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Reovirus-Induced Apoptosis Is Mediated by TRAIL

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Members of the tumor necrosis factor (TNF) receptor superfamily and their activating ligands transmit apoptotic signals in a variety of systems. We now show that the binding of TNF-related, apoptosis-inducing ligand (TRAIL) to its cellular receptors DR5 (TRAILR2) and DR4 (TRAILR1) mediates reovirus-induced apoptosis. Anti-TRAIL antibody and soluble TRAIL receptors block reovirus-induced apoptosis by preventing TRAIL-receptor binding. In addition, reovirus induces both TRAIL release and an increase in the expression of DR5 and DR4 in infected cells. Reovirus-induced apoptosis is also blocked following inhibition of the death receptor-associated, apoptosis-inducing molecules FADD (for FAS-associated death domain) and caspase 8. We propose that reovirus infection promotes apoptosis via the expression of DR5 and the release of TRAIL from infected cells. Virus-induced regulation of the TRAIL apoptotic pathway defines a novel mechanism for virus-induced apoptosis.

Studies using mammalian reoviruses have provided fundamental insights into the molecular and genetic basis of viral pathogenesis and virus-induced cell death. Reovirus infection induces apoptosis in cultured cells in vitro (13, 15, 26) and in target tissues in vivo, including the central nervous system, heart, and liver (12, 13). Reovirus induces apoptosis by a p53-independent mechanism that involves cellular proteases including calpains (4), is dependent on reovirus-induced NF-κB activation (3), and is inhibited by overexpression of Bcl-2 (15). Strain-specific differences in the capacity of reoviruses to induce apoptosis are determined by the viral S1 gene (26) and require viral binding to cell surface receptors but not completion of the full viral replication cycle (15). Reovirus-induced apoptosis correlates with pathology in vivo and is a critical mechanism by which disease is triggered in the host (12). Inhibition of apoptosis in vivo reduces the extent of tissue injury (R. L. Debiasi et al., Am. Soc. Virol. Sci. Program Abstr., abstr. W52-1, 1999), emphasizing the importance of apoptosis in reovirus pathogenesis. We have thus used reovirus infection to study mechanisms of virus-induced apoptosis.

Cellular death receptors (DRs) transmit apoptosis-inducing signals initiated by specific death ligands, most of which are primarily expressed as biologically active type II membrane proteins that are cleaved into soluble forms. Fas ligand (FasL) activates Fas/CD95/Apo1, tumor necrosis factor (TNF) activates TNFR1 (TNF receptor 1), Apo 3L/TWEAK activates DR3, and TRAIL (for TNF-related apoptosis-inducing ligand; also called Apo2L) activates DR4 (TRAILR1) and DR5 (TRAILR2/TRICK2). Ligand-mediated activation triggers a cascade of events that begins with DR oligomerization and the close association of their cytoplasmic death domains (DDs). This is followed by DD-associated interaction with adapter molecules and cellular proteases critical to DR-induced apoptosis (reviewed in reference 1). In this paper we describe a novel mechanism for virus-induced cell death involving the upregulation of DR5, the release of TRAIL from infected cells, and subsequent TRAIL-mediated apoptosis.

MATERIALS AND METHODS

Cells, virus, and inhibitors. HEK293 cells (ATCC CRL1573) were grown in Dulbecco's modified Eagle's medium supplemented with 100 U each of penicillin and streptomycin per ml and containing 10% fetal bovine serum. HeLa cells (ATCC CCL2) were grown in Eagle's minimal essential medium supplemented with 2.4 mM L-glutamine, nonessential amino acids, 60 U each of penicillin and streptomycin per ml, and containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, Md.). FADD-DN cells express amino acids 80 to 208 of the Fas-associated DD (FADD) cDNA (with the addition of an AU1 epitope tag at the N terminus), from the cytomegalovirus promoter from pcDNA3 (Invitrogen, Carlsbad, Calif.). Reovirus (type 3 Abney [T3A]) is a laboratory stock which has been plaque purified and passaged (twice) in L929 (ATCC CCL1) cells to generate working stocks (27). Virus growth was determined by plaque assay as previously described (25).

