ORIGINAL ARTICLE

Oncolytic reovirus induces intracellular redistribution of Ras to promote apoptosis and progeny virus release

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Reovirus is a naturally oncolytic virus that preferentially replicates in Ras-transformed cells and is currently undergoing clinical trials as a cancer therapeutic. Ras transformation promotes reovirus oncolysis by enhancing virion disassembly during entry, viral progeny production, and virus release through apoptosis; however, the mechanism behind the latter is not well understood. Here, we show that reovirus alters the intracellular location of oncogenic Ras to induce apoptosis of H-RasV12-transformed fibroblasts. Reovirus infection decreases Ras palmitoylation levels and causes accumulation of Ras in the Golgi through Golgi fragmentation. With the Golgi being the site of Ras palmitoylation, treatment of target cells with the palmitoylation inhibitor, 2-bromopalmitate (2BP), prompts a greater accumulation of H-RasV12 in the Golgi, and a dose-dependent increase in progeny virus release and subsequent spread. Conversely, tethering H-RasV12 to the plasma membrane (thereby preventing its movement to the Golgi) allows for efficient virus production, but results in basal levels of reovirus-induced cell death. Analysis of Ras downstream signaling reveals that cells expressing cycling H-RasV12 have elevated levels of phosphorylated JNK (c-Jun N-terminal kinase), and that Ras retained at the Golgi body by 2BP increases activation of the MEK1/MKK4/JNK signaling pathway to promote cell death. Collectively, our data suggest that reovirus induces Golgi fragmentation of target cells, and the subsequent accumulation of oncogenic Ras in the Golgi body initiates apoptotic signaling events required for virus release and spread.

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INTRODUCTION

Reovirus is a naturally oncolytic virus that preferentially replicates in cells harboring an activated Ras signaling pathway.^{1,2} Constitutive Ras activity is implicated in 30% of all human cancers, and its associated downstream effectors increase that frequency to 50%.³ Based on its demonstrable ability to target and kill human cancer cells both *in vitro* and *in vivo*, reovirus is currently undergoing clinical trials as a cancer therapeutic.^{4–6} We previously established that Ras transformation promotes reovirus oncolysis by enhancing proteolytic disassembly of the parental virion during entry, production of infectious viral progeny, virus release through apoptosis and subsequent cell-to-cell virus spread through inhibited interferon production.^{7,8} Although these studies identified specific aspects of the reovirus life cycle that were enhanced through Ras activity, they did not consider any changes to the Ras protein itself in the context of reovirus oncolysis.

There are three human *ras* genes that encode four proteins: Harvey (H)-Ras, Neuroblastoma (N)-Ras, Kirsten (K)-Ras4A and K-Ras4B.^{9,10} Ras proteins function as molecular switches and bind GDP or GTP to cycle between inactivated and activated states, respectively. Each Ras protein requires posttranslational modifications to increase its hydrophobicity and enable an otherwise globular and hydrophilic protein to participate effectively in intracellular trafficking, membrane binding, and biological activation.¹¹ The C terminus of each Ras protein contains a CAAX motif that directs the protein for irreversible prenylation by the cytosolic farnesyltransferase.¹² Farnesylation facilitates Ras to

interact transiently with cellular membranes, and as such, it is at the endoplasmic reticulum (ER) that the Ras CAAX sequence is further modified by endoproteolytic cleavage and carboxymethylation.^{13–15} Ras isoforms require a second signal to associate stably with membranes and this occurs in the form of an additional lipid modification or inherent amino-acid charge within their sequence.¹⁶ With the exception of K-Ras4B, which uses polybasic lysine residues in its C terminus for stable membrane binding, the remaining Ras proteins undergo palmitoylation by the palmitoyl acyltransferase, DHHC9-GCP16, at the Golgi body.¹⁷ The Ras palmitoyl acyltransferase adds one or two palmitoyl groups to the cysteine residues directly upstream of the modified CAAX sequence. Palmitoylation increases Ras membrane affinity by > 100-fold and allows Ras to transit from the Golgi body to the plasma membrane via vesicular transport.¹⁸ Unlike farnesylation, palmitoylation is reversible. To leave the plasma membrane, Ras must first be primed by FKBP12 before it undergoes depalmitoylation by the putative acyl-protein thioesterase-1.^{19,20} Depalmitoylated Ras is then forced to cycle back to the Golgi body for another round of palmitoylation.^{21,22} To this extent, GTP-bound Ras is able to interact with both plasma membrane microdomains and intracellular membranes to signal to specific downstream effectors and regulate essential cellular processes, such as growth, differentiation and death.^{23–25} Ras proteins linked to human cancers often harbor single amino-acid substitutions, typically at sites 12, 13 and 61, resulting in mutated Ras proteins that remain GTP-bound and constitutively activated.²⁶ As a result, signaling to

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downstream effectors becomes deregulated and can lead to cell transformation and cancer development.

