Opposing Effects of ERK and JNK-p38 MAP Kinases on Apoptosis

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Apoptosis plays an important role during neuronal development, and defects in apoptosis may underlie various neurodegenerative disorders. To characterize molecular mechanisms that regulate neuronal apoptosis, the contributions to cell death of mitogen-activated protein (MAP) kinase family members, including ERK (extracellular signal–regulated kinase), JNK (c-JUN NH_2-terminal protein kinase), and p38, were examined after withdrawal of nerve growth factor (NGF) from rat PC-12 pheochromocytoma cells. NGF withdrawal led to sustained activation of the JNK and p38 enzymes and inhibition of ERKs. The effects of dominant-interfering or constitutively activated forms of various components of the JNK-p38 and ERK signaling pathways demonstrated that activation of JNK and p38 and concurrent inhibition of ERK are critical for induction of apoptosis in these cells. Therefore, the dynamic balance between growth factor–activated ERK and stress-activated JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis.

During mammalian development and in the mature organism, various extracellular stimuli act to promote survival and suppress cellular suicide programs (*1*). Because there is competition for limiting amounts of target-derived survival factors in the nervous system, approximately half of all neurons produced by neurogenesis die during development (*1*, *2*). The cells that die typically undergo apoptosis, a morphologically and biochemically distinct form of programmed cell death. The processes of both cell survival and cell death involve highly regulated signaling pathways that are currently the subject of intense investigation.

The signaling pathways that lead to apoptosis are beginning to be defined, and a number of proteins have been identified that either induce or prevent apoptosis (*1*). For example, recent studies involving the microinjection of neutralizing antibodies or the expression of dominant-interfering mutants have suggested a critical role for a transcription factor, the proto-oncogene encoding c-JUN, in apoptosis induced by NGF withdrawal from cultured sympathetic neurons (*3*, *4*). However, the mechanism by which NGF withdrawal triggers c-JUN activation and how c-JUN itself contributes to apoptosis are presently unclear. Studies of c-JUN regulation in other systems have identified several signaling pathways that regulate c-JUN expression and activity. For example, activation of JNK (also termed SAPK) causes phosphorylation of c-JUN at serine residue 63 (Ser^{63}) and Ser^{73} and increases its transcriptional activating potential (*5*–*9*). Once activated, c-JUN stimulates its own expression by interacting with two AP-1 sequence elements within its promoter (*10*). c-JUN can function as a homodimer or as a heterodimer with partner proteins such as ATF2. Like c-JUN, ATF2 is phosphorylated and activated by JNK (*11*, *12*). ATF2 is also phosphorylated and activated by the p38 mitogen-activated protein (MAP) kinase (*13*). ATF2 therefore serves as a mechanism for integration of the stress-regulated signaling pathways that activate JNK or p38.

Activation of JNK and p38 by treatments that induce apoptosis. To elucidate the mechanism of c-JUN activation during apoptosis induced by NGF withdrawal, we examined the potential role of JNK and p38 in this process. Although JNK and p38 are activated by several forms of environmental stress that are known to induce apoptosis under certain circumstances (*1*), the physiological functions of JNK and p38 in mammalian cells are unknown (*6*, *7*, *14*, *15*). A role for p38 in the regulation of the small heat shock protein HSP27 (*16*) and in the biosynthesis of inflammatory cytokines (*17*)

