Regulation of Protein Phosphatase 2A Activity by Caspase-3 during Apoptosis*

Maxine F. Santoro‡, Robert R. Annand‡, Molly M. Robertson‡, Yun-Wen Peng‡, Matthew J. Brady§, John A. Mankovich‖, Maria C. Hackett‖, Tariq Ghayuri†, Gernot Walter**, Winnie W. Wong‡‡, and David A. Giegel§§

From the ‡Department of Biochemistry and ¶§Department of Cell Biology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105; the †Department of Molecular Biology, the ‡Department of Biochemistry, and the **Department of Immunology, BASF Bioresearch Corporation, Worcester, Massachusetts 01605, and the ††Department of Pathology, University of California at San Diego, La Jolla, California 92039

Although the available evidence suggests that whereas the caspase family plays a major role in apoptosis, they are not the sole stimulators of death. A random yeast two-hybrid screen of a lymphocyte cDNA library (using caspase-3 as the bait) found an interaction between caspase-3 and the regulatory subunit Aα of protein phosphatase 2A. This protein was found to be a substrate for caspase-3, but not caspase-1, and could compete effectively against either a protein or synthetic peptide substrate.

In Jurkat cells induced to undergo apoptosis with anti-Fas antibody, protein phosphatase 2A (PP2A) activity increased 4.5-fold after 6 h. By 12 h, the regulatory Aα subunit could no longer be detected in cell lysates. There was no change in the amount of the catalytic subunit. The effects on PP2A could be prevented by the caspase family inhibitors acetyl-Asp-Glu-Val-Asp (DEVD) aldehyde or Ac-DEVD fluoromethyl ketone. The mitogen-activated protein (MAP) kinase pathway is regulated by PP2A. At 12 h after the addition of anti-Fas antibody, a decrease in the amount of the phosphorylated forms of MAP kinase was observed. Again, this loss of activated MAP kinase could be prevented by the addition of DEVD-cho or DEVD-fmk. These data are consistent with a pathway whereby induction of apoptosis activates caspase-3. This enzyme then cleaves the regulatory Aα subunit of PP2A, increasing its activity. These data show that the activated PP2A will then effect a change in the phosphorylation state of the cell. These data provide a link between the caspases and signal transduction pathways.

A role for the caspase family in apoptosis was discovered when a gene of Caenorhabditis elegans was shown to be required for apoptosis during development in this organism. Additionally, this gene was found to have a protein sequence that was homologous to caspase-1. When either ced-3 or the cDNA for caspase-1 was injected into rat-1 fibroblasts, the cells underwent programmed cell death (1). This finding opened the field of caspase biology to more than just the processing of pre-interleukin-1β. Since then, many more members of the caspase family have been cloned, and their essential roles in apoptosis are beginning to be elucidated. Several mechanisms by which the caspases elicit their effect in apoptosis can be postulated. These mechanisms include proteolytic cleavage and inactivation of key substrates involved in maintenance of DNA, the cell cycle, or structural elements.

After caspase-1, caspase-3 is the most extensively studied of all the caspase family members and may be one of the more downstream apoptotic effector molecules (2). One theory to summarize the role of caspase-3 in apoptosis revolves around the premise that this enzyme can cleave key enzymes involved in DNA repair. These enzymes include poly-(ADP)ribose polymerase and DNA-dependent protein kinase. This cleavage then renders these repair enzymes inactive (3–8). But because PARP1 knock-out mice (9) and severe compromised immunodeficient mice lacking the active DNA-PK catalytic subunit can develop normally, it can be concluded that inactivation of these enzymes is not the only requirement for apoptosis.

Caspase-3 has been demonstrated to cleave additional substrates during cellular apoptosis such as the U1 70-kDa small nuclear ribonucleoprotein (which is involved in RNA splicing) (10), structural proteins such as fodrin, non-heme spectrin (11), lamin (12), gas 2 (13), and cell kinases, such as retinoblastoma-associated protein Rb (14). The role of these substrates in apoptosis remains unclear. It may be necessary for each caspase family member, with their different substrate specificities, to cleave many essential substrates in a cooperative fashion for cell death to occur. However, it is apparent that caspase-3 plays an essential role in apoptosis since caspase-3 knock-out mice have a problem with apoptosis and development in the brain (15). Like caspase-1, other family members may have nonapoptotic roles in the cell as well.

Although the available evidence suggests that the caspase-1 homologs play a major role in apoptosis, they are not the sole stimulators of death. Fraser et al. (16) concluded that almost everything, including oncoproteins, tumor suppressor proteins, cytokines, and signaling proteins, seems to both induce and suppress apoptosis. However, little is known about how all of

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§§ To whom correspondence should be addressed: Biochemistry Dept., Parke-Davis Research, 2800 Plymouth Rd., Ann Arbor, MI 48105. Tel.: 734-622-5844; Fax: 734-622-5875; E-mail: Giegeld@aa.wl.com.
these pathways are connected. Protein phosphorylation provides
the cell with a basis for the control of growth, metabolism,
differentiation, and possibly programmed cell death. The phos-
phorylation state of cells relies on a very complex but carefully
orchestrated set of kinases and phosphatases. These phosphata-
eses are broadly classified into two groups, those preferring phosphorylated tyrosine and those preferring phosphorylated serine or threonine. The serine/threonine phosphatases are
grouped into classes based on their substrates and sensitivity
to inhibitors. The functions of these protein phosphatases in the
cell are extensive. It has been reported that the Ser/Thr phosphatases
play roles in metabolism, meiosis, mitosis, and the cell cycle (17). It has been suggested that these phosphatases also play a role in apoptosis (18).

Song and Lavin (19) demonstrated that the inhibitors of protein phosphatases 1 and 2, calcineurin A and okadaic acid, could inhibit apoptosis in the irradiated Burkitt’s lymphoma cell line BM13674. Recently, Morana et al. (20) have demonstrated that regulation of protein phosphatase 1 activity is essential in regulating apoptosis via activation of a caspase-1/3- CED-3 protease, intracellular acidification, and DNA digestion. With regard to the caspase-phosphatase interaction, they demonstrated that incubation of ML-1 cells with 1 μM okadaic acid inhibited DNA fragmentation and caspase-induced cleavage of PARP in cells treated with etoposide. Thus, a plausible link between cell cycle stimulators of death and the caspases may be found in signaling pathways via the serine/threonine phosphatases.

