Regulation of polymeric immunoglobulin receptor expression by reovirus

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Polymeric immunoglobulin receptor (pIgR) transcytoses dimeric IgA and IgA-coated immune complexes from the lamina propria across epithelia and into secretions. The effect of reovirus infection on regulation of pIgR expression in the human intestinal epithelial cell line HT-29 was characterized in this report. Both replication-competent and UV-inactivated reovirus at m.o.i. equivalents of 1–100 p.f.u. per cell upregulated pIgR mRNA by 24 h post-infection and intracellular pIgR protein was increased at 48 h following exposure to UV-inactivated virus. Binding of virus to HT-29 cells was required, as pre-incubating virus with specific antisera, but not non-immune serum, inhibited reovirus-mediated pIgR upregulation. Endosomal acidification leading to uncoating of virus is a required step for pIgR upregulation, as ammonium chloride or bafilomycin A1 pre-treatment inhibited virus-induced pIgR upregulation. Inhibition experiments using the calpain inhibitor N-acetyl-leucyl-leucyl-norleucinal suggested that calpains are involved in reovirus-mediated pIgR upregulation. Upregulation of pIgR following virus infection appears to be an innate immune response against invading pathogens that could help the host clear infection effectively. Signalling induced by microbes and their products may serve to augment pIgR-mediated transcytosis of IgA, linking the innate and acquired immune responses to viruses.

INTRODUCTION

A hallmark feature of adaptive mucosal immune responses is the production and secretion of secretory immunoglobulin A (sIgA) (Brandtzaeg et al., 1997; Lamm, 1997; Kato et al., 2001). In the intestine, polymeric IgA produced by plasma cells in the lamina propria is transported across the adjacent mucusal epithelium into the lumen by the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface of intestinal epithelial cells (Brandtzaeg, 1978, 1985; Brandtzaeg et al., 1997). At the apical surface, pIgR is cleaved by an endopeptidase, releasing slgA into luminal secretions (Musil & Baenziger, 1987). The rate of IgA transcytosis depends on the level of pIgR expression, as one molecule of pIgR must be synthesized for each molecule of transported IgA (Mostov & Deitcher, 1986; Song et al., 1995; Tamer et al., 1995; Norderhaug et al., 1999). Thus, pIgR plays a role in mucosal host defence, and factors that influence expression of pIgR could affect mucosal immunity. A number of cytokines, including gamma interferon (IFN-γ) (Denning, 1996; Ackermann et al., 1999; Schjerven et al., 2000), tumour necrosis factor alpha (TNF-α), interleukin 1β (IL1β) (Blanch et al., 1999) and IL4 (Denning, 1996; Ackermann et al., 1999; Schjerven et al., 2000), increase pIgR expression in epithelial cells. However, little is known about the regulation of pIgR during enteric virus infection.

Mammalian orthorovirus (reovirus) is classified into three serotypes, designated T1, T2 and T3, based on expression of the viral haemagglutinin, which serves as the viral ligand for cellular receptors (Weiner & Fields, 1977). Reovirus infects the gastrointestinal tract in a wide range of mammalian species, including mice and humans (Organ & Rubin, 1998). Although both T1 and T3 infect hosts via the intestine, significant differences exist in the capacity of the serotypes to bind to intestinal epithelial cells and replicate in the intestine. Wolf et al. (1981, 1983, 1987) demonstrated that both T1 and T3 bind to microfold (M)-cells that overlie Peyer’s patches (PP) in the small intestine. This specific binding and uptake by PP exposes the virus to innate and adaptive immune cells in the intestine (Weltzin et al., 1989). Reovirus T3, but not T1, also binds to the apical surfaces of absorptive intestinal epithelial cells (IECs) and is endocytosed (Kauffman et al., 1983). It is unclear whether this is a ‘dead-end’ pathway for T3 virus. Endocytosed viruses appear to become trapped in lysosomes in IECs and basolateral transport has not been uniformly.
observed (Kauffman et al., 1983). On the other hand, at least in young animals, T3 infection induces villus shortening and mild mononuclear infiltration in the lamina propria (Branksi et al., 1980). Alterations in IECs from T3-infected neonates include reduction of lactase and enterokinase activities and increased maltase and leucine aminopeptidase activities (Branksi et al., 1980). Together, these results indicate that reovirus T3 can bind to and be taken up by IECs and affect IEC function, even though replication and virus shedding are limited.

