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Calcineurin Binds the Transcription Factor NFAT1 and Reversibly Regulates Its Activity*

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NFAT1 (previously termed NFATp) is a cytoplasmic transcription factor involved in the induction of cytokine genes. We have previously shown that the dephosphorylation of NFAT1, accompanied by its nuclear translocation and increased DNA binding activity, is regulated by calcium- and calcineurin-dependent mechanisms, as each of these hallmarks of NFAT1 activation is elicited by ionomycin and blocked by the immunosuppressive drugs cyclosporin A and FK506 (Shaw, K. T.-Y., Ho, A. M., Raghavan, A., Kim, J., Jain, J., Park, J., Sharma, S., Rao, A., and Hogan, P. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 11205–11209). Here we show that the activation state of NFAT1 in T cells is remarkably sensitive to the level of calcineurin activity. Addition of cyclosporin A, even in the presence of ongoing ionomycin stimulation, results in rephosphorylation of NFAT1, its reappearance in the cytoplasm, and a return of its DNA binding activity to low levels. Similar effects are observed upon removal of ionomycin or addition of EGTA. We also demonstrate a direct interaction between calcineurin and NFAT1 that is consistent with a direct enzyme-substrate relation between these two proteins and that may underlie the sensitivity of NFAT1 activation to the level of calcineurin activity. The NFAT1-calcineurin interaction, which involves an N-terminal region of NFAT1 conserved in other NFAT family proteins, may provide a target for the design of novel immunosuppressive drugs.

NFAT1 (previously termed NFATp) (1–3) is a member of the NFAT family of transcription factors that play a key role in the regulation of cytokine gene transcription during the immune response (4–6). Other members of the NFAT family include NFATc (7), NFATx (also known as NFAT4 or NFATc3) (8–10), and NFAT3 (9). NFAT family proteins are highly homologous in their DNA-binding domains, which show a weak similarity to the DNA-binding domains of Rel family proteins (9, 11, 12). They also contain a second region (~300 amino acids) of moderate sequence conservation, located immediately N-terminal to the DNA-binding domain, that we have termed the NFAT homology region.[1] The NFAT homology region contains several serine- and proline-rich sequence motifs whose presence and location within the protein are conserved in all four NFAT family proteins (8–10).[1]

A central feature of the regulation of NFAT family proteins was inferred from their pronounced sensitivity to the immunosuppressive drugs cyclosporin A (CsA)[2] and FK506. These drugs (which are potent inhibitors of cytokine gene transcription in activated T cells, B cells, mast cells, and natural killer cells) inhibit NFAT-dependent reporter gene transcription as well as the nuclear appearance of NFAT DNA binding activity (1, 14–23). CsA and FK506 act by binding to intracellular immunophilin receptors and inhibiting the activity of the calmodulin-dependent phosphatase calcineurin (24, 25). Overexpression of calcineurin replaces the calcium requirement for NFAT-dependent transactivation in T cells and renders the transactivation less sensitive to CsA and FK506 (15, 26, 27). These results prompted the suggestion that calcineurin plays a major role in inducible gene transcription during the immune response, in part by preventing the nuclear translocation of NFAT family proteins (28, 29).

Detailed analysis of the pre-existing family member, NFAT1, has provided more insight into the molecular mechanisms by which calcineurin regulates NFAT activity (30, 31).[3] NFAT1 is present in the cytoplasm of resting T cells and translocates into the nucleus in response to stimulation with ionomycin, antigen, or anti-CD3. The nuclear translocation is preceded by a rapid dephosphorylation that correlates with a significant increase in the DNA binding affinity of NFAT1. Each of these three hallmarks of activation (dephosphorylation, nuclear translocation, and increase in affinity for DNA) is elicited by calcineurin-dependent mechanisms since each is blocked by CsA and FK506 (30, 31).[3] NFAT1 is also effectively activated by stimulation through the T cell receptor in that it is rapidly dephosphorylated, it translocates to the nucleus, and it shows an increased ability to bind DNA. However, this phase of activation is followed by a return of the bulk of the NFAT1 to its resting state, a process that correlates with the decline in intracellular free calcium levels due to feedback mechanisms triggered by the T cell receptor.[3] Furthermore, treatment of stimulated T cells with CsA results in a loss of occupancy of the NFAT sites within the murine interleukin-2 promoter, as assessed by *in vivo* footprinting studies (33). These observations prompted us to inves-

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[1] C. Luo, E. Burgeon, J. A. Carew, T. M. Badalian, P. G. McCaffrey, W. S. Lane, P. G. Hogan, and A. Rao, submitted for publication.

[2] The abbreviations used are: CsA, cyclosporin A; PAGE, polyacrylamide gel electrophoresis; CaM, calmodulin; GST, glutathione *S*-transferase.

[3] C. Loh, J. A. Carew, J. Kim, P. G. Hogan, and A. Rao, submitted for publication.

tigate whether the activation of NFAT1 is dependent on the sustained activity of calcineurin.

Here we demonstrate that the activation state of NFAT1 is indeed critically dependent on calcineurin activity. We further show a direct interaction between calcineurin and NFAT1 that may underlie the responsiveness of NFAT1 to the level of calcineurin activity in T cells. The interaction is not influenced by the activation state of NFAT1 and is not inhibited by immunosuppressive concentrations of CsA and FK506. The specificity of signaling pathways is often conferred by direct interactions of signaling enzymes with their substrates or with specific targeting proteins within the cell (34–40). The NFAT1-calcineurin interaction, which provides a dramatic illustration of this mechanism, may be used to identify new classes of immunosuppressive drugs that block this interaction without inhibiting the enzymatic activity of calcineurin.

MATERIALS AND METHODS

Cells and Reagents— The Ar-5 murine T cell clone, responsive to arsonate-conjugated ovalbumin in the context of the murine major histocompatibility complex Class II molecule IA^d, was cultured and grown as described previously (41). Cyclosporin A, FK506, and rapamycin were kindly donated by Sandoz, Fujisawa, and Wyeth Ayerst, respectively. Ionomycin was purchased from Calbiochem. Purified calcineurin was purchased from Sigma. Trypsin was purchased from Boehringer Manheim. FKBP12 and cyclophilin A were kind gifts of Dr. S. Schreiber's and Dr. C. Walsh's laboratories, respectively.