Protein phosphatase 2A (PP2A), the most abundant serine/threonine-specific phosphatase in mammals, plays a role in many fundamental cellular processes, including cell division, signal transduction, gene expression, and development. PP2A consists of three subunits, the catalytic C subunit and the 65-kDa regulatory A subunit, which together form the core enzyme, and the regulatory B subunit, which binds to the core enzyme yielding the holoenzyme. The A and C subunits both exist as two isoforms (α and β) and the B subunit as multiple isoforms, which are subdivided into three families, B, B’, and B”, unrelated to each other by primary sequence. The A subunit polypeptide consists of 15 nonidentical repeats that form a rod-shaped molecule. The B subunit binds to repeats 1–10 and the C subunit to repeats 11–15 of the A subunit. Binding of the rod-shaped molecule. The B subunit binds to repeats 1–10 and the C subunit to repeats 11–15 of the A subunit. Binding of the C subunit to the A subunit occurs in the absence of the B subunit, whereas binding of the B subunit requires the presence of both the A and C subunit for stability (17). The purpose of the studies reported here was to demonstrate an interaction between caspase-3 and the Ser/Thr phosphatases.

The yeast two-hybrid system (21) has been used to find unknown protein ligands that bind with known receptors. The data presented here will demonstrate that the yeast two-hybrid system can also be used to detect an interaction between an enzyme and a substrate and thus was used to find a new putative substrate for caspase-3. This interaction provides a link between the cell cycle, metabolic control, and the tumor necrosis factor/FAS-derived death pathways.

MATERIALS AND METHODS
Yeast Two-hybrid Screen—A yeast two-hybrid screen was performed according to the method of Field and Song (22) utilizing the Matchmaker Two-hybrid System (CLONTECH). The yeast strains used in the screen were HFF1 (MATa, ura3-52, his3-200, leu2-3, 112, gal-4, trp1-901, URA3::[GAL 17-mers (CYCI)::lacZ]) as the primary strain and Y190 (MATa, ura3-52, his3-200, leu2-3, 112, gal-4, trp1-901, URA3::[GAL 17-mers (CYCI)::lacZ]) as the secondary screening strain. Yeast were grown in YPD media until transformation. After transformation the yeast were maintained in the appropriate selection media. The p29 form of caspase-3β was removed from the plasmid pMCH1 and cloned in-frame to the Gal4 binding domain in pGBT9 at the EcoRI and BamHI sites. The active site cysteine to alanine mutant of caspase-3 was constructed by a PCR strategy which involved the use of a complimentary set of 3′- and 5′-active site mutant primers. In brief, the 5′-mutant primer was mixed with a 3′-wild type primer in one reaction and a 3′-mutant and a 5′-wild type primer in a second reaction with the wild type caspase-3 gene contained in the vector pK9. In a third reaction, 5 μl of each of the first two reactions were mixed and the 5′- and 3′-wild type primers were extended. The DNA PCR product was cut with EcoRI and BamHI and cloned directly into pGBT9. The mutation was confirmed by DNA sequence analysis.

A yeast two-hybrid screen was constructed by cutting the full-length cDNA at the unique HindIII site at base 417 within the coding region and at a unique BamHI site that had been incorporated at the 3′-end of the clone after the end of the coding region. An EcoRI-HindIII linker was used to place this fragment in-frame with the binding domain in pGBT9. pGBT9 has a selectable trp 1 marker and fuses the Gal4 binding domain protein with the bait protein. Caspase-3 was also cloned into pGAD424. pGAD424 contains a selectable leu2 marker and fuses the Gal4 activating domain to an in-frame sequence. Human leukemia, fetal brain, and lymphocyte Matchmaker cDNA libraries were also supplied by CLONTECH. These libraries all expressed the cDNA fused to the Gal4-activating domain protein. The cDNAs were at least 600 base pairs in length.

A biological screen entailed coexpression of an HFF7C with 100 μg of amplified library plasmid DNA and 100 μg of pGBT9-caspase-3 or pGBT9-caspase-3. Colonies expressing the His3 reporter gene, and thus interacting proteins, were selected by rapid growth on agar plates that did not contain leucine, tryptophan, or histidine. Positive colonies were screened in a secondary filter-lift assay for expression of the LacZ gene (23). The binding domain plasmid in the β-galactosidase-positive colonies was lost by growth in media without leucine. Colonies that had lost the binding domain plasmid were selected by loss of the ability to grow on agar lacking leucine and tryptophan. The DNA from these colonies was extracted and propagated in the Escherichia coli strain DH1S. The DNA was then sequenced using the Perkin-Elmer ABI Prism automated sequencing kit (Perkin-Elmer) according to manufacturer’s instructions. The sequencing results were compared with the sequences in the GenBank database using the BLAST algorithm to identify sequence homologies. The length, and at times the identity of the obtained clones, was determined by PCR. PCR analysis was performed using the Perkin-Elmer Amplitaq kit according to the manufacturer’s instructions.

Expression of Caspase-3 and Caspase-1—The His-tagged, 29-kDa form of caspase-1 was expressed from the cDNA cloned into the E. coli expression vector pMCH-1 as described previously (24). The vector was maintained in the host strain MM294A and propagated at 28–30 °C to avoid induction of the protein. Cells were grown in a 2-liter fermentor in Superbroth (Digene Diagnostics Inc., Beltsville, MD) supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin to an optical density at 600 nm of 9. Expression of the protein was induced by rapidly raising the temperature to 40 °C for 100 min. Full-length caspase-1 was cloned into the E. coli expression vector pMCH-1, maintained in MM294A, and expressed as above.

Purification of the His-tagged caspase-3 was accomplished by resuspending the pellet cells in 25 ml of buffer containing 50 mM HEPES, 0.2 mM NaCl, 10% glycerol, 0.1% CHAPS, 0.2 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 10 μM pepstatin at pH 7.5. The cell suspension was stirred at 4 °C until smooth and then lysed in a French press at 18,000 p.s.i. The lysate was clarified by centrifugation at 31,000 × g for 30 min and at 4 °C. The supernatant was stirred with an equal volume of lysate buffer and loaded onto a column packed with 10 ml of Ni-NTA resin (Qiagen, Chatsworth, CA) that had been previously equilibrated in lysate buffer. The column was washed with lysate buffer containing 5 mM Imidazole and then again with lysate buffer containing 25 mM Imidazole. The protein was then eluted with five, 10-ml aliquots of lysate buffer containing 125 mM Imidazole. All fractions were collected and analyzed by SDS-PAGE before pooling peak fractions and quantitating protein concentration and enzyme activity. The enzyme was stored frozen at −80 °C. Caspase-3 was purified essentially as described by Thorneycroft (25).

