting altered virions (called 'A-particles') are believed to be essential intermediates in an uncoating process that results in release of viral RNA and production of 80S empty capsids (reviewed in Hogle, 2002). The resulting 80S capsids then are disassembled or are otherwise degraded. It is not known whether A-particle formation and RNA release occur by a single concerted mechanism or occur at different times, at different cellular locations, or in response to different stimuli.

To examine the structure of virions at different stages of infection, we permitted ³⁵S-labeled virus to attach at 16°C, raised the temperature to 37°C, and recovered virions at various times. Analysis of recovered virions by sucrose gradient sedimentation suggested that virus underwent conversion to 135S by 30 min p.i. (a time when virus remained on the cell surface), and that most virus persisted in this form at least until 3 h p.i. (when virus was detected within intracellular vesicles) (Supplementary Figure 1A and B). At 4 h p.i., when we no longer detected virus within the cell by immunofluorescence (Figure 1C), the 135S peak had virtually disappeared; at the same time, there was accumulation of very small material at the very top of the gradient (fractions 1–3, likely representing degraded capsids that had already released their RNA) (Supplementary Figure 1B). We did not detect 80S empty capsids at any time point. This could be because they are never formed in these cells; alternatively, they may be very short-lived. These results suggest that virus converts to 135S particles on the cell surface, enters the cell, and persists in this form (which has not released RNA) within the cell until 3 h p.i. Between 3 to 4 h p.i., the virion undergoes further changes and is degraded to smaller material (presumably having released its RNA in the same interval).

PV internalizes with PVR

At 0 h, virus (detected by staining for VP1) colocalized with PVR along the apical surface, and in the junctional complex (Figure 2A, top). Within 2.5 h p.i., PV was concentrated in vesicular structures containing PVR (Figure 2A, bottom). We

detected no significant entry of PVR into the cytoplasm between 30 min and 2 h p.i. (not shown), consistent with the results of the fluorescence-based virus internalization assay (Figure 1C and E). Thus, although formation of 135S particles occurs rapidly on the cell surface (within 30 min p.i.; Supplementary Figure 1A), PV remains in close proximity to PVR throughout its internalization (up to 3 h p.i.).

PV infection requires dynamin and caveolin

Immunofluorescent staining revealed that VP1- and PVR-containing vesicles colocalized with caveolin (Figure 2B) but not the endosomal markers early-endosome antigen-1 (EEA1), Rab7, or LAMP-II (not shown). To confirm that caveolin was, in fact, responsible for PV internalization, we used specific inhibitors (pharmacological agents, siRNAs, and dominant-negative mutants). In cells transfected with dominant-negative mutants of caveolin-1 or caveolin-3, infection was markedly inhibited (Figure 2C). Furthermore, expression of dominant-negative dynamin II, a GTPase involved in budding of both clathrin-coated and caveolin-containing vesicles, also inhibited infection (Figure 2C). In contrast, dominant-negative forms of Eps15, Rab5, or Rab7, proteins involved in clathrin-mediated endocytosis and endosomal trafficking, did not inhibit infection. siRNAs targeting caveolin-1, -2, and dynamin II inhibited infection, but siRNA-knockdown of clathrin heavy chain, the clathrin adaptor protein AP2 μ , EEA1, Rab7, or flotillin-1 had no effect (Figure 2D). As expected, both the clathrin heavy chain and AP2 μ siRNAs inhibited transferrin uptake (Supplementary Figure 3C and D). Using the fluorescence-based internalization assay described above, we determined that caveolin-1, caveolin-2, and dynamin II siRNAs significantly decreased PV internalization, but that a clathrin heavy chain siRNA had no effect (Supplementary Figure 4). Taken together, these results indicate that caveolin and dynamin, but not components associated with clathrin-mediated endocytosis, are involved in PV internalization.

Pharmacological modulation of lipid rafts often perturbs the membrane dynamics required for caveolin-mediated endocytosis (reviewed in Simons and Toomre, 2000). Although some of these drugs may disrupt other endocytic mechanisms (Rodal *et al*, 1999), the cholesterol sequestering agents filipin and nystatin specifically disrupt caveolae and caveola-like structures (Rothberg *et al*, 1992; Orlandi and Fishman, 1998). Although these agents had no effect on transferrin uptake (Supplementary Figure 3E and F), we found that both filipin and nystatin inhibited PV infection (Figure 2E) and internalization (Supplementary Figure 5). Since filipin and nystatin did not inhibit PV infection when added after viral entry had occurred (3 h p.i.) (Figure 2E), this would suggest that caveolae or caveola-like membranes are required for PV internalization, not a later event in infection.