Antibodies—Anti-67.1 antibody, a rabbit polyclonal antibody to the 67.1 peptide (AISSPSGLAYPDDVLDYGL) in the N-terminal region of NFAT1, is referred to as anti-NFAT1 antibody (42). Anti-calcineurin antibody is a rabbit polyclonal antibody raised against the peptide (YITSFEEAKGLDRINERMPPRRDAMPSD) corresponding to the autoinhibitory domain of the calcineurin A chain, conjugated via the N-terminal tyrosine to keyhole limpet hemocyanin.[4]

Immunofluorescence—Ar-5 T cells (1×10^6) were attached to coverslips previously coated with poly-D-lysine, fixed in 3% paraformaldehyde, permeabilized with 0.1% Nonidet P-40, and stained with anti-NFAT1 antibody.[3] The cells were photographed under 63× magnification with a Zeiss MC80 microscope and Kodak Tri-X Pan ASA400 film.

Whole Cell Extracts and Electrophoretic Mobility Shift Assay—Ar-5 T cells (25×10^6/lane), left unstimulated or stimulated, were resuspended in 50 μl of buffer (10 mM Tris, pH 7.4, 120 mM sodium pyrophosphate, 20 mM EDTA, and 0.4 mM EGTA supplemented with 80 μM leupeptin, 40 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 8 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol), followed by addition of 50 μl of 5 × gel shift buffer (1 M KCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.4, 5 mM EGTA, and 20% glycerol) and incubation on ice for 30 min. Following centrifugation at 4 °C in a microcentrifuge, supernatants were transferred to fresh microcentrifuge tubes and diluted on ice with 100 μl of water. For electrophoretic mobility shift assay, the binding reaction consisted of whole cell extract with 200 μg/ml poly(dI)·poly(dC) as nonspecific competitor and 10,000 cpm (~0.1–0.5 ng) of end-labeled oligonucleotide corresponding to the distal NFAT site in the murine interleukin-2 promoter (1–3) in a final volume of 15 μl containing 80 mM NaCl, 80 mM KCl, 20 mM Hepes, pH 7.4, 0.6 mM $MgCl_2$, 0.6 mM EGTA, 2.5 mM EDTA, and 6% glycerol. All binding reactions were incubated at room temperature for 20 min. The resulting DNA-protein complexes were separated by electrophoresis on a 4% nondenaturing gel (1, 2).

SDS Whole Cell Lysates and Western Blotting—After the various stimulations and treatments, the cells (1×10^6/lane) were resuspended in 30 μl of buffer (40 mM Tris, pH 8, 60 mM sodium pyrophosphate, and 10 mM EDTA) and lysed by addition of an equal volume of 10% SDS, followed by boiling for 20 min in reducing sample buffer. The lysates were analyzed by 6% SDS-PAGE followed by Western blotting with anti-NFAT1 antibody. The bands were visualized using enhanced chemiluminescence (Amersham Corp.).

Anti-NFAT1 Immunoprecipitation and Tryptic Peptide Mapping—Ar-5 T cells (20×10^6/sample) were labeled with [^{32}P]orthophosphate essentially as described by Shaw *et al.* (30). Briefly, the cells were incubated overnight at 37 °C in phosphate-free T cell medium (41) containing 0.25–0.5 mCi/ml [^{32}P]orthophosphate (DuPont NEN). One-third of the cells were left unstimulated, one-third were stimulated for 5 min with 1 μM ionomycin, and one-third were treated with 1 μM CsA for 30 min, followed by stimulation for 5 min with 1 μM ionomycin. In the case of ionomycin-stimulated cells treated with CsA, the cells were incubated for 30 min in phosphate-free Dulbecco's modified Eagle's medium containing 0.25–0.5 mCi/ml [^{32}P]orthophosphate and then stimulated with 1 μM ionomycin for 5 min. CsA was then added (1 μM final concentration), and the cells were allowed to incubate at 37 °C for a further 30 min. All cells were harvested, washed twice, and lysed on ice with cold radioimmune precipitation assay buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8, 150 mM NaCl, and 0.5 mM EDTA supplemented with 10 mM iodoacetamide, 20 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 20 μM leupeptin, 10 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, and 2 μM phenylmethylsulfonyl fluoride). Lysates were subjected to immunoprecipitation with 2 μg of affinity-purified anti-NFAT1 (anti-67.1) antibody and 50 μl of packed protein A-Sepharose beads at 4 °C for 3 h. The immunoprecipitates were washed and then boiled in Laemmli sample buffer, and NFAT1 was separated on a 6% SDS-polyacrylamide gel, eluted from the gel, and digested with trypsin (43). The samples were electrophoresed at 1000 V for 45 min in pH 1.9 buffer in the first dimension, followed by chromatography for 5 h in butanol/pyridine/acetic acid/water (4:2:1:3) in the second dimension (43).

CaM-Sepharose Binding Assay— Ar-5 T cells (10×10^6/sample) were lysed in 1% Triton X-100 buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, and 1% Triton X-100 supplemented with 10 mM iodoacetamide, 20 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 20 μM leupeptin, 10 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, and 2 μM phenylmethylsulfonyl fluoride) and supplemented with 1 mM $CaCl_2$ or 5 mM EGTA. CaM-Sepharose beads were preactivated by washing three times in 10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM $CaCl_2$. The beads were aliquoted into individual microcentrifuge tubes and spun, and all of the supernatant was removed. The packed beads (50 μl) were incubated with each lysate for 1 h at 4 °C. The beads were then washed three times in wash buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, and protease inhibitors supplemented with 1 mM $CaCl_2$ or 5 mM EGTA) and once in 10 mM Hepes, pH 7.4, and then boiled in Laemmli reducing buffer. The samples were analyzed by 6% SDS-PAGE followed by Western blotting with anti-NFAT1 and anti-calcineurin antibodies. In the experiment of Fig. 5*A*, Triton X-100 lysates were made from COS cells transfected with a plasmid encoding the N-terminal 415 amino acids of NFAT1 [1] using a DEAE-dextran method (44). The COS cell lysates were incubated with CaM-Sepharose beads as described above. In the experiments of Fig. 5 (*B* and *C*), GST-NFAT1-(1–415) (the N-terminal 1–415 amino acids of human NFAT1 [1] fused to GST in a pGEX2T vector) derived from 100 ml of bacterial culture was bound to 100 μl of glutathione-Sepharose beads (Pharmacia Biotech Inc.). Bound protein was eluted from the beads by addition of 1.5–3 volumes of 1% Triton X-100 buffer containing 30 mM glutathione. Eluted GST-NFAT1-(1–415) was incubated with CaM-Sepharose beads in the presence or absence of purified calcineurin in 1% Triton X-100 buffer supplemented with 1 mM $CaCl_2$ or 5 mM EGTA. In the experiment of Fig. 5*C*, purified calcineurin was incubated for 1 h on ice with a 10-fold molar excess of CsA, cyclophilin A, FK506, FKBP12, or combinations of the immunophilin/immunosuppressants that had been formed by preincubation for 1 h on ice; the mixture was then added to eluted GST-NFAT1-(1–415) and CaM-Sepharose beads. The CaM-Sepharose beads were then washed and treated as described above.

