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ARTICLES

Calcium- and FK506-Independent Interaction Between the Immunophilin FKBP51 and Calcineurin

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Abstract FKBP51 is a member of the immunophilin family having intrinsic peptidyl-prolyl cis-trans-isomerase (PPIase) activity. Its enzymatic activity is inhibited by binding either immunosuppressive agent FK506 or rapamycin. Similar to FKBP12, but at higher concentrations of FK506, FKBP51 has been shown to inhibit the serine/threonine phosphatase activity of calcineurin in the presence of calcium and calmodulin. Here we show that a glutathione S-transferase (GST) fusion protein of FKBP51 on glutathione-Sepharose beads precipitated both purified calcineurin from bovine brain and calcineurin from murine T cell lysates. Surprisingly, the binding of GST-FKBP51 to calcineurin was FK506-independent and independent of a requirement for calcium or exogenous calmodulin. Unlike FKBP12, FKBP51 transiently expressed in COS-7 cells was precipitated by calcineurin bound to calmodulin-Sepharose beads in the absence of either FK506 or rapamycin. Unlike FKBP12, however, overexpression of FKBP51 in Jurkat T cells did not significantly affect the transcriptional activation of nuclear factor of activated T cells (NFAT) upon physiological stimulation, nor did it affect the ability of FK506 to inhibit NFAT-driven transcription. We generated a series of FKBP51 mutations to map the interaction of FKBP51 with calcineurin. Deletion of the aminoterminal, FKBP12-like domain of FKBP51 did not affect the ability of FKBP51 to bind to purified calcineurin, while deletion of the FKBP51 carboxyterminal domain abrogated the ability of FKBP51 to bind to calcineurin. Taken together, these results demonstrate a novel interaction between calcineurin and the immunophilin FKBP51 that is independent of calcium, calmodulin, and drug. The binding site on calcineurin for FKBP51 is separable from the immunophilin PPIase-active and drug-binding site. J. Cell. Biochem. 84: 460–471, 2002. Published 2001 Wiley-Liss, Inc.†

Key words: immunosuppression; transplantation; FKBPs; cyclosporin; rapamycin; calcineurin; immunophilin

Immunophilins are characterized by drug-inhibitable peptidyl-prolyl cis-trans-isomerase (PPIase, also termed rotamase) activity (reviewed in Galat [1993] and Kay [1996]): the enzymatic activity of the family of FK506-binding proteins (FKBPs) is inhibited by FK506 and rapamycin, while the activity of the cyclophilins (CyPs) is inhibited by cyclosporin A (CsA) (reviewed in Bierer [1994] and Marks [1996]). However, while these agents inhibit the PPIase activity of the immunophilins, their ability to function as immunosuppressive drugs is independent of this feature. The mechanism of immunosuppression in T lymphocytes has been demonstrated to be the inhibition of calcineurin phosphatase activity by the drug/immunophilin complex [Clipstone and Crabtree, 1992; Fruman et al., 1992; O'Keefe et al., 1992]. Calcineurin (protein phosphatase 2B) is a calcium (Ca^{2+})/calmodulin-dependent serine/threonine phosphatase that consists of a catalytic A subunit and a regulatory, calcium-binding B subunit. In T lymphocytes and other cells, calcineurin is able to dephosphorylate members of the NFAT family of transcription factors. NFAT dephosphorylation is required for its nuclear translocation and subsequent transcriptional activation of a number of cytokine and other genes (reviewed in Rao et al. [1997]). We previously characterized an immunophilin FKBP51 expressed during adipocyte differentiation of mouse 3T3-L1 cells

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[Yeh et al., 1995]. The FKBP51 cDNA predicted a 456-amino acid polypeptide of approximately 51 kDa. The amino acid sequences of mouse FKBP51 and FKBP52 [Schmitt et al., 1993] were 54% identical while the aminoterminal region (amino acids 32–138; termed the FKBP12-like domain) of mouse FKBP51 was 47% identical to mouse FKBP12, an abundantly expressed 12-kDa cytosolic FKBP and the principal receptor important for drug-dependent inhibition of calcineurin activity. FKBP51 was shown to be an active PPIase; its enzymatic activity was inhibitable by either FK506 or rapamycin [Yeh et al., 1995]. Independently, Baughman et al. [1995] cloned mouse FKBP51 and demonstrated its ability to inhibit calcineurin phosphatase activity in the presence of FK520, an analogue of FK506, albeit ~260-fold less potently than human FKBP12. Human FKBP51 is 90% identical to its murine counterpart and was also shown to be an FK506-sensitive peptidylprolyl isomerase [Nair et al., 1997]. Human FKBP51 is also able to inhibit calcineurin phosphatase activity weakly [Baughman et al., 1997].

Given the sequence similarity between FKBP12 and the aminoterminal domain of FKBP51, we compared the interaction of calcineurin with FKBP51 and FKBP12. Here we report that, unlike FKBP12, the interaction between FKBP51 and calcineurin is independent of a requirement for drug. A glutathione S-transferase (GST) fusion protein of FKBP51 (GST-FKBP51) was used to bind both purified and T cell-derived calcineurin; neither calcium nor calmodulin were required for binding. Overexpression of wild-type FKBP51 in Jurkat T cells, however, did not significantly affect the transcriptional activation of NFAT stimulated by treatment of the cells with PMA and ionomycin nor did it modulate the ability of FK506 to inhibit the transcriptional activation of NFAT. Interestingly, by mutagenesis analysis, we show that the carboxyterminal region, not the FKBP12-like domain, of FKBP51 mediated the drug-independent binding to calcineurin.

