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Characterization of a Mutant Calcineurin Aα Gene Expressed by EL4 Lymphoma Cells

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The calmodulin-stimulated phosphatase calcineurin plays a critical role in calcium-dependent T-lymphocyte activation pathways. Here, we report the identification of a missense mutation in the calcineurin Aα gene expressed by EL4 T-lymphoma cells. This mutation changes an evolutionarily conserved aspartic acid to asparagine within the autoinhibitory domain of the calcineurin Aα protein. A comparison of wild-type and mutant autoinhibitory peptides indicates that this amino acid substitution greatly reduces inhibition of calcineurin phosphatase activity. Additional peptide inhibition studies support a pseudosubstrate model of autoinhibitory function, in which the conserved aspartic acid residue may serve as a molecular mimic of either phosphoserine or phosphothreonine. Expression of the mutant calcineurin appears to affect cellular signal transduction pathways, as EL4 cells can be activated by suboptimal concentrations of calcium ionophore in the presence of phorbol esters. Moreover, this phenotype can be transferred to Jurkat T cells by transfection of the mutated calcineurin gene. These findings implicate a conserved aspartic acid in the mechanism of calcineurin autoinhibition and suggest that mutation of this residue is associated with aberrant calcium-dependent signaling in vivo.

Calcineurin (protein phosphatase 2B) regulates the activity of several transcription factors involved in the induction of lymphokine gene expression in activated T lymphocytes. Inhibition of calcineurin phosphatase activity by the immunosuppressive drugs cyclosporin A (CsA) and FK506 and by analogs of these compounds correlates with inhibition of lymphokine gene activation (8, 9, 20, 21). In addition, overexpression of calcineurin in T cells reduces sensitivity to CsA and FK506 (4, 26). Calcineurin is a heterodimeric protein consisting of a catalytic subunit (CnA) and a calcium (Ca^{2+})-binding subunit (CnB) (31). The amino-terminal catalytic domain of CnA is followed by segments that mediate binding to CnB and to calmodulin (CaM). The carboxy-terminal portion contains a negative regulatory domain that was originally identified by experiments demonstrating that limited proteolysis of calcineurin resulted in increased phosphatase activity (22). Later, a 25-amino-acid peptide derived from the regulatory domain of CnA was shown to inhibit calcineurin phosphatase activity in vitro (16, 28), suggesting that this domain acts by autoinhibition. Stimuli that induce a rise in the intracellular calcium concentration are thought to increase calcineurin activity by two mechanisms. First, the binding of Ca^{2+} to CnB lowers the K_m of CnA for its substrates by an unknown mechanism (29, 32). Secondly, Ca^{2+} ions also bind to CaM, increasing its affinity for the CnA catalytic subunit. The binding of CaM to CnA increases the V_{max} of the phosphatase (29, 32), perhaps because of the displacement of the autoinhibitory domain. The Ca^{2+} dependence of calcineurin is thought to explain the selective action of CsA and FK506 on cellular activation pathways involving a rise in intracellular Ca^{2+} concentration.

In most T lymphocytes, T-cell receptor-mediated induction of interleukin-2 (IL-2) gene expression requires two signals (34) and may be mimicked pharmacologically by treatment with phorbol esters and a Ca^{2+} ionophore (33). In the human Jurkat T-cell line, the requirement for a Ca^{2+} ionophore can be partially relieved by transient expression of a CaM-independent form of calcineurin (26–28, 35). In the murine EL4 T-lymphoma cell line, some IL-2 production can be triggered by treatment with phorbol esters in the absence of a Ca^{2+} ionophore (7), suggesting that calcineurin may be aberrantly regulated in these cells. We have determined that EL4 cells express a calcineurin Aα gene with a missense mutation in a region encoding the autoinhibitory domain. Peptide inhibition studies suggest that the aspartic acid residue altered in EL4 CnAα may be a central determinant of a pseudosubstrate mechanism of autoinhibition. The importance of this mutation is demonstrated by the observation that expression of the mutated calcineurin correlates with increased ionomycin responsiveness in T lymphocytes.

MATERIALS AND METHODS

Cell culture. The EL4 murine T-lymphoma cell line and the Jurkat human T-leukemia cell line were cultured in RPMI 1640 medium containing 10% fetal calf serum (GIBCO/BRL, Grand Island, N.Y.), 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μM 2-mercaptoethanol. CTLL-20 cells were cultured in the same medium supplemented with 1% conditioned medium from concanavalin A-stimulated rat spleen cells.

IL-2 assays. EL4 cells or Jurkat cells (1.5×10^5) were cultured in 48-well plates in the presence or absence of phorbol 12-myristate 13-acetate (PMA) and/or ionomycin, in a total volume of 0.6 ml per well. The concentrations of the drug diluent ethanol (EtOH) were identical in all wells. Twenty to twenty-four hours after initiation of the culture, supernatants were harvested and frozen at

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−20°C. Samples were thawed at 37°C, and the amount of IL-2 in serial dilutions of the samples was quantitated by using the IL-2-dependent murine cell line CTLL-20, which proliferates in the presence of both murine and human IL-2 (11). One unit was defined on the basis of recombinant human IL-2 added to each assay as a standard.

Measurement of intracellular free calcium concentration. Intracellular free Ca^{2+} concentrations were determined by in vivo fluorescence-activated cell sorting analysis of EL4 cells and Jurkat cells loaded with the Ca^{2+}-sensitive dye indo-1 and were calibrated by the method of Chused et al. (3).

