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MEK/ERK Signaling Pathway Regulates the Expression of Bcl-2, Bcl-X_L, and Mcl-1 and Promotes Survival of Human Pancreatic Cancer Cells

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Abstract *Background and aims:* Growth factors are well known for their participation in the regulation of cell proliferation and survival. However, the intracellular signaling pathways by which growth factors promote survival are still poorly understood. In the present study, using the MIA PaCa-2 cell line, a well-established model of pancreatic cancer cells, we analyzed the roles of ERK1/2 activities in the regulation of cell survival and investigated some of the mechanisms involved. *Methods:* The ability of the MEK inhibitor PD98059 to modulate survival of the MIA PaCa-2 cells was evaluated, and the responses were correlated with expression of Bcl-2 homologs and caspases 1, 3, 6, 8, and 9 activities. Results. Herein, we showed that inhibition of ERK1/2 activities caused (1) a G1 arrest; (2) a down-regulation of the expression levels of the anti-apoptotic homologs Bcl-2, Mcl-1, and Bcl-X_L without affecting the pro-apoptotic levels of Bax and Bak; (3) a promotion of caspases 3, 6, 8, and 9 activities; (4) a stimulation of PARP cleavage; and (5) a programmed cell death by apoptosis. *Conclusion:* Our data suggest that activation of the ERK pathway functions to protect pancreatic tumor cells from apoptosis as well as to regulate their progression in the cell cycle. J. Cell. Biochem. 79:355–369, 2000. © 2000 Wiley-Liss, Inc.

Apoptosis, a form of programmed cell death is an intrinsic biological process that plays an essential role in organogenesis, tissue development and tissue repair [Green, 1998]. The typical death throes of a cell undergoing apoptosis include DNA fragmentation, nuclear condensation and cell shrinkage [Green, 1998]. The Bcl-2 homolog family represents a critical decisional checkpoint within most apoptotic pathways, acting upstream of such irreversible damage to cellular constituants [Adams and Cory, 1998]. At least 15 family members have been identified so far in mammalian cells, sharing in three or four conserved domains (BH1-4) and functioning either as pro-apoptotic (e.g., Bax, Bak, Bad) or anti-apoptotic (e.g., Bcl-2, Mcl-1, Bcl-X_L) regulators [Adams and Cory, 1998; Farrow and Brown, 1996]. These proteins form heterodimers of anti- and pro-apoptotic members, thereby titrating one another's function [Adams and Cory, 1998]. The ratio of anti-apoptotic (Bcl-2, Mcl-1, Bcl-X_L) and pro-apoptotic (Bax, Bad, Bak) proteins determines in part how cells will respond to apoptotic or survival signals [Farrow and Brown, 1996].

Furthermore, post-translational modifications of Bcl-2 homologs, as well as interactions with other molecules (such as the anti-apoptotic protein Bag-1), add greater complexity to the regulation of Bcl-2 homolog functions and to the apoptotic pathway overall [Farrow and Brown, 1996; Gajewski and Thompson,

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1996; Wang et al., 1996b; Anderson, 1997; Adams and Cory, 1998].

While the exact mechanisms by which Bcl-2 homologs perform their pro- or anti-apoptotic functions remain unclear, it is becoming increasingly evident that part of their role is to act at the activation level of the apoptotic machinery [Adams and Cory, 1998; Farrow and Brown, 1996]. The major signaling pathway involved in apoptotic cell death includes the intracellular caspases, a family of structurally related cysteine proteases [Casciola-Rosen et al., 1996; Salvesen and Dixit, 1997]. So far, 13 members have been identified that are grouped into three subfamilies (e.g., CASP-1, CASP-3, and CASP-8), according to specific amino acid motifs preferentially recognized and cleaved. Caspases are expressed as precursors with little, if any, catalytic activity. Their proteolytic activation occurs in a cascade-like fashion whereby these proteases serve as one another's substrate, thereby allowing efficient propagation and enhancement of pro-apoptotic stimuli. In a final step, key substrates are proteolytically cleaved. To date, at least 40 different caspases'substrates have been identified including the DNA repair enzyme poly-(ADP ribosyl) polymerase (PARP) [Casciola-Rosen et al., 1996], actin [Salvesen and Dixit, 1997], lamin A [Salvesen and Dixit, 1997], the caspase-activated DNAse (CAD) inhibitory protein (I^{CAD}) [Sakahira et al., 1998], as well as the anti-apoptotic Bcl-2 and Bcl-X_L homologs [Thornberry and Lazebnik, 1998]. Although it is very likely that their selective cutting and that of other key proteins is detrimental to the cell, it is still unclear which of these substrates must be preferentially cleaved to commit the cell to die by apoptosis.

Over the past few years, major advances have been made in our understanding of the molecular mechanisms that trigger apoptosis. Growth factors are well known for their participation in the regulation of cellular processes such as proliferation and survival [Raff, 1997]. The intracellular signaling pathways by which growth factors promote survival are still poorly understood. Members of the MAP kinase family such as ERK1/2 (extracellular-regulated kinase) and c-jun N-terminal kinases (JNKs) appear to play important roles in the regulation of cell survival [Anderson, 1997; Parrizas et al., 1997; Xia et al., 1995]. For example, ERK1/2 activation promotes the anti-apoptotic functions of Bcl-2 and cell survival in neuronal PC12 cells, whereas activation of JNKs leads to their death by apoptosis [Anderson, 1997; Xia et al., 1995]. Similarly, stimulation of ERK1/2 by FGF-2 protects L929 cells from TNF-α-induced apoptosis [Gardner and Johnson, 1996]. More recently, studies in HeLa cells showed that inhibition of basal ERK1/2 activity was sufficient to trigger apoptosis [Berra et al., 1998]. However, the molecular mechanisms by which ERK1/2 regulate cell survival remains poorly understood, although the well-characterized functions in gene expression of these MAP kinases are likely to be involved [Marshall, 1995].

Recent molecular studies have identified a variety of growth factors and growth factor receptors that are overexpressed in human pancreatic cancer in vivo and in vitro [Friess et al., 1996]. The regulation of apoptotic cell death is often altered in transformed cells. The most common mutations in pancreatic cancer cells are not surprisingly found among genes implicated in the regulation of apoptosis, including the p53 and K-*ras* genes [Friess et al., 1996]. It is believed that the combination of overexpression of growth-promoting factors and the alteration or loss of inhibiting pathways may give pancreatic cancer cells a major growth advantage, which clinically results in rapid tumor progression, formation of metastases, and subsequently the early death of the patient. In the present study, we analyzed for the first time the potential role of MEK/ERK signaling pathway in the regulation of pancreatic cancer cell survival, and elucidated some of the mechanisms implicated. The MIA PaCa-2 cells used in this study exhibit activating mutations in the small GTPase Ki-Ras and overexpress the EGF receptor and its ligands [Friess et al., 1996]. Consequently, Ras-dependent signaling cascades may already be activated in these cells. Herein, we showed that inhibition of basal ERK1/2 activities causes: (1) a G1 arrest; (2) a down-regulation of the expression levels of anti-apoptotic homologs (namely, Bcl-2, Mcl-1, Bcl-X_L) without affecting pro-apoptotic levels; (3) a promotion of CASP-3 and CASP-8 subfamily caspase activities; (4) a stimulation of PARP cleavage; and (5) a programmed cell death by apoptosis. Our data suggest that activation of the ERK pathway functions to protect pancreatic tumor cells from apoptosis as well as to regulate their progression in the cell cycle.

