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Mammalian orthoreoviruses are currently being investigated as novel cancer therapeutics, but the cellular mechanisms that regulate susceptibility to reovirus oncolysis remain poorly understood. In this study, we present evidence that virion disassembly is a key determinant of reovirus oncolysis. To penetrate cell membranes and initiate infection, the outermost capsid proteins of reovirus must be proteolyzed to generate a disassembled particle called an infectious subviral particle (ISVP). In fibroblasts, this process is mediated by the endo/lysosomal proteases cathepsins B and L. We have analyzed the early events of infection in reovirus-permissive and -resistant cells. We find that, in contrast to susceptible glioma cells and Ras-transformed NIH3T3 cells, reovirus-resistant cancer cells and untransformed NIH3T3 cells restrict virion uncoating and subsequent gene expression. Disassembly-restrictive cells support reovirus infection, as in vitro-generated ISVPs establish productive infection, and pretreatment with poly(I:C) does not prevent infection in cancer cells. We find that the level of active cathepsin B and L is increased in tumors and that disassembly-restrictive glioma cells support reovirus oncolysis when grown as a tumor in vivo. Together, these results provide a model in which proteolytic disassembly of reovirus is a critical determinant of susceptibility to reovirus oncolysis.

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INTRODUCTION

Reoviruses are members of the Reoviridae family of non-enveloped, double-stranded RNA (dsRNA) viruses. The mammalian reovirus, commonly found in stagnant water and sewage, infects a wide range of species including chimpanzees, monkeys, pigs, cattle, cats, sheep, mice, and humans. Because of its ubiquity in the natural environment, up to 50% of adults have had a previous exposure to reovirus as evidenced by anti-reovirus antibodies. In immunocompetent animals, reovirus infection produces few clinical symptoms and the virus is not linked to any known human disease (hence the designation “orphan”). Reovirus is an enteric virus that is transmitted mainly through the fecal–oral route and principally infects the gastrointestinal and respiratory tracts of mammals. To gain entry into the target cells of its host organism, reovirus virions must undergo proteolytic disassembly (uncoating). Uncoating results in the degradation of the outermost capsid protein σ3 and the subsequent cleavage of the underlying viral protein μ1/μ1C to generate an infectious subviral particle (ISVP). This processing gives the subviral particle the capacity to penetrate the endosome/lysosome or the plasma membrane and thereby gain entry to the cytoplasm where reovirus replication can occur. Work in animal models suggests that, during natural enteric infections, σ3 is proteolytically degraded extracellularly within the intestinal lumen. In cell culture, however, reovirus takes advantage of endosomal and lysosomal proteases for its proteolytic conversion into ISVP. The necessity of reovirus uncoating for infectivity in vivo and in vitro is further demonstrated by the capacity of protease inhibitors to block enteric infection in mice and restrict reovirus uncoating and infection in cell culture.

Recently, reovirus type-3 Dearing has been investigated as an oncolytic agent against a variety of human cancers (reviewed in ref. 22). The oncolytic properties of reovirus are also being investigated in clinical trials (http://www.oncolyticsbiotech.com). These studies revealed that reovirus infects all but a few cancer cells while leaving normal cells relatively unaffected. Although it has been proposed that reovirus requires an activated Ras signaling pathway in order to mediate oncolysis, other studies have demonstrated that active Ras alone does not control susceptibility of cells to reovirus infection. Several cell lines, including the highly susceptible L929 mouse fibroblasts, are very susceptible to reovirus despite low levels of Ras activity (T.A. and P.A.F., unpublished results). Furthermore, we have observed that the addition of proteases into the culture medium or direct infection with ISVPs of restrictive cells can render them permissive for reovirus infection. These observations suggest that the protease-mediated disassembly of reovirus’ capsid may determine whether or not a cell or tumor is permissive for reovirus infection and that this may be independent of elevated Ras activity.

