The RelA(p65) Subunit of NF-κB is Essential for Inhibiting Double-stranded RNA-induced Cytotoxicity

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Running title: Inhibition of dsRNA-induced cytotoxicity by NF-κB
Double-stranded RNA (dsRNA) molecules generated during virus infection can initiate a host antiviral response to limit further infection. Such a response involves induction of antiviral gene expression by the dsRNA-activated protein kinase (PKR) and the NF-κB transcription factor. In addition, dsRNA can also induce apoptosis by an incompletely understood mechanism which may serve to further limit viral replication. Here we demonstrate a novel role for the RelA subunit of NF-κB in inhibiting dsRNA-induced cell death. dsRNA treatment resulted in caspase 3 activation and apoptotic morphological transformations in mouse embryonic fibroblasts (MEFs) derived from RelA-/- mice but not from RelA+/+ mice. Such dsRNA-induced killing could be inhibited by expression of either a dominant-negative mutant of PKR or wild-type RelA. Interestingly, caspase 3 activated following dsRNA treatment of RelA-/- MEFs was essential for apoptotic nuclear changes but dispensable for cytotoxicity. A broader specificity caspase inhibitor was also unable to inhibit dsRNA-induced cytotoxicity, suggesting that caspase activation is not essential for the induction of cell death by dsRNA in MEFs. However, combined inhibition of caspase 3 and reactive oxygen species (ROSs) production resulted in complete inhibition of dsRNA-induced cytotoxicity. These results demonstrate an essential role for NF-κB in protecting cells from dsRNA-induced apoptosis and suggest that NF-κB may inhibit both caspase-dependent and ROSs-dependent cytotoxic pathways.
Virus replication within infected cells results in generation of double-stranded RNA (dsRNA) molecules which can trigger host antiviral responses (1). Such dsRNA-activated responses can be mediated by dsRNA-dependent enzymes such as the interferon-inducible protein kinase (PKR) which phosphorylates key cellular substrates (e.g., eIF-2α) (2). PKR can also activate the NF-κB transcription factor (see below) resulting in induction of type I interferon gene expression which can prevent further virus infection (3). In addition, dsRNA can also induce apoptosis in a PKR-dependent manner (4-6). Thus PKR is required for both dsRNA-mediated induction of gene expression and induction of apoptotic cell death. PKR-mediated apoptosis of virus-infected cells by dsRNA may thus limit virus infection by preventing virus replication within host cells.

Apoptosis is a genetically controlled process that plays an essential role in regulating homeostasis and in protecting the host against microbial infections (7,8). Apoptotic cells manifest characteristic morphological changes, such as nuclear condensation and fragmentation, which are mediated by proteases belonging to the caspase family (9,10). Although caspases are normally present in an inactive form, they can be activated by proteolysis triggered by cell death inducers (10,11). Activated caspases cleave key cellular substrates and thus provide a safe and efficient mechanism for eliminating surplus or infected cells. Although inhibitors of caspase proteases have been shown to prevent apoptosis induced by many different agents (10,12,13), recent studies have demonstrated that in certain cell-lines, inhibition of caspase activity induces necrotic killing of cells by TNFα (14-16). Such necrosis can occur in cell-lines that are normally resistant to TNFα-killing, is accompanied by increased production of reactive oxygen species (ROSs) and can
be prevented by antioxidants (14-16). Importantly, similar to TNFα, dsRNA also induces necrosis in the presence of caspase inhibitors in dsRNA-resistant wild-type fibroblasts (16). Both TNFα and dsRNA-induced necrosis may represent host strategies for eliminating cells infected with viruses encoding caspase inhibitors (16).

The NF-κB family of transcription factors are key regulators of genes involved in immune and inflammatory responses (17,18). Recent studies have also demonstrated a critical role for NF-κB in regulating apoptotic cell death. Mice deficient in the RelA (p65) subunit of NF-κB die prenatally because of massive hepatocyte apoptosis (19), which appears related to the cytotoxic effect of TNFα (20). Fibroblasts or macrophages derived from RelA-/- mice or cells over-expressing a super-repressor form of the inhibitory IκB protein are also highly susceptible to TNFα-killing (21-24). These studies have demonstrated an essential role for NF-κB in protecting cells from TNFα-induced killing. In addition, NF-κB can also mediate pro-apoptotic effects. A recent report has shown that NF-κB is critically important for mediating p53-induced apoptosis (25). Our recent studies have demonstrated an essential role for RelA in induction of the death-receptor Fas expression and in subsequent apoptosis after Fas ligation (26). Taken together, these studies suggest that NF-κB mediated anti-apoptosis and pro-apoptosis may be context-dependent.

The NF-κB proteins also play a key role in mediating cellular responses to conserved microbial structures, such as dsRNA and LPS (17). Stimulation of cells with dsRNA or LPS results in rapid nuclear translocation of NF-κB proteins and induction of NF-κB target gene expression. Although, both dsRNA-mediated NF-κB activation and
apoptosis require PKR activity, less is known about mechanisms important for regulating
dsRNA-induced apoptosis. Recent studies have however suggested that PKR-dependent
induction of Fas expression may be involved in dsRNA-induced apoptosis (6,27,28). Since
NF-κB regulates Fas expression, these studies suggest that NF-κB may play a pro-
apoptotic role in dsRNA-induced cell death.

