c-Jun NH2-terminal kinases (JNKs) potentiate transcriptional activity of c-Jun by phosphorylating serines 63 and 73. Moreover, JNK and c-Jun can modulate apoptosis. However, an involvement of nitric oxide (NO)-induced phosphorylation of c-Jun on Ser-63 and Ser-73 in apoptosis has not been explored. We report that in SH-Sy5y neuroblastoma cells, NO induced apoptosis following JNK activation and phosphorylation of c-Jun almost exclusively on Ser-63. Importantly, NO-induced apoptosis and caspase-3 activity were inhibited in cells stably transformed with dominant-negative c-Jun in which Ser-63 is mutated to alanine (S63A), but not in cells transformed with dominant-negative c-Jun (S73A). Ser-63 of c-Jun (but not Ser-73) was required for NO-induced, c-Jun-dependent transcriptional activity. NO-induced apoptosis, Ser-63 phosphorylation of c-Jun, and caspase-3 activity were all inhibited in SH-Sy5y cells transformed with dominant-negative JNK. A caspase-3 inhibitor prevented apoptosis but not c-Jun phosphorylation. In a different neuroblastoma cell line, NO-induced Ser-63 phosphorylation of c-Jun and apoptosis were blocked by a specific JNK inhibitor. We conclude that NO-inducible apoptosis is mediated by JNK-dependent Ser-63 phosphorylation of c-Jun upstream of caspase-3 activation in neuroblastoma cells.

In the central nervous system, NO generation results in part from successive events of enhanced glutamate release, N-methyl-D-aspartate receptor stimulation, Ca2+ influx, and NO synthase activation (1–3). There is much evidence that excessive NO generation during strokes, ischemia, or neurodegenerative diseases contributes to neuronal cell death (4, 5). NO can exert its cytotoxic effects in diverse cell types via generation of highly reactive free radicals such as peroxynitrite, which damages DNA, proteins, and lipids by oxidation (3, 6, 7). Such damage in turn triggers downstream signal transduction pathways, which lead to apoptosis or necrosis (6). However, the death pathways that are activated in neurons in response to massive NO production are not well understood. Because NO can stimulate the activity of the transcription factor AP-1 in neurons (8, 9), one such death pathway might involve the AP-1-dependent regulation of cell death or survival genes.

c-Jun, a prominent member of the AP-1 transcriptional factor family, has been implicated in the regulation of a wide range of biological processes including apoptosis, which it can promote or counteract, depending on the tissue, the developmental stage and the nature of the death stimulus (10, 11). Its transcriptional activities are regulated by changes in the level of c-Jun expression as well as post-translational modifications of the c-Jun protein. In particular, phosphorylation of Ser-63 and Ser-73 in the NH2-terminal transactivation domain of c-Jun, which is mediated primarily by the c-Jun NH2-terminal kinases (JNKs) (12), substantially enhances the activity of c-Jun as a transcriptional factor (13, 14). c-Jun NH2-terminal phosphorylation on Ser-63 and Ser-73 can be either pro- or antiapoptotic (15, 16). c-Jun phosphorylation is thought to be required for the antiapoptotic function of c-Jun during hepatic necrosis (17). The precise role of c-Jun phosphorylation in genotoxic-induced apoptosis (UV irradiation, DNA-damaging agents) is controversial, but c-Jun phosphorylation is proapoptotic in neurons subjected to kainate, a low potassium concentration, or nerve growth factor deprivation (15, 18–20). Bim, Hrk, and Fas ligand are among the proteins whose up-regulation upon neurotrophin withdrawal is transcriptionally controlled, at least in part, by c-Jun phosphorylation (19, 21, 22).