Enzymatic Activity Assays—Caspase-3 activity was assayed by monitoring the release of p-nitroaniline from the synthetic substrate Ac-DEVD-pNA at 380 nm. The total reaction volume was 200 μl and contained HGE buffer (100 mM HEPES, pH 7.4, 20% glycerol, 0.1 mM EDTA), 5 mM DTT, 0.05% BSA, 100 μM Ac-DEVD-pNA, and 5 mM caspase-3. Inhibition of caspase-3 activity by PP2A was measured by varying the concentration of PP2A holoenzyme (Upstate Biotechnology
Inc., Lake Placid, NY) in each of the wells of a microtiter dish. The reactions were initiated by the addition of the caspase-3. The assays proceeded for 30 min and were linear throughout the entire time course.

Inhibition of Caspase-3-mediated PARP Cleavage by PP2A—The cDNA for PARP, contained on the T7 driven expression vector pKK, was in vitro transcribed and translated using the T7-coupled reticulocyte lysate system (Promega Corp., Madison, WI) according to the manufacturer’s protocol. [32P]Metionine (1000 Ci/mmol, Amershams Pharmacia Biotech) was substituted for cold methionine in the reaction and resulted in the production of approximately 15 μg of [32P]-labeled PARP. The labeled PARP was diluted 75-fold with HGE and used without further purification. In a 20-μl reaction 15 μl of caspase-3 (1 μM) was mixed with 2 μl of caspase-3 (to give a final concentration of 500 μg of caspase-3), 0.5 mg/ml BSA in HGE containing various concentrations of PP2A in a total of 3 μl of PP2A buffer (50 mM HEPES, 50% glycerol, 1 mM EDTA, 0.1% 2-mercaptoethanol, pH 7.5). The reactions were incubated at 30 °C for 90 min and quenched by addition of an equal volume of 2× SDS-PAGE sample buffer (Integrated Separation Systems, Natick, MA) and then heated for 10 min at 95 °C. The quenched reaction was loaded onto a 10–20% denaturing acrylamide gradient gel and electrophoresed in a Tris-Tricine buffer system (Novex, San Diego, CA). The gel was dried and imaged using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the cleavage products were quantitated by densitometry using the PhosphorImager software. Data are expressed as a percent of the 32 9- or 25-kDa products found in the control reaction with no PP2A.

Cleavage of PP2A by Caspase-3—PP2A holoenzyme (1 μg), isolated from rabbit skeletal muscle (Upstate Biotechnology Inc, Lake Placid, New York), or 10 μg of recombinant A subunit (26) was incubated in 100 μl of modified HGE buffer (containing only 5% glycerol) and 5 mM DTT and initiated by the addition of 40 nM caspase-3 or caspase-1 (as a negative control) at time 0 or after a 15-min preincubation of the phosphatase. Control reactions either minus PP2A or minus caspase-3 were also included. The incubations were carried out at 37 °C for 60 min, and the reaction was terminated by the addition of 5 volumes of acetone. The samples were chilled at −20 °C for 2 h and then centrifuged at 14,000 × g for 30 min. The samples were resuspended in 20 μl of 2× SDS-PAGE sample buffer, electrophoresed as above, and transferred to nitrocellulose or PVDF membranes. For immunostaining, nitrocellulose membranes were blocked with a mixture of nonfat dried milk and bovine serum albumin (2% of each) in phosphate-buffered saline that contained 0.05% Tween 20. The PP2A regulatory A subunit was visualized by probing with a 10,000-fold dilution of the monoclonal antibody H2G3, followed by enzyme-linked chemiluminescence. Membranes were washed with phosphate-buffered saline that contained 0.05% Tween 20. This antibody was previously demonstrated to recognize the 15th repeat in the C-terminal portion of the regulatory A subunit (27). For N-terminal sequencing, the PVDF membranes were washed with phosphate-buffered saline that contained 0.05% Tween 20. This antibody was previously demonstrated to recognize the 15th repeat in the C-terminal portion of the regulatory A subunit (27). For N-terminal sequencing, the PVDF membranes were washed with phosphate-buffered saline that contained 0.05% Tween 20. This antibody was previously demonstrated to recognize the 15th repeat in the C-terminal portion of the regulatory A subunit (27).

Phosphatase Assays—PP2A activity was determined in one of two ways. Assay-3 on purified PP2A activity from human red blood cells was determined using a Ser/Thr phosphatase assay kit (Boehringer Mannheim, Indianapolis, IN). The enzyme to the 25-μl reaction was added to 2 munits/ml and resulted in the production of approximately 15 μg of 32Pi released from 32P-labeled phosphorylase C. Phosphatase activity in cell lysates was performed by the method of Cohen et al. (28) and involved the quantitation of trichloroacetic acid-soluble 32Phosphate released from 32P-labeled phosphorylase a. Treated cells were harvested from a 96-well tissue culture dish by centrifugation and then snap-frozen in liquid nitrogen. The cell pellets were resuspended in 100 μl of assay buffer that contained 60 mM HEPES, pH 7.2, 2 mM EDTA, 2 mg/ml glycogen, 2% 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 μg/ml aprotinin and then lysed by sonication. Duplicate assays were run in the presence and absence of 3 mM okadaic acid. The 10-min reactions were initiated by the addition of [32P]phosphorylase a and quenched by the addition of 90 μl of ice-cold 20% trichloroacetic acid. Samples were chilled for 10 min, and the insoluble protein was cleared by centrifugation at 14,000 × g for 2 min. Half of the soluble fraction was counted in a scintillation counter. All experiments were repeated three times. PP2A activity was determined by subtracting the counts from the okadaic acid-insensitive fraction from the total counts in the soluble fraction. In these experiments, at least 95% of all measured phosphatase activity was due to PP1 and PP2A activity as determined in a preliminary experiment using 500 nm okadaic acid which will totally inhibit both PP1 and PP2A (29).

Apoptosis Assays—Jurkat cells were seeded at a concentration of 1 × 106 cells/ml and grown overnight in RPMI 1640 media supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (100 units/ml and 10,000 μg/ml, respectively). Cells were harvested by centrifugation and aliquoted into a 96-well tissue culture dish at a density of 5 × 103 cells per 200 μl. Cells were treated with anti-Fas antibody (63 μg/ml) (Upstate Biotechnology Inc., Lake Placid, NY) with or without DEVD-fmk, FA-FMK (Enzyme Systems Products, Dublin, CA), or DEVD-cho (Bachem Biosciences Inc., King of Prussia, PA) added at time 0, or at various times after anti-Fas addition. Samples were harvested at various times after the addition of anti-Fas antibody. Cells were analyzed for protein phosphatase activity, as described above, and the ability to metabolize the dye AlamabBlue (Alamar Bio-Sciences, Sacramento, CA). After incubation, 10% volume of AlamarBlue dye was added to each well of the 96-well plate. The plates were incubated for 3 h at 37 °C. The reaction was monitored at an excitation wavelength of 584 nm and an emission wavelength of 512 nm, on a fluorometric plate reader ( Molecular Devices, Sunnyvale, CA).