In this study we investigate the requirement for reovirus uncoating in the glioma cell lines U87 and U118 as well as in the
NIH3T3 cell line and its Ras-transformed derivatives. We demonstrate a lack of effective reovirus disassembly and penetration in the reovirus-resistant glioma U118 and the non-transformed NIH3T3 cell lines. Protease-generated ISVP particles efficiently infect these disassembly-restrictive cells and other reovirus-resistant cancer cells. We also find that synthetic dsRNA does not protect transformed cells from infection. These results support a model in which the requirement for proteolytic uncoating determines the susceptibility of cancer cells to reovirus oncolysis. Our finding that reovirus can productively infect and mediate in vivo oncolysis of tumors that restrict disassembly in vitro suggests that the microenvironment of tumors and the cellular environment of cancer cells, perhaps through elevated cathepsin B and L activity, facilitate the extracellular conversion of reovirus virions into particles that can penetrate cell membranes and mediate oncolysis.

RESULTS
Reovirus disassembly is ineffective in resistant U118 glioma and NIH3T3 cell lines
Reovirus infects the majority of glioma cell lines, and only a few lines are resistant to reovirus infection. Until now, reovirus disassembly has not been addressed as a factor in the resistance of glioma cells to reovirus oncolysis. To examine reovirus capsid disassembly in susceptible and resistant cells, [35S]-labeled reovirus particles were exposed to the glioma cell lines U87 and U118, the Ras-transformed NIH3T3 (NIH3T3-Ras), and the empty vector pBabe-transfected NIH3T3 (NIH3T3). We found that [35S]-labeled reovirus is proteolytically processed in the susceptible cell lines but not in resistant cell lines. Removal of σ3 and cleavage of μ1C to δ, indicative of ISVP formation, can be readily observed 3 hours post infection (h.p.i.) in the U87 cells but not in the reovirus-resistant U118 cells even up to 24 h.p.i. (Figure 1a). Processing, although minimal when compared to the susceptible glioma U87 and the L929 cells, was also observed in the Ras-transformed NIH3T3 cells but not in the NIH3T3 controls (Figure 1b). Reovirus disassembly has previously been reported to be inhibited when cells are exposed to the cysteine protease inhibitor E64. To verify that this compound could also block reovirus uncoating in our susceptible cells, permissive cells (U87, NIH3T3-Ras, and L929) were exposed to E64 (100 μmol/l) 1 hour before [35S]-labeled reovirus exposure as just described. We found that the degradation of σ3 protein and cleavage of μ1C into δ was completely inhibited in the presence of E64 up to 24 hours after exposure to the virus (Figure 1c). Similar results were obtained in a subsequent experiment by Western blotting with a specific anti-μ1 reovirus antibody that recognizes both μ1C and δ (Figure 1d). These results demonstrate that the proteolytic conversion of reovirus virions into ISVPs is deficient in cells resistant to reovirus infection (U118 glioma and the non-transformed NIH3T3 cell line) and that such ineffective reovirus uncoating could explain, in part, the resistance of these cells to infection and oncolysis.

In resistant cell lines, reovirus binds, accumulates within lysosomes, but is not efficiently transcribed
To investigate whether the underlying cause of the defective disassembly in resistant cells reflects differences in the binding and entry of the virus, we used radiolabeled reovirus to compare the binding efficiency of the permissive and resistant cells for the virus. Cells were incubated with [35S]-labeled reovirus for 1 hour at 4°C before collecting the cell lysate for quantitative measurements by scintillation counting. We found that different amount of reovirus could bind to resistant and susceptible cells (Figure 2a). L929 cells were the most efficient at binding reovirus, followed by the highly susceptible glioma cell line U87 and NIH3T3 cells. The reovirus resistant U118 glioma cells had the lowest binding for reovirus. Although inferior binding with the glioma U118 and the NIH3T3 cells may reduce the permissiveness of these cells to reovirus infection, virions do bind onto these cells. Moreover, the binding measured for the resistant NIH3T3 cells was the same as for the susceptible Ras-transformed NIH3T3, further suggesting that the level of binding on these cells does not mediate differential susceptibility. E64 treatment did not affect the level of reovirus binding in any cell line tested, which excludes the possibility of reduced susceptibility via interference with binding.