MATERIALS AND METHODS

Material

[^{3}H]-methyl-thymidine was obtained from Amersham (Oakville, Ontario). The MEK1/2 inhibitor PD98059 was purchased from New England Biolabs, Inc. (Mississauga, Ontario). The p38 inhibitor SB203580, the CASP-1 subfamily inhibitor zVAD-FMK and the CASP-3 subfamily inhibitor zDEVD-FMK were purchased from Calbiochem (Mississauga, Ontario). The fluorogenic substrates of caspase-1, 3, 6, 8, and 9 were from Pharmingen (Mississauga, Ontario, Canada). All other materials were obtained from Sigma unless otherwise stated.

Antibodies. The anti-p27^{KIP1} monoclonal antibody was from Transduction Laboratories (Mississauga, Ontario). Antiserum E1B that specifically recognizes ERK1 and ERK2 on Western blots was a kind gift from Dr. Fergus McKenzie and Dr. Jacques Pousségur (Université de Nice, Nice, France). The anti-cytokeratin-18 antibody was from Sigma. Monoclonal antibody $C_{II}10$ that recognizes the 89 kDa apoptotic fragment and the 113 kDa noncleaved fragment of poly (ADP-ribose) polymerase (PARP) was a kind gift from Dr. G.G. Poirier (Laval University, Québec, Canada). Rabbit polyclonal antibodies against the phosphorylated and active forms of ERK1/2 were from New England Biolabs, Inc. (Mississauga, Ontario). Primary rabbit polyclonal antibodies used in the present study were Ab 1682, directed against Mcl-1 [Krajewski et al., 1994a; Krajewski et al., 1995]; Ab 1695 directed to human/mouse Bcl-X_L [Krajewski et al., 1994c]; Ab 1701 [Krajewski et al., 1994a] and Ab PC68 (Calbiochem, San Diego, CA), both directed against human Bcl-2; Ab 1712 [Krajewski et al., 1994b] and Ab PC66 (Calbiochem), both directed to human Bax; Ab 1764, directed against human Bak [Krajewski et al., 1994b]; Ab 1-19 (Santa Cruz, Santa Cruz, CA), directed to human/mouse Bak; Ab PC67 (Calbiochem), directed to human Bcl-X_L; Ab K-20 (Santa Cruz, Santa Cruz, CA), directed against human/mouse Mcl-1; and Ab 9292 (New Englands Biolabs, Beverly, MA) and Ab R-20 (Santa Cruz, Santa Cruz, CA), both directed to human Bad. Primary mouse monoclonals used were mAb K56C8 [Takayama et al., 1995; Wang et al., 1996b] directed against human Bag-1.

Methods

Cell culture: The human pancreatic tumoral cell line MIA PaCa-2 (American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium (DMEM from Gibco/BRL, Burlington, Ontario) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in an humid atmosphere (5% CO_2, 95% air).

DNA synthesis reinitiation. Cells were seeded in 24-well dishes and subjected to serum-starvation for 24 h. Cells were then stimulated with 1% serum (FBS) ± 20 μM PD98059 for 22 h at 37°C and [^{3}H]-thymidine (1 μCi/500 μl) was added during the last 2 h. The cells were then fixed and washed twice with ice-cold trichloroacetic acid (5%). Cells were then harvested with 0.1 N NaOH and the radioactivity incorporated counted as previously described [Rivard et al., 1996].

Colony formation assay. Cells were seeded in 6-well dishes and subjected to serum-starvation for 24 h. Cells were then stimulated with 1% serum (FBS) ± 20 μM PD98059 at 37°C. Visible colonies that developed after 4 days were then stained with Giemsa [Rivard et al., 1996; Chen et al., 1996].

Immunoblotting. Cells were lysed in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.005% bromophenol blue and 5% 2-β-mercaptoethanol) except for the PARP expression analysis where cells were lysed in Laemmli buffer supplemented with 6M urea. Cell lysates were sonicated, boiled and separated by SDS-Polyacrylamide gel electrophoresis. Proteins were detected immunologically following electrotransfer onto nitrocellulose membrane. The blots were incubated with specific antibodies in blocking solution for 2–4 h at 25°C and then incubated with horseradish peroxidase-conjugated secondary antibodies. The blots were visualized by the Amersham ECL system. Protein were measured using a modified Lowry procedure [Peterson, 1977]. Quantitative analyses of protein levels were performed by scanning of the autoradiograms and densitometric analyses and are representative of more than two independent experiments.

DNA laddering assay. This procedure was performed as previously described [Vachon et al., 1996]. An equal volume of each sample was

loaded on a 1.5% agarose gel for electrophoresis, and fragmented DNA was vizualised by ethidium bromide staining.

Caspase protease assays. The assay to measure caspase activities was as described by Pharmingen. Briefly, MIA PaCa-2 cells were harvested in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 15 mM NaCl, 20 mM $MgCl_2$, 5 mM EGTA, 0.1 mM PMSF, 1 μg/ml leupeptin, 1 μM pepstatin A, 1% Triton X-100 for 15 min at 4°C. Insoluble material was removed by centrifugation at 12,000 × g for 2 min at 4°C. Proteins from lysates (25 μg) were incubated with 20 μM of the corresponding fluorogenic substrates for caspase 1, 3, 6, 8, and 9 for 2 h at 37°C. The AMC liberated from the substrates was measured with a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm for caspases-1 and -3 and an excitation wavelength of 400 nm and an emission of 505 nm for caspases 6, 8, and 9.

Data presentation. The data presented are representative experiments performed at least twice.

RESULTS

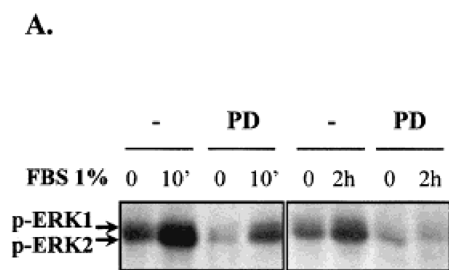
The ERK Cascade Is Involved in MIA PaCa-2 Cell Cycle Progression