Reoviruses induce robust mucosal and systemic mucosal immune responses (London et al., 1987; Cuff et al., 1993; Fan et al., 1998; Major et al., 1998). The intestinal cellular immune response is dominated by T-helper (Th) type 1 responses, resulting in local production of IFN-γ (Fan et al., 1998; Mathers & Cuff, 2004) and development of virus-specific CTL responses in the intestine and periphery (London et al., 1987, 1989, 1990; Fulton et al., 2004).

Rubin et al. (1985) reported that reovirus grows best in the rapidly developing cells of the crypts. In this model, virus is released from cells deep in the intestinal crypts and interacts with adjacent epithelial cells or is shed in the faeces. Productive infection is limited to immature crypt enterocytes, perhaps due to a requirement for an activated ras signalling pathway (Coffey et al., 1998; Strong et al., 1998).

Virus infection induces a number of changes in host-cell biology that could influence cell function. Interaction of reovirus cell attachment protein σ1 with its respective cell-surface receptors, including sialic acid and junctional adhesion molecule (JAM), triggers a number of signalling cascades, including increased activation of calpains (DeBiasi et al., 1999, 2001) and activation of c-Jun N-terminal kinase (JNK) and extracellular signal-related kinase (ERK) (Clarke et al., 2001). These signalling cascades result in upregulation of NF-κB activation (Tyler et al., 1995; Connolly et al., 2000; Hamamdzic et al., 2001) and phosphorylation of c-Jun (Clarke et al., 2001). Whilst these signalling pathways are involved in inducing apoptosis in infected cells, particularly during infection with T3 (Tyler et al., 1995), their roles in other reovirus-induced cellular changes have not been characterized extensively, although one report (Hamamdzic et al., 2001) has linked increased NF-κB activation with production of chemokines and cytokines during reovirus exposure. Significantly, most or all of these changes in cell signalling in vitro occur independently of virus replication, inasmuch as UV-inactivated virus mediates the observed effects (Tyler et al., 1995; DeBiasi et al., 1999; Hamamdzic et al., 2001). These studies indicate that, at least in vitro, replication-incompetent virus can have many effects on cellular processes.

Virus–host-cell interactions and some of the possible cell-signalling pathways involved in reovirus-mediated plgR upregulation are characterized in this report. Reovirus upregulated plgR expression in the transformed human intestinal epithelial cell line HT-29, and this upregulation was independent of virus replication. Binding of the virus to its cellular receptors was required for reovirus-mediated plgR upregulation, as treatment of virus with reovirus-specific serum abolished the increase in plgR expression. Endosomal acidification leading to uncoating of virus was necessary for virus-mediated plgR upregulation, as ammonium chloride blocked the process. Furthermore, treatment of HT-29 cells with a specific inhibitor of calpain abrogated reovirus-mediated plgR upregulation. These observations support the hypothesis that IECs upregulate plgR expression following exposure to enteric virus, possibly by altering cell-signalling pathways that control plgR expression.

**METHODS**

**Cells.** HT-29 (ATCC HTB-38) cells were cultured in McCoy’s 5A Modified Medium (ATCC) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Calu-3 (ATCC HTB-55) cells were cultured in Eagle’s minimum essential medium (ATCC) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated in T-25 flasks or 12-well plates and incubated at 37 °C for 24 h to allow the formation of adherent monolayers and then exposed to virus or UV-inactivated virus and/or inhibitors.