Cell lysates were also examined for the presence of PP2A and MAP kinase. In brief, cells from two independent wells were pooled and harvested by centrifugation at 1000 × g for 2 min. The cells were resuspended in 2× SDS-PAGE buffer, and proteins from 106 cells were fractionated on denaturing 10–20% Tris-Tricine polyacrylamide gels. Proteins were transferred to nitrocellulose or PVDF (Novex, San Diego, CA) membranes and probed as described above for protein phosphatase 2A using an N-terminal recognizing antibody (Upstate Biotechnology, Lake Placid, NY). Total MAP kinase (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and active MAP kinase (Promega Inc., Madison, WI) antibodies were used at a dilution of 1:2000. Visualization of the proteins was accomplished by enzyme-linked chemiluminescence (Amershams Pharmacia Biotech). Quantitation of imaged bands was performed with Bio-Rad Molecular Analyser image analysis software (Bio-Rad).

RESULTS

Yeast Two-hybrid Screening—In a study by Estojak et al. (30), it was demonstrated that protein interactions with disso- ciation constants as weak as 1–20 μM could be detected by yeast two-hybrid methodology. Recently, Margolin et al. (31) determined that the Kd of pre-IL-1β for caspase-1 is 4 μM. Based on these data, an attempt was made to identify substrates for the members of the caspase family with the yeast two-hybrid system. Positive interactions in the yeast two-hybrid system acti- vate transcription of the His3 and LacZ genes. p32 caspase-1 and p29 caspase-3 were expressed in the pGBT9 vector as the mature Gal4 binding domain fusion protein and used as the bait in separate screens. Gal4 binding domain fusion proteins were screened against Gal4 activating domain fusion libraries as well as with themselves cloned into activating domain vec- tors. Transformation with caspase-1- and caspase-3-containing vectors produced no toxic effects and would routinely transform Hf7C with an efficiency between 104 and 1.5 × 104 colonies/μg DNA. Cotransformation frequencies of caspase and random libraries varied but were usually between 105 and 2 × 106 colonies/μg DNA.

In the first screen, caspase-1 was cotransformed with a phor- bide-12-myristate 13-acetate-stimulated human leukemia cDNA library, subcloned IL-1β expression is induced under the con- ditions (32). Over 3 × 108 clones were screened on LTH- plates. Out of 23 LTH-positive clones, six were also positive in the β-galactosidase filter assay, and all six clones contained cDNA inserts encoding pre-IL-1β. From this screen, it was apparent that the two-hybrid screen methodology was sensitive enough to pick up the interaction between an enzyme and its substrate.
A cross-screen cotransforming HF7C with a vector containing caspase-1 in the binding domain construct, and caspase-1 and caspase-3 in the activating domain construct, revealed that caspase-1 interacted with both itself as well as caspase-3. This is consistent with previously reported data (33) demonstrating that caspase-1 can interact with itself and caspase-3 either through a dimer-dimer or enzyme-substrate interaction. These data demonstrate that these proteases are behaving in the yeast two-hybrid system consistent with interactions that have been observed using purified enzyme systems.

An unstimulated lymphocyte cDNA library was utilized in a second screen using p32 caspase-1 fused to the Gal4-binding domain. This library minimized the chance of picking up interactions with pre-IL-1β since, in the absence of stimulation, the level of transcription of pre-IL-1β is very low in lymphoid tissue (25). Out of 1.5 million clones screened, 103 were LTH1 and only 9 were also β-galactosidase+. PCR analysis using primers to the 3′- and 5′-ends of human pre-IL-1β revealed that none of the nine clones contained sequences expressing this cDNA (data not shown).

The initial results with the caspase-1 screen suggested the possibility of identifying substrates for the other caspase family members using the yeast two-hybrid system. Thus, the 29-kDa form of caspase-3 was cloned into the Gal4-binding domain vector for expression of the Gal4 fusion protein in yeast. A human lymphocyte cDNA library was screened utilizing the caspase-3 fusion protein as the bait. Several cotransformations were necessary in order to screen a representative portion of the cDNA library. Further support for validity of utilizing the yeast two-hybrid system for detecting interactions with caspase family members came from this random screen. In this instance, one of the interacting proteins that came out of this random screen was caspase-3 itself. It is known from the crystal structure that this enzyme forms heterodimers with itself (34).

A total of 2.1 × 10⁶ colonies were screened from this lymphocyte library, and a match to the regulatory A0 subunit of protein phosphatase 2A came up three independent times. Upon DNA sequence analysis it was demonstrated that the entire sequence of the regulatory subunit was obtained, except for the first 5′, 63–72 nucleotides (depending on the clone). Cotransformation of yeast with this cDNA and either caspase-3 or caspase-1 revealed an efficient interaction between caspase-3 and the PP2A A0 subunit but no interaction between this cDNA and caspase-1. To examine the interaction between caspase-3 and the A0 subunit of protein phosphatase 2A further, an active site cysteine to alanine mutant was created and used in cotransformations with the PP2A regulatory subunit. In these experiments, the transformation efficiency of the mutant was 50% less than the wild type. This is suggestive of a dysfunctional catalytic complex and thus a dissociation constant that is weaker than that for the wild type caspase-3.

Because of the high occurrence, and the importance of such an interaction if it were biologically relevant, the interaction between caspase-3 and PP2A was examined more closely. An analysis of the full-length amino acid sequence of the A0 regulatory subunit (35) reveals a caspase-3 DXXD consensus sequence, [DEQD]²¹⁵²¹⁸ (36). This cleavage site would be contained within the regulatory domain of the PP2A regulatory subunit. This cleavage is consistent with previously reported data (33) demonstrating that the phosphatase was preincubated for 15 min in the absence of caspase. When visualized with antibodies directed against the 65-kDa regulatory subunit, a 42-kDa C-terminal fragment of the regulatory subunit could be observed (Fig. 1). The 42-kDa piece is the correct calculated size for the C-terminal fragment of the regulatory subunit. This cleavage is consistent with cleavage occurring at the putative cleavage site, DEQD. The regulatory subunit of PP2A purified from rabbit skeletal muscle also cleaves at this same point (data not shown), thus the cleavage site is present in at least one other species. N-terminal sequencing of the 42-kDa cleavage piece yielded the sequence, [DEQD]²¹⁵²¹⁸ SVRLAVEACVNIAQ²³¹, which confirms the site of digestion of the A subunit by caspase-3. In addition sequence analysis demonstrated that cleavage also occurred at the N terminus of the protein after Asp9. Whereas preincubation of the PP2A holoenzyme was necessary in order for appreciable cleavage to occur, this was not true for the recombinant A subunit. The likely reason is that DEQDS is covered by the regulatory B subunit in the holoenzyme that binds to repeats 1–10. Since caspase-3 can only cleave the site when it is uncovered, the preincubation gives the B subunit time to dissociate. Caspase-1 did not cleave the regulatory subunit with or without the preincubation of PP2A.

