Calcineurin Functions in Ca^{2+}-activated Cell Death in Mammalian Cells

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Abstract. Calcineurin is a calcium-dependent protein phosphatase that functions in T cell activation. We present evidence that calcineurin functions more generally in calcium-triggered apoptosis in mammalian cells deprived of growth factors. Specifically, expression of epitope-tagged calcineurin A induces rapid cell death upon calcium signaling in the absence of growth factors. We show that this apoptosis does not require new protein synthesis and therefore calcineurin must operate through existing substrates. Co-expression of the Bcl-2 protooncogene efficiently blocks calcineurin-induced cell death. Significantly, we demonstrate that a calcium-independent calcineurin mutant induces apoptosis in the absence of calcium, and that this apoptotic response is a direct consequence of calcineurin's phosphatase activity. These data suggest that calcineurin plays an important role in mediating the upstream events in calcium-activated cell death.

Apoptosis is an active process by which a multicellular organism eliminates defective, destructive, or redundant cells. This process is invoked in many steps of development, proliferation, immunological tolerance, and disease (Ellis et al., 1991; Martin et al., 1994; Reed, 1994). Genetic studies have uncovered a set of genes regulating the onset of apoptosis during programmed cell death. The *ced-3* and *ced-4* genes of *Caenorhabditis elegans* are necessary for the commitment to programmed cell death. *Ced-9*, which encodes a protein with structural and functional homology to mammalian Bcl-2, counteracts *ced-3* and *ced-4* (Yuan and Horvitz, 1990; Hengartner and Horvitz, 1994). The over-expression of Bcl-2 is thought to contribute to B cell lymphomas by promoting cell survival despite aberrant proliferation signals (Tsujimoto et al., 1984; Bakhshi et al., 1985; Cleary and Sklar, 1985). In experimental systems, over-expression of Bcl-2 has been shown to prevent apoptosis induced by elevated calcium levels, or by c-*myc* and p53 over-expression (Hockenbery et al., 1990; Sentman et al., 1991; Strasser et al., 1991; Reed, 1994).

Many of the stimuli leading to cell death support the view that apoptosis is a mechanism to remove cells which experience inappropriate or contradictory signals, such as in transformation or viral infection (Evan et al., 1992; Debbas and White, 1993; Harrington et al., 1994). Thus c-*myc* over-expression, which is generally associated with cell proliferation, activates cell death in the absence of coincident growth factor stimulation (Evan et al., 1992). The different fates of immature and mature T cells responding to T cell receptor (TCR)[1] activation underscore the complex interactions between apoptotic and growth pathways. Stimulation of the TCR on immature T cells drives them into apoptosis rather than the proliferative state assumed by mature T cells (Smith et al., 1989; Ucker et al., 1989). Moreover, even mature T cells undergo apoptosis when confronted with continuous TCR stimulation, suggesting a wide use of cell death at many stages of the immune response (Singer and Abbas, 1994).

Calcium signaling is upstream of certain pathways that lead to apoptosis, including neuronal cell death via glutamate-induced excitotoxicity and cell death in T cells (Choi, 1992; Reed, 1994). Additionally, calcium ionophores cause apoptosis in a number of experimental systems, implying that elevated intracellular calcium influences the decision to enter apoptosis. The ability of Bcl-2 to block most cases of calcium-induced apoptosis suggests that calcium stimulates a common pathway affecting the commitment to cell death (Barr and Tomei, 1994; Reed, 1994). While the mechanism of Bcl-2 action is uncertain, it has been implicated in antioxidant pathways to prevent the generation of reactive oxygen species associated with cell death (Hockenbery et al., 1993). More recently, Bcl-2 has been implicated in regulation of calcium efflux from the endoplasmic reticulum, and may thereby influence calcium-dependent apoptotic pathways (Lam et al., 1994).

Despite extensive studies linking calcium to cell death, the immediate targets of this calcium flux remain largely unknown. One potential mediator of calcium signaling during apoptosis is the family of calcium-activated phosphatases known as calcineurin (protein phosphatase 2B;

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1. *Abbreviations used in this paper*: CnA, calcineurin A; CsA, cyclosporin A; *HA*, hemagglutinin; TCR, T cell receptor; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated UTP nick end-labeling.

Klee et al., 1988; Guerini and Klee, 1989). We show here that expression of this calcium-activated phosphatase in mammalian cells greatly potentiates the cell death due to calcium signaling. Moreover, a constitutively active, calcium-independent calcineurin mutant completely bypasses the requirement for calcium signaling in this cell death.

Materials and Methods

Cell Culture

BHK-21 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a 5% CO_2 atmosphere at 37°C in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone Labs., Logan, UT), 2 mM glutamine (GIBCO BRL), and 100 μg/ml of penicillin-streptomycin (GIBCO BRL).

Construction of Calcineurin A and B Expression Vectors

The cDNA encoding human calcineurin A (CnA β-isotype; Guerini and Klee, 1989), and human calcineurin B (Guerini et al., 1989) were obtained from a T cell λgt10 library using oligonucleotides as a hybridization probes (Maniatis et al., 1982). CnA was cloned into the mammalian expression vector pCMV1 (Pharmacia, Piscataway, NJ) 200-bp '5-untranslated sequence from human lamin A and an NH_2-terminal influenza hemagglutinin (*HA*)-tag using oligonucleotides and PCR techniques (Heald et al., 1993). CnA lacking the autoinhibitory domain and the CaM binding domain (ΔCnA) was constructed using PCR to introduce a stop codon after N_{407}. CnB was cloned into pCMV1 after the lamin untranslated region but without an epitope tag. The Bcl-2 expression vector, pCMV-*bcl-2*, was a generous gift of Stanley Korsmeyer (Washington University School of Medicine, St. Louis, MO).

DNA Transfections

DNA transfections were performed as described previously (Heald et al., 1993). 16,000 cells were plated onto each coverslip in 400 μl of medium. On day 1, a total of 2 μg of cesium chloride-purified plasmid DNA was added to 30 μl of 0.2 M $CaCl_2$ and precipitated by adding 30 μL of 2× Hepes-buffered saline over 15 s with stirring. After 20 min, 350 μl of complete medium was added to the DNA precipitate, and the mixture applied to the cells. Each coverslip was allowed to incubate in the tissue culture incubator for 4 h. The coverslips were then washed twice with complete medium and returned to the incubator for an additional 12 h.