RNA isolation, cDNA synthesis, and PCR. Cytoplasmic RNA was isolated from different cell lines as described elsewhere (1). RNA (20 μg) was heated at 95°C for 10 min, chilled on ice, and used as a template for synthesis of first-strand cDNA in a volume of 40 μl, with Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) used according to the manufacturer's instructions. PCR was carried out (with a kit from Perkin Elmer, Branchburg, N.J.) in a total volume of 100 μl containing 5 μl of cDNA preparations, 1 μl of each deoxynucleoside triphosphate (10 mM stocks), 10 μl of 10× PCR buffer, 1 μl of AmpliTaq DNA polymerase, and 2 μl of each primer (10 pmol/μl). To amplify the carboxy-terminal portion of CnAα containing the CaM-binding domain and the autoinhibitory domain, the sequences of the forward and reverse primers were TCCGACGATGAACTGGGGTCA and CCATCATGCCCTGCAGCT CAA, respectively. To amplify a full-length cDNA including restriction sites on the ends, the forward and reverse primers used were CGGGATCCTGTGCAG TCGGACGGGACGA and CCGAATTCCCATCATGCCCTGCAGCTCAA, respectively. The PCR parameters followed were 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min, with a 10-min incubation at 72°C after completion of the cycles.

Sequencing. PCR products were purified from agarose gels with a GeneClean kit (Bio 101, La Jolla, Calif.) and sequenced either directly or after subcloning into the vector pMH-Neo (14). Direct sequencing with the ^{32}P-end-labeled primer GAAGATGGATTTGACGGA was carried out with a PCR-based Fmol Sequencing kit (Promega, Madison, Wis.). The full-length CnAα in pMH-Neo was sequenced by the use of ^{35}S-labeled dATP with a Sequenase kit (U.S. Biochemical, Cleveland, Ohio). Sequencing reaction products were resolved on denaturing 6% polyacrylamide-urea gels with Sequagel reagents (National Diagnostics, Manville, N.J.).

Peptides. The calcineurin autoinhibitory peptides ITSFEEAKGLDRINERM PPRRDAMP (WT), ITSFEEAKGLNRINERMPPRRDAMP (D477N), ITSF EEAKGLDRINERMPPRRNAMP (D488N), and ITSFEEAAGLNRINERMP PRRDAMP (K474A) were synthesized and purified by high-pressure liquid chromatography (HPLC) at the Dana-Farber Cancer Institute (Boston, Mass.) Molecular Biology Core Facility. Peptides were dissolved in water to an approximate concentration of 2 mM, and the concentrations were precisely determined by amino acid analysis before use in bioassays. The CaM-binding peptide KRRWKKNFAIAVSAANRFKKISSSGAL, derived from the CaM-binding domain of myosin light chain kinase (2, 18), was provided by C. Klee (National Institutes of Health, Bethesda, Md.).

Calcineurin assays. Phosphatase assays using purified calcineurin were carried out in a total volume of 60 μl containing 50 nM calcineurin (Upstate Biotechnology Inc., Lake Placid, N.Y.), 200 nM CaM (Sigma Chemical Co., St. Louis, Mo.), and 5 μM ^{32}P-labeled phosphopeptide substrate (10) in an assay buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 0.4 mM $CaCl_2$, 0.5 μM dithiothreitol, and 100 μg of bovine serum albumin per ml. When appropriate, calcineurin autoinhibitory peptides or a CaM-binding peptide was included in the reaction mixture. Although CaM is thought to activate calcineurin by displacing the intramolecular autoinhibitory domain, free peptides derived from this domain can bypass this effect and inhibit CaM-stimulated phosphatase activity (16, 28). The reactions were terminated after 15 min at 30°C, and the released P_i was quantitated as described elsewhere (10). Phosphatase assays using cell lysates were performed as described earlier (10).

Plasmids. The reporter plasmid IL2pLUC, provided by T. Williams (University of New Mexico), contains the human IL-2 promoter/enhancer driving expression of firefly luciferase. The control plasmid pRSVβGal, provided by T. Hemesath (Dana-Farber Cancer Institute), contains the β-galactosidase (β-Gal) gene linked to the Rous sarcoma virus promoter, which drives constitutive gene expression in Jurkat cells. The pSRα expression vector containing the wild-type CnAα cDNA and the ΔCaM-AI truncation mutant were provided by R. Kincaid (Human Genome Sciences, Bethesda, Md.) and have been described previously (26). ΔCaM-AI, derived by insertion of a stop codon in the cDNA encoding CnAα (26), was designed to mimic proteolyzed forms of Cn that are CaM independent in vitro. The inserts from these plasmids were subcloned into the pMH-Neo expression vector by using flanking *Eco*RI sites to generate pMH-CnAα and pMH-ΔCaM-AI, originally for the purpose of generating stable transfectants. The wild-type CnAα cDNA is a splice variant (including 30 bp, and encoding 10 amino acids between the CaM-binding domain and autoinhibitory domains) that is absent in some cDNA clones (19). EL4 cells express both CnAα splice variants, although the smaller isoform predominates in PCR studies (see Fig. 2B). The plasmid pMH-CnAα(D477N), containing a guanine-to-adenine substitution at position 1429 of CnAα, was constructed by site-directed mutagenesis of pMH-CnAα with a Transformer Mutagenesis kit (ClonTech, Palo Alto, Calif.). The sequences of the selection and mutagenic primers were CGGCA GCGTGCCGCGGTTTGCAAAAG and GGCCAAGGGCTTAAACCGAAT TAACGAG, respectively. Plasmids carrying the mutation were identified by direct sequencing with an Fmol Sequencing kit, and the whole cDNA was sequenced by automated procedures (Dana-Farber Cancer Institute Molecular Biology Core Facility) to verify that no other mutations had been introduced. The insert from this plasmid was excised with *Eco*RI and subcloned back into the pSRα expression vector for use in transient transfections.

