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# **Coxsackievirus Entry across Epithelial Tight Junctions Requires Occludin and the Small GTPases Rab34 and Rab5**

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# SUMMARY

The major group B coxsackievirus (CVB) receptor is a component of the epithelial tight junction (TJ), a protein complex that regulates the selective passage of ions and molecules across the epithelium. CVB enters polarized epithelial cells from the TJ, causing a transient disruption of TJ integrity. Here we show that CVB does not induce major reorganization of the TJ, but stimulates the specific internalization of occludina TJ integral membrane component-within macropinosomes. Although occludin does not interact directly with virus, depletion of occludin prevents CVB entry into the cytoplasm and inhibits infection. Both occludin internalization and CVB entry require caveolin but not dynamin; both are blocked by inhibitors of macropinocytosis and require the activity of Rab34, Ras, and Rab5, GTPases known to regulate macropinocytosis. Thus, CVB entry depends on occludin and occurs by a process that combines aspects of caveolar endocytosis with features characteristic of macropinocytosis.

# INTRODUCTION

Group B coxsackieviruses (CVB) are common human pathogens that are transmitted by the fecal-oral route and must cross the intestinal mucosa to initiate infection. The intestines are lined by polarized epithelial cells, with distinct apical and basolateral surfaces; intercellular tight junctions (TJs) regulate the flow of ions and macromolecules, including viruses, across the intact epithelium (reviewed in Schneeberger and Lynch, 2004). The primary CVB receptor, the coxsackievirus and adenovirus receptor (CAR), is a transmembrane component of the TJ (Cohen et al., 2001). We previously found that CVB entry into polarized epithelial cells depends on interaction with a second receptor, decay-accelerating factor (DAF) on the apical cell surface (Coyne and Bergelson, 2006). Virus attachment to DAF triggers intracellular signals that permit virus to move to the TJ, interact with CAR, and enter the cell by a caveolin-dependent, but dynamin-independent, mechanism.

CVB entry from the TJ is accompanied by a transient disruption of TJ barrier function (Coyne and Bergelson, 2006). TJs are composed of a complex of integral membrane proteins whose adhesive interactions bring adjacent cell membranes into close apposition, and a network of cytoplasmic scaffolding proteins that provide a link to the cytoskeleton and to intracellular signaling molecules. Barrier function is modulated under a variety of physiological and pathological conditions, and modulation often depends on the reorganization or endocytosis of one or more TJ proteins. In some instances, the specific internalization of a single family of TJ proteins is sufficient to cause marked changes in barrier function (Sonoda et al., 1999).

We have examined the process by which CVB modulates the barrier function of the TJ to enter polarized cells. We find that the junction does not undergo major reorganization, but that virus entry is accompanied by the specific internalization of occludin, a transmembrane component of the TJ. Strikingly, although occludin does not interact directly with the virus, its expression is critical for CVB infection-depletion of occludin prevents CVB entry into the cytoplasm. Internalization of both CVB and occludin are blocked by inhibitors of macropinocytosis and require the function of the GTPases Rab34 and Rab5, important regulators of macropinocytosis. The results suggest that internalization of occludin as well as CVB entry occur by distinctive mechanisms that depend on both caveolar endocytic pathways and processes more typical of macropinocytosis.

# RESULTS

# **Occludin Is Internalized in Response to CVB Entry**

When polarized epithelial monolayers are exposed to CVB, there is a rapid loss of TJ barrier function (within 15 min), evident as loss of transepithelial electrical resistance and increased permeability to small and moderate sized dextrans (Coyne and Bergelson, 2006). In an effort to understand the leakiness of TJs that we observed during virus entry, we looked for rearrangements of TJ-associated proteins. The integral membrane protein occludin

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# Figure 1. Occludin Is Internalized and Tyrosine Phosphorylated in Response to CVB Entry

(A) Monolayers exposed to CVB or EV7 (100 PFU/cell) were fixed and stained for ZO-1 (red) and occludin (green) 90 min p.i. Indicated in white is the percentage of total cells containing occludin within the cytoplasm (as determined as described in the Experimental Procedures).

(B) Quantification of the internalization of CVB and occludin at 30, 60, and 90 min p.i. (shown as the percentage of total cells containing CVB and occludin in the cytoplasm).

(C) Monolayers exposed to CVB (100 PFU/cell) were fixed and stained for claudin-1, JAM-1, or CAR at 90 min p.i.

(D) Lysates of monolayers exposed to CVB (100 PFU/cell) for the time indicated were immunoprecipitated with anti-occludin pAb. Immunoprecipitates were immunoblotted with anti-phosphotyrosine 4G10 mAb, stripped, and reprobed with anti-occludin mAb.

(E) Monolayers expressing GFP-tagged dominant-negative (DN) caveolin-1 or HA-tagged dominant-negative dynamin II were exposed to CVB (100 PFU/cell) for 90 min, fixed, and stained for occludin.

