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Reovirus-Induced G_2/M Cell Cycle Arrest Requires σ1s and Occurs in the Absence of Apoptosis

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Serotype-specific differences in the capacity of reovirus strains to inhibit proliferation of murine L929 cells correlate with the capacity to induce apoptosis. The prototype serotype 3 reovirus strains Abney (T3A) and Dearing (T3D) inhibit cellular proliferation and induce apoptosis to a greater extent than the prototype serotype 1 reovirus strain Lang (T1L). We now show that reovirus-induced inhibition of cellular proliferation results from a G_2/M cell cycle arrest. Using T1L × T3D reassortant viruses, we found that strain-specific differences in the capacity to induce G_2/M arrest, like the differences in the capacity to induce apoptosis, are determined by the viral S1 gene. The S1 gene is bicistronic, encoding the viral attachment protein σ1 and the nonstructural protein σ1s. A σ1s-deficient reovirus strain, T3C84-MA, fails to induce G_2/M arrest, yet retains the capacity to induce apoptosis, indicating that σ1s is required for reovirus-induced G_2/M arrest. Expression of σ1s in C127 cells increases the percentage of cells in the G_2/M phase of the cell cycle, supporting a role for this protein in reovirus-induced G_2/M arrest. Inhibition of reovirus-induced apoptosis failed to prevent virus-induced G_2/M arrest, indicating that G_2/M arrest is not the result of apoptosis related DNA damage and suggests that these two processes occur through distinct pathways.

Reovirus infection of cultured cells results in inhibition of cellular proliferation (10, 17–19, 21, 24–27, 38, 40, 41, 44). Serotype 3 prototype strains type 3 Abney (T3A) and type 3 Dearing (T3D) inhibit cellular DNA synthesis to a greater extent than the serotype 1 prototype strain type 1 Lang (T1L) (40, 44). Studies using T1L × T3A and T1L × T3D reassortant viruses indicate that the S1 gene is the primary determinant of DNA synthesis inhibition (40, 44). Earlier studies suggested that reovirus-induced inhibition of cellular proliferation results from inhibition of the initiation of DNA synthesis, consistent with a G_1-S transition block (10, 19, 26, 27, 38).

Reovirus infection also results in apoptosis (11, 36, 37, 44, 45). Reovirus strains T3A and T3D induce apoptosis to substantially greater extent than T1L (44, 45). A significant correlation exists between the capacities of both T1L × T3A (r = 0.937) and T1L × T3D (r = 0.772) reassortant viruses and reovirus field isolate strains (r = 0.851) to inhibit cellular proliferation and induce apoptosis (44). Like strain-specific differences in DNA synthesis inhibition, strain-specific differences in apoptosis induction also segregate with the S1 gene (36, 44, 45).

The viral S1 gene segment is bicistronic, encoding the viral attachment protein, σ1, and a non-virion-associated protein with no known function, σ1s, from overlapping reading frames (20, 30, 39). Using a σ1s-deficient virus strain, it was shown that σ1s is not required for reovirus growth in cell culture and is dispensable for the induction of apoptosis (37). These observations in conjunction with the genetic mapping studies suggest that σ1 is the primary determinant of strain-specific differences in apoptosis induction. The S1 gene product associated with reovirus-induced inhibition of cellular DNA synthesis has not been identified.

We conducted experiments to further investigate the relationship between reovirus-induced cellular DNA synthesis inhibition and apoptosis. We found that inhibition of cellular proliferation in response to reovirus infection is caused by an arrest in the G_2/M phase of the cell cycle. Reovirus strains differ in the capacity to induce G_2/M arrest, and we used reassortant viruses to demonstrate that these differences segregate with the S1 gene. A reovirus σ1s mutant fails to induce G_2/M arrest but retains the capacity to induce apoptosis. Inducible expression of σ1s results in the accumulation of cells in G_2/M phase. Inhibition of reovirus-induced apoptosis does not affect reovirus-induced G_2/M arrest. These results indicate that the σ1s protein is required for reovirus-induced G_2/M arrest and suggest that reovirus-induced inhibition of cellular proliferation and induction of apoptosis involve independent pathways.

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted mouse L929 cells (ATCC CCL1) were grown in Joklik's modified Eagle's minimal essential medium (JMEM) supplemented to contain 5% heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, Md.) and 2 mM L-glutamine (Gibco). Human embryonic kidney (HEK293) cells (ATCC CRL1573), Madin-Darby canine kidney (MDCK) cells (ATCC CCL34), C127 cells (ATCC CRL1616), and HeLa cells (ATCC CCL2) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented to contain 10% heat-inactivated fetal bovine serum (HEK293, MDCK, and C127) or 10% non-heat-inactivated fetal bovine serum (HeLa), 2 mM L-glutamine, and 100 U of penicillin and 100 μg of streptomycin per ml (Gibco). IκB-ΔN2 cells are HEK293 cells expressing a strong dominant-negative IκB mutant lacking the phosphorylation sites that regulate signal-dependent activation of NF-κB (7).

Reovirus strains T1L, T3A, and T3D are laboratory stocks. T1L × T3D reassortant viruses were grown from stocks originally isolated by Kevin Coombs, Bernard Fields, and Max Nibert (4, 9). The reovirus field-isolate strain type 3 clone 84 (T3C84) was isolated from a human host, and T3C84-MA was isolated

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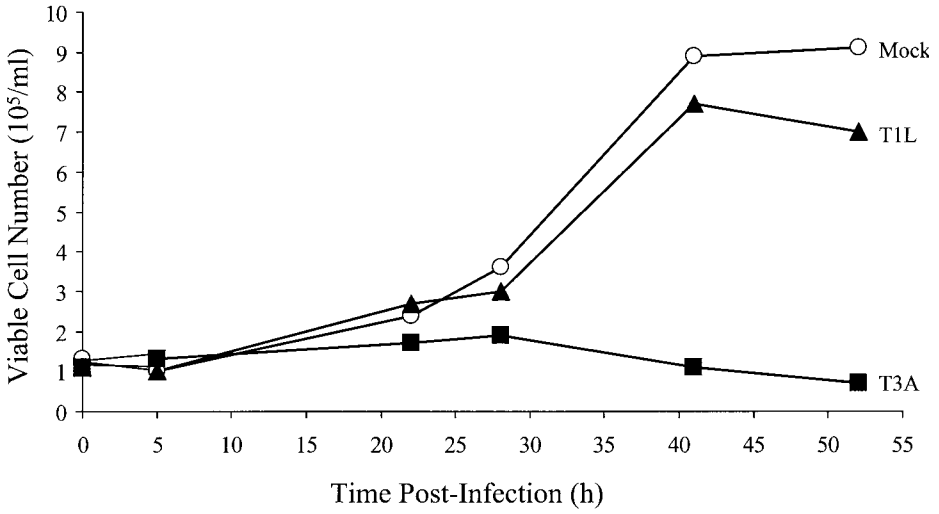

FIG. 1. Reovirus inhibits cellular proliferation. Asynchronous, subconfluent monolayers of L929 cells were either mock infected (circles) or infected with T1L (triangles) or T3A (squares) at an MOI of 100 PFU per cell. Cells were harvested at the indicated times postinfection and counted. Cells that excluded trypan blue were scored as viable. Results are presented as the number of viable cells $\times 10^5$ per ml. The results from a representative experiment of three independent experiments are shown.

as previously described (6, 12). Viral strains were plaque purified and passaged two to three times in L929 cells to generate working stocks as previously described (43).