Transfections. Jurkat cells were suspended at a density of 2×10^7 cells per ml in RPMI 1640 medium containing 10% fetal calf serum at room temperature. Cell suspension (0.5 ml) was added to a 0.4-cm-diameter electroporation cuvette along with 30 μl of sterile TE buffer (10 mM Tris [pH 8], 1 mM EDTA) containing 2 μg of IL2pLUC, 2 μg of pRSVβGal, and 20 μg of calcineurin expression plasmid. After a 10-min incubation at room temperature, electroporation was carried out with a Hoefer Progenitor II apparatus (250 V, 980 μF, 35 ms). The electroporated cells were incubated for an additional 10 min at room temperature before transfer to flasks containing 25 ml of complete medium. When large numbers of cells were needed, multiple electroporation cuvettes with the same DNA combinations were prepared and the cells were pooled together afterwards. After 16 to 18 h, cells were counted and 10^6 cells were removed for assessment of β-Gal expression by fluorescence-activated cell sorting (25). The remaining cultures were divided into aliquots of 2×10^6 cells and mixed with an equal volume of stimuli prepared at a 2× final concentration in prewarmed complete medium. Stimulation flasks were incubated for 6 h at 37°C before the harvesting for luciferase measurement. CnB cDNAs were not cotransfected, because overexpression of both wild-type CnA and CnB has been reported to increase responsiveness to ionomycin (4). Although transient overexpression of CnA does not cause a significant increase in expression of endogenous CnB, it is thought that a portion of the exogenous CnA binds to endogenous CnB to form functional heterodimers (28).

Luciferase assays. Luciferase activity was determined with an Enhanced Luciferase Assay Kit and a luminometer from Analytical Luminescence Laboratories (San Diego, Calif.) used according to the manufacturer's instructions. The background luminescence value of untransfected Jurkat cells was subtracted from all luciferase measurements to give a net luciferase value. Net values were divided by the transfection efficiency determined by β-Gal assay. To determine the fold increase in IL-2 promoter activity, the β-Gal-adjusted values were divided by the value obtained for the vector-transfected, EtOH-treated control. Net luciferase values in unstimulated Jurkat cells were generally 1,000 to 3,000 U, indicating that the IL-2 promoter-luciferase construct displays basal activity in Jurkat cells.

RESULTS

EL4 cells produce IL-2 in the presence of PMA and suboptimal concentrations of ionomycin. EL4 cells were compared with human Jurkat T-leukemia cells which, like peripheral blood T lymphocytes (34), produced IL-2 when stimulated with PMA and ionomycin, a Ca^{2+} ionophore (Fig. 1A). Unlike Jurkat cells, EL4 cells produced a small amount of IL-2 in response to PMA (20 ng/ml) alone (Fig. 1A), as reported previously (7). The amount of IL-2 induced by PMA in EL4 cells was only 3.3% ± 0.4% (mean ± standard error of the mean [SEM]; $n = 3$) of the amount induced by the combination of PMA and 500 nM ionomycin. To examine further the activation requirements of EL4 cells, we measured IL-2 production in cells treated with a range of ionomycin concentrations in the presence of PMA. Importantly, the PMA response in EL4 cells could be greatly increased by the addition of low concentrations of ionomycin (10 to 200 nM) that were suboptimal for stimulation of Jurkat cells (Fig. 1A). For example, treatment with PMA plus 100 nM ionomycin resulted in approximately 35% of the maximal IL-2 production in EL4 cells but less than 5% of the maximal activity in Jurkat cells.

Calcineurin phosphatase activity in EL4 lysates can be detected at low concentrations of Ca^{2+} and CaM. The ability of EL4 cells to respond to suboptimal concentrations of ionomycin may be explained by basal levels of free Ca^{2+}, CaM, and/or calcineurin in the EL4 cytoplasm higher than those in Jurkat cells. However, the concentrations of cytoplasmic free Ca^{2+} were similar in unstimulated EL4 cells and Jurkat cells (275 ± 37 versus 276 ± 35 U [mean ± SEM; $n = 3$]), as assessed by the Ca^{2+}-sensitive fluorescent dye indo-1. In addition, the levels of CaM expression were comparable in EL4 and Jurkat cells, as determined by both immunoblotting and phenyl-Sepharose chromatography (data not shown). Furthermore,

