Identification of a Physical Interaction between Calcineurin and Nuclear Factor of Activated T Cells (NFATp)*

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In T lymphocytes, the calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin, plays a pivotal role in transducing membrane-associated signals to the nucleus. One of the putative targets of calcineurin is the pre-existing, cytosolic component of the nuclear factor of activated T cells (NFATp; also referred to as NFAT1), which is one of several transcription factors required for the expression of interleukin 2. Inhibition of calcineurin by the immunosuppressive drugs cyclosporin A and FK506 prevents dephosphorylation of NFATp and its translocation to the nucleus. However, a physical interaction between calcineurin and NFATp has not been demonstrated. Here we demonstrate the binding of NFATp from lysates of T cells to immobilized calcineurin. Stimulation of T cells with calcium ionophore induced a shift in the molecular weight of NFATp that is due to its dephosphorylation. This dephosphorylation was inhibited by treatment of T cells with cyclosporin A or FK506 prior to stimulation. Of note, both the phosphorylated and the dephosphorylated form of NFATp bound to calcineurin. Furthermore, the binding of both forms of NFATp to calcineurin was inhibited by pretreatment of calcineurin with a complex of FK506 and its ligand FKBP12. Taken together these data strongly suggest a direct interaction of calcineurin with NFATp and that this interaction does not depend upon the phosphorylation sites of NFATp affected by activation.

The calcium/calmodulin-dependent serine/threonine phosphatase calcineurin (PP2B) plays a crucial role in T cell activation. Calcineurin (CN) is a ubiquitously expressed heterodimer, composed of a 59-kDa calmodulin-binding catalytic subunit and a 19-kDa Ca2+-binding regulatory subunit that is required for enzymatic activity. The central role of calcineurin in T cell signaling was appreciated by its identification as the target of the immunosuppressive drugs cyclosporin A (CsA) and FK506 (1, 2). The phosphatase activity of CN is inhibited by either drug when complexed to intracellular binding proteins (immunophilins), i.e. CsA to cyclophilin and FK506 to the FK506-binding protein 12 (FKBP12), respectively. Neither drug nor immunophilin alone bind to or affect the activity of CN (2). In T lymphocytes both CsA and FK506 inhibit a variety of T cell responses. A well characterized example of the effect of CsA and FK506 is the inhibition of T cell receptor-mediated expression of cytokine genes (3–5).

Among the putative targets of calcineurin is the nuclear factor of activated T cells (NFAT) (6), which regulates, in part, the expression of a number of cytokine genes including IL-2, IL-3 (7), IL-4 (8, 9), granulocyte-macrophage colony-stimulating factor (7), and tumor necrosis factor α (10). Within the IL-2 promoter, NFAT consists of a pre-existing, cytoplasmic component and an ubiquitously expressed nuclear component, which contains members of the AP1 family of transcription factors (11, 12). NFATp is one of several isoforms of the cytosolic component of NFAT (13–16). Increases in intracellular calcium induce the translocation of the cytosolic component of NFAT to the nucleus, where NFATp interacts with the AP1 elements Fos and Jun and binds to its cognate DNA-binding site. The calcium-dependent dephosphorylation and nuclear translocation of NFATp cytoplasmic components are inhibited by CsA and FK506 whereas the generation of the nuclear components is unaffected (11, 17–20). It has been further demonstrated that CN dephosphorylates NFATp in vitro and that the dephosphorylated form of NFATp binds with a higher affinity to an NFAT oligonucleotide (20, 21). Moreover, it has been shown that CN phosphatase activity correlates with T cell receptor-induced IL-2 expression in vitro and in vivo (22-24). Thus it appears that CN plays a central role in transducing T cell receptor-mediated signals to the nucleus. However, it has not been determined whether the influence of CN on the nuclear translocation of the cytosolic forms of NFAT is due to a direct or indirect interaction.