METHODS

Cells, Media, and Reagents

Murine T hybridoma cells (By155.16; Ref. [Bierer et al., 1990]) and SV40 large T antigen-transformed human Jurkat T cells [Lin et al., 1998] were cultured in RPMI-1640 (MediaTech, Herndon, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma, St. Louis, MO and Life Technologies, Gaithersburg, MD), 100 U/ml of penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, and 50 μM 2-mercaptoethanol (termed 10%-RPMI). Monkey kidney COS-7 cells were cultured in Dulbecco Modified Eagles Medium (DMEM, Life Technologies) supplemented with 10% heat-inactivated FCS (Life Technologies), 100 U/ml of penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (termed 10%-DMEM). All cells were cultured at 37°C in 5% CO_2-in-air. Phosphate-buffered saline (PBS) was purchased from Life Technologies. FK506 (Alexis Biochemicals, San Diego, CA), rapamycin (Biological Modifiers Resources Program, NIH, Bethesda, MD), phorbol-12-myristate-13-acetate (PMA; Calbiochem, San Diego, CA), and ionomycin (Calbiochem) were reconstituted in pure ethanol. Both calcineurin and calmodulin, purified from bovine brain, were purchased from Sigma. Glutathione-conjugated Sepharose 4B (glutathione-Sepharose beads), calmodulin-conjugated Sepharose 4B (calmodulin-Sepharose beads), and DEAE-Dextran were obtained from Pharmacia Biotech (Piscataway, NJ). Purified rabbit anti-calcineurin A antibody [Parsons et al., 1994] and mouse anti-calmodulin mAb were purchased from Veritas, Inc. (Potomac, MD) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Purified rabbit anti-FKBP12 antibody [Fruman et al., 1995] and anti-FKBP51 antiserum [Yeh et al., 1995] have been previously described. Both horseradish peroxidase (HRP) linked donkey anti-rabbit Ig and sheep anti-mouse Ig antibodies were obtained from Amersham (Arlington Height, IL).

Generation and Purification of Wild Type and Mutated Fusion Proteins of GST-FKBP51

A purified DNA fragment containing the entire mouse FKBP51 coding sequence was isolated from a FKBP51 cDNA clone [Yeh et al., 1995] by *Eco* RI digestion and was further digested with *Ban* I to eliminate the 5′-untranslated region and the first 21 bps of FKBP51 coding sequence. The obtained *Ban* I/*Eco* RI fragment was ligated with both the pGEX-2T expression vector (Pharmacia Biotech) linearized by *Bam* HI/*Eco* RI digestion and a *Bam* HI/*Ban* I linker designed to restore the first 21 bps

of the FKBP51 coding sequence. The ligation mixture was transformed into *E. coli* DH5α. Plasmids (pGEX-2T-FKBP51) isolated from transformants were verified by restriction digestion and DNA sequence analysis. To generate the deletion mutations FKBP51 Δ1-134, FKBP51 Δ44-134, and FKBP51 Δ134-456 of wild-type GST-FKBP51, the pGEX-2T-FKBP51 plasmid was digested by *Bam* HI/*Sac* I, *Spe* I/*Sac* I, and *Sac* I/*Eco* RI, respectively. After restriction digestion, each of the linearized plasmids was treated with T4 DNA polymerase followed by self-ligation. All constructs were sequenced to insure fidelity of the ligation. The wild-type and mutant clones were transformed into *E. coli* BL21, and the recombinant GST fusion proteins were induced by IPTG (Sigma) and purified by glutathione-Sepharose beads as previously described [Frangioni and Neel, 1993]. BL21 transformed with untreated pGEX-2T was used to generate GST as the fusion protein control. All of the purified GST fusion proteins on glutathione-Sepharose beads were prepared as a 50% slurry in storage buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol, and 10% glycerol) and kept at −80°C. The GST-fusion proteins were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie blue (Bio-Rad, Hercules, CA) staining.

Overexpression of FKBP51 and FKBP12 in COS-7 Cells

The FKBP51 coding sequence with 3′-untranslated region isolated from a mouse FKBP51 cDNA clone [Yeh et al., 1995] was subcloned into the mammalian expression vector pCAGGS [Niwa et al., 1991] to generate the pCAGGS-FKBP51 construct. The construct pCMV5-FLAG-FKBP12 [Chen et al., 1997] expressing the FLAG-tagged FKBP12 protein was kindly provided by J. Massagu (Memorial Sloan-Kettering Cancer Center, New York, NY). FKBP51 or FLAG-tagged FKBP12 proteins was overexpressed in COS-7 cells by transient transfection. For transfection, COS-7 cells (approximately 50–70% confluence) were incubated with 0.67 μg/ml DNA in the presence of 100 μM chloroquine (Sigma) and 400 μg/ml DEAE-Dextran at 37°C for 3 h. After incubation with DNA, cells were treated with 10% DMSO (Sigma) in PBS at room temperature for 2 min. Cells were washed with PBS and cultured in 10%-DMEM for 48 h prior to analysis.

Precipitation and Immunoblotting

Murine T hybridoma cells were washed in RPMI-1640 and lysed in buffer A (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10 μM $CaCl_2$, 10 mM NaF, 20 mM sodium pyrophosphate, 1 mM Na_3VO_4, 60 μg/ml aprotinin, 60 μg/ml leupeptin, 200 μg/ml soybean trypsin inhibitor, 2 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100). Cell lysates were clarified by centrifugation at ∼19,000*g* for 10 min at 4°C. For glutathione-dependent precipitation, GST-FKBP12 [Jin et al., 1992], GST-FKBP51, or GST on glutathione-Sepharose beads were incubated with either T cell lysates or purified calcineurin, calmodulin, and/or FK506, as indicated, at 4°C for 1.5–2.5 h. After incubation, beads with precipitated proteins were washed five times in buffer B (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 5 mM NaF, 20 mM sodium pyrophosphate, 1 mM Na_3VO_4, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 30 μg/ml soybean trypsin inhibitor, 10% glycerol, and 0.1% Triton X-100). The precipitated proteins were then separated by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA) or nitrocellulose (Bio-Rad) membrane. Proteins on membranes were immunoblotted with specific antibodies as indicated, followed by incubation with HRP-linked secondary antibodies. Enhanced chemiluminescence (ECL) detection system (Amersham) was used according to manufacturer's instructions. In some experiments, following ECL detection, the membranes were stained by Coomassie blue to visualize total proteins.

