UV irradiation-induced apoptosis leads to activation of a 36-kDa myelin basic protein kinase in HL-60 cells

MICHAEL L. LU*, MITSUHIRO SATO, BOLIAN CAO, AND JEROME P. RICHIE

Division of Urologic Surgery, Brigham and Women's Hospital, Department of Surgery, Harvard Medical School, 75 Francis Street, Boston, MA 02115

Communicated by Arthur B. Pardee, Dana-Farber Cancer Institute, Boston, MA, May 24, 1996 (received for review March 21, 1996)

ABSTRACT UV irradiation induces apoptosis (or programmed cell death) in HL-60 promyelocytic leukemia cells within 3 h. UV-induced apoptosis is accompanied by activation of a 36-kDa myelin basic protein kinase (p36 MBP kinase). This kinase is also activated by okadaic acid and retinoic acid-induced apoptosis. Irrespective of the inducing agent, p36 MBP kinase activation is restricted to the subpopulation of cells actually undergoing apoptosis. Activation of p36 MBP kinase occurs in enucleated cytoplasts, indicating no requirement for a nucleus or fragmented DNA in signaling. We also demonstrate the activation of p36 kinase in tumor necrosis factor- α - and serum starvation-induced cell death using the human prostatic tumor cell line LNCap and NIH 3T3 fibroblasts, respectively. We postulate that p36 MBP kinase is a common component in diverse signaling pathways leading to apoptosis.

Apoptosis, or programmed cell death, is a highly regulated physiological process. There is ample evidence that proteases play a central role in apoptosis, but the specific steps along the signal transduction pathway have not been delineated. Several kinases are implicated in transduction of apoptotic signals. For example, activation of Cdc2 kinase is associated with granzyme B-induced apoptosis in lymphoma cells, while Wee1, an inhibitor of Cdc2, represses programmed death in baby hamster kidney cells (1, 2). Another cyclin-dependent kinase (CDK)-related kinase, p58 PITSLRE, initiates apoptosis when overexpressed in Chinese hamster ovary cells and becomes activated during Fas-induced apoptosis in T cells (3). A 68-kDa double-stranded RNA-activated protein kinase (4) and a 160-kDa calmodulin-dependent serine/threonine kinase (5) are involved in the signaling pathway for interferon-induced cell death. However, it remains unclear whether activation of these kinases reflects signal specific transduction events or components of a conserved apoptotic signal transduction machinery.

Exposing mammalian cells to UV irradiation or other DNA-damaging agents results in the activation of a cellular UV response (6). This response involves a signaling pathway that originates in the plasma membrane, through Ras and Raf and culminates in the activation of transcription factors such as AP-1 and NF κ B. Activation of a newly identified c-Jun N-terminal kinase (JNK), also known as stress activated protein kinase (SAPK), links this response pathway to stress activated signaling pathways (7). The UV response represents a protective mechanism that is likely to enhance the DNA repair capacity of exposed cells (8). The commitment of cells to enter the death program may lay further downstream from these early events. Despite progress in establishing the early stress-induced signaling cascade, it remains undetermined whether any direct relationship exists between the activation of JNK/SAPK and the subsequent initiation of the cell death program.

In the present study, we employ a previously established UV irradiation-induced HL-60 promyelocytic cell apoptosis system in conjunction with a kinase zymographic technique to examine candidate signaling components that may be more directly involved in the apoptotic signaling cascade. We report the activation of a p36 myelin basic protein (MBP) kinase specifically associated with cells undergoing apoptosis. The kinetics of the activation of this kinase precedes DNA fragmentation in UV-induced HL-60 apoptosis. Furthermore, p36 MBP kinase is specifically activated in HL-60 cells treated with other apoptosis-inducing agents (okadaic acid, retinoic acid) but not by phorbol ester, an activator of mitogen-activated protein (MAP) kinase. Additionally, p36 MBP kinase is activated in apoptosis induced by TNF- α in the prostatic cancer cell line LNCap and by serum starvation in mouse NIH 3T3 fibroblasts. Thus, p36 MBP kinase appears to be a common component of the apoptosis signal transduction pathway that is induced by diverse stimuli.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma unless specified. Monoclonal antibody against pan-extracellular signal-regulated kinase (ERK) and Raf were obtained from Transduction Laboratories (Lexington, KY). Monoclonal antibody against map kinase kinase, JNK, Cdc2, Cdk4, PKC- α , PKC- δ , and PITSLRE were from Santa Cruz Biotechnology. Polyclonal antibody against p38 MAP kinase was kindly provided by R. J. Ulevitch, Scripps Research Institute (La Jolla, CA).