(F) Quantification of the effects of wild-type

(WT) or dominant-negative caveolin-1 (Cav-1), dynamin II (Dyn II), or Eps15 on occludin internalization. Data are shown as percentage of occludin internalized compared to nontransfected controls. Data are representative of at least three independent experiments performed at least three times. Data in (B) and (F) are presented as means  $\pm$  standard deviation. \*p < 0.05.

moved from the TJ and relocalized to cytoplasmic vesicles by 90 min postinfection (p.i.) (Figure 1A). Occludin internalization was pronounced-94% ± 7% of cells exposed to CVB contained occludin within cytoplasmic vesicles, compared to only 1% ± 0.6% of control cells. This effect was specific for CVB; occludin did not internalize in response to the entry of another picornavirus, echovirus 7 (Figure 1A), which enters and infects polarized cells, but does not require relocalization to the TJ (C.B.C. and J.M.B., unpublished data). The kinetics of occludin internalization were consistent with those for CVB; virus and occludin do not enter the cytoplasm until 60-90 min p.i. (Figure 1B). The internalization of occludin was specific, as other major TJ transmembrane proteins (CAR, JAM-1, and claudin-1 [Figure 1C]) and the major cytoplasmic TJ proteins (ZO-1 [Figure 1A], ZO-2, and ZO-3 [data not shown]) all remained at the junction. We also observed no internalization of the adherens junction proteins β-catenin or E-cadherin (data not shown).

Tyrosine phosphorylation has been linked with increased TJ permeability in polarized epithelial cells (Staddon et al., 1995), and occludin itself undergoes tyrosine phosphorylation in response to a variety of stimuli (reviewed in Feldman et al., 2005). We observed a significant increase in the tyrosine phosphorylation of occludin in response to CVB (Figure 1D); no phosphorylation was evident in cells pretreated with the general tyrosine kinase inhibitor genistein (data not shown). In contrast, we observed no change in the serine or threonine phosphorylation of occludin (data not shown), modifications that

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have been associated with alterations in TJ permeability (Sakakibara et al., 1997; Wong, 1997).

Occludin internalization, like internalization of CVB (Coyne and Bergelson, 2006), required functional caveolin; dominant-negative caveolin-1 (Figures 1E and 1F) and caveolin-1 siRNA (data not shown) blocked occludin internalization. However, occludin internalization was not blocked by dominant-negative dynamin II (Figures 1E and 1F) or Eps15 (Figure 1F); as expected, both of these dominant-negative mutants inhibited endocytosis of transferrin (data not shown). Although the GTPase dynamin has been reported to be essential for the internalization of several caveolar ligands (reviewed in Parton and Simons, 2007), both occludin and CVB were internalized by a caveolin-mediated, but dynamin-independent, process.

# **Occludin Is Required for CVB Internalization**

At 90 min p.i., internalized occludin colocalized with caveolin-1 (Figure S2B) and CVB (Figure 2A; Figure S2B) in cytoplasmic and perinuclear vesicles. However, CVB did not induce the internalization of claudin-1 (Figure 2B). Surprisingly, depletion of occludin with siRNA blocked virus entry (Figure 2F) and infection (Figure 2C), leaving virus trapped at the TJ (only  $11\% \pm 4\%$  of virus had entered the cytoplasm in occludin siRNA-transfected cells compared to  $93\% \pm 5\%$  in control transfected cells). Consistent with previously published data (Yu et al., 2005), we found that occludin siRNA had no significant effect on barrier properties of the TJ (Figures S1A and S1B). Occludin siRNA also had no effect on the expression (Figure 2D) Coxsackievirus Entry Requires Occludin and Rab34



#### Figure 2. Occludin Is Required for CVB Entry and Infection

(A) Monolayers exposed to CVB (100 PFU/cell) were fixed and stained for VP1 (red) and occludin (green) 90 min p.i.

(B) Cells expressing EGFP-claudin-1 and mRFP-occludin were exposed to CVB (100 PFU/cell) for 90 min and stained for VP1 (blue). For clarity, VP1 staining is pseudocolored red, and fluorescent occludin is pseudocolored blue.

(C) Cells transfected with the indicated siRNA were exposed to CVB (1 PFU/cell) and stained for VP1 at 7 hr p.i.

(D) Western blot analysis of monolayers 48 hr after transfection with the indicated siRNAs. Blot was stripped and reprobed with the indicated antibody. Densitometry was performed with ImageJ to determine efficacy (% reduction).

(E) Cells transfected with CON or OCC siRNAs were stained for CAR (in green) and occludin (in red) 48 hr after transfection.

(F) Cells transfected with control (CON) or occludin (OCC) siRNAs were exposed to CVB (100 PFU/cell) and stained for VP1 90 min p.i. The relative amount of CVB internalized is shown in white (93% ± 8% for CON siRNA and 11% ± 4% for OCC siRNA).

(G) CHO cells transfected with control (GFP) or human CAR or occludin plasmids were exposed to S<sup>35</sup>-labeled virus (25,000 cpm). Cells were lysed following binding at 16°C, and radioactivity was determined. (Inset) Western blot analysis for CAR (top), occludin (middle), or GAPDH (bottom) in transiently transfected CHO cells to control for transfection efficiency. Data are representative of at least three independent experiments performed at least three times.

Data in (C) and (F) are shown as mean  $\pm$  standard deviation. \*p < 0.05.

or localization of CAR (Figure 2E) or other TJ-associated proteins (data not shown). SiRNA depletion of ZO-1, claudin-1, and JAM-1 had no effect on entry (data not shown) or infection (Figure 2C). These results indicate that virus entry from the TJ depends in some way on occludin and suggest that virus internalization may depend on a cellular mechanism(s) that function to internalize occludin.

To confirm that occludin was not directly interacting with CVB, we measured CVB attachment to CHO cells transiently transfected with human CAR or occludin. CVB is incapable of binding to or infecting CHO cells in the absence of receptor expression (Bergelson et al., 1997). CHO cells expressing CAR, but not control or occludin transfectants, bound radiolabeled CVB (Figure 2G). In addition, CHO cells expressing CAR became infected by CVB, whereas control or occludin transfectants did not (data not shown). Furthermore, occludin siRNA did not inhibit CVB binding to Caco-2 cells (Figure S1C).