RESULTS

Sensitivity of NFAT1 to Calcineurin in Vivo—When immunosuppressive concentrations of CsA were added to previously activated T cells, interleukin-2 gene transcription was shut off within 30 min (45). To determine if this effect reflected the deactivation of NFAT family proteins, we asked whether addition of CsA to previously activated T cells would alter the subcellular localization and function of NFAT1. As shown previously (30), stimulation of the murine T cell clone Ar-5 with 1 μM ionomycin for 15 min resulted in quantitative translocation of NFAT1 from the cytoplasm to the nucleus, as assessed by immunocytochemistry with an NFAT1-specific antibody (Fig. 1, *A* and *B*). Preincubation of the cells with cyclosporin A inhibited this nuclear translocation (Fig. 1*D*). Addition of CsA to the

[4] C. Luo and A. Rao, unpublished data.

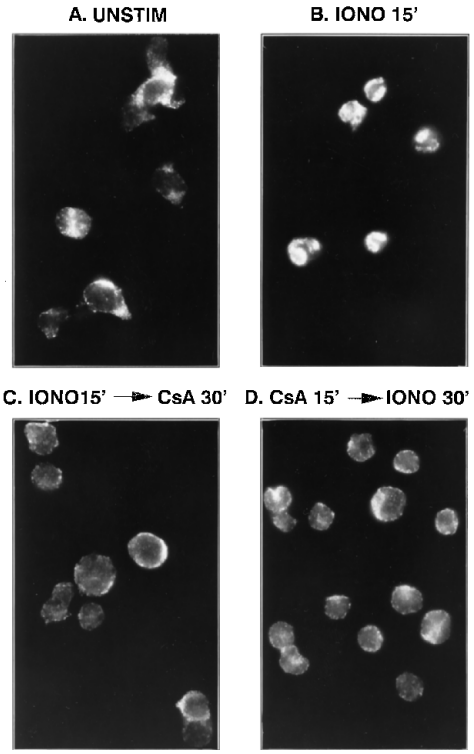

FIG. 1. **The nuclear localization of NFAT1 is reversed by cyclosporin A.** Ar-5 T cells were left unstimulated (*A*) or were stimulated with 1 μM ionomycin (*IONO*) for 15 min (*B*); stimulated with 1 μM ionomycin for 15 min, followed by addition of 1 μM CsA and incubation at 37 °C for a further 30 min (*C*); or incubated in 1 μM CsA for 15 min prior to stimulation with 1 μM ionomycin for 30 min (*D*).

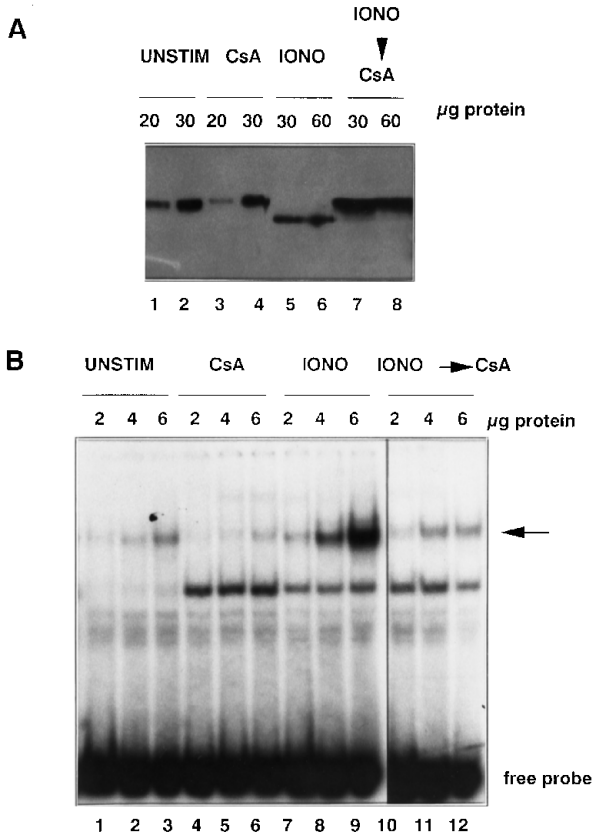

FIG. 2. **The ionomycin-induced increase in NFAT1 DNA binding activity is reversed by cyclosporin A.** *A*, Ar-5 T cells were left unstimulated (*lanes 1* and *2*) or were treated for 15 min with 1 μM CsA (*lanes 3* and *4*); stimulated with 1 μM ionomycin (*IONO*) for 15 min (*lanes 5* and *6*); or stimulated with 1 μM ionomycin for 15 min, followed by addition of 1 μM CsA and a further 30-min incubation at 37 °C (*lanes 7* and *8*). Whole cell extracts were analyzed for NFAT1 content by 6% SDS-PAGE and Western blotting with anti-NFAT1 antibody. The total protein loaded in each lane is indicated at the top. NFAT1 present in extracts from cells treated with CsA following ionomycin stimulation (*lanes 7* and *8*) was rephosphorylated and comigrated with the bands present in unstimulated and CsA-treated cells (*lanes 1–4*). *B*, the whole cell extracts were analyzed for NFAT1 DNA binding activity by electrophoretic mobility shift assay. The *arrow* indicates the specific NFAT1-DNA complex, so defined as addition of an antibody specific for NFAT1 in the binding reactions completely supershifted the complex (data not shown). The band migrating below the specific NFAT1-DNA complex is nonspecific. The total protein used in the binding reactions is indicated.

ionomycin-stimulated cells resulted in the reappearance of NFAT1 in the cytoplasm within 30 min (Fig. 1*C*). It is well documented that CsA is a potent inhibitor of calcineurin activity when added to cells or when added as CsA-cyclophilin complexes to purified calcineurin *in vitro* (25, 46). Thus, the reappearance of NFAT1 in the cytoplasm of cells treated with CsA, even in the presence of ongoing ionomycin stimulation, suggests that the subcellular location of NFAT1 is continuously responsive to the level of calcineurin activity. We cannot, however, exclude the possibility that CsA might be acting to downregulate NFAT1 activity by inhibiting other signaling pathways not involving calcium or calcineurin.