Caspase-3 and PP2A—The in vitro cleavage of purified PP2A was examined utilizing catalytic quantities of purified, recombinant caspase-3. Since the amount of activated caspase-3 in the cell is probably small, it would not be relevant to use large quantities of enzyme which might artificially cleave any DXXD motif. Therefore, all of the in vitro studies in this report used concentrations of caspase-3 in the range of 4–40 nM.

Caspase-3 Cleaves PP2A—To examine the ability of caspase-3 to cleave PP2A, 40 nM caspase-3 was incubated with PP2A for 1 h. Caspase-1 was used as a negative control, since this caspase family homolog prefers a hydrophobic amino acid in the p4 pocket and thus should not cleave the regulatory subunit A0 at this site. In preliminary experiments, it was determined that maximal cleavage of PP2A by caspase-3 was observed within 2 h. In addition, it was observed that the order of addition of the phosphatase and the protease was important. Thus the phosphatase was preincubated for 15 min in the absence of caspase. When visualized with antibodies directed against the 65-kDa regulatory subunit, a 42-kDa C-terminal fragment of the regulatory subunit could be observed (Fig. 1). The 42-kDa piece is the correct calculated size for the C-terminal fragment of the regulatory subunit. This cleavage is consistent with cleavage occurring at the putative cleavage site, DEQD. The regulatory subunit of PP2A purified from rabbit skeletal muscle also cleaves at this same point (data not shown), thus the cleavage site is present in at least one other species. N-terminal sequencing of the 42-kDa cleavage piece yielded the sequence, [DEQD]²¹⁵²¹⁸ SVRLAVEACVNIAQ²³¹, which confirms the site of digestion of the A subunit by caspase-3. In addition sequence analysis demonstrated that cleavage also occurred at the N terminus of the protein after Asp9. Whereas preincubation of the PP2A holoenzyme was necessary in order for appreciable cleavage to occur, this was not true for the recombinant A subunit. The likely reason is that DEQDS is covered by the regulatory B subunit in the holoenzyme that binds to repeats 1–10. Since caspase-3 can only cleave the site when it is uncovered, the preincubation gives the B subunit time to dissociate. Caspase-1 did not cleave the regulatory subunit with or without the preincubation of PP2A.

Caspase-3 and PP2A—The in vitro cleavage of purified PP2A was examined utilizing catalytic quantities of purified, recombinant caspase-3. Since the amount of activated caspase-3 in the cell is probably small, it would not be relevant to use large quantities of enzyme which might artificially cleave any DXXD motif. Therefore, all of the in vitro studies in this report used concentrations of caspase-3 in the range of 4–40 nM.

Caspase-3 Cleaves PP2A—To examine the ability of caspase-3 to cleave PP2A, 40 nM caspase-3 was incubated with PP2A for 1 h. Caspase-1 was used as a negative control, since this caspase family homolog prefers a hydrophobic amino acid in the p4 pocket and thus should not cleave the regulatory subunit A0 at this site. In preliminary experiments, it was determined that maximal cleavage of PP2A by caspase-3 was observed within 2 h. In addition, it was observed that the order of addition of the phosphatase and the protease was important. Thus the phosphatase was preincubated for 15 min in the absence of caspase. When visualized with antibodies directed against the 65-kDa regulatory subunit, a 42-kDa C-terminal fragment of the regulatory subunit could be observed (Fig. 1). The 42-kDa piece is the correct calculated size for the C-terminal fragment of the regulatory subunit. This cleavage is consistent with cleavage occurring at the putative cleavage site, DEQD. The regulatory subunit of PP2A purified from rabbit skeletal muscle also cleaves at this same point (data not shown), thus the cleavage site is present in at least one other species. N-terminal sequencing of the 42-kDa cleavage piece yielded the sequence, [DEQD]²¹⁵²¹⁸ SVRLAVEACVNIAQ²³¹, which confirms the site of digestion of the A subunit by caspase-3. In addition sequence analysis demonstrated that cleavage also occurred at the N terminus of the protein after Asp9. Whereas preincubation of the PP2A holoenzyme was necessary in order for appreciable cleavage to occur, this was not true for the recombinant A subunit. The likely reason is that DEQDS is covered by the regulatory B subunit in the holoenzyme that binds to repeats 1–10. Since caspase-3 can only cleave the site when it is uncovered, the preincubation gives the B subunit time to dissociate. Caspase-1 did not cleave the regulatory subunit with or without the preincubation of PP2A.
Caspase-3 prebound with DEVD-cho and DEVD-cho (1 nM) were used to cleave and inactivate a regulatory subunit A of phosphatase activity. These data are consistent with the presence of the regulatory subunit, there is a significant basal phosphatase activity by itself. It should be noted that even in the absence of caspase-3, the inhibitor Ac-DEVD-cho inhibited the stimulation observed by addition of caspase-3. The inhibitor did not influence phosphatase activity by itself. These data indicate that PP2A activity should be observed after incubation with caspase-3, provided a suitable substrate is being used. In this assay, PP2A activity was measured by the release of Pi from the synthetic PP2A substrate KIpTIRR. The phosphatase activity assay was linear over 30 min with this substrate (data not shown). Upon incubation of 40 nM caspase-3 with PP2A for 20 min, a 40% stimulation of PP2A activity over the basal level was observed (Fig. 2). This stimulation was not observed by incubation with 40 nM caspase-1. Prebinding caspase-3 with the inhibitor Ac-DEVD-cho inhibited the stimulation observed by addition of caspase-3. The inhibitor did not influence phosphatase activity by itself. It should be noted that even in the presence of the regulatory subunit, there is a significant basal level of phosphatase activity. These data are consistent with the cleavage and inactivation of a regulatory subunit Aα by caspase-3.