Interaction of CVB with CAR induces conformational changes in the capsid that are believed to be essential for the uncoating process that culminates in release of viral RNA into the cytoplasm (reviewed in Hogle, 2002).

Native virions, which sediment at 160S in sucrose velocity gradients, are converted to 135S altered (A)-particles; the release of RNA leads to the appearance of 80S empty capsids. We have shown previously that transfection of cells with CAR siRNA prevents A-particle formation and prevents virus entry into the cytoplasm (trapping 160S particles in the TJ) (Coyne and Bergelson, 2006). Although occludin siRNA also trapped virus at the TJ (Figure 2F), virions recovered from occludin siRNA-transfected cells sedimented at 135S, and thus were trapped as A-particles. In control cells, virions entered the cytoplasm and recovered virions underwent further conversion to faster-sedimenting 80S empty capsids (Figure S1D).

Taken together, these results indicate that. although occludin does not directly bind CVB, and is not needed for A-particle conversion, it is nonetheless required for subsequent events in CVB entry.

# **Occludin Is Internalized in Macropinosomes**

Macropinocytosis is a dynamin- and receptor-independent endocytic mechanism responsible for nonselective fluid and solute uptake, characterized by the closure of



#### Figure 3. Occludin Is Internalized in Macropinosomes, and Inhibitors of Macropinocytosis Block CVB Entry

(A) The rate of macropinocytosis (as assessed by FITC-dextran uptake) was determined following exposure to CVB (100 PFU/cell) at the indicated times.

(B) Cells exposed to CVB (in the absence or presence of FITC-dextran) for 90 min were fixed and stained for occludin (red), and actin (green, top) or dextran uptake was visualized (green, bottom).

(C) Monolayers pretreated with control (DMSO), EIPA, or rottlerin were exposed to CVB (100 PFU/cell) in the presence of FITC-dextran (1 mg/ml). At 90 min p.i., cells were fixed and the extent of FITC-dextran uptake was determined by microscopy.

(D) The rate of macropinocytosis (as determined by FITC-dextran uptake) and the percentage of cells with internalized occludin was determined in cells treated with DMSO (no inhibitor), EIPA, or rottlerin and exposed to CVB (100 PFU/cell) for 90 min at 37°C or in cells exposed to no virus. Left y axis, rate of macropinocytosis; right y axis, occludin internalization.

(E) Monolayers treated with control (DMSO), EIPA, or rottlerin were exposed to CVB (100 PFU/cell) and stained for VP1 at 90 min p.i. The percentage of CVB internalized is indicated in white.

(F) Cells were treated with control (DMSO), EIPA, or rottlerin prior to exposure to CVB (1 PFU/cell) and stained for VP1 at 7 hr p.i. Inhibitor was added to cultures 1 hr prior to infection (pre-treat) or was added 90 min p.i.

(G) Cells treated with control (DMSO) or EIPA were exposed to CVB (100 PFU/cell) in the presence of FITC-dextran, then fixed and stained for occludin (red) at 90 min p.i. Data are representative of at least three independent experiments performed three times.

Data in (A), (D), and (F) are shown as mean  $\pm$  standard deviation. \*p < 0.05.

cell surface ruffles to form actin-coated intracellular vesicles of variable size (reviewed in Swanson and Watts, 1995). CVB entry stimulated macropinocytosis by 60–90 min p.i., as evidenced by a significant increase in the uptake of fluorophore-conjugated dextran (Figure 3A). While we observed occludin in cytoplasmic and perinuclear caveolin-1-positive vesicles (Figure S2B), we also saw occludin in typical macropinosomes—large actin-coated cytoplasmic vesicles, heterogeneous in size, in which labeled dextran was concentrated (Figure 3B). We also observed both CVB and occludin within vesicles enriched in dextran within the cytoplasm (Figure S2A).

# Inhibitors of Macropinocytosis Block Both Occludin Internalization and CVB Entry

Because we observed that CVB entry stimulated macropinocytosis and the internalization of occludin in macropinosomes, we examined the role of macropinocytosis in Coxsackievirus Entry Requires Occludin and Rab34



#### Figure 4. Rab34 Localizes to Tight Junctions and Mediates Occludin Internalization

(A) Monolayers were exposed to CVB (100 PFU/cell) for 30 min at 37°C and then fixed and stained for occludin (red) and Rab34 (green).
(B) Cells expressing wild-type EGFP-Rab34 were fixed and stained for ZO-1 (red).

(C) Colocalization of EGFP-Rab34 and DsRed-caveolin-1. Insert, 3× zoomed image.

