NRAGE, a p75 Neurotrophin Receptor-interacting Protein, Induces Caspase Activation and Cell Death through a JNK-dependent Mitochondrial Pathway*

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The p75 neurotrophin receptor (p75NTR) mediates signaling events leading to activation of the JNK pathway and cell death in a variety of cell types. We recently identified NRAGE, a protein that directly interacts with the p75NTR cytosolic region and facilitates p75NTR-mediated cell death. For the present study, we developed an inducible recombinant NRAGE adenovirus to dissect the mechanism of NRAGE-mediated apoptosis. Induced NRAGE expression resulted in robust activation of the JNK pathway that was not inhibited by the pharmacological mixed lineage kinase (MLK) inhibitor CEP1347. NRAGE induced cytosolic accumulation of cytochrome c, activation of Caspases-3, -9, and -7, and caspase-dependent cell death. Blocking JNK and c-Jun action by overexpression of the JNK-binding domain of JIP1 or dominant-negative c-Jun ablated NRAGE-mediated caspase activation and NRAGE-induced cell death. These findings identify NRAGE as a p75NTR interactor capable of inducing caspase activation and cell death through a JNK-dependent mitochondrial apoptotic pathway.

The neurotrophins are a family of growth factors involved in the survival, development, and death of specific populations of neurons and non-neuronal cells. Their effects are mediated by binding to cell surface tyrosine-kinase receptors and to the p75 neurotrophin receptor (p75NTR). Roles for tyrosine-kinase receptors in neurotrophin-dependent growth, survival, and synaptic function are well established, but the precise physiological functions of p75NTR are still being defined. p75NTR can act as a co-receptor for tyrosine-kinase receptors, but p75NTR is also expressed in many cells that lack catalytically active tyrosine-kinase receptors, where it functions autonomously to mediate neurotrophin signaling events (1).

Numerous studies over the last several years have shown that p75NTR can induce apoptosis in a variety of settings. For example, p75NTR-dependent cell death has been observed in cultured trigeminal neurons (2), embryonic hippocampal neurons (3), neonatal sympathetic neurons (4), neonatal Schwann cells (5), neuroblastoma cells (6), and within explants of otic vesicles (7). In vivo, overexpression of the intracellular domain of p75NTR in transgenic mouse neurons results in widespread apoptosis of peripheral and central neurons (8). Accordingly, disruption of nerve growth factor binding to p75NTR reduces apoptosis in the retina (9); mice lacking full-length p75NTR exhibit decreased apoptosis in the developing spinal cord and retina (10) and display increased numbers of sympathetic and sensory neurons following the normal period of naturally occurring cell death (4, 11).

The precise signaling events that link p75NTR activation to apoptotic cascades remain uncertain, but several findings suggest that activation of the JNK pathway may play a key role. A number of studies have shown that p75NTR-dependent apoptosis correlates with an increase in JNK activity (3, 4, 12–14), and some studies have demonstrated that blockade of the JNK pathway with chemical inhibitors (3, 13) or dominant-negative forms of JNK (15) attenuates p75NTR-dependent death in oligodendrocytes and hippocampal neurons.

Several p75NTR-interacting proteins have been recently identified (1). Some of these, including NRIF (16) and NADE (17), appear to facilitate p75NTR-dependent apoptosis, but to date, p75NTR interactors have not been linked to specific apoptotic pathways. We recently demonstrated that NRAGE, a novel member of the MAGE family, binds to p75NTR under physiological conditions and facilitates p75NTR-dependent cell death (18). For the present study, we developed an overexpression paradigm to examine signaling pathways activated by NRAGE. Our data indicate that NRAGE is a potent apoptotic inducer that activates a mitochondrial death pathway involving cytochrome c release and activation of Caspase-9, -7, and -3. NRAGE-induced apoptosis correlates with MLK-independent activation of JNK and with c-Jun phosphorylation. Accordingly, blockers of JNK activity or c-Jun-mediated transcription inhibit NRAGE-dependent caspase activation and reduce apoptosis. Together, these data demonstrate that the p75NTR interactor NRAGE activates a mitochondrial apoptotic cascade through a JNK- and c-Jun-dependent pathway.