Calmodulin-Sepharose beads were charged with Ca^{2+} by pre-incubation with 2 mM $CaCl_2$ prior to precipitation. Transfected COS-7 were harvested, washed in DMEM, and lysed in buffer A. Cell lysates were clarified by centrifugation ∼21,000*g* for 10 min at 4°C and incubated with calmodulin-Sepharose beads in the presence of 4 μg purified calcineurin at 4°C for 2 h. After incubation, beads with precipitated proteins were washed five times in buffer B. Precipitated proteins were then separated by 15% SDS–PAGE and transferred to PVDF membranes. The membranes were immunoblotted and detected using ECL as described above.

NFAT Luciferase Assays

The luciferase reporter construct p3xNFAT-luc [Lin et al., 1998] containing three tandem

repeats of the distal NFAT sequence from the IL-2 promoter was used to analyze transcriptional activation of NFAT. Transfection efficiency was controlled by co-transfection with the Renilla luciferase control vector, pRL-TK (Promega, Madison, WI), containing the promoter of herpes simplex virus thymidine kinase and the coding sequence of Renilla luciferase. SV40 large T antigen-transformed Jurkat cells (1×10^7) were transiently transfected with 10 μg pCAGGS or pCAGGS-FKBP51 in the presence of 1 μg pRL-TK and 10 μg p3xNFAT-luc by electroporation using CELL-PORATOR (Life Technologies) at 250 V and 800 μF. Transfected cells were cultured in 10%-RPMI media at 37°C for 24 h. Cells were pretreated with FK506 or equivalent ethanol diluent for 1–1.5 h and then stimulated with PMA, ionomycin, PMA plus ionomycin, or ethanol diluent at the indicated concentrations for an additional 6 h. After stimulation, cells were harvested, washed with PBS, and prepared for the luciferase assay using the Dual-Luciferase Reporter Assay System (Promega). Cell pellets derived from cells treated with ethanol control were frozen to ensure protein expression by Western blot analysis. For the luciferase assay, each cell pellet was lysed in 40–50 μl lysis buffer; 15–20 μl lysate was used to measure luminescence using a liquid scintillation and luminescence counter (1450 Microbeta) from Wallac (Gaithersburg, MD). NFAT luciferase activity for each sample was calculated as a ratio of the luminescence counts per second (LCPS) of firefly to Renilla luciferase.

RESULTS

Association of the Fusion Protein GST-FKBP51 With Purified Calcineurin

The serine/threonine phosphatase calcineurin is a cellular target of the immunophilin/drug complex FKBP12/FK506 [Liu et al., 1991]. In the presence of FK506 or its analogue FK520, FKBP51 was shown to inhibit the phosphatase activity of calcineurin in vitro, although the FKBP51/FK506 complex was shown to be ~250-fold less effective than FKBP12/FK506 in the inhibition of calcineurin activity [Baughman et al., 1995, 1997]. To study the interaction of calcineurin with FKBP51, we generated the GST fusion protein GST-FKBP51 and compared it to GST-FKBP12. Purified material was used to precipitate associating proteins on glutathione-Sepharose beads (Fig. 1). As expected [Liu et al., 1991], in the absence of exogenous calmodulin, GST-FKBP12 bound to purified bovine calcineurin only in the presence of FK506 (Fig. 1, lane 2). While GST-FKBP12 did not precipitate calmodulin (Fig. 1, lane 3), the amount of calcineurin retained by the complex of GST-FKBP12/FK506 was markedly increased in the presence of added calmodulin (Fig. 1, lane 4). There was no binding of calcineurin to GST alone on glutathione-Sepharose beads (data not shown). Notably, GST-FKBP51 on glutathione-Sepharose beads precipitated purified calcineurin in the absence of either FK506 or calmodulin (Fig. 1, lane 6). Neither FK506 (Fig. 1, lane 8) nor calmodulin (Fig. 1, lane 9 and data not shown) affected the amount of calcineurin bound by GST-FKBP51. In addition, the binding of GST-FKBP51 to purified calcineurin was not affected by the presence of 1 mM DTT, pH 8.0, arguing that the interaction between FKBP51 and calcineurin did not result from disulfide linkage (data not shown). The observation that the addition of calmodulin had no effect on retention of calcineurin by GST-FKBP51 suggested that calmodulin played a minimal role, if any, in the interaction between FKBP51 and calcineurin.

It has been reported that calmodulin binding to calcineurin is required for calcineurin activity presumably by inducing a conformational change that results in the unfolding of the autoinhibitory domain of calcineurin and the exposure of the active site. Binding of calmodulin to calcineurin is dependent on the concentration of the cation calcium (reviewed in Klee et al. [1998] and Rusnak et al. [1999]). To insure that trace amounts of calmodulin had not co-purified with bovine calcineurin, we compared the association of FKBP51 to calcineurin in the absence or presence of the calcium chelator EGTA, using the Ca^{2+}- and FK506-dependent binding of GST-FKBP12 to calcineurin as a control (Fig. 2). As expected, in the presence of calmodulin, the FK506/GST-FKBP12 complex precipitated calcineurin (Fig. 2, lane 2); the addition of 5 mM EGTA to the reaction buffer, however, completely abolished the binding of calcineurin (Fig. 2, lane 4) and calmodulin (data not shown) to the GST-FKBP12/FK506 complex. The addition of 5 mM EGTA completely abrogated the association of calmodulin with the GST-FKBP12/FK506/calcineurin complex