Since reovirions bind to both resistant and susceptible cells, we next evaluated whether reovirus accumulated in lysosomes within our disassembly-restrictive cells. Previous studies have described how reovirus capsid disassembly is necessary...
to perforate either the endosomal/lysosomal or the plasma membrane and thus to penetrate the cytoplasm.\textsuperscript{9–11} In order to do this, we labeled reovirus with Cy3-maleimide monofunctional dye and exposed resistant and susceptible cells grown on glass slides to this labeled reovirus. Immunofluorescence was then performed to detect lysosomal associated membrane protein 2, frequently used as a functional marker of lysosomes.\textsuperscript{35} Our results show that, in the susceptible U87 glioma cells and in the Ras-transformed NIH3T3 cells, the Cy3-labeled reovirus is present not only within areas where the lysosomal associated membrane protein 2 staining is found but also appears diffusely throughout the cytoplasm, suggesting that perforation of the lysosome and penetration of the virus occurred in these cells. On the other hand, in resistant cells and E64 disassembly inhibited susceptible cells, a punctuate Cy3 fluorescence pattern was observed that was restricted to the same compartment as the lysosomal associated membrane protein 2, indicating lysosomal localization (Figure 2b).

Since it is proposed that reovirus transcription is not initiated until the virus has been disassembled and its core has entered the cytoplasm of cells,\textsuperscript{36} we next addressed whether reovirus transcription occurs in our susceptible and resistant cells, using Northern blotting as a quantitative approach. S1 reovirus transcripts 24 h.p.i. were detected only in the susceptible U87 and Ras-transformed NIH3T3 cells and were blocked when these cells were treated with E64. However, when cells were exposed to ISVPs, the presence of S1 transcripts were detected in both resistant and susceptible cells even in the presence of

Figure 2 Assessment of reovirus binding, internalization, and viral transcription in susceptible and resistant cell lines. (a) \textsuperscript{[35S]}-labeled reovirus was allowed to bind on cells in minimal medium for 1 hour at 4 °C. Cells were washed twice in phosphate-buffered saline and lysed in a sonification buffer. Samples were subjected to scintillation counting, which was performed in triplicate. (b) Cy3-labeled reovirus at a multiplicity of infection (MOI) of 5,000 was added for 24 hours to cells grown on glass slides, after which cells were fixed in 4% formaldehyde. Immunofluorescence with the lysosomal marker lysosomal associated membrane protein 2 (LAMP2) antibody followed by secondary fluorescein isothiocyanate (FITC) was performed; slides were then mounted with a 4',6-diamidino-2-phenylindole (DAPI) mounting medium (VECTOR) and photographed by multiple acquisition with a fluorescent Zeiss microscope (magnification ×400). (c) Total RNA from cells were purified using the RNeasy RNA extraction kit; equal RNA amounts were subjected to Northern blotting using a digoxygenin-labeled probe against the positive strand of reovirus S1 transcripts. ISVP, infectious subviral particle.
E64 (Figure 2c). These results are consistent with a lack of penetration of reovirus particles in the restrictive U118 glioma cell line and in the non-transformed NIH3T3 cells.

**ISVPs infect reovirus–resistant cancer and non-transformed cell lines**

We next examined whether infection with in vitro-generated ISVPs could bypass this disassembly block and result in a productive infection in the resistant cells. According to the model of the molecular basis of reovirus oncolysis, Ras signaling in susceptible cancer cells overcomes a translational block of reovirus transcripts found in resistant, non-transformed cells. Therefore, this model would predict that infection by ISVP particles should also be restrained in the resistant NIH3T3 and U118 cell lines through a translation block. In contrast, we found that ISVPs efficiently infected both the resistant glioma cell line U118 and the non-transformed NIH3T3 cells (Figure 3a). Whereas reovirus protein synthesis from reovirion infection was apparent in the susceptible cell lines U87 and Ras-transformed NIH3T3—though not when these cells were treated with the disassembly inhibitor E64 (or the weak base ammonium chloride (data not shown))—viral protein synthesis following ISVP infection occurred in the resistant U118 and NIH3T3 cell lines as well as the susceptible cell lines even when treated with E64. Similar results were obtained by immunofluorescence using a rabbit anti-reovirus type-3 antibody followed by FITC–conjugated goat anti-rabbit IgG to detect positive reovirus infection (green fluorescent cells) (Figure 3b). Increased viral progeny was also measured in conditions where productive reovirus infection was observed (Figure 3c). We next examined the viability of the resistant and susceptible cells following reovirus or ISVP infection with or without treatment with E64 as described previously (Figure 3d). We found that the protease inhibitor E64 efficiently prevented oncolysis 48 h.p.i. of our susceptible cancer cells infected with reovirus but not with ISVPs. ISVP infection also resulted in a significant decrease in viability of the reovirus-resistant glioma U118 cells. The non-transformed NIH3T3 cells generally remained viable at 48 h.p.i.