To determine a possible role for NF-κB in dsRNA-induced apoptosis, we have
utilized embryonic fibroblasts and macrophages from mice deficient in the RelA subunit of
NF-κB. We show here that dsRNA-induced Fas expression was dramatically reduced in
RelA-/- MEFs. Surprisingly, dsRNA specifically induced apoptosis in NF-κB RelA-/- but
not in RelA+/+ MEFs. In addition, inhibition of macromolecule synthesis also rendered
RelA+/+ MEFs susceptible to dsRNA-induced killing. These results suggest that dsRNA-
induced apoptosis may be prevented by NF-κB mediated induction of anti-apoptotic gene
expression, rather than induced by expression of pro-apoptotic genes, such as Fas. These
results demonstrate the existence of a novel anti-apoptotic role for NF-κB in the dsRNA-
induced cell death pathway.
MATERIALS AND METHODS

Cells and materials.

Mouse embryonic fibroblasts, fetal liver macrophages and 3T3 fibroblasts were derived as described previously (21). Fibroblasts were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing L-glutamate (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and calf serum (10%). Human TNFα was obtained from R&D Systems and used at concentration of 10 ng/ml in all experiments. dsRNA [poly(I-C)] was purchased from Sigma and used at a final concentration of 100 µg/ml. Caspase inhibitors, z-DEVD-fmk, z-VAD-fmk and biot-VAD-fmk, (Enzyme System Products) were dissolved in dimethyl sulfoxide at 20 mM and used at 100 µM and 1 µM respectively. Actinomycin D and antioxidant butylated hydroxy-anisole (BHA) were obtained from Sigma and used at 2 µg/ml and 100 µM respectively. The nuclear dye 4’,6´-diamidino-2-phenylindole (DAPI) and the reactive oxygen species dye dihydrorhodamine 123 (DHR) were obtained from Molecular Probes. pLPC expression vector was a gift from Dr. S. Lowe (CSHL, NY). pRelA was constructed by cloning the mouse RelA cDNA into pLPC. pPKRDN, which has a six-amino acid deletion as previously described (29), was a gift from Dr. A. García-Sastre (Mount Sinai Medical Center, NY).

Analysis of cell death.

Nucleus morphology. Cells in tissue culture plates were rinsed with PBS, fixed with 3.7% formaldehyde and permeabilized with 0.2% triton X-100 for 5 min. They were then washed
and incubated with a DAPI labeling solution (2 µg/ml in PBS) for 5 min and examined under a fluorescence microscope.

**Cell viability experiments.** Approximately 2x10⁵ cells were plated on each well of a six-well plate 1 day before the experiments. The caspase inhibitors, z-DEVD-fmk or z-VAD-fmk, or macromolecule synthesis inhibitor, actinomycin D, was added 1 h before the addition of dsRNA. After the indicated periods, the cells were trypsinized (fibroblasts) or scraped (macrophages) and viable cells were counted by trypan blue exclusion. Four independent readings within a single experiment were used to calculate the standard deviation.

**Transfection experiments.**

RelA-/- MEFs were cotransfected with a GFP expression vector (0.5 µg) and pLPC, pRelA or pPKRDN (0.5 µg) using Fugene 6 (Roche). 24 h later, cells were either left untreated or treated with dsRNA for 12 h. Viable GFP-positive cells from 4 randomly chosen fields were counted and used to calculate standard deviation.

**Determination of reactive oxygen species levels.**

DHR was added to a final concentration of 2 µM before RelA-/- MEFs were treated with agents indicated. 12 h later, cells were trypsinized, washed and resuspended in PBS before FACS analysis.

**EMSA, Northern blots, affinity blots and Western blots.**

Electrophoretic mobility shift assay (EMSA) was carried-out as described previously (19).
RelA-specific antisera were purchased from Santa Cruz Biotechnology. Northern blotting was carried out as described (26) with probes generated from cDNA fragments by RT-PCR using gene-specific primers. Affinity blotting was performed essentially as described previously (30). Briefly, approximately 5x10^6 cells were harvested after the treatments indicated. The cells were then washed once with PBS and pelleted, and the pellet was snap-frozen on dry ice. An equal volume of 1 µM biot-VAD-fmk in MDB buffer (50 mM NaCl, 2 mM MgCl2, 5 mM EGTA, 10 mM HEPES, 1 mM DTT, [pH 7]) was added to the cell pellet, and the cells were lysed by three cycles of freezing and thawing. The lysates were incubated at 37°C for 15 min and centrifuged. 20 µg of protein lysates from the supernatant were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20) supplemented with 2% nonfat dry milk (NFDM) for 30 min and then incubated in avidin-Neutralite (Molecular Probes) at 1 µg/ml in TBST supplemented with 1% NFDM for 1 h. The membrane was then washed and incubated in biotinylated horseradish peroxidase (Molecular Probes) at 25 ng/ml in TBST for 1 h. The labeled protein was visualized by ECL (Amersham). For Western blotting, membranes were blocked with TBST supplemented with 0.5% casein. Caspase 3-p17 antibody (New England Biolabs), which specifically recognizes the large subunit of activated caspase 3, was used at 1 to 10,000 dilution in subsequent steps.