Cell Culture. Human promyelocytic leukemia cells HL-60 were cultured in RPMI 1640 supplemented with 20% fetal bovine serum. The prostatic cancer cell line, LNCap, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, and NIH 3T3 fibroblasts were grown in DMEM supplemented with 10% calf serum. Cells were kept in a humidified 5% CO₂ incubator at 37°C.

UV Irradiation. HL-60 cells were harvested and resuspended in PBS at 2×10^6 cells/ml. UV irradiation (254 nm, UVC) was performed with a UV gene linker equipped with an energy output control (Bio-Rad). Cells were irradiated with 34 mJ/cm² of UVC light and then centrifuged at $800 \times g$ for 10 min. The cells were then resuspended in complete media and treated as described below.

MBP Kinase Assay. The *in vitro* kinase assays were carried out as described previously (9). Briefly, 2×10^6 cells were treated with either UV or phorbol 12-myristate 13-acetate (PMA) (20 nM) for various times followed by homogenization in 200 μ l of lysis buffer [10 mM KH₂PO₄/1 mM EDTA/1 mM EGTA/10 mM MgCl₂/1 mM Na₃VO₄/50 mM β -glycerolphosphate (pH 7.2) supplemented with 1 mM phenylmethylsulfonyl

Abbreviations: CDK, cyclin-dependent kinase; JNK, Jun N-terminal kinase; SAPK, stress activated protein kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; H&E, hematoxylin and eosin.

*To whom reprint requests should be addressed. e-mail: mllu@bics.bwh.harvard.edu.

fluoride/1 mM diisopropyl fluorophosphate/1 μ M pepstatin A]. Cell lysates were centrifuged at $14,000 \times g$ for 30 min at 4°C, and the supernatant (postorganelle supernatant) was retained for the kinase assay. The MBP kinase reaction was performed by incubating 10 μ g of postorganelle supernatant with 1 μ g of MBP in 40 μ l of reaction buffer {50 mM Tris (pH 7.5)/15 mM MgCl₂/10 μ M ATP/1 μ Ci of [γ -³²P]ATP} at 30°C for 20 min. The kinase reaction was terminated by the addition of 25 μ l of SDS/PAGE sample buffer to the reaction mixture. Relative MBP kinase activity was determined using a PhosphorImager (Molecular Dynamics) for quantitation of radioactive bands.

In-Gel MBP Kinase Assay. An in-gel MBP kinase assay was performed as described previously for calmodulin-dependent protein kinase II (10). In brief, SDS/polyacrylamide gels were prepared with 0.5 mg/ml of MBP co-polymerized in the separation gel. Postorganelle supernatants containing 15 μ g of protein were loaded in each lane and resolved by electrophoresis. The MBP polyacrylamide gel was incubated in two changes of washing buffer for 1 h each [50 mM Tris (pH 7.4)/5 mM β -mercaptoethanol] containing 20% isopropanol at room temperature. The gel was denatured by incubating in two changes of 6 M guanidine HCl in wash buffer for 1 h each, followed by renaturation by incubating the gel overnight in 0.04% Tween 40 in wash buffer at 4°C. The renatured gel was then equilibrated with kinase reaction buffer [40 mM Hepes (pH 7.4)/5 mM MgCl₂/2 mM dithiothreitol/0.1 mM EGTA] for 10 min at room temperature. The kinase reaction was initiated by the addition of 50 μ Ci of [γ -³²P]ATP at room temperature for 60 min. The reaction was terminated and the gel was washed with 5 changes of 1% pyrophosphate in 5% trichloroacetic acid. The gel was then dried and subjected to autoradiography.

Jun N-Terminal Kinase Assay. The JNK assay was performed as described (11). Cell lysates from experimental groups were incubated with glutathione *S*-transferase-Jun (1–97) immobilized on glutathione-Sepharose beads. After the beads were extensively washed with kinase buffer, kinase reactions were performed in the same buffer at 30°C for 20 min. The reactions were stopped by removing the beads and heating them to 100°C for 5 min in SDS/PAGE sample buffer. Phosphorylated glutathione *S*-transferase-Jun fusion protein was separated by gel electrophoresis and kinase activity visualized by autoradiography. Relative kinase activity was calculated based upon quantitative data obtained from a PhosphorImager.