Finally, to determine whether the ability of a cell to process reovirus is a key determinant of infectivity in a broader range of cells, we tested other reovirus-resistant cells: the glioma U343, the Burkitt lymphoma cell line Daudi, and the normal human foreskin fibroblast HS68. In all cases we found that infection with ISVPs—as measured by immunofluorescence

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**Figure 3 Continued**
or metabolic SDS-PAGE to detect viral protein synthesis and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoium bromide (MTT) or 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1) assay to measure viability—also resulted in positive infection and cell lysis (Figure 3e). Restriction in reovirus disassembly was also observed in these cells (Figure 3e, right panel). Given that ISVPs readily infect both susceptible and disassembly-restrictive cancer and normal cells, these results further suggest that the critical factor of resistance is at the level of reovirus penetration into the cytoplasm.

**Reovirus protein synthesis is not inhibited by synthetic dsRNA treatment in transformed cell line**

It has been proposed that resistance to reovirus infection is the direct result of inhibition of viral translation. In contrast, our data using ISVPs show that “resistant” cells (i.e., resistant to infection with virions) support a productive infection; ISVPs bypass the limitations of viral entry, uncoating, and penetration into the cytoplasm. To further examine the proposed viral translation block in resistant cells, we assessed whether pretreatment of cells before reovirus infection with synthetic dsRNA (poly(I:C) (20 μg/ml)), a potent activator of the dsRNA-dependent pathways of interferon and other anti-viral mechanisms, could inhibit viral protein synthesis. We found that viral protein synthesis was reduced in the non-transformed cells (NIH3T3 and HS68 cells) that were pretreated with poly(I:C) and infected with ISVPs. However, exposure of dsRNA did not prevent reovirus/ISVP infection of the susceptible transformed cells (U87 glioma, Ras-transformed NIH3T3, and the Burkitt lymphoma Raji cell lines). In addition, ISVP infection of the reovirus resistant glioma U118 and Burkitt lymphoma Daudi was not prevented by poly(I:C) treatment (Figure 4a).

Moreover, reovirus exposure to NIH3T3 cells 6 hours before ISVP infection did not result in the inhibition of viral protein synthesis that synthetic dsRNA provided (Figure 4b). Reovirus may be insufficient to stimulate an anti-viral response against ISVP infection in NIH3T3 cells owing to a lack of penetration by the virus. These data support a general model whereby cancer cells—but not normal cells—have defects in their anti-viral
They also suggest that, in reovirus-resistant cancer cells, the foremost factor in the resistance of these transformed cells is at the level of reovirus disassembly and not at the level of a viral messenger RNA translation block. Normal cells may benefit from the additional level of protection provided by an activated anti-viral pathway, but it appears that their primary resistance to reovirus resides in the inability to efficiently uncoat reovirus.

**Figure 4** Susceptible and resistant cell lines exposed to synthetic dsRNA before reovirus ISVP infection. (a) Cells with or without 20 μg/ml of synthetic dsRNA poly(I:C) treatment 6 hours before reovirus exposure were pulse-labeled with [35S]methionine for 6 hours at 18 hours after infection with a multiplicity of infection (MOI) of 20 of reovirus or ISVPs (reovirions processed in 200 μg/ml of chymotrypsin for 30 minutes at 37°C). Cells were lysed, and reovirus proteins were immunoprecipitated from the lysate using rabbit polyclonal anti-reovirus antibodies and then analyzed by SDS-PAGE and autoradiography. (b) NIH3T3 cells were pretreated with poly(I:C) or reovirus at an MOI of 20, 6 hours before reovirus/ISVP infection at an MOI of 20, and then processed as in b.