EXPERIMENTAL PROCEDURES

Materials—zVAD-fmk was purchased from Enzyme System Products. CEP1347 was obtained from Aegera Therapeutics. Cell culture reagents were purchased from BioWhittaker, unless otherwise indicated. The JNK1 antibody (C-17, catalogue no. sc-474) and IκBα antibody (C-12, catalogue no. sc-71) were purchased from Santa Cruz Biotechnology, Inc.
PharMingen (catalogue no. 556433) and anti-HA antibody (12CA5, from Clontech (catalogue no. C3015)) were purchased from Sigma or ICN Biochemicals, unless otherwise indicated. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Anti-FLAG antibody (M2, catalogue no. F-3165) was obtained from Cell Signaling Technology. Anti-FLAG-tagged dominant-negative c-Jun, and HA epitope-tagged MLK-3 were provided by Dr. Aviva Tolkovsky, Aegera Therapeutics, and David Kaplan, respectively, and their construction has been described elsewhere (19–21). To produce recombinant adenovirus driving the Tet-inducible expression of Myc epitope-tagged full-length NRAGE, full-length rat NRAGE cDNA was subcloned into the vector cytomegalovirus 5-TetO, and recombinant virus was generated and plaque purified in 293A cells. All viruses were amplified in 293A cells and purified on a sucrose gradient, as previously described (14). Viruses were then titrated by optical density and by using the tissue culture infectious dose 50 (TCID50) assay in 293A cells (22). Titters are expressed as plaque-forming units.

Cell Culture and Infection—The PC12^{ΔTA} cell line was purchased from Clontech (catalogue no. C3015–1) and maintained in 10% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Clontech), 5% horse serum, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were plated 18–24 h prior to infection. Where indicated, 1 μg/ml doxycycline was added to the plates at time of infection.

Caspase Activity Assay—Forty-eight hours post-infection, cells were harvested on ice, washed with cold phosphate-buffered saline, and caspase activity was measured at room temperature using the fluorescein substrate DEVD-amino-methyl-coumarin (AMC) at a concentration of 10 μM as described previously (23).

Cytoskeleton Release Assay—Cytosol-enriched subcellular fractions were prepared using a modification of a previously described protocol (24). In brief, 5,000,000 cells were harvested, washed once in Tris-buffered saline (10 mM Tris, pH 9.0), lysed with 0.5 ml 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μM sodium orthovanadate, and then resuspended in 500 μl of Buffer B (Buffer A plus 5% Percoll, 0.01% digitonin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, and 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). A sample of this suspension was retained as total cell lysate. The remainder was incubated on ice for 15 min and then centrifuged at 2500 × g for 10 min to remove intact cells and nuclei. The supernatant was then centrifuged at 15,000 × g for 15 min to pellet mitochondria. The final supernatant was designated cytosol.

Immunoblotting—Cells were lysed in RIPA buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) and analyzed for protein content using the BCA assay (Pierce). Samples were normalized for protein content, suspended in Laemmli sample buffer, separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose. Blocking and secondary antibody incubations of immunoblots were performed in Tris-buffered saline/Tween (10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Tween 20) supplemented with 5% (w/v) dried skim milk powder. All primary antibody incubations were performed in the blocking solution, except for those involving phospho-specific antibodies, which were performed in Tris-buffered saline/Tween supplemented with 5% (w/v) bovine serum albumin. Immunoreactive bands were detected by chemiluminescence (PerkinElmer Life Sciences) according to the manufacturer’s instructions.

Survival Assay—Analysis of cell survival was performed by MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which was added at a final concentration of 1 μg/ml for the last 4 h of a 24-h infection. The reaction was ended by the addition of 1 volume of solubilization buffer (20% SDS, 10% dimethylformamide, and 20% ascoric acid). After overnight solubilization, specific and nonspecific absorbances were read at 570 and 690 nm, respectively. In each experiment, each data point was performed in triplicate or quadruplicate, and experimental results were analyzed by multiple analyses of variance with statistical probabilities assigned using the Tukey test for multiple comparisons. Each experiment was run out independently at least three times. In the case of MTT assays that were accompanied by parallel lactate dehydrogenase (LDH) experiments, cells were treated as described below prior to infection.

Death Assay—Analysis of cell death was performed by the LDH assay using the cytotoxicity detection kit (Roche, catalogue no. 1644798) according to the manufacturer’s instructions. In brief, prior to infection, cells were washed three times with an equal volume of PC12{ΔTA} medium, and then the medium serum concentration was reduced by 20% by the addition of Dulbecco’s modified Eagle’s medium. Forty hours after infection, cell cultures were centrifuged at 500 × g, and the supernatant was added to an equal volume of LDH reaction mixture supplied by the manufacturer (Roche). After 15–30 min of incubation, specific and nonspecific absorbances were read at 490 (A<sub>490 nm</sub>) and 690 (A<sub>690 nm</sub>), respectively. Values reported were calculated using the following formula: (A<sub>490 nm</sub> − A<sub>690 nm</sub>) − (A<sub>490 nm</sub> of media alone − A<sub>690 nm</sub> of media alone). Each data point was performed in triplicate or quadruplicate, and experimental results were analyzed by multiple analyses of variance with statistical probabilities assigned using the Tukey test for multiple comparisons. Each experiment was run out independently at least three times.