**Virus and antibodies.** Third-passage stocks of reovirus T3, strain Dearing (T3/D; originally obtained from ATCC), were prepared in L929 cells and purified by 1,1,2-trichloro-1,2,2-trifluoroethane (freon) extraction and CsCl gradient centrifugation (Smith et al., 1969). The concentration of virions in purified preparations was determined by spectrophotometry, where 1 absorbance unit at 260 nm = 2.1 × 1012 particles ml⁻¹ (Smith et al., 1969), and by plaque assays (Cuff et al., 1990). T3/D was exposed to UV light at 100 μl cm⁻² for 2 min at a distance of 15 cm at room temperature. UV inactivation reduced infectivity by approximately 1000-fold, as determined by a single-cycle infection assay. Polyclonal anti-T3/D antiserum was obtained from mice infected with 3 × 10⁶ p.f.u. purified T3/D virions 1 month previously. One millilitre of serum completely neutralized a minimum of 1 × 10⁹ p.f.u. virus, as assessed by in vitro neutralization (Cuff et al., 1990). In some experiments, UV-irradiated, virus-free L929-cell (L-cell) lysate (used at 4 × 10⁶ cell equivalents ml⁻¹ or 2 × 10⁴ cell equivalents per culture) or wheatgerm agglutinin (50 μg ml⁻¹; Biomedia Corporation) was used to treat HT-29 cells.

**RNA extraction and quantitative RT-PCR for plgR mRNA.** Total cellular RNA was extracted from HT-29 cells by using an RNasy Mini kit (Qiagen) and RNA was reverse-transcribed into cDNA by using Superscript RNase H⁻ reverse transcriptase (Invitrogen Life Technologies) according to the manufacturers’ protocols. Real-time PCR was performed by using a Lightcycler (Roche Molecular Biochemicals). Taqman primers and probes for human plgR (Applied Biosystems) were produced from published sequences (Blanch et al., 1999) and human β-actin was obtained from Biosource International. Reactions were carried out in a total volume of 20 μl and 2-5 μg BSA was added to each sample. PCR conditions were optimized for the human plgR and human β-actin primer pairs as follows: denaturation for 1 cycle at 95 °C for 15 s; enzyme activation for 1 cycle at 50 °C for 2 min, followed by 95 °C for 10 min; amplification for 55 cycles at 94 °C for 30 s, followed by 60 °C for 1 min; cool down for 1 cycle at 40 °C for 1 min. Data were analysed by determining ‘crossing points’ or the cycle number at which newly synthesized PCR product was first detected. Samples were analysed in duplicate. plgR expression was normalized with respect to β-actin expression by subtracting the β-actin crossing point from the plgR crossing point for each sample. Preliminary
experiments demonstrated that mRNA could be reverse-transcribed quantitatively to cDNA and amplified by PCR over at least a 64-fold range of concentrations (see Supplementary Fig. S1, available in JGV Online). The fold induction of plgR in virus-treated cells compared with the control was determined by using the following equation: fold change = K_mRNA \Delta C_T, where K_mRNA is the amplification coefficient for the plgR gene and ΔC_T is the difference in crossing point between the normalized non-treated and virus-treated HT-29 cells (Schjerven et al., 2000). The theoretical value of K_mRNA is 2 and preliminary experiments indicated that the K_mRNA value for this system ranged from 1.9 to 2.0 (see Supplementary Fig. S1 in JGV Online). Therefore, a K_mRNA value of 2 was used for data analysis.

**ELISA for cell-associated plgR.** Concentrations of plgR protein in cell lysates were determined by ELISA as described previously (Chintalacharuvu et al., 1991). Briefly, 96-well EIA/RIA (Costar) flat-bottom plates were coated with guinea pig anti-human secretory component (SC). Human SC purified from colostrum (Kobayashi, 1971) was used to generate standard curves. Purified SC was diluted in cell-lysis buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, with 1 mM PMSF and 5 μg aprotinin ml⁻¹ as protease inhibitors). Bound SC was detected with rabbit anti-human SC, followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and 100 μl 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (ABTS; Sigma) (0.3 mg ml⁻¹ in 0.1 M citric acid, pH 4.5), supplemented with 10 μl 30% H₂O₂ per 10 ml ABTS. Colour development was assessed by measuring A₄₀₅. Total protein concentration in cell lysates was determined by using a Bicinchoninic Acid Protein (BCA) Assay Reagent kit (Pierce Biotechnology) according to the manufacturer’s protocol. Data were expressed as ng plgR (mg total protein)⁻¹.