The Interaction between Caspase-3 and PP2A during Apoptosis—In order to determine if the interaction between PP2A and caspase-3 is biologically relevant, a series of experiments in Jurkat T cells, induced to undergo apoptosis by ligation of the Fas receptor by anti-Fas antibody, were performed. Jurkat cells were used because caspase-3 is highly expressed and has been shown to be activated in cells of lymphocytic origin (37, 38). This suggests the potential for caspase-3 playing a pivotal role in cells induced to undergo apoptosis. The caspase-3 inhibitors DEVD-fmk and DEVD-cho were used to link the interaction of caspase-3 with PP2A in this cell-based system. YVAD-cho and FA-FMK were used as negative control peptides. The first set of peptides have been demonstrated previously to inhibit anti-Fas-induced apoptosis in Jurkat cells (39, 40). In these studies, apoptosis was monitored by metabolism of the dye AlamarBlue (41). Recently, Vasilakos et al. (42), demonstrated that cell death data obtained with AlamarBlue correlated well with data obtained in the same experiment by monitoring trypan blue exclusion as an index of cell viability. In addition, positive correlation between AlamarBlue and several other parameters of apoptosis, such as caspase-3 activation, DNA laddering, and nuclear condensation, were demonstrated.

The data presented in Fig. 3 are representative of three separate experiments. After 6, 12, and 24 h, cells treated with anti-Fas antibody (63 ng/ml) demonstrated a significant decrease in the ability to metabolize AlamarBlue dye (30, 50, and 70%, respectively) (Fig. 3A). This apoptosis was totally inhibited by 20 μM DEVD-fmk and 100 μM DEVD-cho at 6 and 12 h. Little protective effect was seen in cells incubated with the control peptide FA-FMK; thus, this is not a nonspecific effect of an irreversible modifying agent. Little decrease in the ability to metabolize the dye could be seen at the 3-h time point (data not shown). This suggests that in this model system, cell death begins between 3 and 6 h after treatment with anti-Fas antibody. By 24 h, DEVD-cho showed a decrease in its effectiveness at blocking apoptosis. Since DEVD-fmk is an irreversible inhibitor, it is possible that it is modifying other caspase family members inside the cell, which could account for the differential effectiveness between DEVD-cho and DEVD-fmk. In the absence of anti-Fas antibody, the inhibitors by themselves did not appear to have any negative effect on the ability of the cells to metabolize the dye. In fact at 6 h post anti-Fas treatment, the inhibitors actually appeared to significantly stimulate the metabolism of the AlamarBlue dye.

In order to correlate the increase in phosphatase activity with the onset of apoptosis, a study was done to determine the time at which the cells become committed to apoptosis. This was done by the induction of apoptosis at time 0 and rescuing cells from apoptosis by the addition of either 100 μM DEVD-cho or YVAD-cho at times 0, 3, 6, and 12 h after the addition of anti-Fas antibody. The cells were then incubated for an additional 6 h before the addition of AlamarBlue dye in order to allow the cells time to recover. When added at time 0, cells were completely rescued from apoptosis by DEVD-cho only. In this study, YVAD-cho had no ability to prevent apoptosis at any time (Fig. 3B). However, partial rescue of cells occurred when DEVD-cho was added after 3, 6, and 12 h post anti-Fas antibody. These data also demonstrate that after 3 h 13%, 6 h 16%, and after 12 h only 29% of the cells were actually committed to apoptosis versus 50% of the cells that died with no inhibitor treatment after 18 h.

If caspase-3 is induced during anti-Fas-induced apoptosis, and the regulatory subunit Aα of PP2A is cleaved, then upon induction of cell death at least a transient increase in PP2A activity should be seen. By 6 h an approximate 2-fold increase in total Ser-Thr phosphatase activity could be demonstrated. By subtraction of the okadaic acid inhibitable activity (PP1 activity), the portion of the radioactive counts due to PP2A activity could be demonstrated. This suggests that the total increase in phosphatase activity was due to PP2A, since no effect could be seen for PP1 activity. By 12 h there was a smaller (3.5-fold) but significant increase in PP2A activity (Fig. 3D) and a 45% decrease in PP1 activity. By 24 h (Fig. 3E), only a modest (40%) increase in the activity of PP2A could be observed. Again, there was no significant effect on PP1 activity in inhibited versus noninhibited cells, although there was a downward trend in PP1 activity over time. This increase in PP2A activity was inhibited by both DEVD-cho and DEVDFmk at each time point examined. The inhibitors themselves had no significant effect on PP2A activity. The time course for the increase in PP2A activity correlated with commitment of cells to apoptosis.

These data are consistent with the hypothesis that after the regulatory A subunit is cleaved, there is an up-regulation of PP2A catalytic activity. However, to demonstrate a more direct
link between caspase-3 and PP2A, whole cell lysates were probed with antibodies to PP2A. This was done to demonstrate cleavage of the regulatory A subunit in the anti-Fas-treated cell lysates. The antibody to the regulatory A subunit nonspecifically recognized a 55-kDa protein, as well as the 65-kDa regulatory subunit. In cells treated with anti-Fas antibody for 12 and 24 h, the regulatory subunit of PP2A could no longer be observed, whereas cells treated with anti-Fas antibody plus...
inhibitor retained their intact regulatory subunit (Fig. 4). It is not known why the cleavage fragment could not be detected. Attempts to visualize the cleavage fragment with the C-terminal recognizing antibody yielded the same result as with the N-terminal recognizing antibody (data not shown). It is possible that once cleaved, the regulatory subunits fragments dissociate from the catalytic subunit and are then rapidly degraded. A slight effect could be demonstrated in cells treated for 6 h. This suggests that removal of only a small proportion of the A subunit could result in a significant change in PP2A activity. At these same times, levels of the catalytic subunit remained unchanged.

A role for PP2A in the MAP kinase pathway has been demonstrated (43–45). The increase in PP2A activity would result in a decrease in the pool of phosphorylated, active MAP kinase. If the PP2A activity increases during apoptosis, then the total amount of active MAP kinase should decrease as well, with the total pool of MAP kinase protein remaining unchanged. Probing a cell lysate with antibodies directed against either activated or total MAP kinase demonstrated a decrease in the amount of phosphorylated MAP kinase in cells undergoing apoptosis (Fig. 5). Presumably, the small amount of activated MAP kinase observed in these cells was due to the presence of residual growth factors in the medium. This small amount of activated MAP kinase is observed by others (43, 46). Since the signal was small the blots were quantitated. The decrease in activated MAP kinase was partially inhibited by the caspase-3 inhibitor DEVD-fmk. The total pool of MAP kinase is unchanged after 12 h of incubation with anti-Fas. Again, this indicates that nonspecific proteolysis is not a cause of the differences in the presence of the activated MAP kinase band.

DISCUSSION

These studies were stimulated by the desire to know and understand more about the biology and the natural substrates for several of the members of the caspase family. There have been many reports of substrates for caspase-3, with the most common one being PARP. The evidence supporting this, however, is indirect. During programmed cell death, the cell undergoes changes in morphology as well as metabolism. What has not been elucidated to date is the link between the caspase family and the signal transduction process that could lead to the changes observed in the cell during apoptosis. Since there is a delicate balance in the cell between life and death, it seems possible that any protein with a role in cell proliferation might also, if stimulated or destroyed, play a role in apoptosis.