Caveolins provide structural support to the caveolar membrane and are necessary for vesicle formation and function. In addition, caveolins mediate intracellular signal transduction by interacting with signaling molecules through a domain in the C-terminus referred to as the 'scaffolding domain' (Li *et al*, 1996). Caveolin scaffolding domain peptides are known to interfere with a number of signaling events mediated by the scaffolding domain (Okamoto *et al*, 1998; Bucci *et al*, 2000; Bernatchez *et al*, 2005). We treated HBMEC with a cell-permeable caveolin-1 scaffolding domain peptide

Figure 2 PV internalizes with PVR and requires functional caveolin and dynamin II for entry. HBMEC exposed to PV (100 PFU/cell) were stained with anti-PVR polyclonal antibody (pAb) and anti-VP1 mAb (A), or with anti-PV mAb and anti-caveolin pAb (B), at the indicated times and were examined by confocal microscopy. (C) Monolayers expressing dominant-negative or wild-type forms of caveolin-1, caveolin-3, dynamin II, Eps15, Rab5, or Rab7 were exposed to PV (1 PFU/cell), and stained for VP1 at 14 h p.i. Data are presented as the percentage of transfected cells expressing VP1. (D) Effect of siRNAs targeting components of caveolin-, clathrin-, and lipid raft-mediated endocytic processes; cells transfected with the indicated siRNAs were exposed 48 h later to PV (1 PFU/cell), and stained for VP1 at 14 h. The percentage of cells infected was determined and expressed relative to the percentage of cells infected in monolayers exposed to control (CON) siRNA (indicated by the dashed line). (E) Monolayers treated with lipid raft destabilizing agents (filipin or nystatin) were exposed to PV (1 PFU/cell) and stained for VP1 at 14 h to detect infection. Inhibitor was added to cultures 1 h before infection (pre-treat) or was added 3 h p.i. Dashed line indicates the infection level of control-treated cells. (F) Cells treated with caveolin scaffolding domain or negative control peptide were exposed to PV (100 PFU/cell) and stained for VP1 (green) and caveolin (red) at 2.5 h. Data are representative of at least three independent experiments performed three times. Data in panel C, D, or E are shown as mean $\pm$ standard deviation. Asterisks indicate $P < 0.01$.

(amino acids 82–101) or a scrambled negative control peptide, and then exposed them to virus. Scaffolding domain peptide reduced infection by $78 \pm 4.6\%$ (not shown) and blocked viral entry into the cytoplasm, trapping virus on the cell surface (Figure 2D). Scaffolding domain peptide also blocked disruption of conformationally altered virions (Supplementary Figure 1C), but did not significantly inhibit transferrin internalization (Supplementary Figure 3E and F). The results suggest that the caveolin scaffolding domain may

interact with signaling molecules or other proteins important for viral entry.

PVR triggers RhoA-mediated actin rearrangements

The actin cytoskeleton participates in endocytic processes by providing the driving force for vesicular budding and movement and by stabilizing membrane invaginations at the cell surface. We observed extensive rearrangements of the actin cytoskeleton—in particular a dramatic increase in actin stress fibers—by 60 min p.i. (Figure 3A). We also observed an

Figure 3 The actin cytoskeleton and RhoA GTPase are required for PV entry and infection. **(A)** Phalloidin staining of the actin cytoskeleton in HBMEC exposed to PV (100 PFU/cell) or no virus for 60 min at 37°C. **(B)** Monolayers exposed to PV (100 PFU/cell) were lysed (at 15, 30, or 45 min p.i.), and GTP-bound Rho family proteins were isolated as described in Coyne and Bergelson (2006), and immunoblotted with antibodies specific for RhoA, Rac1, or Cdc42. Equal amounts of whole-cell lysate were blotted to evaluate GTPase expression. **(C)** Cells pretreated with latrunculin A (LatA), jasplakinolide (Jas), toxin B (ToxB), Rac Inhibitor NSC23766, or C3 exoenzyme (C3 Exo) were exposed to PV (1 PFU/cell) and stained for VP1 at 14 h p.i. **(D)** Cells transfected with control (CON), RhoA, Rac1, or Cdc42-siRNA were exposed to PV (1 PFU/cell) and stained for VP1 at 14 h p.i. Data are presented as percent infection of CON siRNA-transfected cells. **(E)** Monolayers treated with LatA, ToxB, C3 Exo, or control (DMSO) were exposed to PV (100 PFU/cell) and stained 2.5 h p.i. with anti-VP1 mAb (green) and anti-caveolin pAb (red). Data are representative of at least three independent experiments performed three times. Data in panels C and D are shown as mean $\pm$ standard deviation. Asterisks indicate $P < 0.01$.

increase in junctional permeability and disruption of junction proteins at this time (Figure 4). Both actin rearrangements (Supplementary Figure 6A) and modulation of junctional permeability (Figure 4A and B) were initiated by exposure of cells to anti-PVR mAb D171, and were not seen in cells transfected with PVR siRNA (not shown). Treatment of cells with latrunculin A, which sequesters actin monomers, or

with jasplakinolide, which stabilizes actin polymers, decreased infection (Figure 3C) and significantly reduced virus internalization (Supplementary Figure 5), trapping virus on the plasma membrane in caveolin-enriched clusters (Figure 3E). Taken together, these results indicate that actin rearrangements are involved in PV infection, and that they are initiated when PVR is ligated by PV or anti-PVR mAb.