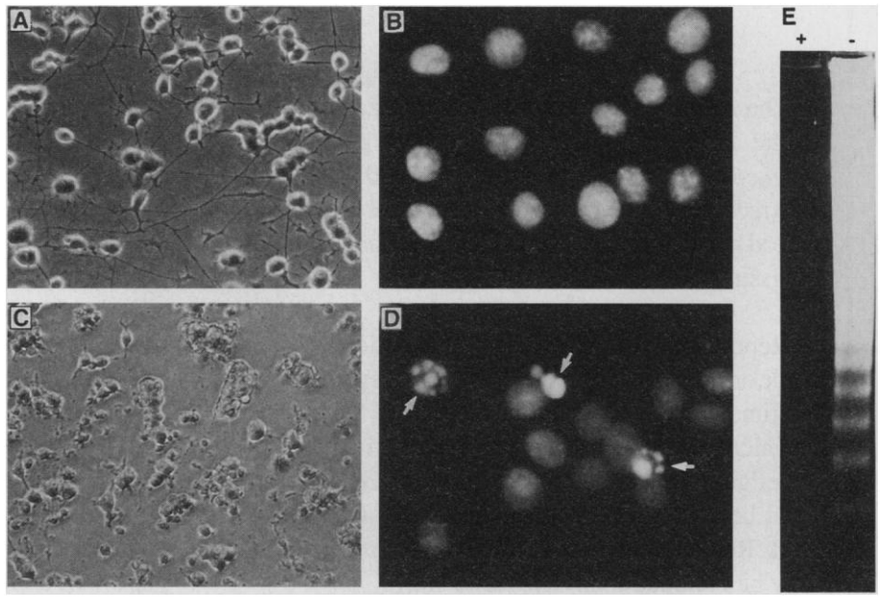

Fig. 1. Apoptosis of NGF-differentiated PC-12 cells after NGF withdrawal. (**A**) Phase-contrast photomicrograph (magnification, ×200) of PC-12 cells differentiated into sympathetic-like neurons with stable neurites after culture in the presence of NGF (50 ng/ml) for 1 to 2 weeks (*42*). (**B**) Nuclei of NGF-treated PC-12 cells with extended neurites stained uniformly with Hoechst 33258 (magnification, ×600), indicating intact nuclei (*43*). (**C**) Phase-contrast photomicrograph (magnification, ×200) of NGF-differentiated PC-12 cells 12 hours after NGF withdrawal (*42*). (**D**) Hoechst staining (magnification, ×600) after NGF withdrawal showing apoptotic nuclei (condensed or fragmented, indicated by arrows) in a fraction of cells with retracted neurites. Dead cells loosely adherent to the tissue culture dish (C) detached from cover slips during the fixation and staining process. (**E**) DNA fragmentation in NGF-differentiated PC-12 cells after NGF withdrawal. Agarose gel electrophoresis of soluble cytoplasmic DNA from NGF-differentiated PC-12 cells was performed in the presence of NGF (+) or after NGF withdrawal (−). Soluble cytoplasmic DNA was isolated from 4 × 10^6 cells 17 hours after NGF removal and subjected to electrophoresis (*44*). Hoechst staining revealed that approximately 1% of the cells in this experiment were apoptotic in the presence of NGF. This very low level of apoptosis was not detectable by DNA fragmentation analysis because of the lower sensitivity of this assay. However, upon NGF withdrawal, DNA fragmentation was detectable and provided a clear indication that there was an increase in apoptosis.

and a role for JNK in T cell activation and RAS-induced transformation (*18*) have been proposed. We induced PC-12 pheochromocytoma cells to differentiate by treatment with NGF and examined the function of the JNK and p38 signaling pathways in apoptosis caused by withdrawal of NGF. Differentiated PC-12 cells undergo pronounced and well-characterized apoptosis upon NGF withdrawal that resembles apoptosis in cultured sympathetic neurons (*19*).

PC-12 cells differentiate into cells that resemble sympathetic neurons after culturing in serum-free media in the presence of NGF for 9 to 14 days (*19, 20*). Addition of NGF to PC-12 cells induces various cellular responses, including ERK activation and the induction of immediate-early genes (*21, 22*). Morphological changes also occur, including membrane ruffling, flattening of cells, enlarged cell bodies, and the formation of stable neurites (*19, 20*) (Fig. 1A). The nuclei of NGF-treated PC-12 cells stained uniformly with Hoechst 33258, which indicated that the cells were alive and the nuclei were intact (Fig. 1B). Once the cells were differentiated in the presence of NGF, the withdrawal of NGF caused morphological changes that are characteristic of apoptosis, including retraction of neurites, shrinkage of the cell bodies, and blebbing of plasma membranes (Fig. 1C). Removal of NGF also caused fragmentation and condensation of nuclei (Fig. 1D) and DNA cleavage (Fig. 1E). The percentage of apoptotic cells detected at different times after NGF withdrawal was generally about 15%. This is clearly an underestimate of the percentage of cells in the population that undergo apoptosis upon NGF withdrawal because cells that have progressed into the later stages of apoptosis detach from the tissue culture dish and are not scored in the assay.

To determine if JNK and p38 MAP kinases are stimulated when NGF is removed and PC-12 cells undergo apoptosis, we quantitated the activities of these kinases at various times after NGF removal (Fig. 2, A and B). JNK and p38 activities increased during the first 6 hours after NGF removal [5.1-fold ± 0.4 (SEM) and 3.2-fold ± 0.6 (SEM), respectively, from three experiments]. In contrast, in control cells that were washed once in NGF-free medium and then incubated in NGF-containing medium, only a small increase in JNK and p38 MAP kinase activities was detected within the first 6 hours (one- to twofold). This increase in kinase activity in the control cells was likely to be the result of stress during washing. The magnitude of the activation of JNK detected upon NGF withdrawal was comparable to that seen when differentiated PC-12 cells were exposed to ultraviolet (UV) light or heat shock (*23*), two stimuli that are known to be effective activators of the JNK and p38 pathways.

The kinetics of apoptotic progression upon NGF withdrawal were compared with those of the activation of the JNK and p38 MAP kinases (Fig. 2, A and B). Stimulation of JNK and p38 preceded the induction of apoptosis, which suggests that activation of these kinases may contribute to cell death. In addition to NGF withdrawal, staurosporine, another inducer of apoptosis (*24*), also induced JNK activity in undifferentiated PC-12 cells (Fig. 2C). Staurosporine-induced apoptosis was rapid and was preceded