**Inhibition of endosomal acidification.** Ammonium chloride (Sigma) is an inhibitor of endosomal acidification that reduces intracellular digestion of reovirus outer-capsid proteins and blocks reovirus-induced apoptosis (Canning & Fields, 1983; Sturzenbecker et al., 1987; Connolly & Dermody, 2002). Bafilomycin A1 (Sigma) is a specific inhibitor of vacuolar protein ATPas (Bowman et al., 1988; Yoshimori et al., 1991; Hacker et al., 1998) and blocks reovirus replication (Martinez et al., 1996). Adherent monolayers of HT-29 cells grown at 37°C in 12-well plates were pre-treated for 1–5 h with various doses of ammonium chloride, or for 1 h with bafilomycin A1 or DMSO (vehicle control). Inhibitor-containing medium was then removed and the cells were cultured in the presence or absence of UV-inactivated reovirus at an m.o.i. equivalent of 50 p.f.u. per cell for 1 h. The m.o.i. equivalent was calculated based on the concentration of replication-competent virus prior to UV inactivation. Virus-exposed cells were then incubated in medium that contained inhibitor. mRNA was isolated 24 h after exposure to virus. Inhibitors used at the doses indicated were not visibly toxic to the cells.

**Inhibition of calpains.** Calpain inhibitor 1 [N-acetyl-leucyl-leucyl-norleucinal (aLLN); Calbiochem] is a modified peptide that competes for the active site of calpain (Wang & Yuen, 1994) and was prepared as a 25 mM stock in DMSO. Adherent monolayers of HT-29 cells were pre-treated with the indicated doses of aLLN or DMSO for 1 h. Inhibitor-containing medium was then removed and cells were exposed to UV-inactivated reovirus at an m.o.i. equivalent of 50 p.f.u. per cell. After incubation for 1 h, inhibitors were added back to each culture (DeBiasi et al., 1999) and mRNA was isolated at 24 h after exposure to virus. Intracellular plgR protein was isolated at 48 h after exposure to virus. The inhibitor aLLN used at the doses indicated was not visibly toxic to the cells.

**Immunofluorescence analysis.** HT-29 cells were grown on coverslips or in T-25 flasks overnight and treated with 50 mM NH₄Cl or 10 μM aLLN at 37°C. Cells were pulsed with virus at a particle-to-cell ratio of approximately 10⁵ and then fixed for 30 min and kept cold during subsequent staining. Additionally, all stains and washes were performed in solutions containing 0.04% (w/v) sodium azide to inhibit endocytosis. Cells were analysed by using a Becton Dickinson FACScan and Cell Quest Pro software (BD Biosciences).

For intracellular staining experiments, virus-infected cells were incubated for 1–2 h at 37°C. Cells on coverslips were fixed for 15 min in PBS supplemented with 4% paraformaldehyde and 1% Triton X-100 and then stained for 30 min with a 1:500 dilution of rabbit anti-reovirus antisera diluted in 1% Triton X-100/PBS. The rabbit antisera was previously raised in this laboratory. Coverslips were then washed in 1% Triton X-100/PBS and stained for 15 min with a 1:100 dilution of Alexa 488-anti-rabbit IgG (Molecular Probes) diluted in 1% Triton X-100/PBS. Coverslips were dried, mounted and analysed with a laser-scanning confocal microscope (Zeiss LSM 510).

**Statistical analysis.** Data from multiple experiments were expressed as the mean ± SEM. The statistical significance of differences between mean values was assessed by either two-way analysis of variance (ANOVA) followed by Tukey’s test or one-way ANOVA followed by orthogonal contrast to test for both a trend in dose response and the threshold at which the effect appeared. A value of P<0.05 was considered significant.

**RESULTS**

**Reovirus upregulates plgR mRNA and protein in HT-29 cells**

To determine whether reovirus is capable of modulating plgR expression in epithelial cells, infectious reovirus T3/D or UV-inactivated virus was adsorbed to confluent monolayers of HT-29 cells at an m.o.i. equivalent of 1, 10 or 100 p.f.u. per cell and plgR mRNA was measured 24 h post-virus exposure by RT-PCR. No cytopathic effect was observed in cultures of cells exposed to replication-competent or UV-inactivated virus for up to 48 h culture. Cytopathic effect was observed at 72 h post-infection with replicating virus; no cytopathic effect was observed with UV-irradiated virus. Infectious virus increased plgR mRNA levels by two- to eightfold over several experiments. However, UV-inactivated virus was substantially more effective at upregulating plgR mRNA expression at equivalent amounts of virus (Fig. 1). No significant increase in plgR mRNA was detected in cells treated with UV-irradiated L-cell lysates or wheatgerm agglutinin (Fig. 2), a ligand for sialic acid that serves as one of the cellular receptors for reovirus.