Figure 4 PV entry modulates junctional integrity and causes relocation of junction-associated proteins. PV entry and D171 binding increase paracellular permeability (P_{app}) (A) and decrease transendothelial resistance (R_T) (B): HBMEC monolayers on transwell filters were exposed to PV (or no virus control) or D171 (or MOPC control) antibody, and flux of ¹⁴C-labeled dextran (10 kDa) (A) or R_T (B) was determined as described in the Supplementary data. Staining for ZO-1 (red) and claudin-1 (CL-1, green) (C), or ZO-1 (red) and E-cadherin (green) (D), in HBMEC 90 min following PV infection. Data in panels A and B are representative of nine transwells from three separate experiments and are shown as mean ± standard deviation. Asterisks indicate $P < 0.01$.

We investigated the role of Rho family GTPases in PV entry. The levels of activated (GTP-bound) RhoA, Rac1, and Cdc42 (Figure 3B) increased substantially at 15–30 min p.i. D171 mAb also activated Rho family GTPases (Supplementary Figure 6B). No activation of Rho family GTPases was seen in cells transfected with PVR siRNA (Supplementary Figure 6C). *Clostridium difficile* toxin B, which inactivates Rho, Rac, and Cdc42, decreased infection and also significantly reduced virus internalization (Figure 3C and E and Supplementary Figure 5). Although a specific inhibitor of Rac1 (NSC23766) had no effect, cell-permeable C3 exoenzyme, a selective Rho inhibitor, decreased infection (Figure 3C) and reduced virus internalization (Supplementary Figure 5A and C), leading to accumulation of virus on the apical and basolateral surfaces (Figure 3E). Knockdown of Rac1 and Cdc42 with specific siRNAs had no effect, but an siRNA targeting RhoA reduced infection by $59 \pm 3.7\%$ (Figure 3D). These results indicate that activation of RhoA by PV is involved in virus entry and infection.

Tyrosine kinases (but not Src kinases) are required for PV entry

We found previously that activation of Src kinases is required for caveolar entry of another picornavirus in a different cell type (Coyne and Bergelson, 2006). To determine if Src kinases are activated by PV entry, we measured the total catalytic activity of Src family kinases (Src, Fyn, and Yes) by *in vitro* kinase assay and by immunoblotting with an antibody that recognizes the activated form (pTyr⁴¹⁸) of all Src family members. A decrease in kinase activity and decreased phos-

phorylation of Tyr⁴¹⁸ were seen 15–30 min after cells were exposed to PV (Figure 5C and D) or D171 mAb (not shown). In contrast, clustering of another virus receptor, decay accelerating factor, activated Src kinases (not shown). Thus, PVR engagement leads to the deactivation of Src kinases. Consistent with this, PP2 (a specific Src kinase inhibitor) had no effect on virus entry (Figure 5B and Supplementary Figure 5). In addition, a specific inhibitor of Abl tyrosine kinase (Gleevec) had no effect on PV infection (Figure 5A).

Nonetheless, other tyrosine kinases are required for entry and infection. Genistein, a broad-specificity tyrosine kinase inhibitor, reduced PV entry (Figure 5B and Supplementary Figure 5A and C), prevented the disruption of virions (Supplementary Figure 1D), and significantly reduced infection (most potently when added pre-entry) (Figure 5A). Genistein also blocked RhoA activation following PVR ligation (Figure 6C), pointing to a role for tyrosine kinases upstream of RhoA-mediated actin rearrangements.