Immunofluorescence

Cells on coverslips were fixed in 3% formaldehyde (Baker Co., Inc., Sanford, ME) in PBS for 10 min and then washed three times with 0.1% NP-40 (Sigma Chem. Co., St. Louis, MO) in PBS (PBS-NP-40). Primary antibodies were incubated on the coverslips for 30 min, followed by four rapid rinses with PBS-NP-40. The 9E10 anti-c-*myc* epitope monoclonal antibody was obtained from the American Type Culture Collection, and the anti-*HA* epitope monoclonal antibody (12CA5) purchased from BAbCO (Berkeley, CA). The polyclonal antibody against the *HA* epitope was purchased from MBL International Corporation (MCI, Watertown, MA). Cy^3-conjugated secondary antibodies (Jackson ImmunoResearch Labs., Inc., West Grove, PA) were incubated on the coverslips for an additional 30 min. DNA was labeled using Hoechst dye 33258 (Sigma Chem. Co.) at 10 μg/ml PBS-NP40 for 1 min. Coverslips were mounted on glass slides in 90% glycerol, 20 mM Tris-HCl (pH 9.35).

Western Blotting

Coverslips were washed twice with ice-cold PBS, and the cells were extracted with 200 μl of pre-heated (70°C) 2× SDS sample buffer containing 10% 2-mercaptoethanol. The extract was transferred to a microcentrifuge tube, boiled for 5 min, and centrifuged at 10,000 *g* for 10 min. 25 μl of each sample was analyzed by 8% SDS gel electrophoresis and electrophoretically transferred to Immobilon membranes (Millipore Corp., Bedford, MA). The membranes were blocked overnight in 0.1% Tween-20 PBS (0.1% Tween-PBS) containing 5% nonfat dry milk and incubated in primary antibody for 1 h at room temperature. After four 5-min washes with blocking solution, membranes were incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs., Inc.) for 1 h. Membranes were then washed and developed using chemiluminescence reagents (ECL; Amersham Corp., Arlington Heights, IL) and exposed to XAR-5 film (Kodak).

DNA Nick End Labeling (TUNEL Method)

To detect DNA fragmentation in situ, we modified the previously described TUNEL method (Tilly and Hsueh, 1993; Surh and Sprent, 1994) as follows. Cells were fixed and washed with PBS-NP-40 and TdT buffer (0.5 M cacodylate, 25 mM Tris, pH 6.8, 150 mM NaCl, 5 mM $CoCl_2$, 0.5 mM DTT, and 0.05% BSA), and then incubated for 1 h at 37°C with 2–5 μM digoxigenin-conjugated dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 5-10 U TdT (terminal transferase; Promega Biotec, Madison,WI) in 50 μl TdT buffer per coverslip. After washing and blocking, cells were incubated with 0.5 μg/ml anti-digoxigenin mouse monoclonal antibody (Boehringer Mannheim Biochemicals), washed, and then incubated with an FITC-labeled anti-mouse antibody (Jackson ImmunoResearch Labs., Inc.).

Immunoprecipitations and Calcineurin Phosphatase Assays

Transfected cells on coverslips were lysed in 200 μl of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% Tween-20, 0.5 mg/ml BSA, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin), transferred to a microfuge tube, and centrifuged at 10,000 *g* for 5 min at 4°C. The supernatant was transferred to a new tube and 5 μl of the anti-*HA* epitope (12CA5) monoclonal antibody (BAbCO) and 30 μl of protein G–Sepharose (1:1 slurry; Sigma Chem. Co.) were added. Tubes were rotated at 4°C for 1 h. The Sepharose beads were pelleted by centrifugation and washed three times in buffer A. Phosphatase activity associated with the immunoprecipitates was determined by following [^{32}P]phosphate released from the RII peptide essentially as described (Milan et al., 1994).

Calcineurin activity from whole cell lysates of transfected and mock-transfected cells was determined by a modification of the assay developed by Fruman and colleagues (Fruman et al., 1992). Cells were scraped from coverslips in 200 μl of PBS, pelleted at 10,000 *g* for 15 s, resuspended in 50 μl hypotonic lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM $CaCl_2$, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM DTT), and subjected to three rounds of freeze–thaw. After removing cell debris by centrifugation, 50 μl of phosphatase buffer (100 mM Tris-HCl, pH 7.4, 1 mM $MnCl_2$, 0.1 mM $CaCl_2$, 0.5 mg/ml BSA, 100 nM calmodulin, and 0.5 mM DTT) was added to the supernatant. 500 nM okadaic acid was added to the reactions to suppress endogenous protein phosphatases PP1 and PP2A (Fruman et al., 1992). The ^{32}P-labeled RII peptide was incubated in the extracts for 30 min at 30°C. To determine the specific contribution of ΔCnA to the phosphatase activity of the lysates, 1 mM EGTA was added prior to the start of the reaction to suppress endogenous calcineurin activity. The effects of immunophilin–drug complexes on calcineurin activity of these lysates was determined using 0.5 μM cyclosporin A (CsA) and 1 μM human cyclophilin B (Price et al., 1991). Reactions were stopped by adding 500 μl of stop buffer (10% TCA, 0.1 M sodium phosphate) and 10 μl of 10 mg/ml BSA to each tube. These tubes were incubated on ice for 10 min, centrifuged at 10,000 *g* for 10 min, and the released [^{32}P]phosphate was determined as described (Milan et al., 1994).

Results

Calcineurin Induces Calcium-dependent Cell Death in the Absence of Growth Factors

To assess the effect of exogenous calcineurin expression on mammalian cells, we transfected BHK cells with vectors expressing an *HA*-epitope-tagged catalytic subunit of calcineurin (β-isotype of CnA) and its regulatory subunit, CnB (Guerini and Klee, 1989; Guerini et al., 1989; Milan et al., 1994; Fig. 1 *A*). Co-transfection of the two calcineurin subunits appears to be essential for efficient calcineurin expression in mammalian cells, as immunoblot-