Isolation and characterization of T3C84-MA/σ1s+. T3C84-MA/σ1s+ was isolated following serial passage of T3C84 in MEL cells as previously described (6). To isolate a sialic acid binding MEL cell-adapted variant derived from T3C84 that retains the capacity to express σ1s, virus isolates from a fifth-passage murine erythroleukemia (MEL) cell lysate stock were plaque purified twice on L929 cell monolayers. Plaques were amplified twice in L929 cell cultures and used to infect L929 cells (10^7) at a multiplicity of infection (MOI) of 10 PFU per cell. Cytoplasmic extracts were prepared 24 h following infection as previously described (8). Protein (100 μg) was electrophoresed in a 14% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. An immunoblot for σ1s was performed as previously described (37). The S1 gene of a fifth-passage isolate that expresses σ1s, termed T3C84-MA/σ1s+, was sequenced as previously described (6). T3C84-MA/σ1s+ contains the mutation at nucleotide 616 that results in a tryptophan-to-arginine substitution at residue 202 of the σ1 protein, which is also present in the S1 gene of T3C84-MA and confers the capacity to bind sialic acid but does not contain the mutation that results in the introduction of a stop codon following amino acid six in the σ1s protein.

Cellular proliferation. L929 cells were seeded in six-well plates (Costar, Cambridge, Mass.) at 10^5 cells per well in a volume of 2.5 ml in JMEM supplemented to contain nonessential amino acids, 5% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. After 24 h of incubation, when cells were 10 to 20% confluent, the medium was removed, and cells were infected with viral strains at an MOI of 100 PFU per cell in a volume of 100 μl at 37°C for 1 h. After viral infection, 2.5 ml of fresh medium was added to each well. At various times postinfection, cells were harvested, resuspended in 2 ml of phosphate-buffered saline (PBS), and counted using a hemacytometer. Cell viability was determined by trypan blue exclusion. Results are presented as the viable cell numbers per milliliter.

Flow cytometry. L929, HEK293, MDCK, and HeLa cells were seeded in either 12-well plates (Costar) at 10^5 cells per well in a volume of 1 ml per well or 24-well plates (Costar) at 3.7×10^4 cells per well in a volume of 0.5 ml per well and then infected with reovirus as described above. Cells were harvested, washed once with PBS, and stained at 4°C overnight with Krishan's stain containing 3.8 mM trisodium citrate (Sigma Chemical Co., St. Louis, Mo.), 70 μM propidium iodide (Sigma), 0.01% Nonidet P-40 (Sigma), and 0.01 mg of RNase A (Boehringer Mannheim Co., Indianapolis, Ind.) per ml (33). Cell cycle analysis was performed using a Coulter Epics XL flow cytometer (Beckman-Coulter, Hialeah, Fla.). Alignment of the instrument was verified daily using DNA check beads (Coulter). Peak versus integral gating was used to exclude doublet events from the analysis. Data were collected for 10,000 events. The Modfit LT program (Verity Software House, Topsham, Maine) was used for cell cycle modeling.

Cell synchronization. L929 cells were seeded in 24-well plates at 3.7×10^4 cells per well in a volume of 0.5 ml per well. After 24 h, cells were treated with 1 μM amethopterin (methotrexate) (Sigma) and 50 μM adenosine (Sigma) for 16 h. Cells were washed twice with PBS, infected with reovirus, and incubated with fresh JMEM supplemented to contain 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 2 mg of thymidine (Sigma) per ml. At various times after infection, cells were harvested, washed once with PBS, and stained at 4°C overnight with Krishan's stain as described above.

Quantitation of apoptosis by acridine orange staining. L929, HEK293, MDCK, and HeLa cells were seeded and infected with reovirus as described above. The percentage of apoptotic cells was determined at 48 h postinfection as previously described (16, 45). Cells were harvested, washed once with PBS, resuspended in 25 μl of cell culture medium, and stained with 1 μl of a dye solution containing 100 μg of acridine orange (Sigma) per ml and 100 μg of ethidium bromide (Sigma) per ml. Cells were examined by epifluorescence microscopy (Nikon Labophot-2; B-2A filter; excitation, 450 to 490 nm; barrier, 520 nm; dichroic mirror, 505 nm) and scored as apoptotic if their nuclei contained uniformly stained condensed or fragmented chromatin (16, 45).

Apoptosis inhibitors. L929 cells were seeded in 24-well plates at 3.7×10^4 cells per well in a volume of 0.5 ml per well. After 24 h of incubation, cells were incubated with the calpain inhibitor PD150606 (Parke-Davis Pharmaceutical Research, Ann Arbor, Mich.) (50 μM, L929 cell), the caspase 3 inhibitor DEVD-CHO (Clontech, Palo Alto, Calif.) (100 μM, HEK293), or anti-TRAIL antibody (Affinity Bioreagents, Golden, Colo.) (30 μM, HEK293) for 1 h. Cells were then infected with T3A at an MOI of 100 PFU per cell at 37°C for 1 h. Following infection, media containing the apoptosis inhibitor was added. Cells were harvested and analyzed for either apoptosis or cell cycle arrest at 48 h postinfection.

Inducible expression of σ1s. C127 stable transformants expressing T3D σ1s (BPX-6) from the mouse metallothionein promoter and vector control (BPV-12) were provided by Aaron Shatkin (21). BPX-6 and BPV-12 cells were seeded in 24-well plates at 3.0×10^4 cells per well in a volume of 0.5 ml per well. After 24 h of incubation, cells were incubated with 1 μM $CdCl_2$ to induce σ1s expression (22) and harvested at various times postinduction for cell cycle analysis.