In the present study, we investigate whether reovirus infection alters the intracellular location of oncogenic Ras to induce apoptosis of Ras-transformed fibroblasts, thereby promoting progeny virus release and viral spread. We found that reovirus-induced fragmentation of the Golgi body and caused oncogenic Ras to accumulate in this organelle. The use of a palmitoylation inhibitor prompted further accumulation of Ras in the Golgi body and acted synergistically with reovirus infection to promote cell death and virus release through activation of the MEKK1/MKK4/JNK (MAP kinase kinase kinase 1/MAPK kinase 4/c-Jun N-terminal kinase) signaling pathway. Preventing the movement of Ras to the Golgi body through plasma membrane tethering or FKBP12 inhibition reduced virus-related death to levels comparable to the non-transformed control. This study identifies the mechanism behind reovirus oncolysis, and sheds further light on Ras compartmentalized signaling and the importance of Ras intracellular location on the outcome of the cell.

RESULTS

Reovirus infection redistributes oncogenic Ras through decreased palmitoylation

We first assessed whether reovirus infection caused any variations to Ras localization. Using immunofluorescence, H-RasV12 was clearly detected in the Ras-transformed cells and showed distinct plasma membrane and endomembrane localization (Figure 1a, top panels). In contrast, endogenous Ras in non-transformed cells appeared uniformly distributed within the cytoplasm (Supplementary Figure S1A). Reovirus infection of H-RasV12-transformed cells altered the normal distribution of oncogenic Ras by causing it to accumulate in non-overlapping, punctate structures, whereas no change in Ras distribution was observed in non-transformed cells (Figure 1a (bottom panels) and Supplementary Figure S1A (upper two panels)). Furthermore, fractionation of reovirus-infected cells into membrane and soluble fractions indicated an increase in cytosolic Ras during the first 18 h of infection (Figure 1b). Co-immunoprecipitation analyses using anti-Ras and anti-reovirus antibodies did not show any association between Ras and reovirus proteins during reovirus infection of Ras-transformed cells (Supplementary Figure S1B).

To determine if Ras activity fluctuated during reovirus infection, the level of GTP-bound Ras at various times postinfection was assessed.²⁷ Expression of total Ras levels, inclusive of all GDP- and GTP-bound Ras isoforms, remained constant during infection; however, activated (GTP-bound) Ras levels were slightly upregulated in reovirus-infected cells when compared with the uninfected control (Figure 1c). The most notable change was the large gel mobility shift that occurred between 6 and 12 hpi (hour postinfection) (Figure 1c, left panel). As lipidated Ras migrates faster than its non-lipidated form during sodium dodecyl sulfate–polyacrylamide gel electrophoresis owing to the increased hydrophobicity provided by the lipid group,^{16,28} an upward gel mobility shift of H-RasV12 implies the removal of lipid groups from the oncogenic protein. If this was the case, then treatment of the pull-down samples with the reducing agent, β ME (β -mercaptoethanol), should cleave the thioester linkage between H-RasV12 and its palmitoyl groups, and abolish the gel mobility shift.²⁹ This was indeed found to be the case (Figure 1c, middle panel), suggesting that reovirus infection likely modulates Ras palmitoylation.

Recently developed acyl-biotin exchange chemistry was then performed to quantify the amount of Ras palmitoylation following reovirus infection.³⁰ H-RasV12xx was engineered to be palmitoylation-deficient (C181S and C184S) and served as a control for the experiment. As expected, hydroxylamine removed

the palmitoyl groups from H-RasV12 and caused the protein to migrate to levels comparable to its palmitoylation-deficient counterpart (Figure 1d, left). Furthermore, when compared with the uninfected control, reovirus infection of H-RasV12-transformed cells decreased Ras palmitoylation levels by 29% (Figure 1d (right) and Supplementary Figure S1C).

Reovirus induces Golgi fragmentation and accumulation of Ras in the trans-Golgi network

As Ras is palmitoylated at the Golgi body, we next examined the structural integrity of this organelle in reovirus-infected cells using confocal microscopy. The typical perinuclear, crescent formation of the Golgi body was clearly discernible in uninfected cells; however, the perinuclear stacks were broken up into punctate structures in reovirus-infected cells, which is an indication of early Golgi fragmentation (Figure 2a and Supplementary Figure S2A). Golgi fragmentation is usually observed during mitosis, but it can also occur as an early apoptotic event or through cytoskeletal interference.^{31–33} In support of the latter, uninfected H-RasV12 cells were treated with nocodazole to disrupt microtubules and induce their depolymerization. Similar to reovirus infection, the Golgi complex formed punctate structures in the presence of the drug (Supplementary Figure S2B). It is well known that reovirus uses cytoskeletal elements to build viral inclusion bodies and that the structural protein, μ 2, associates with microtubules.^{34,35} It is likely the formation of perinuclear reovirus factories and the lytic nature of the virus that contribute to Golgi fragmentation.