DNA Fragmentation. DNA was isolated from viable cell populations as described previously (12). Briefly, PBS-washed cells were resuspended in DNA extraction buffer [50 mM Tris (pH 8.0)/10 mM EDTA/0.5% *N*-laurosarcosine/1 mg/ml proteinase K] and incubated for 1 h in a 50°C water bath. DNase-free RNase was then added to a concentration of 250 μ g/ml, and samples were incubated at 50°C for another hour followed by ethanol precipitation of DNA. The resulting DNA preparations were resuspended in TE buffer [10 mM Tris (pH 8.0)/1 mM EDTA], and electrophoresis was performed in a 1.2% agarose gel. DNA ladders were visualized by ethidium bromide staining.

Percoll Gradient Isolation of Cells Undergoing Apoptosis. Isolation of apoptotic cells by Percoll gradient centrifugation was performed as described previously (13, 14). Percoll solutions of densities 1.08 g/ml (1 ml), 1.078 g/ml (2 ml), 1.076 g/ml (2 ml), 1.074 g/ml (2 ml), 1.072 g/ml (2 ml), and 1.070 g/ml (2 ml) were made as recommended by the manufacturer (Pharmacia), and each component of the gradient was successively layered in a 15-ml conical centrifuge tube. HL-60 cells were pelleted and resuspended at 1×10^6 cells/ml. Two ml of cell suspension was overlaid onto the Percoll gradient followed by centrifugation at $600 \times g$ for 30 min at 4°C. Cells were resolved into three subpopulations; dead cells (top of the gradient), normal cells, live cells (at the 1.060 and 1.070

interface), and apoptotic cells (at 1.075 to 1.080 interface and bottom of the gradient). Cell status was confirmed by trypan blue exclusion assay and morphological evaluation after hematoxylin and eosin (H&E) staining.

Enucleation of HL-60 Cells. Isolation of enucleated cytoplasts was carried out as described previously (15). In brief, log-phase HL-60 cells were harvested and resuspended to 2×10^7 cells/ml in 12.5% Ficoll containing complete media supplemented with cytochalasin B (10 μ g/ml). Three milliliters of this cell suspension was layered onto a Ficoll density gradient that had been prepared previously as follows. Round-bottomed ultracentrifuge tubes were filled with the following layers of Ficoll: 2 ml of 25%, 2 ml of 17%, 0.5 ml of 16%, 0.5 ml of 15%, and 2 ml of 12.5% Ficoll prepared in complete media supplemented with 10 μ g/ml of cytochalasin B and pre-equilibrated in a CO₂ incubator for at least 6 h. Gradients were centrifuged in a prewarmed (32°C) Beckman SW41 swing bucket rotor for 60 min at 25,000 rpm. The enucleated cytoplasts were collected at the interface between the 16 and 17% Ficoll layer. Cytoplasts were then washed with complete media and allowed to recover at 37°C for 2 h before UV irradiation.

RESULTS

To study the biochemistry of apoptotic signal transduction, a previously established experimental system was employed where promyelocytic leukemia cells (HL-60) are induced to undergo apoptosis by irradiation with ultraviolet light (12, 16). Irradiation of HL-60 cells with UV light causes the cells to undergo morphological changes characteristic of apoptosis, including chromatin condensation, nuclear fragmentation, and apoptotic body formation. As shown in Fig. 1*A*, the number of apoptotic HL-60 cells increases from $24.5 \pm 7\%$ by 60 min to a plateau of greater than $93 \pm 1.9\%$ after 120–150 min exposure to UV irradiation, as established by H&E staining. DNA fragmentation (Fig. 1*B*, left), as demonstrated by nucleosomal DNA ladder formation, is also observed in HL-60 cells in response to UV-induced apoptosis. The kinetics of DNA fragmentation parallel the observed morphological changes. Although the exact mechanism remains unclear, Zn²⁺ is known to inhibit UV irradiation-induced apoptosis (12). The inclusion of 0.5 mM ZnCl₂ in the incubation media protects 70–80% of HL-60 cells from cell death during the 3-h time course of the experiment (Fig. 1*A* and *B*, Right). The inability of cyclohexamide to inhibit UV-induced apoptosis suggests that protection does not require new protein synthesis (data not shown) but involves posttranslational events instead.