The activation of NFAT1 is also reflected by an increase in its ability to bind to DNA (30).[3] We investigated the calcineurin sensitivity of this effect by testing the DNA binding activity of whole cell extracts from Ar-5 T cells treated with ionomycin and/or CsA (Fig. 2). Western analysis indicated that each of the extracts contained NFAT1 (Fig. 2*A*). The DNA binding activity of NFAT1 from ionomycin-stimulated cells showed a significant increase over the DNA binding activity of NFAT1 from unstimulated or CsA-treated cells (Fig. 2*B*, compare *lanes 7–9* with *lanes 1–3* and *4–6*). Prior treatment with CsA prevented the ionomycin-induced increase in the DNA binding activity of NFAT1 (data not shown). Likewise, treatment with CsA after ionomycin stimulation returned the DNA binding activity of NFAT1 to a resting level (compare *lanes 10–12* with *lanes 1–3*), again suggesting that the DNA binding activity of NFAT1 is continuously regulated by the level of calcineurin activity.

The nuclear translocation and increased DNA binding activity of NFAT1 are preceded by a rapid dephosphorylation of the protein (30) that is apparent as an increase in its electrophoretic mobility on SDS gels (Fig. 3*A*, *lanes 1* and *2*) and is prevented by preincubation of the cells with CsA (*lane 7*). CsA reversed the mobility shift within 15 min when added to ionomycin-stimulated cells (*lanes 8–10*). This effect was observed even in cells pretreated with protein synthesis inhibitors (data not shown), indicating that the reversal was not due to degradation of the modified NFAT1 and its replacement by newly synthesized protein, but rather reflected the rephosphorylation of previously dephosphorylated NFAT1.

The rephosphorylation of NFAT1 mediated by addition of CsA to ionomycin-stimulated cells was confirmed by tryptic mapping of phosphopeptides (Fig. 3*B*). Two major labeled tryptic peptides (*arrowheads*) that were observed in NFAT1 immunoprecipitated from unstimulated cells (*left panel*) were absent in NFAT1 from ionomycin-stimulated cells (*middle panel*), consistent with previous reports that NFAT1 is partially dephosphorylated in response to ionomycin (30, 31) and suggesting that only certain sites within NFAT1 are dephosphorylated. As expected, this selective dephosphorylation was inhibited by treatment of cells with CsA prior to ionomycin stimulation (data not shown). Treatment of ionomycin-stimulated cells with CsA resulted in the reappearance of the two major labeled peptides (*arrowheads* in *right panel*), indicating that following