Western blot analysis and antibodies. Twenty-four hours following infection with reovirus, cells were pelleted by centrifugation, washed twice with ice-cold phosphate-buffered saline, and lysed by sonication in 200 μl of a buffer containing 15 mM Tris (pH 7.5), 2 mM EDTA, 10 mM EGTA, 20% glycerol, 0.1% NP-40, 50 mM β-mercaptoethanol, 100 μg of leupeptin and 2 μg of aprotinin per ml, 40 μM Z-D-DCB, and 1 mM phenylmethylsulfonyl fluoride. The lysates were then cleared by centrifugation at 16,000 × *g* for 5 min, normalized for protein amount, mixed 1:1 with sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris [pH 6.8], 2% SDS, 300 mM β-mercaptoethanol, 30% glycerol, 5% pyronine Y), boiled for 5 min, and stored at −70°C. Proteins were electrophoresed by SDS–10% polyacrylamide gels and probed with polyclonal antibodies directed against DR4 (366891N [PharMingen, San Diego, Calif.] and sc-6823 [Santa Cruz Biotechnology, Santa Cruz, Calif.]), DR5 (210-730-C100 [Alexis Corporation, Pittsburgh, Pa.] and sc-7191 [Santa Cruz Biotechnology]), DCR-2 (33060-100; Biovision, Palo Alto, Calif.), Fas (sc-714-G; Santa Cruz Biotechnology), and actin (CP01; Oncogene, Cambridge, Mass.). Additional antibodies directed against FasL (sc-834-G; Santa Cruz Biotechnology) and TRAIL (3210-732-R100 [Alexis Corporation] and antibody from Affinity Bioreagents, Golden, Color.) were used for antibody blocking experiments. Autoradiographs were quantitated by densitometric analysis using ImageQuant (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.).

Apoptosis assays and reagents. Forty-eight hours after infection with reovirus, cells were harvested and stained with acridine orange, for determination of nuclear morphology, and ethidium bromide, to distinguish cell viability, at a final concentration of 1 μg/ml each (5). Following staining, cells were examined by epifluorescence microscopy (Nikon Labophot-2; B-2A filter; excitation, 450 to 490 nm; barrier, 520 nm; dichroic mirror, 505 nm). The percentage of cells containing condensed nuclei and/or marginated chromatin in a population of 100 cells was recorded. The specificity of this assay has been previously established in

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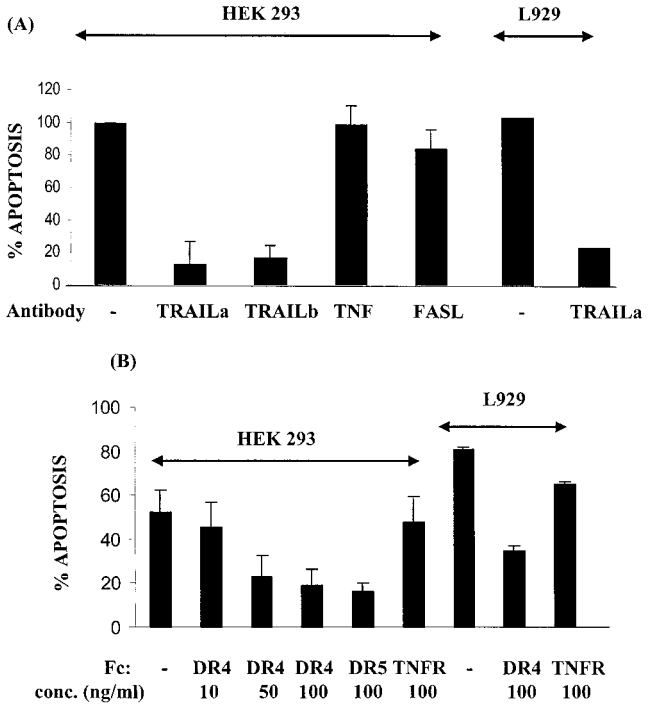