In this report we show that purified bovine CN immobilized to calmodulin Sepharose beads precipitates NFATp from lysates of a murine T cell line. Of note, CN associated with both the phosphorylated as well as the dephosphorylated form of NFATp. The interaction of CN with NFATp was inhibited by the complex of FK506:FKBP12 but not by either FK506 or FKBP12 alone. The finding of a physical association of CN with NFATp supports a model whereby CN is directly involved in dephosphorylation and nuclear translocation of NFATp.

MATERIALS AND METHODS

Cdls—The CD4+ CD8- murine T cell hybridoma BYDP (25) was grown in RPMI 1640 complete medium (RPMI 1640 supplemented with 5% fetal bovine serum, 5% iron-supplemented calf serum, 2 mM l-glutamine, 100 units of penicillin/streptomycin, and 2 x 10⁻⁵ M 1-mercaptoethanol). Four hours prior to stimulation, BYDP cells were washed and placed in serum-free medium (same as complete RPMI 1640 medium, except without serum).

Inhibition and Stimulation of T Cell Hybridoma—BYDP (10⁷ cells/sample) were washed once and transferred to 1.5-ml microcentrifuge tubes and incubated with FK506 (0.1 μM) with or without rapamycin.
(10 μM) or with ethanol as control for 30 min at 37 °C. Subsequently the cells were stimulated with PMA (5 ng/ml or as indicated) and/or ionomycin (2 μM) for 5 min at 37 °C. Control samples were adjusted to a similar concentration of the diluent ethanol. The cells were pelleted by a pulse spin, washed once in phosphate-buffered saline, and lysed in lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.6, 150 mM NaCl, 10 μM CaCl2, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na3VO4, and proteinase inhibitors (60 μg/ml aprotinin, 60 μg/ml leupeptin, 10 μg/ml pepstatin A, 200 μg/ml trypsin inhibitor, 17 μg/ml calpain inhibitor I, 7 μg/ml calpain inhibitor II, 50 μg/ml antipain, 50 μg/ml chymostatin, 25 μg/ml bestatin, 10 μg/ml E64, 100 μg/ml phosphoramidon, and 2 μM phenylmethylsulfonyl fluoride)).

 Precipitation and Immunoblotting—Caldesmon-Sepharose beads were washed in Tris-buffered saline containing 1 mM CaCl2 and preincubated with 4 μM (or as indicated) FK506 and/or 400 μM rapamycin and 4 μM FKBP12 or GST-FKBP12 and 0.2 μM calcineurin for 60 min at 4 °C in 250 μl of lysis buffer. Subsequently 500-μl lysates of 107 cells were added and incubated for 1 h at 4 °C. Beads were washed four times with precipitation wash buffer (0.2% Triton X-100, 20 mM HEPES, pH 7.4, 10% glycerol, 1 mM CaCl2, 5 mM NaF, 1 mM Na3VO4, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 1 μg/ml pepstatin A, 30 μg/ml trypsin inhibitor). For postincubation of beads with FKBP12-FK506, beads were washed three times before adding 40 μM FK506 and 4 μM FKBP12 for 20 min at 4 °C in 250 μl of precipitation wash buffer and washed additionally two times with the same buffer. Proteins bound to calmodulin-Sepharose beads were separated by 8–12% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), immunoblotted with the indicated antibodies, and developed by the enhanced chemiluminescence (ECL) system (Amersharm Corp.).

Reagents—CsA is produced by Sandoz (East Hanover, NJ) and FK506 by Fujisawa (Méross Park, IL) and rapamycin was kindly provided by Dr. J. Johnson (NIH, Bethesda, MD). Ionomycin was purchased from Calbiochem and PMA from Sigma. Proteinase inhibitors (the exception of trypsin inhibitor which was purchased from Sigma, were acquired from Boehringer Mannheim (Mannheim, Germany). Calmodulin-Sepharose beads were obtained from Pharma Biotech Inc. Bovine brain calcineurin and human, recombinant FKBP12 were obtained from Sigma. The fusion protein GST-FKBP12 was prepared as described (26). Rabbit anti-peptide antisera specific for FKBP12 (amino acids 1–16) was kindly provided by Dr. M. Harding (Vertex Pharmaceuticals, Cambridge, MA). The rabbit antiserum against NFATp (R59) was generated by immunizing rabbits with purified recombinant NFATp and generously provided by Dr. A. Rao (13).