RESULTS

Reovirus strains T1L and T3A differ in the capacity to inhibit cellular proliferation. We have previously shown that T1 and T3 reovirus strains differ in the capacity to inhibit cellular DNA synthesis as measured by [^{3}H]thymidine incorporation (40, 44). To determine whether reovirus-induced DNA synthesis inhibition is associated with inhibition of cellular proliferation, we infected L929 cells with either T1L or T3A at an MOI of 100 PFU per cell. At various intervals after infection, viable cells were counted (Fig. 1). Infection with T3A resulted in complete inhibition of cellular proliferation. A modest reduction in proliferation was observed for cells infected with T1L compared to mock-infected controls. Therefore, strain-specific differences in inhibition of cellular proliferation parallel those previously reported for DNA synthesis inhibition.

T3 reoviruses induce G_2/M arrest. To identify the phase in the cell cycle that T3 reoviruses inhibit cellular proliferation,

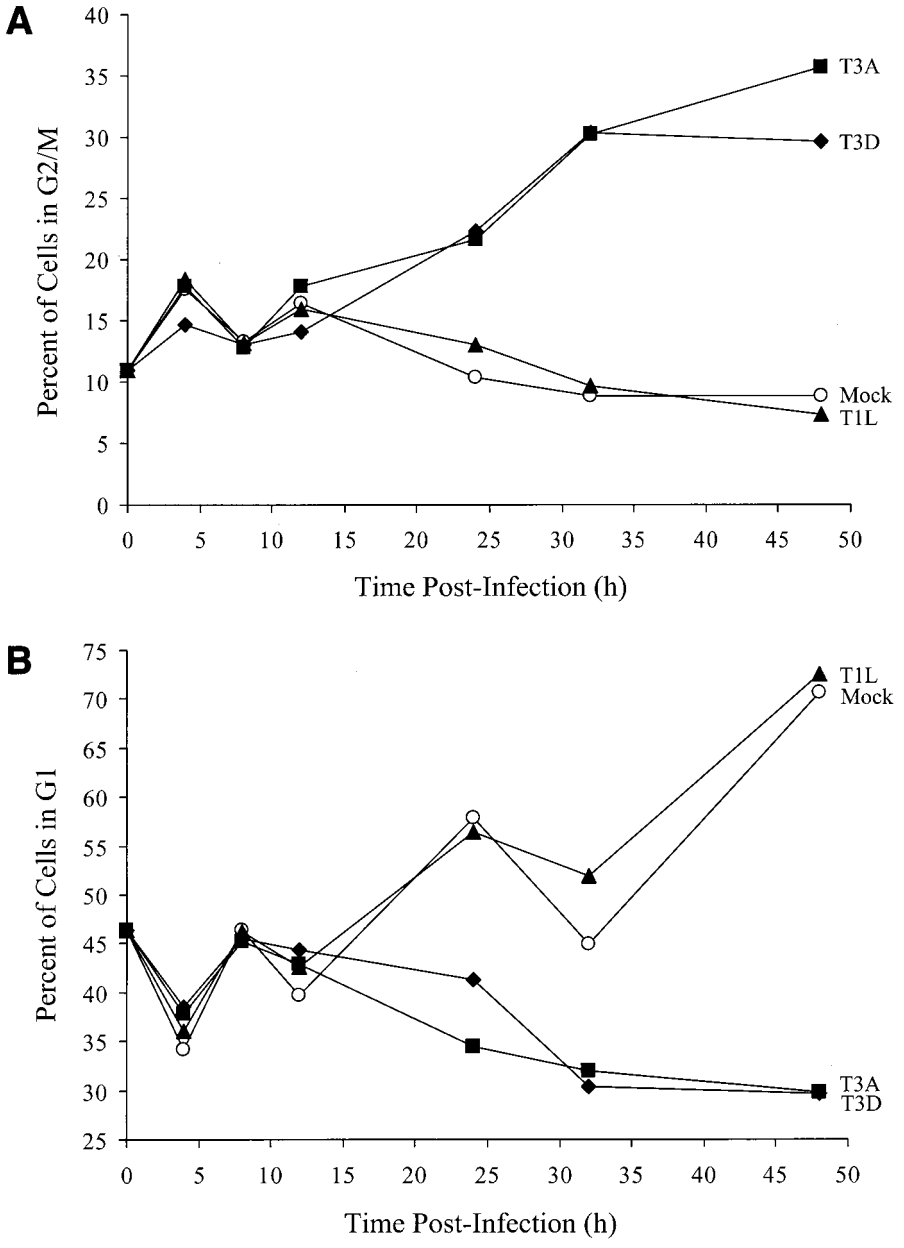

FIG. 2. T3 reovirus induces an increase in the percentage of cells in the G_2/M phase of the cell cycle. Asynchronous, subconfluent monolayers of L929 cells were either mock infected (circles) or infected with T1L (triangles), T3A (squares), or T3D (diamonds) at an MOI of 100 PFU per cell. Cells were harvested at the indicated times postinfection, stained with Krishan's stain, and analyzed for DNA content using flow cytometry. Results are presented as the percentage of cells in G_2/M phase (A) or G_1 phase (B) of the cell cycle. Results of a representative experiment of three independent experiments are shown. (C) L929 cells were synchronized with 1 μM methotrexate and 50 μM adenosine for 16 h. Cells were released using fresh media containing 2 mg of thymidine per ml and either mock infected or infected with T1L or T3A at an MOI of 100 PFU per cell. Cells were harvested at the indicated times postinfection, stained with Krishan's stain, and analyzed for DNA content using flow cytometry. Results are presented as the cell cycle distribution following either mock, T1L, or T3A infection at the indicated times postinfection.

we analyzed reovirus-infected cells using flow cytometry. L929 cells were infected with T1L, T3A, or T3D at an MOI of 100 PFU per cell and stained with Krishan's stain (33) containing propidium iodide to determine cellular DNA content at various intervals postinfection. The results were converted to the percentage of cells in G_2/M phase of the cell cycle using Modfit LT software (Fig. 2). Infection with either T3A or T3D resulted in a substantial increase in the percentage of cells in the G_2/M phase of the cell cycle compared to T1L-infected or mock-infected cells by 24 h postinfection (Fig. 2A). There also was a corresponding decrease in the percentage of cells in G_1 phase following infection with either T3A or T3D compared to T1L-infected or mock-infected cells (Fig. 2B). To confirm these results, L929 cells were synchronized with methotrexate prior to reovirus infection and assessed for cell cycle progression (Fig. 2C). Similar to findings with unsynchronized cells, T3A induced a significant increase in the proportion of cells in the G_2/M phase of the cell cycle compared to T1L or mock infection. The increase in the proportion of cells in G_2/M was first seen at 12 h postinfection and was maintained throughout the observation period (48 h). These findings indicate that the inhibition of proliferation induced by T3 reoviruses is caused by a block in the G_2/M phase of the cell cycle. Following T1L or mock infection, cells traverse the cell cycle, proliferate, and