The mitogen-activated protein (MAP) kinases include the JNKs and the p38 MAP kinases, which are activated by diverse cellular stress including inflammatory cytokines, heat shock, and UV irradiation (10, 23, 24). JNK and p38 activities have been implicated in cell death associated with glutamate excitotoxicity (25, 26). Two previous reports suggested that NO activates p38 MAP kinase, triggering significant apoptosis in neuronal cells (27, 28). JNK-mediated c-Jun phosphorylation is important for apoptosis of starved neuronal cells (19), and the JNK3 isoform is required for kainate-induced cytotoxicity in the central nervous system (29). Mouse fibroblasts derived from jnk1−/− jnk2−/− double knock-out embryos that lack all JNK activity are less sensitive to apoptosis induced by UV irradiation. The brains of these embryos exhibit altered morphologies because of deregulated apoptosis, which surprisingly is increased in some brain regions but decreased in others (30). Thus, much evidence suggests that c-Jun phosphorylation is often but not always proapoptotic, particularly in neuronal cells.

We reported recently that in SH-Sy5y cells, the constitutive activity of c-Jun/AP-1 in the absence of detectable AP-1 DNA binding is required for the expression of the neural cell adhe-
tion molecule NCAM-140 (31). This basal c-Jun/AP-1-dependent
expression of NCAM-140 counteracts NO-induced apoptosis.
Here, we investigated whether NO induces c-Jun phosphoryla-
tion and regulates apoptosis through the JNK-c-Jun pathway,
as do other cellular stressors. Notably, we found that JNK-de-
pendent phosphorylation of c-Jun on Ser-63 promotes NO-induc-
duced apoptosis of neuroblastoma cells. A speculative model is
proposed which can account for the pro- and antiapoptotic action of c-Jun/AP-1 within a single neuroblastoma cell.

EXPERIMENTAL PROCEDURES

Materials and Plasmid Constructions—The human neuroblastoma cell lines SH-Sy5y and SHEP were obtained from Eva Feldman (Uni-
versity of Michigan, Ann Arbor) and from Evelyne Goïllo (Laboratoire d’Immunologie, Centre Leon Berard, Lyon, France), respectively. The polyclonal antibodies against phospho-c-Jun, phospho-JNK, c-Jun, and JNK were from Cell Signaling Technology. The antibody against actin was from Santa Cruz Biotechnology, Inc. DEVD-ac and z-DEVD-fmk were obtained from BACHEM. Lipofectin and Opti-MEM were pur-
chased from Invitrogen. The cell proliferation reagent WST-1 was pur-
chased from Roche Applied Science. The D-TAT and D-JNK1 peptides were from Alexis Biochemicals (Switzerland). The dual luciferase assay kit and β-galactosidase assay kit were from Promega. All the other reagents used in this research were from Sigma Chemical Company.

The plasmid encoding dominant-negative c-Jun (denoted JunAA) was obtained from Dan Mercola (Sidney Kimmel Cancer Center, San
Diego). The plasmids bearing the dominant-negative S63A or S73A mutations in c-Jun were constructed by PCR-based mutagenesis based on
the plasmid JunAA. The primers used were: S63A (forward), 5’-GCT
CAA GCC GGC GGA GG-3’; S63A (reverse), 5’-CCG GCT CGG
GAG AGG TGA GGA GG-3’; S73A (forward), 5’-GTT CAC CTC
TCC CTG AGG AGG TGA GGA GG-3’; S73A (reverse), 5’-CGT
CGG GAG AGG TGA GGA GG-3’. The Gal4-c-Jun transactivator and
Gal4-luciferase reporter plasmids were purchased from Stratagene. The modified Gal4-c-Jun plasmids (S63A or S73A) were constructed by
cloning the transactivation domain of c-Jun (amino acids 1–221) bear-
ing either the S63A or S73A mutations into the pFA-CMV vector from
Stratagene. The pGL3-AP1 reporter plasmid and RPL-TK plasmid were kindly provided by S. Dhakshinamoorthy (Institute of Molecular
and Cell Biology, Singapore). The plasmid encoding dominant-negative (DN)-JNK1 was provided by Roger J. Davis (Howard Hughes Medical
Institute, University of Massachusetts Medical School, Worcester), and the plasmid encoding DN-JNK2 was provided by Dr. Shengcai Lin
(Institute of Molecular and Cell Biology, Singapore).