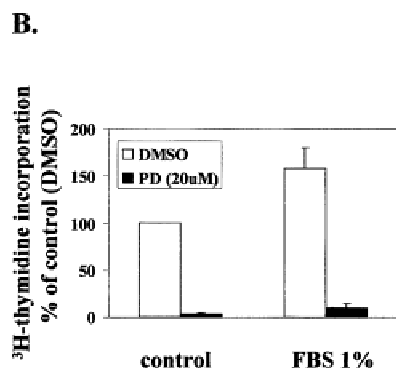
It was previously shown that sustained activation of ERK1/2 cascade was required to pass the G1 restriction checkpoint and enter the S phase in different cell types [Pagès et al., 1993; Marshall, 1995]. In fibroblasts, agents that elicit a short term ERK activation (5–10 min) are not mitogenic, whereas all mitogenic stimuli elicit a longer term activation (over 2 h) of ERK [Meloche et al., 1992]. Therefore, the ERK cascade is likely to regulate some mid-late changes in gene expression that are rate-limiting events for S phase entry during the G1 progression of the cell cycle [Brunet et al., 1999; Lavoie et al., 1996]. In agreement with this model, serum stimulation of MIA PaCa-2 cells exhibited rapid and maximal activation of ERK1/2 within 10 min which persisted for 2 h (Fig. 1A). Addition of 20 μM PD98059, a specific inhibitor of MEK-1/2, to these MIA PaCa-2 cells efficiently blocked both basal and serum-stimulated ERK1/2 activities for up to 2 h. These data are consistent with the reported ability of the drug to inhibit the MEK-ERK pathway [Alessi et al., 1995]. We then next evaluated the effect of PD98059 on serum DNA synthesis induction. It is noteworthy that the MIA PaCa-2 cells can significantly incorporate [^{3}H]-thymidine into DNA and grow in serum-free medium [Douziech et al., 1998]. Figure 1B shows that treatment of the MIA PaCa-2 cells with PD98059 abolished [^{3}H]-thymidine incorporation into DNA in both serum-deprived and serum-stimulated MIA PaCa-2 cells. The role of the MEK/ERK signaling pathway in MIA PaCa-2 cell proliferation was also examined in a colony formation assay. As shown in Figure 1C, treatment of the MIA PaCa-2 cells with PD98059 strongly attenuated basal and 1% serum-stimulated colony formation after 4 days. As cell cycle progression is governed by cyclin-dependent kinases (Cdks) counterbalanced by Cdk inhibitors [Sherr and Roberts, 1995], we investigated whether treatment of the MIA PaCa-2 cells with PD98059 could affect expression of one or several cell cycle regulators. Treatment of serum-starved or serum-stimulated MIA PaCa-2 cells with PD98059 had no effect on the expression of cyclin D1, cyclin E nor $p21^{Cip}$ (data not shown). However, as shown in Figure 1D, addition of PD98059 to serum-starved cells significantly increased $p27^{Kip1}$ expression by 40%, 41%, and 27%, 16, 24, and 48 h, respectively, after its addition. Moreover, the down-regulation of $p27^{Kip1}$ induced by serum was totally abolished by PD98059 when compared to controls. These results clearly establish that ERK1/2 are involved in $p27^{Kip1}$ down-regulation and in G1/S phase transition in response to serum stimulation in MIA PaCa-2 cells.

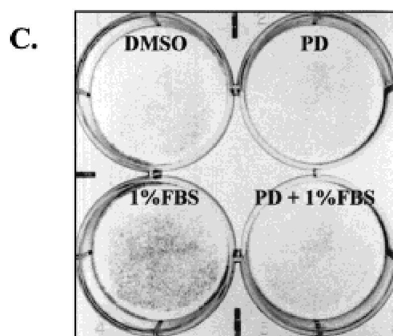
The MEK/ERK Cascade Promotes MIA PaCa-2 Cell Survival

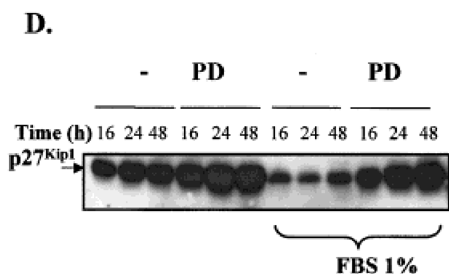
To evaluate the role of MEK/ERK in controlling cell survival, we performed DNA laddering assays to visualize DNA fragmentation, a key biochemical event in cells undergoing apoptosis [Loo and Rimmela, 1998] after inhibition of MEK/ERK activities. As shown in Figure 2A, treatment of serum-deprived MIA PaCa-2 cells with PD98059 markedly reduced ERK1/2 activities after 24 and 48 h. These changes in ERK activities took place without changes in ERK1/2 protein levels. DNA fragmentation remained undetectable in 24 and 48 h serum-starved cells (Fig. 2A). On the contrary, significant DNA fragmentation was observed 48 h after treatment of serum-starved cells with 20 μM PD98059, a result consistent with the notion that basal ERK activity may provide survival signals for serum-deprived MIA

PaCa-2 cells. The PD98059 treatment (> 24 h) also induced some morphological changes indicative of apoptosis: cell rounding, nucleus breakdown, followed by detachment and resulting in floating apoptotic bodies [Boucher et al., manuscript in preparation]. Similar experiments performed in other pancreatic tumoral cell lines (BxPC-3 and AR4-2J) confirmed this result (data not shown). Interestingly, addition of 1% or 10% serum to PD98059-treated MIA PaCa-2 cells did not restore ERK1/2 activities but prevented the induction of DNA fragmentation (Fig. 2A). This observation suggests that serum prevented apoptosis by a still unknown mechanism other than the ERK1/2 pathway. However, in serum-deprived cells, a dose-response experiment confirmed the strict correlation between inhibition of ERK1/2 activities and appearance of DNA laddering, with DNA degradation appearing at 15 μM PD98059 coincidental with maximal ERK1/2 inhibition (Fig. 2B). Interestingly, 24 h of PD98059 treatment was not sufficient to cause MIA PaCa-2 cell apoptosis after 48 h because no DNA fragmentation could be observed when PD98059 was removed from the medium after 24 h (Fig. 2C). This suggests that after 24 h of PD98059 treatment, the cells were arrested in G1 and were not already in an apoptotic process.









Inhibition of ERK1/2 Down-Regulates Anti-Apoptotic Bcl-2 Homolog Expression Levels

Since inhibition of MEK/ERK activities resulted in apoptosis of MIA PaCa-2 cells, we analyzed the expression levels of pro-apoptotic (Bak, Bax, Bad) and anti-apoptotic (Bcl-2, Mcl-1, Bcl-X_L) homologs, to verify whether MEK/ERK activities influenced this apoptotic checkpoint. As illustrated in Figure 3 (A and

Fig. 1. The ERK cascade is involved in MIA PaCa-2 cell cycle progression. **A:** Inhibition of ERK1/2 activities by PD98059 in MIA PaCa-2 cells. MIA PaCa-2 cells were serum-starved for 24 h and then stimulated with or without (0) 1% FBS for 10 min and 2 h in the presence of either vehicle (DMSO, -), or 20 μM PD98059 (PD). Equal amounts of whole cell lysates were separated by SDS-PAGE, and proteins were electrotransferred onto nitrocellulose. Western blot for active ERK1/2 activities were performed as described in experimental procedures. **B:** Inhibition of DNA synthesis by PD98059. MIA PaCa-2 cells were arrested for 24 h in serum-free DMEM medium. Reinitiation of DNA synthesis in response to 1% FBS +/- 20 μM PD98059 (PD) was measured as described in experimental procedures. Results are the mean ± S.E. of at least three separate experiments. **C:** Inhibition of cell proliferation by PD98059. Serum-starved cells were stimulated with 1% serum (FBS) ±20 μM PD98059 at 37°C. Visible colonies that developed after 4 days were then stained with Giemsa. **D:** Up-regulation of $p27^{Kip1}$ expression in PD98059-treated MIA PaCa-2 cells. MIA PaCa-2 cells were serum-starved for 24 h and then stimulated with or without 1% FBS for 16, 24, and 48 h in the presence of either vehicle (DMSO, -), or 20 μM PD98059 (PD). Cell lysis and Western blotting for $p27^{Kip1}$ expression were performed as described in A.