**Identification of p18.**

Fifty 10-cm plates of RelA-/- 3T3 fibroblasts grown to 1-2x10^7/plate were either left untreated or treated with TNFα (10 ng/ml) for 6 h. Cells were collected by centrifugation,
lysed in the presence of 1 μM bio-VAD-fmk to label caspases and the lysates were clarified by centrifugation at 100,000 g for 1 h at 4°C. The protein concentrations of the pooled supernatant were 4.7 mg/ml for untreated and 2.9 mg/ml for TNFα-treated samples. To remove unbound bio-VAD-fmk, the lysates were dialyzed with four changes of MDB buffer (see above) supplemented with a proteinase-inhibitor cocktail (Roche). Dialyzed extracts were run through an Immunopure Immobilized Streptavidin Column (Pierce). The column was washed with 150 ml of washing buffer (50 mM sodium phosphate, 0.4 M urea, 50 μM PMSF [pH 7]) and bound proteins were eluted by boiling in the washing buffer with 2% SDS. The eluted proteins were precipitated with 0.25 volume of TCA solution (100% trichloroacetic acid, 0.4% sodium deoxycholate) and washed twice with acetone. The precipitates were resuspended in 50 μl of the SDS-loading buffer. 5 μl of either the untreated or TNFα-treated samples were resolved by SDS-PAGE electrophoresis. The gel was silver-stained to test for the purity and amount of p18 (approximate 10 ng). The rest of the samples were also subjected to SDS-PAGE and transferred to PVDF membrane (Problott, Applied Biosystem). p18 was excised and wetted with 1 μl of methanol. The band was reduced and alkylated with isopropylacetamide followed by digestion in 20 μl of 0.05 M ammonium bicarbonate containing 0.5% Zwitergent 3-16 (Calbiochem) with 0.2 μg of trypsin (Frozen Promega Modified) at 37°C for 17 h. Peptides generated from in situ tryptic digests were seperated on a C18 0.18 x 150 mm capillary column (LC Packing, Inc.). The HPLC consisted of a prototype capillary gradient HPLC system (Waters Associates) and a model 783 UV detector equipped with a Z-shaped flow cell (LC Packing, Inc.). A 30 cm length of 0.025 mm ID glass capillary was connected to the outlet of the Z-shaped cell inside.
the detector housing to minimize the delay volume. Solvent A was 0.1% aqueous TFA and B
was acetonitrile containing 0.08% TFA. Peptides were eluted using a linear gradient of 0 to
80% B in 60 min and detected at 195 nm. Fractions were collected automatically by a BAI
Protocol onto pre-made spots of matrix (0.5 µl of 20 mg/ml α-cyno-4-hydroxycinnamic
acid + 5 mg/ml nitrocellulose in 50% acetone/50% 2-propanol) on the target plate. Ions
were formed by matrix-assisted laser desorption/ionization with a nitrogen laser, 337 nm.
Spectra were acquired with a PerSeptive Biosystems Voyager Elite time-of-flight mass
spectrometer, operated in reflector delayed extraction mode. Peptides detected by MALDI-
TOF MS were subjected to collision-induced dissociation (CID) in an ion trap mass
spectrometer (LCQ, Finnigan MAT). A 1 µl aliquot (5%) of the P150 tryptic digest was
loaded onto a 100 µm i.d., 360 µm o.d., 30 cm length of fused silica capillary packed with 15
cm of POROS 10R2 reverse phase beads (PerSeptive Biosystems). Peptides were eluted with
an acetonitrile gradient at a flow rate of 500 nl/min for 15 min. A data-dependent
experiment was performed to obtain structural information for selected peptides. Ions with
m/z values corresponding to peptides observed by MALDI-TOF MS were monitored in full
mass range scans and automatically subjected to CID as each eluted from the capillary
column. Peptide masses and selected b and y series fragments were used to search an in-
house protein and DNA sequence database with an enhanced version of the FRAGFIT (31)
and the SEQUEST program. The mouse caspase 3 was identified by a database search of
data obtained from an LC/MS/MS analysis of a tryptic digest of the 18 kDa band. MS/MS
analysis of MH+ 1118.9 was found to correspond to residues 65-75 (SGTDVDAANLR) of
mouse capase 3. MALDI MS analysis identified additional eight masses that matched with
the caspase 3 protein.
RESULTS

The RelA subunit of NF-κB is required for dsRNA-induced gene expression.

Our recent studies have revealed a critical role for the RelA subunit of NF-κB in regulation of TNFα and LPS-induced Fas expression (26). Previous studies have also suggested that dsRNA-induced apoptosis could be mediated by induction of Fas expression (6,27). We therefore tested the possible involvement of NF-κB in dsRNA-dependent induction of Fas expression and apoptosis. To this end, we first determined whether RelA was a component of NF-κB complexes activated by dsRNA. RelA+/− mouse embryonic fibroblasts (MEFs) (19) were treated with dsRNA for 2 hours, after which nuclear extracts were tested for κB-site binding activity by EMSA. As expected, dsRNA strongly activated NF-κB (Fig. 1A). Activated NF-κB was supershifted by antisera generated against RelA (Fig. 1A), demonstrating the presence of RelA in dsRNA-activated NF-κB complexes. Furthermore, as previously observed following TNFα treatment (19), dsRNA-treated RelA−/− MEFs showed a significantly lower level of NF-κB activation (Fig. 1A; compare lane 2 and lane 5).