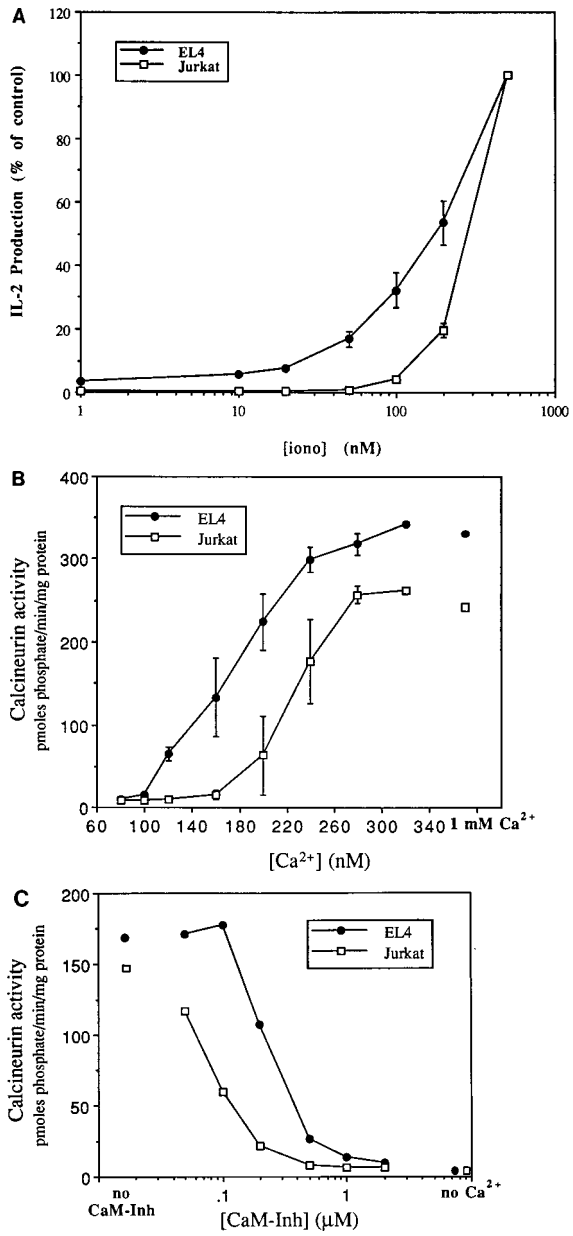

FIG. 1. (A) Comparison of IL-2 production in EL4 cells and Jurkat cells stimulated with PMA and various concentrations of ionomycin. Cells were stimulated with PMA (20 ng/ml) alone, ionomycin (iono) alone (500 nM), or with a combination of PMA and the indicated concentrations of ionomycin. Twenty-one hours after the initiation of culture, the supernatants were harvested and IL-2 was quantitated by using the IL-2-dependent CTLL-20 cell line (11). The amount of IL-2 produced (in units per milliliter) was converted to percent control IL-2 production, defined as the amount of IL-2 produced in the presence of PMA and 500 nM ionomycin. Data are expressed as the means ± SEM (error bars) ($n = 3$). The control IL-2 production values for EL4 and Jurkat cells were 53.9 ± 3.6 and 81.0 ± 24.2 U/ml, respectively. (B) Lower concentrations of exogenous Ca^{2+} can activate calcineurin activity in EL4 lysates than in Jurkat lysates. Cells were lysed under hypotonic conditions, and calcineurin activity in the lysates was measured in the presence of okadaic acid, an inhibitor of phosphatases 1 and 2A (10). The amount of free Ca^{2+} added to the phosphatase assay preparations was titrated with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid]-$CaCl_2$ mixtures (10). Shown is the average ± SEM of triplicate determinations in a single assay that was repeated three times with comparable results. (C) Higher concentrations of a CaM inhibitor are needed to inhibit calcineurin activity in EL4 lysates than Jurkat lysates. Calcineurin activity in hypotonic lysates was measured in duplicate in the absence or presence of various concentrations of a specific peptide inhibitor of calmodulin (termed CaM-Inh) derived from the CaM-binding domain of myosin light chain kinase (10). Similar results were observed in two additional experiments.

the levels of expression of CnA and CnB in EL4 cells were similar to those in other T-cell lines, as assessed by immunoblotting with two different anti-Cn antisera (data not shown).

To analyze calcineurin regulation directly, we assessed the Ca^{2+} dependence and CaM dependence of calcineurin activity in EL4 cells. The CaM-dependent phosphatase activity of crude cell extracts can be assayed by measuring the release of P_i from a ^{32}P-labeled phosphopeptide substrate in the presence of okadaic acid, an inhibitor of cellular phosphatases 1 and 2A (10). Under these conditions, phosphatase activity in Jurkat cells and EL4 cells was strictly dependent on the concentration of Ca^{2+} in the assay buffer (Fig. 1B). However, phosphatase activation in EL4 cell extracts was seen at a significantly lower concentration of exogenous Ca^{2+} than that for Jurkat cells (Fig. 1B). A high-affinity peptide inhibitor of CaM (2) effectively inhibited Ca^{2+}-dependent phosphatase activity in both Jurkat and EL4 cell extracts (Fig. 1C). However, approximately fourfold more CaM inhibitor was required to inhibit EL4 calcineurin activity (50% inhibitory concentration, 0.3 μM) than Jurkat calcineurin activity (50% inhibitory concentration, 0.08 μM) (Fig. 1C). Insofar as the levels of CaM protein expression were similar in Jurkat cells and EL4 cells, these results suggested that calcineurin activity in EL4 cells could be activated by reduced concentrations of CaM.