RESULTS

An Inducible NRAGE Adenovirus Mediates Cell Death—To investigate signaling pathways involved in NRAGE-mediated cell death, we constructed a recombinant adenovirus driving the expression of full-length NRAGE (AdNRG). Previous attempts to produce virus in which expression of an NRAGE fragment was driven by a constitutively active cytomégalovirus promoter had proven unsuccessful, presumably because of the pro-apoptotic effect of NRAGE. Therefore, we designed an adenovirus that would allow for the doxycycline-inducible expression of full-length NRAGE (tagged with an amino-terminal myc epitope, myc-NRAGE) in cells co-expressing a doxycycline-regulated trans-acting factor, rtTA. For our studies, PC12 cells stably expressing the doxycycline-regulated trans-acting factor were used (PC12<sup>ΔTA</sup>). Fig. 1A shows that NRAGE expression in AdNRG-infected cells is minimal in the absence of doxycycline but is strongly induced following addition of 1 μg/ml doxycycline. A small amount of ‘leaky’ NRAGE expression occurs, even in the absence of doxycycline, but only at high multiplicities of infection (m.o.i.).

To determine whether NRAGE induces cell death, PC12<sup>ΔTA</sup> cells were infected with AdNRG in the absence or presence of 1 μg/ml doxycycline. AdNRG-infected PC12<sup>ΔTA</sup> that were treated with doxycycline displayed dose-dependent cytoplastic condensation, cell shrinkage, and detachment from the plate (data not shown). MTT viability assays performed to quantify this effect indicated that cells infected with AdNRG and treated with doxycycline displayed a significant loss in cell viability at all m.o.i. tested (Fig. 1B). In contrast, cells infected with a control virus expressing enhanced green fluorescence protein (AdGFP) showed no significant loss of viability in presence or absence of doxycycline (Fig. 1B). As expected (14), cells treated with a virus that constitutively expresses a myristoylated form of the p75<sup>NTR</sup> intracellular domain showed reduced viability that was unaffected by doxycycline (Fig. 1B). Together, these data indicate that NRAGE overexpression induces PC12<sup>ΔTA</sup> cell death.

NRAGE-induced Cell Death Is Caspase-dependent—Most forms of apoptosis converge on caspases as downstream effectors (25). To determine whether caspases are activated during NRAGE-mediated cell death, a fluorogenic synthetic peptide (DEVD-amino-methyl-coumarin) cleavage assay (26) was used to assess caspase activity (Fig. 2A). Doxycycline treatment had 48044 NRAGE-mediated Apoptosis
no effect on caspase activity in control-uninfected cells, and cells infected with a control AdGFP virus did not display increased caspase activity relative to control-uninfected cells in either the absence or presence of doxycycline. In contrast, cells infected with AdNRG and treated with doxycycline showed a robust induction of DEVD cleavage activity.

Caspases are present within the cell in zymogen forms that, following an apoptotic signal, undergo proteolytic cleavage to produce active caspases (25). To identify specific caspases activated by NRAGE overexpression, cells were either left uninfected or were infected with AdGFP or AdNRG in the absence or presence of doxycycline. Cells were then lysed and analyzed by immunoblot for levels of the intact Caspase-9 zymogen and for cleavage/activation of effector Caspases-3 and -7. Fig. 2B shows that doxycycline-induced NRAGE expression results in the reduction in levels of the Caspase-9 zymogen, a corresponding increase in activated Caspase-3 and -7, and the cleavage of PARP, a dual Caspase-3/7 substrate (25). Uninfected cells or cells infected with AdGFP showed no evidence of caspase activation in either the absence or presence of doxycycline. Together, these results indicate that NRAGE is a potent inducer of Caspase-9, an initiator caspase, and Caspases-3 and -7, downstream effector caspases.

To confirm that caspase activation is necessary for NRAGE-mediated cell death, the viability of cells overexpressing myc-NRAGE was examined after treatment with either Boc-Asp-OMe (BAP) or zVAD-fmk, both broad-spectrum inhibitors of caspase activity (26). MTT survival and LDH death assays both revealed that NRAGE-induced death is significantly inhibited in the presence of BAF or zVAD-fmk (Fig. 2C), confirming a role for caspase activity in NRAGE-induced cell death. Together, these data establish that NRAGE induces cell death through an apoptotic mechanism involving caspase activation.