FIG. 1. UV irradiation-induced HL-60 apoptosis. (A) Time course study of UV-induced HL-60 cell apoptosis. HL-60 cells were exposed to UV (254 nm) at 34 mJ/cm² followed by incubation at 37°C for various times. Cells undergoing apoptosis were determined by scoring apoptotic nuclei of cytospin preparations morphologically from HL-60 cells stained either by propidium iodide or H&E stain. The solid bar indicates UV irradiation alone and hollow bar indicates Zn²⁺ inhibiting UV-induced apoptosis. (B) DNA ladder formation of HL-60 cells in response to UV-induced apoptosis of the same experiments described above.

MBP was used as an exogenous protein kinase substrate to monitor protein kinase activation in UV-induced cell death. Postorganelle supernatants isolated from HL-60 cells at various time points after UV irradiation were evaluated with an *in vitro* MBP kinase assay. As shown in Fig. 2A (Left), substantially delayed up-regulation of cytoplasmic MBP kinase activity in response to UV irradiation was observed. Activation of MBP kinase was apparent by 60 min and was maximal by 150 min, with a total 5.5-fold increase in activity. As a control, HL-60 cell lysates were also treated with PMA (20 nM), which does not induce apoptosis during the 3-h time course of the experiments. Consistent with previous reports (17, 18), PMA induced rapid MBP kinase activation, which peaked after 5 min of stimulation (5.6-fold; Fig. 2A, Right). The kinetics of MBP kinase activation was distinct from that of UV-induced activation. Intriguingly, UV-induced activation of MBP kinase follows similar kinetics to morphological changes and DNA fragmentation described above.

UV irradiation activates JNK/SAPK and MAP kinase (also termed ERK) (11, 19). Both kinases can phosphorylate MBP *in vitro*, and up-regulation of both leads to the activation of distinctive downstream signal cascades (7, 20–22). The possibility that one of these two kinases might account for the elevated MBP kinase activity detected in the UV-treated group was examined. ERK is activated upon phosphorylation of specific threonine and tyrosine residues (11, 19). Modification of ERK by phosphorylation retards its migration in polyacrylamide gels and is readily detectable by probing Western blots with monoclonal antibody raised against ERK. Cytoplasmic fractions from UV- and PMA-treated HL-60 cells were evaluated by this procedure. As shown in Fig. 2B, no activation of ERK was observed in response to UV irradiation. Instead, a down-regulation of ERK kinase activity was detected 30 min after UV irradiation (Fig. 2B, Upper). Consistent with results presented in Fig. 2A showing PMA activation of MAP kinase, a typical transient MAP kinase (ERK) activation

FIG. 2. (A) UV- or PMA (TPA)-induced postorganelle supernatant MBP kinase activation. Postorganelle supernatants of HL-60 cells treated with either UV or PMA (20 nM) for various times were subjected to *in vitro* MBP kinase assays. (B) Activation of ERK 2 (MAP kinase pp42) in TPA-treated cells but not in UV-treated cells. Activated ERK 2 (*) was detected by molecular weight shift of nonactivated ERK2 (•) on immunoblots with an anti-Pan ERK antibody. (C) Activation of JNK by UV and PMA treatment.

was observed when cells were treated with phorbol ester (Fig. 2B, Lower). As demonstrated previously, both UV and PMA transiently activated JNK/SAPK activity (11) but with slightly different kinetics (Fig. 2C). In the UV-treated group, activation of JNK was detected within 5 min, but activity abruptly returned to base line by 60–90 min. This timing coincides with the massive onset of UV-induced apoptosis. The apparent difference in the activation kinetics between the total cytoplasmic MBP kinase in response to UV-irradiation and the ERK and JNK/SAPK indicates that neither ERK nor JNK/SAPK accounts for the elevated activity of the total MBP kinase observed in HL-60 cells.

To further investigate the kinase species responsible for the elevation of cytoplasmic MBP kinase activity during UV-induced apoptosis, a myelin basic protein zymogram in-gel kinase assay was used. Cytoplasmic fractions of HL-60 cells were isolated from UV-irradiated cells at various time points and subjected to electrophoresis in an MBP-impregnated polyacrylamide gel. An in-gel kinase reaction was performed by denaturation and renaturation of the kinase in the polyacrylamide gel and incubation with [γ - 32 P]ATP as described (10). In Fig. 3A (Left), UV irradiation is shown to induce activation of a 36-kDa MBP kinase. The p36 MBP kinase activity plateaus 90–120 min after UV irradiation. In contrast,