**Figure 5** Activity and involvement of the proteases cathepsin B and L for reovirus oncolysis. (a) The specific inhibitors CA-074 (Cathepsin B) and Inhibitor III (Cathepsin L) (Invitrogen) were added to cells at a concentration of 10 μmol/l for 6 hours before lysis. An equal amount of protein was assessed for cathepsin L or B activity as measured by fluorescence, following the procedure recommended by the manufacturer (Invitrogen). Results are presented as percentages of the total activity of untreated cells measured in relative fluorescence units. (b) Susceptible cells were treated with 10 μmol/l of either or both cathepsin B and L inhibitors for 1 hour before infection with reovirus or ISVP with an MOI of 20. Reovirus oncolysis was assessed by MTT 48 hours after infection; the remaining viability is presented.
Cathepsin B and L inhibitors prevent reovirus oncolysis of susceptible cancer cells

To characterize the proteolytic requirement of susceptible cancer cells to reovirus, we first investigated the proteases cathepsin B and L. Both have been reported to mediate reovirus disassembly and permit reovirus infection. In addition, these proteases have been implicated in the progression of various cancers, including gliomas, and are elevated by Ras activation in NIH3T3 cells. We determined whether cathepsin B and L are critical in promoting permissiveness to reovirus in susceptible cell lines. Reovirus-susceptible cells treated or untreated for 6 hours with 10 μmol/l of the cathepsin B (CA-074) and L (Inhibitor III) (Invitrogen) were lysed, and equal amounts of protein from each sample were assayed for cathepsin B or L protease activity, respectively. Both inhibitors strongly impeded the activity of their respective protease and also had some inhibiting effect on the activity of the other cathepsin (Figure 5a). We then found that inhibition of cathepsin B and L 1 hour before reovirus infection significantly protected susceptible cells against reovirus oncolysis 48 h.p.i. (Figure 5b). The combination of both inhibitors had the strongest effect in blocking reovirus oncolysis in the susceptible cell lines U87, Ras-transformed NIH3T3, and L929. As anticipated, ISVP infection was unaffected by these inhibitors. Furthermore, this result was specific to cathepsins because we did not find protection from infection when these cell lines were treated with the broad-spectrum synthetic matrix metalloproteinase inhibitors AG3340 or BB-94 (data not shown).

Productive reovirus infection in tumors of the in vitro disassembly-restrictive U118 glioma cell line

Since the microenvironment of tumors in vivo contains a number of proteases that may help promote reovirus disassembly, we decided to challenge the reovirus-restrictive U118 cell line grown as a tumor in mice with a single intratumoral injection of reovirus. U118 tumors were allowed to grow until the establishment of a palpable mass and were then challenged with 1 × 10⁷ plaque forming units (pfu) of live reovirus or saline intratumorally. We observed a lack of tumor growth in animals treated with reovirus (Figure 6a). Hematoxylin and eosin stained sections showed necrosis of tumor cells after live reovirus treatment, and immunohistochemistry with a polyclonal anti-reovirus antibody showed the presence of reovirus (brown) in the U118 tumor; uninfected tumors showed no staining (Figure 6b). We then determined the levels of cathepsin B and L activity from in vivo glioma tumor samples grown in severe combined immunodeficiency mice compared to their respective glioma cells grown in culture, finding elevated cathepsin activity for both U87 and U118 tumors in vivo (Figure 6c). These results provide additional evidence that cell lines resistant to reovirus oncolysis in vitro can be rendered permissive once grown in animals and that this may be due to the proteolytic microenvironment of the tumor. The permissiveness of the U118 cells in vivo (in contrast to their resistance in vitro) was not a consequence of upregulated Ras in the in vivo setting, since Ras–GTP levels from U118 tissue lysates remained low—as in U118 cells grown in culture (Figure 6d).

DISCUSSION

Reovirus is currently being evaluated in clinical trials as a novel anti-cancer therapeutic. The majority of cancer cell lines are susceptible to infection and killing by reovirus, and only a small number are resistant. In spite of intensive preclinical studies, the mechanisms underlying the susceptibility of cancer cells are not fully understood. In this study, we focused on host-mediated proteolytic reovirus disassembly as an important mediator of susceptibility. Using radiolabeled, fluorescently labeled, and reovirus virions and infectious subviral particles, we found that reovirus-resistant cancer cells and non-transformed cells restrict infection in vitro because they fail to mediate viral disassembly and penetration. Our data show that proteolysis of the reovirus outer-capsid
protein is necessary for the selective infection and killing of cancer cells.

