The Activation of p38 and Apoptosis by the Inhibition of Erk Is Antagonized by the Phosphoinositide 3-Kinase/Akt Pathway*

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Considerable attention has recently been focused on the role played by different kinase cascades in the control of apoptosis. The triggering of stress-activated kinases concomitant with the inhibition of the extracellular signal-regulated kinase (ERK) pathway has been observed in a number of cell systems undergoing programmed cell death. In addition, the activation of the phosphoinositide 3-kinase (PI 3-kinase)-Akt signaling cascade has been shown to protect from apoptosis. Here we have explored the potential role played by the inhibition of ERK in the activation of the stress kinases as well as the possible cross-talk with the PI 3-kinase pathway in HeLa cells. We show that the simple inhibition of ERK basal activity is sufficient to trigger apoptosis and p38 activation with no changes in Jun N-terminal kinase/stress-activated protein kinase. This is a process dependent on the caspases and is completely abrogated by serum. The incubation with wortmannin or the transfection of dominant negative mutants of p85 or Akt block the inhibitory function of serum, suggesting the involvement of the PI 3-kinase-Akt system. Consistent with this, expression of active mutants of PI 3-kinase and Akt inhibits p38 activation and apoptosis. We also show here that the inhibition of ERK triggers the caspase system, which is abolished by serum in a wortmannin-dependent manner. Collectively, these results demonstrate a link between ERK and the p38 apoptotic pathway that is modulated by the survival PI 3-kinase-Akt module, acting upstream the caspase system.

Signal transduction pathways controlled by kinase modules regulate critical cellular functions such as cell growth, differentiation and apoptosis. Three major kinase cascades have recently been identified that culminate in the activation of three different sets of mitogen-activated protein kinases: the extracellular signal-related kinase (ERK),1 JNK/SAPK, and p38 (1, 2). The three have been implicated in the control of apoptosis. ERK is activated by mitogens and survival factors, whereas JNK/SAPK and p38 are stimulated by stress signals (1, 2). A number of findings indicate that the activation of JNK/SAPK and p38 may play decisive roles in the control of cell death. Thus, the JNK/SAPK pathway is critical during ceramide and stress-induced apoptosis (3–5) as well as in the Daxx-mediated Fas cascade (6). Also in favor of a role of JNK/SAPK and p38 in apoptosis are the results reported by Xia et al. (7) who demonstrated that the transfection of a constitutively active mutant of MKK3/6 (the physiological activator of p38) along with p38 is sufficient to induce apoptosis in PC-12 cells. In addition, transfection of dominant negative mutants of MKK3/6 in PC-12 cells or of p38 in NIH-3T3 fibroblasts dramatically inhibits apoptosis by nerve growth factor withdrawal (7) and UV irradiation (8), respectively. Consistent with this notion is the lack of excitotoxicity-induced apoptosis in the hippocampus of JNK3 knock-out mice (9). However, the simple transient activation of the stress kinase cascades may not always be sufficient to induce apoptosis, because, for example, tumor necrosis factor α promotes a significant induction of JNK/SAPK but does not invariably induce apoptosis (10). In this regard, the concomitant inactivation of survival signals may be a prerequisite for JNK/SAPK and p38 to induce cell death (11). Interestingly, deprivation of neurotrophic factors in PC-12 or UV-irradiation of NIH-3T3 cells not only activates the stress kinase cascades but also leads to a dramatic inhibition of the ERK pathway (7, 8). This is particularly relevant because ERK has been shown to be required for survival signaling in response to fibroblast growth factor (12) and insulin-like growth factor 1 (13). Furthermore, the overexpression of ERK in NIH-3T3 cells impairs a large part of the UV-induced apoptotic response (8). Thus, it seems that the ability of a cell to die or survive may be dictated by a critical balance between the ERK and the JNK/SAPK and p38 pathways (11).