Cloning and sequencing of calcineurin Aα cDNAs. We cloned the calcineurin Aα cDNA from EL4 cells by reverse transcription and PCR. The EL4 calcineurin Aα sequence contained a single missense mutation when compared to the published sequence of mouse calcineurin Aα (Fig. 2A), which was cloned from a National Institutes of Health Swiss (Swiss/N) mouse brain library (19). The mutation observed in EL4 CnAα was not an artifact due to the use of a low-fidelity polymerase for PCR, since it was observed in eight independent PCR products derived from three EL4 cDNA preparations. In addition, the mutation was observed in a separate line of EL4 cells obtained independently from the American Type Culture Collection. Since the EL4 cell line was derived from a C57BL/6N mouse (12), it was possible that this guanine-to-adenine change at position 1429 of the coding sequence was indicative of a mouse strain polymorphism. However, no nucleotide changes were observed in CnAα cDNAs cloned from splenocytes of closely related C57BL/6J mice or from a murine T-cell hybridoma (By155.16 [30]) derived by fusion of C57BL/6J splenocytes with the AKR/J-derived thymoma BW5147 (data not shown).

The nucleotide substitution in EL4 eliminates a *Dde*I restriction site from the CnAα gene (Fig. 2A), allowing PCR products to be screened by restriction digestion rather than sequencing. Thus, *Dde*I digestion of EL4 PCR products gave rise to a 245-bp band in place of two smaller bands (158 and 87 bp) observed in digests of C57BL/6J and By155.16 PCR products (Fig. 2B). Two other bands of 114 and 102 bp were observed in all samples. PCR products containing the wild-type sequence or a mixture of guanine and adenine at position 1429 were never observed when EL4 cDNA was used, suggesting either that EL4 is homozygous for the mutation or that the wild-type allele is not expressed in EL4 cells.

Calcineurin mutations decrease autoinhibitory potency in vitro. The missense mutation in the EL4 calcineurin CnAα gene changes an aspartic acid residue (D) to an asparagine (N) at position 477 of the polypeptide (Fig. 2A). The D477 residue, which lies near the center of the 25-amino-acid minimal autoinhibitory peptide defined by in vitro studies (16), is one of several residues in this domain that are highly conserved throughout evolution (Fig. 2A). To determine if the D477N substitution affected inhibitory function, wild-type (WT) and

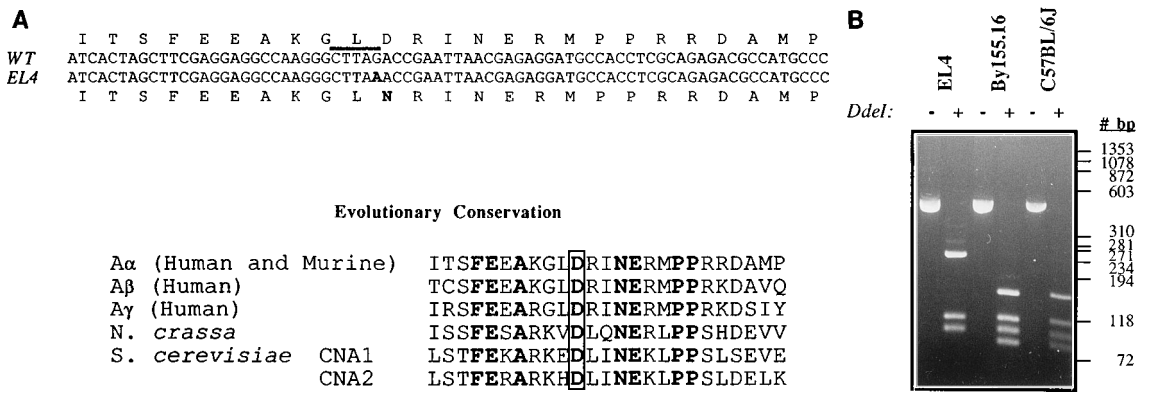

FIG. 2. A mutation in the EL4 calcineurin Aα gene changes a highly conserved aspartic acid residue to asparagine in the autoinhibitory domain. (A) Nucleotide and predicted amino acid sequences of the 25-amino-acid minimal autoinhibitory peptide of wild-type and EL4 CnAα. The wild-type (*WT*) sequences (nucleotides 1399 to 1473 and amino acids 467 to 491) and numbering are derived from the published sequence of the murine CnAα cDNA including a 30-nucleotide alternatively spliced exon (19). The boldface letters represent the point mutation and predicted change in the amino acid sequence of EL4 CnAα. The underline indicates a *Dde*I restriction site (CTNAG) that is eliminated by the G-to-A transition in the EL4 sequence. The amino acid sequences of calcineurin AI domains expressed in mice (19), humans (13, 19, 24), *Neurospora crassa* (17), and *Saccharomyces cerevisiae* (5) are indicated. Several amino acid residues are absolutely conserved (boldface print) including the aspartic acid residue (box) that is altered in EL4 calcineurin. (B) Sizes and *Dde*I restriction map of CnAα PCR products. The carboxy-terminal portion of the CnAα cDNA was amplified by reverse transcription PCR from RNA expressed by EL4 cells, By155.16 cells, or C57BL/6J splenocytes. PCR products were purified, incubated for 1 h at 37°C in the absence or presence of 10 U of *Dde*I restriction enzyme (New England Biolabs), and electrophoresed in a 2.5% agarose gel. The positions of the size markers (ϕX-*Hae*III digest) are shown on the right.