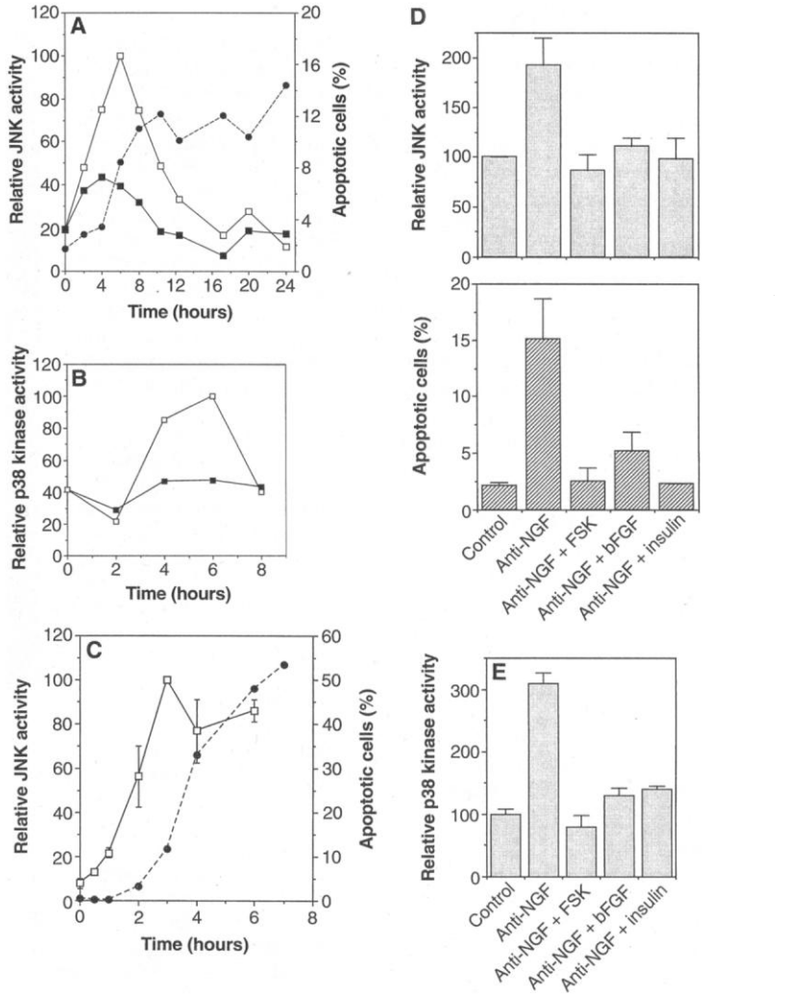

Fig. 2. Activation of JNK and p38 MAP kinase before apoptosis. (**A**) Time course of JNK activation and apoptosis after NGF withdrawal from NGF-differentiated PC-12 cells (*42, 43*). Open squares, kinase activity after NGF withdrawal; closed squares, kinase activity in control cells; closed circles, percentage of cells undergoing apoptosis. Data shown are representative of five independent experiments. (**B**) Time course of p38 MAP kinase activation in control (closed squares) or NGF-depleted PC-12 cells (open squares) (*42*). Data shown are representative of three independent experiments. (**C**) Induction of apoptosis and JNK activation by staurosporine (1 μM) in undifferentiated PC-12 cells (*42, 43*). It was not necessary to differentiate PC-12 cells with NGF in this experiment because undifferentiated PC-12 cells were responsive to staurosporine. Data shown are averages of three independent experiments, and reported errors are SEM. Open squares, JNK activity in the presence of staurosporine; closed circles, percentage of apoptotic cells. (**D**) Inhibition of JNK activation by agents that prevent apoptosis caused by NGF withdrawal. NGF-differentiated PC-12 cells were deprived of NGF (Anti-NGF) (*42*) in the presence of forskolin (FSK, 10 μM), bFGF (40 ng/ml), or insulin (5 μM) and assayed 6 hours later for JNK activity (*42*) or 12 hours later for apoptosis (*43*). The data are averages from five independent experiments. Error bars represent SEM. (**E**) Inhibition of p38 activation by agents that prevent apoptosis caused by NGF withdrawal. NGF-differentiated PC-12 cells were deprived of NGF (*42*) in the presence of forskolin (FSK, 10 μM), bFGF (40 ng/ml), or insulin (5 μM) and assayed for p38 activity (*42*). Error bars represent SEM.

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by increased JNK activity (13-fold). The kinase activities of JNK and p38 were also measured in the presence of several agents that prevent apoptosis. These agents included adenosine 3′,5′-monophosphate (cAMP), basic fibroblast growth factor (bFGF), and insulin (*19*, *25*). All three agents prevented apoptosis induced by NGF withdrawal and inhibited the activation of JNK and p38 kinase (Fig. 2, D and E). Taken together, these initial experiments suggested that activation of JNK or p38 MAP kinase or both may contribute to the induction of apoptosis in PC-12 cells.

Role of the MEKK1-MKK4-JNK signaling pathway in apoptosis. Extracellular stimuli, such as tumor necrosis factor–α (TNF-α) or interleukin 1 (IL-1), activate JNK through a kinase cascade in which MAP kinase kinase kinase 1 (MEKK1) phosphorylates and activates a dual specificity Thr-Tyr protein kinase (MKK4, also termed SEK1 or JNKK), which then phosphorylates and activates JNK (*26*–*31*). By a similar mechanism, extracellular stimuli such as UV light, osmotic shock, TNF-α, or IL-1 activate MKK3, a protein kinase related to MKK4 that phosphorylates and activates p38 (*13*, *30*).

To test the hypothesis that activation of JNK or p38 MAP kinase or both contributes to the induction of apoptosis, we introduced constitutively activated MEKK1, or a catalytically inactive mutant in which Lys^{432} was changed to Ala [MEKK1(K432A)], into NGF-differentiated PC-12 cells by transient transfection. The cells were cotransfected with an expression vector encoding β-galactosidase (CMV–β-Gal) as a marker for transfection. The presence of MEKK1, but not that of the kinase inactive mutant, increased the number of apoptotic cells 2.7-fold over that in control transfected cultures in the presence of NGF (Fig. 3, A and B), which suggests that the active kinase induces apoptosis. Because transfected MEKK1 activates p38 MAP kinase poorly in vivo (*30*, *31*) and preferentially activates JNK over the ERKs (*27*, *28*), the apoptotic activity of MEKK1 is most likely caused by the activation of the JNK signaling pathway.