NRAGE Induces Caspase-independent Release of Cytochrome c from Mitochondria—Cleavage of the Caspase-9 zymogen requires formation of a complex consisting of Caspase-9, Apaf-1, and cytosolic cytochrome c (25). Release of cytochrome c from mitochondria into the cytosol is a key regulatory step in this process (27). To determine whether cytochrome c is released during NRAGE-induced apoptosis, cells were left uninfected or were infected with either AdNRG or with a control adenovirus driving constitutive expression of β-galactosidase (AdLacZ) in the presence of doxycycline (Fig. 2D). Cells were then lysed, subjected to subcellular fractionation, and cytosolic fractions were analyzed for cytochrome c levels by immunoblot. Cytochrome c was not detected in the cytosol of uninfected cells or in cells infected with AdLacZ. However, high levels of cytochrome c were detected in the cytosol of cells expressing myc-NRAGE (Fig. 2D), indicating that NRAGE overexpression induces cytochrome c release from the mitochondria.

In some models of apoptosis, cytochrome c release may be a secondary event that occurs after caspase activation (25, 28). To determine whether NRAGE-induced release of cytochrome c from mitochondria is independent of caspase activation, the effect of the caspase inhibitor zVAD-fmk on the accumulation of cytosolic cytochrome c was examined. Fig. 2D shows that zVAD-fmk did not reduce NRAGE-mediated cytosolic cytochrome c accumulation, indicating that NRAGE-induced cytochrome c release is not a consequence of caspase activation but instead lies upstream of caspase activation.

NRAGE Activates JNK through an MLK-independent Pathway—Activation of Jun kinase (JNK) and consequent transcriptional events play a necessary role in several neuronal cell death paradigms that involve cytochrome c release (20, 29, 30), such as p75NTR-dependent apoptosis (3, 15). To determine whether JNK is activated during NRAGE-induced apoptosis, PC12<sup>rta</sup> cells were left uninfected or were infected with AdNRG or the control viruses AdGFP or AdLacZ in the presence or absence of 1 μg/ml doxycycline, then lysed, and analyzed by immunoblot for JNK phosphostatus. Fig. 3 shows that NRAGE expression induced a robust increase in JNK phosphorylation with no increase in total JNK1 protein levels. JNK activation results in phosphorylation of c-Jun, which in turn leads to transcriptional activation of target promoters, including the promoter for c-Jun itself (31, 32). Therefore, to confirm that NRAGE-mediated phosphorylation of JNK was an indication of JNK signaling pathway activation, phosphostatus and protein levels of c-Jun were determined. NRAGE overexpression resulted in a dramatic increase in both the phosphorylation of c-Jun (visualized by phospho-Jun Ser<sup>63</sup> immunoblot and by slower migrating band in the total c-Jun immunoblot) and in total c-Jun levels (Fig. 3). In contrast, cells infected with AdGFP or AdLacZ showed no alteration in the levels or phosphostatus of JNK or c-Jun. Under these infection conditions, EGFP is highly expressed by AdGFP, and it accumulates to much higher levels than myc-NRAGE (Fig. 3, bottom panel), indicating that the effects of NRAGE on the JNK pathway do
**Fig. 2.** NRAGE expression induces cytochrome c release and caspase-dependent cell death. 

**A**, NRAGE expression induces caspase activity. PC12<sup>rtTA</sup> cells were left uninfected or were infected with 50 m.o.i. of recombinant adenovirus expressing myc-NRAGE (NRG) or EGFP (GFP) in the presence or absence of 1 μg/ml doxycycline. Two days after infection, cells were lysed, and 50 μg of cytosolic extracts were assayed for caspase activity using DEVD-amino-methyl-coumarin as substrate. The cleavage activity is shown as arbitrary fluorescence units per min. 

**B**, NRAGE expression leads to caspase zymogen cleavage. PC12<sup>rtTA</sup> cells were infected and incubated under the same conditions as in panel A. Thirty hours after infection, cells were lysed, normalized for protein levels, and analyzed by immunoblotting for levels of NRAGE and full-length Caspase-9, and, using cleavage-specific antibodies, for levels of cleaved Caspases-3 and -7 and cleaved PARP. LeBa levels were analyzed by immunoblot to confirm equal loading between lanes. 