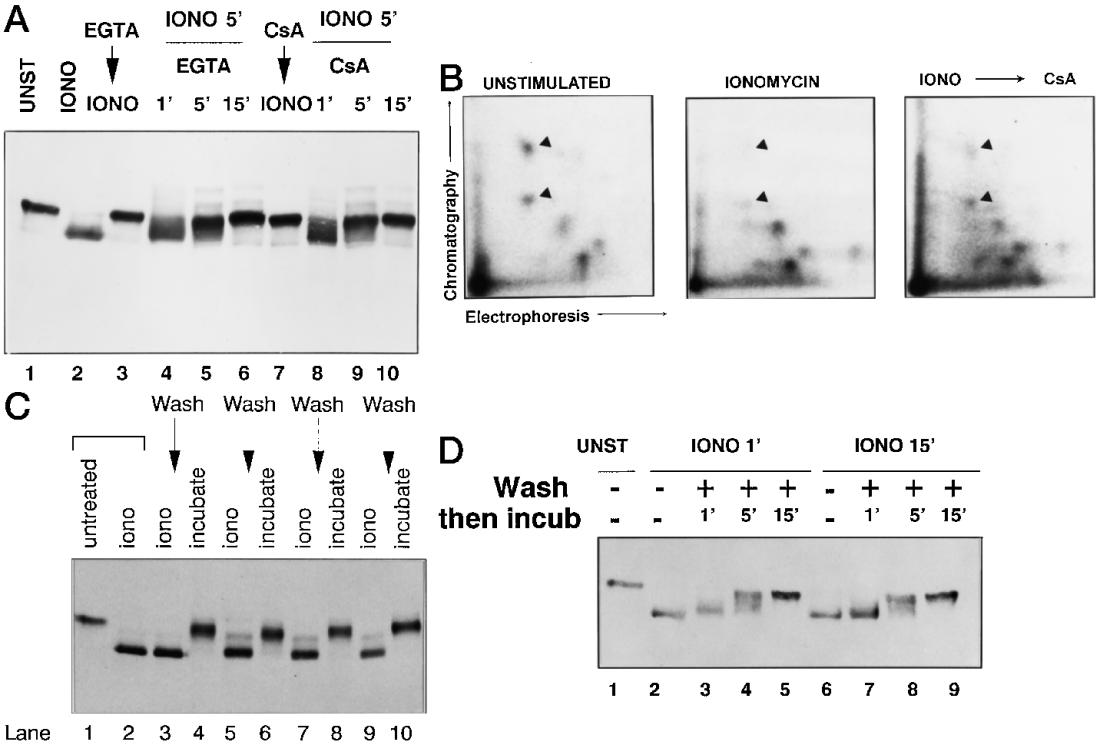

FIG. 3. **The activation-dependent dephosphorylation of NFAT1 is reversed by removal of ionomycin, addition of EGTA, or treatment with CsA.** *A*, addition of CsA or EGTA. Ar-5 T cells were left unstimulated (*lane 1*) or were stimulated with 1 μM ionomycin (*IONO*) for 5 min at 37 °C (*lanes 2*, *4–6*, and *8–10*). EGTA was added to a final concentration of 2 mM, and the cells were incubated at 37 °C for a further 1, 5, or 15 min (*lanes 4–6*, respectively). CsA was added to a final concentration of 1 μM, and the cells were incubated at 37 °C for a further 1, 5, or 15 min (*lanes 8–10*, respectively). In *lanes 3* and *7*, cells were pretreated with 2 mM EGTA or 1 μM CsA, respectively, for 15 min, followed by stimulation with 1 μM ionomycin for 5 min at 37 °C. *B*, tryptic phosphopeptide mapping of NFAT1. Ar-5 T cells were labeled with [^{32}P]orthophosphate as detailed under "Materials and Methods" and then left unstimulated (*left panel*) or stimulated with 1 μM ionomycin for 5 min (*middle panel*) or stimulated for 5 min with 1 μM ionomycin, followed by a further 30-min incubation in the presence of 1 μM CsA (*right panel*). Lysates from these cells were subjected to anti-NFAT1 immunoprecipitation. NFAT1 was separated on a 6% SDS-polyacrylamide gel, eluted from the gel, and digested with trypsin. The samples were electrophoresed in pH 1.9 buffer in the first dimension, followed by chromatography in the second dimension. Tryptic phosphopeptides were visualized by autoradiography. *Arrowheads* indicate the two major tryptic phosphopeptides that are stimulation-sensitive. *C*, removal of ionomycin. Ar-5 T cells were exposed to one cycle of stimulation with 1 μM ionomycin (*iono*) for 2 min at 37 °C (*lane 3*), followed by washing and incubation in fresh medium without ionomycin for 15 min at 37 °C (*lane 4*). *Lanes 5–10* show cells exposed to subsequent cycles of ionomycin stimulation (2 min), washing, and incubation (15 min) in fresh medium lacking ionomycin. *Lanes 1* and *2* show NFAT1 in Ar-5 T cells that were left unstimulated or were treated with ionomycin for the entire period of the experiment, respectively. *D*, kinetics of rephosphorylation after removal of ionomycin. Ar-5 T cells were left unstimulated (*lane 1*) or were stimulated with 1 μM ionomycin (*IONO*) for 1 min (*lanes 2–5*) or 15 min (*lanes 6–9*) at 37 °C. The stimulated cells (*lanes 3–5* and *7–9*) were centrifuged briefly in a microcentrifuge, resuspended in fresh medium without ionomycin (*Wash*), and incubated at 37 °C for 1, 5, or 15 min.

addition of CsA, NFAT1 is rephosphorylated on the same sites that had been dephosphorylated in response to ionomycin.

NFAT1 that had reverted to its original electrophoretic mobility could be repeatedly dephosphorylated and rephosphorylated (Fig. 3*C*). For this experiment, we took advantage of the reversibility of ionomycin action. Ionomycin induces the immediate release of calcium stores and stimulates capacitative calcium entry; conversely, removal of ionomycin allows the replenishment of calcium stores, thereby deactivating capacitative calcium entry and returning $[Ca^{2+}]_i$ to resting levels within a few minutes (47, 48). NFAT1 that had been dephosphorylated during a 2-min stimulation of Ar-5 T cells with ionomycin (Fig. 3*C*, *lane 3*) reverted to the phosphorylated form upon washing away the ionomycin and incubating the cells in fresh medium (*lane 4*). Upon readdition of ionomycin, the rephosphorylated NFAT1 was again dephosphorylated (*lane 5*). This successive rephosphorylation and dephosphorylation was observed for up to five successive cycles of washing the cells and restimulating them with ionomycin (*lanes 3–10*) (data not shown), again indicating that rephosphorylation returns NFAT1 to a state similar (if not identical) to that found in resting cells.

We examined the kinetics of NFAT1 rephosphorylation in Ar-5 cells stimulated with ionomycin for 1 min, at which time NFAT1 has not yet translocated to the nucleus, or for 15 min, by which time NFAT1 is in the nucleus of all cells (30). Removal of ionomycin by washing resulted in both cases in the rapid rephosphorylation of NFAT1 (Fig. 3*D*, *lanes 3–5* and *7–9*). The simplest explanation of these results is that the dephosphorylated NFAT1 is accessible to cellular kinases whether it is located in the nucleus or in the cytoplasm. Chelation of extracellular calcium with EGTA reversed the ionomycin-induced dephosphorylation of NFAT1 with kinetics very similar to those seen for reversal by CsA (Fig. 3*A*, compare *lanes 4–6* with *lanes 8–10*), indicating that the rephosphorylation was unlikely to be mediated by Ca^{2+}-dependent kinases, whose activity declines rapidly after Ca^{2+} is withdrawn (49).

Calcium-dependent Association of NFAT1 with Calcineurin in T Cell Lysates—We hypothesized that a physical association of NFAT1 with calcineurin might explain the striking sensitivity of NFAT1 to calcium and calcineurin. To address this point, we asked whether calcineurin would coprecipitate with NFAT1. A series of experiments using a variety of anti-NFAT1 and anti-calcineurin antibodies yielded equivocal results. However, when T cell extracts were incubated with CaM-Sepharose beads in Ca^{2+}-containing buffers, both NFAT1 and calcineurin were consistently found associated with the beads (Fig. 4*A*). This association was unaffected whether NFAT1 was derived from unstimulated or ionomycin-stimulated cells (compare