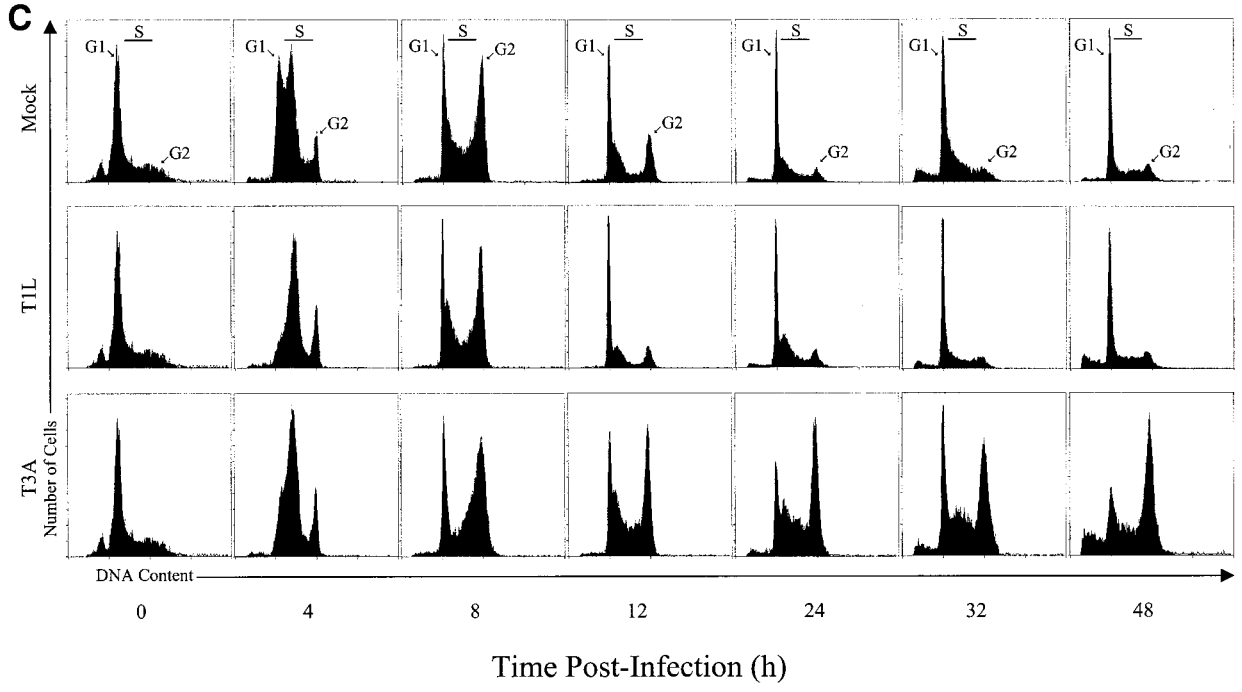

FIG. 2—*Continued.*

reenter the cell cycle. Conversely, T3-infected cells enter the cell cycle, stall in G_2/M phase, and do not proliferate.

T3 reovirus-induced G_2/M arrest is dose dependent. To investigate the relationship between MOI and the induction of G_2/M arrest, we infected L929 cells with T3A at MOIs of 1, 10, and 100 PFU per cell. Cells were harvested at 48 h postinfection, stained with Krishan's stain (33), and analyzed for DNA content by flow cytometry (Fig. 3). T3A infection induced a greater percentage of cells in G_2/M than mock infection at each MOI tested, and the effect was dose dependent.

G_2/M arrest occurs in a variety of cell lines following T3 reovirus infection. To determine whether the capacity of reovirus to block cell cycle progression is cell type dependent, L929, MDCK, C127, HEK293, and HeLa cells were either mock infected or infected with T1L or T3A at an MOI of 100 PFU per cell. Cells were harvested at 48 h postinfection, stained with Krishan's stain (33), and analyzed for DNA content by flow cytometry (Fig. 4). T3A infection induced a greater percentage of cells in G_2/M than either T1L or mock infection in all cell lines tested. However, the magnitude of the strain-specific difference was greatest in L929 (Fig. 4A), MDCK (Fig. 4B), and C127 (Fig. 4C) cells. Therefore, reovirus-induced G_2/M arrest is not cell type specific and likely requires non-cell-type-specific factors to mediate G_2/M arrest.

T3 reovirus G_2/M arrest phenotype is dominant. To determine whether G_2/M arrest resulting from T3 reovirus infection could be overcome by T1 reovirus infection, we coinfected L929 cells with equivalent MOIs of T1L and T3A and measured the percentage of cells in G_2/M by flow cytometry at 48 h postinfection. The percentage of cells in G_2/M after coinfection with T1L and T3A was identical to that of T3A alone and significantly greater than that of T1L alone (Fig. 5). These results indicate that the G_2/M arrest phenotype of T3 reovirus is dominant.

G_2/M arrest by T1L × T3D reassortant viruses. To identify viral genes associated with differences in the capacity of T1L and T3D to induce G_2/M arrest, we tested 12 T1L × T3D reassortant viruses for the capacity to induce G_2/M arrest in unsynchronized and synchronized L929 cells (Table 1). The results demonstrate a significant association between the capacity of reassortant viruses to induce G_2/M arrest in unsynchronized L929 cells and the S1 gene segment (Student *t* test, $P = 0.004$; Mann-Whitney, $P = 0.007$). No other viral genes were significantly associated with G_2/M arrest in this analysis (*t* test and Mann-Whitney, all $P > 0.05$). However, when L929 cells were synchronized prior to infection, the results demonstrate a significant association between the capacity of reassortant viruses to induce G_2/M arrest and the derivation of the S1 gene segment (Student *t* test, $P = 0.007$; Mann-Whitney, $P = 0.016$) and the M2 gene segment (Student *t* test, $P = 0.007$; Mann-Whitney, $P = 0.016$). We used parametric stepwise linear regression analysis to determine whether the S1 and M2

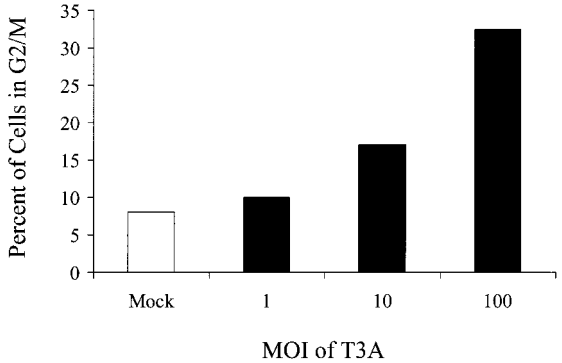

FIG. 3. G_2/M arrest induced by T3 reovirus is dose dependent. Asynchronous, subconfluent monolayers of L929 cells were either mock infected or infected with T3A at MOIs of 1, 10, and 100 PFU per cell. Cells were harvested at 48 h postinfection, stained with Krishan's stain, and analyzed for DNA content using flow cytometry. Results are presented as the percentage of cells in G_2/M phase.