Recently, considerable attention has been focused on the role of PI 3-kinase as a source of survival signals. Yao and Cooper (14) have shown that inhibition of PI 3-kinase in PC-12 cells is sufficient to induce apoptosis. PI 3-kinase also plays a critical role in anoikis and apoptosis induced by myc and UV irradiation (15–17). Interestingly, the expression of permanently active mutants of PI 3-kinase or Akt (a target of PI 3-kinase) is sufficient to inhibit apoptosis (15, 17–19). Collectively, these results suggest that the PI 3-kinase-Akt pathway is required and sufficient for cell survival. However, this does not seem to always be the case. Thus, for example, inhibition of PI 3-kinase does not induce apoptosis in cerebellar granule neurons maintained in serum and K+-rich medium (18). The inhibition of PI

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‡ The abbreviations used are: ERK, extracellular signal regulated kinase; CPP32, caspase-3 or cysteine protease 32; FADD, Fas-associated death domain protein; JNK, Jun N-terminal kinase; MEK, mitogen-activated protein kinase kinase; MKK3/6, p38 mitogen-activated protein kinase kinase; Par-4, prostate apoptosis response 4; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; SAPK, stress-activated protein kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling.
3-kinase only has an impact in this system when cells are incubated in serum-free low K⁺ medium in the presence of insulin-like growth factor 1, which partially supports neuron survival (18). In addition, and consistent with these observations, the inhibition of PI 3-kinase is not sufficient to induce apoptosis in superior cervical neurons (20), although the overexpression of active mutants of PI 3-kinase or Akt can support survival of these cells in the absence of nerve growth factor (20).

Together these results suggest that multiple pathways are important for cell survival and that the comprehension of the cross-talk existing between these cascades will greatly help in the understanding of this important pathophysiological process. In the present study, we have addressed the role that the inhibition of ERK plays in the activation of the JNK/SAPK and p38 pathways as well as the possible cross-talk between this and the PI 3-kinase-Akt signaling cascade.

**MATERIALS AND METHODS**

**Plasmids and Expression of Recombinant Fusion Protein.—** The pCDNA3-myc-pCMV-R01 have previously been described (Diaz-Meco et al. (21)). The p38 expression vectors (pCMV-Flag-p38 and the dominant negative pCMV-Flag-p38GFP) were provided by Dr. Roger Davis. pRK5-C2Dp110mmy, containing a permanently active mutant of PI 3-kinase was a gift from Dr. Cantrell (ICRF, London). The pCDNA3-myc-Δp85 is a mouse-targeted version of plasmid pRSΔp85, generously provided by Dr. Kasuga. The FADD dominant negative (pCDNA3AU1FADD) was provided by Dr. Dixit (Genentech, San Francisco). The expression vectors for the permanently active and the kinase defective mutants of Akt were a gift from Dr. Julian Doward (ICRF, London). The pGEX-2TK-E1K construct was transformed into Escherichia coli JM101, and the expression and purification of the glutathione S-transferase fusion protein on glutathione-Sepharose was carried out according to the manufacturer’s procedures.

**Cell Culture and Transfections—** HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin G (100 μg/ml), and streptomycin (100 μg/ml) (Life Technologies, Inc.) in a CO₂ incubator (5% CO₂) at 37 °C. Subconfluent cells were transfected with different plasmid DNAs by the calcium phosphate method (CLONTECH, Inc.). Some experiments were performed in the presence of the MEK inhibitor PD98059 (Calbiochem), the interleukin 1β converting enzyme (ICE) inhibitor z-VAD-fmk (Bachem), actinomycin D (Sigma), or wortmannin (Sigma).

**Immunoblot Analysis—** HeLa cells either untreated or incubated with different stimuli were extracted with lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, protease inhibitors), and cellular extracts (20 μg) were resolved in SDS-polyacrylamide (10%) gels. Afterward, they were electrophoretically transferred into a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) and incubated with the specific antibodies: ERK (Pharmingen); p38, phospho-specific ERK, phospho-specific p38, and JNK/SAPK (New England Biolabs); anti-Fas ligand (Santa Cruz Biotechnology); anti-FADD (Santa Cruz Biotechnology); anti-Flag (Eastman Kodak Co.); anti-CPP32 (Tandus Laboratories); or anti-myc. The bands were visualized with the ECL system (Amersham).

**p38 Activity—** For the p38 assay, HeLa cells transfected with Flag-p38 were extracted with lysis buffer (20 mM Tris (pH 7.5), 10% glycerol, 1% Triton X-100, 137 mM NaCl, 2 mM EDTA, protease inhibitors) and immunoprecipitated with anti-Flag antibody (2 μg/ml of protein extract). The activity was determined by phosphorylation of 5 μg of glutathione S-transferase-Elk-1 in 25 mM Hepes (pH 7.5), 25 mM β-glycerophosphate, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate. The kinase reaction was terminated after 30 min at 30 °C by the addition of Laemmli sample buffer. The phosphorylation of the substrate protein was examined after SDS-polyacrylamide gel electrophoresis followed by exposure and quantitation in an InstantImager (Packard).