Cell Culture and Transfection—Both SH-Sy5y and SHEP cells were
maintained in Dulbecco’s modified medium containing 10% fetal bovine
serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The sub-
strates that ensure well plating is prepared to be extracellular matrix
proteins, either exogenous or derived from the bovine serum. Normally
the cells were split after 48 h in culture. The JunAA, S63A, S73A, DN-
jjnk1, and DN-jnk2, plasmids were transfected into SH-Sy5y cells using Lipofectin following the manufacture’s instructions. The stably
transfected cells were selected and maintained in medium with 100 μg/ml hygromycin (JunAA, S63A, S73A), 500 μg/ml G418 (DN-jnk1),
and 100 μg/ml zeosin (DN-jnk2).

Reporter Assays—For the Gal4-c-Jun reporter assay, 50 ng of Gal4-
c-Jun activator plasmids (wild type, S63A, S73A, or JunAA), 1 μg of
Gal4-luciferase reporter plasmid, and 10 ng of β-galactosidase plasmid
plasmids were cotransfected into SH-Sy5y cells in 6-well plates (1×10^5
cells/plate). 40 h later, cells were treated with 2 μM SNP for the indicated times. Medium was removed, and cells were washed three
times with ice-cold phosphate-buffered saline. Cells were harvested with 400 μl of reporter lysis buffer (provided in the β-galactosidase
assay kit) and subjected to a short spin. 20 μl of supernatant was added to 100 μl of luciferase substrate, and the luciferase activity was mea-
sured immediately in a TD-20e luminometer. An aliquot of the same
sample was used to determine β-galactosidase activity for normalizing
luciferase activity obtained above. For the AP-1 reporter assay, 1 μg of
pGL3-AP1 reporter plasmid and 10 ng of RPL-TK plasmid were co-
transfected in S63A or S73A or vector control cells in 6-well plates. 40 h
later, cells were treated with 2 μM SNP for the indicated times and harvested above. Lysates were diluted 10 times and 2 μl of each was added
in 100 μl of firefly luciferase substrate. The Renilla luciferase activity, as
an internal control, was used to correct for variations in transfection.

Cell Death Assays—To measure cell death by WST-1 or lactate de-
hydrogenase release assay, cells (1×10^5/well) were plated in 96-well plates and treated with SNP for up to 15 h. WST-1 was added to the
culture medium at a 1:10 dilution and incubated at 37 °C for 1 h or until
the color of the medium turned red (incubation time can vary according
to the cell number in the culture). The absorbance was measured at
a wavelength of 420 nm. To carry out the lactate dehydrogenase release
assay, the supernatants of the cells were collected, and the cell layer
was lysed with an equal volume of lysis buffer (Dulbecco’s modified
Eagle’s medium plus 0.1% Triton X-100). Lactate dehydrogenase activ-
ity in the supernatant and the lysate was quantitated. The cytotoxicity
was calculated as percentage of lactate dehydrogenase release by the
ratio of supernatant/lysate (lysatelysis + supernatant).

RESULTS

NO Induces JNK Activation, c-Jun Phosphorylation on Ser-
63, and Apoptosis in SH-Sy5y Cells—Various NO donors have
been used widely to study oxidative stress and cellular re-
sponses by mimicking endogenous NO generation (33). SH-
Sy5y neuroblastoma cells are highly sensitive to cell death
induced by various NO donors including SNP at concentrations
in the range 0.5–2.5 μM, and the mode of cell death under these
conditions is apoptosis (28, 31, 34). As described previously
(31), a time-dependent increase in cell death was observed
beginning around 8 h after the addition of SNP to the SH-Sy5y
cells (Fig. 1A), and this increase correlated with the appearance
of significant JNK activity at 6–7.5 h after the addition of SNP
(Fig. 1B, upper panel).