To investigate further the role of MEKK1 and JNK in apoptosis, we transfected NGF-differentiated PC-12 cells with dominant-interfering mutants of c-JUN, a downstream target of the MEKK1-JNK signaling pathway. Because JNK phosphorylates and activates c-JUN, dominant-interfering forms of c-JUN might be expected to block apoptosis induced by MEKK1 or NGF withdrawal in PC-12 cells. Two different dominant-interfering c-JUN expression vectors, pCDNA1-FlagΔ169 (*4*) and CMV-TAM67 [Δ3-122] (*32*), were individually introduced into NGF-differentiated PC-12 cells. Both c-JUN mutants act as dominant-interfering mutants because of a deletion in the NH_2-terminal transactivation domain that includes the binding site for JNK. For pCDNA1-FlagΔ169, immunohistochemical analysis confirmed that the transfected c-JUN mutant was efficiently expressed in NGF-differentiated PC-12 cells and correctly localized to the nucleus (*23*). Expression of either dominant-interfering c-JUN construct blocked apoptosis induced by MEKK1 or NGF withdrawal (Fig. 3, C and D), which suggests a central role for the MEKK1-MKK4-JNK signaling pathway in mediating these apoptotic processes. Expression of MKK4(Ala), a dominant-interfering mutant of JNK kinase, also prevented apoptosis induced by NGF withdrawal (*23*), which is consistent with the hypothesis that the MEKK1-MKK4-JNK pathway is required for this form of cell death.

Role of the p38 MAP kinase signaling pathway in apoptosis. To investigate the importance of the p38 MAP kinase pathway for apoptosis, we examined whether ectopic expression of the kinases in this signaling pathway induced cell death. To

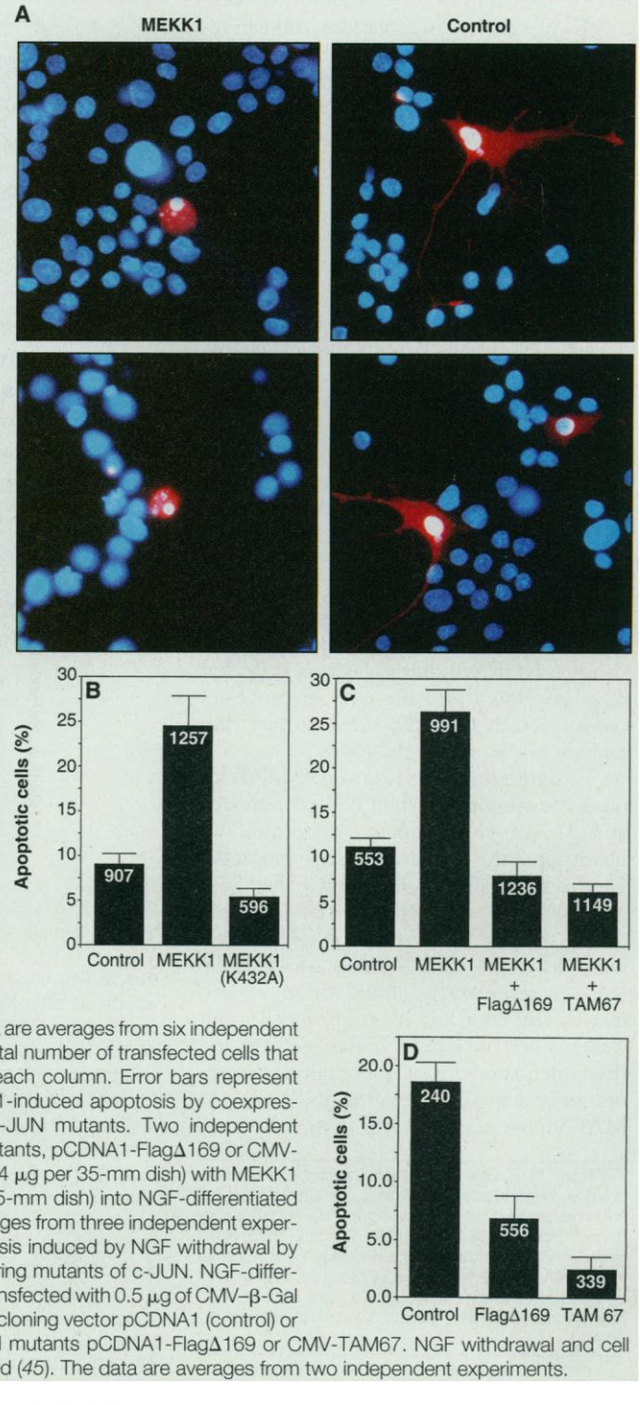

Fig. 3. Induction of apoptosis after activation of the MEKK1-MKK4-JNK signaling pathway. (**A**) Representative immunofluorescence photomicrographs (magnification, ×400) of cells transfected with constitutively activated MEKK1 undergoing apoptosis. NGF-differentiated PC-12 cells were transfected (*45*) with 0.3 μg of pCMV5-MEKK1 or the empty cloning vector pCMV5 (control) (*46*). Transfected cells were detected by cotransfection with 0.3 μg of an expression vector encoding β-galactosidase (CMV–β-Gal) (*45*). The nuclei were stained blue with Hoechst 33258 (*43*). In transfected cells (red), the nuclei appear white because of the colocalization of weak red nuclear staining from anti–β-galactosidase and blue Hoechst nuclear staining. (**B**) Induction of apoptosis in NGF-differentiated PC-12 cells after expression of MEKK1, but not of the catalytically inactive mutant MEKK1(K432A). Cells were transfected with 0.3 μg of the empty cloning vector (pCMV5; control), MEKK1 expression vector (MEKK1), or a vector encoding a catalytically inactive mutant of MEKK1, MEKK1(K432A). Transfected cells were detected by cotransfection with 0.3 μg of CMV–β-Gal. The data are averages from six independent experiments. The respective total number of transfected cells that were counted is given within each column. Error bars represent SEM. (**C**) Prevention of MEKK1-induced apoptosis by coexpression of dominant-interfering c-JUN mutants. Two independent dominant-interfering c-JUN mutants, pCDNA1-FlagΔ169 or CMV-TAM67, were cotransfected (1.4 μg per 35-mm dish) with MEKK1 and CMV–β-Gal (0.3 μg per 35-mm dish) into NGF-differentiated PC-12 cells. The data are averages from three independent experiments. (**D**) Inhibition of apoptosis induced by NGF withdrawal by expression of dominant-interfering mutants of c-JUN. NGF-differentiated PC-12 cells were cotransfected with 0.5 μg of CMV–β-Gal and 1.5 μg of either the empty cloning vector pCDNA1 (control) or the dominant-interfering c-JUN mutants pCDNA1-FlagΔ169 or CMV-TAM67. NGF withdrawal and cell staining were done as described (*45*). The data are averages from two independent experiments.