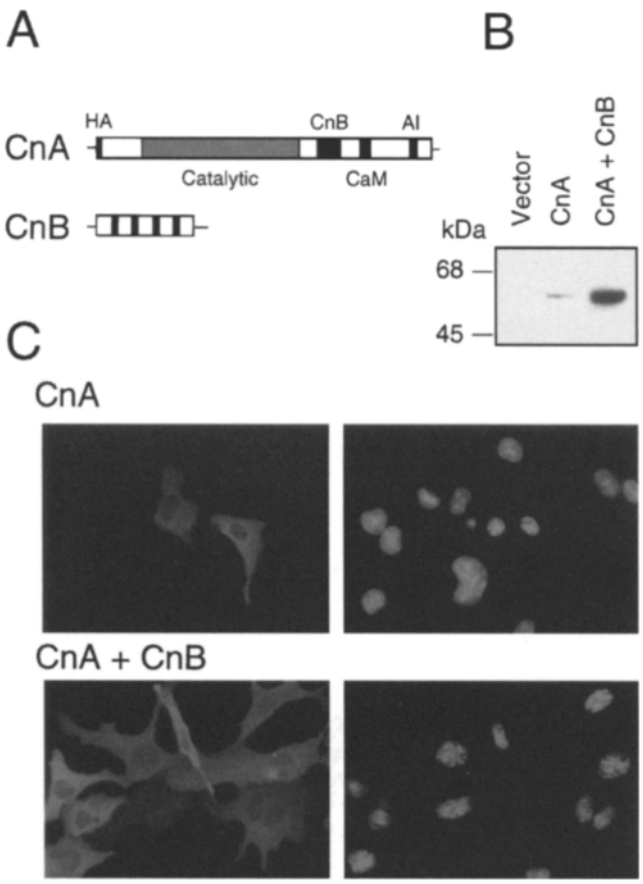

Figure 1. Expression of human CnA and CnB in mammalian cells. (*A*) Domain structure of human calcineurin A (*CnA*), ΔCnA, and calcineurin B (*CnB*). The cDNA encoding human CnA (β-isotype) was modified by an insertion of an *HA*-epitope coding sequence at its NH_2 terminus. HA, Catalytic, CnB, CaM, and AI represent the hemagglutinin-epitope, catalytic domain, calcineurin B-binding domain, calmodulin-binding domain, and auto-inhibitory domain, respectively. The human CnB cDNA encodes four Ca^{2+}-binding domains (*black boxes*) and was expressed in its wild type form. (*B*) Total cell lysates prepared from BHK cells transiently transfected with control vector alone (lane *1*), CnA alone (1 μg CnA, 1 μg control vector, lane *2*), both CnA and CnB (1 μg each, lane *3*) were fractionated by polyacrylamide gel electrophoresis, transferred to Immobilon membranes, and probed with the anti-*HA*-epitope antibody to detect CnA. The molecular weight markers indicate the mobility of bovine serum albumin (*68 kD*) and ovalbumin (*45 kD*). (*C*) Immunofluorescence localization and expression efficiency of calcineurin subunits in BHK cells. BHK cells were co-transfected with the epitope-tagged CnA cDNA and either a control vector or one expressing CnB. The subcellular localization and transfection efficiency of CnA were determined by using an anti-*HA*-epitope polyclonal antibody and is shown in the left micrograph. Nuclei were stained with Hoechst dye 33258 and are shown in the right panel. Cells expressing CnA alone are shown in the upper panel, and those co-expressing CnA and CnB are shown in the lower panel.

ting with anti-*HA* antibodies revealed considerably higher levels of CnA in cells co-expressing CnA and CnB, compared to those expressing CnA alone (Fig. 1 *B*). It is likely that the assembly of CnB onto its binding site on CnA acts to protect CnA from proteolytic degradation in the cell (Milan et al., 1994). Indirect immunofluorescence using the anti-*HA* monoclonal antibody detected *HA*-tagged CnA in the cytoplasm of transfected cells, and showed that co-expressing CnB along with CnA yields more cells with detectable levels of CnA (Fig. 1 *C*). The indirect immunofluorescence also revealed that the vast majority of BHK cells grown in normal media appears to tolerate the co-expression of *HA*-CnA and -CnB. However, approximately 3% of transfected cells displayed hypercondensed nuclei and cytoskeletal changes reminiscent of cells undergoing apoptosis (Kerr et al., 1994). Since calcineurin activity is highly dependent on a transient rise in intracellular calcium (Klee et al., 1988), we asked whether stimulating calcineurin activity by exposing cells to calcium ionophores would provoke increased rates of apoptosis in calcineurin-transfected cells. Cells co-expressing *HA*-CnA and -CnB for 16 h were treated with the calcium ionophore ionomycin, and scored for the apoptotic phenotype. Despite a 4-h incubation with ionomycin (0.25 μM), calcineurin-transfected cells showed only a modest increase in the rate of apoptosis (Fig. 2 *A*). We then considered the possibility that, as with c-*myc* over-expression (Evan et al., 1992), growth factor pathways inhibit apoptosis in calcineurin-transfected cells. We therefore stimulated calcineurin-transfected cells with calcium ionophores after 4 h of serum deprivation. Significantly, more than 60% of the transfected cells entered apoptosis within 4 h (Fig. 2 *A*). The process of apoptosis in these cells was very rapid: cytoplasmic retraction was apparent within 15 min of calcium ionophore treatment, and nuclear condensation was obvious at 30 min (Fig. 2 *B*). By 4 h, cells were rounded and had nuclei with multiple bodies of hypercondensed chromatin.

To further characterize the apparent apoptotic events, calcineurin-transfected cells were treated with calcium ionophores with and without serum deprivation, and genomic DNA subsequently probed for the presence of strand breaks using the terminal deoxynucleotidyl transferase (TdT)-mediated UTP nick end-labeling (TUNEL) method (Fig. 3; Tilly and Hsueh, 1993). Calcium-activated cells grown in high serum showed no incorporation of labeled nucleotides (Fig. 3). In contrast, apoptotic nuclei of calcium activated, serum-deprived cells showed strong incorporation of labeled nucleotides indicative of multiple nicks in nuclear DNA (Fig. 3). These results demonstrate that transfected calcineurin induces apoptosis in a calcium-dependent manner and that this process of cell death is blocked by growth factors.

We also note that non-transfected BHK cells, or those transfected with the regulatory subunit CnB alone, undergo apoptosis when serum deprived for 4 h and subsequently treated with ionomycin. However, the percentage of apoptotic cells is only 7.5% compared to 65% in CnA/CnB-transfected cells (not shown).