FIG. 1. TRAIL mediates reovirus-induced apoptosis. Anti-TRAIL antibodies and soluble TRAIL receptors (Fc:DR4 and Fc:DR5) specifically inhibit reovirus-induced apoptosis. HEK293 and L929 cells were pretreated for 1 h with two different anti-TRAIL (TRAILa and TRAILb) antibodies (A) or increasing concentrations of soluble TRAIL receptors (B) before being infected with reovirus (MOIs of 10 and 50, respectively, for antibody and receptor experiments). After infection cells were incubated in media containing antibody or receptor for 48 h before cells were harvested and the percentage of apoptotic cells was determined. The graphs show percent apoptosis compared to untreated cells in reovirus-infected minus mock-infected cells (A) and the actual percent apoptosis in reovirus-infected minus mock-infected cells (B). Error bars represent standard error of the mean. Antibodies directed against TNF and FasL were used as controls in the antibody blocking experiments. Soluble TNFR (Fc:TNFR) was used as a control in the receptor experiments.

reovirus-infected cells using DNA laddering techniques and electron microscopy (26). Soluble TRAIL was obtained from Upstate Biotechnology, Lake Placid, N.Y. Soluble DRs Fc:DR4, Fc:DR5, and Fc:TNFR were obtained from Alexis Corporation. Z-IETD-FMK (granzyme B inhibitor III), a specific inhibitor of caspase 8 activity, was obtained from Clontech, Palo Alto, Calif.

RESULTS

Reovirus-induced apoptosis is mediated by TRAIL. We investigated the role of ligand-mediated apoptosis in reovirus-induced cell death using two separate polyclonal antibodies directed against TRAIL and antibodies directed against FasL and TNF to block ligand binding during reovirus infection. HEK293 cells were pretreated with antiligand antibodies (30 μg/ml) for 1 h before viral infection (multiplicity of infection [MOI] of 10) and were maintained in antibody-containing media following infection with reovirus. Antibody was not present during viral infection. The percentage of apoptotic cells was determined at 48 h postinfection. Anti-TRAIL antibodies, but not antibodies directed against FasL (TRAIL versus FasL, $P = 0.008$) or TNF (TRAIL versus TNF, $P = 0.003$) significantly inhibit reovirus-induced apoptosis (Fig. 1A). Thus, anti-TRAIL antibodies specifically inhibit reovirus-induced apoptosis. Anti-TRAIL antibody also inhibits reovirus-induced apoptosis in L929 cells (Fig. 1A), indicating that TRAIL-mediated apoptosis is likely to be a general feature of reovirus-induced apoptosis. Both anti-TRAIL antibodies bound soluble ligand in Western blot analysis (results not shown).

TRAIL binding was further shown to be essential for reovirus-induced apoptosis using the soluble TRAIL receptors Fc:DR4 and Fc:DR5 (Fig. 1B). These molecules contain the extracellular domain of DR4 or DR5 fused to the Fc portion of human immunoglobulin G and inhibit TRAIL-induced apoptosis by preventing TRAIL binding to DR4 and DR5 present on the cell surface (7). Cells were pretreated with soluble receptor for 1 h before virus infection (MOI of 50) and were maintained in receptor-containing media following infection. Soluble receptor was not present during viral infection. Treatment of cells with Fc:DR4 or Fc:DR5 (not shown) produces a dose-dependent inhibitory effect on reovirus-induced apoptosis (Fig. 1B). Thus, Fc:DR4 and Fc:DR5 appear to be similar in potency for TRAIL binding. Fc:DR4 (100 ng/ml) and Fc:DR5 (100 ng/ml) reduced reovirus-induced apoptosis by 65% (from 54% to 19%, $P = 0.048$) and by 70% (from 54% to 16%), respectively. Soluble TNFR (Fc:TNFR; 100 ng/ml) does not significantly inhibit reovirus-induced apoptosis, indicating that the inhibition is specific for the TRAIL-associated receptors DR4 and DR5. In L929 cells, Fc:DR4, but not Fc:TNFR, also significantly inhibited reovirus-induced apoptosis by 57% (from 81% to 35% [Fig. 1B]), again indicating that TRAIL-mediated apoptosis is likely to be a general feature of reovirus-induced apoptosis. To confirm that antibody or soluble receptor-mediated inhibition of apoptosis was not due to any effect of these reagents on viral replication, we measured viral yield in anti-TRAIL and soluble receptor-treated cells and found no significant difference compared with untreated cells (results not shown).