To determine whether RelA was important for dsRNA-induced Fas expression, we treated RelA+/− or RelA−/− MEFs with dsRNA for 6 hours, after which Fas mRNA expression was determined. Similar to TNFα and LPS, dsRNA-induced Fas expression was dramatically reduced in RelA−/− cells (Fig. 1B). In addition, dsRNA-mediated induction of the neutrophil-specific chemokine, MIP-2, was also found to be dependent on RelA (Fig. 1B). These results thus demonstrate an important role for the RelA subunit of NF-κB in mediating dsRNA-induced gene expression.
RelA is essential for inhibiting dsRNA-induced cytotoxicity.

We wanted to determine whether Fas expression was responsible for controlling susceptibility of MEFs to dsRNA-induced cell death. Surprisingly, a 12-hour dsRNA treatment significantly reduced viability of RelA-/- but not RelA+/+ MEFs (Fig. 2A). These results demonstrate the existence of a previously unrecognized function of RelA in inhibiting dsRNA-induced cytotoxicity. Recent studies have demonstrated a critical role for RelA in protecting cells from TNFα-induced killing (21), through induction of survival gene expression (32-36). Consistent with a role for survival gene expression in preventing TNFα-induced killing, inhibition of RNA or protein synthesis sensitizes normally resistant cells to TNFα-induced killing (37). Similar to TNFα, treatment of RelA+/+ MEFs with an RNA synthesis inhibitor actinomycin D (thus resulting in inhibition of RelA-mediated transcription) also rendered them susceptible to dsRNA-induced killing (Fig. 2A). TNFα is cytotoxic to both RelA-/- fibroblasts and macrophages (21). To determine whether dsRNA could also induce cytotoxicity to RelA-/- macrophages, we generated fetal liver macrophages from embryonic day 14 (E14) RelA+/+ and RelA-/- mice. As seen with MEFs, RelA-/- macrophages readily lost viability in the presence of dsRNA while RelA+/+ cells were not affected (Fig. 2B). Taken together, these results demonstrate a new role for RelA in preventing dsRNA-induced cytotoxicity, which similar to TNFα may also be mediated by regulation of survival gene expression. They also suggest that induction of pro-apoptotic genes, such as Fas, may not play an important role in mediating dsRNA-induced killing.

These results have revealed strikingly similar mechanisms for inhibiting cell
death induced by TNFα and dsRNA. However, it is also possible that dsRNA-induced killing of RelA-/- cells is somehow mediated by the TNFα signaling pathway. This could be accomplished, e.g., by dsRNA-induced release of pre-synthesized TNFα or by dsRNA-dependent mechanisms which could lead to activation of TNF receptors. Two TNFα receptors have been identified and named TNFR1 and TNFR2 (38,39). Unlike TNFR2, TNFR1 contains a death-domain that can induce cytotoxicity in many cell types (40). To determine a possible involvement of TNFR1-mediated signaling in dsRNA-induced cytotoxicity, we tested the sensitivity of both TNFR1+/+RelA-/- and TNFR1-/-RelA-/- MEFs to dsRNA or TNFα-induced cell death (41). As expected, TNFα induced significant cytotoxicity in TNFR1+/+RelA-/- but not in TNFR1-/-RelA-/- MEFs (manuscript submitted) (Fig. 2C). In contrast, dsRNA treatment efficiently killed both cell types. These results thus suggest that dsRNA induces a cell death pathway that does not depend on TNFR1-induced signaling.

**dsRNA-induced cell death requires PKR activity.**

To determine whether dsRNA-induced killing of RelA-/- cells was in fact due to the absence of RelA, we tested whether ectopic expression of RelA was sufficient to protect RelA-/- MEFs from dsRNA-induced cytotoxicity. A GFP-expressing vector (to identify transfected cells) was co-transfected with either a control pLPC vector or a vector expressing RelA (pRelA). 24 hours later, cells were left untreated or treated with dsRNA for another 12 hours after which the viability of GFP-positive cells was determined. As expected, RelA-/- MEFs transfected with the control pLPC vector readily lost viability after
dsRNA treatment (Fig. 3). In contrast, co-transfection of pRelA significantly protected RelA-/- MEFs from dsRNA-induced cytotoxicity (Fig. 3). These results suggest that RelA plays a direct role in inhibiting dsRNA-induced cell death.

PKR activity is thought to be important for induction of cell death by dsRNA (6). We were therefore also interested in determining whether PKR was important for dsRNA-induced killing of RelA-/- MEFs. To this end, we cotransfected RelA-/- MEFs with a construct encoding a PKR dominant-negative mutant, pPKR-DN. Importantly, expression of PKR-DN significantly enhanced viability of RelA-/- MEFs following dsRNA treatment (Fig. 3). These results thus suggest that PKR is required for induction of cell death by dsRNA in RelA-/- MEFs.

dsRNA induces apoptosis and caspase activation in RelA-/- MEFs.