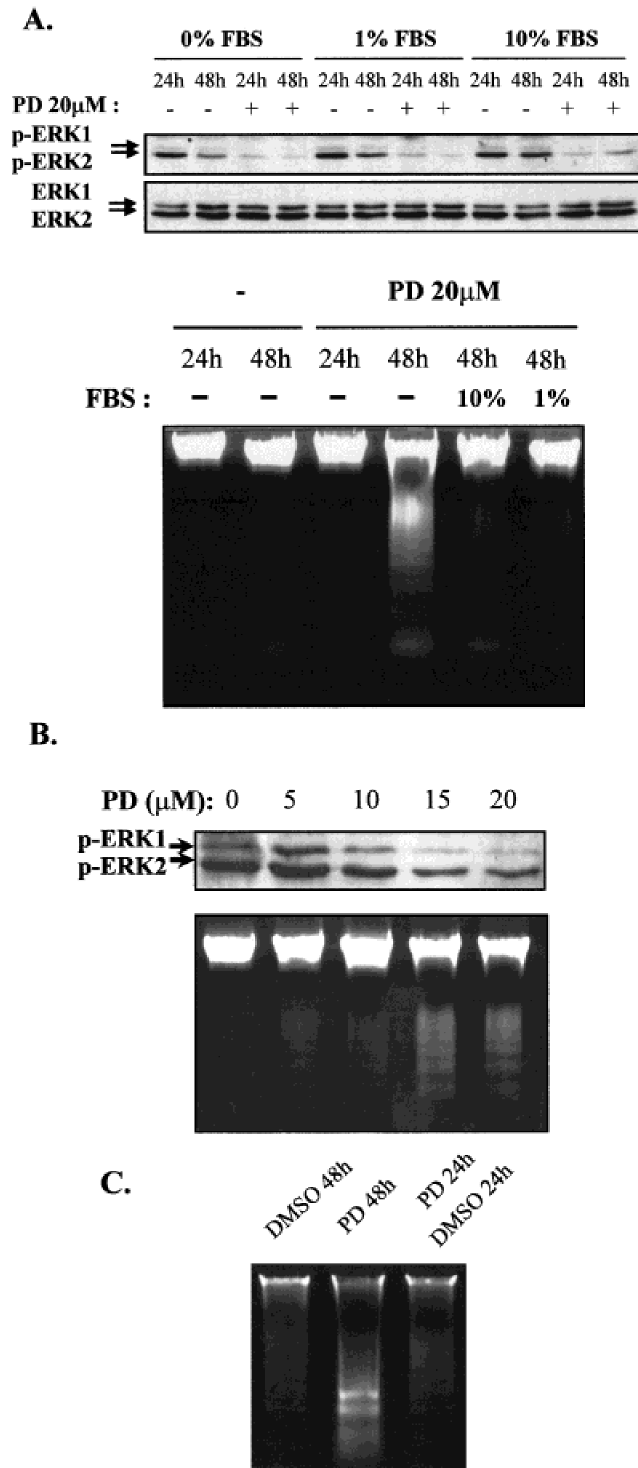


Fig. 2. Persistent inhibition of ERK1/2 activities induces DNA fragmentation in MIA PaCa-2 cells. **A:** Time course of PD98059-induced ERK1/2 inhibition and DNA fragmentation. Serum-starved, 1 and 10% serum-stimulated MIA PaCa-2 cells were incubated in the presence of either vehicle (DMSO, -), or 20 μM PD98059 (PD) for 24 and 48 h. Cell lysis and western blotting were performed as described in Fig. 1A and DNA laddering assay was performed as described in experimental procedures. **B:** Dose-dependence of PD98059-induced ERK1/2 inhibition and appearance of DNA fragmentation. Serum-starved MIA PaCa-2 cells were incubated in the presence of either vehicle (DMSO, -), or 5, 10, 15, or 20 μM PD98059 (PD) for 48 h. Cell lysis and Western blotting were performed as described in Fig. 1A and DNA laddering assay was performed as described in experimental procedures. **C:** 24 h of PD98059 treatment is not sufficient to induce DNA fragmentation. Serum-starved MIA PaCa-2 cells were incubated in the presence of either vehicle (DMSO), or 20 μM PD98059 (PD) for 24 h. Thereafter, cultures were washed with PBS and subsequently changed to DMEM without serum in presence of DMSO (lanes 1 and 3) or 20 μM PD98059 (lane 2). DNA laddering assay was performed 24 h later as described in experimental procedures.

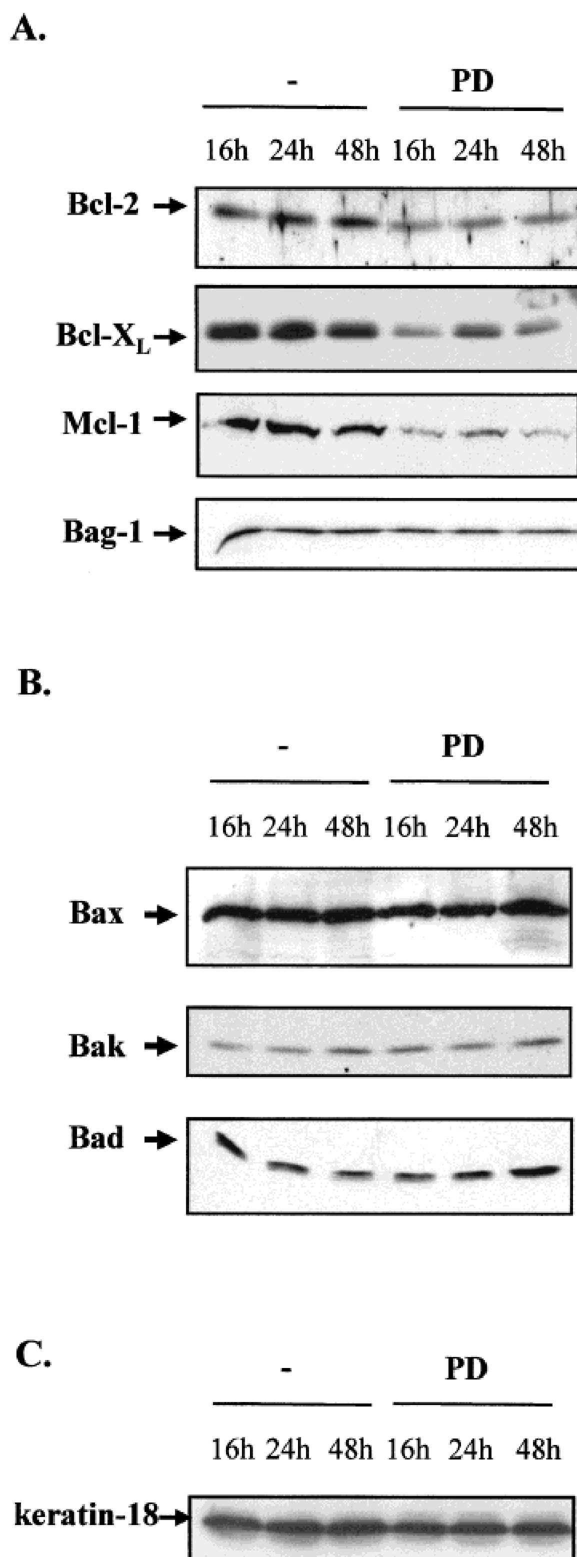


Fig. 3. Expression of Bcl-2 homologs in PD98059-treated MIA PaCa-2 cells. **A and B:** Serum-starved MIA PaCa-2 cells were incubated in the presence of either vehicle (DMSO, -), or 20 μM PD98059 (PD) for 16, 24, and 48 h. Cell lysis and western blotting were performed as described in Fig. 1A. **C:** Endogenous expression of cytokeratin-18 is shown as internal control.