RESULTS AND DISCUSSION

Stimulation of T Cells with Calcium Ionophore Induces a Shift in Relative Mobility of NFATp—Murine hybridoma T cells (BYDP) were stimulated for 5 min with the calcium ionophore, ionomycin, the protein kinase C-activating phorbol ester, PMA, or with both agents. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with an antiserum raised against NFATp (R59) was generated by immunizing rabbits with purified recombinant NFATp and generously provided by Dr. A. Rao (13).

The cytoplasm and is rephosphorylated.2 Calcineurin Associates with Both M, Forms of NFATp in Vitro—Calcineurin is thought to be involved in the dephosphorylation and subsequent translocation of NFATp from the cytoplasm to the nucleus since inhibition of calcineurin activity by the immunosuppressive drugs FK506 and CsA inhibits nuclear translocation of cytosolic NFAT (11, 17). Therefore, we investigated whether NFATp, in T cell lysates (BYDP cells), associates with exogenous calcineurin in vitro. T cell lysates were incubated with purified calcineurin from bovine brain immobile to calmodulin-Sepharose (CaM beads). In order to inhibit phosphate activity of CN we included the phosphatase inhibitors sodium fluoride and sodium pyrophosphate during incubation. In lysates of unstimulated T cells, CN bound to the higher (phosphorylated) M, form of NFATp whereas in lysates of T cells stimulated with PMA and ionomycin, CN associated with the lower (dephosphorylated) form (Fig. 2). Thus, immunoblotted CN bound both phosphorylated and dephosphorylated forms of NFATp. CN activity in T cells was inhibited in vivo by preincubating T cells with FK506, and this resulted in the inhibition of the activation-induced dephosphorylation of NFAT. When rapamycin was added in a 100-fold excess to FK506, the FK506-mediated inhibition of NFATp dephosphorylation was reversed (Fig. 2). Since rapamycin and FK506 bind competitively to FKBP12, but the complex of rapamycin FKBP12 does not bind to CN (2), the reversal by rapamycin of FK506-mediated inhibition of the stimulation-induced shift of NFATp supports the involvement of CN in the dephosphorylation of NFATp in vivo.

Preincubation of CN/CaM Beads with Exogenous FK506-FKBP12 in Vitro Inhibits NFATp Binding to CN—It has previously been demonstrated that the complex of FK506 with its intracellular ligand FKBP12 binds to and inhibits the phosphate activity of CN in vitro (2). Since we could demonstrate a physical interaction of CN with NFATp, we investigated the effect of the FK506-FKBP12 complex on the association of CN and NFATp in vitro. CaM beads were incubated with purified

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CN in the presence of FK506 and purified FKBP12 before adding lysates of stimulated or unstimulated T cells. It was observed that once the complex of CN, FKBP12, and FK506 was formed, NFATp from either unactivated or activated T cell lysates could no longer bind to CN (Fig. 2). Interestingly, once NFATp was associated with CN in vitro, the addition of the FK506FKBP12 complex could not disrupt this interaction. Specifically, NFATp bound to CN/CaM beads could not be "eluted" with FK506FKBP12 (Fig. 2).

In order to assess the specificity of the FK506FKBP12-mediated inhibition of NFATp to CN, rapamycin was added in 100-fold excess to FK506 during preincubation of CN/CaM beads and GST-FKBP12 with or without FK506. After preincubation, lysates of activated or nonactivated T cell were added, and proteins associated with CN/CaM beads were resolved by SDS-PAGE and blotted with anti-NFATp, anti-CN (data not shown), or anti-FKBP12 antibodies (Fig. 3). Preincubation of CN/CaM beads with FK506 and FKBP12 (GST-FKBP12) led to the binding of FK506FKBP12 to CN and the inhibition of the interaction of CN with NFATp. Only the combination of both FKBP12 plus FK506 blocked the binding of NFATp to CN; neither drug nor immunophilin alone had any effect on the association nor did rapamycin FKBP12. Inhibition by FK506FKBP12 was mediated by blocking the binding of CN to NFATp and not by disturbing the interaction of CN with CaM, since immunoblots for CN revealed no loss of CN (precipitated via CaM beads) in the presence of FK506FKBP12 (data not shown). Addition of rapamycin in 100-fold excess to FK506 restored the association of NFATp to CN, thus reversing the effect of FK506FKBP12 on the CN/NFATp interaction (Fig. 3). Although the presence of FK506 is necessary for optimal binding of FKBP12 to CN some binding of FKBP12 alone to CN/CaM beads was noted.