Tyrosine phosphatase activity is required for entry and infection

The dephosphorylation of Src-Tyr⁴¹⁸ suggested a possible role for PTPases during virus entry. PTPases constitute a family of membrane-associated and cytoplasmic enzymes that both positively and negatively regulate cellular processes by dephosphorylating tyrosine residues in target proteins. Engagement of PVR by PV or anti-PVR mAb D171 led to a dramatic increase in general PTPase activity (Figure 6A). There was no PTPase activation in cells transfected with PVR siRNA (Supplementary Figure 7B). Exposure of cells to

Figure 5 Tyrosine kinases are required for PV entry and infection. **(A)** Monolayers treated with genistein (Gen), PP2, or Gleevec were exposed to PV (1 PFU/cell), and stained for VP1 at 14 h p.i. Inhibitor was added to cultures 1 h before infection (pre-treat), or was added 3 h p.i. Dashed line indicates the infection level in control-treated cells. **(B)** Genistein blocks PV internalization: monolayers treated with PP2, Gen, or control medium were exposed to PV and stained for VP1 (green) at 2.5 h. **(C, D)** Src kinase activity (by phosphorylation of Src substrate peptide **(C)** or immunoblotting with mAb specific for Src family-pTyr⁴¹⁸ **(D)**). Data are presented as fold activation relative to no virus control (dashed line indicates activity in control cells). In both panels C and D, virus (100 PFU/ml) was adsorbed at 16°C, cells were washed to remove unbound virus, and kinase activity or phosphorylation determined (as described in Materials and methods) following incubation at 37°C for the indicated times (time 0 corresponds to no incubation at 37°C). Data are representative of at least three independent experiments performed three times. Data in panels A and C are shown as mean $\pm$ standard deviation. Asterisks indicate $P < 0.01$. Double asterisks in panels A indicate statistically significant difference from cells exposed to genistein at a post-entry time point (3 h).

an Fab fragment of D171 did not induce PTPase activation (Supplementary Figure 7C), indicating that clustering of PVR is required. Pervanadate (VO_4) and phosphatase inhibitor IV (PI-IV) reduced virus infection (Figure 6B), decreased entry of virus into the cytoplasm (Figure 6E and Supplementary Figure 5), and prevented the disruption of virions (Supplementary Figure 1E); in contrast, the serine/threonine phosphatase inhibitor okadaic acid had no effect (Figure 6B). VO_4 and PI-IV did not inhibit PV infection when added after viral entry had occurred (3 h p.i.) (Figure 6B). These data indicate that PTPases become activated by ligand binding to PVR, and that their activity is required for PV entry.

PVR-induced SHP-2 activation is required for virus entry

Whereas VO_4 inhibits a wide variety of PTPases, PI-IV acts on a limited number, including SHP-2 and PTP1B (Huang *et al*, 2003). SHP-2, an SH-2 domain-containing PTPase, is known to mediate cellular responses to growth factors and cytokines, and to regulate processes such as cell proliferation and differentiation (reviewed in Qu, 2000), but it has not been implicated in endocytosis or in virus entry. Both PV and D171 mAb led to activation of SHP-2 (Figure 7A), but not other PTPases (PTP1B, PTP μ , LAR, and CD45, not shown). SHP-2 activation is often accompanied by tyrosine phosphorylation of its C-terminus (Poole and Jones, 2005). We observed a significant increase in the tyrosine phosphorylation of SHP-2 in response to PV (Figure 7C, top) and D171 mAb engage-

ment (Figure 7C, bottom); no phosphorylation was evident in cells pretreated with genistein (Figure 7C). Consistent with SHP-2 activation, several downstream substrates of SHP-2 (cortactin and focal adhesion kinase, FAK) were dephosphorylated after PV attachment (Supplementary Figure 7A). These results demonstrate that SHP-2 is activated and undergoes tyrosine phosphorylation in response to PVR engagement.

Whereas PTP1B inhibitor had no effect on entry or infection (not shown), a selective inhibitor of SHP-1/2 (Chen *et al*, 2006) reduced virus infection by $75 \pm 9\%$ and decreased entry (Figure 7D and Supplementary Figure 5A and C). Both a dominant-negative SHP-2 mutant (SHP-2 C>S) and SHP-2 siRNA dramatically reduced levels of PV infection (Figure 7B), confirming a role for SHP-2 activity in PV entry and infection.

PV induces interaction of SHP-2 with PVR

SHP-2 activation at 45 min p.i. correlated with its relocalization to the junctional complex, where it colocalized with PVR (not shown) and PV (Figure 8B). SHP-2 interacts with phosphorylated tyrosine residues within immunoreceptor tyrosine-based inhibition motifs (ITIMs). The PVR α isoform contains an ITIM within its C-terminus and, when overexpressed in transfected cells, interacts with SHP-2 (Oda *et al*, 2004). To determine if PVR and SHP-2 interact during PV entry, cells were exposed to PV in the absence or presence