B), MIA PaCa-2 cells express the anti-apoptotic members Bcl-2, Mcl-1 and Bcl-X_L along with the pro-apoptotic members Bak, Bad and Bax. The anti-apoptotic protein Bag-1 was detected as well (Fig. 3A). Interestingly, inhibition of ERK1/2 activities by PD98059 resulted in significant reductions in the expression of all the anti-apoptotic molecules studied to different extent and with different time courses (Fig. 3A). Indeed, treatment with the PD98059 inhibitor significantly decreased the expression of Bcl-2 by 32% after 16 h, by 38% after 24 h and by 39% after 48 h. The expression of Bcl-X_L was significantly and maximally inhibited by 69% after 16 h, 47% after 24 h and 52% after 48 h of treatment with PD98059. Treatment with the PD98059 inhibitor also decreased Mcl-1 expression by 66% after 16 h, by 33% after 24 h, and by 51% after 48 h. The expression of Bag-1 was reduced by 42%, 18%, and 26% respectively, after 16, 24, and 48 h of PD98059 treatment. In contrast, as shown in Fig. 3B, the expression of Bak and Bax was not significantly affected by the PD98059 treatment whereas the expression of Bad was significantly increased by 54% only after 48 h. Equal protein loading of each lane was confirmed by using an anti-keratin-18 antibody (Fig. 3C). These results indicate that the expression of Bcl-2, Mcl-1, and Bcl-X_L are regulated through an ERK-mediated signal transduction pathway, and that inhibition of ERK activity results in an overall increased ratio of pro-apoptotic Bcl-2 homologs.

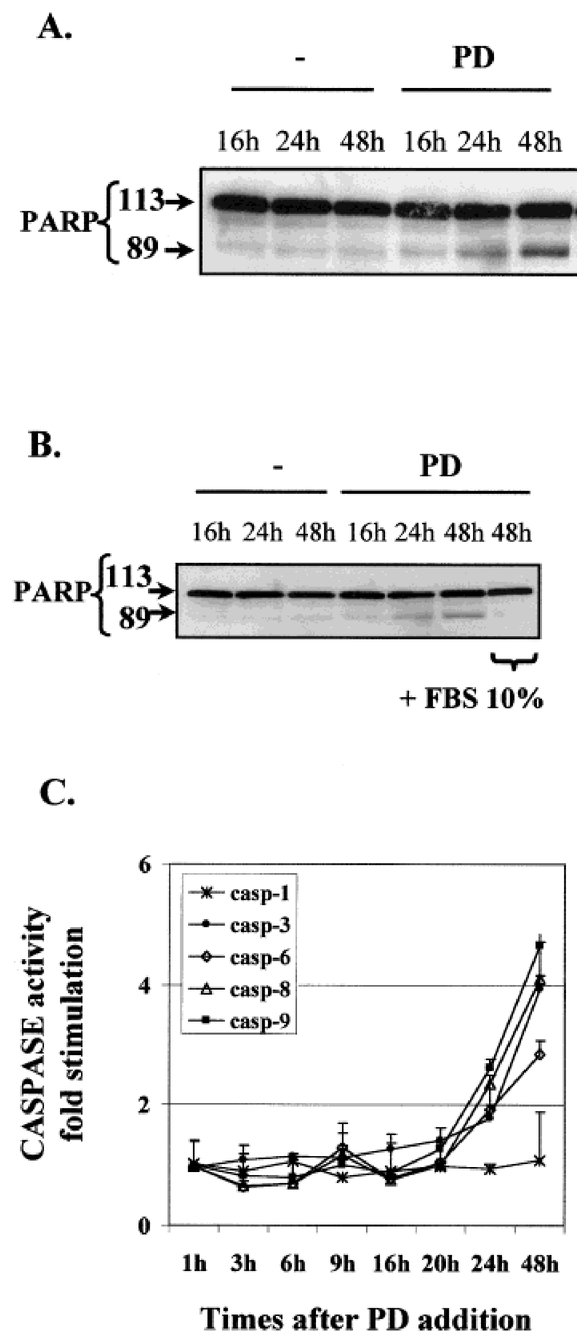
Activation of Caspases 3, 6, 8, and 9 and PARP Degradation During PD98059-Induced MIA PaCa-2 Cell Apoptosis

To analyze the relationship that might exist between the inhibition of ERK cascade and the molecular events regulating apoptosis, we measured the expression of PARP, a well known substrate for caspase-3 [Casciola-Rosen et al., 1996] in cells treated with the PD98059 compound. As shown in Figure 4A, a 24-h treatment with PD98059 slightly induced the cleavage of PARP revealed by the appearance of its 89 kDa fragment. This cleavage was more pronounced after 48 h of treatment. However, this PARP cleavage was totally prevented by the addition of 10% serum (Fig. 4B). In addition, by using fluorogenic substrate assays to measure caspase activities, enhanced activities of caspase-3, 6, 8, and 9 cleavage were observed, significant 24 h after PD98059 with

maximal activation at 48 h. No CASP-1 subfamily activity was detected at any time points after PD98059 addition to the cells (Fig. 4C).

Inhibition of PD98059-Induced MIA PaCa-2 Cell Apoptosis by Caspase Inhibitors

To further characterize the apoptotic machinery components involved in PD98059-induced apoptosis, MIA PaCa-2 cells were treated with PD98059 alone or in combination with the irreversible caspase inhibitors zVAD-fmk (a general inhibitor of caspases [Vercammen et al., 1998] or zDEVD-fmk (a selective inhibitor of caspase 3) [Villa et al., 1997]. As shown in Figure 5 (lanes 3–5), both inhibitors significantly reduced the apoptosis-inducing effect of PD98059 as they prevented DNA fragmentation. Interestingly, the combination of both inhibitors, 10 μM zVAD-fmk and 50 μM zDEVD-fmk (lane 7), totally abolished the pro-apoptotic effect of PD98059. Complete inhibition of PD98059-induced apoptosis was also observed with concentrations of zVAD-fmk 50 μM and higher (lane 6).



SB203580-Induced ERK1/2 Activities and MIA PaCa-2 Cells Protection Against PD98059-Induced Apoptosis

Recent data demonstrated that inhibition of ERK basal activity in Hela cells with the MEK inhibitor was sufficient to trigger apoptosis and p38 activation [Berra et al., 1998]. In MIA PaCa-2 cells, treatment with the PD98059 compound had no effect on p38 and JNK activities (data not shown). However, as shown in Figure 6A, treatment of serum-deprived MIA PaCa-2 cells with SB203580, a known p38 inhibitor [Lee et al., 1994], resulted in a significant and sustained activation of ERK1/2, an activation blocked by the addition of PD98059. This result is consistent with two previous studies which recently reported that SB203580 can directly stimulates Raf-1 kinase activity and its downstream effectors, the MEK-ERK kinases [Hall-Jackson et al., 1999; Kalmes et al., 1999]. No significant DNA fragmentation was visualized after 48 h of treatment with SB203580 alone (Fig. 6B, lane 3), even though the p38 MAP kinase activity was significantly

Fig. 4. Effect of PD98059 on PARP cleavage and caspase activities in MIA PaCa-2 cells. A and B. PARP cleavage is observed in PD98059-treated cells. Serum-starved MIA PaCa-2 cells were stimulated with or without 10% FBS for 16, 24, and 48 h in the presence of either vehicle (DMSO, -), or 20 μM PD98059 (PD). Cell lysis and western blotting were performed as described in Fig. 1A. **C:** CASP-3 and CASP-8 subfamily caspases are activated in PD98059-treated cells. Serum-starved MIA PaCa-2 cells were incubated in the presence of either vehicle (DMSO, -), or 20 μM PD98059 (PD) for the indicated time periods. Cell lysis and caspase assays were performed as described in experimental procedures and according to the Pharmingen protocol. All data are the mean ± S.E. of at least three experiments. Variations in caspase activities were estimated relative to the basal activity of the control (DMSO) at each time period.