FIG. 3. Identification of activated p36 MBP kinase with an in-gel MBP kinase assay. Postorganelle supernatant extracts from HL-60 cells treated with either UV or PMA (TPA) were subjected to in-gel MBP kinase assay. (A) Activation of p36 MBP kinase was detected in UV-treated cells but not in PMA-treated cells. PMA stimulated the activation of pp42 ERK 2. (B) Inhibition of UV induced p36 MBP kinase activation by including Zn^{2+} in the incubation media. (C) Activation of p36 MBP kinase is specifically associated with the apoptotic cell population. HL-60 cells were induced to undergo apoptosis by UV (34 mJ/cm², 90 min postirradiation), retinoic acid (1 μ M, 7 days), and okadaic acid (20 nM, 16 h). Three cell populations were isolated from different positions in a Percoll gradient (i.e., top, dead cells; middle, live, normal cells; bottom, apoptotic cells). MBP in-gel kinase assays were performed with cell extracts from apoptotic, live, and dead cells.

the same cells treated with phorbol ester only demonstrate ERK-2 kinase activation (Fig. 3A, *Right*) with the same activation pattern as observed on the anti-ERK Western blot shown in Fig. 2B. The p36 MBP kinase is clearly distinct from ERK (pp44/42) and JNK/SAPK (pp46/56), based upon its molecular weight and activation kinetics. The possibility of p36 MBP kinase being a CDK was also excluded, since CDK requires association with a cyclin partner to become an active holoenzyme (23), whereas activity of p36 MBP kinase was detected by the in-gel zymogram assay that involved denaturing SDS-polyacrylamide gel electrophoresis.

It is difficult to rule out the possibility that p36 MBP kinase is not a proteolytic fragment of a previously characterized protein kinase. We have, however, evaluated its reactivity to a battery of antibodies raised against other protein kinases. Partially purified p36 MBP kinase is not recognized by antibodies specific to PKC- α , PKC- δ , Cdc2, Cdk2, Cdk4, PITSLRE, p38 MAPK, JNK-1, Raf, or map kinase kinase (data not shown).

The correlation between UV-induced apoptosis and activation of p36 MBP kinase prompted further characterization of the association of these two events. The experimental system that uses Zn^{2+} to inhibit UV-induced apoptosis was employed. P36 MBP kinase activation was determined in HL-60 cells induced to undergo apoptosis by UV irradiation in the presence or absence of $ZnCl_2$ treatment. As shown in Fig. 3B, UV-induced p36 MBP kinase activation was inhibited significantly by Zn^{2+} (Fig. 3B, compare the *Left* and *Right* panels). The low p36 kinase activity detected at later time points in the Zn^{2+} -treated group is consistent with the results shown in Fig. 1A, which indicate that Zn^{2+} does not completely inhibit UV-induced apoptosis. Inclusion of Zn^{2+} does not alter the activation of ERK in PMA-stimulated HL-60 cells (data not shown).

To further illustrate the association between apoptosis and p36 kinase activation, we employed a Percoll gradient to specifically separate the apoptotic cells from the live and dead cells. Different populations of cells are separated based upon differences in buoyant density (12, 14). Centrifugation in a Percoll gradient facilitates the separation of three subpopulations of cells. (i) Dead cells that have proceeded through apoptosis are concentrated at the top of the percoll gradient. (ii) Normal, live cells are enriched in the middle layer of the gradient, and (iii) cells actively undergoing apoptosis are collected at the bottom of the gradient. After 90 min of UV irradiation, more than 60% of cells were isolated at the bottom of a Percoll gradient with clear apoptotic characteristics, including condensed chromatin and formation of apoptotic bodies. These cells also exhibited a highly fragmented DNA pattern (data not shown). Nearly 35% of the cells were isolated as normal live cells, and less than 3% were dead cells as determined by trypan blue staining. Additionally, two previously described apoptotic inducing agents, okadaic acid (24) and retinoic acid (12), were used for the induction of apoptosis to determine whether activation of p36 MBP kinase is a UV-specific event. While UV treatment is a physical inducer of apoptosis, okadaic acid is a chemical inducer. Retinoic acid does not directly induce apoptosis in HL-60 cells. Instead, it induces HL-60 cells to undergo granulocytic differentiation in 6–8 days, which subsequently leads to apoptosis (12). This phenomenon is considered to parallel terminal differentiation of aging peripheral blood granulocytes. Normal, apoptotic, and dead cells obtained from all three apoptosis-inducing agents were isolated and subjected to an in-gel MBP kinase assay. As shown in Fig. 3C, p36 MBP kinase is preferentially activated in the apoptotic cells but not in the nonapoptotic cells. These results suggest a specific association between the activation of p36 MBP kinase and the onset of apoptosis. Furthermore, they also suggest that activation of p36 kinase is not UV-specific but appears to represent a common compo-

nent of the apoptotic signaling pathway. The rapid, intense induction of apoptosis by UV irradiation may account for the residual p36 activity in gradient fractions that comprised dead and normal cells as shown in Fig. 3C.