Oncogenic H-Ras is typically associated with the plasma membrane, but when its palmitate group is removed, it cycles back to the Golgi body for another round of palmitoylation. Based on the above observation that reovirus infection decreased the palmitoylation levels of H-RasV12 and caused Golgi fragmentation, it was likely that Ras accumulation in reovirus-infected cells occurred at the ER or the Golgi. Confocal immunofluorescence microscopy of reovirus-infected H-RasV12-transformed cells revealed colocalization of Ras punctate structures with the trans-Golgi marker, TGN38 (Figure 2b). Therefore, reovirus-induced fragmentation of the Golgi body leads to the accumulation of H-RasV12 in this organelle.

2BP enhances accumulation of H-RasV12 in the Golgi body and targets Ras palmitoylation to promote reovirus spread

With reovirus infection decreasing Ras palmitoylation levels and causing H-RasV12 to accumulate in the Golgi body, it was speculated that use of a palmitoylation inhibitor could provide synergistic effects to reovirus oncolysis. 2-Bromopalmitate (2BP) is commonly used as a Ras palmitoylation inhibitor and is believed to target the Ras palmitoyl acyltransferase, DHHC9-GCP16.³⁶ Although it is more effective at preventing the palmitoylation of monopalmitoylated N-Ras, 2BP can alter the membrane localization of oncogenic H-Ras by ~20%.^{23,37} Using confocal microscopy, 2BP treatment of reovirus-infected H-RasV12 cells resulted in two times the amount of Ras puncta formation, which once again showed colocalization with the Golgi body (Figures 3a and b). Uninfected cells treated with 2BP also showed Ras-TGN colocalization; however, the distinct punctate formation was not observed. Reovirus-infected cells treated with 2BP were also stained with the ER marker, calnexin, and despite the formation of numerous Ras puncta, ER colocalization was not observed (Figure 3a, lower panels). Additionally, neither reovirus infection nor 2BP treatment caused puncta formation in non-transformed cells (Supplementary Figure S1A).

To assess the effect of 2BP on reovirus oncolysis, plaque assays were performed. Non-transformed and H-RasV12-transformed cells were infected with reovirus and treated with increasing concentrations of 2BP (Figure 3c). Consistent with previous data, reovirus spread was enhanced in Ras-transformed cells.⁷ In the

Figure 1. Reovirus infection modifies oncogenic Ras and increases its level of depalmitoylation. H-RasV12-transformed cells were mock- or reovirus-infected (multiplicity of infection (MOI) 20) and collected at the indicated hpi. **(a)** At 12 hpi, the fixed cells were probed using anti-Ras (green) and anti-reovirus (red) antibodies, and visualized under 1000x magnification by confocal microscopy. White squares highlight regions that are enlarged under the 'Zoom' heading. Arrows indicate Ras plasma membrane localization in uninfected (UI) cells and punctate formation following reovirus infection. Scale bar: 10 μ m. **(b)** At the indicated hpi, the cells underwent membrane/cytosolic fractionation, and subsequent western blot probing for Ras. Whole-cell lysates were probed for reovirus proteins (denoted as: λ , μ and σ). **(c)** Mock- or reovirus-infected cell lysates were incubated with Raf-1 RBD-conjugated agarose beads to pull-down GTP-bound Ras. The resulting pull-down was treated $\pm$ β -mercaptoethanol and a subset of the pull-down (10% of total) underwent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and anti-Ras western blot probing. Whole-cell lysates were used to detect the total amount of Ras and reovirus proteins at each time point. β -Actin served as a loading control. Arrows indicate the processed (P) and unprocessed (UP) forms of Ras. **(d)** H-RasV12- and H-RasV12xx-transformed cells were mock- or reovirus-infected (MOI 100) and collected at 12 hpi for acyl-biotin exchange chemistry. Cell lysates were immunoprecipitated using anti-Ras antibodies and treated with either phosphate-buffered saline (PBS) (control) or hydroxylamine (H) to remove palmitate groups from Ras. All samples underwent acyl-biotin exchange chemistry and the resulting western blots were probed with anti-Ras and streptavidin, and analyzed by densitometry (paired *t*-test $\pm$ s.e.; $n = 3$). C, beads+anti-Ras (no cell lysate); UI, uninfected; V, reovirus infected.

presence of 2BP, reovirus plaque size markedly increased in a concentration-dependent manner in H-RasV12-transformed cells. In contrast, no enhancement was observed in the non-transformed cells. Collectively, the data suggest that 2BP causes further accumulation of H-RasV12 in the Golgi body of

reovirus-infected cells and that this correlates with enhanced reovirus oncolysis and viral spread.