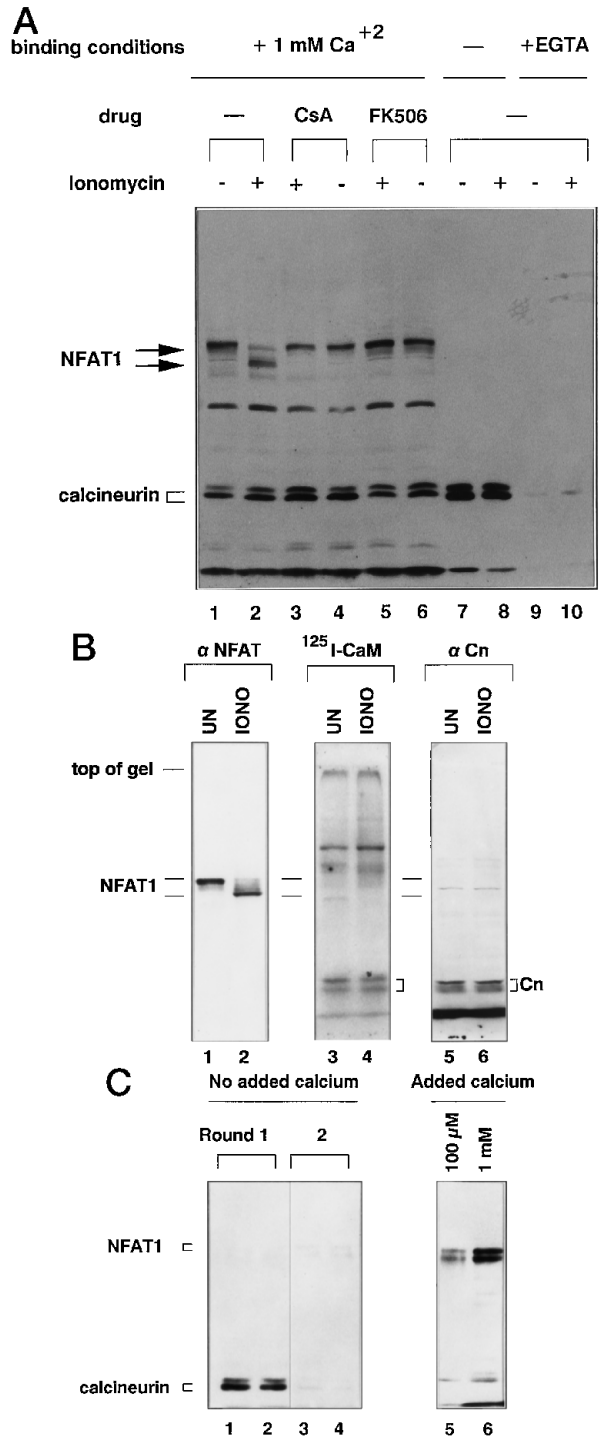

FIG. 4. **NFAT1 coprecipitates with calcineurin on CaM-Sepharose beads.** *A*, Ar-5 T cells (10×10^6) were left unstimulated (−) or were stimulated with ionomycin (+) in the absence or presence of 1 μM CsA or FK506 and lysed in 1% Triton X-100 buffer without added calcium (*lanes 7* and *8*) or supplemented with 1 mM $CaCl_2$ (*lanes 1–6*) or with 5 mM EGTA (*lanes 9* and *10*). Lysates were incubated with CaM-Sepharose beads, and the bound proteins were analyzed by 7.5% SDS-PAGE followed by Western blotting with a mixture of anti-NFAT1 and anti-calcineurin antibodies. *B*, SDS lysates of unstimulated (*UN*) or ionomycin-stimulated (*IONO*) Ar-5 T cells were analyzed by 7.5% SDS-PAGE followed by Western blotting with anti-NFAT1 antibody (*α NFAT*; *lanes 1* and *2*). The blot was stripped and incubated with ^{125}I-calmodulin (*^{125}I-CaM*); the bands were visualized by autoradiography (*lanes 3* and *4*). The blot was once again stripped and reprobed with anti-calcineurin antibody (*α Cn*). *C*, Triton X-100 lysates of unstimulated Ar-5 T cells without added calcium in the buffer were incubated with CaM-Sepharose beads for three successive rounds of 1 h each at 4 °C. The lysate was then supplemented with 100 μM or 1 mM $CaCl_2$ and subjected to one further incubation with CaM-Sepharose beads before washing, boiling, and analysis as described for *A*. Note that given the presence of EDTA, sodium pyrophosphate, and NaF in the buffers, the free calcium concentration in the buffers in *lanes 5* and *6* is much lower than the total concentration indicated and is plausibly in the physiological range.

lanes 1 and *2*). Inhibition of calcineurin activity with maximally immunosuppressive concentrations of CsA or FK506 did not affect the association of either calcineurin or NFAT1 with the CaM-Sepharose beads (*lanes 3–6*), consistent with the finding that the calmodulin-binding region of the calcineurin A chain is distinct from the B chain-binding α helix involved in the interaction with drug-immunophilin complexes (51). In buffer without added calcium, calcineurin bound to the CaM-Sepharose beads, whereas NFAT1 did not (*lanes 7* and *8*); the binding of calcineurin was eliminated by addition of EGTA (*lanes 9* and *10*). These results indicate that the calcium concentration in this nominally calcium-free buffer is at least 0.1–1 μM, the K_d of CaM for Ca^{2+} (53); the exact concentration is difficult to estimate due to the presence of sodium fluoride and sodium pyrophosphate included to inactivate cellular phosphatases.

The binding of NFAT1 to CaM-Sepharose beads did not appear to reflect a direct interaction between NFAT1 and CaM. When a Western blot of whole cell lysates was used in an "overlay" experiment (39) with iodinated or biotinylated CaM, there was no detectable binding of CaM to NFAT1, even at high calcium concentrations (Fig. 4*B*, compare *lanes 1* and *2* with *lanes 3* and *4*). In contrast, CaM bound strongly to calcineurin and to other unidentified CaM-binding proteins under the same conditions (compare *lanes 3* and *4* with *lanes 5* and *6*). These results suggest that NFAT1 binds indirectly to CaM-Sepharose beads, through an ability to interact with calcineurin or other CaM-binding proteins. The specific involvement of calcineurin is suggested by the experiments of Fig. 5 (*B* and *C*) below.

To investigate the calcium requirement for binding of the putative NFAT1-calcineurin complex, we depleted CaM-binding proteins from cell extracts by incubating them with CaM-Sepharose beads in buffer without added Ca^{2+} (Fig. 4*C*, *lanes 1* and *2*). This process removed >95% of the calcineurin (and presumably other CaM-binding proteins) capable of binding under these conditions (compare *lanes 1* and *2* with *lanes 3* and *4*). After an additional cycle of depletion (not shown), Ca^{2+} was added to replicate samples of the depleted lysates to achieve total Ca^{2+} concentrations of 100 μM and 1 mM (*lanes 5* and *6*, respectively; again, the free Ca^{2+} concentrations in these buffers are likely to be much lower and are plausibly in the physiological range). These experiments showed a striking Ca^{2+}-dependent increase in the level of NFAT1 bound to the beads; concomitantly, an increase in the level of bound calcineurin was observed (*lanes 5* and *6*). Addition of exogenous calcineurin to the extracts did not increase the level of NFAT1 binding to the CaM-Sepharose beads (data not shown). These results suggest that the fraction of calcineurin that coprecipitates with NFAT1 under these conditions has properties distinct from the bulk of the calcineurin in that its binding to CaM-Sepharose requires higher concentrations of calcium or trace concentrations of other ions present in our Ca^{2+} stocks.