TRAIL is released from cells following infection with reovirus. Having shown that TRAIL is required for reovirus-induced apoptosis, we next wanted to determine whether cleaved, soluble TRAIL is released from in reovirus-infected cells.

Following infection of HEK293 cells with reovirus (MOI of 100), the supernatant was collected and transferred onto HeLa cells, which are sensitive to TRAIL-induced apoptosis (Fig. 2). Supernatants collected from virus-infected HEK293 cells 24, 36, and 48 h postinfection induce apoptosis (18, 30, and 68%, respectively) when transferred onto HeLa cells. Apoptotic

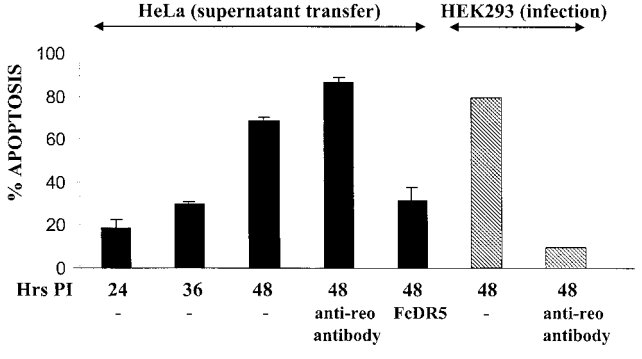

FIG. 2. TRAIL is released from reovirus-infected cells. HEK293 cells were either mock infected or infected with reovirus (MOI of 100). At various times postinfection (PI), supernatant from infected HEK293 cells was transferred onto TRAIL-sensitive HeLa cells. Apoptosis was assayed in HeLa cells 24 h following supernatant transfer. The graph shows the percent increase of apoptotic nuclei in HeLa cells following treatment with supernatants taken from reovirus-infected, compared to mock-infected, HEK293 cells. Error bars represent standard errors of the mean. Soluble DR5 (Fc:DR5) and an antireovirus (anti-reo) antibody were used as TRAIL specificity controls. The shaded bars demonstrate that reovirus-induced (MOI of 100) apoptosis is blocked by the antireovirus antibody in HEK293 cells.

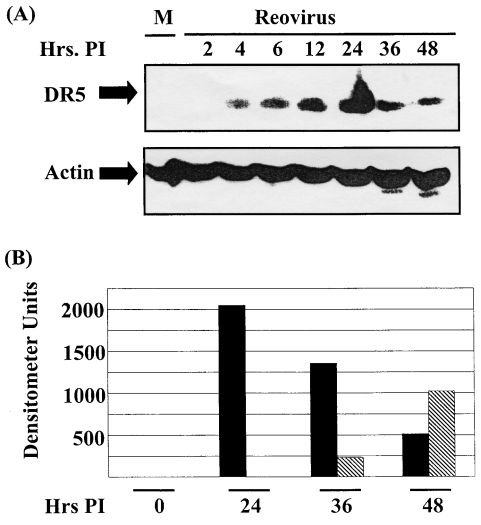

FIG. 3. DR5 is up-regulated during reovirus-induced apoptosis. Cell lysates were prepared and examined by Western blotting using antibodies directed against DR5 and actin (A). Following autoradiography, densitometric analysis was performed (B). The graph shows the increase in signal observed in reovirus-infected compared to mock-infected cells for DR5 (black columns) and DR4 (shaded columns). M, mock infection; PI, postinfection.

HeLa nuclei were assayed 24 h following treatment with supernatant from reovirus-infected HEK293 cells. Supernatant-induced apoptosis of HeLa cells is inhibited 64% (from 68% to 31%, $P = 0.001$) by soluble DR5 (Fc:DR5; 100 ng/ml [Fig. 2]) and by soluble DR4 (Fc:DR4; 100 ng/ml [results not shown]), indicating that the apoptosis seen in the HeLa cells following supernatant transfer is TRAIL specific. TRAIL is thus released from reovirus-infected cells and induces apoptosis in HeLa cells. The apoptotic effects of infected cell supernatants are not due to the presence of infectious virus in the transferred supernatant since addition of a neutralizing polyclonal antireovirus antiserum that blocks apoptosis induced by infectious virus (26) does not block apoptosis induced in HeLa cells by supernatant transfer (Fig. 2). This antibody inhibits reovirus (MOI of 100)-induced apoptosis in HEK293 cells (Fig. 2).