It was important to determine whether 2BP was acting on Ras palmitoylation, and furthermore, which stage(s) it was enhancing in the reovirus life cycle. To this end, we used cell

Figure 2. Reovirus infection causes Golgi fragmentation and accumulation of H-RasV12 in the trans-Golgi network. H-RasV12-transformed cells were mock- or reovirus-infected (multiplicity of infection (MOI) 20). The cells were fixed at 12 hpi and stained with antibodies against (a) Golgin97 (red) and reovirus (blue) proteins or (b) anti-Ras (green), anti-reovirus (blue) and anti-TGN38 (red) antibodies. Imaging was performed at x630 magnification by confocal microscopy. White squares denote regions that were enlarged under the 'Zoom' heading. The arrow indicates further enlargement. Scale bar: 20 μm.

lines transformed by H-RasV12, N-Ras61K, or K-RasV12. Characterization of these cell lines can be found in Supplementary Figures S3A and C. 2BP did not affect the number of cells infected by the virus (Supplementary Figure S3D), the amount of viral proteins synthesized (Supplementary Figure S3E) or the total amount of infectious virus produced (Figure 4a, left). However, 2BP did increase the amount of progeny virus being released (Figure 4a, right). This increase correlated with the palmitoylation status of the oncogenic Ras present. The largest increase in reovirus release was observed in N-Ras61K cells, as it is monopalmitoylated and highly susceptible to palmitoylation inhibition. This was followed by a smaller increase in cells transformed by dually palmitoylated H-RasV12. No change was observed in cells transformed by palmitoylation-null K-Ras4B. Differences in progeny virus release were also reflected in plaque size analysis. 2BP greatly enhanced

plaque size on N- and H-Ras-transformed cells, whereas only a minimal change was observed on K-Ras-transformed cells (Figure 4b).

Retention of H-RasV12 at the plasma membrane results in basal levels of reovirus-induced cell death

To investigate the importance of Ras redistribution during reovirus oncolysis, constructs were generated to tether oncogenic H- and N-Ras to the plasma membrane by using the transmembrane domain of the CD8α receptor, as well as render them palmitoylation-deficient. A schematic of the various Ras constructs and sites of mutagenesis is shown in Figure 5a. Characterization of the additional cell lines in terms of Ras activity and expected localization can be found in Supplementary Figures S4A and E.

Figure 3. 2BP promotes Golgi-associated Ras and increases reovirus spread. H-RasV12-transformed cells were mock- or reovirus-infected (multiplicity of infection (MOI) 20) and treated with 2BP (50 μ M). **(a)** The cells were fixed at 12 hpi and stained with anti-Ras (green), anti-reovirus (blue) and anti-TGN38 (red) antibodies (upper panels) or anti-Ras (green), anti-reovirus (blue) and anti-calnexin (red) antibodies (lower panels). Imaging was carried out by confocal microscopy at $\times 400$ magnification. White squares highlight regions that are enlarged under the 'Zoom' heading. Scale bar: 20 μ m. **(b)** The percentage of cells with Ras puncta resulting from reovirus infection $\pm$ 2BP treatment (paired t -test $\pm$ s.e.; $n = 3$). **(c)** Reovirus plaque size assay on non- and H-RasV12-transformed cells $\pm$ increasing concentrations of 2BP (25–75 μ M). Immunocytochemistry was performed to visualize reovirus plaques.

Plasma membrane-tethered H-RasV12 (termed CD8-H-RasV12xx) and palmitoylation-deficient H-RasV12 (termed H-RasV12xx) primarily localized to the bulk plasma membrane and ER, respectively. We showed above that reovirus caused distinct Golgi fragmentation and TGN colocalization of H-RasV12 with puncta formation. In the case of H-RasV12xx (palmitoylation-deficient) and CD8-H-RasV12xx (tethered and palmitoylation-deficient) cells, reovirus triggered Golgi fragmentation in both cell lines, but no Ras puncta were detected (Supplementary Figures S5A and B). Additionally, use of 2BP on these cell lines had no effect on reovirus plaque size or Ras puncta formation (Figure 4b and Supplementary Figure S5B).