**Apoptosis Assay—** Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis was performed by using an in situ cell death detection kit (Boehringer Mannheim). The β-galactosidase co-transfection assays for determination of cell death was performed as described previously (21).

**RESULTS**

**The Inhibition of ERK Activates p38 but Not JNK/SAPK—** We initially determined whether the simple inhibition of ERK leads to the activation of p38 and JNK/SAPK. HeLa cells cultured either in the presence or in the absence of serum were incubated with the MEK inhibitor PD98059, and the activity of ERK, JNK/SAPK, and p38 was determined by immunoblot analysis with antibodies that recognized the activated phosphorylated forms of the three kinases in cell extracts obtained at different times. The levels of phospho-ERK were very similar in cells that were incubated either with or without serum (Fig. 1), indicating that the basal levels of ERK are not dramatically affected by the presence of serum when cells are cultured asynchronized. The addition of the MEK inhibitor rapidly and profoundly reduced the phospho-ERK signal with no effect on ERK protein levels (Fig. 1). This is consistent with the reported ability of this drug to inhibit the MEK-ERK pathway. This inhibition was detected in cells maintained both in serum-containing and in serum-free media (Fig. 1). Interestingly, subsequent to the inhibition of ERK, there is a dramatic activation of p38 in cell cultures kept in serum-free media, as determined by immunoblot analysis with an anti-phospho-p38 antibody (Fig. 1) or in anti-p38 immunoprecipitates (not shown). This effect takes place with no changes in p38 protein levels (Fig. 1). This increase in p38 activity is detectable at 2 h after ERK inhibition and remained elevated at least until 12 h, the maximum time point measured (Fig. 1). These changes in ERK and p38 activities took place with little or no effect on the activity or levels of JNK/SAPK (not shown). Therefore, the inhibition of ERK leads to the activation of p38 in cell cultures maintained in serum-free media. However, the presence of serum, although unable to affect the inhibition of ERK by the MEK inhibitor, completely abrogated the stimulation of p38 (Fig. 1). This indicates that signals activated by the presence of serum blocked, in an ERK-independent manner, the ability of p38 to be activated by the inhibition of the ERK cascade.

**The Inhibition of the MEK-ERK Pathway Induces Apoptosis by a Caspase-Dependent Mechanism—** Consistent with the notion that ERK may provide survival signals are the results of Fig. 2A, demonstrating that the incubation of HeLa cells with the MEK inhibitor PD98059 induces apoptosis in cell cultures maintained in the absence of serum. The presence of serum or the incubation with the caspase inhibitor z-VAD, completely inhibited that effect. This suggests that the mechanism whereby the inhibition of the MEK-ERK cascade induces apoptosis involves the caspase system and that the presence of serum not only inhibits the activation of p38 but also the induction of apoptosis. Immunoblot analysis of parallel ex-
were treated for 12 h with 50 HeLa cells incubated either in the absence or in the presence of serum Fas to induce apoptosis but also that of tumor necrosis factor contains just the death domain, not only inhibits the ability of FLICE (25, 26). A dominant negative mutant of FADD, which ent experiments with incubations in duplicate.

![Image](68x457 to 278x729)

**FIG. 2.** The inhibition of the ERK pathway induces apoptosis. HeLa cells incubated either in the absence or in the presence of serum were treated for 12 h with 50 μM PD98059 with or without 100 μM caspase inhibitor z-VAD. The induction of apoptosis was determined by TUNEL analysis (A). Results are the means ± S.D. of three independent experiments with incubations in duplicate. B, parallel extracts were immunoblotted with antibodies to phospho-ERK, ERK, or CPP32. Essentially identical results were obtained in another two experiments.

tracts demonstrate that the MEK inhibitor actually blocked the ERK pathway and activated CPP32 in a serum-inhibitable and z-VAD-dependent manner (Fig. 2B). The loss of CPP32 represents cleavage and activation of the caspase.

**Caspase Inhibition Blocks the Activation of p38 in PD89059-treated Cells**—In the next series of experiments we determined whether the stimulation of p38 is mediated by the caspase system. Thus, HeLa cells maintained in serum-free medium for 24 h were incubated or not with the MEK inhibitor either in the absence or in the presence of z-VAD. Results of Fig. 3A demonstrate that the activation of p38 is completely blocked by the inhibition of caspases.