(D) Cells expressing wild-type (WT) or constitutively active (Q111L) EGFP-Rab34 were fixed and stained for occludin (red) and ZO-1 (blue). (E) Quantification of occludin internalization in cells transfected with a GFP control plasmid (CON), or wild-type (WT), constitutively active (Q111L) EGFP-Rab34, dominant-negative (T66N) Rab34. Cells were either exposed to no virus (–CVB) or exposed to CVB (+CVB) for 90 min at 37°C. Data are representative of at least three independent experiments performed three times. Data in (E) are shown as mean  $\pm$  standard deviation. \*p < 0.05.

occludin and CVB internalization. Macropinocytosis, but not clathrin-mediated or caveolar endocytosis, is blocked by amiloride (an inhibitor of epithelial Na<sup>+</sup>/H<sup>+</sup> exchange) (Meier et al., 2002; West et al., 1989), and by rottlerin (a nonspecific inhibitor of protein kinase C and other kinases) (Sarkar et al., 2005). Whereas EIPA (ethyl-isopropyl-amiloride) and rottlerin had no effect on internalization of labeled transferrin or cholera toxin, markers of the clathrin and caveolin-mediated pathways (data not shown), both inhibitors prevented occludin internalization and dextran uptake (Figures 3C, 3D, and 3G), consistent with occludin entry in macropinosomes. In addition, both EIPA and rottlerin decreased CVB entry (Figure 3E) and infection (Figure 3F), leaving virus trapped at the TJ. EIPA and rottlerin did not inhibit CVB infection when added after viral entry had occurred (90 min p.i.) (Figure 3F), suggesting that macropinocytosis or a macropinocytosis-like process is required for CVB internalization, not a later event in infection. Although we have shown previously that CVB entry depends on functional caveolin (Coyne and Bergelson, 2006), these result suggest a role for macropinocytosis in CVB internalization and infection.

# Rab34, a GTPase Required for Macropinocytosis, Is Required for Occludin and CVB Internalization

Individual Rab GTPases are targeted to specific vesicular compartments and control specific events in endocytosis and endosomal trafficking (reviewed in Zerial and McBride,

2001). Rab34 has been linked specifically with macropinocytosis (Sun et al., 2003). In CVB-exposed Caco-2 cells, we observed endogenous Rab34 associated with occludin-rich membranes at the junction within 30 min p.i. (Figure 4A), but not at the apical or basolateral surfaces (data not shown). Endogenous (data not shown) and wild-type EGFP-Rab34 were expressed at the TJ of uninfected Caco-2 cells and colocalized with the TJ marker ZO-1 (Figure 4B). In addition to being concentrated at the TJ, EGFP-Rab34 was localized within cytoplasmic vesicles that colocalized with caveolin-1 (even in the absence of virus) (Figure 4C). Expression of either wild-type or constitutively active (Q111L) Rab34 led to pronounced internalization of occludin in resting cells (Figures 4D and 4E)-but not other junctional proteins (claudin-1, claudin-4, CAR, JAM-1, β-catenin, E-cadherin, and ZO-1 [Figure 4D and data not shown]) - and led to increased permeability to solutes through the paracellular space (Figure S3C). Upon exposure to CVB, internalization of occludin was further increased in Q111L-Rab34 transfected cells (from  $43\% \pm 12\%$  to  $82\% \pm 9\%$ ) (Figure 4E).

Both dominant-negative (T66N) Rab34 and Rab34 siRNA prevented CVB-induced macropinocytosis (Figures S3A, S3B, and S3D) but did not affect either transferrin or cholera toxin uptake (data not shown). Consistent with this, occludin internalization and CVB infection were markedly inhibited by T66N-Rab34 (Figures 4E and 5A). In addition, Rab34 siRNA, but not Rab7 siRNA, decreased

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# Figure 5. Rab34 Is Required for CVB Entry and Infection

(A) Monolayers expressing dominant-negative (T66N), constitutively active (Q111L), or wildtype forms of EGFP-Rab34 were exposed to CVB (1 PFU/cell), and stained for VP1 at 7 hr p.i. Data are presented as the percentage of transfected cells expressing VP1.

(B) Western blot analysis of Caco-2 cells 48 hr after transfection with the indicated siRNAs. All blots were stripped and reprobed with GAPDH pAb to control for equal protein loading. Densitometry was performed with ImageJ to determine efficacy.

(C) Cells transfected with control (CON), Rab34, Rab7, or Rab13 siRNAs were exposed to CVB (1 PFU/cell) and stained for VP1 at 7 hr p.i.

(D) Quantification of the effects of CON, Rab34, Rab7, and Rab13 siRNAs on CVB and occludin internalization. Data are shown as percentage of cells containing internalized CVB and occludin. (E) Cells transfected with control (CON), Rab34, or Rab13 siRNAs were exposed to CVB (100 PFU/cell) and stained for VP1 at 90 min p.i. Data are representative of at least three independent experiments performed three times. Data in (A), (C), and (D) are shown as mean  $\pm$  standard deviation. \*p < 0.05.

CVB infection (Figure 5C), reduced CVB-induced dextran uptake (Figure S3D), and significantly impaired occludin and CVB entry into the cytoplasm (Figures 5D and 5E). Although Rab13 has been shown to be involved in the continuous recycling of occludin from the tight junction into the cytoplasm (Morimoto et al., 2005), we found that Rab13 siRNA did not prevent CVB-induced macropinocytosis (Figure S3D), or inhibit occludin or CVB internalization (Figures 5D and 5E). These data suggest a role for Rab34, a component of macropinocytosis, in the entry of occludin and CVB.