**C**, caspase activity is required for NRAGE-mediated cell death. PC12<sup>rtTA</sup> cells were infected with 50 m.o.i. of recombinant adenovirus expressing myc-NRAGE (NRG) or β-galactosidase (LacZ) in the presence of 1 μg/ml doxycycline or were treated with camptothecin (100 μM) as a positive control for caspase-dependent cell death. In addition, cells were incubated with increasing concentrations of zVAD-fmk (zVAD) or Boc-Asp-fmk (BAF), broad spectrum caspase inhibitors, as indicated. After 40 h, cells were assayed for survival using the MTT assay and for death using the LDH assay, where untreated cells (Untr'd) and cells treated with 1% Triton X-100 (Triton) were used to delineate the output range of the assays. Results are normalized relative to those obtained with 1% Triton for the LDH assay or untreated cells for the MTT assay and represent the mean ± S.D. of a representative experiment performed in triplicate. (*, p < 0.0025 relative to LacZ + Dox at 0 μM caspase inhibitor for all gray bars. **, p < 0.0025 relative to AdNRG + Dox at 0 μM for all black bars. **, p < 0.0025 relative to camptothecin (Campto.) at 0 μM caspase inhibitor for all white bars). 

**D**, NRAGE expression results in caspase-independent accumulation of cytochrome c in the cytosol. PC12<sup>rtTA</sup> cells were left uninfected or were infected with 50 m.o.i. of AdNRG (NRG) or as a control with 50 m.o.i. of recombinant adenovirus expressing β-galactosidase under the control of an unmodified cytomegalovirus promoter (LacZ). Cells were maintained in 1 μg/ml doxycycline for 30 h after infection. The cytosolic fraction was then isolated as described under "Experimental Procedures." This procedure was performed in the absence (DMSO) or presence (zVAD) of 100 μM zVAD-fmk as indicated. The cytosolic fractions were normalized for protein content and were subjected to gel electrophoresis, along with a sample of the total cell lysate (Total cell) obtained from the uninfected cells maintained in the presence of doxycycline and containing the same amount of protein, followed by immunoblotting with a cytochrome c-specific antibody. The level of LeBa was also analyzed by immunoblotting with an LeBa-specific antibody to demonstrate equal loading of the cytosolic fractions. In the bottom of panel D, total cell lysate samples obtained prior to the fractionation procedure were normalized for protein content and immunoblotted with an NRAGE-specific antibody.
not reflect overexpression artifacts. Together, these data demonstrate that NRAGE specifically increases JNK activity, leading to the phosphorylation of c-Jun and activation of c-Jun transcriptional activity.

Mixed lineage kinases (MLKs) are MAP kinase kinase kinases (MAP3Ks) that have been identified as the upstream activators of the JNK pathway necessary for neurotrophin withdrawal-induced apoptosis (33–37). CEP1347, an indolocarbazole of the K252a family, is a potent inhibitor of MLKs (35) and blocks neuronal cell death in several neuronal apoptosis paradigms (33–37). To determine whether CEP1347 blocks NRAGE-dependent activation of the JNK pathway, PC12<sub>rtTA</sub> cells were infected with 50 m.o.i. of recombinant adenovirus expressing myc-NRAGE (NRG), EGFP (GFP), or β-galactosidase (LacZ) in the presence or absence of 1 μg/ml doxycycline as indicated. Thirty hours after infection, cells were lysed, normalized for protein levels, and analyzed for levels of phospho-Thr<sup>183</sup>/Tyr<sup>185</sup>-JNK and total JNK, phospho-Ser<sup>63</sup> Jun and total c-Jun, as well as IκBα and NRAGE, by immunoblotting. Total proteins on the immunoblot were shown by Ponceau staining of a protein blot to confirm equal loading and allow for comparison of myc-NRAGE and EGFP expression levels.

**Fig. 3.** NRAGE expression induces the activation of the JNK pathway. PC12<sub>rtTA</sub> cells were left uninfected or were infected with 50 m.o.i. of recombinant adenovirus expressing myc-NRAGE (NRG), EGFP (GFP), or β-galactosidase (LacZ) in the presence or absence of 1 μg/ml doxycycline as indicated. Thirty hours after infection, cells were lysed, normalized for protein levels, and analyzed for levels of phospho-Thr<sup>183</sup>/Tyr<sup>185</sup>-JNK and total JNK, phospho-Ser<sup>63</sup> Jun and total c-Jun, as well as IκBα and NRAGE, by immunoblotting. Total proteins on the immunoblot were shown by Ponceau staining of a protein blot to confirm equal loading and allow for comparison of myc-NRAGE and EGFP expression levels.