Expression of DR5 is up-regulated following infection with reovirus. Reovirus-induced apoptosis thus requires TRAIL binding, and TRAIL is released from reovirus-infected cells. We next investigated the expression of TRAIL receptors in reovirus-infected cells. HEK293 cells were infected with reovirus (MOI of 100), harvested at various times postinfection, and examined by Western blot analysis. DR5 is detected in lysates extracted from reovirus-infected but not mock-infected HEK293 cells. DR5 expression is first detected at 4 h postinfection. Expression peaks at 24 h postinfection and then declines (Fig. 3). The expression of DR4 also increases in reovirus-infected cells, but with much less magnitude and only at late times after infection (Fig. 3B). DR5 thus appears to be the TRAIL receptor that is predominantly up-regulated following reovirus infection.

Decoy receptor 1 (DcR-1; also called TRAILR3/TRID/LIT) and DcR-2 (TRAILR4) compete with DR4 and DR5 for TRAIL binding. These decoy receptors do not contain active intracellular DDs do not transduse apoptotic signals, and have antiapoptotic effects (6, 17). Neither DcR-1 nor DcR-2 expression is significantly altered in reovirus-infected cells (results not shown).

Reovirus infection sensitizes cells to TRAIL-induced apoptosis. TRAIL-induced apoptosis is enhanced in cells demonstrating an increase in the surface expression of DR4 and DR5 (7). Having shown that reovirus infection results in increased expression of DR5 and to a lesser extent DR4, we next wished to determine whether these increases occur at the cell surface by demonstrating that reovirus sensitizes cells to TRAIL-induced apoptosis. Cells were infected with reovirus (MOI of 10), treated with TRAIL (200 ng/ml) at various times postinfection and assayed for apoptosis 24 h later. Mock-infected HEK293 cells do not undergo apoptosis when treated with TRAIL. However, following infection with reovirus, HEK293 cells become sensitive to TRAIL-induced apoptosis (Fig. 4), and the percentage of apoptotic nuclei in TRAIL-treated, reovirus-infected cells is greater than that in cells treated with reovirus alone (Fig. 4). At 12, 24, 30, and 48 h after infection with reovirus, TRAIL-treated cells demonstrated 2.4-, 3.7-, 3.3-, and 1.8-fold increases in apoptosis, respectively, compared to TRAIL-treated mock-infected cells. Since TRAIL-induced apoptosis is apparent 12 h following infection with

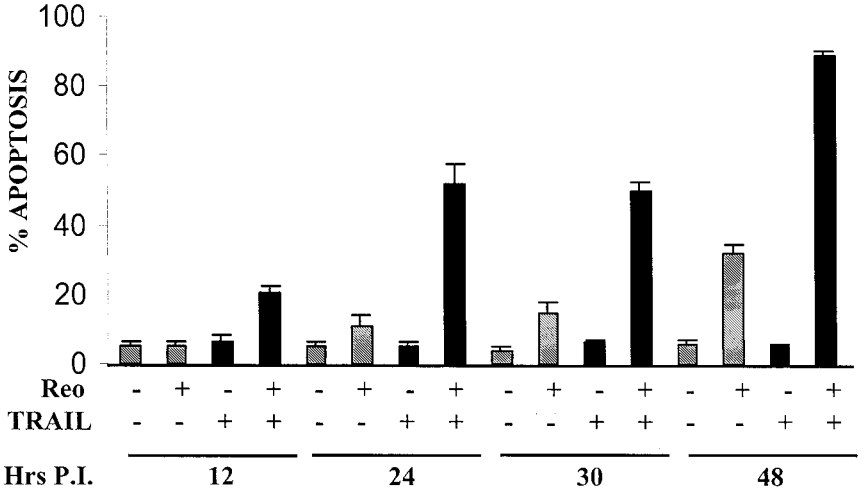

FIG. 4. Reovirus infection sensitizes cells to TRAIL-induced apoptosis. The effectiveness of TRAIL (200 ng/ml)-induced apoptosis was assayed in mock (−)- or reovirus (+; MOI of 10)-infected cells. Cells were treated with TRAIL (black bars) or left untreated (shaded bars). At various times postinfection (P.I.), cells were assayed for the presence of apoptotic nuclei. The graph shows the mean percentage of apoptotic nuclei. Error bars represent standard errors of the mean.