To determine whether the increased mRNA levels resulted in increased plgR protein production, HT-29 cells were cultured in the presence or absence of infectious virus or UV-inactivated virus at an m.o.i. equivalent of 50 p.f.u. per cell and intracellular plgR protein was measured in cell lysates by ELISA (Fig. 3). Increased levels of intracellular plgR were detected by 48 h reovirus exposure. Thus, the elevation in protein levels followed the increased mRNA
levels by 24 h. As with the mRNA, UV-inactivated virus had a more pronounced effect than replicating virus. Immunoblot analysis additionally demonstrated up-regulation of pIgR protein by UV-treated reovirus or IFN-\(\gamma\) (see Supplementary Fig. S2 in JGV Online).

Virus-specific antiserum blocks the effect of reovirus on pIgR upregulation

To ascertain whether increased pIgR mRNA requires virus binding to the host cells, UV-inactivated T3/D was pre-incubated with T3/D-immune or non-immune mouse serum prior to use on HT-29 cells. T3/D-immune but not non-immune serum blocked the virus-induced up-regulation of pIgR, indicating that virus binding to HT-29 cells is required to upregulate pIgR (Fig. 4).

Inhibition of endosomal acidification blocks reovirus-mediated pIgR upregulation

Reovirus is endocytosed following binding to its cellular ligands, sialic acid and JAM (Borsa et al., 1979, 1981; Sturzenbecker et al., 1987; Rubin et al., 1992; Barton et al., 2001b; Forrest et al., 2003; Prota et al., 2003). Acidification leads to partial uncoating of the virus inside endosomes, resulting in production of intermediate subviral particles that translocate to the cytoplasm and initiate transcription of viral genes (Borsa et al., 1979, 1981; Sturzenbecker et al., 1987). To examine whether endosomal acidification is
required for reovirus-mediated plgR upregulation, HT-29 cells were pre-treated for 90 min with various doses of ammonium chloride. Inhibitor-containing medium (or control medium) was then removed and the cells were cultured in the presence or absence of UV-inactivated reovirus at an m.o.i. equivalent of 50 p.f.u. per cell. Ammonium chloride was then added back to each well and plgR mRNA levels were measured 24 h after exposure to virus. Ammonium chloride blocked virus-mediated pIgR mRNA upregulation in a dose-dependent manner (Fig. 5), suggesting that endosomal acidification leading to uncoating of virus plays a role in plgR upregulation. Bafilomycin A1, another inhibitor of endosomal acidification that acts through inhibition of vacuolar proton ATPase (Bowman et al., 1988; Yoshimori et al., 1991; Martinez et al., 1996; Hacker et al., 1998), also blocked reovirus-induced plgR upregulation (Fig. 6).

Calpain inhibition abrogates reovirus-mediated plgR upregulation

Calpains, calcium-dependent cysteine proteases, induce NF-κB activation through the degradation of its cytosolic...
upregulation (Fig. 7a). Under similar culture conditions, UV-inactivated T3/D failed to upregulate intracellular plgR protein in cells treated for 48 h with 1–10 μM aLLN (Fig. 7b, and Supplementary Fig. S3 in JGV Online), suggesting a role for calpain activation in plgR upregulation.

Neither ammonium chloride nor aLLN affected binding of virus to cellular receptors, as determined by flow-cytometric analysis of cells pulsed with virus at 4°C in the presence of sodium azide (see Supplementary Fig. S4 in JGV Online). Additionally, when inhibitor-treated, virus-infected cells were incubated at 37°C to allow endocytosis, bright intracellular perinuclear staining of reovirus was observed (Fig. 8).