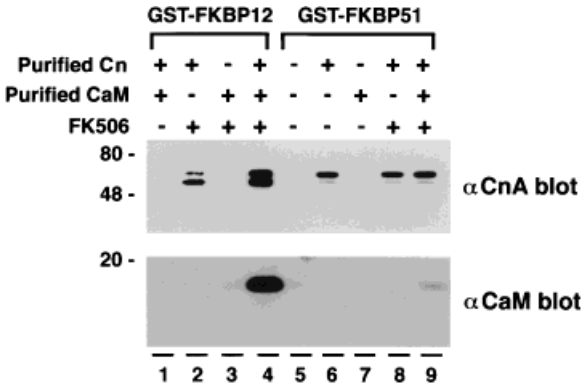

Fig. 1. FK506-independent association of GST-FKBP51 with purified calcineurin. GST-FKBP51 (~100 nM) or GST-FKBP12 (~100 nM) on glutathione-Sepharose beads was incubated with (+) or without (−) 50 nM purified calcineurin (Cn), 100 nM purified calmodulin (CaM), 100 nM FK506, or equivalent ethanol diluent, as indicated, at 4°C for 2 h. After incubation, beads were washed. The precipitated proteins were separated on 13% SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was cut appropriately, immunoblotted with the anti-calcineurin A (CnA) (top panel) antibody or anti-calmodulin mAb (bottom panel), and developed by ECL. Molecular weight markers (in kDa) are shown on the left.

and with the GST-FKBP51/calcineurin complex (data not shown), confirming the requirement for Ca^{2+} in calmodulin binding. Surprisingly, however, the addition of 5 mM EGTA did not quantitatively affect the binding of purified calcineurin to GST-FKBP51 on beads (Fig. 2, compare lanes 6 and 9 with 7 and 10). Taken together, our data suggested that the interaction of FKBP51 and calcineurin was not only independent of FK506, but also independent of Ca^{2+} and calmodulin.

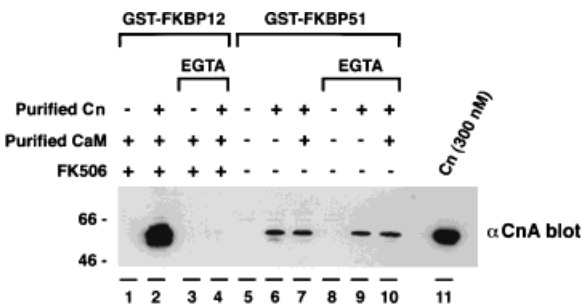

Fig. 2. Ca^{2+}/calmodulin-independent association of GST-FKBP51 with purified calcineurin. GST-FKBP51 (~85 nM) or GST-FKBP12 (~120 nM) on glutathione-Sepharose beads was incubated with (+) or without (−) 50 nM purified calcineurin (Cn), 100 nM purified calmodulin (CaM), 100 nM FK506, or equivalent ethanol diluent in the absence or presence of 5 mM EGTA, as indicated, at 4°C for 2.5 h. After incubation, beads were washed. The precipitated proteins and purified calcineurin were separated on 13% SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was cut, immunoblotted with the anti-calcineurin A (CnA) antibody or anti-calmodulin mAb (not shown), and developed by ECL. Molecular weight markers (in kDa) are shown on the left.

Interaction of FKBP51 With Calcineurin in Cell Lysates

Having demonstrated the association of GST-FKBP51 and calcineurin in vitro, we sought to determine whether GST-FKBP51 was able to bind endogenous calcineurin from T cell lysates (Fig. 3). Either GST or GST-FKBP51 on glutathione-Sepharose beads was incubated with buffer (Fig. 3A, lanes 1 and 2), murine T cell lysates (Fig. 3A, lanes 3 and 4), or purified bovine calcineurin (Fig. 3A, lanes 5 and 6). GST-

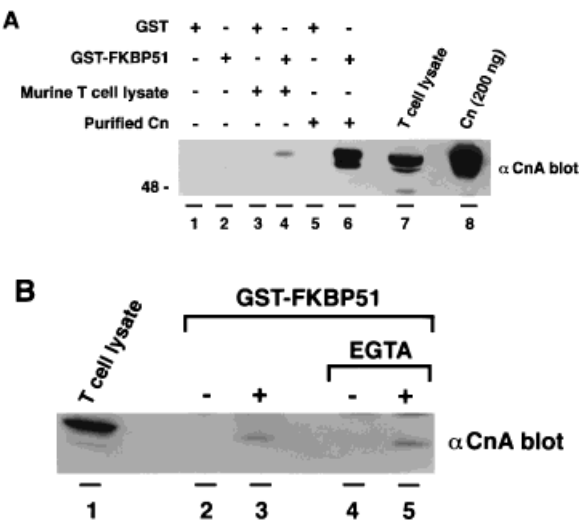

Fig. 3. Association of GST-FKBP51 with calcineurin from T cells. **A**: GST-FKBP51 (~30 μg) or GST (~10 μg) control protein on glutathione-Sepharose beads was incubated with buffer (**lanes 1–2**), cell lysate derived from 5×10^7 murine hybridoma cells (**lanes 3–4**), or 12.5 nM purified calcineurin (**lanes 5–6**) at 4°C for 2 h. After incubation, the beads were washed. The precipitated proteins were separated on 10% SDS–PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with anti-calcineurin A (CnA) antibody and developed by ECL. T cell lysate isolated from 5×10^5 cells (**lane 7**) and purified calcineurin (**lane 8**) were run as controls to ensure detection. Molecular weight markers (in kDa) are shown on the left. **B**: GST-FKBP51 (~20 μg) on glutathione-beads was incubated with buffer (−) or cell lysate (+) derived from 6×10^7 murine hybridoma cells in the absence or presence of 5 mM EGTA, as indicated, at 4°C for 2 h. After incubation, the beads were washed. The precipitated proteins were separated on 10% SDS–PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with the anti-calcineurin A (CnA) antibody and developed by ECL. T cell lysate isolated from 3×10^5 cells (lane 1) was run as a control to ensure detection. Molecular weight markers (in kDa) are shown on the left.

FKBP51 retained endogenous T cell calcineurin (Fig. 3A, lane 4), albeit modestly compared to purified bovine calcineurin (Fig. 3A, lane 6). We tested the effects of Ca^{2+} and calmodulin on the interaction of GST-FKBP51 and T cell-derived calcineurin. The addition of 5 mM EGTA had no quantitative effect on the precipitation of calcineurin from T cell lysates by GST-FKBP51 on beads (Fig. 3B, lanes 3 and 5).