A recent report by Martins et al. (47) indicates that caspase-3 may be found in the nucleus of the cell during apoptosis. This translocation to the nucleus may be the method by which PARP is cleaved. However, the cleavage of this DNA repair enzyme may not be sufficient to cause the morphological and metabolic changes that the cell undergoes during apoptosis. Indeed, mice that have had the PARP gene deleted appear to develop normally, unlike the mice where caspase-3 has been deleted (9).

A recent report by Ghaury et al. (48) reported that caspase-3 cleaved protein kinase C-δ, which resulted in up-regulation of its activity. Overexpression of the active catalytic kinase fragment in cells is associated with chromatin condensation, nuclear fragmentation, induction of sub-G₁ phase DNA, and lethality. These pieces of information would indicate that caspase-3 may interact in a pathway that is more central to cellular metabolism. In order to answer the question of what additional cellular substrates caspase-3 might interact with directly, a random yeast two-hybrid screen was performed with caspase-3 acting as the bait.

Until now, substrates of caspase-3 have been identified mostly by indirect evidence indicating that they are cleaved during apoptosis (49). The best characterized interaction is that between PARP and caspase-3, detailed in recent publications by Margolin et al. (31) and Nicholson et al. (6). DNA-dependent protein kinase catalytic subunit has also been demonstrated to be cleaved by caspase-3 in the cell (8, 50). Additional putative substrates are comprehensively reviewed in a recent report by Matsuda et al. (51).
article by Schwartz and Milligan (51). The yeast two-hybrid system, however, is a random selection method that should be able to directly find proteins that interact with caspases.

When caspase-3 was used as the bait in several random screens, an interaction between this protease and the regulatory subunit Aα of protein phosphatase 2A was found. Upon examination of the amino acid sequence for this protein, the recognition sequence for a caspase-3 cleavage site (DXXD) was found (DEQD specifically). This sequence is similar to the cleavage sequence found in PARP (DEVD) and many of the other putative caspase-3 substrates. This sequence is also comparable to the optimal cleavage sequence suggested by the work of Talianian et al. (36). Upon analysis of all entries in the Entrez protein data base for the PP2A 65-kDa regulatory subunit Aα, the DXXD cleavage site is conserved in frog and pig as well as in rabbit and human.

The data presented in this report demonstrate that PP2A is a substrate for caspase-3. PP2A will compete with either small synthetic substrates (like Ac-DEVD-pNA) or protein substrates (like PARP) for caspase-3. Additionally, caspase-3 was shown to cleave the PP2A regulatory A subunit (Fig. 1). This cleavage resulted in approximately a 40% increase in phosphatase activity in vitro after 20 min. The PP2A core and holoenzymes are similarly abundant in cells but differ in substrate specificity (27). The holoenzyme is more active than the core enzyme toward substrates phosphorylated by cyclin-dependent kinases, and the core enzyme is more active against these substrates than the free C subunit. Thus, with the cyclin-dependent kinase substrates, the A and B subunits have a stimulatory effect on the C subunit. On the other hand, the core enzyme is equally or more active than the holoenzyme toward most other substrates, indicating that the B subunit (Bo being the most frequently studied) is inhibitory (52–56). With respect to core enzyme and free catalytic subunit, the situation is also complex. Depending on the substrate size, type of substrate, and reaction conditions, the A subunit either inhibits or stimulates the catalytic subunit (57).

Since the various forms of PP2A differ so markedly in substrate specificity, it is important to discuss how accessible the core and holoenzymes are to cleavage by caspase-3. According to the previous model of PP2A (58, 59), the B subunit binding region on the A subunit includes the caspase-3 recognition sequence DEQD in repeat 6 of the A subunit. This was suggested by the finding that mutation of this sequence destroyed binding of the B subunit to the A subunit, whereas binding of the C subunit was intact (58). Therefore, we would predict that in holoenzyme, the A subunit is protected by the B subunit from cleavage by caspase-3, whereas in core enzyme it can be cleaved. This explains the observation that cleavage of the holoenzyme, but not of free A subunit, by caspase-3 required preincubation, resulting in dissociation of the B subunit from the holoenzyme and generation of core enzyme. The finding that the A subunit completely disappeared from cells after induction of apoptosis, whereas C subunit was stable, suggests that holoenzyme was slowly converted to core enzyme and that all core enzyme was cleaved by caspase-3. It may not be surprising that the generation of a high amount of free C subunit, which normally does not exist in cells, has dramatic effects on cell growth, signal transduction, and apoptosis.

In order to answer the question of relevancy in the cell, we examined the activity of phosphatases during anti-Fas antibody-induced apoptosis in Jurkat cells. The ability of several caspase family inhibitors to block apoptosis was correlated with their ability to block the increase in PP2A activity. These data show that by 6 h of anti-Fas treatment, cells had reduced ability to metabolize AlamarBlue and PP2A activity had increased 4.5-fold when compared with untreated control cells. In fact, after 6 h, cells treated with only inhibitor demonstrated a significant increase in their ability to metabolize the dye. This suggests that these cells may have a basal amount of apoptosis which is inhibited by the caspase-3 inhibitors. The fact that this increase in phosphatase activity is completely inhibited by both DEVD-fmk and DEVD-cho suggests the involvement of caspase family members in this up-regulation of PP2A activity. In this cell model, PP1 activity was not up-regulated by either anti-Fas or inhibitor.

These data partially agree with the data of Morana et al. (20), demonstrating that several inhibitors of serine/threonine phosphatases, including okadaic acid, will also completely inhibit etoposide-induced apoptosis. However, they concluded that a decrease in phosphorylation of a PP1 substrate, Rb...
protein, during apoptosis was not due to up-regulation of PP1 activity. They did not observe an increase in PP2A activity either. In addition they did observe a decrease in the level of PP1 protein during apoptosis without subsequent loss of PP1 activity. It is possible that the one time point at which they examined phosphatase activity was insufficient, since this is likely a temporal response and relies on the model system being tested. In fact, in the studies reported here, by 24 h after anti-Fas administration, the PP2A activity was not significantly elevated compared with control. Song and Lavin (19) have also demonstrated that inhibitors of PP1 and PP2A (calcium and okadaic acid) will prevent apoptosis in a Burkitt’s lymphoma cell line. The authors concluded that there was a role for up-regulation of phosphatase activity in apoptosis. Wolf et al. (60) reported a correlation between Rb dephosphorylation as an index of phosphatase activity and apoptosis. In that report, PARP cleavage, intracellular acidification, and DNA digestion, all indicators of apoptosis, were inhibited by the caspase family inhibitor, benzamido-carbonyl-VAD-fmk. However, Rb dephosphorylation was not prevented by this same inhibitor. This observation suggested that the phosphatases play a role upstream of the caspases. However, since VAD-fmk is not a specific caspase-3 inhibitor, this does not rule out the ability of caspase-3 to affect directly or indirectly Rb protein and thus lie upstream of the phosphatases. Although many reports have suggested the connection between phosphatases and caspase family members, no direct link has been identified. Therefore, the interaction between caspase-3 and PP2A is significant.