distinguish the effects of p38 from those of JNK, it was necessary to induce the activity of p38 by cotransfection with its activator MKK3 because other methods of increasing p38 kinase activity, such as osmotic shock and UV treatment, also stimulate JNK activity. MKK3(Glu) is a constitutively activated form of the p38 MAP kinase kinase (*30*) that activates cotransfected p38 MAP kinase in vivo (*33*). MKK3(Ala) is a dominant-interfering mutant of MKK3. NGF-differentiated PC-12 cells were cotransfected with Flag-MKK3(Glu) and Flag-p38 MAP kinase and scored for apoptosis.

In a control experiment, cells were cotransfected with Flag-MKK3(Ala) and pCMV5, the empty cloning vector for p38 MAP kinase. The expression of MKK3-(Glu), MKK3(Ala), and p38 MAP kinase was confirmed by immunostaining with an antibody to the Flag-epitope tag. Apoptotic cells were detected by Hoechst 33258 staining. Coexpression of Flag-MKK3(Glu) with Flag-p38 MAP kinase led to a marked increase in p38 kinase activity in transfected cells (*33*) and induced a 4.5-fold increase in the number of apoptotic cells in the presence of NGF as compared to that in cultures of control transfected cells (Fig. 4A). Intermediate increases in p38 MAP kinase activity resulting from expression of Flag-MKK3(Glu) with pCMV5, or expression of Flag-MKK3(Ala) with Flag-p38 MAP kinase (*33*), also induced apoptosis (twofold over the control), but the effect was not as large as when MKK3(Glu) and p38 were cotransfected. In contrast, the dominant-interfering mutant MKK3(Ala) blocked cell death induced by NGF withdrawal (Fig.

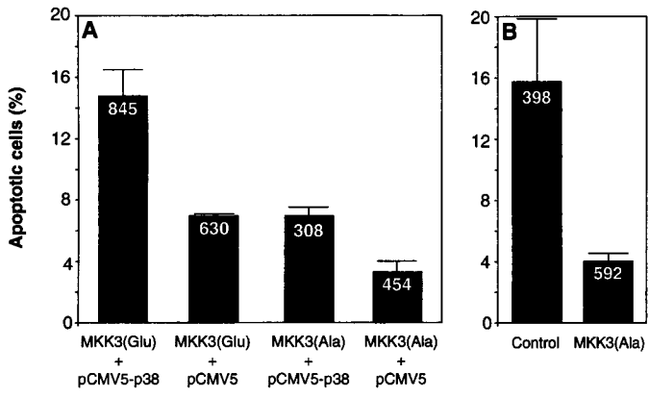

Fig. 4. Induction of apoptosis by activation of the p38 MAP kinase signaling pathway. (**A**) Induction of apoptosis in NGF-differentiated PC-12 cells by expression of constitutively activated MKK3 together with the wild-type p38 MAP kinase. Cells were transfected with various combinations of 1 μg each of pRc/RSV-Flag-MKK3-(Glu), pRc/RSV-Flag-MKK3(Ala), pCMV-Flag-p38 MAP kinase, and pCMV5 (the cloning vector for p38 MAP kinase). Cells were fixed for immunostaining with M2 antibody to Flag 48 hours after transfection (*45*). The data are averages from two independent experiments. The respective total number of transfected cells that were counted is given within each column. Error bars represent SEM. (**B**) Inhibition of apoptosis induced by NGF withdrawal after expression of a dominant-interfering mutant of MKK3. NGF-differentiated PC-12 cells were transfected with 0.5 μg of CMV–β-Gal together with 1.5 μg of either the empty cloning vector pRc/RSV (control) or the dominant-interfering MKK3 mutant pRc/RSV-Flag-MKK3(Ala). NGF withdrawal and cell staining were done as described (*45*). The data are averages from three independent experiments.