**Fig. 4.** NRAGE-mediated JNK activation is not blocked by CEP1347. A, effect of short-term CEP1347 treatment on JNK phosphorylation induced by NRAGE or MLK-3. PC12<sub>rtTA</sub> cells were left uninfected or were infected with 50 m.o.i. of recombinant adenovirus expressing myc-NRAGE (NRG), or HA epitope-tagged mixed lineage kinase-3 (MLK-3) or EGFP (GFP), in the presence or absence of 1 μg/ml doxycycline (DMSO) for an additional 60 min and were then lysed, normalized for protein content, and analyzed for levels of phospho-Thr<sup>183</sup>/Tyr<sup>185</sup>-JNK and total JNK, phospho-Ser<sup>63</sup> Jun and total c-Jun, as well as IκBα and NRAGE, by immunoblotting. Total proteins on the immunoblot were shown by Ponceau staining of a protein blot to confirm equal loading and allow for comparison of myc-NRAGE and EGFP expression levels.
ated transcriptional events that result in mitochondrial cytochrome c release, Caspase-9 activation, and the activation of effector Caspases-3 and -7. To directly address the requirement of JNK activation in NRAGE apoptotic signaling, we employed an adenovirus (AdJBD) encoding the JNK binding domain of the JNK-interacting protein-1, which acts to sequester JNK and thereby inhibit JNK-dependent c-Jun phosphorylation (19). To examine the role of c-Jun-dependent transcription on NRAGE-induced apoptosis, we used an adenovirus encoding a c-Jun dominant-negative mutant (AdΔJun) that lacks the c-Jun transactivation domain and functions as an inhibitor of AP-1 activity (20, 38).

To determine whether overexpression of either the c-Jun dominant-negative mutant or the JNK binding domain of JNK-interacting protein-1 (JIP1) reduces NRAGE-mediated apoptosis, PC12×<sup>TTA</sup> cells treated with AdNRG or AdLacZ were coinjected with either AdΔJun, AdJBD, or AdLacZ, and their viability was then assessed by MTT survival or LDH death assays (Fig. 5C). These experiments were complicated by the finding that these inhibitors of the JNK pathway caused a reduction in PC12×<sup>TTA</sup> cell viability on their own (Fig. 5C, bars d–i), indicating that some basal level of JNK activity is important for viability of the PC12 cell line, as suggested by others (39–41). Nonetheless, in both assay types, myc-NRAGE expressing cells co-infected with AdΔJun or AdJBD showed a significant decrease in cell death when compared with myc-NRAGE expressing cells treated with the control virus (Fig. 5C, compare k, l to j, or n, o to m). In fact, the viability of myc-NRAGE expressing cells treated with AdΔJun and AdJBD is indistinguishable from that of cells treated with AdΔJun and AdJBD in the absence of AdNRG co-infection (Fig. 5C, compare n and o to h and i), indicating that expression of these JNK pathway inhibitors completely masks the effects of NRAGE expression on viability. Therefore we conclude that activation of the JNK pathway is required for the induction of NRAGE-mediated apoptosis.

Biochemical assays were performed in parallel to confirm that the blockade of JNK signaling attenuates the NRAGE apoptotic pathway. PC12×<sup>TTA</sup> cells were infected with AdNRG together with the JNK pathway inhibitors, lysed, and then analyzed by immunoblot for activation of the JNK pathway and for induction of caspases. As expected, NRAGE expression resulted in the phosphorylation and accumulation of c-Jun, the activation of Caspase-3, and the cleavage of PARP (Fig. 5A). As predicted, co-expression of NRAGE with the JNK binding domain of JIP1 blocked NRAGE-induced c-Jun phosphorylation and prevented the accumulation of c-Jun protein. Similarly, co-expression of NRAGE with dominant-negative c-Jun prevented the NRAGE-induced accumulation of c-Jun protein. A reduction in the level of NRAGE-induced phosphorylated c-Jun was also detected, which is likely secondary to the reduction in the level of total c-Jun. Most importantly, NRAGE-dependent Caspase-3 activation and PARP cleavage were completely blocked when the JNK pathway was inhibited, demonstrating that the JNK pathway is necessary for mediation of NRAGE-induced apoptosis.