To establish whether the movement of Ras from the plasma membrane to the Golgi body was a requirement of efficient reovirus oncolysis, H-RasV12-transformed cell lines were equally infected with reovirus for cell death analysis (Figure 5b and Supplementary Figure S5C). Reovirus-induced cell death and virus release were highest in H-RasV12 cells, whereas cells transformed by plasma membrane-tethered and/or palmitoylation-deficient H-RasV12 exhibited levels comparable to the non-transformed control (Figures 5c and d). It was recently discovered that the

inhibitor, FK506, alters the normal distribution of Ras.¹⁹ FKBP12 is involved in priming Ras for depalmitoylation, and inhibition of FKBP12 by FK506 enriches the amount of H-Ras on the plasma membrane while decreasing Ras levels at the Golgi. With reovirus infection causing basal levels of cell death in plasma membrane-tethered CD8-H-RasV12xx cells, it was postulated that FK506 treatment would produce similar results. In equally infected cells, FK506 had no effect on reovirus-induced cell death or plaque size in non-transformed, CD8-H-RasV12xx or H-RasV12xx cells (Figures 5e and f and Supplementary Figure S5D). In contrast, FK506 decreased reovirus-induced cell death and plaque size in cycling H-RasV12 cells to levels and sizes comparable to its plasma membrane-tethered form. Therefore, restricting Ras to the plasma membrane during reovirus infection reduces the ability of the virus to induce apoptosis and progeny release.

Reovirus induces cell death through activation of the MEKK1/MKK4/JNK pathway

With different H-RasV12 isoforms inducing varying degrees of reovirus-induced cell death, it was important to determine any

Figure 4. Increase in reovirus release by 2BP is dependent on the degree of Ras palmitoylation. **(a)** Total and release titers from reovirus-infected H-, N- and K-Ras-transformed cells $\pm$ 2BP (50 μ M) at 24 hpi (paired *t*-test $\pm$ s.e.; *n* = 3). **(b)** Reovirus plaque size assay on non- and Ras-transformed cells $\pm$ 2BP treatment (50 μ M). Immunocytochemistry was performed to visualize reovirus plaques. Scale bar: 2 mm. The diameter of individual plaques was used to obtain reovirus plaque size (nested analysis of variance (ANOVA) with Tukey's test $\pm$ s.e.; *n* = 3).

differences in the ability of the targeted constructs to activate well-known Ras downstream effector pathways. In comparison with the non-transformed control, western blot analysis revealed that uninfected H-RasV12-transformed cells had elevated levels of phosphorylated JNK and Akt, but similar levels of p38 (Figure 6a). Cells expressing cycling H-RasV12 were more effective at activating JNK and Akt, and were the only cell line with elevated levels of phosphorylated Erk. These results were similar to the ones obtained by Matallanas *et al.*²⁵ in their study of site-specific

Ras activation in NIH3T3 cells. Following infection, activated Erk and Akt levels rose slightly in all cell lines, whereas p38 was significantly upregulated (Figure 6a) as observed previously.⁸ Interestingly, it was the activation of JNK, which has a key role in inducing cell apoptosis, that caused the most notable difference. A slight increase in phosphorylated JNK was noted in the non-transformed and CD8-H-RasV12xx-transformed cells, whereas a much more significant increase occurred in the H-RasV12 and H-RasV12xx cells lines.

Figure 5. Retention of H-RasV12 at the plasma membrane results in basal levels of reovirus-induced cell death. **(a)** Schematic of the various Ras constructs. X's indicate locations of site-directed mutations that resulted in cysteine to serine exchanges within the Ras amino-acid sequence. **(b–e)** Non-transformed and H-RasV12-transformed cell lines were mock- or reovirus-infected (multiplicity of infection (MOI) 20–80). **(b)** At 12 hpi, FACS analysis was used to determine the percentage of cells infected by the virus (analysis of variance (ANOVA) with Tukey's test $\pm$ s.e.; $n = 3$). At 24 hpi, the cells were collected for **(c)** cell death analysis, and **(d)** reovirus release titration (ANOVA with Tukey's test $\pm$ s.e.; $n = 3$; *significantly different). **(e)** Mock- and reovirus-infected non-transformed and H-RasV12-transformed cell lines were also treated $\pm$ FK506 (10 μ M) and collected at 24 hpi for cell death analysis (two-sample t -test $\pm$ s.e.; $n = 2$). **(f)** Reovirus plaque size assay on non-transformed and H-RasV12-transformed cell lines $\pm$ FK506 treatment. Immunocytochemistry was performed to visualize reovirus plaques (nested ANOVA with Tukey's test $\pm$ s.e.; $n = 3$).

Figure 6. JNK activation is required in reovirus-induced cell death of H-RasV12 cells. Non-transformed and H-RasV12-transformed cell lines were mock- or reovirus-infected (multiplicity of infection (MOI) 20–80). **(a)** Whole-cell lysates were collected at 8 hpi to assess total and phosphorylated levels of JNK, Akt, p38 and Erk by western blot. Reovirus and β -actin served as controls. Densitometry was used to determine the relative levels of phosphorylation ($n = 3$). **(b)** and **(c)** Mock- and reovirus-infected non-transformed and H-RasV12-transformed cell lines were treated $\pm$ JNK inhibitor II (50 μ M) or $\pm$ U0126 (40 μ M) at 2 hpi. At 24 hpi, the cells were collected for cell death analysis (two-sample t -test $\pm$ s.e.; $n = 2$). UI, uninfected; V, reovirus infected.