**DISCUSSION**

plgR contributes to the host defence of mucosal surfaces by mediating efficient transport and secretion of IgA. How mucosal pathogens affect plgR expression to the advantage of either the host or the pathogen is not clear. These studies are the first to show that reovirus increases plgR mRNA and protein in mucosal epithelial cells. The results indicate that virus binding to cellular ligands and uncoating in acidified endosomes are required steps for virus-mediated plgR upregulation through a calpain-mediated pathway. UV-inactivated T3/D reovirus induced higher levels of plgR expression than infectious virus, possibly because replicating virus usurps or inhibits normal host-cell mRNA and protein synthesis to produce infectious virions (Kudo & Graham, 1966; Ensminger & Tamm, 1969; Zweerink & Joklik, 1970; Sharpe & Fields, 1981, 1982).

Preliminary studies indicated that both replication-competent and UV-inactivated T1 (strain Lang) was less efficient than T3/D at inducing plgR upregulation (data not shown). It has long been thought that, during T1 infection, viral entry into IECs occurs through the basolateral surface (Rubin et al., 1985), probably as a result of T1 binding to linked sialic acid on M-cell apical surfaces (Helander et al., 1983, 1987). Together, these observations imply that perhaps sialic acid-mediated binding and entry are critical for plgR upregulation. However, it has recently been reported that T1 can also bind to glycoconjugates containing α-2-3-linked sialic acid on M-cell apical surfaces (Helander et al., 2003). Therefore, the role of sialic acid binding in mediating plgR upregulation remains to be determined. Approaches to assess the role of sialic acid include using reoviruses deficient in the ability to bind sialic acid and pre-treating HT-29 cells with sialidase. Replication of virus in host cells is not required for reovirus-mediated plgR upregulation, implying that this innate immune response can occur in the absence of significant virus replication.

Reovirus infection of the respiratory tract induces various types of pathology, including acute pneumonia in juvenile mice (Morin et al., 1994, 1996). Reovirus also upregulated plgR mRNA expression 15-fold in the transformed human

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**Fig. 7.** Calpain inhibition abrogates reovirus-mediated plgR upregulation in HT-29 cells. (a) HT-29 cells were pre-treated for 60 min with the indicated doses of the calpain inhibitor aLLN. Medium containing inhibitor (or control DMSO) was then removed and cells were cultured in the presence or absence of UV-inactivated reovirus at an m.o.i. equivalent of 50 p.f.u. per cell. After 1 h incubation, inhibitors were added back to each well and plgR mRNA was quantified at 24 h post-exposure. Data are expressed as fold increases in plgR mRNA in response to virus compared with cells receiving no virus at each dose of aLLN (mean±SEM). Data were compiled from three independent experiments. Asterisks indicate that the mean is significantly different from that of control cells (no pre-treatment with aLLN) as determined by one-way ANOVA followed by orthogonal contrast (P<0.05). (b) Under identical culture conditions, cells were lysed at 48 h after exposure to virus and plgR protein levels were determined by ELISA. Data are from a single experiment. Data from an additional similar experiment can be found as Supplementary Fig. S3 in JGV Online.

Repressor, inhibitory κBz (κBζ). DeBiasi et al. (1999, 2001) demonstrated that calpain inhibitors reduced reovirus-mediated calpain activation in vitro and in vivo. To determine whether calpains play a role in reovirus-mediated upregulation of plgR, HT-29 cells were pre-treated for 1 h with the calpain I inhibitor aLLN at doses of 0.001–10 μM or with the vehicle control DMSO. Medium containing inhibitor (or control medium) was then removed and the cells were cultured in the presence or absence of UV-inactivated reovirus at an m.o.i. equivalent of 50 p.f.u. per cell. After 1 h incubation, inhibitors were added back to each well and plgR mRNA was measured 24 h after exposure to virus. Virus-mediated upregulation of plgR mRNA was inhibited by aLLN in a dose-dependent manner, suggesting that calpains play a role in virus-induced plgR.
airway epithelium cell line Calu-3, whereas UV-inactivated virus upregulated plgR mRNA expression by as much as 25-fold under similar conditions (see Supplementary Fig. S5 in JGV Online), suggesting that plgR upregulation in epithelial cells from both the respiratory tract and the intestine could clear infection more effectively by increasing transport of plgA in the mucosal secretions.