The experiments described above were performed using recombinant GST-fusion proteins purified from *E. coli*. The addition of the GST fusion protein may have affected calcineurin binding or other properties of FKBP51. Therefore, to insure native folding of FKBP51, we overexpressed wild-type murine FKBP51 (expressed in the absence of fusion partners) in monkey kidney COS-7 cells by transient transfection and examined the interaction of FKBP51 with calcineurin (Fig. 4). For comparison, we transiently transfected the mammalian expression plasmid pCMV5-FLAG-FKBP12 in COS-7 cells to express FLAG-tagged FKBP12 (FLAG-FKBP12) protein. Transfected cells were further treated with FK506, rapamycin, or equivalent ethanol diluent prior to precipitation. The amount of overexpressed FKBP51 was similar among the transfected cells (Fig. 4B, lanes 2, 4, and 6), as was the amount of overexpressed FLAG-FKBP12 (Fig. 4B, lanes 7, 8, and 9). As expected, purified calcineurin retained on calmodulin-Sepharose beads was able to precipitate both FLAG-FKBP12 and endogenous FKBP12 in FK506-treated, but not rapamycin- nor ethanol-treated cells (Fig. 4A). In contrast, precipitation of overexpressed FKBP51 by purified calcineurin on calmodulin-Sepharose beads was independent of treatment with either FK506 or rapamycin (Fig. 4A, compare lane 2 with lanes 4 and 6).

Overexpression of FKBP51 Did Not Alter the Transcriptional Activation of NFAT

There have been several endogenous cellular proteins that have been shown to interact with calcineurin and appear to function as potential regulators of calcineurin phosphatase activity. The ability of these proteins (e.g., the 79 kDa A-kinase-anchoring protein, cain/cabin 1, calcineurin homologous protein) to inhibit NFAT transcriptional activation has been used as a sensitive and robust measure of calcineurin regulatory activity (discussed below). We therefore examined whether overexpression of FKBP51 affected the transcriptional activation of NFAT in T cells (Fig. 5). SV40 large T-antigen-transformed Jurkat T cells (Fig. 5B, lane 1) were cotransfected with the NFAT luciferase reporter and either the pCAGGS vector (Fig. 5B, lanes 2 and 4) or pCAGGS-FKBP51 (Fig. 5B, lanes 3 and 5). Overexpression of FKBP51 in Jurkat T cells did not significantly affect the transcriptional activation of NFAT induced by PMA and ionomycin (Fig. 5A), nor did it affect the ability of FK506 to inhibit or enhance NFAT-driven transcription at optimal or suboptimal concentrations of stimulation (data not shown).

Calcineurin-Binding Domain of FKBP51

The observation that FKBP51 bound to calcineurin independent of FK506 and that overexpression of FKBP51 did not appear to regulate calcineurin-dependent NFAT transcription prompted us to analyze the structural requirements of FKBP51 responsible for calcineurin binding. The aminoterminal domain (amino acids 32–138) of mouse FKBP51 shares the highest degree (47% identity) of sequence similarity to mouse FKBP12 [Baughman et al., 1995; Yeh et al., 1995]. Three deletion mutants of GST-FKBP51 (GST-FKBP51 Δ44-134, GST-FKBP51 Δ134-456, and GST-FKBP51 Δ1-134) were generated and are shown schematically in Figure 6A. The aminoterminal FKBP12-like domain was deleted in both mutants GST-FKBP51 Δ44-134 and GST-FKBP51 Δ1-134; the mutant GST-FKBP51 Δ134-456 retained the FKBP12-like domain and deleted the carboxyterminal domain (amino acids 134–456) including the tetratricopeptide repeat (TPR) region [Barent et al., 1998]. Calcineurin binding using the wild-type and mutated GST-FKBP51 proteins, in the absence or presence of FK506, was performed (Fig. 6B).

As expected, wild-type GST-FKBP51 bound to calcineurin in the absence of FK506; the addition of FK506 did not significantly modify calcineurin binding. Both GST-FKBP51 Δ44-134 (Fig. 6B, lanes 5 and 6) and GST-FKBP51 Δ1-134 (Fig. 6B, lanes 11 and 12) retained the ability to precipitate purified calcineurin, suggesting that the FKBP12-like domain was not required to mediate binding. Deletion of the carboxyterminal domain of FKBP51 abrogated the ability of GST-FKBP51 Δ134-456 to bind to calcineurin (Fig. 6B, lanes 8 and 9). The