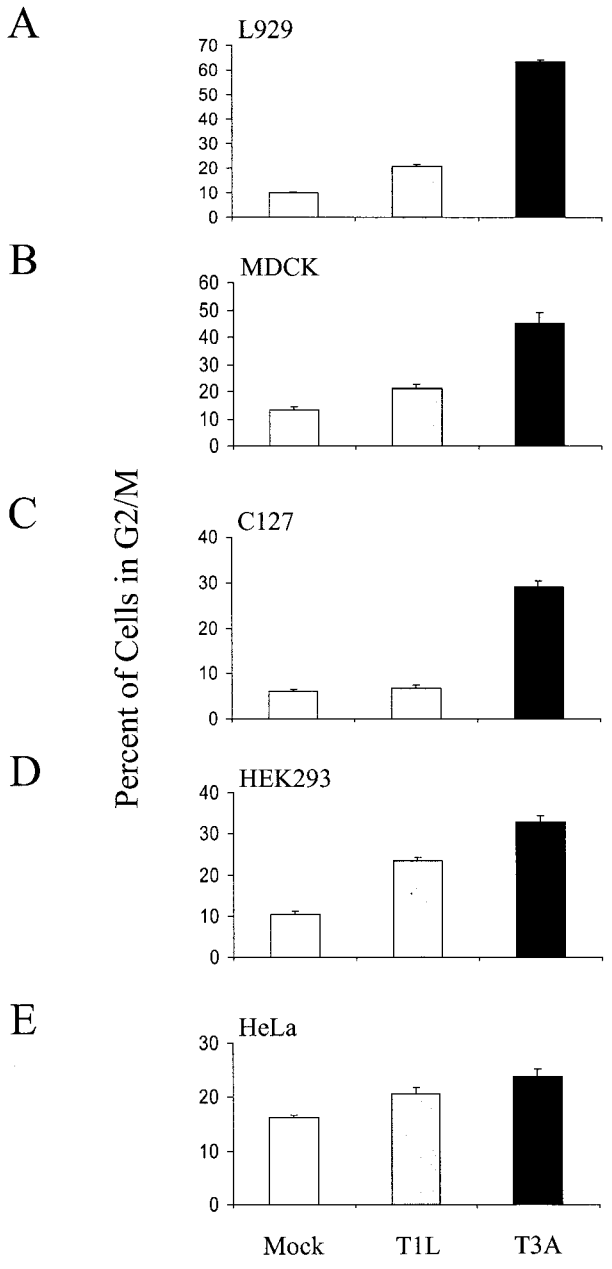

FIG. 4. T3 reovirus induces G_2/M arrest in murine, canine, and human cells. Asynchronous, subconfluent monolayers of L929 (A), MDCK (B), C127 (C), HEK293 (D), and HeLa (E) cells were either mock infected (white) or infected with T1L (gray) or T3A (black) at an MOI of 100 PFU per cell. Cells were harvested at 48 h postinfection, stained with Krishan's stain, and analyzed for DNA content using flow cytometry. Results are presented as the mean percentage of cells in G_2/M phase for three independent experiments. The error bars indicate the standard errors of the mean. A significantly greater percentage of T3A-infected cells were in G_2/M than mock-infected cells in all cell lines tested ($P < 0.01$ to 0.001). A significantly greater percentage of T3A-infected cells were in G_2/M than T1L-infected cells in all cell lines tested ($P < 0.01$ to 0.001) except HeLa. A significantly greater percentage of T1L-infected cells were in G_2/M than mock-infected cells in L929 and HEK293 cells ($P < 0.001$).

genes contributed independently to the capacity of T1L × T3D reassortant viruses to induce G_2/M arrest. We obtained R^2 values of 91.3 and 96.7% for the regression equation using all 10 reovirus genes for unsynchronized and synchronized L929 cells, respectively: 52.2% ($P = 0.004$) for S1 in unsynchronized L929 cells and 84.9% ($P < 0.001$) for S1 and M2 and 53.5% ($P = 0.007$) for the S1 gene alone in synchronized L929 cells. These results indicate that the S1 gene segment is the primary determinant of strain-specific differences in reovirus-induced G_2/M arrest.

G_2/M arrest induced by T3 reovirus. The S1 gene segment encodes two proteins, the viral attachment protein σ1 and the nonstructural protein σ1s (20, 30, 39). To determine whether σ1s is required for G_2/M arrest, we infected L929 cells with reovirus strain T3C84-MA, which does not express σ1s (37) (Fig. 6). The percentage of cells in G_2/M following infection with T3C84-MA was significantly less than the percentage of cells in G_2/M following infection with the σ1s-expressing parental virus, T3C84. T3C84-MA failed to induce G_2/M arrest, even at an MOI 10-fold greater than T3C84. T3C84-MA/σ1s+, a MEL-cell-adapted strain that does not contain the point mutation in S1 that results in an early stop codon in σ1s but contains the tryptophan-to-arginine substitution at position 202 in σ1, induced a level of G_2/M arrest that was significantly greater than T3C84-MA at an MOI of 100 in L929 cells ($P = 0.002$; percentage of cells in G_2/M following T3C84-MA/σ1s+ infection, 23.02 ± 1.1%). These findings indicate that functional σ1s is required for reovirus-induced G_2/M arrest.

Expression of T3 σ1s induces an increase in the percentage of cells in G_2/M phase. To determine whether σ1s alone is sufficient to induce the accumulation of cells in G_2/M phase, we analyzed the DNA content of C127 cells engineered to express the T3D σ1s protein. Expression of σ1s from the mouse metallothionein promoter was induced by 1 μM $CdCl_2$ (21) however, levels of σ1s were substantially less than levels found following natural virus infection (data not shown). The percentage of cells in G_2/M following induction was significantly greater in cells expressing σ1s than in vector control cells at 45 and 55 h postinduction ($P = 0.03$ and $P = 0.005$, respectively) (Fig. 7). These results provide additional evidence that σ1s expression is involved in the accumulation of cells in the G_2/M phase of the cell cycle.