Analysis of cell lysates by antibodies directed to the PP2A regulatory A subunit demonstrated the loss of this subunit after 12 h. In inhibitor-treated cells, there was no loss of this protein subunit. At the same time, there was no decrease in the amount of the catalytic subunit. These data strongly indicate that the up-regulation of PP2A activity is due to removal of the regulatory A subunit. Cleavage and subsequent proteolytic degradation is one explanation for these data. However, due to the lack of visualization of a cleavage fragment, other mechanisms cannot be ruled out. It also suggests that the catalytic subunit is stable in cells in the absence of the regulatory A subunit.

If the effect on PP2A activity observed has biological relevance, a corresponding change should be seen in the phosphorylation state of a PP2A substrate. The caspase-3-inhibitable decrease in the phosphorylation state of only the activated MAP kinase satisfies this requirement. In a recent report by Cardone et al. (61), it was demonstrated that caspases were required to activate apoptosis via the JNK pathway, by cleavage and activation of MEKK-1. These data, in combination with the data reported here, provide convincing support for the involvement of caspases in the regulation of cell signaling events. Caspases appear to work in concert to turn on the stress-activated and turn off the growth factor-activated cell signaling pathways.

Involvement of PP2A in apoptosis would directly link two lines of evidence suggesting that both regulation of the cell cycle and the caspase family play a role in apoptosis. This would also suggest an upstream role in the induction of cell death. Recently, Dou et al. (62) have reported that induction of a protein serine/threonine phosphatase is responsible for the anti-cancer drug-induced Rb hypophosphorylation and consequent G1 arrest and apoptosis in two p53-null human leukemic cell lines, HL60 and U937. Cells unable to hypophosphorylate Rb protein were resistant to drug-induced cell death. In a subsequent paper, An and Dou (63) reported that hypophosphorylated Rb protein is cleaved during DNA damage-induced apoptosis by a caspase-3-like protease. These data strongly suggest a relationship between the caspase-3-like subfamily members and protein phosphatases at both the level of dephosphorylation and cleavage of dephosphorylated substrates. It is intriguing to note that many of the caspase-3 substrates have cleavage sites containing a serine either in or near the P1’ position of the cleavage sequence. These substrates include the regulatory subunit of PP2A, (DEQDS), Rb protein (DSIDS and DEADGS), protein kinase Cδ (DMQDNSS), and GDP dissociation inhibitor protein (DELDs).

The interaction between PP2A and caspase-3 may provide a partial explanation for the phenotype of the caspase-3 knockout mouse, since the proportion of PP2A in brain tissue is much higher in the developing brain than in the adult brain (64). Thus the interaction between caspase-3 and PP2A may be more important in developing brains than in other tissues.

In summary, this is one of the first reports of direct evidence for a caspase-3 substrate. These data support a model where caspase-3 may also act as an upstream initiator of apoptosis. Activation of caspase-3 then causes up-regulation of PP2A activity. This increase in phosphatase activity is carried out by cleavage and inactivation of the regulatory A subunit of PP2A. Our data suggest this causes the hypophosphorylation of the PP2A substrate, MAP kinase. However, this is just one PP2A substrate. As the role of PP2A in the cell is further elucidated, the significance of the interaction with caspase-3 should become apparent. Further work is needed to identify additional critical substrates for both proteolysis and dephosphorylation and to determine what role phosphatase activation plays in the commitment of the cells to apoptosis.

Acknowledgments—We thank Michael Skidmore for providing preliminary data. We also thank Frank Bourbonais for providing PP2A substrate and Drs. Jack Dixon, Hamish Allen, and Daniel Tracey for critically reviewing this manuscript.

REFERENCES

1. Miura, M., Zhu, H., Rotello, R., Hartwig, E. A., and Yuan, J. (1993) Cell 73, 635–660.
2. Orth, K., O’Rourke, K., Salvesen, G. S., and Dixit, V. M. (1996) J. Biol. Chem. 271, 20977–20980.
3. Tewari, M., Quan, L. T., O’Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Pouirier, O. G., Salvesen, G. S., and Dixit, V. M. (1995) Cell 81, 801–809.
4. Tewari, M., Beidler, D. R., and Dixit, V. M. (1995) J. Biol. Chem. 270, 18738–18741.
5. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Pouirier, G. G., and Earnshaw, W. C. (1994) Nature 371, 346–347.
6. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gureau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Iaji, S., Vaudry, H., Lewis, G. G., Munday, S. N., and Poulson, D. E. (1994) J. Biol. Chem. 269, 3401–3408.
7. An, B., Jin, J. R., Lin, P., and Dou, Q. P. (1996) J. Biol. Chem. 271, 2721–2729.
8. Song, Q. Z., Leesmiller, S. P., Kumar, S., Zhang, N., Chan, D. W., Smith, G. C. M., Jackson, S. P., Almenri, E. S., Litwack, G., Khanna, K. K., and Levin, M. F. (1996) EMBO J. 15, 3238–3246.
9. Wang, Z.-Q., Auer, B., Sting, L., Berghammer, H., Haidacher, D., Schweiger, M., and Wagner, E. F. (1995) Genes Dev. 9, 509–520.
10. Casciola-Rosen, L. A., Miller, D. K., Anhalt, G. J., and Rosen, A. (1994) J. Biol. Chem. 269, 30757–30764.
11. Martin, S. J., and Green, D. R. (1995) Cell 82, 349–352.
12. Lazebnik, Y. A., Takahashi, A., Moir, R. D., Goldman, R. D., Pouirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9042–9046.
13. Brancolini, C., Benedetti, M., and Schneider, C. (1995) EMBO J. 14, 5179–5190.
14. An, B., Jin, J. R., Lin, P., and Dou, Q. P. (1996) FEBS Lett. 399, 158–162.
15. Kuida, K., Zheng, T. S., Na, S., Kuan, C.-Y., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R. A. (1996) Nature 384, 36–372.
16. Fraser, A., McCarthy, N., and Evans, G. I. (1995) Curr. Opin. Neurobiol. 5, 71–80.
17. Mumby, M. C., and Walter, G. (1993) EMBO J. 12, 9042–9046.
18. Fields, S., and Song, O. (1989) Nature 340, 245–246.