Calcineurin Acts on Existing Substrates to Promote Apoptosis

To determine whether calcineurin-induced apoptosis in BHK cells requires new protein synthesis, we treated cells co-expressing CnA and CnB with the protein synthesis inhibitor cycloheximide (36 μM) immediately prior to serum deprivation. 1 h after calcium ionophore treatment in the

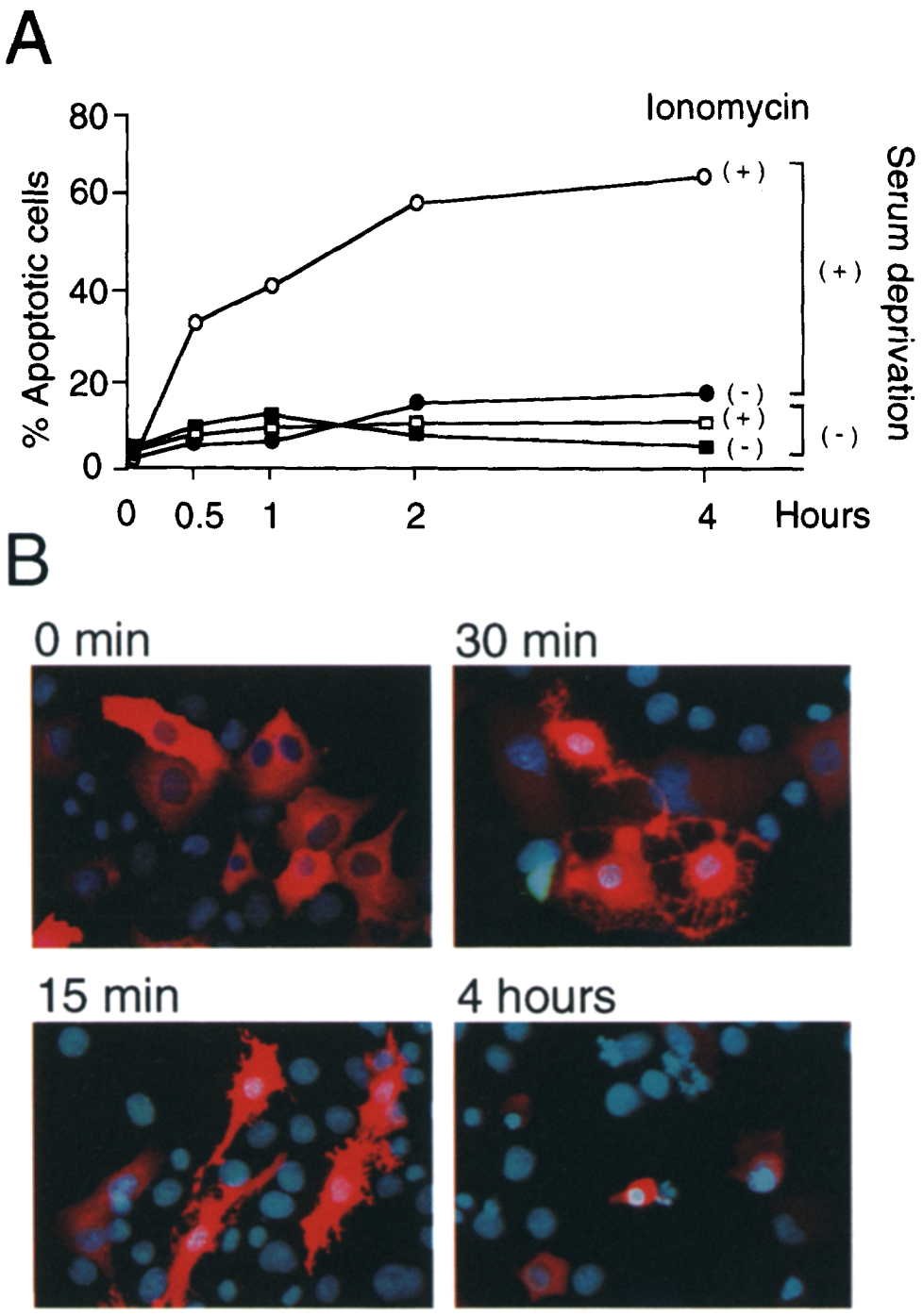

Figure 2. Induction of apoptosis in BHK cells transfected with CnA and CnB.(*A*) Effect of serum deprivation and ionomycin on induction of apoptosis in CnA- and CnB-transfected cells. BHK cells were transfected with CnA (1 μg) and CnB (1 μg) cDNAs and cultured overnight in normal media containing 10% FCS. The next day, cells were serum deprived by incubation in low serum medium (0.1% FCS) for 4 h, and then incubated in low serum medium with ionomycin (0.25 μM, *open circles*) or without ionomycin (*closed circles*). Identical coverslips of transfected cells were incubated in media containing 10% FCS containing ionomycin (*closed rectangles*) or without ionomycin (*open rectangles*). Transfected cells were scored for the apoptotic phenotype including changes in cell shape and hypercondensed chromatin as judged by Hoechst 33258 staining. A minimum of 1,000 transfected cells was scored for each time point. (*B*) Apoptotic events in cells coexpressing CnA/CnB. BHK cells transfected with CnA and CnB were serum-deprived for 4 h, and then exposed to ionomycin (0.25 μM) in the same media. The morphological changes of cell shape and nuclei were observed by using both the anti-*HA* epitope antibody to detect CnA/CnB-transfected cells and Hoechst 33258 dye at the indicated time: 0 min, 15 min, 30 min, and 4 h of ionomycin treatment.

presence or absence of cycloheximide, cells were scored for changes in cytoplasmic and nuclear structure characteristic of apoptosis. Cycloheximide showed no effect on the progression of serum-deprived, calcineurin-transfected cells toward apoptosis upon calcium ionophore treatment, an indication that calcineurin stimulates cell death by acting on existing substrates in the cell (Fig. 4 *A*, lane *3*). Direct measurement of the inhibition of protein synthesis by 36 μM cycloheximide revealed an 83% decrease in the incorporation of [^{35}S]methionine into polypeptides (not shown). Significantly, cycloheximide treatment in the absence of ionomycin did not promote cell death in either the serum deprived cells or those grown in 10% FCS (Fig. 4 *A*, lanes *1* and *4*). In contrast, cycloheximide appears to neutralize the ability of high serum to block calcineurin-induced cell death (Fig. 4 *A*, lane *6*). In this case it is likely that cycloheximide is preventing the synthesis of factors required by the cell for survival.

