Calcineurin Is Downstream of the Inositol 1,4,5-Trisphosphate Receptor in the Apoptotic and Cell Growth Pathways*

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The inositol 1,4,5-trisphosphate receptor (IP₃R) is a calcium (Ca²⁺) release channel found on the endoplasmic reticulum of virtually all types of cells. Human T lymphocytes (Jurkat) that are made deficient in IP₃R do not generate Ca²⁺ signals in response to T cell receptor stimulation, fail to translocate the nuclear factor for activated T cells to the nucleus, and are remarkably resistant to induction of apoptosis with CD95 (Fas), dexamethasone, γ irradiation, and T cell receptor stimulation using anti-CD3 antibody. Expression of constitutively active calcineurin A in IP₃R-deficient T cells restored nuclear factor for activated T cells translocation to the nucleus and dephosphorylation of Bad and rendered the cells sensitive to apoptotic inducers. Induction of apoptosis required both active calcineurin A (ΔCnA) and activation-dependent colocalization of CnA with its substrate. Thus, the Ca²⁺²⁺ dependent phosphatase calcineurin (CnA) is downstream of the IP₃R in both the cell growth and apoptotic signaling pathways.

Elevation of [Ca²⁺], signals diverse functions in T cells including cell growth and apoptosis (1–4). Previous work has shown that the IP₃R is necessary for both cell growth (5) and cell death (3). It has been proposed that one of the key downstream targets of IP₃-mediated Ca²⁺ flux in the growth pathway is calcineurin A (CnA) (6). Moreover, sustained Ca²⁺ elevation is required for CnA-dependent dephosphorylation of NF-AT and the subsequent translocation of this important transcription factor into the nucleus of T cells (7). Because IP₃R-deficient cells are resistant to apoptosis (3) and FK506, a CnA inhibitor, prevents apoptosis (8–10) we reasoned that CnA could be a Ca²⁺²⁺ dependent enzyme downstream of IP₃R in the apoptotic and cell growth signaling pathways.

CnA is a serine-threonine phosphatase regulated by Ca²⁺ and the Ca²⁺ calmodulin complex (11). It is sensitive to immunosuppressive drugs such as FK506 and cyclosporin A. CnA inhibition results in the failure to translocate NF-AT to the nucleus, preventing activation of several cytokine genes, including interleukin-2, that are required for T cell growth. It has been shown that T cell activation requires a sustained increase in cytosolic [Ca²⁺] to activate interleukin-2 gene transcription. IP₃ receptor-deficient Jurkat T cells are defective in intracellular Ca²⁺ release and are resistant to apoptosis (3).

We now show that ΔCnA restores NF-AT translocation and the apoptotic phenotype in IP₃R-deficient T cells. The identification of CnA as a target for intracellular Ca²⁺ release suggests that Ca²⁺²⁺ dependent dephosphorylation of downstream targets plays a role in the biochemical events triggering apoptosis and cell growth pathways.

EXPERIMENTAL PROCEDURES

Cell Culture and Apoptosis Induction—IP₃R-deficient T cells (3) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 0.6 mg/ml hygromycin, whereas vector- and ΔCnA-transfected cells were cultured with 0.6 mg/ml hygromycin and 0.4 mg/ml G418. To induce apoptosis, both vector-transfected and ΔCnA-transfected cells were washed three times with serum-free RPMI 1640 medium and cultured in 10% RPMI 1640 medium in the presence and absence of plate-bound αCD3 (10 μg/ml) as described (3). After the indicated periods, the cells were washed and analyzed as described previously (3).

In addition, the apoptotic cells were identified by the annexin V-fluorescein staining as per the manufacturer’s instructions (CLONTECH Laboratories, Palo Alto, CA). The annexin assay and the cell death assays were used as complementary techniques to determine apoptotic cell death; both assays were performed, and representative results are shown.

Transfections—A 1.2-kilobase Smal-SalI fragment containing the coding region of the constitutively active CnA was cloned into pEGFP-C3 (CLONTECH Laboratories). 3.2 μg of either vector or vector containing CnA DNA was used to transfect IP₃R-deficient cells with the Lipofectin reagent (Life Technologies, Inc.). 36 h after transfection with either GFP vector alone or GFP vector containing ΔCnA, transfected cells were pelleted by centrifugation at 1500 rpm for 5 min, resuspended in 1.5 ml of RPMI 1640 medium, layered over an equal volume of a Ficoll-Paque gradient (Amersham Pharmacia Biotech), and centrifuged for 20 min at 1500 rpm. The viable cells at the interphase were collected, washed in RPMI 1640 medium, and used for the experiments. The viability of these cells was determined using the trypan blue exclusion test. To induce apoptosis viable cells were cultured on aCD3-coated plates as described (3). In all experiments only viable cells expressing levels of ΔCnA comparable with the levels of CnA in the parental cells (as determined by immunoblotting; e.g. see Fig. 1D) were used. The relatively low expression of ΔCnA was similar in all cells used for all experiments.