As the Ras cell lines varied in their ability to activate JNK following infection, a JNK inhibitor was used to determine if it could explain the contrasting death and reovirus release data from the spatially distinct Ras-transformed cells. JNK inhibition significantly decreased reovirus-induced cell death in H-RasV12- and H-RasV12xx-transformed cells, but had no effect on non-transformed or CD8-H-RasV12xx cell lines (Figure 6b; controls in Supplementary Figures S6A and B), thereby supporting the above western blot data. In contrast, use of the MEK1/2 inhibitor, U0126, had little effect on reovirus-induced cell death (Figure 6c; controls in Supplementary Figures S6C and D). Collectively, the data reinforces the importance of JNK activation in reovirus-induced cell death,³⁸ and suggests that efficient JNK activation is dependent on Ras localization.

With the above demonstrating that the palmitoylation inhibitor, 2BP, caused further accumulation of H-RasV12 in the Golgi body and increased reovirus-induced cell death and virus release, we then determined if 2BP treatment activated similar downstream signaling pathways. One of the Ras downstream effectors linked to JNK activation is MEKK1.³⁹ MEKK1 initiates a kinase cascade that leads to MKK4 activation and subsequent JNK phosphorylation. In comparison with uninfected cells, western blot analysis suggested that reovirus infection caused an upregulation of both MKK4 and JNK phosphorylation (Figure 7a). Interestingly, when H-RasV12-transformed cells were treated with 2BP alone, MKK4 and JNK activation increased. This was further elevated in the presence of reovirus and 2BP. 2BP had little to no effect on uninfected or reovirus-infected CD8-H-RasV12xx-transformed cells.

In light of 2BP-induced JNK activation in H-RasV12-transformed cells, reovirus-induced cell death was assessed in the context of JNK inhibition and 2BP treatment. As expected, JNK inhibition reduced virus-related cell death in H-RasV12-transformed cells, whereas 2BP enhanced it (Figure 7b; controls in Supplementary Figures S6E and F). Treatment of H-RasV12 cells with both the JNK inhibitor and 2BP decreased reovirus-induced cell death significantly, but not to levels of the JNK inhibitor alone. CD8-H-RasV12xx-transformed cells were used as a negative control, as individual or combination treatments with 2BP and/or JNK inhibitor II did not alter the basal levels of reovirus-induced cell death. It is well known that reovirus infection triggers caspase-dependent cell death and that activated JNK can stimulate the release of cytochrome C and Smac/Diablo from the mitochondria to initiate the caspase cascade.^{38,40,41} As determined by FACS (fluorescence-activated cell sorting) analysis, treatment of reovirus-infected cells with the caspase inhibitor, Z-VAD(OMe)-FMK (ZVAD), inhibited cell death in H-RasV12-transformed cells (Figure 6c). Combination treatment of H-RasV12 cells with 2BP and ZVAD also caused a significant decline in reovirus-induced cell death, linking 2BP treatment to events further downstream of the Ras/MEKK1/MKK4/JNK signaling pathway.

DISCUSSION

Constitutive Ras activity and its associated downstream pathways have an important role in human oncogenesis.³ Ras activation alters the cellular environment and provides an opportunity for adaptive pathogens to become oncolytic. It is well established that reovirus pathogenicity is relatively benign in normal cells, and that it preferentially targets Ras-transformed cells. So far, studies have identified aspects of the reovirus life cycle that are enhanced in cells harboring activated Ras.^{7,8} This study illustrates how reovirus alters both the intracellular distribution and activity of oncogenic Ras itself, resulting in enhanced cell death and subsequent release of progeny virus particles.

Our data demonstrate that reovirus infection causes fragmentation of the Golgi body, likely through its association with microtubules during viral factory formation (Figure 7d). As a result, oncogenic H-Ras accumulates in the Golgi body as it cycles

back for repalmitoylation and initiates cellular apoptosis through activation of the MEKK1/MKK4/JNK signaling pathway. Tethering of Ras to the plasma membrane reduces virus-related cell death to levels comparable to that of the non-transformed control. Alternatively, use of the palmitoylation inhibitor, 2BP, further promotes the association of Ras with the Golgi body and causes additional activation of the MEKK1/MKK4/JNK signaling pathway to initiate higher levels of reovirus-induced cell death. Previous work implicated MEKK1 and JNK activity in reovirus-induced apoptosis;³⁸ however, the exact mechanism remained undetermined until now.