Calcineurin Interacts with the N-terminal Region of NFAT1—To delineate the region of NFAT1 involved in the putative association with calcineurin, COS cells were transfected with expression plasmids encoding full-length NFAT1 or the N-terminal 415-amino acid fragment of NFAT1 (NFAT1-(1–415)), which contains the entire NFAT homology region.[1]

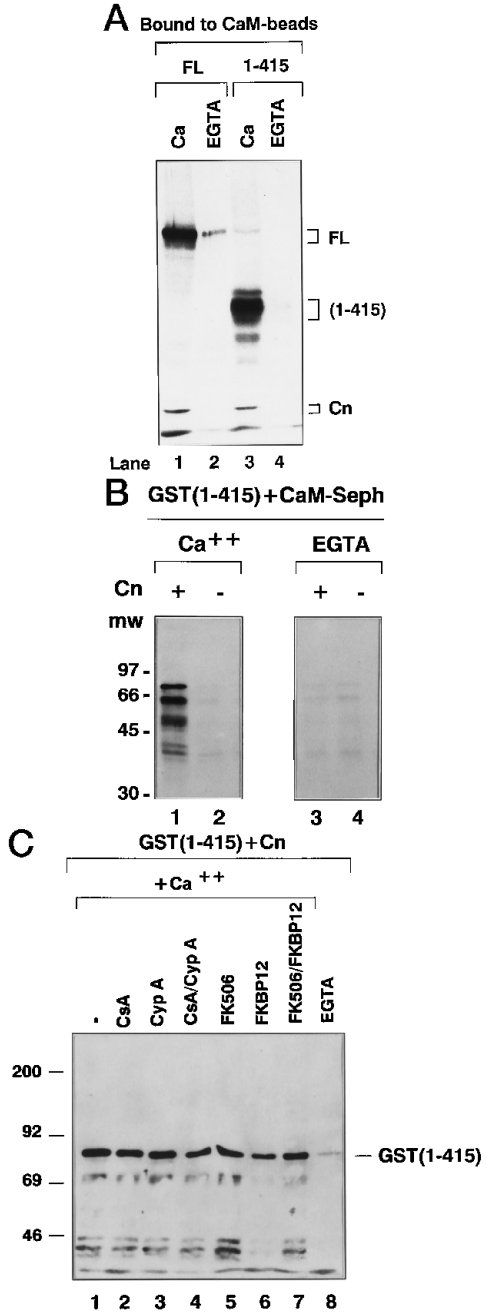

FIG. 5. **The N-terminal 415 amino acids of NFAT1 are sufficient to bind calcineurin.** *A*, COS cells were transfected with plasmids encoding full-length NFAT1 (*FL*) or the N-terminal 1–415 amino acids of NFAT1 (*1–145*). The cells were harvested 48 h after transfection and lysed in 1% Triton X-100 buffer. The lysates were incubated with CaM-Sepharose for 1 h at 4 °C. The beads were washed, boiled in Laemmli reducing buffer, and analyzed by 10% SDS-PAGE followed by Western blotting with a mixture of anti-NFAT1 and anti-calcineurin antibodies. *Cn*, calcineurin. *B*, the GST-NFAT1-(1–415) fusion protein was eluted from glutathione-Sepharose beads and diluted with 3 volumes of 1% Triton X-100 buffer supplemented with 1 mM $CaCl_2$ or 5 mM EGTA as indicated, and 100 μl of this mixture was added to 50 μl of washed, packed CaM-Sepharose beads. In *lanes 1* and *3*, purified calcineurin (0.3 μg) was added. All samples were rocked for 1 h at 4 °C and then washed, boiled in Laemmli reducing buffer, and analyzed by 10% SDS-PAGE followed by Western blotting with anti-NFAT1 antibody. *C*, the GST-NFAT1-(1–415) fusion protein was eluted from glutathione-Sepharose beads and diluted with 1.5 volumes of 1% Triton X-100 supplemented with 1 mM $CaCl_2$ (*lanes 1–7*) or 5 mM EGTA (*lane 8*), and 100 μl of this mixture was added to 50 μl of washed, packed CaM-Sepharose beads. Purified calcineurin (0.3 μg), preincubated alone or in the presence of a 10-fold molar excess of CsA, cyclophilin A (*Cyp A*), FK506, FKBP12, or the appropriate drug-immunophilin complex as indicated, was added to the samples. Following incubation at 4 °C for 1 h, the CaM-Sepharose beads were washed, boiled in Laemmli reducing buffer, and analyzed by 10% SDS-PAGE followed by Western blotting with anti-NFAT1 antibody.

Both full-length NFAT1 and NFAT1-(1–415) bound to CaM-Sepharose beads in the presence of Ca^{2+}, but not in the presence of EGTA (Fig. 5*A*). Calcineurin also bound to the beads under the same conditions, indicating that the N-terminal region of NFAT1 contained the information required for association with calcineurin.

To address the possibility that NFAT1-(1–415) bound to CaM-beads in association with CaM-binding proteins other than calcineurin, we used a fusion protein of GST with the N-terminal 415 amino acids of NFAT (GST-NFAT1-(1–415)). GST-NFAT1-(1–415) did not bind to CaM-Sepharose directly, but was capable of binding in the presence of purified calcineurin and calcium (Fig. 5*B*, compare *lane 1* with *lanes 2* and *3*). Bound GST-NFAT1-(1–415), which is a mixture of several proteolytic fragments formed by degradation within the bacteria (data not shown), was detected with an antibody to a peptide near the N terminus of NFAT1 (*lane 1*). Furthermore, consistent with the results of Fig. 4*A*, the association of purified calcineurin with GST-NFAT1-(1–415) was not disrupted by addition of CsA and purified cyclophilin A (Fig. 5*C*, *lanes 2–4*) or FK506 and purified FKBP12 (*lanes 5–7*). These experiments recapitulate the conditions of the earlier assay shown in Figs. 4*A* and 5*A* and support our interpretation that endogenous NFAT1 in cell lysates binds to CaM-Sepharose beads as a complex with calcineurin.