# **Occludin Entry Depends on Rab5**

Rab5 is an important regulator of clathrin-mediated endocytosis, acting through multiple effectors to control internalization of clathrin-coated vesicles, and their fusion with early endosomes (Zerial and McBride, 2001). Rab5 has also been reported to target caveolar vesicles to early endosomes, and to regulate traffic between caveolae and caveosomes: constitutively active Rab5 leads to abnormal fusion of early endosomes and diverts caveolar traffic from caveosomes to the enlarged endosomes (Pelkmans et al., 2004). In addition, Rab5 signals have recently been implicated in membrane ruffling and macropinocytosis (Lanzetti et al., 2004; Schnatwinkel et al., 2004). We observed endogenous Rab5 localized to the junction and colocalized with internalized CVB (Figure S4A). We found that both dominant-negative (S34N) and constitutively active (Q79L) Rab5 blocked CVB infection (Figure 6A). Rab5 siRNA significantly decreased CVB infection (Figure 6C), as well as virus entry into the cytoplasm (Figure 6D). While Rab5-S34N trapped virus and occludin at the TJ and prevented entry into the cytoplasm, Rab5-Q79L trapped occludin and virus in large Rab5-positive vesicles (Figure 6B) within the cell. (Other cargoes internalized by caveolin-1 mediated mechanisms [such as SV40] are unaffected by Rab5-S34N [Pelkmans et al., 2004].) Rab5-S34N (data not shown) and Rab5 siRNA (Figure S3D) inhibited CVB-induced dextran uptake. Thus, Rab5 is an upstream regulator of CVB-induced macropinocytosis and is required for entry of both virus and occludin.

Rabankyrin-5, a Rab5 effector, has been shown to localize to and stimulate macropinocytosis in polarized epithelial cells (Schnatwinkel et al., 2004). We found that Rabankyrin-5 localized to 82%  $\pm$  13% of macropinosomes induced by CVB (Figure S4B), colocalized with internalized CVB and occludin (Figures S4C and S4D), and siRNA depletion of Rabankyrin-5 decreased CVB-induced macropinocytosis by 78%  $\pm$  12% (Figure S3D) and decreased CVB infection (Figure 6E). Although Rabankyrin-5 has been shown previously to specifically regulate apical macropinocytosis in polarized epithelial cells (Aoki et al., 1999; Schnatwinkel et al., 2004), our results indicate that it may also play a role in macropinocytosis from the TJ.

# Ras Activation Is Required for CVB-Induced Macropinocytosis and CVB Entry

The membrane ruffling required for macropinocytosis is often downstream of Rac and Ras GTPase activation (Bar-Sagi and Feramisco, 1986; Ridley et al., 1992; West et al., 2000). Expression of wild-type Rab34 stimulated macropinocytosis in resting cells and in response to CVB (Figure S3B). Macropinocytosis stimulated by Rab34 was not inhibited by a dominant-negative mutant of Rac1 (T17N) and was not stimulated by a constitutively active Rac1 mutant (Q61L) (Figure 7B). Furthermore,



# Figure 6. Rab5 Is Required for CVB Entry and Infection

(A) Monolayers expressing dominant-negative (S34N), constitutively active (Q79L), or wild-type forms of EGFP-Rab5 were exposed to CVB (1 PFU/ cell) and stained for VP1 at 7 hr p.i. Data are presented as the percentage of transfected cells expressing VP1.

(B) Monolayers expressing dominant-negative (S34N), constitutively active (Q79L), or wild-type forms of EGFP-Rab5 were exposed to CVB (100 PFU/ cell) and stained for occludin (blue) and VP1 (red) at 90 min p.i.

(C) Cells transfected with control (CON) or Rab5 siRNAs were exposed to CVB (1 PFU/cell) and stained for VP1 at 7 hr p.i. (Inset) Western blot analysis of cells 48 hr after transfection with CON or Rab5 siRNAs. Blot was stripped and reprobed with GAPDH pAb to control for equal protein loading. Densitometry was performed with ImageJ to determine efficacy.

(D) Cells transfected with CON or Rab5 siRNAs were exposed to CVB (100 PFU/cell) and stained for VP1 at 90 min p.i. The relative amount of CVB internalized is shown in white ( $95\% \pm 7\%$  for CON siRNA and  $14\% \pm 8\%$  for Rab5 siRNA).

(E) Cells transfected with control (CON) or Rabankyrin-5 siRNAs were exposed to CVB (1 PFU/cell) and stained for VP1 at 7 hr p.i. (Inset) Western blot analysis of cells 48 hr after transfection with CON or Rabankyrin-5 siRNAs. Data are representative of at least three independent experiments performed three times.

Data in (A), (C), and (E) are shown as mean  $\pm$  standard deviation. \*p < 0.05.

inhibitors of Rac1 expression and function (siRNA and NSC23766) had no effect on Rab34-mediated dextran uptake and internalization of occludin in response to CVB (data not shown). We have shown previously that these Rac inhibitors prevent CVB infection by blocking virus movement to the TJ (Coyne and Bergelson, 2006).

Internalized EGFP-Rab34-positive macropinosomes colocalized with coexpressed wild-type Ras in response to CVB (Figure 7A), and expression of dominant-negative Ras (S17N) blocked Rab34-induced macropinocytosis (by  $\sim$ 5-fold) (Figure 7B). In addition, both constitutively active (Q65L) and wild-type Ras expression increased the number of Rab34-positive macropinosomes (by  $\sim$ 2-fold and  $\sim$ 3-fold, respectively) (Figure 7B). These results suggest that Ras and Rab34 act synergistically to promote macropinocytosis in polarized Caco-2 cells, by a mechanism that does not involve Rac.

The level of activated (GTP-bound) Ras increased substantially at 10–20 min following exposure to CVB (Figure 7C), internalized CVB colocalized with Ras

(Figure 7E), and dominant-negative Ras decreased CVB infection (Figure 7D) and decreased virus internalization by 71%  $\pm$  12% (Figure 7E). Taken together, these data suggest that Ras activation is required for CVB entry.