Virus infection of host cells involves multiple steps, including binding, uptake and replication. The requirements of receptor engagement and uptake for virus-mediated plgR upregulation were also investigated. T3/D-immune but non-immune serum blocked the upregulation of plgR, indicating that specific virus binding to HT-29 cells is required to induce plgR upregulation. Whether virus binding through JAM (Barton et al., 2001b; Forrest et al., 2003; Prota et al., 2003), sialic acid (Chappell et al., 1997, 2000; Barton et al., 2001a; Connolly et al., 2001; Connolly & Dermody, 2002; Helander et al., 2003), or both, is required for plgR upregulation is under investigation.

After binding, the virus undergoes receptor-mediated endocytosis (Borsa et al., 1979, 1981; Sturzenbecker et al., 1987; Rubin et al., 1992) and subsequent endosomal acidification leads to partial uncoating of virus inside the

**Fig. 8.** Neither ammonium chloride nor aLLN affects endocytosis of reovirus by HT-29 cells. (a) HT-29 cells grown on coverslips were pre-incubated for 1 h in the presence or absence of 50 mM ammonium chloride or 10 μM ALLN at 37 °C followed by exposure to reovirus for 30 min at a concentration of 10^6 particles per cell. Unbound virus was washed free and cells were incubated for an additional 90 min at 37 °C. Following incubation, cells were fixed with 4% paraformaldehyde and stained with rabbit anti-reovirus followed by Alexa 488–anti-rabbit IgG. Intracellular staining was visualized by using laser-scanning confocal microscopy through multiple z planes. Images shown are taken through central planes on the cells and demonstrate perinuclear staining of virus. Bar, 60 μm. (b) In a separate, similar experiment, HT-29 cells were pre-treated with inhibitors in T-25 flasks, stripped from the flask with trypsin/EDTA, pulsed with reovirus and incubated at 37 °C for 1 h. Cells were then fixed, permeabilized, stained for reovirus antigen and analysed by flow cytometry. Dashed lines indicate uninfected cells and solid lines indicate reovirus-infected cells.
Ammonium chloride blocked virus-mediated plgR upregulation, indicating that endosomal acidification leading to virus disassembly is necessary for plgR upregulation. Ammonium chloride-mediated inhibition of virus disassembly has previously been shown to block reovirus-induced apoptosis (Connolly & Dermody, 2002) without affecting binding or uptake of virus (Sturzenbecker et al., 1987), which was confirmed in this study. Bafilomycin A, an inhibitor of vacuolar proton ATPases, was previously shown to block reovirus replication in cells infected with whole virions, but not in cells infected with intermediate subviral particles (Martinez et al., 1996). Like ammonium chloride, bafilomycin A1 inhibited reovirus-infected plgR upregulation, further supporting the idea that viral uncoating is required for plgR upregulation. It is possible that, following degradation in the endosomes, liberated viral double-stranded RNA (dsRNA) interacts with intracellular toll-like receptor 3 (TLR-3) to induce intracellular signals that lead to plgR upregulation. TLR-3 is expressed constitutively in human intestinal epithelial cells (Cario & Podolsky, 2000), although the precise intracellular location of TLR-3 has not yet been identified. In human dendritic cells, TLR-3 is expressed intracellularly in vesicles and possibly at a low level in late endosomes (Matsumoto et al., 2003; Funami et al., 2004). Thus, TLR-3 signalling in response to reovirus dsRNA might be initiated following fusion of endosomes containing reovirus dsRNA from degraded virions with cellular vesicles containing TLR-3. However, during virus replication, it is possible that dsRNA synthesized inside newly formed capsids is shielded from intracellular TLR-3. Therefore, an alternative activation pathway, such as through TLR-7 in mice or TLR-8 in humans, which recognize viral single-stranded RNA (Diebold et al., 2004; Heil et al., 2004), might be responsible for the observed effects.

Alternatively, reovirus dsRNA may trigger cellular responses via a TLR-independent mechanism. dsRNA may bind to the cellular RNA helicase, retinoic acid-inducible gene 1 (RIG-1) (Li et al., 2003; Sumpter et al., 2005) or melanoma differentiation-associated gene 5 (mda-5) (Kang et al., 2002; Andrejeva et al., 2004) and subsequently activate latent transcription factors including IFN regulatory factor 3 and NF-κB. Activation of NF-κB results in increased transcription of the plgR gene (Ackermann et al., 1999; Schjerven et al., 2001; Ackermann & Denning, 2004). Because RIG-1 and mda-5 activate NF-κB and reovirus upregulates NF-κB activation, it is possible that one or both of these intracellular pathogen-receptor/signalling molecules mediates the effect of reovirus on plgR levels. These hypotheses are currently being investigated. UV inactivation of reovirus damages the dsRNA genome by bonding the pyrimidine molecules, which impairs replication and blocks transcription of early and late genes (Strong et al., 1998). However, it is not known whether there is residual transcription of viral mRNA in the UV-inactivated virus used in the experiments reported here.