Calcineurin-induced Apoptosis Is Suppressed by Bcl-2 Expression

The oncoprotein Bcl-2, and its homolog in *C. elegans* encoded by *ced9*, appear to play a major role in suppressing cell death provoked by a wide variety of stimuli (Sentman et al., 1991; Barr and Tomei, 1994; Reed, 1994). To deter-

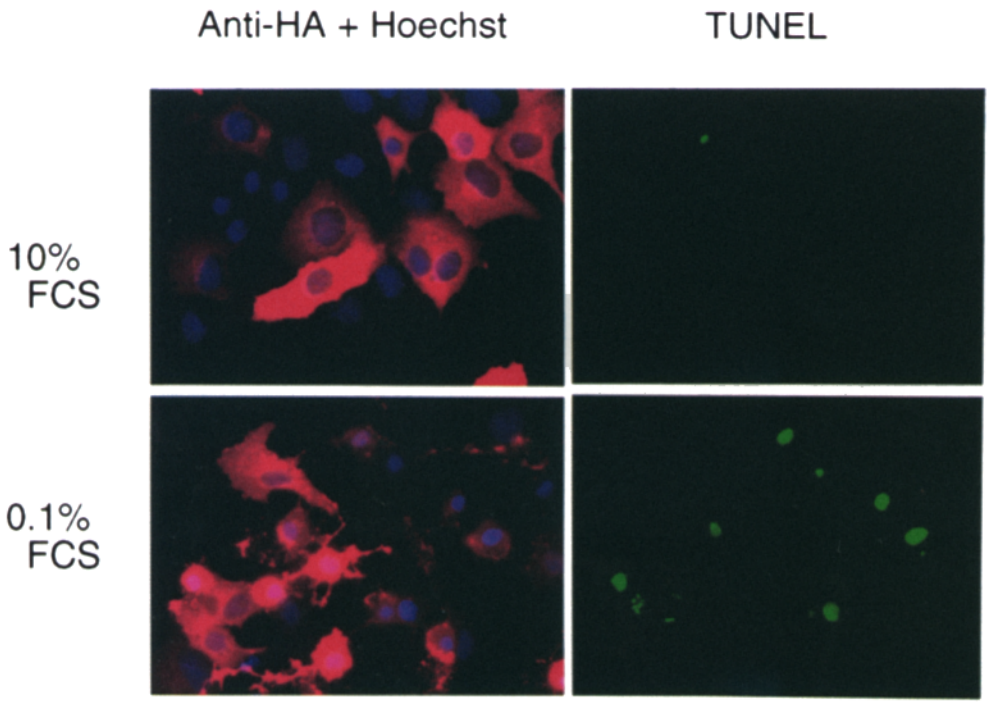

Figure 3. TUNEL staining of CnA/CnB-transfected cells. BHK cells co-expressing CnA and CnB cDNAs for 16 h were incubated in either 10% FCS (*top*) or 0.1% FCS (*bottom*) for 4 h, incubated in ionomycin (0.25μM) in either 10% FCS or low serum for 1 h, and subsequently processed for immunodetection of CnA and fragmented DNA using the TUNEL method. Left panels reveal staining for CnA (*red*) and DNA (*blue*) in cells exposed to ionomycin in high and low serum, respectively. Right panels show fields corresponding to left panels in which DNA was labeled at internal breaks using digoxigenin-modified nucleotides and terminal deoxynucleotide transferase, followed by FITC-labeled anti-digoxigenin antibodies (*green*).

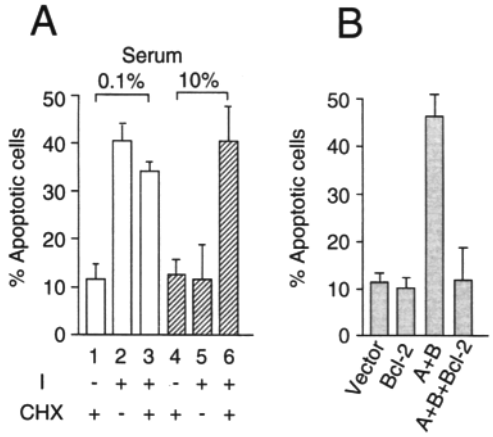

Figure 4. Effect of cycloheximide and Bcl-2 on BHK cells co-transfected with CnA and CnB. (*A*) Effect of the protein synthesis inhibitor cycloheximide on calcineurin-induced apoptosis in BHK cells. Cells were co-transfected with cDNAs encoding CnA and CnB, and subsequently incubated for 4 h in either normal media or low-serum media in the presence (lanes *1*, *3*, *4*, and *6*) or absence (lanes *2* and *5*) of cycloheximide (36 μM) prior to stimulation with ionomycin (0.25 μM) for 1 h. I and CHX represent ionomycin and cycloheximide, respectively. Error bars indicate standard deviation of three experiments in which 800 transfected cells in each experiment were scored. (*B*) Bcl-2 expression prevents apoptosis in calcineurin-transfected cells. BHK cells were transfected with cDNAs encoding CnA and CnB, CnA, CnB, and Bcl-2, or Bcl-2 alone and subsequently serum-deprived for 4 h and stimulated with ionomycin for 1 h. The percentage of apoptotic cells was determined by scoring 800 cells from four experiments. Error bars represent the standard deviation for these determinations. Each column represents as follows: Vector (2.0 μg of pCMV control vector); Bcl-2 (0.6 μg of pCMV control vector + 1.4 μg of pCMV-*bcl-2*); A + B (0.3 μg each of pCMV-CnA and -CnB + 1.4 μg of pCMV control vector); A + B + Bcl-2 (0.3 μg each of pCMV-CnA and -CnB + 1.4 μg of pCMV-*bcl-2*).

mine whether Bcl-2 can regulate calcineurin-induced apoptosis, we assayed the interaction of Bcl-2 and calcineurin in BHK fibroblasts. Cells expressing CnA, CnB, and Bcl-2 were found to be markedly resistant to apoptosis induced by growth factor withdrawal and ionomycin treatment in comparison to cells not transfected with the Bcl-2 expression vector (Fig. 4 *B*). These data suggest that the actions of Bcl-2 are dominant over those of calcineurin in cell death, although they fail to address whether Bcl-2 is acting upstream or downstream of calcineurin. Regardless, calcineurin appears to function in a pathway of cell death regulated by Bcl-2.