Figure 6 PTPases are required for PV entry and infection. (A) Cells exposed to PV (100 PFU/cell) or to D171 mAb were lysed at the indicated times following incubation at 37°C, and tyrosine phosphatase activity was measured (time 0 corresponds to no incubation at 37°C). Data are presented as fold activation relative to no virus or MOPC mAb controls (dashed line indicates activity in control cells). (B) Monolayers treated with pervanadate (VO₄), PI-IV, or okadaic acid were exposed to PV (1 PFU/cell) and stained for VP1 at 14 h p.i. Inhibitor was added to cultures 1 h before infection (pre-treat), or was added 3 h p.i. Dashed line indicates the infection level of control-treated cells. (C) GTP-bound Rho family proteins were isolated from cells treated with Gen, PI-IV, SHP2 inhibitor, or control (DMSO) before exposure to PV (100 PFU/cell) for the indicated times. An equal amount of whole-cell lysate was blotted to evaluate total RhoA protein levels. (D) Monolayers pretreated with VO₄, PI-IV, or control (DMSO) medium were exposed to PV (100 PFU/cell) and stained for VP1 (green) and caveolin (red) at 2.5 h p.i. Data are representative of at least three independent experiments performed three times. Data in panels A and B are shown as mean ± standard deviation. Asterisks indicate $P < 0.01$.

of VO₄ (to maintain high levels of tyrosine phosphorylation) and PVR was immunoprecipitated with a specific polyclonal antibody. The precipitates were immunoblotted with anti-phosphotyrosine 4G10, anti-SHP-2, and anti-PVR antibodies. There was a significant increase in the level of tyrosine-phosphorylated PVR in response to PV or D171 mAb attachment (Figure 8A). Furthermore, PVR and SHP-2 co-immunoprecipitated only from cells exposed to PV or D171 mAb. The interaction was more robust in VO₄-treated cells, in which tyrosine phosphorylation of PVR was maximal (Figure 8A). The phosphorylation of PVR and the association of PVR and SHP-2 during PV entry were blocked by treatment of cells with genistein (Figure 8A), pointing to a role for tyrosine kinase activity in PVR tyrosine phosphorylation and SHP-2 recruitment. We also found that SHP-2 relocalized to the sites of cell contact in cells exposed to PV (Figure 8B); this effect

was blocked by genistein, but was more robust in VO₄-treated cells (Figure 8C). These results suggest that tyrosine phosphorylation of the PVR C-terminus in response to PV or D171 mAb attachment mediates its association with SHP-2.

Discussion

PVR, like many virus receptors, is thought to participate in virus entry by serving as an attachment site and by inducing alterations in the virion that facilitate genome release. Our results demonstrate that PVR is an active participant in signal transduction events required for entry of PV virions from the cell surface. PVR-induced signals led to rearrangement of actin, modulation of junctional permeability, and activity of tyrosine kinases and phosphatases essential for dynamin-dependent caveolar entry.

Figure 7 SHP-2 is activated by PVR engagement. **(A)** Lysates of cells exposed to PV or D171 mAb were immunoprecipitated with SHP-2 antibody following incubation at 37°C for the indicated times, and phosphatase activity was measured in immunoprecipitates (time 0 corresponds to no incubation at 37°C). Dashed line indicates activity in control cells. **(B)** Cells transfected with wild-type or dominant-negative (C>S) SHP-2 (left panel), or with control (CON) or SHP-2 siRNAs (right panel) were exposed to PV (1 PFU/cell) and stained for VP1 at 14 h p.i. **(C)** Lysates of cells exposed to PV (100 PFU/cell) were incubated with agarose-conjugated anti-SHP-2 pAb. Immunoprecipitates were immunoblotted with anti-phosphotyrosine 4G10 mAb, stripped, and reprobed with anti-SHP-2 mAb. **(D)** SHP2 inhibitor- or DMSO control-pretreated cells were exposed to PV (100 PFU/cell) and stained for VP1 (green) and caveolin (red) at 2.5 h p.i. Data are representative of at least three independent experiments performed three times. Data in panels A and B are shown as mean $\pm$ standard deviation. Asterisks indicate $P < 0.01$.

We observed pronounced rearrangements of the actin cytoskeleton in response to PVR ligation, and an intact actin network was required for both PV entry and infection. While the specific role of actin remains unclear, PV-containing vesicles likely require reorganization of the cortical actin network beneath the plasma membrane in order to enter the cell. Although the actin rearrangements were accompanied by activation of RhoA, Rac1, and Cdc42 (Figure 4B), only RhoA siRNA inhibited PV infection, suggesting a specific role for RhoA in virus entry. RhoA localizes to caveolae (Michaely *et al*, 1999) and GTP-bound RhoA increases their formation (Senda *et al*, 1997), which may suggest that it participates in the formation of caveolar vesicles required for PV internalization or directly in actin rearrangements involved in PV entry.

We also observed an increase in junctional permeability and rearrangement of junctional proteins early in the course of PV infection. Because these changes were triggered by anti-PVR mAb in the absence of virus, they are likely to have resulted from a signal initiated by PVR. PVR-induced modulation of endothelial tight junctions, as well as cytolytic infection of endothelial cells themselves, may result in loss of endothelial integrity, thus permitting virus entry.