Reovirus-induced apoptosis can be dissociated from reovirus-induced G_2/M arrest. Previous studies indicate that the capacity of reovirus to inhibit DNA synthesis correlates with the capacity to induce apoptosis (44). Like strain-specific differences in reovirus-induced G_2/M arrest, differences in the capacity of reovirus strains to inhibit DNA synthesis and induce apoptosis are determined by the S1 gene (40, 44). To determine whether apoptosis-associated disruption of cellular DNA is required for reovirus-induced inhibition of cellular proliferation, L929 cells or HEK293 cells were either mock infected or infected with T3A in the presence or absence of inhibitors of reovirus-induced apoptosis (7, 8, 11). Treatment of cells with the calpain inhibitor PD150606 (11), the caspase inhibitor DEVD-CHO (D. J. Kominsky, personal communication), or anti-TRAIL antibody (7) blocks reovirus-induced apoptosis, as does expression of an IκB mutant that blocks NF-κB activation (7, 8). G_2/M arrest was evaluated by flow cytometry at 48 h postinfection (Fig. 8). Treatment with the calpain inhibitor PD150606 (Fig. 8A), the caspase 3 inhibitor DEVD-CHO (Fig. 8B), or anti-TRAIL antibody (Fig. 8C) using conditions that inhibit reovirus-induced apoptosis, had no effect on T3A-induced G_2/M arrest, nor did inhibition of NF-κB by expression of a dominant-negative IκB (7, 8) (Fig. 8D). Therefore, inhibitors of reovirus-induced apoptosis do not inhibit reovirus-induced G_2/M arrest. These findings indicate that apoptosis induced DNA damage is not required for reovirus-induced G_2/M arrest.

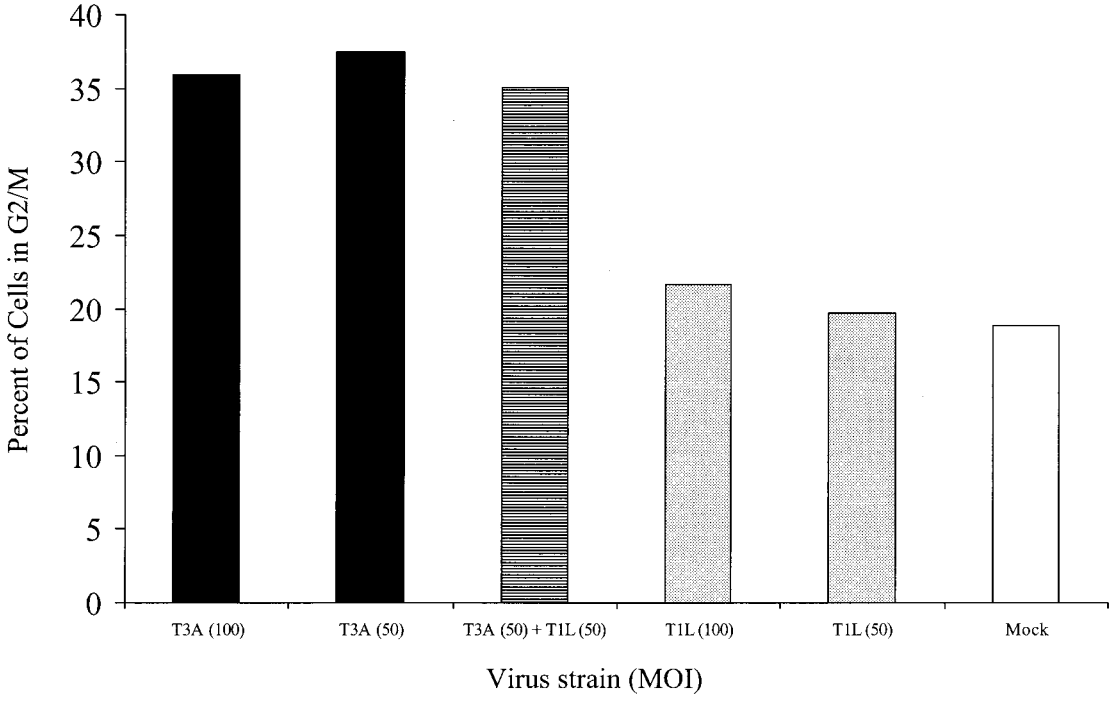

FIG. 5. T3A-induced G_2/M arrest phenotype is dominant. L929 cells were either mock infected (white), coinfected with equivalent MOIs of T1L and T3A (the MOI of each virus was 50 PFU per cell) (hatched), or infected with T1L (shaded) or T3A (solid) alone at MOIs of 50 or 100 PFU per cell. L929 cells were harvested at 48 h postinfection and analyzed using flow cytometry. The results are presented as the percentage of cells in the G_2/M phase of the cell cycle.

DISCUSSION

T3 reovirus strains inhibit host cell proliferation, as measured by cellular DNA synthesis inhibition, to a substantially greater extent than T1 reovirus strains (40, 44). It had been suggested, based on extrapolation of results obtained using [^{3}H]thymidine incorporation, that T3 reoviruses induce cell cycle arrest at the G_1-to-S transition. We now show, using flow cytometry to directly analyze cell cycle progression in reovirus-infected cells, that reovirus-induced inhibition of cellular pro-

TABLE 1. Capacities of T1L × T3D reassortant viruses to induce G_2/M arrest

Virus strain	Genome segment[a]										% Cells in G_2/M[b]	
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	Unsynchronized	Synchronized
EB138	3D	1L	1L	3D	3D	1L	3D	3D	1L	1L	ND	24.56
EB28	3D	3D	1L	3D	3D	3D	3D	1L	3D	3D	38.13	28.59
KC150	3D	1L	1L	1L	3D	1L	3D	3D	1L	3D	33.91	36.47
EB97	3D	3D	1L	3D	3D	3D	3D	3D	3D	1L	30.30	28.35
G2	1L	3D	1L	1L	1L	1L	3D	1L	1L	1L	29.09	13.92
H41	3D	3D	1L	1L	1L	3D	1L	1L	3D	1L	26.56	ND
T3D	3D	3D	3D	3D	3D	3D	3D	3D	3D	3D	25.71	38.51
H15	1L	3D	3D	1L	3D	3D	3D	3D	3D	1L	24.95	31.63
EB127	3D	3D	1L	1L	3D	1L	1L	3D	3D	1L	23.54	ND
H9	3D	3D	1L	3D	1L	1L	3D	3D	3D	3D	23.11	17.17
EB85	1L	1L	1L	1L	1L	3D	1L	3D	1L	1L	21.88	ND
T1L	1L	1L	1L	1L	1L	1L	1L	1L	1L	1L	19.23	5.56
EB145	3D	3D	3D	3D	3D	1L	1L	3D	3D	3D	15.72	14.72
EB121	3D	3D	1L	3D	1L	3D	1L	3D	3D	3D	14.98	9.45
EB1	1L	3D	1L	1L	3D	1L	1L	1L	3D	1L	11.89	16.19
Significance (P)[c]												
Unsynchronized L cells												
t test	0.30	0.84	0.60	0.85	0.46	0.36	**0.004**	0.78	0.58	0.66		
MW	0.30	1	0.77	0.95	0.41	0.38	**0.007**	0.80	0.73	0.85		
Synchronized L cells												
t test	0.25	1	0.27	0.73	**0.007**	0.16	**0.007**	0.18	0.67	0.53		
MW	0.28	1	0.28	0.76	**0.016**	0.2	**0.016**	0.21	0.57	0.48		