DISCUSSION

Although it has been well documented that CsA prevents the calcineurin-mediated activation of NFAT (15, 18–20), thus far, there has been no reason to expect that NFAT activation would be continuously dependent on the level of calcineurin activity. We show here that the activation of NFAT1 requires a sustained increase in the level of cytoplasmic $[Ca^{2+}]_i$ and calcineurin activity *in vivo*. Inhibition of calcineurin by addition of CsA or chelation of extracellular calcium by EGTA, even in the constant presence of ionomycin, reverses NFAT1 activation by permitting its rephosphorylation, its reappearance in the cytoplasm, and the return of its DNA binding activity to a low level. It is likely that all NFAT family proteins that are expressed at detectable levels are similarly responsive to the level of calcineurin activity since CsA completely inhibits the induction of nuclear NFAT-binding complexes in a variety of immune system cells (1, 15, 17–23). Our results are consistent with the ability of CsA to block ongoing interleukin-2 transcription (13, 33, 45). They suggest a central function for NFAT family proteins in cytokine gene transcription and explain the remarkable potency of CsA and FK506 as immunosuppressive drugs.

We have also shown that calcineurin interacts directly with NFAT1 *in vitro*. A fraction of total cellular calcineurin is coprecipitated with NFAT1 on calmodulin-Sepharose beads under conditions distinct from those required for binding of the bulk of calcineurin to the same beads. The results are consistent with the hypothesis that a fraction of the calcineurin is tethered to NFAT1 in a pre-existing complex; formation of the complex may be stabilized at the higher cytoplasmic free calcium concentrations that prevail in activated cells. The likelihood of an NFAT1-calcineurin interaction provides an attractive explanation for the sensitivity of NFAT1 to the level of calcineurin activity. Moreover, it suggests that calcineurin dephosphorylates at least some of the relevant phosphorylated residues of NFAT1, implying that NFAT1 is a physiological

substrate for calcineurin and hence an immediate secondary target of CsA and FK506 (1, 2, 50). The N-terminal 415 amino acids of NFAT1, which suffice for calcineurin binding, include the ~300 amino acids of the NFAT homology region[1]; it remains to be determined whether the conserved sequence motifs identified in this region (8–10)[1] play a role in calcineurin binding. The CaM-bound state of calcineurin is not required for the interaction between calcineurin and NFAT1, as purified calcineurin can be directly coprecipitated with GST-NFAT1-(1–415) on glutathione-Sepharose beads.[5] In addition, the NFAT1-calcineurin interaction observed in cell lysates is not affected by pretreatment of cells with maximally immunosuppressive concentrations of CsA and FK506. One explanation is that NFAT1 binds to a region of calcineurin distinct from the B chain-binding α helix that interacts with drug-immunophilin complexes (51, 52), thus rendering it feasible to screen for drugs that block the NFAT1-calcineurin interaction without interfering with calcineurin phosphatase activity.

Our results suggest that the phosphorylation state (and concomitantly the activation state) of NFAT1 is regulated by a dynamic equilibrium between calcineurin and basally active kinases. Tryptic phosphopeptide mapping indicates that NFAT1 is selectively dephosphorylated via calcineurin-dependent mechanisms, as at least two of several phosphopeptides of NFAT1 disappear when cells are stimulated with ionomycin. Moreover, these same two peptides reappear upon addition of CsA to ionomycin-stimulated cells, suggesting that kinases rapidly rephosphorylate NFAT1 following inhibition of calcineurin activity by CsA-cyclophilin. The phosphorylation state of several other phosphopeptides remains unchanged. It is possible that at least two classes of kinases act on NFAT1, one or more basally active kinases that phosphorylate the stimulation-insensitive sites and one or more kinases that phosphorylate only those sites that are dephosphorylated following T cell stimulation.

Our experiments do not address the intracellular location of the NFAT1-modifying enzymes in resting and activated cells. NFAT1 remains dephosphorylated and in the nucleus for long periods under conditions in which calcineurin activity is maintained, and dephosphorylated NFAT1 retains the ability to bind calcineurin directly; thus, one possibility is that a fraction of calcineurin translocates to the nucleus together with NFAT1. Likewise, dephosphorylated NFAT1 is accessible to kinases whether it is in the cytoplasm or the nucleus, suggesting either that the kinases are distributed throughout the cell or that they translocate with NFAT1 into the nucleus. However, it is equally possible that calcineurin and other relevant kinases and phosphatases function exclusively in the cytoplasm; if both dephospho-NFAT1 and phospho-NFAT1 constantly shuttled between the nucleus and the cytoplasm at rates determined by their phosphorylation state, both forms would be fully accessible to cytoplasmic enzymes.

The interaction of calcineurin with NFAT1 is a novel illustration of a more general principle, that the specificity of signaling pathways is achieved in many cases by targeting signaling proteins to their downstream effectors or substrates (34–40). This targeting may be accomplished by stabilizing the normally transient interactions of enzymes with their substrates through protein-protein interactions, by using specialized binding domains such as SH2 and SH3 domains (36, 37) to recruit effector proteins, or by directing nonspecific enzymes to specific intracellular locations by means of targeting proteins or subunits (38, 39). Several of these strategies may be utilized by the NFAT1-calcineurin complex; in particular, the NFAT1-calcineurin interaction is not dependent on the phosphorylation state of the substrate and thus resembles the stable interaction of Jun N-terminal kinase with both phosphorylated and dephosphorylated c-Jun proteins (34). However, additional proteins may be involved in targeting, as shown for calcineurin itself and for other serine/threonine phosphatases (38, 39). The catalytic subunit of protein phosphatase 1, which shows relatively low substrate specificity *in vitro*, is directed toward its substrates (the enzymes involved in glycogen metabolism) by a glycogen-binding targeting subunit (32, 38). Likewise, the protein kinase A anchor protein AKAP-79 is a targeting protein that binds both to calcineurin and to protein kinase A (39), thus bringing into proximity two enzymes that have mutually opposing actions on a variety of cellular processes (38). By analogy, the NFAT1-calcineurin complex may include an additional targeting protein and also the kinases that deactivate NFAT1. "Scaffolding" proteins may also play a role. In yeast, multiprotein complexes comprising independent signaling modules, which contain enzymes associated with specific signaling pathways, have been described (40), and similar large signaling complexes may regulate signal transduction in mammalian cells. Analysis of the NFAT1-calcineurin interaction represents the first step in delineating the regulatory components of this unique signaling pathway, in which calcineurin activation is directly coupled to inducible gene expression.

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Note Added in Proof—An NFATI-calcineurin interaction has also been reported by Wesselborg *et al.* (54).

[5] K. T.-Y. Shaw and A. Rao, unpublished data.

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