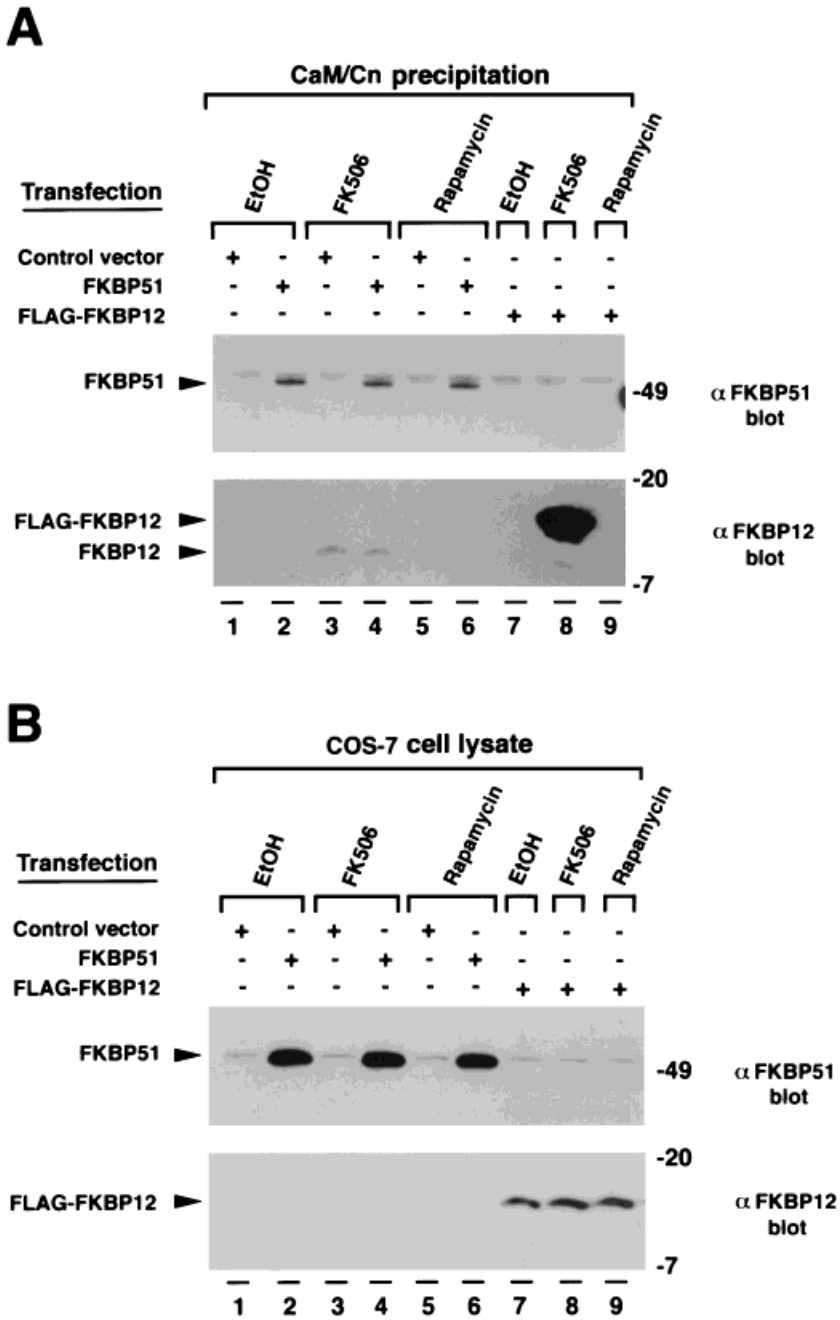

Fig. 4. FK506- and rapamycin-independent binding of COS-7-expressed FKBP51 to calcineurin. COS-7 cells were transfected with 5 μg of control vector pCAGGS, pCAGGS-FKBP51, or pCMV5-FLAG-FKBP12, as indicated (+). After 48 h, COS-7 cells were treated with 1 μM FK506, 1 μM rapamycin, or equivalent ethanol diluent (1:1,000) at 37°C for 1 h. Cells were harvested and lysed. The cell lysate was collected and 10 μl cell lysate from each sample was set aside. The remaining cell lysate was incubated with calmodulin-Sepharose beads in the presence of 4 μg purified bovine calcineurin (CaM/Cn precipitation), at 4°C for 2 h. After incubation, beads were washed. **A:** The precipitated proteins and (**B**) 10 ml COS-7 cell lysates were separated on 15% SDS–PAGE and transferred to PVDF membranes. The membranes were cut appropriately, immunoblotted with the anti-FKBP51 antiserum (top panels) or anti-FKBP12 antibody (bottom panels), and developed by ECL. Molecular weight markers (in kDa) are shown on the right.

concentration of fusion proteins was approximately equivalent in each lane (Fig. 6B, bottom panel). Thus, the carboxyterminal domain of FKBP51 containing the TPR region, but not the aminoterminal FKBP12-like domain, appeared to mediate the ability of FKBP51 to bind to calcineurin, binding that was independent of FK506, Ca^{2+}, and calmodulin.

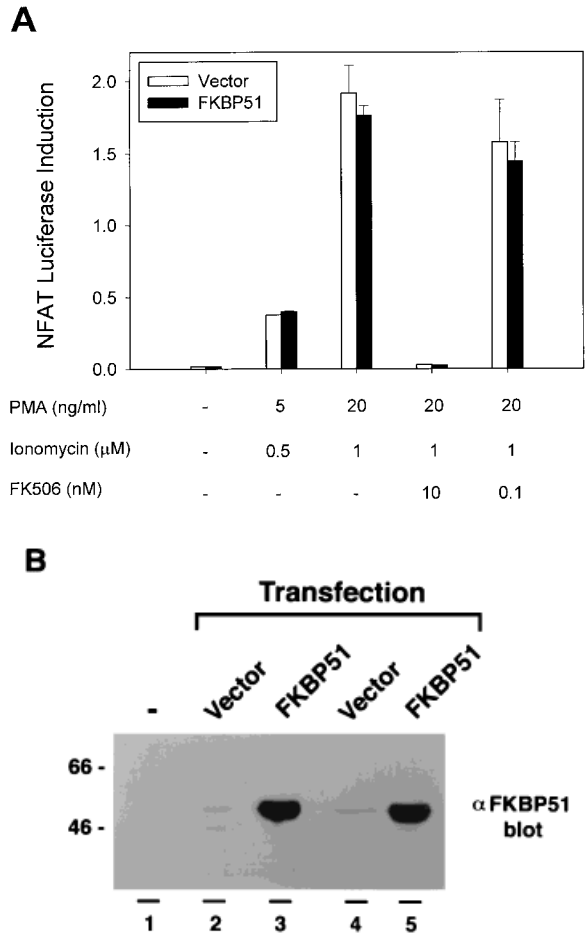

Fig. 5. Effect of FKBP51 overexpression on the transcriptional activity of NFAT. **A**: SV40 large T-antigen-transformed Jurkat T cells were transiently transfected with p3xNFAT-luc and pRL-TK in the presence of the control vector pCAGGS (vector, open bars) or pCAGGS-FKBP51 (FKBP51, closed bars) for 24 h. After 24 h, transfected cells (1×10^6/ml) in duplicates were then pretreated with equivalent ethanol diluent (–) or FK506 (10 or 0.1 nM, as indicated) for 1.5 h, followed by stimulation with equivalent ethanol diluent (–) or PMA plus ionomycin, as indicated, at 4°C for 6 h. Cells were harvested and prepared for luciferase assay. The values of NFAT luciferase induction corrected for transfection efficiency (Renilla luciferase) was the average ± SD from duplicates and is representative of three experiments. **B**: Total proteins in whole cell lysates harvested from cells before transfection (–, **lane 1**), transfected cells before stimulation (**lanes 2–3**), and transfected cells after stimulation (with ethanol control, **lanes 4–5**) were separated on 10% SDS–PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with anti-FKBP51 antiserum and developed by ECL. Molecular weight markers (in kDa) are shown on the right.