mutant (D477N) peptides were tested for the ability to inhibit calcineurin phosphatase activity in vitro. The WT autoinhibitory peptide inhibited the phosphatase activity of purified calcineurin in a dose-dependent manner, with an 50% inhibitory concentration of approximately 20 μM (Fig. 3A). The D477N peptide was much less potent, inhibiting calcineurin by only 30% at a concentration of 100 μM (Fig. 3A). The change from aspartic acid to asparagine is a nonconservative substitution resulting in the loss of an acidic amino acid residue. The peptide D488N, in which another evolutionarily conserved aspartic acid residue (Fig. 2A) was changed to asparagine, inhibited calcineurin activity with a potency similar to that of the WT peptide (Fig. 3A). This finding suggested that loss of activity by the D477N peptide was not simply due to a reduced overall negative charge but was likely the result of a specific change in its interaction with the calcineurin catalytic domain or with other residues within the autoinhibitory domain. The difference in inhibitory potency was not an artifact due to the use of a bovine calcineurin enzyme, since calcineurin activity in EL4 cell extracts showed a similar difference in sensitivity to the two peptides (Fig. 3B).

CsA and FK506 inhibit calcineurin by forming complexes with intracellular binding proteins and subsequently binding to the CnB subunit (23), an interaction which results in noncompetitive inhibition of phosphatase activity. In contrast, the calcineurin autoinhibitory domain appears to be a competitive inhibitor of phosphatase activity (28), perhaps acting as a pseudosubstrate (16, 29). The reduced inhibitory activity of the D477N peptide is consistent with such a model in that the conserved aspartic acid, by virtue of its negative charge, may serve as a nonhydrolyzable analog of phosphoserine or phosphothreonine. In vitro studies suggest that optimal phosphopeptide substrates of calcineurin contain a basic amino acid at position −3 relative to the phosphorylated amino acid (6). Interestingly, calcineurin autoinhibitory domains of a variety of species contain a lysine or an arginine at position −3 relative to the conserved aspartic acid (Fig. 2A). A peptide in which the conserved lysine in mammalian CnAα was changed to alanine (peptide K474A) was approximately three- to fourfold weaker as an inhibitor of calcineurin than the wild-type peptide (Fig. 3C). The K474A peptide retained greater inhibitory activity than the D477N peptide. These findings are consistent with a pseudosubstrate model of autoinhibitory action, in which the negatively charged aspartic acid residue is a primary determinant of peptide recognition while adjacent residues contribute to a secondary structure similar to that of phosphopeptide substrates.

Expression of calcineurin mutant protein in Jurkat cells. The peptide inhibition studies described above supported the hypothesis that the increased ionomycin responsiveness of EL4 cells could be explained by aberrant regulation of calcineurin. Transient transfections were performed to determine if expression of the CnAα mutant protein in Jurkat cells could confer a phenotype similar to that of EL4 cells. Jurkat cells were cotransfected with a reported plasmid consisting of the firefly luciferase gene linked to the human IL-2 promoter and a control plasmid containing the β-Gal gene linked to a constitutively active Rous sarcoma virus promoter. In addition, cells were cotransfected with calcineurin expression plasmids carrying cDNAs encoding either wild-type CnAα(WT), the mutated CnAα(D477N), or a truncated form of CnAα (termed ΔCaM-AI) previously shown to confer responsiveness to PMA alone (26–28, 35). The percentage of β-Gal-expressing cells in each transfection group, determined by fluorescence-activated cell sorting (25), served as a measure of transfection efficiency. Cells from each transfection group were incubated with various stimuli for 6 h and then were harvested for measurement of luciferase activity.

In the presence of PMA, D477N transfectants were substantially more responsive at all ionomycin concentrations tested than WT or vector transfectants (Fig. 4A). PMA plus 50 nM ionomycin, a treatment which resulted in minimal (about twofold) activation of the IL-2 promoter in vector and WT transfectants, induced a 30- to 40-fold activation in D477N transfectants (Fig. 4). Inhibition by coincubation with CsA (Fig. 4B) demonstrated that this response was calcineurin dependent. These results provided strong evidence that the sensitivity of EL4 cells to suboptimal doses of ionomycin in the presence of PMA was attributable to the D477N mutation in their CnAα gene. As reported previously (26–28, 35), IL-2 promoter activ-