The demonstration that proteolytic disassembly of reovirus can be a primary determinant of susceptibility requires a revision of the proposed model,25 which we call the "reovirus-ras" model, for the molecular basis of reovirus oncolysis. According to the reovirus-ras model, ras signaling (through an unknown mechanism perhaps through p38 signaling27) releases a block in translation of viral transcripts by the dsRNA-activated protein kinase found in resistant cells. In support of the model, viral transcripts were found to be equivalent with respect to both susceptible and resistant cells. However, we have four observations that are inconsistent with the model. First, we found a restriction of reovirus uncoating in reovirus-resistant cancer cell lines and non-transformed cells. Infection by reovirus requires the disassembly of its outermost capsid in order to penetrate the host and initiate viral gene transcription.9–11 Second, we found that ISVPs efficiently infected "reovirus-resistant" cells. Golden et al. also found that the addition of proteases to the media of resistant cells rendered them susceptible to infection.11 Third, the infection of reovirus-resistant cells by ISVPs was not prevented in cells transformed by pretreatment with synthetic dsRNA. The reovirus-ras model predicts that this treatment would activate PKR, resulting in a translation block and an unproductive infection. Finally, consistent with the ineffective penetration of the virus in resistant cells, we found a lack of reovirus S1 transcripts in resistant cells rather than the equivalent levels of transcripts predicted by that model. Taken together, these results suggest that reovirus disassembly is a critical determinant for oncolysis of cancer cells and a primary factor in resistance to reovirus oncolysis.

We have no data that explain the discordance between our observations and those that led to the development of the reovirus-ras model. That model, which was largely based on studies of murine fibroblasts transfected with high levels of oncogenic ras, was never proposed to account for all cases of susceptibility. Our studies demonstrate that NIH3T3 cells have very low levels of binding and entry of reovirus, so the reovirus-ras model may apply most directly to cell lines with inefficient viral entry; its generalization to the signaling milieu of human cancers remains uncertain. Since reovirus infects a vast majority of cancer cell lines and since most of these do have ras activation, it follows that mutations leading to activated ras may contribute to reovirus susceptibility by increasing protease activity40 (e.g., by increasing expression or reducing expression of an inhibitor) or otherwise facilitating viral entry and disassembly. Studies that address these questions are currently underway in our laboratories.

Many other viruses (adenovirus, Newcastle disease virus, measles, severe acute respiratory syndrome, Ebola, etc.) also rely on proteolytic processing of a capsid or envelope protein to permit cell entry and replication.42–46 In this study, we found that specific inhibitors of cathepsin B and L block reovirus oncolysis but that broad-spectrum matrix metalloproteinase inhibitors did not. Others have also reported that a number of different proteases (cathepsin S, neutrophil elastase, tpsyn, and others) are capable of converting intact reovirus virions into ISVPs that can enter cells without additional proteolysis.43–45 Elevated protease expression and/or activity are common consequences of tumorigenesis (e.g., invasion, angiogenesis, and metastasis).48,49 Consistent with this, we found that tumors expressed more cathepsin B and L activity than their in vitro counterparts. The in vivo proteolytic microenvironment may explain why a highly resistant cell line became susceptible to reovirus-induced oncolysis when established as a tumor in vivo.

There are three broad implications of our study for the use of oncolytic viruses in the clinic. First, it is encouraging that the efficiency of some viruses might be enhanced in vivo by the protease-rich environment of the tumor. However, this complicates predictions of efficacy based on in vitro assays because cells that are resistant in vivo may be susceptible in patients. Second, improving disassembly and viral entry may be important strategies to employ in the design and use of oncolytic viruses for clinical use. Isolation of reovirus variants that disassemble more efficiently or require cancer-type specific proteases for uncoating may increase the efficacy of reovirus in vivo and provide further specificity. A related strategy has been used with a measles virus variant specifically engineered to selectively fuse to and infect cancer cells that overexpress metalloproteinases.44 Finally, a better understanding of the molecular basis of reovirus oncolysis will allow us to properly select the patients who are most likely to benefit from this treatment and optimize its use.