Apoptotic cell death results in characteristic morphological changes such as membrane blebbing, nuclear fragmentation and chromatin condensation (42). DAPI staining of dsRNA-treated RelA-/- MEF nuclei revealed significant nuclear fragmentation and chromatin condensation (Fig. 4A), suggesting that dsRNA induces apoptotic cell death in RelA-/- MEFs. Activation of cysteine proteinases belonging to the caspase family has been shown to be critically important for induction of apoptosis (43). To determine whether caspase proteases are activated in RelA-/- MEFs following dsRNA treatment, we used a biotinylated caspase inhibitor, biot-Val-Ala-Asp(OMe)-fmk (biot-VAD-fmk). This inhibitor can covalently associate with activated caspases and thus provides a sensitive method for detection of caspase activation (9). dsRNA treatment of RelA-/- MEFs resulted
in a time-dependent increase of a biot-VAD-fmk binding-activity of approx. 18 kDa (p18)(Fig. 4B). Importantly, the molecular weight of p18 corresponded to that of the larger subunit of most activated caspases (also see below). Significantly, p18 could also be induced by TNFα treatment of RelA-/- MEFs (Fig. 4B), suggesting that both TNFα and dsRNA may induce a common caspase in these cells. In contrast, dsRNA treatment of RelA+/+ MEFs did not lead to increased levels of p18 but co-treatment with actinomycin D resulted in dramatic increase of p18 (Fig. 4C). These observations thus demonstrate a critical role for RelA in inhibiting dsRNA and TNFα-induced caspase activation.

Caspase 3, a major caspase activated by dsRNA and TNFα, is essential for apoptotic nuclear fragmentation but dispensable for dsRNA-induced cytotoxicity.

To further characterize p18, we used a streptavidin affinity column to purify p18. After extensive washing to remove activities bound non-specifically to streptavidin, an 18 kDa activity could be detected in TNFα-treated extracts subjected to SDS-PAGE (Fig. 5A). Peptide sequencing and mass spectrometry analysis (not shown) of this 18 kDa protein revealed that the sequence of one peptide was identical to residues 65 to 75 of mouse caspase 3 (SGTDVDAANLR). Western analysis using an antibody specific for the p17 subunit of activated caspase 3 confirmed its activation following both TNFα and dsRNA treatment (Fig. 5B) (the apparent molecular weight difference between p18 and p17 is likely due to association of biot-VAD-fmk, which has a molecular weight of 672, to p18). These results thus demonstrate that caspase 3 is a major caspase activated by both dsRNA and TNFα treatment of RelA-/- MEFs.
Caspase 3 has been proposed to be a major downstream effector caspase responsible for executing the apoptotic cell death program (10,44,45). To determine the functional significance of caspase 3 in dsRNA-induced apoptosis, we used the caspase 3 inhibitor, z-DEVD-fmk (9). z-DEVD-fmk completely inhibited binding of p18 (caspase 3) to biot-VAD-fmk induced by dsRNA in RelA-/- MEFs (Fig. 6A). Interestingly, z-DEVD-fmk did not affect processing of caspase 3 (Fig. 6B) suggesting that z-DEVD-fmk does not inhibit upstream caspases that are responsible for proteolytic processing of caspase 3, but rather specifically inhibits caspase 3 activity. Although z-DEVD-fmk completely inhibited caspase 3 activity, z-DEVD-fmk did not protect RelA-/- MEFs from dsRNA-induced cytotoxicity (Fig. 6C). These results suggest that caspase 3 activity is not essential for dsRNA-induced killing of RelA-/- MEFs. However, z-DEVD-fmk completely inhibited nuclear fragmentation (Fig. 6D, compared to Fig. 4A). These results thus demonstrate that caspase 3 activity is essential for apoptotic nuclear changes but dispensable for dsRNA-induced cytotoxicity in RelA-/- MEFs.

Combined treatment of RelA-/- MEFs with z-DEVD-fmk and antioxidants completely inhibits dsRNA-induced cytotoxicity.

z-DEVD-fmk is not a broad specificity caspase inhibitor, raising the possibility that the inability of z-DEVD-fmk to inhibit killing of RelA-/- MEFs was due to incomplete blockage of caspase activity. We therefore tested the effect of the broader specificity z-VAD-fmk caspase inhibitor (9) on dsRNA-induced caspase activation and killing of RelA-/- MEFs. Unlike z-DEVD-fmk, z-VAD-fmk inhibited caspase 3 processing (Fig. 7A,
compared to Fig. 6B), demonstrating its ability to inhibit the activity of upstream caspases responsible for caspase 3 processing and activation. However, z-VAD-fmk did not inhibit, but rather significantly enhanced, cytotoxicity to RelA-/- MEFs following dsRNA treatment (Fig. 7B). In addition, both z-DEVD-fmk and z-VAD-fmk did not affect NF-κB activation by dsRNA (data not shown), suggesting that the enhanced cytotoxicity by z-VAD-fmk was not due to inhibition of NF-κB. This is consistent with our recent observations showing that z-VAD-fmk could sensitize RelA+/+ fibroblasts to dsRNA-induced necrotic killing (16). These results also indicate that caspase inhibition may not be sufficient to protect RelA-/- MEFs from dsRNA-induced cytotoxicity.