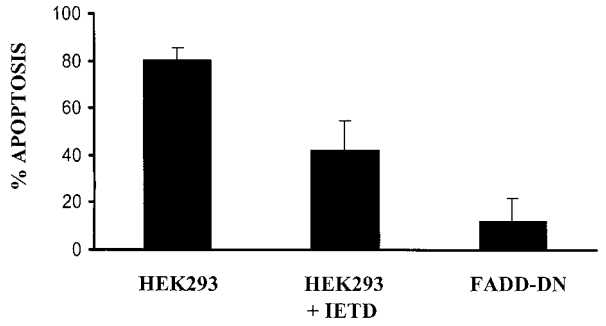

FIG. 5. Reovirus-induced apoptosis involves FADD and caspase 8 activity. HEK293 cells expressing FADD-DN or HEK293 cells treated with a specific inhibitor of caspase 8 (IETD) were infected with reovirus (MOI of 50). Apoptosis was assayed 48 h postinfection. The graph shows the mean percentage of apoptotic nuclei. Error bars represent standard errors of the mean.

reovirus and since the up-regulation of DR4 is not seen until 24 h postinfection, this again suggests that it is the increased expression of DR5 rather than DR4 that is the primary TRAIL receptor involved in reovirus-induced apoptosis.

FADD and caspase 8 are involved in reovirus-induced apoptosis. DRs mediate apoptosis through receptor-associated DD containing adapter proteins, exemplified by FADD (also called Mort 1). These adapter molecules contain their own DDs that bind to the clustered receptor DDs, resulting from receptor-ligand binding (reviewed in reference 1). Studies with dominant negative (DN) mutants of FADD (28) and cells derived from FADD gene knockout mice (31) indicate that FADD is necessary for apoptosis mediated by Fas, TNFR1, and DR3 (1, 9, 28). Apoptotic signals induced by DR4 and DR5 also appear to be mediated either by FADD or a FADD-like adapter molecule (1, 29). We constructed a HEK293 cell line expressing DN FADD (FADD-DN) in order to inhibit FADD and therefore DR-mediated apoptosis. Reovirus-induced apoptosis in HEK293 cells (and in HEK293 cells expressing vector alone [not shown]) is reduced by 85% (from 80.3% to 12%, $P = 0.0012$) in reovirus-infected HEK293 cells expressing FADD-DN (Fig. 5). These results confirm our findings that reovirus-induced apoptosis involves cellular DRs.

DR-induced, FADD-mediated apoptosis requires the activity of caspase 8. Activation of caspase 8 requires association of its death effector domains with those of FADD. Activated caspase 8 then activates the downstream effector caspases, including caspase 3 (reviewed in references 16 and 23). To further support the role of the TRAIL/DR pathway in reovirus-induced apoptosis, we demonstrate that IETD-fmk (50 μM), a specific inhibitor of caspase 8, reduces reovirus-induced apoptosis by 48% (from 80.3% to 42%, $P = 0.372$), indicating that caspase 8 is involved in reovirus-induced apoptosis (Fig. 5).

DISCUSSION

We have shown that reovirus-induced apoptosis requires TRAIL binding to its apoptosis-inducing receptors DR5 and/or DR4. However, exogenous TRAIL (200 ng/ml) does not induce apoptosis in uninfected HEK293 cells since these cells do not express sufficient cell surface DR4 or DR5. To induce apoptosis, reovirus must therefore up-regulate both TRAIL and a death-associated TRAIL receptor. We therefore determined that there is both an increase in the release of TRAIL and an increase in the expression of DR5, and to a lesser extent DR4, in reovirus-infected cells. It seems unlikely that the up-regulation of both DR4 and DR5 is required for TRAIL-mediated expression in reovirus-infected cells. The quicker and more dramatic increase in DR5 expression compared to DR4 expression suggests that DR5 is the major receptor involved in triggering apoptosis. Furthermore, the increased sensitivity of reovirus-infected HEK293 cells to TRAIL-induced apoptosis is detectable 12 h following infection, whereas the alteration in expression of DR4 does not occur until 24 h postinfection. These results suggest that the contribution of DR4 to reovirus-induced apoptosis may be a secondary event and that its up-regulation in reovirus-infected cells may function to amplify the effects of TRAIL/DR5 regulation.