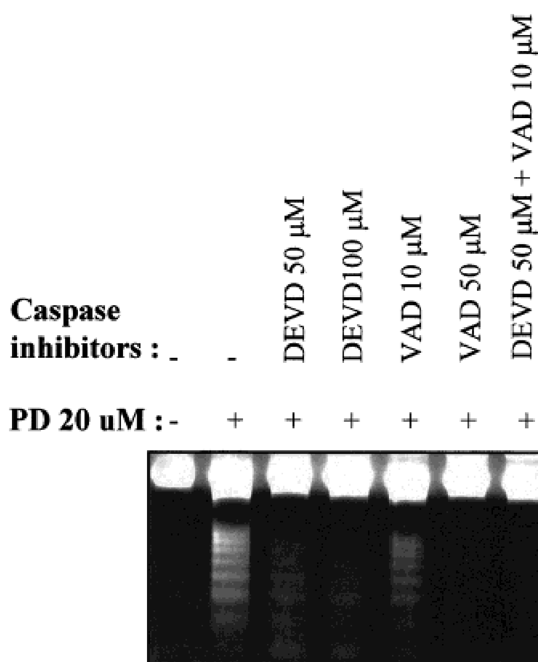


Fig. 5. Inhibition of PD98059-induced MIA PaCa-2 cell apoptosis by caspase inhibitors. Serum-starved MIA PaCa-2 cells incubated with or without 20 μM PD98059 (PD) for 48 h in the presence of either vehicle (DMSO, -), or 50 or 100 μM zDEVD-fmk (DEVD) or 10 and 50 μM zVAD-fmk (VAD). DNA laddering assay was performed as described in experimental procedures.

inhibited (data not shown). Interestingly, the SB203580 compound enhanced the expression of Bcl-2 by about 1.5-fold, Bcl-X_L by about 2-fold, and Mcl-1 by about 4-fold after 24 and 48 h of treatment (Fig. 6C). Conversely, SB203580 greatly attenuated the effect of PD98059 on DNA fragmentation (Fig. 6B, lane 4) and on the expression of Bcl-2, Bcl-X_L and Mcl-1 (Fig. 6C). However, the expression of pro-apoptotic homologs Bak, Bad, and Bax remained largely unaffected by the SB203580 treatment (data not shown). These data confirm that basal ERK activity observed in serum-starved MIA PaCa-2 cells regulates the expression of Bcl-2, Mcl-1 and Bcl-X_L, and therefore cell survival.

COX-1 and COX-2 Are Not Involved in PD98059-Induced MIA PaCa-2 Cell Apoptosis

The PD98059 inhibitor was initially described as a specific MEK inhibitor. However, a recent study indicated that this compound also inhibited purified cyclooxygenase-1 and -2 (COX-1 and COX-2) [Börsch-Haubold et al., 1998], two rate-limiting enzymes involved in the conversion of arachidonic acid into prostaglandins. COX-2 inhibition was shown to induce apoptosis of human prostate cancer cells [Liu et al., 1998], gastric cancer xenografts [Sawaoka et al., 1998] and intestinal epithelial cells [Tsujii and DuBois, 1995]. To exclude the potential involvement of COX-1 and COX-2 in apoptosis induced by the PD98059 treatment, we verified the effect of NS-398, a specific COX-2 inhibitor, and of indomethacin, a non-selective COX inhibitor, on MIA PaCa-2 cell survival. As shown in Figure 7, a 48-h treatment of cells with 10 and 50 μM NS-398 or 1 and 5 μM indomethacin, concentrations known to completely inhibit both COX activities [Lora et al., 1998], did not induce PARP cleavage as PD98059 did. Moreover, these COX inhibitors had no effect on DNA synthesis reinitiation (data not shown) nor did they affect cell morphology (data not shown). These results indicate that COX-1 and COX-2 activities are not implicated in the regulation of MIA PaCa-2 cell survival, and confirm MEK/ERK-specific effect of PD98059 in our model system.

PD98059-Induced MIA PaCa-2 Cell Apoptosis Appears to Be Dependent on Proteasome

The relatively long lag-interval (24–48 h) between PD98059 addition and the induction of apoptosis suggests that additional intermediate steps dependent either on protein synthesis or protein degradation are required prior to the onset of apoptosis. In this respect, a recent study demonstrated that Bcl-2 was specifically degraded after stimulation of human endothelial cells with TNF-α in a process inhibited by specific proteasome inhibitors [Dimmeler et al., 1999]. Simultaneous stimulation of MIA PaCa-2 cells with PD98059 and the proteasome inhibitor LLnL (N-acetyl-leucyl-leucylnorleucinal, 15 μM) [Palombella et al., 1994] markedly suppressed the effects of the MEK inhibitor on DNA fragmentation (Fig. 8A). Conversely, the ability of PD98059 to enhance caspase 3 and 9 activities, but not caspase 6 and 8 (data not shown), were totally abolished by a 48-h proteasome inhibitor treatment (Fig. 8B). Interestingly, the repressive effect of PD98059 compound on the expression of Bcl-X_L, but not those of Bcl-2 and Mcl-1, was almost totally restored by the proteasome inhibitor after 16 h of LLnL treatment (Fig. 8C). These data indicate a major participation of proteasome function in the activation of caspases 9 and 3 and in the onset of apoptosis through inhibition of MEK/ERK activities.

Unfortunately, we were unable to ascertain the role of protein neosynthesis because cycloheximide alone induced apoptosis in the MIA PaCa-2 cells (data not shown).

DISCUSSION

In the present study, we analyzed the roles of ERK1/2 pathway in the regulation of cell survival in the human pancreatic tumoral cell line MIA PaCa-2 cells. Specifically, we provided several evidences for a direct correlation between elevated ERK1/2 activities and MIA PaCa-2 cell proliferation and survival, whereby inhibition of ERK1/2 activities results in (1) an induction of $p27^{Kip1}$ expression and G1 arrest; (2) a down-regulation of expression levels of anti-apoptotic Bcl-2 homologs (namely, Bcl-2, Bcl-X_L, Mcl-1) concomitant with an up-regulation (Bad) or steady (Bax, Bak) expression levels of pro-apoptotic Bcl-2 homologs; and (3) an activation of caspase 3, 6, 8, and 9, along with cleavage of PARP and nucleosomal DNA fragmentation. Interestingly, proteasome function appears to be a major component of the apoptotic machinery activated by ERK1/2 inhibition.

Stimulation of the ERK signaling pathway by growth factors is well known to be implicated in the regulation of numerous cellular physiological processes including cell prolifer-

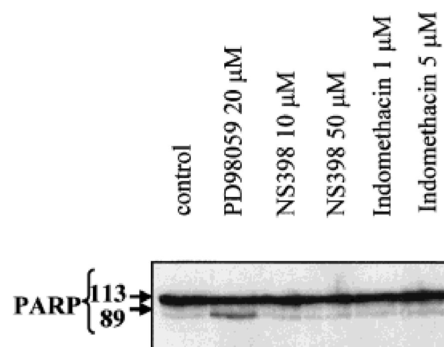
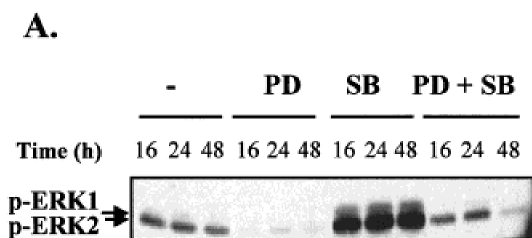
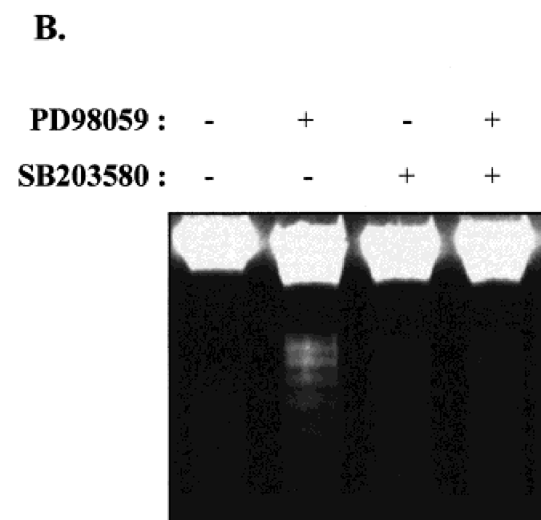