[a] The parental origin of each genome segment in the reassortants strains: 1L, genome segment derived from T1L; 3D, genome segment derived from T3D.
[b] Unsynchronized or synchronized L cells were infected with viral strains at an MOI of 100 PFU per cell and analyzed by flow cytometry at 48 h postinfection. ND, not determined.
[c] As determined by two-sample parametric Student *t* test (*t* test) and Mann-Whitney nonparametric analysis (MW). Values in boldface are statistically significant.

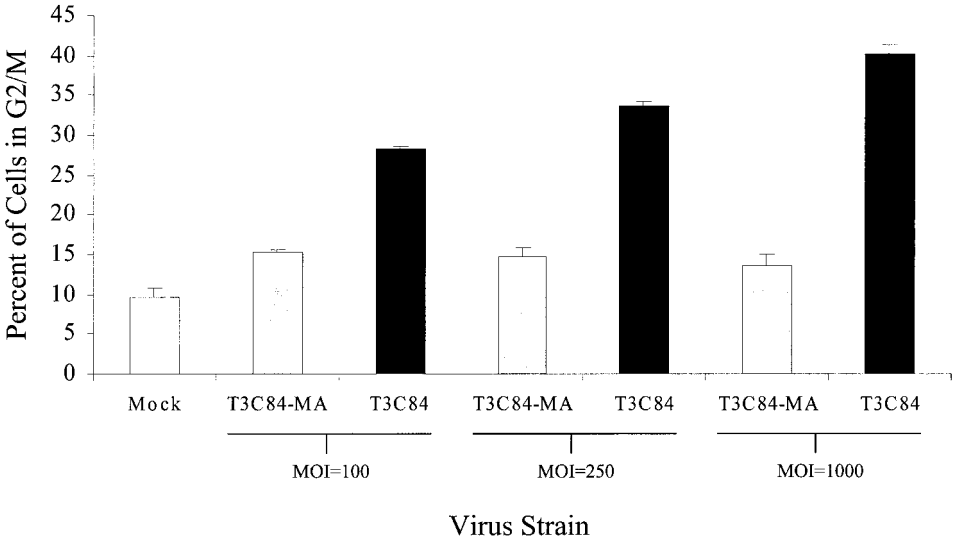

FIG. 6. Reovirus-induced G_2/M arrest requires σ1s. L929 cells were either mock infected (white) or infected with wild-type T3C84 (black) or σ1s-null mutant T3C84-MA (gray) at MOIs of 100, 250, or 1,000 PFU per cell. Cells were harvested 48 h postinfection, stained with Krishan's stain, and analyzed using flow cytometry. The results are presented as the mean percentage of cells in G_2/M phase of the cell cycle for six independent experiments at an MOI of 100 and three independent experiments at MOIs of 250 and 1,000. The error bars indicate the standard errors of the mean. A significantly greater percentage of T3C84-infected cells were in G_2/M than T3C84-MA-infected cells at each MOI tested ($P < 0.001$).

liferation results from G_2/M arrest. This effect is not cell type specific and is dominant in strains that block cell cycle progression.

Differences in the capacity of reovirus strains to inhibit cellular proliferation are determined by the viral S1 gene (40, 44). Our results indicate that the same is true for G_2/M arrest. The reovirus S1 gene is bicistronic, encoding the structural protein σ1 and the nonstructural protein σ1s using overlapping, alternative reading frames (20, 30, 39). As a result of this coding strategy, there is no sequence similarity between the σ1 and σ1s proteins (12). To determine which of the two S1-encoded proteins are required for G_2/M arrest, we examined the capacity of the σ1s null mutant T3C84-MA to induce G_2/M arrest. T3C84-MA and its σ1s expressing parent, T3C84, produce equivalent yields of viral progeny in L929 cells, and both viruses are equally effective in inducing apoptosis (37). However, T3C84-MA fails to induce G_2/M arrest. This finding suggests that σ1s is required for blockade of cell cycle progression following T3 reovirus infection. It is also possible that differences in the capacity of T3C84 and T3C84-MA to induce cell cycle arrest are influenced by other sequence differences. The mutation in the S1 gene that introduces a termination codon in the σ1s open reading frame also results in a lysine-to-isoleucine substitution at residue 26 in the deduced amino acid sequence of σ1. The T3C84-MA S1 gene also contains an additional mutation that results in a tryptophan-to-arginine substitution at residue 202 in σ1, which determines the capacity of this strain to bind sialic acid. To exclude the possibility that sialic acid binding influences cell cycle arrest, we isolated and characterized an additional T3C84-MA variant, T3C84-MA/σ1s+, that binds to sialic acid and expresses σ1s. In contrast to T3C84-MA, which binds sialic acid but does not express σ1s, T3C84-MA/σ1s+ induces G_2/M arrest. Therefore, it is unlikely that the capacity to bind sialic acid influences the efficiency of cell cycle arrest induced by T3 reoviruses.

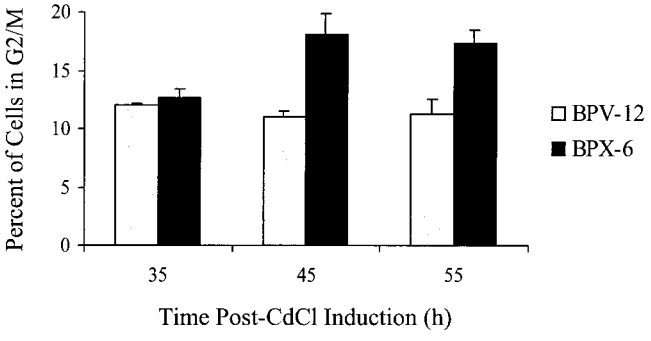

FIG. 7. σ1s expression induces an increase in the percentage of cells in G_2/M phase. C127 cells stably transfected with σ1s (BPX-6) or vector control (BPV-12) under the control of the mouse metallothionein promoter were induced with $CdCl_2$, harvested at the indicated times postinduction, and analyzed for DNA content by flow cytometry. The results are presented as the mean percentage of cells in the G_2/M phase of the cell cycle for three to six independent experiments. The error bars indicate the standard errors of the mean. The percentage of cells in G_2/M was significantly greater in the σ1s-expressing cells than in the vector-control cells at 45 h ($P = 0.03$, $n = 4$) and 55 h ($P = 0.005$, $n = 6$) postinduction.