Because JNKs are the main upstream kinases for c-Jun NH₂-
terminal phosphorylation (12), we next tested whether the Ser-63 and Ser-73 residues of c-Jun were phosphorylated after SNP treatment of SH-Sy5y cells by using phosphoserine 63- and phosphoserine 73-specific antibodies. A strong and
sustained c-Jun phosphorylation on Ser-63 was observed at
6–8 h after the addition of SNP, whereas phosphorylation on Ser-73 was virtually undetectable (Fig. 1C). In contrast, UV
irradiation of SH-Sy5y cells resulted in similar levels of Ser-63
and Ser-73 phosphorylation (Fig. 1C). Thus, JNK activation and selective phosphorylation of c-Jun on Ser-63 both occurred
around the onset of NO donor-induced apoptosis. Ser-63 phos-
phorylation and the death of SH-Sy5y cells both occurred at 1.5
and 2 μM SNP. Concentrations of SNP lower than 1.5 μM
neither induced cell death nor elicited Ser-63 phosphorylation.
Ser-63 and Ser-73 have been found previously to lead to c-Jun- and AP-1-mediated transactivation (14), and accordingly mutation of Ser-63 and Ser-73 of c-Jun (data not shown), indicating that c-Jun is phosphorylated only at toxic concentrations of SNP. Excessive concentrations of SNP higher than 2.5 mM resulted in detectable Ser-73 phosphorylation that closely correlated with the onset of appreciable necrosis, indicating that predominant Ser-63 phosphorylation is an apoptosis-related phenomenon (data not shown).

**NO-induced Apoptosis Is Blocked in S63A and JunAA Stable Cells but Not in S73A Stable Cells**—To investigate whether c-Jun phosphorylation contributes to NO-induced apoptosis, we stably transfected SH-Sy5y cells with plasmids encoding various dominant-negative forms of c-Jun. In one form, Ser-63 was mutated to alanine (denoted S63A), and in another Ser-73 was mutated to alanine (denoted S73A). In a third form, both Ser-63 and Ser-73 were mutated to alanines (JunAA), which compromises the ability of c-Jun to transactivate target genes (15).

To exclude the possibility that highly overexpressed S63A, S73A, or JunAA might quench JNK activity by sequestering JNK in an abortive complex, we chose for further analysis independent clones in which S63A (Fig. 2A, *top panel*) or JunAA (Fig. 2A, *lower panel*) or S73A (data not shown) are expressed at levels only slightly in excess of endogenous c-Jun. Normal phosphorylation of endogenous c-Jun on Ser-63 in response to UV irradiation was still observed in two independent clones of S63A stable cells (Fig. 2B); and as expected, the endogenous Ser-73 phosphorylation of c-Jun in response to UV irradiation was more intense in S63A stable cells compared with the vector control cells (Fig. 2C). Analogous results were obtained in UV irradiation-treated S73A cells (data not shown). In addition, NO-induced phosphorylation of endogenous c-Jun on Ser-63 still occurred in two independent JunAA clones (Fig. 2D). These data indicate that the expression of c-Jun mutated to S63A and/or S73A did not compromise endogenous JNK activity.

We then compared the sensitivities of the above three different stable cells and vector control cells to NO donors and UV radiation. At any concentrations of SNP which were sufficient to induce apoptosis, several independent clones of S63A and JunAA stable cells showed markedly increased resistance to cell death compared with vector control cells (Fig. 3A). Importantly, various S73A stable cell lines failed to show resistance to NO compared with vector control cells (Fig. 3A). In contrast, neither S63A nor S73A stable cells were resistant to UV irradiation-induced cell death, whereas JunAA cells only showed a marginal increase in resistance to UV irradiation (Fig. 3B). These data provide evidence that Ser-63 phosphorylation of c-Jun is important in NO-induced, but not UV irradiation-induced cell death.