Fig. 7. Inhibition of COX-1 and COX-2 did not induce PARP cleavage. Serum-starved MIA PaCa-2 cells were incubated in the presence of either vehicle (DMSO, -), or 20 μM PD98059 (PD), 10 or 50 μM COX-2 inhibitor NS398, or 1 or 5 μM COX1/2 inhibitor indomethacin for 48 h. Cell lysis and western blotting for PARP were performed as described in Fig. 1A.





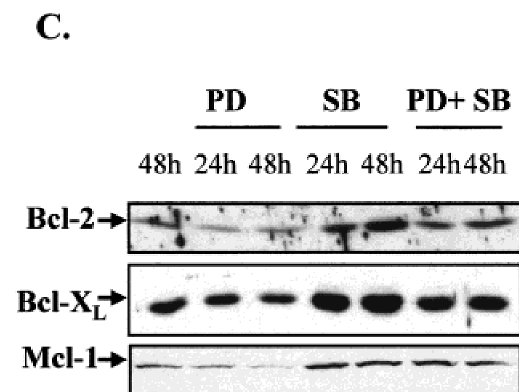


Fig. 6. Up-regulation of ERK1/2 activities and anti-apoptotic Bcl-2 homologs expressions by the p38MAPK inhibitor SB203580. **A and C:** Effect of SB203580 on ERK1/2 activities and Bcl-2 homologs. Serum-starved MIA PaCa-2 cells were incubated in the presence of either vehicle (DMSO, -), or 20 μM PD98059 (PD), 20 μM SB203580 (SB) or 20 μM PD + 20μM SB for 16, 24, and 48 h. Cell lysis and Western blotting for active ERK1/2 and Bcl-2 homologs were performed as described in Fig. 1A. **B:** Effect of SB203580 on DNA fragmentation. Serum-starved MIA PaCa-2 cells incubated with or without 20 μM PD98059 (PD) for 48 h in the presence of either vehicle (DMSO, -), or 20 μM SB203580 (SB). DNA laddering assay was performed as described in experimental procedures.

ation and differentiation [Marshall, 1995]. While the existence of an intimate link between quiescence, differentiation, and programmed cell death has been demonstrated in numerous cell types [Saini and Waeber, 1998], evidence for a similar linkage between cell proliferation and apoptosis has begun to emerge only recently [Evan and Littlewood, 1998]. Indeed, numerous cell cycle regulatory proteins (e.g., p53, pRb, $p21^{Cip}$, $p16^{INK4A}$) have been shown to induce apoptosis [Evan et al., 1995; Tan and Wang, 1998]. $p27^{Kip1}$ has also been added to this growing number of cell cycle regulators that are implicated in the control of cell survival [Wang et al., 1997]. However, such relationship between proliferation and apoptosis remains poorly understood and appears to be stimulus- and cell-type linked [Evan and Littlewood, 1998]. In this respect, our observation that inhibition of ERK1/2 in MIA PaCa-2 cells leads to G1 arrest, elevated $p27^{Kip1}$ expression and apoptosis suggests the existence of a proliferation-apoptosis linkage in pancreatic tumor cells; however, treatment of the MIA PaCa-2 cells with forskolin, which increased intracellular cAMP concentrations, blocked DNA synthesis along with induced $p27^{Kip1}$ protein levels without inducing apoptosis [Boucher et al., manuscript in preparation]. Therefore, this finding indicates that MIA PaCa-2 cell apoptosis by inhibition of ERK1/2 activities is not a consequence of the G1 arrest and $p27^{Kip1}$ elevation observed, and that the effect of ERK1/2 inhibition on cell cycle progression and survival in these cells constitutes two separate but concomitant processes regulated by the same pathway. Moreover, enhanced expression of $p27^{Kip1}$ was reported to lead to G1 cell cycle arrest. We [Rivard et al., 1996] and others [Coats et al., 1996] have recently demonstrated that overexpression of $p27^{Kip1}$ antisense cDNA allowed cells to grow for several generations in medium supplemented with insulin and transferrin or in me-

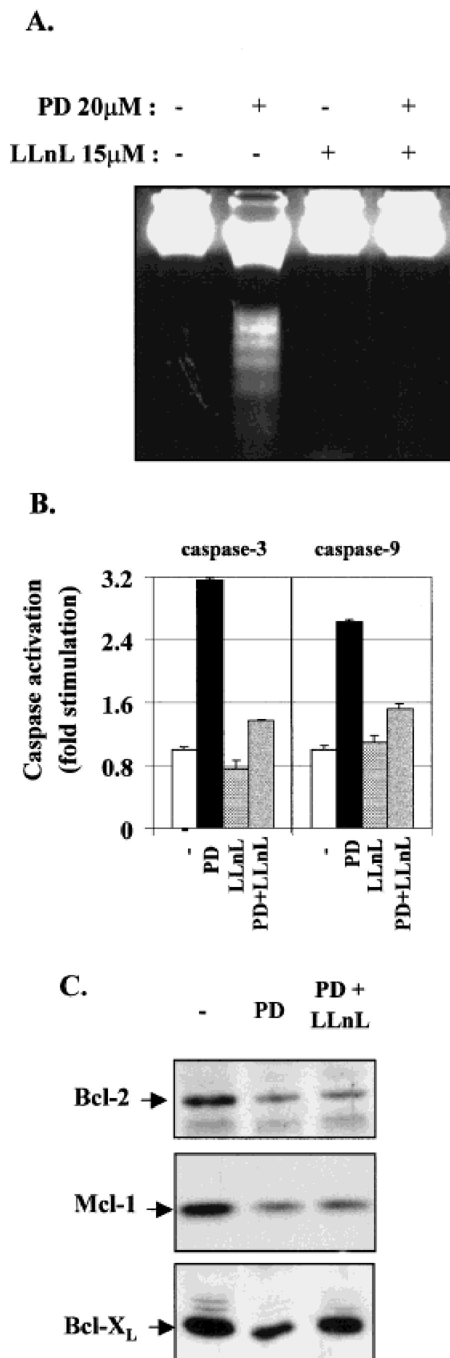


Fig. 8. PD98059-induced MIA PaCa-2 cell apoptosis is dependent on proteasome. A: Inhibition of proteasome protects cells against PD98059-induced DNA fragmentation. Serum-starved MIA PaCa-2 cells were incubated with or without 20 μM PD98059 (PD) for 48 h in the presence of either vehicle (DMSO, -), or 15 μM proteasome inhibitor LLnL. DNA laddering assay was performed as described in experimental procedures. B: PD98059-induced caspase 3 and 9 activities are inhibited by LLnL. Serum-starved MIA PaCa-2 cells were incubated with or without 20 μM PD98059 (PD) for 48 h in the presence of either vehicle (DMSO,-) or 15 μM proteasome inhibitor LLnL. Cell lysis and caspase assays were performed as described in experimental procedures and according to the Pharmingen protocol. All data are the mean ± S.E. of at least three experiments. Variations in caspase activities were estimated relative to the basal activity of the control at 48 h, set at 1. C: Expression of Bcl-2 homologs in LLnL-treated cells. Serum-starved MIA PaCa-2 cells incubated with or without 20 μM PD98059 (PD) for 16 h in the presence of either vehicle (DMSO, -), or 15 μM LLnL. Cell lysis and Western blotting for Bcl-2 homologs were performed as described in Fig. 1A.