As mentioned above, the exact mechanism of Zn^{2+} protection of cells from UV-induced apoptosis remains to be determined. Nevertheless, it was postulated that Zn^{2+} may act by inhibiting nuclease activity, suggesting that apoptosis may be a nucleus-dependent process. This hypothesis raises the question as to whether activation of p36 kinase is a nucleus-dependent event, or if p36 MBP kinase is activated by fragmented DNA. To address this question, we used an enucleated cell system. Enucleated cytoplasts were isolated from cytochalasin B-treated cells by a Ficoll step gradient as described (17, 25). Cytochalasin B disrupts the actin cytoskeleton, which facilitates enucleation by centrifugation. More than 95% of the isolated cytoplasts were devoid of nuclei as determined by H&E staining (data not shown). The isolated cytoplasts were allowed to recover at 37°C for 2 h before they were exposed to UV irradiation and then subjected to an MBP in-gel kinase assay. As shown in Fig. 4, activation of p36 MBP kinase was observed in UV-irradiated cytoplasts, whereas no activation of the kinase was detected in non-UV irradiated cytoplasts or cytochalasin B-treated whole cells. After UV irradiation, more than 90% of the cytoplasts exhibited key morphological features of apoptosis including membrane blebbing and cytoplasmic condensation (data not shown). This is consistent with previous observations by Schulze-Osthoff and colleagues that the cell's nucleus and DNA fragmentation are not required for apoptosis (25). Activation of p36 kinase may thus be either a parallel or upstream event of DNA fragmentation.

We find that the activation of p36 MBP kinase is not restricted to apoptosis in HL-60 cells. The prostate cancer cell line, LNCap undergoes apoptosis upon exposure to TNF- α (26). Activation of p36 MBP kinase is readily detected in LNCap cells after 8 h of exposure to 50 ng/ml TNF- α (Fig. 5). NIH 3T3 cells are known to undergo apoptosis in response to serum deprivation (27). Upregulation of p36 MBP kinase activity is apparent in this cell line after 24 h of serum starvation (Fig. 5). The kinetics of p36 MBP kinase activation coincides with release of fragmented DNA in both cell lines (refs. 26 and 27 and unpublished observations).

DISCUSSION

The functional designation of p36 MBP kinase in apoptotic signal transduction remains to be established. The nucleus-independent activation of p36 MBP kinase observed is consistent with the concept that apoptosis involves cytoplasmic

FIG. 4. Activation of p36 kinase in UV-treated enucleated HL-60 cytoplasts. Cytoplasts were irradiated with UV and incubated at 37°C for 90 min followed by MBP in-gel kinase assay. WC, cytochalasin B-treated whole cells; cyto., cytoplasts.

FIG. 5. Activation of p36 kinase in prostatic cancer cell line, LNCap, and NIH 3T3 fibroblasts undergoing apoptosis. Apoptosis is induced in LNCap cells using 50 ng/ml TNF- α for the indicated periods of time. NIH 3T3 fibroblasts are induced to undergo apoptosis by serum starvation. Postorganelle supernatant extracts were subjected to in-gel MBP kinase assays as described in *Materials and Methods*.

signaling events. The most prominent feature of apoptosis is the disintegration of the nucleus. This process was thought to be initiated by proteolytic degradation of nuclear lamins (28). It was demonstrated by Lazebnik *et al.* (29) that an inhibitable pRICE activity in the S/M-phase extract induces isolated nuclei to undergo nuclear breakdown characteristic of apoptosis. Recently, Nicholson *et al.* (30) demonstrated that pRICE is in fact CPP-32. They also demonstrated that in terms of induction of nuclear apoptotic morphology changes, the presence of CPP-32 is necessary but not sufficient. Incubation of isolated normal nuclei with CPP-32 alone does not initiate nuclear breakdown. This raises the possibility that unidentified factor(s) may be involved in the process. Dissolution of the nuclear lamina in mitosis is regulated by phosphorylation of lamin proteins by cdc2 kinase and is a reversible process. It is conceivable that during the process of apoptotic nuclear breakdown, the nuclear lamina proteins may undergo a similar phosphorylation to facilitate the subsequent disassembly of the nucleus, possibly by rendering nuclear matrix proteins more accessible to proteases. While this is purely a speculation, a recent report by Masumi-Fukazawa *et al.* (31) describes an enhanced phosphorylation of nuclear 21- and 34-kDa proteins in TNF- α -induced apoptosis. The significance of this phosphorylation remains to be established. Interestingly, our preliminary results using biochemically purified p36 MBP kinase also demonstrates phosphorylation of nuclear proteins with similar molecular weight (unpublished observations). It will be intriguing to determine if any of these observations correlate with one another.