**Ser-63 of c-Jun Is Required for c-Jun- and AP-1-mediated Transactivation in Response to NO**—Dual phosphorylation of Ser-63 and Ser-73 has been found previously to lead to c-Jun-dependent transactivation (14), and accordingly mutation of both serines reduces the ability of c-Jun to transactivate target genes (15). Because we found that NO caused c-Jun phosphorylation predominantly on Ser-63, and since S63A and JunAA stable cells showed markedly increased resistance to cell death, we next asked whether Ser-63 phosphorylation alone can potentiate c-Jun and AP-1 transactivation. Using a Gal4-c-Jun reporter system, we found that Gal4-c-Jun (wild type) as well
as Gal4-c-Jun (S73A) were transactivated up to 4-fold in SH-Sy5y cells upon NO stimulation (Fig. 4A). However, Gal4-c-Jun (S63A) and Gal4-c-Jun (JunAA) were completely inactive in transactivation (Fig. 4A). In parallel experiments, transient transfections with AP-1 reporter plasmids revealed that NO-induced AP-1 activation of up to ~2.7-fold occurred in SH-Sy5y and S73A cells, but was absent in S63A cells (Fig. 4B). Thus, our combined data from the c-Jun and AP-1 reporter assays indicate that the presence of Ser-63 (but not Ser-73) is required for NO-induced c-Jun/AP-1 transactivation. These results also indicate that S63A and the JunAA constructs function as dominant-negatives by inhibiting gene transcription mediated by endogenous c-Jun.

**Caspase-3 Contributes to NO-induced Cell Death and Is Inhibited in S63A Stable Cells**—Caspase-3 was found to be important for NO-induced apoptosis of SH-Sy5y cells because prevention of caspase-3 activity by z-DEVD (a selective caspase-3 inhibitor) promoted cell survival (Fig. 5A). At z-DEVD concentrations that reduce cell death by 50% (Fig. 5A) and completely inhibit caspase-3 (Fig. 5B), the levels of NO-induced Ser-63 phosphorylation of c-Jun were similar to those in the absence of z-DEVD (Fig. 5C), indicating that caspase-3 activity is downstream of c-Jun phosphorylation. Two bands of phospho-c-Jun were occasionally observed, (Figs. 5C and 6) as has been noted previously (19, 35). We next assayed caspase-3 activity in S63A and S73A stable cells after NO donor treatment and found that caspase-3 activity was inhibited efficiently in S63A cells compared with vector control cells, whereas S73A cells showed similar (or slightly enhanced) caspase-3 activity under the same conditions (Fig. 5D). Because we showed that caspase-3 contributes to NO-induced apoptosis of SH-Sy5y cells, these results provide additional evidence that c-Jun phosphorylation on Ser-63 (but not Ser-73) mediates NO-induced apoptosis and indicate that caspase-3 contributes to apoptosis downstream of c-Jun phosphorylation.

**Phosphorylation of c-Jun on Ser-63 and Apoptosis Are Blocked in DN-jnk Stable Cells**—To provide more direct proof that JNK(s) play a role in c-Jun phosphorylation in response to NO, we stably transfected SH-Sy5y cells with DN-jnk1 or DN-jnk2 plasmids. At least two independent clones of both DN-jnk1 and DN-jnk2 stable cells exhibited a greatly diminished or absent phosphorylation of endogenous c-Jun on Ser-63 (Fig. 6, A and B), indicating that JNKs are responsible for NO donor-stimulated c-Jun phosphorylation in SH-Sy5y cells. Various independently isolated DN-jnk1 and DN-jnk2 clones also showed increased resistance to apoptosis at three concentrations of NO donor (Fig. 7A), that was quantitatively similar to that observed in S63A cells (Fig. 3A). A marked decrease of caspase-3 activity, indicative of increased survival, was also observed in these DN-jnk1 and DN-jnk2 cells (Fig. 7B). This combined evidence suggests that inhibition of JNK leads to...
enhanced cell survival through the suppression of JNK-dependent Ser-63 phosphorylation of c-Jun and the inhibition of caspase-3.

Evidence That JNK-mediated c-Jun Phosphorylation on Ser-63 Is a General Phenomenon in NO-induced Apoptosis of Neuroblastoma Cells—It was important to find out whether JNK-mediated c-Jun phosphorylation on Ser-63 also plays a general role in NO-induced apoptosis in neuroblastoma cell lines and to employ an alternative strategy to block JNK. Using SHEP neuroblastoma cells (36, 37), we found strong Ser-63 phosphorylation of c-Jun beginning at 8 h after SNP treatment, whereas Ser-73 phosphorylation was again virtually undetectable (Fig. 8A). As with SH-Sy5y cells, UV irradiation caused both Ser-63 and Ser-73 phosphorylation of c-Jun in SHEP cells (Fig. 8A). A cell-permeable peptide, D-JNKI1, which specifically inhibits JNK activity (38), effectively blocked both Ser-63 phosphorylation (Fig. 8B) and death of SHEP cells (Fig. 8C). In contrast, a control cell-permeable peptide (D-TAT) neither prevented Ser-63 phosphorylation nor the death of SHEP cells (Fig. 8, B and C, respectively). These results offer additional evidence that the JNK family of protein kinases phosphorylates c-Jun in NO-induced apoptosis and argue that JNK-mediated c-Jun phosphorylation on Ser-63 plays an important general role in triggering the death of neuroblastoma cells.