The Activation of p38 Is Independent of FADD—Recent evidence indicates that the stimulation of apoptosis by UV irradiation or myc expression involves the activation of the Fas signaling cascade (22–24). To determine whether the activation of p38 by the inhibition of Erk may depend on the expression of Fas ligand, HeLa cells were incubated with PD98059 either in the absence or in the presence of actinomycin D. Results of Fig. 3B demonstrate that inhibition of transcription has no effect on p38 activation. In addition, reverse transcription-polymerase chain reaction and immunoblot analysis demonstrate that Fas ligand levels were unaffected upon the stimulation of HeLa cells with PD98059 (not shown). It is possible that the ability of PD98059 to activate p38 is due to the stimulation of the Fas receptor by the MEK-ERK pathway through FLICE-like interleukin-1β converting enzyme (FLICE) (25, 26). A dominant negative mutant of FADD, which contains just the death domain, not only inhibits the ability of Fas to induce apoptosis but also that of tumor necrosis factor α (25, 27). Therefore, we next determined whether the activation of p38 by the inhibition of the MEK-ERK pathway is mediated by the FADD signaling cascade. Thus, Flag-p38 was transfected along with an expression vector for a dominant negative FADD mutant (10 μg). Sixteen h post-transfection, cells were shifted to serum-free medium, after which they were incubated with 50 μM PD98059 for 6 h. Afterward, Flag-tagged p38 was immunoprecipitated, and its activity was determined by using recombinant GST-Elk-1 as the substrate. Essentially identical results were obtained in another three experiments.

The PI 3-Kinase-Akt Pathway Regulates the Activation of p38—We next explored whether the blockade by serum of p38 activation depends on PI 3-kinase. HeLa cells were maintained either in the absence or in the presence of serum and were incubated with the MEK inhibitor either with or without wortmannin (an inhibitor of PI 3-kinase). Results of Fig. 5A show that serum can block the activation of p38 by PD98059 is completely abrogated by wortmannin, which has little or no effect on cells incubated in the absence of serum. This suggests that PI 3-kinase is required for the effects of serum on p38 stimulation. The following experiments confirm these findings. Transfection of an active PI 3-kinase mutant inhibited the activation by the MEK inhibitor of a co-transfected Flag-tagged p38 construct (Fig. 5B). Transfection of an active mutant of Akt also inhibits the activation of p38, suggesting that Akt activation accounts for the inhibitory effects of serum and PI 3-kinase on p38 activation. Consistently, transfection of the kinase-inactive mutant of Akt abrogates the inhibitory effect of serum on the activation of p38 by the MEK.
inhibitor. Likewise, expression of a dominant negative mutant of PI 3-kinase produces the same effect (Fig. 5B).

The PI 3-Kinase Pathway Interferes the p38 Cascade at the Level of Caspase Activation—To position the PI 3-kinase negative effects on the PD98059-triggered p38 pathway, we initially determined whether the inhibition of the ERK cascade actually activated the caspase pathway. To this aim, the cleavage of CPP32 was determined in HeLa cell cultures triggered with PD98059 either in the absence or in the presence of serum. This treatment promoted the cleavage of CPP32 in a time-dependent manner that was completely inhibited by the presence of serum (Fig. 6A). The CPP32 cleavage was reproducibly detectable at 10–30 min after ERK inhibition and was maximal at 2 h (Fig. 6A). Of note, wortmannin abrogated the inhibitory effects of serum (Fig. 6B), indicating that PI 3-kinase acts upstream in a pathway leading to activation of the caspases, which are required for p38 stimulation by PD98059.

**The PI 3-Kinase Pathway Inhibits PD98059-induced Apoptosis**

**TABLE I**

| Number of blue cells/well | Control | P38AGF | PI 3-kinase | Akt |
|---------------------------|---------|--------|-------------|-----|
| Control                   | 2200 ± 180 | 2100 ± 200 | 2600 ± 400 | 2250 ± 220 |
| PD98059                   | 790 ± 85  | 1880 ± 150 | 2500 ± 320 | 2200 ± 170 |