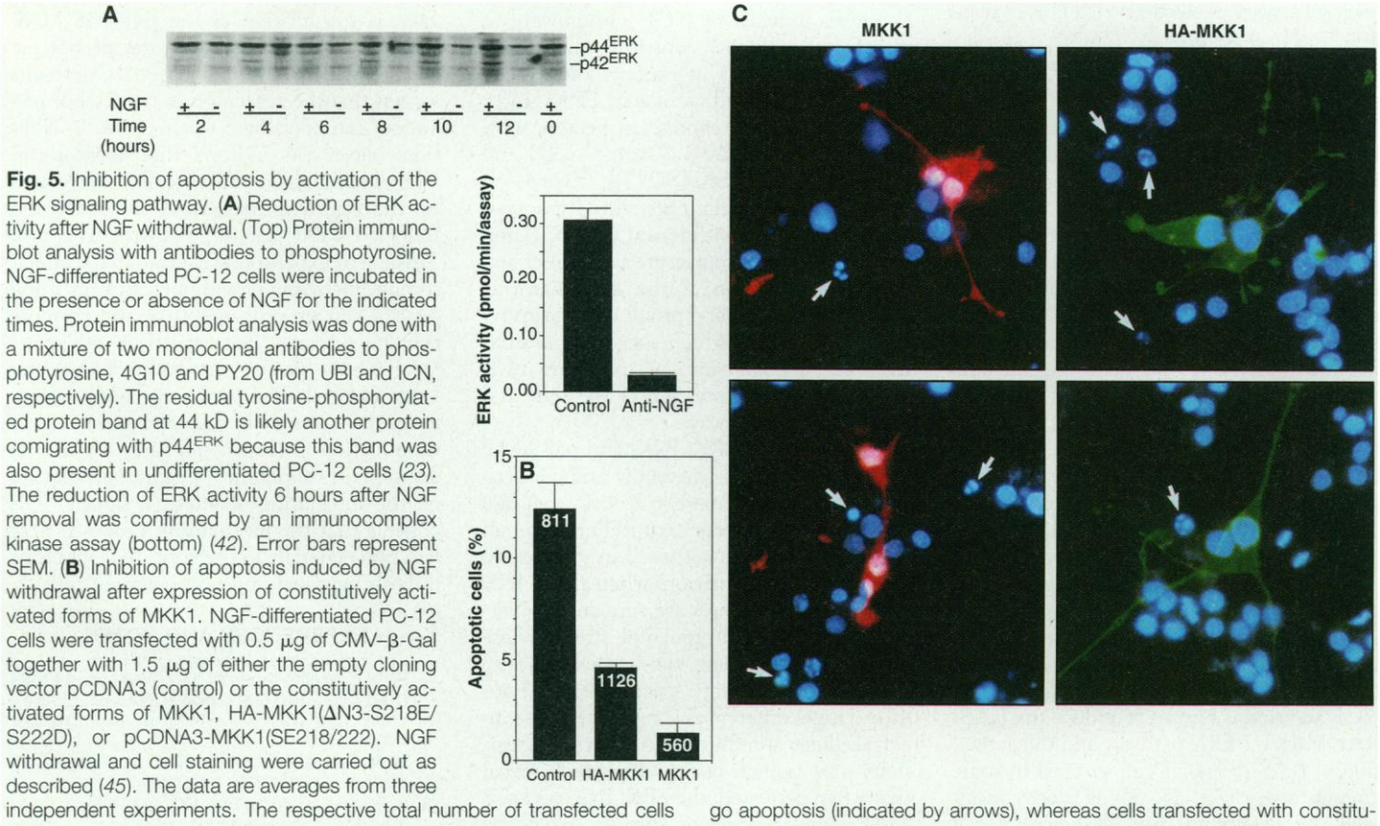

Fig. 5. Inhibition of apoptosis by activation of the ERK signaling pathway. (**A**) Reduction of ERK activity after NGF withdrawal. (Top) Protein immunoblot analysis with antibodies to phosphotyrosine. NGF-differentiated PC-12 cells were incubated in the presence or absence of NGF for the indicated times. Protein immunoblot analysis was done with a mixture of two monoclonal antibodies to phosphotyrosine, 4G10 and PY20 (from UBI and ICN, respectively). The residual tyrosine-phosphorylated protein band at 44 kD is likely another protein comigrating with $p44^{ERK}$ because this band was also present in undifferentiated PC-12 cells (*23*). The reduction of ERK activity 6 hours after NGF removal was confirmed by an immunocomplex kinase assay (bottom) (*42*). Error bars represent SEM. (**B**) Inhibition of apoptosis induced by NGF withdrawal after expression of constitutively activated forms of MKK1. NGF-differentiated PC-12 cells were transfected with 0.5 μg of CMV–β-Gal together with 1.5 μg of either the empty cloning vector pCDNA3 (control) or the constitutively activated forms of MKK1, HA-MKK1(ΔN3-S218E/S222D), or pCDNA3-MKK1(SE218/222). NGF withdrawal and cell staining were carried out as described (*45*). The data are averages from three independent experiments. The respective total number of transfected cells that were counted is given within each column. Error bars represent SEM. (**C**) Representative immunofluorescence photomicrographs (magnification, ×400) of cells transfected with pCDNA3-MKK1(SE218/222) (detected by cotransfected β-galactosidase, red) or HA-MKK1(ΔN3-S218E/S222D) (antibody to HA staining, green). Upon NGF removal, nontransfected cells undergo apoptosis (indicated by arrows), whereas cells transfected with constitutively activated MKK1 are protected. Cells transfected with HA-MKK1(ΔN3-S218E/S222D) were double-immunostained and scored with β-galactosidase antibody and HA antibody (anti-HA). The anti-HA staining directly confirms the expression of HA-MKK1(ΔN3-S218E/S222D). Scoring of cells after antibody to β-galactosidase or anti-HA staining yielded identical results.

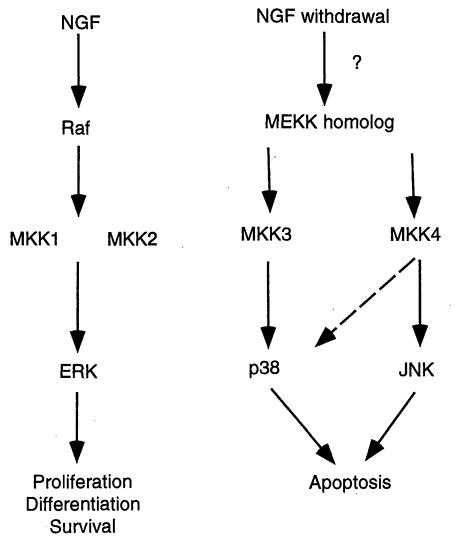

Fig. 6. Model for the roles of the ERK and JNK-p38 kinase pathways in apoptosis. Neuronal survival and induction of cell death may be controlled by the opposing actions of the ERK and JNK-p38 pathways. In the presence of NGF, the survival signaling pathway (through ERK) is activated, whereas the cell death signaling pathways (JNK or p38) are suppressed. When NGF is removed, the ERK signaling pathway is deactivated while the JNK-p38 signaling pathways are activated. Other signaling pathways, including the cAMP and Ca^{2+} signal transduction systems, may also promote neuronal survival by stimulating the ERK or inhibiting the JNK-p38 signaling pathways.