A partnership between tyrosine kinases and PTPases mediates the transduction of signals from cell surface receptors

(reviewed in Tonks, 2006). We found that PV and D171 mAb interaction with PVR activated PTPases, specifically SHP-2. SHP-2 regulates a wide variety of cell biological processes, including cell motility and adhesion (reviewed in Qu, 2000), and has been associated with aberrant signals transduced by *Helicobacter pylori* cagA (Higashi *et al*, 2002). However, we are unaware that SHP-2 has been implicated in endocytosis or participates in entry of viruses into host cells.

We found that both PV and anti-PVR mAb led to tyrosine phosphorylation of PVR and the subsequent recruitment of SHP-2. It is unclear why SHP-2 is required for PV entry. SHP-2 is known to act upstream of RhoA (Schoenwaelder *et al*, 2000), and we observed that PVR-dependent RhoA activation was partly inhibited by an SHP-2 inhibitor (Figure 3D); it is thus possible that SHP-2 is important for virus-induced actin rearrangements. Another interesting possibility is that SHP-2, like other signaling molecules, interacts directly with caveolins to regulate caveolar endocytosis. We found that PV enters the cell cytoplasm in association with caveolin, and that the caveolin scaffolding domain is essential to this process. SHP-2 is known to localize to caveolin-rich domains (Caselli *et al*, 2002) and contains the peptide motif necessary for interaction with the caveolin scaffolding domain (ϕ X ϕ XXXX ϕ XX ϕ , where ϕ is an aromatic residue) (Couet *et al*, 1997a, b). We noted a marked dephosphorylation of caveolins during PV

Figure 8 SHP-2 binds PVR during PV internalization. (A) Monolayers exposed to PV (100 PFU/cell) (left), D171 mAb (center) in the presence of absence of VO₄, or to PV in the presence or absence of genistein (right), were lysed at 45 min p.i., and lysates were immunoprecipitated with agarose-conjugated anti-PVR pAb. Immunoprecipitates were immunoblotted with anti-phosphotyrosine 4G10 mAb, stripped, and reprobed with anti-SHP-2 mAb and anti-PVR pAb. (B, C) PV-exposed monolayers (100 PFU/cell) were fixed at 45 min p.i. and stained with anti-VP1 mAb (red) and anti-SHP-2 pAb (green). Cells were treated with either no inhibitor (B) or with genistein or VO₄ (C). Data are representative of at least three independent experiments performed three times.

internalization (Supplementary Figure 7A) that was blocked by PI-IV (not shown), but it remains unclear whether caveolin dephosphorylation is important for PV entry. Whereas a variety of kinases have been associated with caveolar function, it will be interesting to determine whether SHP-2 or other phosphatases also regulate the formation of caveolae,

mediate their internalization, or control their intracellular movement.

The results presented here suggest a possible mechanism by which PV interaction with endothelial cells may permit blood-borne virus to cross the BBB and enter the CNS. The results demonstrate for the first time that PV depends on

dynamamin-dependent caveolar endocytosis for entry and infection. PVR was one of the first virus receptors to be identified and studied extensively. Only recently has it been demonstrated that virus attachment to receptors trigger essential intracellular signals (reviewed in Marsh and Helenius, 2006). The work presented here provides a dramatic example of specific receptor-mediated signals required for virus entry.

Materials and methods

Cells and viruses

HBMEC were isolated and established as previously described (Stins *et al*, 2001). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10% NuSerum, sodium pyruvate, non-essential amino acids, MEM vitamins, 5 U/ml heparin, and 30 µg/ml endothelial cell growth supplement (BD Transduction Labs, San Diego, CA). Cells were plated in collagen-coated chamber slides (BD Transduction Labs) at a density of 5×10^5 cells/well for immunofluorescence microscopy or on 6-mm Transwell-Col (3 µm pore size) filters for polarity of infection studies. Cells were grown a minimum of 48 h before experiments.

PV Sabin 2 was a kind gift from the Centers for Disease Control and Prevention (Atlanta, Georgia, United States). Virus was expanded by growth in HeLa cells and concentrated by ultracentrifugation through a sucrose cushion, and titers were determined by plaque assay on HeLa cells. Immunofluorescence experiments tracking virus entry or biochemical experiments examining kinase/phosphatase activation were performed at a multiplicity of 100 plaque forming unit (PFU) per cell. Experiments measuring productive virus infection were performed with 1 PFU/cell. The typical picornavirus particle/PFU ratio is approximately 200 (Rueckert, 1996). In all experiments, PV was permitted to attach to HBMEC monolayers at 16°C. The temperature was raised to 37°C for entry/infection.