The calpain inhibitor aLLN abolished virus-mediated plgR upregulation, suggesting an essential role for calpain activation in this process. Calpains are calcium-dependent cysteine proteases distributed widely throughout the cytosol of many cell types (Murachi, 1989). Calpain exists in the cytosol as an inactive pro-enzyme bound to its endogenous inhibitor, calpastatin (Croall & DeMartino, 1991). Upon activation, calpains degrade a variety of substrates, including proto-oncogenes, steroid-hormone receptors, protein kinases and cytoskeletal elements (Croall & DeMartino, 1991). Calpains also regulate a number of cellular transcription factors, including activation of NF-κB, by mediating degradation of its inhibitor, IκB (Liu et al., 1996; Chen et al., 1997). It seems reasonable to speculate that reovirus-induced plgR upregulation is mediated through NF-κB activation via a calpain-mediated pathway and this hypothesis is also under investigation. It has been speculated that aLLN can transiently affect endocytosis (Kamal et al., 1998), but no evidence was found to support that mechanism of action in the experiments reported here.

Enteroinvasive bacteria, including Salmonella dublin, enteroinvasive Escherichia coli and Yersinia enterocolitica can increase NF-κB activity in the human intestinal epithelial cell lines HT-29, Caco-2 and T84. NF-κB activation leads to enhanced transcription of an array of downstream inflammatory genes, including IL8, TNF-α and monocyte chemoattractant protein-1 (Elewaut et al., 1999). Thus, NF-κB serves as a central regulator of the intestinal epithelial cell innate immune responses to infection with enteroinvasive bacteria and perhaps enteric viruses as well.

Although not yet demonstrated, it is reasonable to speculate that reovirus infection leads to plgR upregulation in vivo. Reovirus replicates in the rapidly dividing cells of the intestinal crypts (Rubin et al., 1985) and virus replication appears to be restricted to host cells with an activated ras pathway (Strong et al., 1998). If only replicating virus induced plgR upregulation, then the effect in vivo would be limited to crypt cells. However, reovirus replication is not necessary for upregulating plgR expression, so reovirus could potentially modulate plgR levels in differentiated IECs lacking an activated ras pathway. At least in vitro, reovirus infection results in the production of replication-competent and replication-incompetent virus (Smith et al., 1969). This phenomenon probably occurs in vivo as well and could result in the production of particles that would efficiently induce plgR expression in IECs independently of virus replication. Although it could be questioned whether UV-inactivated virus is equivalent to normally occurring, replication-incompetent virus, UV-inactivated virus has been used as a surrogate for replication-incompetent virus in vitro (Farone et al., 1993; Tyler et al., 1995; DeBiasi et al., 1999; Hamamdzic et al., 2001; Labrada et al., 2002) and in vivo (Rubin et al., 1981).

Upregulation of intestinal plgR mRNA expression has been reported in formerly germ-free mice colonized with Bacteroides thetaiotaomicron (Hooper et al., 2001). In
addition, plgR mRNA and protein expression are upregulated in mammalian epithelial cells in sheep in response to exogenous hormones and glucocorticoid treatment (Rincheval-Arnold et al., 2002b), and this effect could be mediated through an IFN-γ-linked mechanism (Rincheval-Arnold et al., 2002a). Lamina propria mononuclear cell-derived IFN-γ has been shown to upregulate plgR expression in HT-29 cells (Youngman et al., 1994). Thus, along with the direct effects of the virus, reovirus infection induces IFN-γ expression in the intestine (Fan et al., 1998; Mathers & Cuff, 2004), and thus, cytokine-mediated upregulation of plgR might be operative in vivo. Virus-induced upregulation of plgR that augments IgA transcytosis could be an innate host-defence mechanism against mucosal pathogens.

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REFERENCES


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