# DISCUSSION

In the experiments reported here, we found that internalization of CVB occurs by a process that combines aspects of caveolar endocytosis with features more characteristic of macropinocytosis. We found that entry of CVB depended on the TJ protein occludin, and that virus induces internalization of occludin within macropinosomes. Both CVB entry (Coyne and Bergelson, 2006) and occludin internalization (this report) required caveolin but were independent of dynamin; both were blocked by drugs that inhibit macropinocytosis; and both required Rab34, a GTPase implicated in macropinosome formation, and Rab5, a GTPase known to stimulate macropinocytosis.



# Figure 7. Ras Is Required for CVB Entry and Infection

(A) Colocalization in macropinosomes (arrows) of EGFP-Rab34 (green) and constitutively active Ras (red).

(B) Caco-2 monolayers were transfected with plasmids encoding wild-type EGFP-Rab34, as well as constitutively active, or dominant-negative, Ras or Rac. Macropinosomes (defined as actin-coated Rab-34-positive vesicles) were counted. The graph shows the mean  $\pm$  standard deviation for four monolayers. NT, monolayers transfected with Rab 34 without Rac/Ras. \*p < 0.05 compared to NT.

(C) Monolayers exposed to CVB (100 PFU/cell) were lysed (at 0, 10, 20, or 30 min p.i.), and GTP-bound Ras was isolated. Equal amounts of whole-cell lysate were blotted to evaluate total Ras expression.

(D) Monolayers expressing dominant-negative (S17N) or wild-type forms of Ras were exposed to CVB (1 PFU/cell) and stained for VP1 at 7 hr p.i. Data are presented as the percentage of transfected cells expressing VP1.

(E) Monolayers expressing dominant-negative (S17N) or wild-type forms of Ras were exposed to CVB (100 PFU/cell) and stained for VP1 (red) and Ras (green) 90 min p.i. The relative amount of CVB internalized is shown in white ( $89\% \pm 14\%$  for WT Ras and  $26\% \pm 12\%$  for DN Ras). Data are representative of at least three independent experiments performed three times.

Data in (A) and (D) are shown as mean  $\pm$  standard deviation. \*p < 0.05.

The molecular mechanisms underlying macropinocytosis in polarized epithelia are poorly defined. Although macropinocytosis from the apical membrane has been linked to Src kinase activity (Mettlen et al., 2006), we found that a Src siRNA had no effect on CVB entry (Coyne and Bergelson, 2006) or occludin internalization (data not shown) from the TJ, suggesting that different mechanisms control macropinocytosis from the apical and lateral domains.

In fibroblasts, Rab34-mediated macropinocytosis has been shown to require the activity of Rac and the actin nucleation factor WAVE2 (Sun et al., 2003). In Caco-2, cells, we found that Rab34-induced macropinocytosis was Rac independent (Figure 7B) and was unaffected by a WAVE2 siRNA (data not shown). Although Rac was not required for macropinocytosis, CVB-induced activation of Ras was essential for virus entry and infection as well as for macropinocytosis induced by Rab34 (Figures 7B, 7D, and 7E). Ras-mediated signals have also been implicated in Rab5-dependent macropinocytosis (Lanzetti et al., 2004).

Few viruses appear to utilize macropinocytosis to gain entry into host cells. Human immunodeficiency virus-1 (HIV-1) enters both macrophages and brain endothelium by macropinocytosis (or a macropinocytosis-like process) (Liu et al., 2002; Marechal et al., 2001). Adenovirus type 2 (Ad2) utilizes Rac-dependent macropinocytosis not for entry into the host cell, but rather for pH-activated escape from endosomes into the cytoplasm (Meier et al., 2002). Although both Ad2 and CVB induce macropinocytosis, and both interact with CAR, CAR itself does not appear to be essential for induction. In the case of Ad2, macropinocytosis depends on virus interaction with its  $\alpha_v$ -integrin coreceptor (Meier et al., 2002), and we have found that CVB-induced macropinocytosis of occludin was not inhibited by CAR siRNA (data not shown). It may be that CAR and occludin provide different, but equally important,

functions in CVB entry—CAR is required for CVB uncoating (Coyne and Bergelson, 2006), and occludin may serve as a scaffold to recruit and anchor signaling or regulatory molecules in the vicinity of virus entry.

If macropinocytosis is required for CVB and occludin entry, why is caveolin also necessary? The pinching off of caveolar vesicles to internalize caveolar cargoes such as SV40 and cholera toxin depend on dynamin (reviewed in Parton and Simons, 2007), but the internalization of both occludin and CVB occurred even in the presence of dominant-negative dynamin (Figures 1E and 1F; Coyne and Bergelson, 2006). Caveolae have been implicated in the internalization of cargos such as bacteria and lymphocytes that are too large (>1  $\mu$ m for bacteria and > 7  $\mu$ m for lymphocytes) to be contained within a single caveolar vesicle (60-80 nm) (Millan et al., 2006; Mulvey and Hultgren, 2000). The internalization of large cargoes likely requires membrane rearrangements on a scale characteristic of macropinosome or phagosome formation. Caveolin-1 knockout mice display phagocytic defects, consistent with a role for caveolin-1 in endocytic processes distinct from internalization of typical caveolar vesicles (Li et al., 2005). Taken together, these observations have led to the suggestion that caveolin may serve to regulate a variety of endocytic processes (Parton and Simons, 2007).