DISCUSSION

Here we show that FKBP51 is able to bind to calcineurin in the absence of a requirement for drug (FK506 or rapamycin), calcium, or calmodulin. We demonstrate further that transient overexpression of FKBP51 in a responsive T cell line failed to inhibit the stimulation-dependent transcriptional activity of NFAT nor did it affect the ability of FK506 to inhibit NFAT-dependent transcription. Surprisingly, the FKBP12-like domain of FKBP51 was not required for its interaction with calcineurin. The carboxyterminal domain containing three TPR repeats and the putative calmodulin-binding motif was sufficient for calcineurin binding.

FKBP12 has been co-localized with calcineurin, in the absence of FK506, by immunohistochemistry in the nervous system, epidermis, and thymus [Steiner et al., 1992; Dawson et al., 1994; Nishio et al., 2000]. Furthermore, a number of reports have previously shown that immunophilins are capable of binding to calcineurin, although in each case binding was modified either by the addition of drug or by the concentration of calcium. Yeast FKBP12 was shown to be capable of weak binding to calcineurin in vivo [Cardenas et al., 1994], binding that was dramatically enhanced by addition of drug. Neither we (Figs. 1 and 4A) nor others [Liu et al., 1991; Lai et al., 1998] have been able to demonstrate human FKBP12 binding to calcineurin in the absence of FK506. Whether structural differences between the yeast and human polypeptides or different experimental conditions explain the species-specific discrepancies is not clear.

Emerging evidence suggests a role for immunophilins in vivo in the absence of drug. Although the direct molecular interactions are not known, FKBP12 has been shown to associate with the ryanodine receptor (RyR) and the IP_3R, associations that are thought to stabilize these receptors and to regulate Ca^{2+} flux [Brillantes et al., 1994; Cameron et al., 1995]. FKBP12 has also been shown to anchor calcineurin to the inositol 1,4,5-triphosphate receptor (IP_3R) in a calcium-dependent manner [Cameron et al., 1995]; the calcium-dependent association of calcineurin with the IP_3R/FKBP12 complex was inhibited by FK506 [Cameron et al., 1995]. The FK506-independent binding of FKBP51 to calcineurin demonstrated here was neither modulated by nor dependent upon addition of drug (FK506 or rapamycin) or calcium levels, and binding mapped to regions outside of the FKBP12-like domain.

In addition to immunophilins, a number of cellular proteins have been identified that

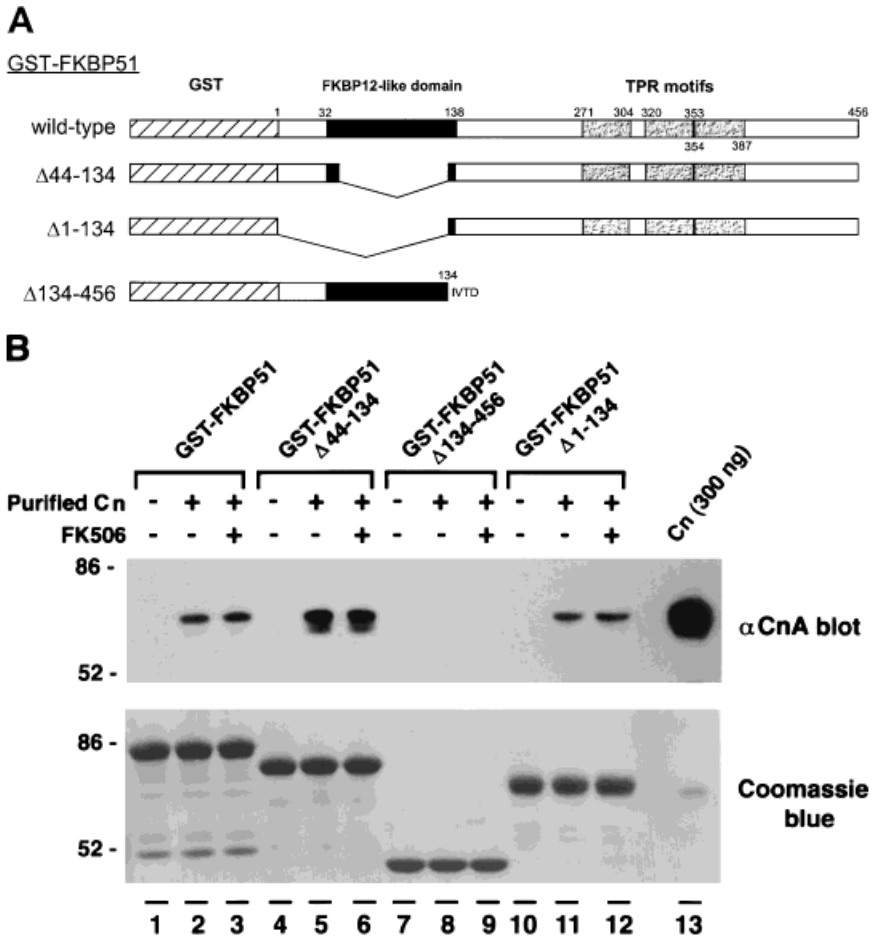

Fig. 6. Calcineurin binding to carboxyterminal domain of FKBP51. **A**: Schematic diagram of GST-FKBP51 mutations. **B**: Wild-type GST-FKBP51, GST-FKBP51 Δ44-134, GST-FKBP51 Δ134-456, or GST-FKBP51 Δ1-134 (~100 nM) on glutathione-Sepharose beads was pre-treated with 20 μM FK506 (+) or equivalent ethanol diluent (−) at 4°C for 1 h. After pre-treatment, the reaction was further incubated with (+) or without (−) 50 nM purified calcineurin (Cn), as indicated, for an additional 2 h, and the beads were washed. The precipitated proteins were separated on 10% SDS–PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with the anti-calcineurin A (CnA) antibody and developed by ECL (top panel). Purified calcineurin was run as a control to ensure detection. After ECL, the membrane was stained with Coomassie blue to visualize the fusion proteins (bottom panel).