MATERIALS AND METHODS

Cells and reovirus. Human malignant glioma cell lines U87, U118, U251, and U343, Burkitt lymphoma cell lines Raji and Daudi, the mouse fibroblasts NIH3T3 cell line, and L929 cells were obtained from American Type Culture Collection and were maintained as described.32,34 Activated Ras constructs in the pBABE retroviral vector were generously provided by P. W. Lee (Dalhousie University, Halifax, Canada), and transfection and selection of NIH3T3 cells were as described.27 The Dearing strain of reovirus serotype 3 and intermediate subviral particles were obtained as described.32 Purified virions containing [35S]-labeled proteins were obtained by culturing the cells with [35S]-methionine (Amersham Biosciences, Arlington Heights, IL) into a medium of plated cells (50 µCi/ml) for 12 hours at 12 h.p. Cells were collected and freeze-thawed three times; [35S]-radiolabeled reovirus was purified as described. For Cy3-labeled reovirus, Cy3 monofunctional reactive dye (Amersham Biosciences, Arlington Heights, IL) was added to purified reovirus virions (5 x 10⁹ particles per ml) and incubated at 25°C for 45 minutes. Conjugated virus was dialyzed at 4°C against phosphate-buffered saline (PBS) (pH 7.0) overnight to remove free dye. Conjugation of reovirus with fluorescent dyes has been shown to yield labeling of viral outer-capsid proteins σ1, σ3, μ1, and λ2 as well as a fivefold decrease in viral infectivity.49 However, we did not detect a significant decrease in infectivity of our conjugated reovirus.

Reovirus binding analysis. Cells were plated in triplicates at a density of 10⁶ per well in a 6-well culture dish and incubated for 24 hours before treatment. [35S]-methionine-labeled reovirions (2.5 x 10⁶ counts per minute/sample corresponding to about 1,000 MOI) were added to cells n minimal medium and allowed to bind to cells for 1 hour at 4°C. Cells were subsequently washed twice with PBS to remove unbound virions, and samples were collected by lysis of the cells (PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, and 1 mmol/l EDTA); samples were then exposed to scintillation counting. For negative binding control, rabbit polyclonal anti-reovirus type-3 serum was added together with labeled virus.

Reovirus disassembly analysis. Cells were treated as described previously. After binding, fresh medium was added to cells, which were then returned to a 37°C incubator. At the indicated times p.i., cells were washed in PBS.
and lysed. Lysates were cleared of debris by centrifugation, and supernatants were submitted to SDS-PAGE followed by autoradiography. For E64 protease inhibitor (Sigma-Aldrich, St. Louis, MO), cells were exposed to 100 μmol/l of E64 in culture medium 1 hour before reovirus infection. After binding, E64 was again added to the cells to yield a final concentration of 100 μmol/l. Western blotting (on equal amounts of protein from lysates) with a specific anti-μI reovirus antibody (10H2) (1/1,000)47 was performed for the same experiment but using unlabeled reovirions.

Reovirus uptake and trafficking. Cells were grown on slides and infected with Cy3-labeled reovirus at an MOI of 5,000 pfu/cell (the minimum amount of virus to detect fluorescence) for 1 hour at 4°C. Cells were subsequently washed in PBS and returned to 37°C in fresh medium for 24 hours. Cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature, followed by four washes with PBS. The fixed cells were treated with 10% goat serum in PBS and then incubated with mouse monoclonal lysosomal associated membrane protein 2 lysosomal marker (1/250) (Abcam, Cambridge, MA) followed with secondary FITC–conjugated rabbit anti-mouse IgG (1/250) (Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature; cells were then photographed with a fluorescent Zeiss microscope using the multiple acquisition software provided by Zeiss (magnification ×400).

Reovirus infection. Approximately 10^5 cells of each cell line were dispensed into 12-well plates or 2-well glass slides and infected with reovirus or ISVPs at an MOI of 20 pfu/cell. For E64 protease inhibitor (Sigma-Aldrich, St. Louis, MO) treatment, cells were exposed to 100 μmol/l of E64 in culture medium 1 hour before reovirus infection. For synthetic dsRNA (poly I:C), Amersham Biosciences, Arlington Heights, IL) treatment, cells were exposed to 20 μg/ml of the synthetic dsRNA in the medium for a period of 6 hours before reovirus or ISVP infection. Virus was allowed to bind for 45 minutes at 4°C, after which E64 was again added to the cells to a final concentration of 100 μmol/l. For metabolic labeling, [35S]-methionine was added to the culture medium at 18 h.p.i. for a period of 6 hours; then viral protein synthesis was assessed as previously described.32,41 For immunofluorescent studies, cells were grown on slides and processed as described but then incubated with rabbit polyclonal anti-reovirus type-3 serum (diluted 1/5,000 in PBS) for 1 hour at room temperature, washed, and incubated with secondary antibody FITC–conjugated goat anti-rabbit IgG diluted 1/250 in PBS (Cedarlane, Hornby, Canada) for 1 hour at room temperature.