**DISCUSSION**

The role played by different signal transduction molecules in the control of cell survival is the subject of intense discussion. The stress-activated kinase cascades, which include the JNK/SAPK and the p38 pathways, are activated in response to different apoptotic stimuli and seem to play a decisive role in...
This process (3, 4, 6–9). Thus, the simple activation of p38 by its physiological stimulant, MKK3/6, is sufficient to induce apoptosis in transfected cells (7). Conversely, expression of MKK3/6 or p38 dominant negative mutants blocked apoptosis induced by nerve growth factor withdrawal (7) and UV irradiation (8). On the other hand, there are two major kinase pathways involved in the induction of cell survival: the ERK and the PI 3-kinase cascades. Inhibition of ERK activity correlates with the activation of JNK/SAPK and p38 as well as with the induction of apoptosis in nerve growth factor-deprived PC-12 cells and UV-irradiated mouse fibroblasts (7, 8). Also, the inhibition of ERK below a basal threshold level triggers apoptosis, suggesting that, in addition to its well established role in cell cycle progression, ERK also controls survival. The balance between the activity of the stress kinases to that of ERK has been proposed to determine the cell fate (11). This is consistent with results demonstrating the antagonistic effects of ceramide versus sphingosine 1-phosphate. The former potently activates the stress kinases, whereas the latter inhibits them and activates ERK (28). This, again, suggests a link between both types of mitogen-activated protein kinase cascades. Our own data shows that UV irradiation or the inhibition of the atypical PKCs leads to an increase in p38 activity and a decrease in ERK activity (8).

We show here that the simple inhibition of the ERK basal activity is sufficient to trigger p38 activation in a caspase-dependent manner. This effect is blocked by the presence of serum through the PI 3-kinase-Akt signaling pathway that acts upstream of the caspase system. Recent evidence suggests that the mechanism whereby certain stimuli induce apoptosis may involve the activation of the Fas pathway (22–24). In this case, however, we demonstrate that the blockade of this pathway has no effect on the ability of the inhibition of ERK to activate p38. Because the strategy employed here (the use of a FADD dominant negative mutant) not only blocks Fas but also tumor necrosis factor alpha signaling to cell death (25, 27), we conclude that neither of these systems is involved in the activation of p38 by PD98059.

We have recently shown that the inhibition of the atypical isoforms of PKC (PKC η and δ) is required for the induction of apoptosis and the activation of p38 in mouse fibroblasts (8, 21). This involves the interaction of PKC and δ/PKC with the protein Par-4, whose expression is sufficient to induce apoptosis in fibroblasts incubated under low serum conditions (8, 21, 29). Interestingly, the ability of Par-4 to induce apoptosis is a caspase-dependent process that correlates with a decrease in ERK activity concomitant with an increase in p38 activity (8). We show here is that there is a link between the inhibition of ERK, the activation of the caspase system, and the stimulation of p38. These results are consistent with the model depicted in Fig. 7, according to which an apoptotic stress signal such as UV irradiation produces at least two actions, one that by inducing Par-4, inactivates the atypical PKCs, which leads to the inhibition of ERK, triggering the caspase system that stimulates p38. Another action blocks PI 3-kinase, which allows the caspase system to be operative. The fact that the atypical PKCs are activated by PI 3-kinase products (30–33) indicates that these PKCs are subjected to a double control mechanism in stress-activated cells: the interaction with Par-4 and the inactivation of PI 3-kinase. From all these results, at least three important issues arise for future studies. One is the mechanism whereby Akt inhibits p38. Because the caspase activation is required for p38 stimulation by PD98059, we are tempted to speculate that Akt may be affecting either directly or indirectly the caspase system. The recent finding that Akt phosphorylates and inactivates Bcl-XL/Bcl-2-associated death promoter (34, 35) coupled with the existence of a link between Bcl-2 and the caspases (36, 37) may explain why PI 3-kinase has to be inhibited for p38 to be activated. The second issue, concerning the mechanism whereby PI 3-kinase may be inhibited by UV irradiation, is less clear and will need further work to be solved. However, the recent discovery that PI 3-kinase is blocked by c-Abl in the genotoxic stress response (38) opens new possibilities for investigation. Finally, the mechanism whereby p38 is activated in response to ERK inhibition remains to be determined. We show here that the caspase system is essential, and preliminary results demonstrate that it is mediated by MKK3/6. Because the upstream activator of MKK3/6 has not been established yet, it is very difficult to address the link existing between this kinase and the caspases. However, other evidences support a connection between the caspases and the stress-activated kinases. Thus, Frisch and co-workers have recently demonstrated that MEK kinase 1 activation during anoikis requires the cleavage of the kinase by caspases, which seems critical for cell death (39).
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