Antibodies

Polyclonal antibody specific for PVR was provided by Merja Roivainen (National Public Health Institute (KTL) Helsinki, Finland). Mouse anti-enterovirus VP1 (Ncl-Enterovirus) was obtained from Novocastra Laboratories (Newcastle upon Tyne, United Kingdom). Monoclonal antibodies against Rac1 and activated Src kinases (phospho-Src (Tyr⁴¹⁶) clone 9A6), and horseradish peroxidase-conjugated anti-phosphotyrosine 4G10 were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-FAK (sc-1688), anti-Src antibody (sc-8056) that detects Src, Fyn, and Yes, and rabbit polyclonal antibodies to RhoA (sc-179), Cdc42 (sc-87), SHP-2 (sc-424), agarose-conjugated SHP-2 (sc-424 AC), FAK (sc-932), cortactin (sc-11408), and HRP-conjugated GAPDH (sc-25778) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Control mAb MOPC 21 was purchased from Sigma (St Louis, MO). Polyclonal anti-caveolin and anti-SHP-2 and caveolin-1 mAbs were purchased from BD Transduction Labs. Monoclonal anti-PVR D171 was obtained from Abcam (Cambridge, MA). AF-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Pharmacological inhibitors

Monolayers were pretreated with inhibitors for 1 h at 37°C, washed, and incubated with PV at a multiplicity of 100 PFU/cell (viral entry studies) or 1 PFU/cell (viral infection studies) in virus binding buffer (DMEM supplemented with 1 mM HEPES) containing inhibitor for 1 h at 16°C. Following washing, virus entry/infection was initiated by shifting cells to 37°C in medium containing inhibitor.

PP2 (5 µM), genistein (20 µM), latrunculin A (5 µg/ml), Rho kinase inhibitor Y-27632 (5 µM), *C. difficile* toxin B (50 ng/ml), sodium orthovanadate (1 mM), PTPase inhibitor IV (10 µM), okadaic acid, *Prorocentrum* sp. (1 µM), PTP1B inhibitor (10 µM), CD45 inhibitor (1 µM), SHP1/2 inhibitor, NSC-87877 (1 µM), caveolin-1 scaffolding domain peptide (and corresponding negative control peptide) (50 µM), and the specific Rac inhibitor NSC232766 (100 µM) were obtained from Calbiochem (San Diego, CA). Filipin (5 µg/ml), nystatin (10 µg/ml), and cytochalasin D (10 µg/ml) were obtained from Sigma (St Louis, MO). Cell-permeable exoenzyme

C3 transferase (1 µg/ml) was purchased from Cytoskeleton Inc. (Denver, CO). Imatinib mesylate (5 µM) (GleevecTM, STI-571) was obtained from Novartis Pharmaceuticals). Doses were based on IC₅₀ values and published reports.

Plasmids, siRNAs, and transfections

Specific plasmids encoding wild-type and dominant-negative effector molecules are described in Supplementary Figure 2, as are the sequences, sources, and efficacies of siRNAs used in these studies. Transfection of HBMEC with siRNAs and plasmids was performed using the Nucleofector System (Amaxa, Gaithersburg, MD) using Nucleofector solution V and program T023. Transfection efficiencies with plasmids (as determined by GFP expression) were 90–100%, and transfection efficiency with Cy5-labeled siRNA was 95–100%.

Immunofluorescence microscopy

Monolayers were exposed to PV in virus binding buffer for 1 h at 16°C, then washed, and virus entry was initiated by shifting cells to 37°C. In all experiments, cells were fixed before staining with ice-cold methanol for 5 min or 4% paraformaldehyde for 10 min at room temperature (RT) followed by permeabilization with 0.1% Triton X-100 in PBS. Cells were incubated with the indicated primary antibodies for 1 h at RT, washed, incubated with fluorochrome-conjugated secondary antibodies for 30 min at RT, washed, and mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2-phenylindole (DAPI). Images were captured with a BX51 Olympus fluorescence microscope (Melville, NY) or a LeicaTCS 4D laser-scanning confocal microscope (Leica, Exton, PA). For three-dimensional analysis, XZ or YZ series stacks were acquired at ~0.5 µm intervals through the total thickness of the cell monolayer (~8–10 µm). A dashed white line through the merged XY image shows the position of the XZ or YZ cross-sections. Scale bar in figures is 10 µm.