Ceramide, an intermediate metabolite from the sphingomyelin pathway, has emerged as a key mediator in the apoptotic signaling pathway as well as in cell differentiation. The downstream target after ceramide generation remains to be determined. Nevertheless, several signal mediators have been identified in the ceramide pathway, including a ceramide-activated protein kinase (32), a ceramide-activated protein phosphatase (33), and PKC ζ (34). The relevance of these signal components in mediating apoptosis is yet to be established. In the present study, three different apoptosis-inducing agents were used for the induction of programmed cell death. The status of ceramide generation in okadaic acid induced cell death remains unclear. Retinoic acid was demonstrated to elicit intracellular accumulation of ceramide. We and others (35) have also

observed an intracellular accumulation of ceramide in UV irradiation-induced apoptosis (unpublished results). This suggests the possibility that p36 kinase activation during apoptosis is a downstream event in the ceramide signaling pathway.

The molecular identity of p36 MBP kinase remains to be determined, it is possible that p36 MBP kinase is a proteolytic fragment of a larger kinase species produced by cleavage of apoptotic proteases, since intracellular proteolysis is known to play a central role in apoptosis (36, 37). Nevertheless, proteolytic processing may be a physiologically relevant event since the removal of regulatory domains can lead to constitutive activation of kinases (38, 39). This is further illustrated by a recent report from Emoto *et al.* (40) demonstrating the proteolytic activation of a 40-kDa PKC δ in ionizing radiation-induced apoptosis. The similarity in molecular weight of the 40-kDa PKC δ proteolytic fragment with the p36 MBP kinase prompted us to determine the potential relationship between these two kinases. Judging from the different activation kinetics (proteolytic activation of PKC δ occurs much later than the activation of p36 MBP kinase in UV-induced apoptosis), apparent differences in kinase inhibitor sensitivity and differences in anion-exchange column elution profiles (unpublished results), p36 MBP kinase does not appear to be related to the proteolytic fragment of PKC δ .

We report the activation of a p36 MBP kinase upon induction of apoptosis in HL-60 promyelocytic leukemia cells. The activation of this kinase is not specific to UV-induced apoptosis. Instead, it appears to be a general phenomenon of apoptosis since it occurs in response to okadaic acid and retinoic acid as well. We have also observed the activation of p36 kinase in other cells such as the human prostate cancer cell line, LNCap cells, and 3T3 fibroblasts using TNF- α or starvation respectively to induce apoptosis. These observations further suggest that p36 MBP kinase plays a general role in apoptotic signal transduction.

We thank Dr. W. F. Patton for many valuable suggestions. This work was supported by National Institutes of Health Grant DK45865.