It has been reported that FK506FKBP12 inhibits the phosphatase activity of CN on protein/peptide substrates but not on small organic substrates such as para-nitrophenyl phosphate (2). This finding suggests that the complex of FK506FKBP12 inhibits substrate binding to CN rather than affecting the phosphatase activity of CN. It further implies that the catalytic domain of CN consists of at least two sites: a substrate-binding site and a catalytic site. Our observation that (i) CN binds both the phosphorylated and dephosphorylated forms of NFATp and (ii) these interactions are inhibited by FK506FKBP12 supports the notion that the major interaction of CN with NFATp occurs via the substrate-binding site rather than the catalytic site. The recently solved x-ray crystallographic structure of FK506FKBP12 bound to CN revealed that the FK506FKBP12 complex binds to the A-chain of CN at a site approximately 10 Å removed from the catalytic site (27). This structural information is consistent with our data, suggesting that the interaction of NFATp with the catalytic site is not a requirement for binding.

Though we were able to demonstrate the binding of NFATp to CN, we could not exclude the possibility of a third protein involved in this interaction. While it has been shown that CsA and FK506 inhibit the dephosphorylation and nuclear translocation of NFATp, it has not yet been demonstrated whether the influence of CN on the nuclear translocation of the cytosolic form of NFAT is due to a direct or indirect interaction. In the direct model, CN dephosphorylates NFAT directly, thereby enabling it to cross the nuclear membrane. Thus, the nuclear translocation of the cytoplasmic component of NFAT would be regulated by dephosphorylation, as has been shown for the regulation of the yeast transcription factor SWI5 (28, 29). Alternatively, the indirect model takes into account that all four isoforms of the cytosolic component of NFAT contain a region similar to the Rel homology domain of the NFκB family (14–16). It has, therefore, been suggested that NFAT in nonactivated T cells might be retained by a cytosolic anchoring protein, in a similar manner as NFκB is retained by IκB (30). Dephosphorylation of the putative anchoring protein by CN would then result in the release of NFAT and its subsequent translocation to the nucleus (31).

The finding that CN associates with phosphorylated and dephosphorylated NFATp favors a model of a direct interaction of CN with NFATp for the following reasons. (i) It has been demonstrated that dephosphorylated NFATp is able to translocate to the nucleus (19, 20). In case NFATp is released from a cytosolic anchoring protein after activation it is unlikely that CN associates with NFATp via a cytosolic anchoring protein, since NFATp would already be released. (ii) The known NFκB anchoring proteins, IκBα and IκBβ, degrade 10 min and 4 h after stimulation, respectively (32). Nuclear translocation of NFATp takes place 5–10 min after stimulation (19, 20), indicating that if NFATp is retained in the cytosol by an IκB-like anchoring protein it must be released from this protein and from CN.
Fig. 4. CN associates with NFATp up to 6 h after stimulation. CN/CaM-Sepharose beads were incubated with 500-μl lysates of T cells stimulated with 2 μM ionomycin for the indicated amount of time. After a 60-min incubation, calmodulin-Sepharose beads were washed, and precipitated proteins were resolved by SDS-PAGE and immunoblotted with NFATp antiserum. The arrow indicates the dephosphorylated form of NFATp.

Therefore, our observation of a physical interaction of CN and NFATp, together with the fact that phosphorylated NFATp can serve as a substrate for CN in vitro (21), supports a model in which CN is directly involved in the dephosphorylation and subsequent nuclear translocation of NFATp.

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