The mechanism of CVB entry may be distinct from those described for other caveolin-dependent viruses because the virus enters through the TJ. TJs themselves share characteristics of lipid rafts, and TJ proteins have been shown to associate with caveolin-rich membranes (Nusrat et al., 2000b). However, the flask-like invaginations typical of caveolae are located primarily at the basolateral surface of resting polarized epithelial cells and are not generally observed at the TJ (Lahtinen et al., 2003; Mora et al., 1999; Vogel et al., 1998). In previous experiments, we did not detect a concentration of caveolin-1 at the TJ of resting Caco-2 cells. However, CVB interaction with DAF on the cell surface rapidly induced the phosphorylation of caveolin-1 on tyrosine 14 and the appearance of phosphorylated caveolin at the TJ, which was necessary for CVB entry (Coyne and Bergelson, 2006). Phosphorylation of tyrosine 14 of caveolin-1 has been associated with the formation and fusion of caveolin-derived vesicles (Aoki et al., 1999). Nonetheless, it remains unclear whether TJ-associated caveolin-1 leads to the formation of typical caveolae, or whether it may serve another function. The physical interaction of caveolin with cellular membranes may facilitate membrane perturbations associated with the formation and/or closure of macropinosomes at the junction. Alternatively, caveolin-1 may act as an anchor to concentrate signaling molecules or other effector molecules in the area of the TJ.

The TJ is a complex of transmembrane proteins (such as occludin) and cytoplasmic scaffolding proteins, both of which serve to link the cell membrane to the actin cytoskeleton and an array of signaling molecules. With such an elaborate network of proteins concentrated at the TJ, why is occludin specifically required for CVB infection? Occludin is not involved in CVB attachment, or in the relocalization of CVB to the TJ, but an occludin siRNA trapped virus at the TJ, suggesting that occludin regulates a key aspect of CVB entry. Occludin interacts directly with signaling molecules (Basuroy et al., 2006; Chen et al., 2002; Nusrat et al., 2000a), including the p85 subunit of PI3K, a known mediator of phagocytosis and macropinocytosis (reviewed in Lindmo and Stenmark, 2006). In addition, occludin interacts with caveolin-1 (Nusrat et al., 2000b), suggesting a possible functional connection. Although occludin has never been implicated as a regulator of endocytosis, it is possible that it promotes CVB entry by recruiting caveolin or other regulatory molecules to the TJ.

CVB enters polarized epithelial cells by a complex mechanism. The complexity may be due to the particular properties of polarized intestinal cells, or to the fact that CVB uses a receptor that is itself a component of the TJ. At least three viruses initiate infection by attaching to receptors within the junctional complex of polarized epithelia. Like CVB (and adenoviruses, which also bind to CAR), reoviruses (Barton et al., 2001) and members of the herpesvirus family (Geraghty et al., 1998) interact with receptors that are normally sequestered within junctions. In addition, recent data support a role for claudin-1 (a transmembrane component of the TJ with structural similarities to occludin) in a postattachment event associated with the entry of hepatitis C virus (HCV) (Evans et al., 2007). Claudin-1 is not known to bind HCV, and HCV infection may also require interaction of the virus with two coreceptors-the tetraspanin CD81 (Pileri et al., 1998) and the scavenger receptor class B member I (Scarselli et al., 2002)-neither of which is sufficient to promote infection. It is therefore conceivable that claudin-1 and occludin facilitate virus internalization by similar mechanisms. Defining the mechanism of CVB entry is likely to provide insights into the physiology of tight junction regulation as well as the entry mechanisms of other viral pathogens.

#### **EXPERIMENTAL PROCEDURES**

#### **Cells and Viruses**

Caco-2 cells, cultured as described (Coyne et al., 2004), were plated in collagen-BD BioCoat Collagen I 8-well culture slides (BD Biosciences, San Jose, CA) at a density of  $1 \times 10^5$  cells/well for immunofluorescence microscopy and in 12 mm Transwell-Col inserts (0.4 m pore size) (Costar) at a density of  $5 \times 10^5$  cells for permeability studies. Cells were grown a minimum of 48 hr prior to study.

CVB3-RD (Reagan et al., 1984) was originally obtained from Dr. Richard Crowell (Hahnemann University). Viruses were 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 were performed at a multiplicity of 100 PFU per cell. Experiments measuring productive virus infection were performed with 1 PFU/cell. The typical picornavirus particle/PFU ratios is approximately 200 (Rueckert, 1996).

## Antibodies

Mouse anti-enterovirus VP1 (NcI-Entero) was obtained from Novocastra Laboratories (Newcastle upon Tyne, UK). Monoclonal antibodies against ZO-1 and occludin and polyclonal antibodies against occludin, ZO-1, JAM, and claudin-1 were purchased from Invitrogen (Carlsbad, California). Polyclonal antibodies specific for Rab34, Ras, and Rab13 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal Rab5 and Rab7 antibodies were purchased from Sigma (St. Louis, MO). Monoclonal anti-phosphotyrosine antibody (4G10) was purchased from Upsate Biotechnology (Lake Placid, NY). Polyclonal anti-Rabankyrin-5 was provided by Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics). Alexa Fluor-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

#### **Pharmacological Inhibitors**

The amiloride analog ethyl isopropyl amiloride (EIPA) and rottlerin were purchased from Sigma and were used at 50  $\mu$ M and 1  $\mu$ M, respectively. Monolayers were pretreated with inhibitors for 1 hr at 37°C, washed, and incubated with CVB 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 hr at 4°C. Following washing, virus entry/infection, dextran uptake, and occluding internalization were initiated by shifting cells to 37°C in medium containing inhibitor. (Although toxicity can be associated with long-term exposure to EIPA, we did not observe an increase in apoptosis [as assessed by Annexin-V staining], or decrease in cell number [as assessed by light microscopy] over the course of treatment.)