Immunofluorescence—Cells were activated for 15 min in the presence of the indicated stimuli, fixed with 3.7% paraformaldehyde, permeabilized with 0.2% Triton X-100 in buffer with divalent (BWD) buffer (125 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 5 mM glucose, 10 mM NaHCO₃, 1 mM MgCl₂, 1 mM CaCl₂, and 20 mM HEPES, pH 7.4), and immunostained as described (12). Anti-NF-ATp antibody (Upstate Biotechnol-ogy Inc., Lake Placid, NY) and rhodamine conjugated to rabbit IgG were used for NF-AT detection. After washing, the cells were mounted and examined at ×100 magnification using a scanning laser confocal attachment (Zeiss LSM 410) mounted on a Zeiss Axiovert 100 TV inverted fluorescence microscope equipped for fluorescence and transmitted-light imaging. Sample excitation and confocal image collection are accomplished using an argon-krypton laser and photomultiplier detectors. For green fluorescent protein, the excitation and barrier filters...
Fig. 1. Expression of constitutively active calcineurin A. A, schematic representation of full-length (CnA) and the truncated, constitutively active CnA (ΔCnA). The phosphatase catalytic domain, calcineurin B binding domain (CnB), calmodulin binding domain (CnM), and the autoinhibitory domain (A) are shown. IP, R-deficient T cells were transfected with (B) GFP vector alone or (C) GFP vector containing ΔCnA and were then imaged using a confocal microscope. D, immunoblot analysis of CnA and ΔCnA expression in control Jurkat cells and in IP, R-deficient T cells transfected with GFP vector or ΔCnA. In the latter, both the endogenous CnA and the ΔCnA are observed.

for the present study the level of ΔCnA expression was comparable with that of the endogenous CnA (Fig. 1D). The growth rate of cells stably expressing low levels of CnA was the same as that of vector-transfected controls (Fig. 2).

To determine whether activation of CnA is a downstream signal required for apoptosis in Jurkat cells, sensitivity to inducers of apoptosis was examined using IP, R-deficient cells that are resistant to apoptosis and that express low levels of ΔCnA (Fig. 3A). Stimulation with αCD3 induced apoptosis in a concentration-dependent manner in IP, R-deficient Jurkat cells expressing ΔCnA but not in control cells (Fig. 3A). Annexin V assays, which are specific for apoptosis, showed that there was no increase in apoptotic cell death after stimulation with αCD3 in IP, R-deficient cells not expressing ΔCnA (Fig. 3B). Thus, Fig. 3, A and B shows that expression of ΔCnA, but not vector alone, rendered the previously resistant cells sensitive to induction of apoptosis by αCD3. This finding suggests that the resistance to apoptosis exhibited by IP, R-deficient cells (3) is causally linked to their inability to release intracellular Ca2+ (5), which is required to activate CnA (7).

It has been shown that the activation state of NF-AT is critically dependent on CnA activity (15). NF-AT activation is required to coordinate the immune response (16). The immunosuppressive drugs cyclosporin A and FK506, which are potent inhibitors of CnA, inhibit NF-AT translocation (15). Because FK506 is also known to inhibit activation-induced apoptosis in T cells, the requirement of CnA in both cellular activation and apoptosis raises the immediate question of whether alternate pathways for cellular activation or cell death share common Ca2+-dependent signaling steps. IP, R-deficient T cells expressing low levels of ΔCnA, but not those expressing vector alone, exhibited translocation of NF-AT in response to stimulation with αCD3 (Fig. 4A). In the cell expressing GFP vector alone NF-AT remains localized to the cytoplasm after stimulation with αCD3 (Fig. 4A). A more stringent inhibitor of CnA, FK506 (1 μM) blocked NF-AT translocation in both constitutively active ΔCnA-expressing cells and in ionomycin-treated cells (Fig. 4B). These findings, coupled with our earlier reports showing that IP, R-deficient cells are resistant to apoptosis (3) and cannot undergo TCR-mediated activation (5), suggest that IP, R-induced intracellular Ca2+ release is required for activation of apoptosis and for NF-AT translocation.

Western Blotting—Cells were pelleted by centrifugation and washed with phosphate buffered saline and then lysed with radioimmunoprecipitation assay buffer (1% Nonidet P-40, 1% deoxycholate, 0.1% SDS supplemented with 10 μg/ml aprotinin, 2 μg/ml leupeptin). After clearing cellular debris using centrifugation at 14,000 × g, protein concentration in the supernatant was determined by Bradford assay (Bio-Rad). 100 μg of protein from each lysate was denatured with SDS sample buffer and separated by 12% SDS-polyacrylamide gel electrophoresis. Following size fractionation using SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes using a wet tank apparatus (Bio-Rad mini wet transfer unit) overnight at 4 °C. Transfer membranes were then incubated with blocking solution (5% dry milk containing 0.5% Tween 20) for 1 h at room temperature followed by incubation with 1 μg/ml primary antibody for 3 h. Membranes were washed four times with Tris-buffered saline with Tween 20 and incubated with the appropriate secondary antibody (1/1000 dilution) for 1 h followed by washing four times. Signal detection was performed with a chemiluminescence kit (Amersham Pharmacia Biotech). The primary antibodies were anti-CnA antibody (PharMingen; antibody recognizes both CnA and ΔCnA) and anti-Bad antibody (Transduction Laboratories, Lexington, KY).