- Shi, L., Nishioka, W. K., Th'ng, J., Bradbury, E. M., Litchfield, D. W. & Greenberg, A. H. (1994) *Science* **263**, 1143–1145.
- Chen, G., Shi, L., Litchfield, D. & Greenberg, A. (1995) *J. Exp. Med.* **181**, 2295–2300.
- Lahtai, J. M., Xiang, J., Heath, L. S., Campana, D. & Kidd, V. J. (1995) *Mol. Cell. Biol.* **15**, 1–11.
- Lee, S. B. & Esteban, M. (1994) *Virology* **199**, 491–496.
- Deiss, L. P., Feinstein, E., Berissi, H., Cohen, O. & Kimchi, A. (1995) *Genes Dev.* **9**, 15–30.
- Angel, P. (1995) in *Inducible Gene Expression and Cytoplasmic/Nuclear Signal Transduction*, ed. Baeuerle, P. (Birkhauser, Boston), pp. 62–92.
- Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. & Woodgett, J. R. (1994) *Nature (London)* **369**, 156–160.
- Schreiber, M., Baumann, B., Cotten, M., Angel, P. & Wagner, E. F. (1995) *EMBO J.* **14**, 5338–5349.
- Wang, H.-C. R. & Erikson, R. L. (1992) *Mol. Biol. Cell* **3**, 1329–1337.
- Kameshita, I. & Fujisawa, H. (1989) *Anal. Biochem.* **183**, 139–143.
- Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M. & Davis, R. J. (1994) *Cell* **76**, 1025–1037.
- Martin, S. J. & T. G. Cotter, (1991) *Int. J. Radiat. Biol.* **59**, 1001–1016.
- Martin, S. J., Bradley, J. G. & Cotter, T. G. (1990) *Clin. Exp. Immunol.* **79**, 448–453.
- Ishida, Y., Furukawa, Y., Decaprio, J. A., Saito, M. & Griffin, J. D. (1992) *J. Cell. Physiol.* **150**, 484–492.
- Wigler, M. H. & Weinstein, I. B. (1975) *Biochem. Biophys. Res. Commun.* **63**, 669–674.
- Bazar, L. S. & Deeg, H. J., (1992) *Exp. Hematol.* **20**, 80–86.

17. Radler-Pohl, A., Sachenmaier, C., Gebel, S., Auer, H.-P., Bruder, J. T., Rapp, U., Angel, P., Rahmsdorf, H. J. & Herrlich, P. C. (1993) *EMBO J.* **12**, 1005–1012.
18. Devary, Y., Gottlieb, R. A., Smeal, T. & Karin, M. (1992) *Cell* **71**, 1081–1091.
19. Hibi, M., Lin, A., Smeal, T., Minden, A. & Karin, M. (1993) *Genes Dev.* **7**, 2135–2148.
20. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R. & Templeton, D. J. (1994) *Nature (London)* **372**, 798–800.
21. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L. & Karin, M. (1994) *Science* **266**, 1719–1723.
22. Derijard, B., Raingeaud, J., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R. J. & Davis, R. J. (1995) *Science* **267**, 682–685.
23. Morgan, D. O. (1995) *Nature (London)* **374**, 131–135.
24. Sakurada, K., Zheng, B. & Kuo, J. F. (1992) *Biochem. Biophys. Res. Commun.* **187**, 488–492.
25. Schulze-Osthoff, K., Walczak, H., Droge, W. & Krammer, P. H. (1994) *J. Cell Biol.* **127**, 15–27.
26. Sensibar, J. A., Sutkowski, D. M., Raffo, A., Buttyan, R., Griswold, M. D., Sylvester, S. R., Kozlowski, J. M. & Lee, C. (1995) *Cancer Res.* **55**, 2431–2437.
27. Wang, E. (1995) *Cancer Res.* **55**, 2284–2292.
28. Neamati, N., Fernandes, A., Wright, S., Kiefer, J. & McConkey, D. J. (1995) *J. Immunol.* **154**, 3788–3795.
29. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. & Earnshaw, W. C. (1994) *Nature (London)* **371**, 346–347.
30. Nicholson, D. W., Ali, A. & Thornberry, N. A. (1995) *Nature (London)* **376**, 37–43.
31. Masumi-Fukazawa, A., Kohase, M. & Fuse, A. (1995) *Eur. J. Biochem.* **232**, 134–140.
32. Kolesnick, R. N. & Hemej, M. R. (1990) *J. Biol. Chem.* **265**, 18803–18808.
33. Dobrowsky, R. T. & Hannun, Y. A. (1992) *J. Biol. Chem.* **267**, 5048–5051.
34. Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L. & Moscat, J. (1994) *J. Biol. Chem.* **269**, 19200–19202.
35. Martin, S. J., Newmeyer, D. D., Mathias, S., Farschon, D. M., Wang, H.-G., Reed, J. C., Kolesnick, R. N. & Green, D. R. (1995) *EMBO J.* **14**, 5191–5200.
36. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvensen, G. S. & Dixit, V. M. (1995) *Cell* **81**, 801–809.
37. Martin, S. J. & Green, D. R. (1995) *Cell* **82**, 349–352.
38. Levine, H. & Sahyoun, N. E. (1987) *Eur. J. Biochem.* **168**, 481–486.
39. Planas-Silva, M. D. & Means, A. R. (1992) *EMBO J.* **11**, 507–517.
40. Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W. W., Kamen, R., Weichselbaum, R. & Kufe, D. (1995) *EMBO J.* **14**, 6148–6156.