To corroborate findings made using viruses that vary in σ1s expression, we also tested the capacity of cells engineered to express σ1s under the control of an inducible promoter to undergo cell cycle arrest. Following induction of σ1s expression, we observed an increase in the percentage of cells in the G_2/M phase of the cell cycle, which suggests that σ1s is capable of mediating cell cycle blockade at the G_2/M checkpoint. This observation suggests that the reovirus σ1s protein is similar to the human immunodeficiency virus (HIV) Vpr protein (2, 28, 31, 35) or the human papillomavirus (HPV) E2 protein (23), which similarly block cell cycle progression at the G_2/M boundary. Thus, our findings indicate that reovirus-induced G_2/M arrest requires σ1s and provide the first evidence of a functional role for this nonstructural protein.

We have previously shown that the capacity of reovirus to induce apoptosis correlates with the capacity to inhibit cellular proliferation and that both properties are determined by the viral S1 gene (44). Our results clearly show that G_2/M arrest can occur in cells treated with potent inhibitors of reovirus-

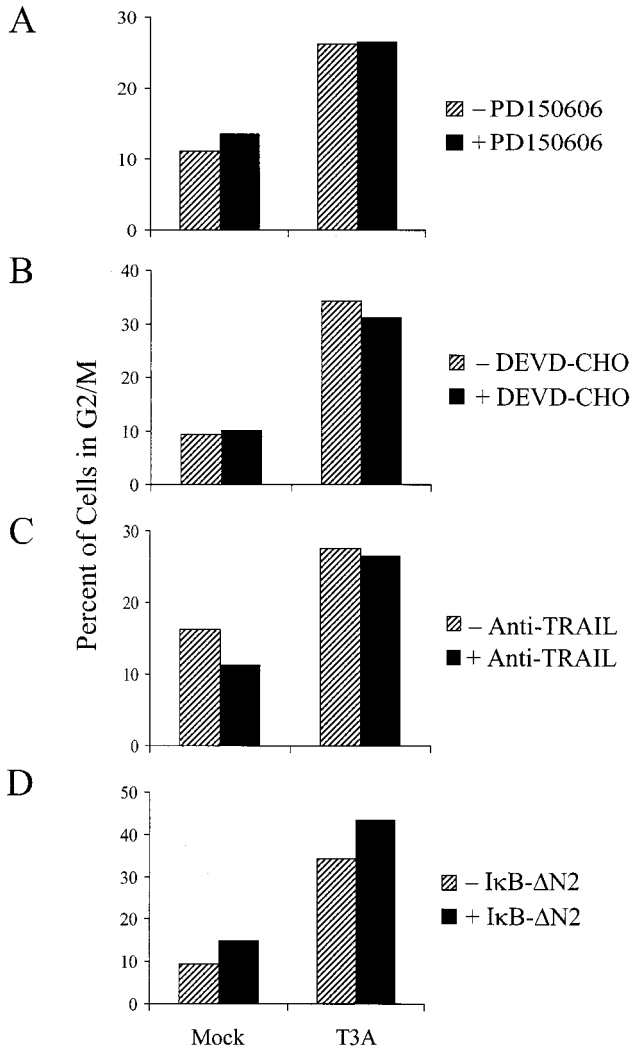

FIG. 8. Inhibitors of reovirus-induced apoptosis do not inhibit reovirus-induced G_2/M arrest. (A) Effect of calpain inhibitor PD150606 on T3A-induced G_2/M arrest. L929 cells were treated with either 25 μM calpain inhibitor PD150606 or an ethanol control and then either mock infected or infected with T3A at an MOI of 100 PFU per cell. (B) Effect of caspase 3 inhibitor DEVD-CHO on T3A-induced G_2/M arrest. HEK293 cells were treated with either 100 μM caspase 3 inhibitor DEVD-CHO or a dimethyl sulfoxide control and then either mock infected or infected with T3A at an MOI of 100 PFU per cell. (C) Effect of anti-TRAIL antibodies on T3A-induced G_2/M arrest. HEK293 cells were treated with either 30 μg of an anti-TRAIL antibody per ml or mock treated as a control and then either mock infected or infected with T3A at an MOI of 100 PFU per cell. (D) Effect of NF-κB inhibition on T3A-induced G_2/M arrest. HEK293 cells expressing a dominant-negative form of IκB (IκB-ΔN2) to inhibit NF-κB activation or untransfected HEK293 cells were either mock infected or infected with T3A at an MOI of 100 PFU per cell. In all cases, G_2/M arrest was assessed 48 h postinfection.

induced apoptosis. These findings indicate that the induction of G_2/M arrest and apoptosis by reovirus are functionally independent at some stage following infection. Moreover, although strain-specific differences in reovirus-induced G_2/M arrest and apoptosis induction segregate with the viral S1 gene, each property is determined by a different S1 gene product. Strain-specific differences in reovirus-induced G_2/M arrest are determined by σ1s, whereas differences in reovirus-induced apoptosis are determined by σ1 (36, 45). The induction of G_2/M arrest by HIV Vpr is apparently required for Vpr-induced apoptosis (42), whereas reovirus-induced apoptosis can occur in the absence of G_2/M arrest (37). These findings suggest that viruses may utilize different mechanisms to induce G_2/M arrest and apoptosis.

The G_2/M transition is regulated by the kinase cdc2/cdk1 (13–15, 32, 34). Expression of HIV Vpr (28, 35) or HPV E2 protein (23) results in inhibition or delayed activation of cdc2 kinase activity resulting in an accumulation of cells in the G_2/M phase of the cell cycle. In contrast, the baculovirus *Autographa californica* nuclear polyhydrosis virus (AcNPV) (3) and herpes simplex virus (HSV) (1, 29) induce G_2/M arrest by a mechanism that is cdc2 independent, since cells infected with either of these viruses maintain high levels of cdc2 kinase activity. HIV, HPV, AcNPV, and HSV require a nuclear phase to replicate, whereas reovirus replicates in the cytoplasm. T3 σ1s has been detected in the nucleus as well as in the cytoplasm following reovirus infection (5, 37), and it is possible that this nuclear localization is required for reovirus-induced G_2/M arrest. Future studies will be aimed at identifying which cell cycle regulatory proteins are involved in reovirus-induced cell cycle perturbation, the role of cellular localization of σ1s in this process, and the significance of cell cycle arrest in reovirus-induced cytopathology and pathogenesis.

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