To further understand mechanisms involved in dsRNA-induced killing of RelA-/- MEFs, we wished to identify non-caspase cytotoxic mechanisms which may be potentially responsible for inducing cell death. One such cytotoxic mechanism involves enhanced generation of reactive oxygen species (ROSs), which is also important in induction of necrotic cell death by TNFα (14-16). We therefore tested whether treatment of RelA-/- MEFs with dsRNA resulted in enhanced ROSs production. However, no significant increase in ROSs production was noticed following dsRNA treatment of RelA-/- MEFs (Fig. 7C). Similarly, dsRNA+z-DEVD-fmk-treated RelA-/- MEFs also showed no significant increase in ROSs production (Fig. 7C). In contrast, treatment of RelA-/- MEFs with dsRNA+z-VAD-fmk significantly enhanced ROSs production (Fig. 7C). Furthermore, z-VAD-fmk, but not z-DEVD-fmk, also enhanced ROSs production and necrotic cell death in RelA+/+ MEFs (data not shown). Thus in the presence of broad-specificity caspase inhibitors such as z-VAD-fmk, dsRNA treatment can enhance ROSs production and induce
necrotic cell death in both RelA+/- and RelA-/- MEFs. Nevertheless, our results suggest that such enhanced ROSs production may not be responsible for killing of RelA-/- MEFs by dsRNA or dsRNA+z-DEVD-fmk.

It was however possible that dsRNA-induced killing of RelA-/- MEFs may result not from increased generation of ROSs but from enhanced susceptibility to constitutively produced ROSs. Importantly, in the presence of the antioxidant butylated hydroxy-anisole (BHA), constitutive generation of ROSs was significantly reduced in both untreated and dsRNA+z-DEVD-fmk-treated RelA-/- MEFs (Fig. 7C). To determine a role for such constitutively generated ROSs in dsRNA-induced killing, we tested whether BHA could inhibit cell death induced by dsRNA. Interestingly, BHA treatment substantially enhanced survival of RelA-/- MEFs suggesting that dsRNA-induced killing may indeed result from enhanced susceptibility to constitutively produced ROSs (Fig. 7D). Importantly, combined treatments with z-DEVD-fmk and BHA resulted in almost complete protection from dsRNA-induced cytotoxicity (Fig. 7D). These results indicate that inhibition of dsRNA-induced cytotoxicity requires inhibition of both caspase-dependent and ROSs-dependent mechanisms and raise the possibility that anti-apoptotic functions of NF-κB are mediated by simultaneous inhibition of both cytotoxic pathways.
DISCUSSION

The results presented here provide evidence for a novel function of the RelA subunit of NF-κB in inhibiting dsRNA-induced apoptosis. Although the role of NF-κB in inhibiting apoptosis by endogenously produced factors such as TNFα (i.e., host-derived) is well established, we provide the first evidence for an NF-κB dependent function in inhibiting apoptosis induced by an exogenous agent (i.e., microbe-derived factor). Our results thus suggest that NF-κB mediated inhibition of apoptosis may be an important mechanism for regulating cell survival during viral infection. Interestingly, and similar to TNFα, we have found that dsRNA also induces caspase activation and apoptotic changes in RelA−/− cells. In addition, and also similar to TNFα, dsRNA also triggers apoptosis in RelA+/+ cells in the presence of an RNA synthesis inhibitor. These results reveal a striking similarity in cellular mechanisms responsible for inhibiting cell death induced by TNFα and dsRNA. These results thus indicate that dsRNA-induced cell death may be inhibited by NF-κB mediated survival gene expression, rather than enhanced by NF-κB activation and induction of Fas expression as previously reported (6,27,28).

dsRNA can be generated during infection with virtually any kind of virus (1). NF-κB activated by dsRNA in infected cells allows activation of antiviral gene expression to limit further infection (46). However, our results suggest that NF-κB mediated induction of survival gene expression may be important for inhibiting apoptosis of infected cells and may thus enhance viral infection and virulence. Consistent with such a function of NF-κB, a previous study has demonstrated a critical role for NF-κB in maintaining virulence of encephalomyocarditis virus (EMCV) by preventing virus-induced apoptosis (47). It will
thus be interesting to determine whether NF-κB mediated inhibition of dsRNA-induced apoptosis is a mechanism important for enhancing viral virulence. However, it is also possible that inhibition of NF-κB dependent anti-apoptosis in virus-infected cells may lead to eradication of infected cells. As shown here, inhibition of macromolecule synthesis sensitized cells to dsRNA-induced killing. Since shutdown of host macromolecule synthesis is one of the key events in late stage virus replication (48), it is likely that under these conditions the NF-κB-induced protective pathway is also inhibited. Under these conditions, dsRNA may thus mediate cytotoxicity to virus-infected cells. Interestingly, TNFα has also been shown to kill vesicular stomatitis virus (VSV)-infected cells (49,50). Since NF-κB also protects cells from TNFα-induced apoptosis, it is possible that killing of virus-infected cells by either dsRNA or TNFα is due to viral inhibition of the NF-κB-induced protective pathway. Additional studies aimed at determining the impact of these NF-κB dependent pathways in controlling viral virulence may thus be required to fully understand the physiological functions of these key anti-apoptotic pathways.