interact with calcineurin in the absence of FKBPs (or CyPs) and in the absence of immunosuppressant drugs. These proteins have been thought to function as endogenous regulators of the serine/threonine phosphatase activity of calcineurin. Using yeast-two hybrid technology, the targeting protein 79 kDa A-kinase-anchoring protein (AKAP79) was shown to interact with calcineurin in a complex that contained cAMP-dependent protein kinase [Coghlan et al., 1995; Klauck et al., 1996]. AKAP79 and calcineurin co-localized in tissue sections. Despite containing a putative calcineurin-binding, FKBP-12 like domain [Coghlan et al., 1995], AKAP79 has been shown to bind to calcineurin at a site distinct from the immunophilin-binding region on calcineurin [Kashishian et al., 1998]. Neither the extreme carboxy- nor the aminoterminal domains of calcineurin A, nor association of calcineurin B, were required for AKAP79 binding. Importantly, Kashishian et al. [1998] demonstrated that overexpression of AKAP79 blocked the transcriptional activation of NFAT and inhibited Ca^{2+}-induced dephosphorylation of NFAT, a property not shared with FKBP51 (this report).

Other calcineurin-interacting proteins have been identified that, like AKAP79, appear to regulate calcineurin phosphatase activity. The phosphoprotein cain/cabin 1, was also identified by interaction with calcineurin in yeast two-hybrid screens. Dephosphorylation of NFAT

and IL-2 transcriptional activation, an NFAT-dependent function, were both inhibited by overexpression of cain/cabin 1 [Lai et al., 1998; Sun et al., 1998]. Similarly, overexpression of calcineurin homologous protein (CHP), a protein identified by its interaction with the Na^+/H^+ exchanger (NHE)-1 [Lin and Barber, 1996], was also shown to inhibit NFAT nuclear translocation and transcriptional activation [Lin et al., 1999], properties attributed to inhibition of calcineurin activity. A conserved family of regulators of calcineurin, termed calcipressins, has been identified in yeast and mammals. Calcipressins regulate calcineurin signaling [Fuentes et al., 2000; Gorlach et al., 2000; Kingsbury and Cunningham, 2000]. Finally, a protein derived from the African swine fever virus coprecipitated with calcineurin specifically from virally-infected cells. In these cells, calcineurin phosphatase activity was inhibited [Miskin et al., 1998]. In each of these examples, calcineurin-interacting proteins appeared to regulate the phosphatase activity of the enzyme, demonstrated directly or by inhibition of NFAT transcriptional activation. In contrast, overexpression of FKBP51 had no functional effect on activation-dependent NFAT-driven transcription, and the cells remained sensitive to inhibition by FK506. It should be noted that FK506 has been reported to bind FKBP51, albeit weakly [Baughman et al., 1997; Nair et al., 1997]. The failure to observe an apparent shift in the dose-response curve to FK506 following FKBP51 overexpression may be explained by the lower affinity of drug for FKBP51 and by the inability to achieve sufficient ($>$ 250-fold) FKBP51 overexpression compared to FKBP12 expression in vivo. Human FKBP51, unlike murine FKBP51, is highly expressed in many tissues and, in some, in excess of FKBP12 [Baughman et al., 1997]. Our findings do not eliminate the possibility that FKBP51 plays a functional role in other cell types or in other downstream functions.

Our data demonstrate that the TPR repeat domains within FKBP51, and not the conserved, FKBP12-like domain, is essential for calcineurin binding. In a number of proteins, the TPR-containing sequences mediate binding to heat shock protein (HSP)90 [Barent et al., 1998; Young et al., 1998]. As expected, both murine and human FKBP51 bind to HSP90 ([Nair et al., 1997; Barent et al., 1998], and data not shown). Among other functions, HSP90 functions as a molecular chaperone required for protein folding and trafficking of steroid receptor heterocomplexes, dynamic complexes that contain FKBPs [Rutherford and Zuker, 1994; Pratt and Toft, 1997]. FKBP51 has been shown to associate with mature steroid receptor complexes [Johnson et al., 1996; Nair et al., 1996; Barent et al., 1998; Reynolds et al., 1999; Graumann and Jungbauer, 2000], preferentially progesterone and glucocorticoid complexes [Barent et al., 1998], and to regulate responsiveness to [Reynolds et al., 1999; Denny et al., 2000] and be regulated by [Baughman et al., 1991, 1997; Kester et al., 1997; Amler et al., 2000], steroid hormones. Glucocorticoids have been shown to inhibit calcineurin- (and NFAT-) dependent transcription [Paliogianni and Boumpas, 1995; Chen et al., 2000]. Reciprocally, gene expression mediated by progesterone- and glucocorticoid- receptors has been shown to be modulated by FK506 [Ning and Sanchez, 1993; Tai et al., 1994; Le Bihan et al., 1998] and that glucocorticoid-dependent DNA-binding is affected by HSP90 [Hutchison et al., 1992] but not by FK506 [Hutchison et al., 1993]. The specific involvement of FKBP51 in these studies was not explored. Whether a role for calcineurin, suggested by its binding to FKBP51, may be demonstrated in these systems is the subject of current investigation.

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