RESULTS

CnA is a heterodimer comprised of a catalytic A subunit and a Ca2+-binding regulatory B subunit (11). Constitutively active (Ca2+-independent) CnA (13) was stably expressed in IP, R-deficient T cells (Jurkat) (Fig. 1A). Jurkat transfected with GFP vector alone exhibited a diffuse fluorescent pattern (Fig. 1B), whereas cells transfected with GFP/ΔCnA exhibited a cytoplasmic localization of the fluorescent signal (Fig. 1C) consistent with the cytoplasmic distribution of ΔCnA. Typically both parental and IP, R-deficient T cells expressing high levels of ΔCnA underwent spontaneous cell death. This finding was consistent with previous reports showing that expression of ΔCnA induces cell death (14). However, it was possible to select cells that expressed low enough levels of ΔCnA to remain viable (Fig. 1D). Immunoblot analysis of endogenous CnA and transfected ΔCnA expression in IP, R-deficient T cells transfected with GFP vector or ΔCnA showed that in the cell lines selected...
The Bcl-2 family of proteins are important regulators of programmed cell death (17–19) and are comprised of both anti- and pro-apoptotic members. It is generally believed that the ratio of pro-apoptotic and anti-apoptotic members of the Bcl-2 family members regulates cell fate by the ability of the members to form a complex with Bcl-2 or Bcl-XL (17, 20). Bad is a member of the Bcl-2 family that is pro-apoptotic when it is associated with Bcl-2 or Bcl-XL. Recent studies have suggested that phosphorylation of Bad by the serine-threonine kinase, Akt (21), causes its release from Bcl-2 or Bcl-XL and eventual degradation after binding to 14-3-3 proteins (22). Thus, phosphorylation of Bad by Akt is anti-apoptotic, and dephosphorylation of Bad is pro-apoptotic.

Akt is an important mediator of the growth-promoting pathways that are linked to growth factor receptor pathways such as those involved in insulin signal-
ing. Thus, signals such as growth factors and cytokines promote cell survival in part by activating pathways that result in Bad phosphorylation by Akt (23).

We hypothesized that Bad might be a downstream target of CnA. TCR-mediated activation of cells expressing low levels of ΔCnA resulted in dephosphorylation of Bad protein (Fig. 5A). In contrast, dephosphorylated Bad was not observed in cells expressing ΔCnA in the absence of αCD3 stimulation or in cells treated with ionomycin (3 μM) alone (without αCD3 stimulation), suggesting that active CnA alone in the absence of an apoptotic inducer is insufficient to dephosphorylate Bad. This was in contrast to the translocation of NF-AT, which is also dependent on activation of CnA (15). We showed that ionomycin (3 μM) alone (without αCD3 stimulation) caused NF-AT translocation. TCR-mediated activation of IP$_3$R-deficient T cells that were not expressing ΔCnA failed to dephosphorylate Bad (Fig. 5A). These findings suggested to us that when ΔCnA was present at physiological levels in cells (as opposed to overexpressed) an additional signal was required to achieve colocalization of ΔCnA with Bad. Indeed, αCD3-mediated TCR stimulation did induce the colocalization of ΔCnA and Bad (Fig. 5B). These data suggest that the resistance to apoptosis in IP$_3$R-deficient T cells is linked to the inability to dephosphorylate Bad because of insufficient CnA activation.

**DISCUSSION**

Elevation of intracellular Ca$^{2+}$ levels can influence a wide variety of biochemical processes including gene transcription. Activation of CnA and the resulting dephosphorylation of NF-AT are downstream events triggered by a rise in [Ca$^{2+}$]. Modulation of CnA activation can alter the immune response. Indeed, viruses have evolved the ability to evade host defense systems by inhibiting CnA activity (24). NF-AT4-deficient thyrocytes display heightened sensitivity to apoptosis mediated through the TCR, indicating that NF-AT4 might control the up-regulation of survival genes (25). It should be noted that NF-AT4 first associates with CnA in the cytoplasm and then both are translocated into the nucleus, where CnA may continue to act to counter the effects of nuclear NF-AT kinases (26). Our data are in agreement with the earlier findings that NF-AT translocation is defective in triple negative (type 1,2,3) cells expressing low levels of active CnA were higher than those observed in normal cells. Interestingly, raising [Ca$^{2+}$], by itself (without ionomycin) was sufficient to induce translocation of NF-AT but not dephosphorylation of Bad. Thus, in addition to the elevation of [Ca$^{2+}$], another signal is required for apoptotic but not necessarily for cell growth pathways. These data suggest that in addition to Ca$^{2+}$-dependent activation of CnA, apoptotic inducers must also trigger localization of CnA to substrates to initiate apoptosis.

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