Progeny virus production. Approximately 2 × 10^5 cells grown in 6-well plates were infected with reovirus at an MOI of 20. At 48 h.p.i., the plates were frozen and stored at −70°C until use. To assay for progeny virus production, the plates were subjected to three rounds of freeze-thaw, and serial dilution of the supernatants were used for plaque titration on L929 cells. All titration experiments were repeated in triplicate.

Northern blot analysis. Digoxigenin-labeled riboprobes were generated from the plasmid pGEM-4Z-S1, which contained the open reading frame of the S1 complementary DNA. The plasmid was linearized by restriction enzyme Sall. Antisense probes to detect positive strand transcript were synthesized using the T7 polymerases and DIG-RNA labeling reagents (Roche Diagnostics, Laval, Canada) according to the manufacturer’s recommendation. Probes were precipitated with ethanol and then assessed for quality by agarose gel electrophoresis. Cells were infected with reovirus at an MOI of 20 pfu and the virus was allowed to bind for 1 hour at 4°C; inoculum was then replaced by fresh medium and put at 37°C before total cellular RNA was extracted 24 h.p.i. using RNeasy according to the manufacturer’s protocol (Qiagen Inc, Mississauga, Canada). Five micrograms of total RNA were resolved on 1.2% agarose-formaldehyde gel and transferred electrophoretically (0.5 A overnight at 4°C) to Hybond-NTM nylon membranes (Amersham Biosciences, Arlington Heights, IL) in 0.5× Tris/acetate/EDTA buffer (pH 7). After prehybridization for 5 hours, the hybrids were carried out at 50–55°C for 24–36 hours, followed by high-stringency washing at 68°C in 0.1× SSC, 0.1% sodium dodecyl sulfate. Anti-DIG-HRP (Roche) was used for detection of the probe using enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL) on film (Kodak, Chalon-sur-Chaone, France).

Viability assay. Cells grown to 50% confluence were infected with reovirus or ISVP at an MOI of 20. Cell viability was measured at 48 h.p.i. by MTT assay (Sigma-Aldrich, St. Louis, MO) and by WST-1 assay (Roche Diagnostic, Laval, Canada) to measure cell viability of the Burkitt lymphoma Daudi cells as previously described.32,48

Cathepsin B and L inhibition and activity. The specific inhibitors CA-074 (Cathepsin B) and Inhibitor III (Cathepsin L) (Invitrogen, Burlington, Canada) were added to cells at a concentration of 10 μmol/l/1 hour before reovirus infection. Cathepsin B or L activity in susceptible and resistant cells, as well as in tumor tissues, was quantified using a proteolytic activity kit (Invitrogen, Burlington, Canada) according to the manufacturer’s protocol.

Severe combined immunodeficiency mice studies. Six to eight-week-old Fox-Chase severe combined immunodeficiency mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The animals were maintained under specific pathogen-free conditions and according to a protocol approved by the University of Calgary Animal Care Committee. As a xenograft model, 1.0 × 10^6 U118 glioma cells were injected subcutaneously in the hind flank of the mice. Once palpable tumors were established (day 0), 1.0 × 10^7 pfu of live reovirus in PBS was administered intratumorally (experimental group) or PBS was administered alone (control group). Two-dimensional tumor measurements were performed with calipers every other day for 25 days or until the animals showed severe morbidity due to excess tumor burden or due to complications arising from viral infection. For histology and immunohistochemistry studies, tumors (or remaining masses) taken from animals on day 20 after intratumoral reovirus (or saline) injection were fixed in 10% neutral buffered formalin, embedded in paraffin for histological analysis, and then processed as described.32,41

Ras activity. Ras–GTP levels from established human glioma cells lines, parental NIH3T3 and NIH3T3 transformed with Ras, L929 cells, and tumor tissues derived subcutaneously from U118 cells grown in severe combined immunodeficiency/non-obese diabetic mice were measured using a Ras activation assay kit (Upstate Biotechnology, Lake Placid, NY) according to manufacturer protocol and as described previously.32

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