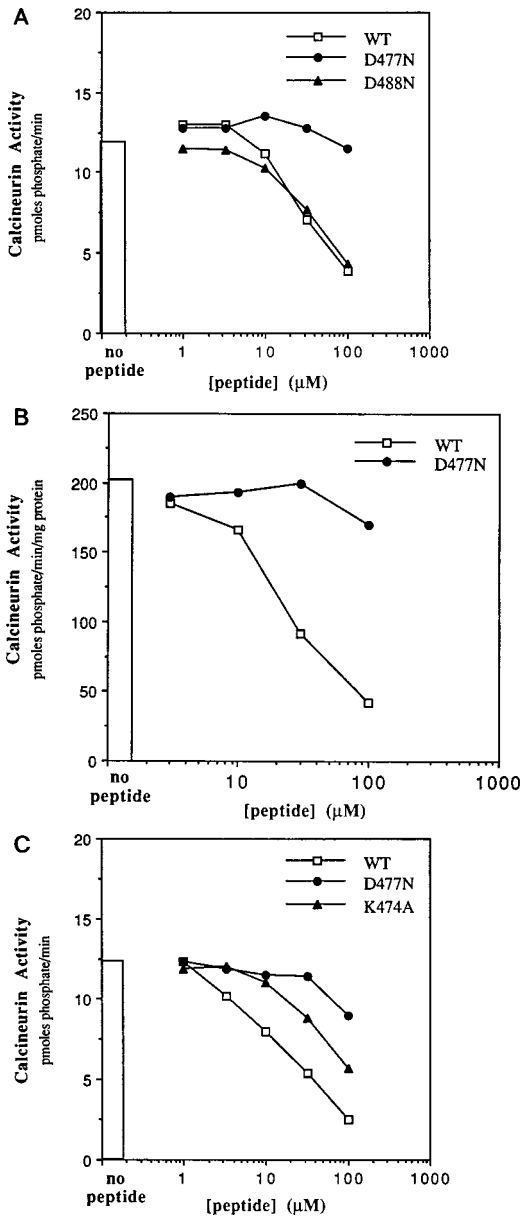

FIG. 3. Structure-function analysis of the autoinhibitory domain in vitro. (A and C) Inhibition of phosphatase activity of purified calcineurin by autoinhibitory peptides. Peptides containing 25 amino acids based on the wild-type (WT) autoinhibitory sequence (Fig. 2A), containing asparagine substitutions for aspartic acid 477 (D477N) or aspartic acid 488 (D488N), or containing alanine for lysine 474 (K474A) were synthesized. Purified bovine brain calcineurin (50 nM) was incubated in the presence of bovine CaM (200 nM) and the indicated concentrations of peptides. Phosphatase activity was measured and expressed as picomoles of phosphate released per minute. (B) Inhibition of calcineurin activity in EL4 cell lysates by autoinhibitory peptides. EL4 cell hypotonic lysates were incubated with okadaic acid and the indicated concentrations of peptides. Phosphatase activity was measured (10) and expressed as picomoles of phosphate released per minute per milligram of protein. Each of these experiments was repeated once with comparable results, and the D477N peptide was compared with the WT peptide in three additional experiments.

ity could be increased by PMA alone in Jurkat cells transfected with ΔCaM-AI but not in cells transfected with CnAα WT or with the expression vector alone (Fig. 4). Incubation with PMA alone did not cause significant activation of the IL-2 promoter in Jurkat cells transfected with D477N (Fig. 4).

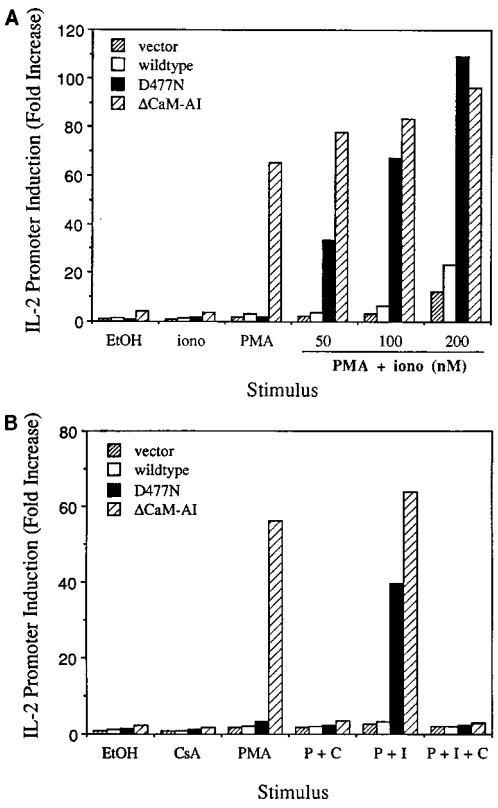

FIG. 4. IL-2 promoter induction in Jurkat cells transfected with various calcineurin expression plasmids. (A) Transfectants incubated in the presence of the drug diluent (EtOH) or stimulated with ionomycin (iono; 500 nM) alone, PMA (20 ng/ml) alone, or PMA plus the indicated concentrations of ionomycin. (B) Cells were incubated with EtOH alone, 100 nM CsA, PMA (20 ng/ml), PMA plus 100 nM CsA (P+C), PMA plus 50 nM ionomycin (P+I), or PMA plus 50 nM ionomycin plus 100 nM CsA (P+I+C). In each experiment, the final concentrations of EtOH were identical in all treatments. After the stimulation period, cells were harvested by centrifugation and luciferase activity was measured. The fold increase in IL-2 promoter activity was expressed relative to that of the vector-transfected, EtOH-treated control (see Materials and Methods). These experiments were repeated twice (A) or once (B) with comparable results.

DISCUSSION

The identification of calcineurin as a target of immunosuppressive agents has revealed a central role for this serine/threonine phosphatase in calcium-dependent signal transduction pathways. Previous studies have suggested that calcineurin phosphatase activity is regulated by an autoinhibitory domain in the carboxy-terminal portion of the catalytic subunit. Increases in the intracellular Ca^{2+} concentration potentiate the binding of CaM to calcineurin, stimulating phosphatase activity by a mechanism thought to involve displacement of the autoinhibitory domain. In this report, we have identified a mutation in the calcineurin Aα gene expressed by EL4 T-lymphoma cells and have shown that the mutation affects the function of the autoinhibitory domain in vitro. In addition, we have demonstrated that expression of the mutant protein correlates with the ability of T cells to respond to suboptimal concentrations of ionomycin in the presence of PMA.