DISCUSSION

Previous reports have indicated that excessive generation of NO might be coupled to the activation of signal transduction cascades involving stressed-activated protein kinases and transcription factors. The activation of p38 MAP kinase occurs in NO-donor induced apoptosis of various neuronal cells (27, 28), which in one case involved p38 acting upstream of Bax to trigger the intrinsic (mitochondria) death pathway (28). There are many reports that NO can regulate AP-1 in the brain (8, 39), and various other studies have demonstrated that c-Jun/AP-1 can modulate apoptosis induced by diverse agents (40–44). However, the existence of NO-induced JNK-c-Jun signal-
ing and subsequent gene regulation in apoptosis has not been explored until now. In neurons, the JNK-c-Jun pathway is proapoptotic during neurotrophin factor withdrawal, kainate treatment, and potassium deprivation (15, 18, 19). This involves Ser-63 and Ser-73 phosphorylation, and it is accepted that the transcriptional activation of c-Jun in cell growth and development depends strictly on the dual phosphorylation of these amino acids (14, 15).

It is, therefore, notable we now show that Ser-63 (but not Ser-73) phosphorylation of c-Jun mediates NO-induced apoptosis of neuroblastoma cells. Our evidence came from several complementary lines of investigation. First, NO induced a strong activation of JNK, and only toxic concentrations of SNP induced phosphorylation of c-Jun on Ser-63 prior to and at the onset of apoptosis. Second, S63A and JunAA stable cells (but not S73A cells) exhibited significantly increased resistance to apoptosis triggered by NO as measured by cell death and caspase-3 assays. Moreover, DN-jnk1 and DN-jnk2 stable cells showed increased resistance to NO correlating with markedly reduced Ser-63 phosphorylation and caspase-3 activation. Our data suggest that JNKs are primarily responsible for phosphorylating c-Jun on Ser-63, which is supported further by our unpublished observation that a specific p38 MAP kinase inhibitor failed to block Ser-63 phosphorylation of c-Jun. Third, in a different approach, a highly specific JNK-inhibitory peptide blocked both exclusive Ser-63 phosphorylation and NO-induced apoptosis of SHEP neuroblastoma cells.

Might one or both of the JNKs (1 and 2) directly phosphorylate substrates other than c-Jun and thereby contribute to apoptosis? Although still controversial, there is evidence that JNK-mediated phosphorylation of p53, p66shcA, or Bel-2 family members is proapoptotic in various different contexts (45–49). However, we showed here that JNK activity is present at normal levels in S63A, JunAA, and S73A cells, arguing that alternative potential JNK death substrates other than c-Jun would still be phosphorylated under conditions in which S63A protects from NO killing. Thus, we believe that Ser-63 of c-Jun is the important target in the NO-inducible killing pathway. It is not known why NO induces only Ser-63 phosphorylation, but it is worth speculating. There are 10 known isoforms of JNKs, and NO might activate one isoform that only targets Ser-63. Alternatively, NO might activate a Ser-73 phosphatase; or putative NO-mediated chemical modification (e.g. nitrosylation) of c-Jun could preferentially block Ser-73 phosphorylation.