# Plasmids, siRNAs, and Transfections

Wild-type GFP-caveolin-1 and dominant-negative caveolin-1-GFP (Pelkmans et al., 2001) were provided by Ari Helenius and Lucas Pelkmans (Swiss Federal Institute of Technology). HA-tagged wild-type or dominant-negative dynamin (van der Bliek et al., 1993) were provided by Sandra Schmid (Scripps Research Institute). mRFP1-occludin and EGFP-claudin-1 plasmids have been described (Shen and Turner, 2005). YFP-Rabankyrin-5 and wild-type, S34N, and Q79L Rab5 plasmids were provided by Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics). EGFP-tagged Rab34 constructs were provided by Wanjin Hong (Institute of Molecular and Cell Biology, Singapore). All Ras constructs were purchased from Upstate Biotechnology.

Rab13 SMARTpool siRNA reagent was obtained from Dharmacon (Chicago, IL). Rab7 siRNA (sc-29460) was purchased from Santa Cruz Biotechnology. Double-stranded siRNAs targeted against human claudin-1 (sense, 5'-UUCCAUAUUGAUGAAGAUGTT-3'), JAM-1 (5'-AGGCAUCACUAUCCCAUC UUUTT-3'), occludin (5'-GCAGCCAUG UACUCUUCACTT-3'), ZO-1 (5'-AAAUGAGGAUUAU CUCGUCTT-3'), and Rabankyrin-5 (5'-CUCUUUAGUGG AAGACAAGUU-3') were synthesized by Integrated DNA Technologies (Coralville, IA).

Transfection of Caco-2 and CHO cells with siRNAs and/or plasmids was performed using the Nucleofector System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol. Following transfection, cells were plated at a density of  $1 \times 10^5$  cells/well in collagen-coated chamber slides and used for virus infection 48 hr later. Transfection efficiencies with plasmids (as determined by GFP expression) were 60%–90%, and transfection with Cy5-labeled siRNA was 80%–100%.

#### Immunofluorescence Microscopy

Monolayers were exposed to CVB in virus-binding buffer for 1 hr at 4°C, then washed, and virus entry was initiated by shifting cells to 37°C. In all experiments, cells were fixed and permeabilized before staining with ice-cold methanol/acetone (3:1) for 5 min at room temperature (RT) or 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100 in PBS. Cells were incubated with the indicated primary antibodies for 1 hr at RT, washed, incubated with Alexa Fluor (AF)-594- or AF-488-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 (Leica, Exton, PA). Images were 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. For three-dimensional analysis, XZ or YZ series stacks were acquired at  ${\sim}0.5~\mu m$  intervals through the total thickness of the cell monolayer ( ${\sim}10~\mu m).$  Scale bar in figures is 10  $\mu m.$ 

Images were processed and quantified using the ImageJ program (http://rsb.info.nih.gov/ij/). Quantification of CVB internalization was determined by measuring the relative fluorescence intensity profiles (in arbitrary units) of CVB in the cytoplasm. Internalization was calculated according to the following equation: internalization = (F<sub>C</sub>)/(F<sub>TOTAL</sub>) x 100%, where F<sub>C</sub> is the relative fluorescence of CVB in the cytoplasm and F<sub>TOTAL</sub> is the total CVB fluorescence. Calculations were based on ~50 cells. Occludin internalization was determined in two ways: cells containing occludin within the cytoplasm were counted, and internalization was expressed as a percentage of the total cell number; alternatively, the fluorescence intensity of occludin within the cytoplasm was measured, and internalization was expressed as described above for CVB. Calculations were based on approximately 50 cells. For quantification of dextran uptake, the number of cells containing FITC-dextran was determined (for approximately 500 cells).

#### Immunoblots

Cell lysates were prepared with RIPA buffer (50 mM Tris-HCI [pH 7.4]; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethanesulfonyl fluoride; 1  $\mu$ g/ml aprotinin, leupeptin, and pepstatin; 1 mM sodium orthovanadate), and insoluble material was cleared by centrifugation for 5 min at 4°C. Lysates (30  $\mu$ g) was loaded onto 4%–15% Tris-HCl gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes. Membranes were blocked overnight in 5% nonfat dry milk or bovine serum albumin, probed with antibodies, and developed with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), and ECL reagents (Amersham Biosciences, Piscataway, NJ).

#### **Ras Activation Assay**

Monolayers were lysed in magnesium lysis buffer (Upstate Biotechnology) supplemented with 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium vanadate, and 1 mM sodium fluoride. To precipitate GTP-bound Ras, lysates (100–200 µg) were incubated with Raf-1 (RBD)-agarose for 1 hr at 4°C. After centrifugation, beads were washed in lysis buffer and then heated at 95°C for 10 min in Laemmli sample buffer. Supernatant was run on 15% Tris-HCl gels (Bio-Rad) and probed as described above. Total lysates (30 µg) were also immuno-blotted to measure the total amount of Ras.

#### **Statistical Analysis**

Data are presented as mean  $\pm$  standard deviation. A one-way analysis of variance (ANOVA) and Bonferroni's correction for multiple comparisons were used to determine statistical significance (p < 0.05).

#### Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and four supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/3/ 181/DC1/.

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