Calcium-independent Calcineurin Mutant Promotes Apoptosis in the Absence of Calcium Signaling

Although calcineurin-transfected cells undergo rapid and efficient apoptosis upon exposure to calcium ionophores, it was formally possible that the calcium influx was activating endogenous enzymes which in turn trigger cell death. To circumvent the requirement for calcium ionophores in this process, we transfected cells with a COOH-terminal deletion mutant (ΔCnA) of calcineurin which is constitutively active even in the absence of elevated calcium (Fig. 5 *A*; Hubbard and Klee, 1989; Clipstone and Crabtree, 1992; O'Keefe et al., 1992). As with cells expressing full-length CnA and CnB, cells expressing ΔCnA and CnB appear normal 16 h after transfection (Fig. 5 *B*, *upper panel*). In contrast to cells expressing the full-length CnA and CnB, those transfected with ΔCnA and CnB initiated apoptosis without exposure to calcium ionophores (Fig. 5 *B*, *lower panel*). Further, these cells reacted positively for fragmented DNA using the TUNEL method (Fig. 5 *B*, *right panels*). The time course for cell death after the start of serum deprivation of ΔCnA/CnB-transfected cells was remarkably rapid and approached 50% by 4 h (Fig. 5 *C*).

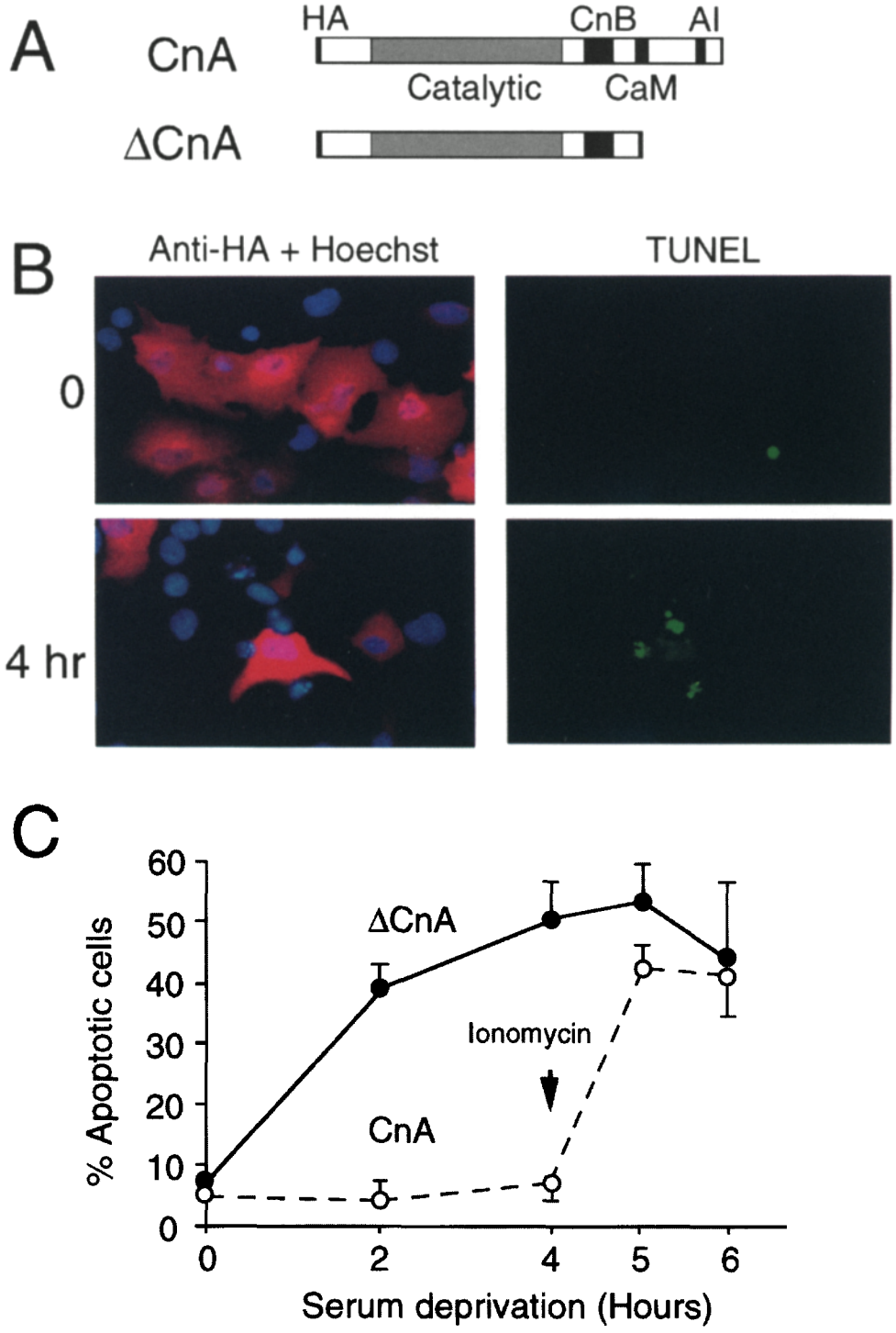

Figure 5. ΔCnA expression results in apoptosis in the absence of ionomycin. (*A*) Schematic representation of full-length (*CnA*) and the truncated, constitutively active calcineurin A (*ΔCnA*). (*B*) Cells co-transfected with ΔCnA and CnB were fixed before and after 4 h of serum-deprivation and processed for immunofluorescence using the anti-*HA* monoclonal antibody to detect ΔCnA and Hoechst dye to label DNA (*right*) and for fragmented DNA using the TUNEL method (*right*). (*C*) BHK cells were co-transfected with CnA and CnB (*open circles*) or ΔCnA and CnB (*closed circles*), and subsequently transferred to low serum media. After 4 h of serum deprivation, cells were incubated with ionomycin (0.25 μM). At the time points indicated, cells were fixed, processed for immunofluorescence using the anti-*HA* monoclonal antibody and Hoechst dye 33258, and analyzed for apoptotic figures. 1,000 cells from each of three experiments were scored for apoptotic nuclei. The vertical bars represent standard deviations.

The absolute percentage of apoptotic cells was not obviously enhanced by the addition of ionomycin at four hours (Fig. 5 *C*). These data indicate that calcineurin can directly stimulate apoptotic pathways without the cooperation of other calcium-activated proteins.