4B). Taken together, these findings support the hypothesis that activation of the MKK3-p38 MAP kinase signaling pathway is required for induction of apoptosis in these cells.

Inhibition of apoptosis by activation of the ERK signaling pathway. The above observations suggest that the sustained overstimulation of the JNK or p38 MAP kinase pathways, or both, is sufficient to induce cell death in PC-12 cells and that these pathways are required for the induction of apoptosis after NGF withdrawal. However, the activation of JNK or p38 MAP kinase does not necessarily lead to apoptosis. For example, although IL-1 is a strong activator of JNK and p38 MAP kinases (*11*, *13*, *16*), IL-1 does not induce apoptosis under the conditions studied (*34*). This information suggests that activation of the JNK or p38 MAP kinase signaling pathways (or the activation of both) may be necessary, but not sufficient, for apoptosis under all conditions. Because NGF addition is known to induce the RAS-RAF-MKK1/2-ERK pathway and the activation of RAS promotes cell survival in some circumstances (*20*, *25*, *35–37*), we considered the possibility that NGF withdrawal may not only trigger JNK and p38 MAP kinase activation but may also concurrently suppress the activity of ERKs. The inactivation of ERKs together with the activation of JNK or p38 or both may be critical for apoptosis. Thus, a greater amount of ERK activity relative to that of JNK or p38 may promote neuronal cell survival, whereas a greater amount of JNK or p38 activity relative to that of ERK may trigger apoptosis.

To test this hypothesis, we examined the effect of NGF withdrawal on $p42^{ERK}$ and $p44^{ERK}$ activities. Extracts prepared from control or NGF-deprived cells were analyzed for the presence of activated ERKs by protein immunoblot analysis with antibodies to phosphotyrosine (Fig. 5A, top). Before NGF withdrawal, the tyrosine-phosphorylated forms of $p42^{ERK}$ and $p44^{ERK}$ were easily detected. NGF withdrawal decreased the amount of tyrosine phosphorylation of both ERKs, which is indicative of decreased ERK activity (*36*). The ERK activity was directly measured by an immunocomplex kinase assay (Fig. 5A, bottom). Removal of NGF for 6 hours resulted in a reduction in ERK activity to one-tenth of previous levels. In addition, agents such as cAMP, bFGF, and insulin, which suppress apoptosis induced by NGF withdrawal (*19*, *25*), stimulate ERK activity (*36*, *38*). These anti-apoptotic agents also blocked the increase in JNK and p38 activity (Fig. 2, D and E).

To obtain evidence that increased signaling through the ERK pathway prevents apoptosis induced by NGF withdrawal, we transfected cells with constitutively activated forms of MKK1, a protein kinase that phosphorylates and activates ERKs (*39*). Two independent expression vectors were used [HA-MKK1(ΔN3-S218E/S222D) and pCDNA3-MKK1(SE218/222)]. Expression of either MKK1 variant prevented apoptosis induced by NGF withdrawal (Fig. 5, B and C). These data demonstrate that direct and selective activation of the ERK pathway prevents apoptosis and promotes the survival of PC-12 cells, which suggests a mechanism for cellular survival involving ERK activation and suppression of the JNK and p38 signaling pathways.

Although the ERK, JNK, and p38 MAP kinases are related structurally and are activated by similar kinase cascades, they are activated by different extracellular stimuli (*40*). The ERKs are activated in response to growth factor stimulation, whereas the JNK and p38 MAP kinases are activated by various forms of environmental stress. These different MAP kinase family members also have distinct substrate specificities (*13*, *36*, *40*). These differences in the response to extracellular stimuli and in substrate specificity may explain our results, which reveal that when activated, the ERK, JNK, and p38 MAP kinases regulate distinct biological functions. Although growth factor–stimulated activation of the ERK signaling pathway is critical for proliferation and differentiation (*35–37*), we found that in NGF-differentiated PC-12 cells, activation of the ERK signaling pathway promotes cell survival.

In contrast, stimulation of the JNK and p38 signaling pathways contributes to cell death. Activation of the ERK signaling pathway by NGF may suppress the activity of JNK or p38 by a direct or indirect mechanism and thereby lead to the promotion of cell survival. By contrast, removal of NGF leads to inactivation of the RAS-RAF-MKK1/2-ERK signaling pathway, which may trigger the activation of JNK and p38 kinase cascades so that cells undergo apoptosis. It is possible that NGF withdrawal directly activates the JNK-p38 MAP kinases independently of its effect on ERK activity. For example, removal of NGF may stimulate signaling by the p75 NGF receptor ($p75^{NGFR}$) and lead to activation of the JNK-p38 MAP kinases, perhaps by a ceramide-dependent mechanism (*41*). NGF withdrawal might also result in the release of cytokines such as IL-1 or TNF-α, which through the activation of their cognate receptors lead to the activation of the JNK-p38 signaling pathways (*11*, *13*, *16*).