This study sheds further light on the molecular basis behind reovirus oncolysis and facilitates the future efficacy of reovirus as a cancer therapeutic. The pathogenesis of other oncolytic viruses, such as vaccinia virus, myxomavirus, coxsackievirus and herpes simplex virus, has also been linked to signaling both up- and downstream of Ras activity.⁴² Of these, herpes simplex virus is known to induce Golgi fragmentation,⁴³ and could potentially use a similar mechanism as reovirus to induce cell death. Efficient viral therapy derives from specific targeting and progeny dissemination. This work reinforces the potential benefits of introducing reovirus into cancer cells specifically expressing constitutively active H- or N-Ras, as well as those harboring mutated EGFR and Src family kinases that can lead to Ras activation. Cancers driven by palmitoylated members of the Src family, such as Lyn, Yes and Lck, could serve as reovirus targets through their association with the Golgi.⁴⁴ Additionally, K-Ras4B mutations are frequently observed in human cancers. Despite K-Ras4B being palmitoylation-null, reovirus may still effectively lyse these cells through their dependency on endogenous, wild-type Ras.⁴⁵ In K-Ras4B-driven cancers, reovirus-induced Golgi fragmentation would sequester endogenous Ras, and likely lead to signal disruption, sensitization of these cells to chemotherapeutic agents and cell death.

It is generally agreed that the future of oncolytic virotherapy will occur in the form of combination strategies. Many of the chemotherapeutic agents currently used in reovirus clinical trials are known to stabilize microtubules and fragment the Golgi body.^{6,46} Thus far, these trials are well tolerated with an effective local response, suggesting that combining reovirus with drugs capable of cytoskeletal interference or Golgi fragmentation would likely produce a synergistic outcome. On the other hand, targeting Ras for cancer therapy has proven difficult. Current strategies focus on interfering with Ras-associated chaperones and modification enzymes.⁴⁷ It has been shown that reovirus exploits activated Ras at multiple levels for its replication and spread. The present study reinforces the need for Ras-specific inhibitors, and in particular, those which retain Ras at the Golgi or alter its palmitoylation status, to further promote reovirus oncolysis and increase its efficacy as a cancer therapy.

MATERIALS AND METHODS

Cell lines and virus

NIH3T3, L929 and 293T cell lines were purchased from ATCC (Manassas, VA, USA) and maintained according to the manufacturer's instructions. Mammalian reovirus Type 3 Dearing stocks were prepared in L929 spinner cultures and purified as described previously.⁴⁸ H-RasV12 in pBABE-puro was kindly provided by C Der (University of North Carolina, NC, USA). N-Ras61K and K-RasV12 (4B variant) in pBABE-puro were purchased from Addgene (Cambridge, MA, USA; catalog nos 12543 and 12544). Plasma membrane-tethered CD8-H-RasV12xx in pCEFL was a gift from Dr Piero Crespo (University of Cantabria, Cantabria, Spain). CD8-H-RasV12xx was cloned into the pBABE-puro vector. N-Ras61K and CD8-H-RasV12xx vectors were used as templates to clone the tethered and/or palmitoylation-deficient forms of activated H- and N-Ras into pBABE-puro. Primers are listed in Supplementary Table S1. Palmitoylation-deficient constructs, denoted with an x or xx, refer to cysteine to serine mutations at sites 181 and 184 within the Ras sequence (C181S and C184S in H-RasV12xx and C181S in N-Ras61Kx). All constructs were verified by sequencing.

Figure 7. 2BP promotes reovirus-induced cell death through activation of the Ras/MEKK1/MKK4/JNK pathway. **(a and b)** H-RasV12- and CD8-H-RasV12xx-transformed cells were infected with reovirus at a multiplicity of infection (MOI) of 20 and 80, respectively. **(a)** At 2 hpi, the cells were treated $\pm$ 2BP (50 μ M). Whole-cell lysates were collected at 8 hpi to assess total and phosphorylated levels of MKK4 and JNK by western blot. Reovirus and β -actin served as controls. Densitometry was used to determine relative levels of MKK4 and JNK phosphorylation ($n = 3$). **(b)** At 2 hpi, the cells were treated with 2BP (1 μ M) and/or JNK inhibitor II (100 μ M). At 24 hpi, the cells were collected for cell death analysis (analysis of variance (ANOVA) with Tukey's test $\pm$ s.e.; $n = 3$; *significantly different). **(c)** H-RasV12-transformed cells were infected with reovirus (MOI of 20) and treated with 2BP (50 μ M) and/or ZVAD (50 μ M). At 24 hpi, the cells were stained with Annexin V-PE and 7-AAD (7-aminoactinomycin D) to determine the level of cell apoptosis and death by FACS analysis (ANOVA with Tukey's test $\pm$ s.e.; $n = 3$). **(d)** Proposed model. Reovirus interacts with microtubules to set up cytoplasmic viral inclusion bodies, leading to Golgi fragmentation and a disruption of vesicular transport. As a result, H-RasV12 is retained in the Golgi body and activates its downstream effector, MEKK1, to initiate cell death through MKK4 and JNK activation. Use of the palmitoylation inhibitor, 2BP, results in further Ras accumulation in the Golgi and even greater levels of reovirus-induced cell death. Tethering of Ras to the plasma membrane generates the opposite effect and produces basal levels of cell death. UI, uninfected; V, reovirus infected.