Correlation between Calcineurin Catalytic Activity and Apoptosis

To probe the relationship between CnA phosphatase activity and cell death, we scored cells transfected with increasing amounts of ΔCnA/CnB for both apoptosis and calcineurin enzymatic activity. Anti-calcineurin antibodies were used in immunoblots to assay calcineurin accumulation in the transfected cells (Parsons et al., 1994). As seen in Fig. 6 *A*, ΔCnA (50 kD) migrates below endogenous calcineurin (60 kD) and its level of expression correlates with the amount of ΔCnA plasmid used in the transfection. To determine if the increased accumulation of ΔCnA is reflected in enhanced calcineurin activity, *HA*-tagged ΔCnA was immunoprecipitated with the anti-*HA* antibody and assayed for activity in vitro. We found a nearly

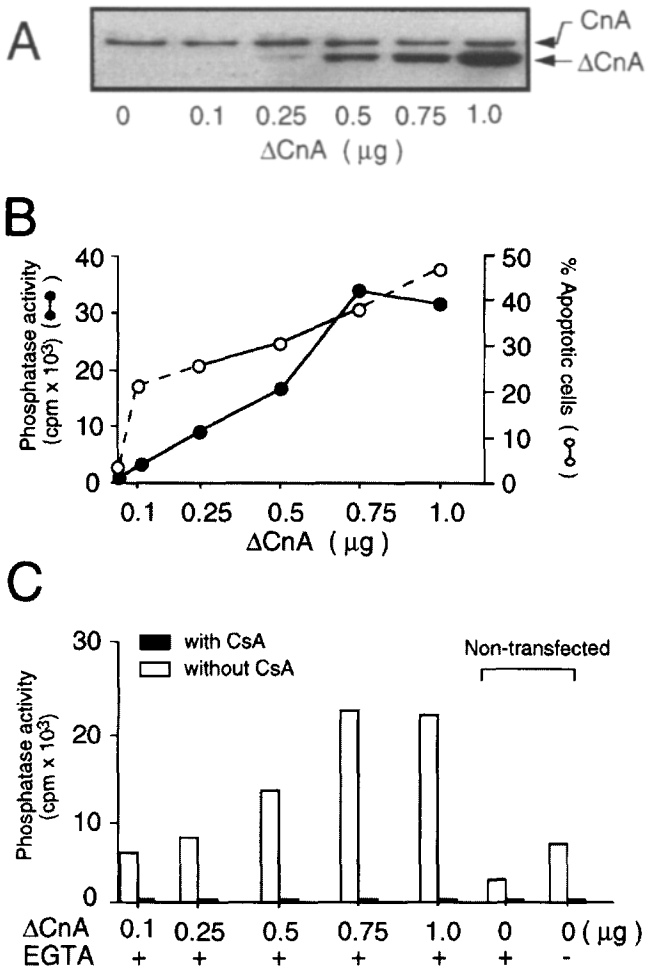

Figure 6. Correlation between calcineurin activity and apoptosis. (*A*) Dose-dependent expression of ΔCnA. BHK cells were transfected with the indicated amounts of ΔCnA/CnB expression vectors (1:1 ratio, adjusted to 2 μg using control vector) and lysed 16 h later. Cell lysates were separated by electrophoresis and transferred to nylon membrane. Endogenous calcineurin A (*CnA*) and transfected ΔCnA were detected by an anti-calcineurin antibody (gift of R. Kincaid, National Institutes of Health, Bethesda, MD). (*B*) BHK cells transfected with different amounts of ΔCnA/CnB were assayed for calcineurin phosphatase activity and scored for apoptosis. To determine calcineurin activity, cells were lysed 16 h after transfection and the *HA*-tagged ΔCnA immunoprecipitated with the 12CA5 monoclonal antibody. Immunoprecipitates were assayed for phosphatase activity against the ^{32}P-labeled RII peptide, as described (Milan et al., 1994). Apoptosis was scored on identical coverslips after 4 h of serum deprivation. Open circles and closed circles represent the percentage of apoptotic cells and phosphatase activity of the expressed calcineurin, respectively. (*C*) To compare the phosphatase activity of transfected ΔCnA with that of endogenous calcineurin, whole cell lysates were prepared from cells transfected with different amounts of ΔCnA/CnB and control vector alone (total 2 μg). Calcineurin-specific phosphatase activity was determined in the presence of 500 nM okadaic acid to suppress PP1 and PP2A (Fruman et al., 1992). Lysates from cells transfected with vector alone displayed calcium-dependent and -independent phosphatase activity, both of which were suppressed by 500 nM CsA and 1 μM cyclophilin B. Lysates made from ΔCnA/CnB-transfected cells, assayed in the absence of calcium, displayed phosphatase activity proportional to the amount of ΔCnA/CnB transfected. The decrease in phosphatase activity at 1 μg ΔCnA/CnB (each 1 μg of ΔCnA and CnB) is likely due to the induction of apoptosis observed even in the presence of 10% FCS (not shown).

linear relationship between the amount of calcineurin plasmids transfected and the phosphatase activity of the immunoprecipitated calcineurin (Fig. 6 *B*). Moreover, these experiments revealed a strong correlation between calcineurin activity and apoptosis in transfected cells (Fig. 6 *B*).

We next asked how this transfected calcineurin activity compared to that of endogenous calcineurin. Cells transfected with a control vector were lysed in extraction buffer and assayed for calcineurin activity in the presence of okadaic acid (500 nM), which inhibits protein phosphatase 1 and 2A (Fruman et al., 1992). In parallel, we performed phosphatase assays on lysates from cells transfected with increasing amounts of ΔCnA/CnB, and found a corresponding increase in phosphatase activity (Fig. 6 *C*), as expected from the immunoprecipitation experiments. At the lowest amount of ΔCnA/CnB transfected, the lysates yielded a twofold higher calcineurin activity than that found in cells transfected with the control vector alone (Fig. 6 *C*). At this level of calcineurin activity, 23% of cells undergo apoptosis after 4 h of serum deprivation, versus 3% for control vector–transfected cells. In transfections involving 0.75 μg ΔCnA/CnB, lysates show approximately eight times the endogenous level of calcineurin catalytic activity, and ~40% of total cells undergo apoptosis. Overall, these results indicate a strong relationship between calcineurin-dependent phosphatase activity and cell death.

Discussion

Calcium signaling has been implicated in a wide variety of apoptotic stimuli and yet the critical effectors of calcium remain obscure. We provide evidence that the activation of calcineurin, a calcium/calmodulin-dependent phosphatase, rapidly provokes apoptosis and is therefore a likely mediator of calcium signaling leading to cell death. We show that calcineurin-induced cell death is abrogated by growth factor stimulation and by Bcl-2 expression. Further, calcineurin was shown to function at a posttranslational level to activate apoptosis. These data support the prospect that calcineurin is acting in a calcium-dependent manner to modify existing substrates important in the commitment to cell death.