Immunofluorescence-based assay for internalized virus

Monolayers were exposed to PV as described above. At the indicated times, cells were fixed in 4% PFA, washed in PBS containing 50 mM NH₄Cl for 5 min, and incubated with monoclonal anti-VP1 antibody (NCL-ENTERO) for 1 h at RT. Cells were then washed and incubated with AF-594-conjugated secondary antibody. Following washing, cells were fixed again in 4% PFA, incubated for 5 min in PBS containing 50 mM NH₄Cl, and permeabilized with 0.1% Triton X-100 for 10 min. Permeabilized monolayers were incubated again with monoclonal anti-VP1 antibody (NCL-ENTERO) for 1 h at RT, washed, and incubated with AF 488-conjugated secondary antibody. Cells were mounted with Vectashield containing DAPI and images captured as follows: to capture the red channel (AF 594), excitation/emission filters were set at band pass 543 nm/long pass 560 nm; green channel (AF 488) images were captured with filters set at band pass 488 nm/long pass 505 nm. Images were subjected to quantification as described below.

Images were processed and quantified using the IMAGE J program (<http://rsb.info.nih.gov/ij/>). Quantification was performed by measuring the relative fluorescence intensity profiles (in arbitrary units) of the red (virus accessible to antibody without permeabilization) and green (virus accessible to antibody with permeabilization) channels. Mean fluorescence was based on quantification of ~400 cells. Index of internalization was calculated according to the following equation: index of internalization = $(F_G - F_R)/(F_G + F_R)$, where F_R is the relative fluorescence from the red channel and F_G is the relative fluorescence from the green channel. Thus, $(F_G - F_R)$ is indicative of the amount of virus internalized (thereby labeled only following permeabilization) and $F_G + F_R$ is equal to the total VP1 fluorescence.

Paracellular permeability

Transendothelial resistance (R_T) measurements were performed with an ohmmeter (EVOM; World Precision Instruments, Sarasota, FL) on cells grown for 72–96 h on 0.4-µm T-col inserts. Permeation of [¹⁴C]dextran (MW 10 kDa) across HBMEC exposed to virus (MOI = 100) was measured for 1 h at 37°C following virus binding at 16°C for 1 h. [¹⁴C]dextran (5 µCi) was added to the apical compartment, and samples (10 µl) were removed from the basolateral compartment. Radioactivity in each sample was counted, and the apparent permeability (P_{app}) coefficient was calculated (Stutts *et al*, 1981).

Analysis of recovered virions

PV was labeled by growth on HeLa cells in the presence of [³⁵S]methionine and purified by cesium gradient centrifugation as described elsewhere (Milstone *et al*, 2005). ³⁵S-labeled virus particles at various stages of internalization were recovered by lysis of cells with sucrose gradient lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium dodecyl sulfate (SDS). Cell lysates were overlaid on linear 15–30% sucrose gradients and centrifuged at 39 000 r.p.m. for 150 min at 4°C in a Beckman SW41Ti rotor. Fractions (400 µl) were collected from the top of the gradient and radioactivity was measured.

Immunoprecipitations and immunoblots

Cell lysates were prepared with RIPA buffer (Supplementary data) and insoluble material was cleared by centrifugation for 5 min at 4°C. Lysate (200 µg–1 mg) was incubated with indicated antibody for 1 h at 4°C. Sepharose G beads were added for an additional 1 h. To directly conjugate CD155 goat polyclonal antibody to protein G-agarose, the Seize X protein G immunoprecipitation kit was used according to the manufacturer's protocol (Pierce Rockford, IL). After centrifugation, the beads were washed 4 × in cell lysis buffer and then heated at 95°C for 10 min in Laemmli sample buffer. Following a brief centrifugation, the supernatant was loaded onto 4–20% Criterion Tris-HCl gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes. Membranes were blocked overnight in TBSTM or TBSTB (Supplementary data), probed with antibodies, and developed with horseradish peroxidase-conjugated antibody to mouse or rabbit immunoglobulin (Jackson ImmunoResearch), and Supersignal West Dura (Pierce). Densitometry was performed with Image J (NIH).

Src kinase assays

Cells were lysed in kinase lysis buffer (Supplementary data) and 400 µg of lysate was subjected to immunoprecipitation with anti-Src family (Src/Fyn/Yes) antibody for 1 h at 4°C followed by protein-G

Sepharose for 1 h. Kinase activity was determined as described (Coyne and Bergelson, 2006).

Assays of Rho, Rac, and Cdc42 activity

Isolation of GTP-bound Rho proteins was performed as described previously (Coyne and Bergelson, 2006).

Assays of PTPase activity

Activity of PTPases and immunoprecipitated SHP-2 was determined using the EnzoLyte pNPP Protein Phosphatase Assay kit (colorimetric) according to the manufacturer's instructions (Anaspec, San Jose, CA).

Statistical analysis

Data are presented as mean ± standard deviation. An unpaired two-tailed Student's *t*-test assuming unequal variance was used to determine statistical significance. Details regarding *P*-values and replicates are provided in the figure legends.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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