dium containing low concentrations of serum. Conversely, it was recently demonstrated that targeted disruption of the murine $p27^{Kip1}$ gene enhanced growth of the mice and led to striking enlargement of their thymus, pituitary, adrenal and gonadal organs [Kiyokawa et al., 1996]. These data suggest that $p27^{Kip1}$ plays a major role in controlling cell cycle exit and thus, may be involved in PD98059-induced G1 arrest in MIA PaCa-2 cells. The observation that serum prevented MIA PaCa-2 cells' apoptosis while ERK1/2 activities were inhibited suggests the involvement of another signaling pathway. It is noteworthy that phosphoinositide 3-kinase is recruited and activated during the intracellular signal transduction of many receptors, an activation associated to signaling induced by survival factors present in serum [Marte and Downward, 1997].

The ERK pathway is known to affect the expression of several genes mostly involved in cell proliferation [Brunet et al., 1999; Lavoie et al., 1996; Pagès et al., 1993]. The fact that the Bcl-2 family and the ERK signaling pathway were both implicated in the control of cell viability suggests that ERK-stimulated enhancement of cell survival might be mediated through its effects on the expression of Bcl-2 or other Bcl-2 family members. In this regard, our data demonstrate that the anti-apoptotic homologs are regulated through an ERK-mediated pathway in human pancreatic MIA PaCa-2 cells. Recent findings have indicated that Mcl-1 expression implicates SRF/Elk-1 transcriptional activity, a known target of ERK1/2 [Townsend et al., 1999]. Previous reports also showed that activation of the Ras pathway by an inducible oncogenic Ras (Ras^{Vall2}) resulted in up-regulation of Bcl-2 and $Bcl\text{-}X_L$ without modulation of Bax expression level in hematopoietic cells [Kinoshita et al., 1995] and this survival-promoting function of Ras seemed to be accomplished by Raf/ERK and rapamycin-sensitive pathways [Kinoshita et al., 1997]. Furthermore, a recent study demonstrated that activation of the Bcl-2 promoter by nerve growth factor was mediated by the ERK cascade [Liu et al., 1999]. However, additional regulatory effects of the ERK pathway in the functions of Bcl-2 homologs cannot be ruled out. Indeed, stimulation of ERK1/2 in some cell types lead to phosphorylation of Bcl-2 and cell survival [Anderson, 1997; Dimmeler et al., 1999].

Aside from demonstrations of a role for the ERK pathway in modulating the balance of pro- and anti-apoptotic homologs as part of the mechanism by which it promotes pancreatic tumor cell survival, our study also provides new insights into the nature of the apoptotic machinery that is activated upon inhibition of this pathway. Indeed, our data demonstrate that inhibition of the ERK pathway activated caspases 3, 6, 8, and 9. A large body of evidence now supports the existence of two pathways of caspase activation: the cell surface death receptor pathway (e.g., TNFα receptor and Fas) and the mitochondria-initiated pathway [Budihardjo et al., 1999]. In the cell surface death receptor pathway, activation of caspase 8 following its recruitment to the death-inducing signaling complex (DISC) is the critical event that transmits the death signal. Activated caspase 8 can then activate downstream caspases by direct cleavage or indirectly by cleaving Bid and inducing cytochrome c release from the mitochondria [Ashkenzi and Dixit, 1998]. In the mitochondria-initiated pathway, caspase activation is triggered by the formation of multimeric Apaf-1/cytochrome c complex that is fully functional in recruiting and activating pro-caspase 9. Activated caspase 9 will then cleave and activate downstream caspases such as caspase 3, 6, and 7. Hence, the specific initiator caspase(s), as well as individual effector caspases, will vary according to the nature of the apoptotic stimulus and the cell studied. Therefore, we put forward a hypothetical model of ERK1/2 inhibition-induced caspase activation with caspases 8 and 9 as initiators and caspase-3 and 6 as effectors. However, at this point, further studies are required to clarify the mechanism by which the inhibition of ERK activates the initiator caspases 8 and 9.

The relatively long lag-interval between PD98059 stimulation and induction of apoptosis is very suggestive of additional intermediate steps requiring protein synthesis or protein degradation. Indeed, inhibition of proteasome functions completely abolished the ERK1/2 inhibition-induced apoptosis in MIA PaCa-2 cells. Activation of caspase 9 and 3 activities was prevented as well. These data indicate a major participation of proteasome function in the activation of caspase 9 and 3 and in the onset of apoptosis through inhibition of MEK/ERK activities. Futhermore, these observations along with others [Grimm et al., 1996;

Sadoul et al., 1996], suggest that the proteasome may either degrade regulatory protein(s) that normally inhibit the apoptotic pathway or may proteolytically activate protein(s) that promote cell death. Bcl-2 homologs (such as Bcl-2 and Bid) and putative caspase inhibitors have been proposed as potential targets of proteasome functions during onset of apoptosis [Dimmeler et al., 1999; Grimm et al., 1996; Sadoul et al., 1996]. Considering that some Bcl-2 homologs such as Bcl-2 and Bcl-X_L bind to Apaf-1 to prevent activation of the caspase cascade [Adams and Cory, 1998; Gross et al., 1999], our observation that inhibition of proteasome functions prevented partially a down-regulation of Bcl-X_L expression levels in ERK1/2 inhibited MIA PaCa-2 cells adds further support for such a role of proteasome in the onset of the apoptosis process. However, other studies indicated that inhibition of the proteasome actually induced apoptosis in proliferating cells but not in quiescent or differentiated cells [Drexler, 1997; Gross et al., 1999; Saini et al., 1998], suggesting that participation of the proteasome in the apoptotic machinery is likely to vary according to the apoptotic stimulus, the cell type, as well as the cell state [Drexler, 1997].

In conclusion, the present finding of an ERK-mediated pathway that regulates expression of Bcl-2, Mcl-1 and Bcl-X_L along with the recent demonstration of post-translational regulation of Bcl-2 [Dimmeler et al., 1999] and Bad [Gross et al., 1999], suggests that a web of phosphorylation pathways acts at multiple levels to regulate Bcl-2 family members and cell death. The particularity of the present report lies in the fact that ERK inhibition was achieved by the MEK inhibitor PD98059 without altering others MAPK cascades (p38 and JNK). Overall, a variety of gene families participates in the control of cell death with members of the Bcl-2 family regulating a cascade of caspase effectors [Anderson, 1997; Farrow and Brown, 1996] and protein kinases including ERK1/2 in turn regulating the Bcl-2 family. This regulation might be emphasized in pancreatic cancer cells in which Ki-Ras is frequently mutated [Friess et al., 1996] and its downstream effectors including ERK1/2 hyperactivated [Douziech et al., 1998]. Hence, alterations in this physiological pathway of cell elimination and tissue homeostasis may be a further mechanism giving pancreatic cancer cells a survival advantage.

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