We have shown here that induction of apoptosis by dsRNA in RelA-/- cells is mediated by activation of caspases. In particular, we have identified caspase 3 as a major caspase activated by dsRNA treatment which was found to be critically important for dsRNA-induced nuclear fragmentation in RelA-/- fibroblasts. These results are consistent with a previous study using caspase 3-deficient cells which demonstrated an important role for caspase 3 in mediating apoptotic nuclear changes by death inducers (44). Importantly, a recent study has identified a caspase 3 substrate, acinus, as the factor responsible for induction of chromatin condensation and fragmentation (51). It will thus be interesting to
test whether acinus is also processed and activated in RelA-/- fibroblasts by dsRNA. Caspase activation involves a cascade of proteolytic events. Upstream initiator caspases, such as caspase 8 or 9 (which are generally activated by oligomerization) (52,53), induce proteolysis and subsequent activation of downstream effector caspases, such as caspase 3 (10,11). Although upstream caspase(s) involved in dsRNA-induced apoptosis are still not known, a recent study has demonstrated an essential role for the FADD protein in dsRNA-induced, PKR-mediated apoptosis (54). FADD is an adapter molecule involved in activation of caspase 8 by both TNFR1 and Fas (55-59). These observations thus suggest a possible involvement of caspase 8 in dsRNA-induced apoptosis of RelA-/- cells and suggest that dsRNA and TNFα may induce apoptosis by similar mechanisms which can be inhibited by NF-κB.

Interestingly, inhibition of caspase 3 by z-DEVD-fmk did not prevent dsRNA-induced cytotoxicity to RelA-/- MEFs. However, combined z-DEVD-fmk and antioxidant treatment resulted in complete protection from dsRNA-induced cytotoxicity, even though dsRNA treatment did not result in a significant increase in ROSs generation. In RelA+/+ MEFs, dsRNA+actinomycin D-induced cytotoxicity was also not inhibited by z-DEVD-fmk alone, while co-treatment with z-DEVD-fmk and BHA resulted in significant suppression of cell death (data not shown). We have also found that inhibition of TNFα-induced killing of RelA-/- MEFs requires simultaneous inhibition of caspase proteases and ROSs generation (unpublished results). Similar to dsRNA, TNFα treatment of RelA-/- MEFs did not enhance ROSs production. These results thus suggest that dsRNA or TNFα treatment may enhance susceptibility of RelA-/- MEFs to constitutively generated ROSs.
These results also suggest that NF-κB regulated anti-apoptotic genes may include those which can provide protection from ROSs-induced cytotoxicity. Such an NF-κB regulated gene may be Mn-dependent superoxide dismutase (MnSOD), which functions as a potent scavenger of superoxide anions, and has previously been shown to inhibit TNFα-induced cell death in certain cell-lines (60). It is also possible that caspases activated during apoptosis can inhibit cellular damage induced by constitutively generated ROSs, perhaps in order to prevent necrotic lysis. In either case, our results suggest that caspase inhibition is not sufficient to inhibit apoptosis of RelA-/- cells and suggest that ROSs may provide an additional mechanism that plays a key role regulation of apoptosis induced by dsRNA and TNFα. Caspase inhibitors are currently being considered for possible therapeutic use in the treatment of tissue degenerative diseases (61). Our results however suggest that combined inhibition of both caspase-dependent and ROSs-dependent pathways may be required for inhibiting cell death induced by certain agents.

In conclusion, we have provided evidence of a novel function for the RelA subunit of NF-κB in preventing dsRNA-induced apoptosis by potentially inhibiting both caspase-dependent and ROSs-dependent mechanisms. These results further underscore the key role played by NF-κB proteins in inhibiting apoptosis induced by diverse agents. Our results also highlight the importance of NF-κB proteins in regulating cellular responses to viral products which may be important for controlling viral virulence and pathogenesis.
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FIGURE LEGENDS

Fig. 1 RelA is critical for dsRNA-induced gene expression in MEFs. A) RelA+/- or RelA-/- MEFs were either left untreated (UT) or treated with dsRNA for 2 h. κB-site binding activity was determined by EMSA. RelA-specific antisera were used to demonstrate the presence of RelA. B) RelA+/- or RelA-/- MEFs were either left untreated (UT) or treated with dsRNA for 6 h after which cellular RNA was tested for the expression of Fas, MIP-2 and GAPDH gene by Northern blotting.