In neuronal death paradigms, JNK activation precedes mitochondrial cytochrome c release and Caspase-9 activation. However, some reports indicate that the JNK pathway can be initiated downstream of caspases (42–44). The data presented in Fig. 5A demonstrate a reduction in Caspase-3 activity and PARP cleavage in myc-NRAGE expressing cells following co-expression of inhibitors of the JNK pathway, suggesting that JNK is an activator, rather than a target, of caspases in the NRAGE-mediated apoptotic pathway. To directly address this possibility, NRAGE-dependent JNK activation was assessed in cells treated with caspase inhibitors. Although both BAFl and zVAD-fmk were potent blockers of NRAGE-induced cell death (Fig. 2), neither inhibitor attenuated the NRAGE-mediated activation of c-Jun or JNK (Fig. 5B), confirming that the observed induction of the JNK pathway lies upstream of caspase activation.

**DISCUSSION**

The specific signaling mechanisms employed by p75NTR to induce apoptosis remain uncertain. We have previously demonstrated that NRAGE is a p75NTR-interacting protein that facilitates p75NTR-induced cell death (18), and in this study we have analyzed the signaling pathways used by NRAGE to induce apoptosis. By using an inducible adenoviral expression system, we found that apoptosis induced by NRAGE occurs through an intrinsic death pathway that involves mitochondrial cytochrome c release and cleavage of Caspase-9, an apical caspase, and activation of effector Caspases-3 and -7. We also demonstrated that NRAGE-induced apoptosis is associated with MLK-independent activation of JNK and subsequent phosphorylation and accumulation of c-Jun. Finally, using adenovirus expressing dominant inhibitory proteins, we established that the activities of JNK and the c-Jun transcription factor are critical elements required for NRAGE-induced apoptosis.

Previous studies have indicated that p75NTR, like NRAGE, also activates the JNK pathway (3, 4, 12, 14, 45). Furthermore, Harrington et al. (15) have demonstrated that dominant-negative JNK can block p75NTR-induced apoptosis in oligodendrocytes. The precise signaling events that link p75NTR-induced JNK activation to caspase activation are not known, but p75NTR-induced death can be blocked by overexpression of Bcl-xL and appears to involve the activation of Caspases-9 and -3 but not Caspase-8 (46–48). Together, these findings indicate that p75NTR induces apoptosis through a JNK-dependent activation of the mitochondrial death pathway, thus pointing to a substantial overlap between the apoptotic pathway utilized by p75NTR and that induced by NRAGE. This overlap suggests that p75NTR and NRAGE lie on the same signaling pathway and adds credence to the hypothesis that NRAGE is an important element in p75NTR-induced apoptosis.

A remarkable aspect of NRAGE-mediated activation of the JNK pathway is its insensitivity to the neuroprotective compound CEP1347. CEP1347 has been shown to reduce apoptosis in several neuronal apoptosis paradigms, including that induced following trophic factor withdrawal (33–37). Recent studies have revealed that CEP1347 is a potent inhibitor of MAP3Ks that lie on the JNK pathway (35). In this study, we confirm that CEP1347 is very effective in blocking JNK activation induced by MLK-3 but also show that NRAGE-dependent activation of the JNK pathway is not affected by this compound, therefore suggesting that in PC12 cells, NRAGE-mediated JNK activation occurs independently of the MLKs. Data reported by Yoon et al. (13) suggest a similar MLK independence in the case of p75NTR-induced JNK activation and subsequent apoptosis of cultured oligodendrocytes. CEP1347 is a potent inhibitor of MLK activity with maximal inhibition of MLK-induced cell death occurring at a concentration of 100 nM (35). However, Yoon et al. found no significant inhibition of p75NTR-mediated death at a CEP1347 concentration of 100 nM and only a partial inhibition at a concentration of 1 μM (13), suggesting that the observed inhibition of apoptosis was because of an MLK-independent effect of CEP1347. In contrast, Friedman (3) reported a complete inhibition of p75NTR-induced death of hippocampal neurons after treatment with 200 nM CEP1347, suggesting a role for MLK activity in the p75NTR-induced death in this cell type. These disparate results hint at

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the existence of cell type-specific differences in the mechanism of the p75NTR-mediated activation of JNK, presumably reflecting cell-specific expression of individual interactor proteins.

NRAGE is a member of the MAGE family of proteins. The MAGE molecules initially identified were exclusively expressed in tumor cells, germ cells, or in the early embryo (49). However, subsequent studies revealed that additional family members, including NRAGE, belong to a distinct class of MAGE proteins expressed in several normal fetal and adult tissues. The molecular function of MAGE proteins still remains ill-defined, but the experiments described above suggest a novel role for MAGE proteins in regulation of cell survival through modulation of the JNK pathway. Others have recently detected an interaction between NRAGE and the anti-apoptotic protein XIAP (50) and have proposed that NRAGE may act to sequester and inactivate this protein, leading to a potentiation of caspase-mediated cell death in a promyeloid leukemic cell