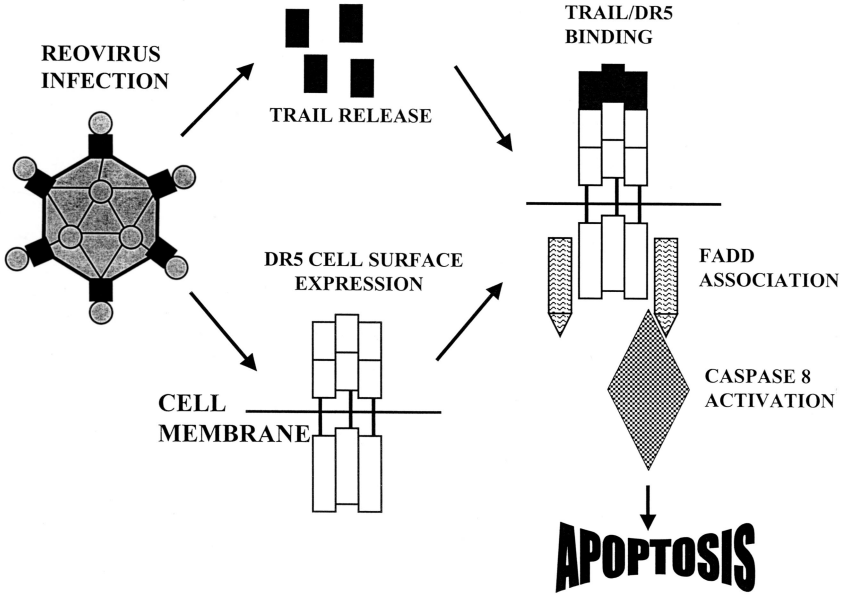

FIG. 6. Reovirus induces the TRAIL apoptotic pathway in infected cells by inducing the release of TRAIL and the up-regulation of DR5. The consequent binding of TRAIL to DR5 then promotes FADD association and the activation of caspase 8.

We propose a model in which reovirus infection results in the up-regulation of DR5 and the release of TRAIL, thereby activating the TRAIL pathway of cell death (Fig. 6). Similar to other DR-mediated apoptotic pathways, reovirus-induced apoptosis requires the participation of an adapter molecule (FADD) and the activation of the caspase cascade since it is reduced in the presence of inhibitors of FADD or caspase 8 activity. We have recently shown that reovirus-induced apoptosis requires the transcription factor NF-κB (3). Future studies will be directed at examining the role of NF-κB in the up-regulation of TRAIL and DR5 in reovirus-infected cells.

Our results demonstrate the involvement of the TRAIL apoptotic pathway in reovirus-induced cell death and provide the first direct evidence for the involvement of this pathway in virus-induced apoptosis. Additional support for the potential role of DR4 and DR5 in virus-induced apoptosis comes from studies suggesting that human immunodeficiency virus (HIV) infection increases the expression of TRAIL and sensitizes T cells to TRAIL-mediated apoptosis (10). Previous studies have suggested that other members of the TNFR DR superfamily may also be involved in apoptosis induced in cells infected with a variety of viruses. Alteration of the cell surface expression of Fas may be involved in virus-induced, or viral regulation of, apoptosis in cells infected with influenza virus (21, 22), herpes simplex virus type 2 (19), bovine herpesvirus 4 (30), adenovirus (24), and HIV type 1 (2, 11). Similarly, apoptosis induced by hepatitis B virus (20), HIV type 1 (8), bovine herpesvirus 4 (30), and parvovirus H-1 (14) may involve the TNFR signaling pathway. TRAIL and TRAIL receptor expression have been shown to mediate gamma interferon-induced antiviral activity (18), although the mechanism by which this occurs is unknown.

We propose that the regulation of TRAIL and its death-promoting receptors is a primary mediator of apoptosis that is induced not only following viral infection but also as a component of apoptosis-inducing stress responses, including chemotherapy (7).

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