Our findings raise questions regarding the activation of the JNK-p38 signaling pathways during neuronal apoptosis. First, what are the physiologically relevant upstream activators of the JNK and p38 signaling pathways? Second, what is the relation of the JNK-p38 MAP kinase signaling pathways to the phenotypic changes associated with apoptosis? Our results suggest that the activation of the JNK or p38 kinases can contribute to apoptosis. In addition, our results indicate that apoptosis in NGF-differentiated PC-12 cells is regulated by the opposing actions of the ERK and JNK-p38 MAP kinase pathways (Fig. 6). This might explain how various extracellular stimuli, including growth factors, Ca^{2+}, and cAMP, can prevent apoptosis and promote cellular survival. Because there are many mechanisms for the regulation of the relevant MAP kinase pathways and various forms of cross talk between these signal transduction pathways probably exist, the decision for cellular life or death may depend on the integration of multiple signals. It is possible that the mechanisms proposed here function generally in the control of apoptosis in both neuronal and non-neuronal cells.

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42. PC-12 cells were differentiated for 9 to 10 days in Dulbecco's modified Eagle's medium supplemented with heat-inactivated horse serum (1%) and NGF (50 ng/ml). NGF withdrawal was accomplished by washing the cells once with NGF-free medium followed by incubation in NGF-free medium containing rabbit neutralizing antibody to 2.5*S* NGF (Sigma) at a 1:400 dilution. Control cells were washed once in NGF-free medium and then incubated in NGF-containing medium. Extracts were prepared from control or NGF-deprived cells (*30*). JNK, p38, or ERK was immunoprecipitated with polyclonal antibodies raised against recombinant human glutathione-S-transferase (GST)–JNK1, GST-p38, or $p42^{ERK2}$, respectively (*13*) [L. B. Rosen, D. D. Ginty, M. J. Weber, M. E. Greenberg, *Neuron* **12**, 1207 (1994)]. Equal amounts of protein were used for immunoprecipitation within each experiment. The JNK, p38, or ERK activity was measured in an immunocomplex kinase assay with recombinant GST–c-JUN (amino acid residues 1 to 79), GST-ATF2 (1 to 109), or rabbit myelin basic protein as substrates, respectively (*6, 13, 30*).
43. The number of apoptotic cells was assessed by nuclei staining (blue) with Hoechst 33258 (2.5 μg/ml) after fixation of cells in paraformaldehyde (4%). Nuclei that were fragmented or condensed were scored as apoptotic.
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45. NGF-differentiated PC-12 cells were plated onto poly-D-lysine and laminin-coated glass cover slips in 35-mm culture dishes. Transient transfection was done with LipofectAMINE (Life Technologies) according to the manufacturer's instructions with the use of 2 μg of total DNA per 35-mm dish. The transfection efficiency was approximately 1 to 5%. With the exception of those shown in Fig. 4A, the cells were cotransfected with 0.3 to 0.5 μg of an expression vector encoding β-galactosidase (CMV–β-Gal) as a marker for transfected cells. NGF withdrawal was done 48 hours after transfection (*42*), and cells were fixed for immunostaining 20 hours after NGF withdrawal. Transfected cells were detected by immunostaining with a polyclonal antibody to β-galactosidase (5 prime→3 prime, Inc., Boulder, CO; 1:500 dilution) and Texas Red–conjugated goat antibody to rabbit immunoglobulin G (IgG). Cells transfected with β-galactosidase stained red. Cells transfected with vectors containing the Flag-epitope tag or the hemagglutinin (HA) epitope tag were immunostained with M2 monoclonal antibody to Flag (IBI-Kodak, 15 μg/ml) or monoclonal antibody to HA (Boehringer Mannheim, 0.8 μg/ml), respectively. These transfected cells were visualized by fluorescein-conjugated goat antibody to mouse IgG and stained green. To visualize the nuclei of transfected cells, we included the dye Hoechst 33258 in the wash after the secondary antibody incubation (*43*). Transfected cells were scored blindly for apoptosis.
46. The following expression vectors have been described: GST–c-JUN (*6*), GST-ATF2 (*11*), pCMV5-MEKK1 and the dominant-interfering pCDNA3-Flag-MKK4(Ala) [A. J. Whitmarsh, P. Shore, A. D. Sharrocks, R. J. Davis, *Science* **269**, 403 (1995)], pCMV-Flag-p38 MAP kinase (*13, 30*), the HA epitope–tagged constitutively active HA-MKK1(ΔN3-S218E/S222D) (*37*), and the c-JUN dominant-interfering mutants pCDNA1-FlagΔ169 (*4*) and CMV-TAM67 (Δ3-122) (*32*). The constitutively active mutant pCDNA3-MKK1(SE218/222) is a double point mutation in which Ser^{218} and Ser^{222} were replaced with Glu residues. The kinase-inactive MEKK1 mutant [pCMV5 MEKK1(K432A)] was made by replacing Lys^{432} with Ala. The Flag-MKK3 (*30*) was cloned into pRc/RSV (Invitrogen) between the Hind III and Spe I sites. The constitutively active pRc/RSV-Flag-MKK3(Glu) and the dominant-interfering pRc/RSV-Flag-MKK3(Ala) mutants are double point mutations in which Ser^{189} and Thr^{193} were replaced by Glu or Ala residues. The mutations were made by overlapping polymerase chain reaction [S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989)] and confirmed by DNA sequencing with an Applied Biosystems model 373A machine.
47. The plasmid pCDNA3-MKK1(SE218/222) was provided by E. G. Krebs. We thank I.-H. Wu and T. Barrett for their technical assistance; B. Dérijard for providing MKK3 and MKK4 cDNA clones; and N. G. Ahn, M. J. Birrer, J. Ham, E. G. Krebs, L. L. Rubin, and R. J. Ulevitch for providing various plasmids. R.J.D. is an investigator of the Howard Hughes Medical Institute. Supported by a Medical Foundation postdoctoral fellowship (Z.X.), National Institutes of Health grants CA43855 (M.E.G.) and CA65861 (R.J.D.), and an American Cancer Society Faculty Research Award (FRA-379) (M.E.G.).

24 August 1995; accepted 3 November 1995