To generate retroviruses containing activated Ras sequences, 293T cells were co-transfected with the appropriate pBABE-puro construct, vesicular stomatitis virus G and pHIT60. Retrovirus-containing NIH3T3 cells were selected with 2 µg/ml of puromycin and maintained for 1 month.

Reagents and antibodies

In general, a working concentration of 50 µM was used for 2BP (Santa Cruz Biotechnology Inc., Dallas, TX, USA). JNK inhibitor II and caspase inhibitor I ZVAD were supplied by Calbiochem (San Diego, CA, USA) and used at a working concentration of 50 µM. U0126 (Cell Signaling, Danvers, MA, USA) and FK506 (Sigma-Aldrich, Oakville, ON, Canada) were used at a concentration of 40 and 10 µM, respectively. Antibody information for all experimental assays can be found in Supplementary Table S2.

Cell fractionation, co-immunoprecipitation and western blot analysis

For fractionation, H-RasV12 cells were syringed (30-gauge) and centrifuged at 700 g for 10 min. The resulting supernatant was ultracentrifuged at 100 000 g for 25 min to isolate the different fractions. The cytoplasmic fraction was stored at –80 °C. The membrane pellet was resuspended in 1x RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Na-deoxycholate, 1% IGEPAL CA630) with PIC, syringed and stored at –80 °C. The samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by western blot. The protocol for co-immunoprecipitation can be found in Supplementary Information. Western blot required the incubation of polyvinylidene fluoride membranes with the appropriate primary and secondary antibodies. Blots were developed using ECLplus (Bio-Rad, Mississauga, ON, Canada) or in the case of fluorochrome-tagged secondary antibodies, simply visualized by the Typhoon 9400 Imager (Amersham, Piscataway, NJ, USA). ImageJ 1.47v (National Institutes of Health (NIH), Bethesda, MD, USA) was used for protein band quantification through densitometry. Experiments requiring statistical analyses were completed using SYSTAT 13 (Systat Software Inc., San Jose, CA, USA).

Ras activity assay and acyl-biotin exchange chemistry

Cells were collected according to the Ras activation assay kit (EMD Millipore, Billerica, MA, USA) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and anti-Ras western blot probing. Acyl-biotin exchange chemistry was performed as described previously.⁴⁹ Total Ras was detected by western blot using an anti-Ras primary antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody. Biotinylated protein, which represents palmitoylated protein, was detected using Cy3-conjugated streptavidin.

Immunostaining, flow cytometry, plaque assay and cell death

Immunocytochemistry was performed on methanol-fixed cells using anti-reovirus and alkaline phosphatase-conjugated secondary antibodies. BCIP/NBT AP Substrate Kit IV was used for visualization (Vector Laboratories, Burlington, ON, Canada). Quantification of reovirus-infected cells by flow cytometry and plaque titration were performed as described previously.⁷ For plaque size analysis, the cells were maintained for 5 days with an agar overlay, then fixed with 4% formaldehyde and stained according to the immunocytochemical conditions described above. The entire well was photographed using the Zeiss Stemi 2000-C stereoscope and AxioCam HRC color camera (Carl Zeiss Canada Inc., Toronto, ON, Canada). The image was enlarged and plaque size was determined by measuring the largest diameter of every plaque within each well.

Cell death was quantified using trypan blue exclusion staining, or Annexin V-PE and 7-AAD (7-aminoactinomycin D) staining for FACS analysis (BD Biosciences, San Jose, CA, USA). In either technique, background cell death (<2%) from uninfected cells was taken into account.

Immunofluorescence microscopy

Cells were grown overnight on gelatin-coated coverslips and either mock- or reovirus-infected. The cells were fixed in 4% paraformaldehyde (in phosphate-buffered saline) for 20 min and stained with the appropriate primary and Cy-conjugated secondary antibodies. Coverslips were mounted onto slides using FluoroShield mounting medium (Sigma-Aldrich) and the cells were visualized using the Zeiss LSM 510 Meta laser

scanning confocal microscope (Carl Zeiss Canada Inc.). The LSM image browser was used for analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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