FIG. 5. JNK activation is necessary for NRAGE-mediated caspase activation and cell death. A, PC12<sup>rT</sup>A cells were infected with 50 m.o.i. of recombinant adenovirus expressing myc-NRAGE (NRG) or β-galactosidase (LacZ) in the presence of 1 µg/ml doxycycline. Cells were also infected with 50 m.o.i. of recombinant adenovirus expressing FLAG-tagged dominant-negative c-Jun (ΔJun), FLAG-tagged JNK binding domain of JIP (FLAG-JBD), or control adenovirus (LacZ). Thirty hours after infection, cells were lysed, normalized for protein levels, and analyzed for levels of cleaved Caspase-3, cleaved PARP, phospho-Ser<sup>63</sup>Jun, total c-Jun, FLAG-tagged dominant-negative c-Jun (FLAG-ΔJun), FLAG-JBD and NRAGE by immunoblotting. Note that FLAG-tagged dominant-negative c-Jun is not detected by the c-Jun antibody, because it lacks the amino-terminal epitopes recognized by this antibody. B, the JNK pathway does not lie downstream of caspase activation in the NRAGE-mediated apoptotic pathway. PC12<sup>rT</sup>A cells were left uninfected or were infected with 50 m.o.i. of recombinant adenovirus expressing β-galactosidase (LacZ) or myc-NRAGE (NRG), treated with 1 µg/ml doxycycline, and incubated in the presence of either 100 µM zVAD-fmk (zVAD), 100 µM Boc-Asp-fmk (BAF), or vehicle Me<sub>2</sub>S0 (DMSO) as indicated. Thirty hours after infection, cells were lysed, normalized for protein levels, and analyzed for levels of phospho-Ser<sup>63</sup> c-Jun and total c-Jun, phospho-Thr<sup>183</sup>/Tyr<sup>185</sup> JNK and total JNK, leBo, and NRAGE by immunoblotting. C, NRAGE-mediated cell death is blocked by inhibitors of the JNK pathway. PC12<sup>rT</sup>A cells were infected with 50 m.o.i. of recombinant adenovirus expressing β-galactosidase (LacZ) or myc-NRAGE (NRG) together with 50 or 100 m.o.i. of recombinant adenovirus expressing FLAG epitope-tagged dominant-negative c-Jun (ΔJun), FLAG (FLAG-JBD), or the control protein β-galactosidase (LacZ) and treated with 1 µg/ml doxycycline as indicated. After 40 h, cells were assayed for survival using the MTT assay and for death using the LDH assay, where untreated cells (Untreated) and cells treated with 1% Triton X-100 (Triton) were used to delineate the output range of the assays. Results are normalized relative to those obtained with 1% Triton for the LDH assay or with untreated cells for the MTT assay and represent the mean ± S.D. of a representative experiment performed in triplicate (*, p < 0.0005 relative to NRG + Dox + 50 m.o.i. LacZ for bars d–f, j–l, *, p < 0.0005 relative to NRG + Dox + 100 m.o.i. AdLacZ for bars g–i, m–o).
line. In this setting, Bel-2 overexpression had no effect on NRAGE activity (50), suggesting that NRAGE may act downstream of cytochrome c release. Together with our results, these observations suggest that NRAGE may contribute to apoptosis through at least two distinct mechanisms.

NRAGE represents the first p75NTR-interacting protein that induces cell death through the activation of the JNK pathway. NRIF, another p75NTR-interacting protein (16), may play a role in mediating the p75NTR apoptotic signal because NRIF knock-out mice show a defect similar to p75NTR knock-outs in the apoptosis of retinal neurons (16). However, mechanistic details remain to be defined, and as yet there is no data directly linking apoptosis mediated by NRIF to that induced by p75NTR. NADE is another p75NTR interactor believed to initiate apoptosis (17). The mechanism of NADE-dependent cell death is also unknown but appears to require a member of the 14–3-3 family (51). Other interactors, such as SC-1 and RhoA, have not been proposed to have a role in p75NTR-mediated apoptosis (52, 53), whereas RIP2 and FAP1 may oppose the p75NTR cell death signal by regulating components of the NFκB pathway (54, 55). TRAF6 has been proposed as a positive regulator of the JNK pathway (54, 56), but its main function as a p75NTR interactor appears to be to promote pro-survival signals via the activation of the NFκB pathway (55, 57, 58).

Therefore, the capacity of NRAGE to mediate JNK-dependent apoptosis indicates that this interactor may be a unique physiological mediator of p75NTR-dependent JNK and apoptotic signaling.

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