What is the pathway by which Ser-63 phosphorylation of c-Jun connects to caspase-3 activation and mediates apoptosis? There are two major possibilities. First, Ser-63 phosphorylation might activate c-Jun, which then transactivates death genes (or suppresses protective genes). This is strongly supported by...
The results of the Gal4-c-Jun and AP-1 reporter assays, which show that the S63A mutation alone abolishes transactivation. These assays also indicate that c-Jun is a crucial component of an AP-1 complex activated by NO, in agreement with antibody supershift experiments demonstrating that c-Jun protein is an AP-1 complex after NO stimulation (31). Speculatively, Ser-63 phosphorylation might regulate a different set of target genes compared with dual phosphorylated or nonphosphorylated c-Jun, resulting in a shift to pro-cell death gene expression. It is worth considering the known c-Jun-regulated genes that are thought to play roles in neuronal apoptosis (11, 50–54). Among the stronger candidates are hrf, bim, and fasL, which are proapoptotic (19, 21, 22, 55–57), but other possible genes are bcl3 and GAP43 (58, 59). The second, perhaps less likely, possibility is Ser-63 phosphorylation may lead to apoptosis through transcriptional repression caused by actions of c-Jun that suppress or antagonize other transcription factors (60).

In our previous study (31), we showed that SH-Sy5y cells expressing a different DN-c-Jun (TAM-67 in which the transcriptional activation of c-Jun is deleted) are more sensitive to NO-induced apoptosis. This is in complete contrast to the S63A, JunAA, and DN-jnk constructs used in this study, all of which render neuroblastoma cells more resistant to apoptosis. It is important to explain the opposing effects of the dominant-negative c-Jun (TAM-67) and S63A/JunAA/DN-jnk neuroblastoma cells in an attempt to understand the role(s) of c-Jun/AP-1 in NO-induced apoptosis. A speculative model is presented in Fig. 9 which can account for this apparent paradox. The TAM-67 dominant-negative protein is known to efficiently inhibit general AP-1-mediated transcription (61, 62). TAM-67 cells become more sensitive to NO at least in part through the inhibition of an important NCAM-140-mediated cell survival pathway activated by a constitutive level of c-Jun/AP-1 function (31) (Fig. 9A). The synthesis of NCAM-140 does not presumably require Ser-63 phosphorylation of c-Jun because it occurs in the absence of NO stimulation, which we showed in the present study is essential to activate the JNK-phospho-c-Jun pathway. Support for a phosphorylation-independent function of c-Jun comes from several other directions. For example, mutant mice in which the c-Jun locus is replaced by JunAA are healthy and fertile, which is in contrast to the embryonic lethality of Jun−/− mice (15, 63, 64). In addition, JunAA itself can regulate transcription by acting as a suppressor and antagonist of other transcription factors (60).

In contrast to TAM-67 cells (31), we found that the NCAM-140 protein is still synthesized at normal levels in NO-resistant S63A, JunAA, and in DN-jnk1 and DN-jnk2 cells. This indicates the constitutive c-Jun/AP-1-dependent NCAM-140 survival pathway is intact in these cells (Fig. 9B). In other words, the S63A, JunAA, and DN-jnk constructs block the proapoptotic JNK-c-Jun pathway without affecting the synthesis of neuroprotective NCAM-140, so the cells are resistant to apoptosis compared with SH-Sy5y cells and TAM-67 cells (Fig. 9B).

Consistent with our previous and present studies, it was reported that c-Jun can protect undifferentiated rat PC12 neuronal cells from apoptosis independently of c-Jun phosphorylation; but in the fully differentiated cells JNK signaling can induce apoptosis, and c-Jun mediates this response (42). However, NO was not one of the apoptosis paradigms used. Together with our earlier report (31), now we have evidence that c-Jun/AP-1 can fulfill opposite functions in a single undifferentiated neuroblastoma cell, which we speculate to occur in the following context. A constitutive or basal activity of c-Jun/AP-1 factor(s) (independent of c-Jun phosphorylation on Ser-63) is able to counteract relatively low levels of NO—in part through the constant expression of neuroprotective NCAM-140 (31). In contrast, a toxic concentration of NO will lead to c-Jun phosphorylation on Ser-63 by JNK that triggers apoptosis via unknown c-Jun targets.
JNK-dependent Phosphorylation of c-Jun on Serine 63 Mediates Nitric Oxide-induced Apoptosis of Neuroblastoma Cells
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