Fig. 2 RelA is essential for protecting MEFs and macrophages from dsRNA-induced cytotoxicity. A) RelA-/- or RelA+/+ MEFs were either left untreated (UT), treated with dsRNA alone, treated with actinomycin D (Act D) alone or treated with dsRNA and actinomycin D for 12 h. Viable cells remaining after treatment are shown as a percentage of viable untreated cells. B) RelA-/- or RelA+/+ macrophages (Mφ) were either left untreated (UT) or treated with dsRNA for 12 h. C) TNFR1+/+RelA-/- or TNFR1-/-RelA-/- MEFs were either left untreated (UT) or treated with dsRNA or TNFα for 12 h.

Fig. 3 PKR activity is critical for dsRNA-induced cytotoxicity in MEFs. RelA-/- MEFs were cotransfected with a GFP expression plasmid and pLPC, pRelA or pPKRDN. 24 h later, cells were either left untreated (UT) or treated with dsRNA for 12 h. Viable GFP-positive cells after treatment are shown as a percentage of viable untreated GFP-positive cells.
Fig. 4 dsRNA induces caspase activation and apoptotic cell death in RelA-/- MEFs. A) RelA-/- MEFs were either left untreated (UT) or treated with dsRNA for 6 h before stained with DAPI. Nuclear morphology of apoptotic cells is indicated by arrows. B) RelA-/- MEFs were either left untreated (UT), treated with dsRNA for 3, 6 or 12 h, or treated with TNFα for 12 h. Extracts were made in the presence of biot-VAD-fmk (1 µM) and lysates (20 µg of protein) were examined by a biot-avidin affinity-blotting. The predominant caspase activated is indicated as p18. C) RelA+/+ MEFs were either left untreated (UT), treated with dsRNA alone, treated with actinomycin D (Act D) alone or treated with dsRNA and actinomycin D for 6 h. Extracts were tested for biot-VAD-fmk binding as in 4B. p18 is indicated by an arrowhead.

Fig. 5 Caspase 3 is the major caspase activated in RelA-/- MEFs by dsRNA or TNFα. A) Bio-VAD-fmk-labeled extracts from either untreated (UT) or TNFα-treated RelA-/- fibroblasts were subjected to purification with a streptavidin affinity column. The purity of the extracts before or after purification was tested by SDS-PAGE followed by silver-staining. p18 is indicated with an arrowhead. B) RelA-/- MEFs were either left untreated (UT) or treated with TNFα or dsRNA for 6 h. Whole cell lysates were analyzed by Western blotting with a caspase 3-specific antibody. The caspase 3 p17 is indicated by an arrowhead.

Fig. 6 Caspase 3 activity is essential for dsRNA-induced apoptotic nuclear changes but dispensable for cytotoxicity in RelA-/- MEFs. A) RelA-/- MEFs were either left untreated (UT), treated with dsRNA alone or treated with dsRNA and z-DEVD-fmk for 6 h. Extracts
were tested for biot-VAD-fmk binding as in 4B. p18 is indicated by an arrowhead. B) RelA-/- MEFs were treated as in 6A. Whole cell lysates were analyzed for caspase 3 activation by Western blotting. C) RelA-/- MEFs were either left untreated (UT), treated with dsRNA alone or treated with dsRNA and z-DEVD-fmk for 12 h before cell viability determined. D) RelA-/- MEFs were treated with dsRNA and z-DEVD-fmk for 6 h before stained with DAPI. Nuclear morphology is indicated by arrows.

Fig. 7 Co-treatment of RelA-/- MEFs with z-DEVD-fmk and BHA prevents dsRNA-induced cytotoxicity. A) RelA-/- MEFs were either left untreated (UT), treated with dsRNA alone or treated with dsRNA and z-VAD-fmk for 6 h. Whole cell lysates were analyzed for caspase 3 activation by Western blotting. B) RelA-/- MEFs were either left untreated (UT), treated with dsRNA alone or treated with dsRNA and z-VAD-fmk for 12 h before cell viability determined. C) RelA-/- MEFs were either left untreated (UT), treated with dsRNA alone, treated with z-DEVD-fmk in the presence or absence of dsRNA, treated with z-VAD-fmk in the presence or absence of dsRNA, treated with BHA alone or treated with BHA, dsRNA and z-DEVD-fmk for 12 h. Intracellular levels of reactive oxygen species (ROSs) were determined by DHR-staining followed by FACS analysis. D) RelA-/- MEFs were either left untreated (UT), treated with dsRNA alone, treated with dsRNA and z-DEVD-fmk, treated with dsRNA and BHA or treated with dsRNA, z-DEVD-fmk and BHA for 12 h before cell viability was determined.
A

|          | RelA+/- MEFs | RelA--/− MEFs |
|----------|--------------|---------------|
|          | UT           | UT            |
| dsRNA 2h | dsRNA 2h+RelA Ab | dsRNA 2h       |

B

|          | RelA+/- MEFs | RelA--/− MEFs |
|----------|--------------|---------------|
|          | UT           | UT            |
| dsRNA 6h | dsRNA 6h     | dsRNA 6h      |

NF-κB

Fas

MIP-2

GAPDH
C

![Viability Graph](chart.png)

D

![Image](image.png)

dsRNA
z-DEVD-fmk
The RelA(p65) Subunit of NF-κB is Essential for Inhibiting Double-stranded RNA-induced Cytotoxicity

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