Role of Calcineurin in Calcium-activated Cell Death

Despite advances in defining proteins which regulate apoptosis, including Bcl-2, Bax, ICE/Ced-3, and Ced-4, it is unclear how these factors are influenced by signal transduction pathways that promote cell death. Calcium signaling is associated with glucocorticoid- and activation-induced cell death in immature T cells, growth factor withdrawal in certain cell lines, and hyperactivation of *N*-methyl-D-aspartate receptors of neurons (Barr and Tomei, 1994; Martin et al., 1994; Reed, 1994). In neurons of the central nervous system, calcium mobilization following glutamate stimulation of *N*-methyl-D-aspartate receptors is strongly implicated in cell death, although the identity of the calcium-

sensitive mediators of apoptosis remains unclear (Choi, 1992). The calcium-sensitivity of nitric oxide synthetase makes this enzyme an obvious candidate for effecting cell death in neurons (Choi, 1992). However, calcineurin is present at very high concentrations (1–2 μM) in the central nervous system (Krinks et al., 1984), and therefore may be a material factor in calcium-activated cell death in neurons.

We provide several lines of evidence that calcineurin activity resulting from elevated intracellular calcium concentration, rather than additional calcium-activated events, is sufficient to induce cell death. For one, over-expression of the catalytic and regulatory subunits of calcineurin in fibroblasts renders these cells highly vulnerable to a rapid form of cell death. As with c-*myc* over-expression, these cells execute cell death processes only in the absence of growth factor stimulation. Second, cells expressing ΔCnA, which displays calcium-independent phosphatase activity, undergo apoptosis in the absence of calcium signaling. Further, the extent of apoptosis in a population of cells appears to correlate well with the phosphatase activity generated by the expressed ΔCnA. That calcineurin is a direct activator of cell death is supported by the observation that calcineurin promotes cell death in the absence of de novo protein synthesis. Similarly, neither c-*myc* nor p53 requires new protein synthesis to trigger apoptotic pathways (Evan et al., 1992; Caelles et al., 1994; Wagner et al., 1994). It is possible, then, that both c-*myc*, as a complex with Max, and p53, function in cell death by repressing genes required for survival. Alternatively, these transcriptional activators may be involved in protein–protein interactions independent of transcriptional regulation to affect cell death decisions (Amati et al., 1993; Caelles et al., 1994; Wagner et al., 1994). Calcineurin may participate in cell death pathways by indirectly altering transcriptional regulation through, for instance, c-*myc* or p53. Alternatively, calcineurin may function by affecting regulators of cell death such as Bcl-2 or Ced-3 (Yuan et al., 1993).

Regulation of Calcineurin-induced Cell Death by Bcl-2 and Growth Factors

The ability of Bcl-2/Ced9 to prevent apoptosis due to a diverse array of stimuli, including ones involving calcium signaling, suggests that it regulates a fundamental step in the commitment to cell death (Sentman et al., 1991; Barr and Tomei, 1994; Hengartner and Horvitz, 1994; Reed, 1994). We find that Bcl-2 efficiently suppresses apoptosis in cells transfected with wild type CnA and CnB, an indication that Bcl-2 blocks steps affected by calcineurin's phosphatase activity. Bcl-2 has been implicated in the regulation of both oxygen radical formation and calcium fluxes from intracellular stores, and conceivably either function could affect calcineurin-induced cell death (Hockenbery et al., 1993; Lam et al., 1994).

Our results also demonstrate that growth factors in serum interfere with calcineurin-induced cell death. IGF-1 has been has been shown to block c-*myc*–induced apoptosis in fibroblasts (Evan et al., 1992; Harrington et al., 1994). Significantly, IGF-1 prevents cell death even in the absence of new protein synthesis, an indication that IGF-1 operates through pathways that directly impact apoptotic decisions (Harrington et al., 1994). Cell death via c-*myc* is known to depend on wild-type p53, as c-*myc* over-expression in $p53^{null}$ cell lines leads to proliferation rather than cell death (Hermeking and Eick, 1994). The ability of c-*myc* expressing $p53^{null}$ cells to avoid cell death is thought to underlie tumor progression in many human cancers (Barr and Tomei, 1994). We are currently testing the possibility that calcineurin, c-*myc*, and p53 interact in a common regulatory scheme to influence decisions on cell death.

Calcineurin Function in Apoptosis is Fundamentally Different from That in T Cell Activation

A role for calcineurin in cell death has been deduced from earlier studies of T cell activation. T cell hybridomas, and in some cases, immature T cells, undergo cell death in a calcium-dependent manner thought to require calcineurin (Smith et al., 1989; Fruman et al., 1992). But several features of cell death in T cell hybridomas suggest that it occurs via pathways distinct from those employed in the calcineurin-dependent apoptosis described here in fibroblasts. For one, T cell hybridomas stimulated by anti-CD3 antibodies appear to undergo the early phases of T cell activation including calcium mobilization, Nuclear Factor in Activated T Cells (NFAT) activation, and cytokine transcription within 30 min, and yet cell death is not evident for another six to eight hours (Shi et al., 1989; Smith et al., 1989; Ucker et al., 1989; Fruman et al., 1994). It is now known that much of this lag is due to the requirement of new protein synthesis for T cell hybridoma death (Ucker et al., 1989; Crispe, 1994). In contrast, the failure of cycloheximide to impede cell death in fibroblasts overexpressing calcineurin suggests that calcineurin acts to modify substrates essential for apoptosis which already exist in the cell. This finding supports previous work which argued that important components of the cell death process are constitutively expressed in the cell (Raff et al., 1993). Moreover, the immunosuppressants CsA and FK506, which block apoptosis in T cell hybridomas, fail to block the more direct cell death in BHK fibroblasts described here (Shibasaki, F., and F. McKeon, manuscript in preparation). This apparent dichotomy underscores the possibility of two distinct, calcineurin-dependent pathways leading to cell death. In T cell hybridomas, CsA blocks the early phase of T cell activation which is necessary to promote the subsequent, Fas-dependent process of cell death (Singer and Abbas, 1994). On the other hand, the more direct, calcineurin-dependent pathways described here may involve substrates favored by calcineurin–CsA–cyclophilin complexes (Liu et al., 1991).

In summary, we have shown that calcineurin can mediate calcium-activated cell death in mammalian cells in a manner regulated by the Bcl-2 oncoprotein and growth factors. The calcineurin expression system described here provides an important model for the analysis of signal transduction pathways that directly influence the decision towards cell death or growth. Finally, these results suggest that calcineurin may act in the calcium-activated cell death of neurons and cells of the immune system.

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