Peptide inhibition studies demonstrated that the mutant peptide, termed D477N, is a substantially weaker inhibitor of calcineurin activity than the wild-type autoinhibitory peptide (Fig. 3). These findings suggest that the D477 residue is an important structural determinant of autoinhibitory function. This hypothesis is supported by the absolute conservation of

this aspartic acid throughout evolution (Fig. 2A) and by a recent study in which replacement of this residue with alanine caused a reduction in peptide inhibition (29). We propose that this negatively charged amino acid may serve as a molecular mimic of a phosphorylated serine or threonine. Perhaps the aspartic acid interacts electrostatically with a basic amino acid within the phosphatase active site that normally binds to a phosphorylated serine or threonine; in the absence of a hydrolyzable phosphate group, the autoinhibitory domain would remain bound. Alternatively, the aspartic acid may interact with a basic residue in another portion of the autoinhibitory domain, such that substitution of the aspartic acid affects the tertiary structure of the domain. In this regard, two arginines about 10 residues downstream of D477 were recently shown to play a critical role in autoinhibitory function (29).

For certain kinases containing autoinhibitory domains or regulatory subunits, inhibition of kinase activity has been shown to involve a pseudosubstrate mechanism whereby the inhibitory peptide sequence is similar to substrate consensus sequences but lacks the critical serine or threonine (15). By analogy, a phosphatase pseudosubstrate should have a primary sequence similar to those of known substrates but without a phosphorylated amino acid. Little is known about primary sequence requirements for calcineurin substrates; however, a recent study found that the most effective peptide substrates of calcineurin contained a basic amino acid three residues upstream of the phosphorylated amino acid (6). Each calcineurin gene that has been cloned encodes a domain similar to the mammalian autoinhibitory domain, and each of these proteins contains a basic amino acid (lysine or arginine) three residues upstream of the conserved aspartic acid (Fig. 2A). A lysine-to-alanine substitution in the peptide derived from the human sequence decreased autoinhibitory potency (Fig. 3), which is consistent with a role for the K474 residue in autoinhibitory function. In summary, analysis of sequence conservation combined with in vitro peptide studies provides considerable evidence for a pseudosubstrate model of calcineurin autoinhibitory domain function. If this model is correct, the autoinhibitory domain of calcineurin should act as a competitive inhibitor of phosphatase activity, a conclusion that is supported by some studies (28) but contradicted by others (16, 29). These reports analyzed inhibition by the 25-amino-acid peptide used in the present study, which is a relatively weak inhibitor of calcineurin in vitro. We are currently evaluating the kinetic mechanism of calcineurin autoinhibition by using larger fragments of the carboxy-terminal domain of CnAα.

The D477N mutation in the CnAα autoinhibitory domain is associated with calcineurin activation in EL4 lysates at reduced concentrations of free Ca^{2+} and CaM (Fig. 1). Similarly, it has been shown that a substitution of alanine for the conserved aspartic acid yields a phosphatase that is less dependent on Ca^{2+} and CaM for activity in vitro (29). These studies suggest that a reduction in autoinhibitory domain affinity is associated with increased CaM binding to the catalytic subunit. A possible explanation for these findings is that the CaM binding site on CnA is sterically hindered by the coassociation of the autoinhibitory and catalytic domains, such that a decrease in the stability of autoinhibitory domain interaction would allow an increased association rate of CaM binding.

The identification of a point mutation affecting the function of the calcineurin autoinhibitory domain provided a possible explanation for the unusual activation requirements of EL4 cells (Fig. 1A). Transient transfection of Jurkat T cells confirmed that expression of CnAα(D477N) is sufficient to confer sensitivity to suboptimal concentrations of ionomycin (Fig. 4). Expression of the mutant calcineurin did not result in a significant PMA response in Jurkat transfectants, suggesting that additional factors in EL4 cells may contribute to their ability to respond to PMA alone. The PMA response in EL4 cells was inhibited only 75 to 80% by CsA and FK506 (data not shown), which is consistent with a role for calcineurin-independent factors in this response. The ability of ΔCaM-AI transfectants to respond to PMA alone may be due to the complete absence of the autoinhibitory domain in the truncated protein, eliminating its requirement for CaM altogether. A similar truncation mutant has been shown to possess increased CaM-independent and Ca^{2+}-independent phosphatase activities in vitro, whereas the D477A point mutation did not increase Ca^{2+}-independent activity (29).

Since EL4 is a lymphoma cell line derived from a mutagenized mouse (12), it is conceivable that aberrant regulation of calcineurin is involved in the transformed phenotype of these cells. Calcineurin has been proposed to be a proto-oncogene on the basis of the ability of ΔCaM-AI to synergize with constitutively active $p21^{ras}$ in the activation of IL-2 gene transcription (35). In preliminary experiments, we have found that the proliferation of EL4 cells is not inhibited by CsA or FK506, suggesting that the activity of dysregulated calcineurin is not required for progression through the cell cycle in EL4 cells. Further experiments are required to determine if calcineurin Aα D477N or other calcineurin mutants possess transforming activity.

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