Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8907–8912, August 1996
Biochemistry

Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import

(protein phosphatase/nuclear localization sequence/immunosuppression/T cell activation/signal transduction)

CHUN LUO*†‡, KAREN T.-Y. SHAW*†§, ANURADHA RAGHAVAN¶, JOSE ARAMBURU*, FRANCISCO GARCIA-COZAR*, BRIAN A. PERRINO‖, PATRICK G. HOGAN¶, AND ANJANA RAO*,**

*Division of Cellular and Molecular Biology, Dana–Farber Cancer Institute and Department of Pathology, and ¶Department of Neurobiology, Harvard Medical School, Boston, MA 02115; and ‖The Vollum Institute, Oregon Health Sciences University, Portland, OR 97201

Communicated by Stephen C. Harrison, Harvard University, Cambridge, MA, May 8, 1996 (received for review February 7, 1996)

ABSTRACT **The nuclear import of the nuclear factor of activated T cells (NFAT)-family transcription factors is initiated by the protein phosphatase calcineurin. Here we identify a regulatory region of NFAT1, N terminal to the DNA-binding domain, that controls nuclear import of NFAT1. The regulatory region of NFAT1 binds directly to calcineurin, is a substrate for calcineurin *in vitro*, and shows regulated subcellular localization identical to that of full-length NFAT1. The corresponding region of NFATc likewise binds calcineurin, suggesting that the efficient activation of NFAT1 and NFATc by calcineurin reflects a specific targeting of the phosphatase to these proteins. The presence in other NFAT-family transcription factors of several sequence motifs from the regulatory region of NFAT1, including its probable nuclear localization sequence, indicates that a conserved protein domain may control nuclear import of all NFAT proteins.**

NFAT1 (nuclear factor of activated T cells 1) (previously termed NFATp) (1–3) is a member of the NFAT family of transcription factors, proteins 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/NFAT4 (8–10), and NFAT3 (9). NFAT-family proteins show strong sequence conservation in their DNA-binding domains (8–12), and moderate sequence conservation in a second region located immediately N terminal to the DNA-binding domain (54).

A central feature of the regulation of NFAT-family proteins is their pronounced sensitivity to the immunosuppressive drugs cyclosporin A (CsA) and FK506 (13, 14). These drugs, which are potent inhibitors of cytokine gene transcription in activated T cells, B cells, mast cells, and NK cells (15–18), act by binding to intracellular immunophilins and inhibiting the activity of the calmodulin-dependent phosphatase calcineurin (19, 20). Inhibition of calcineurin with the immunosuppressive drugs prevents the appearance of NFAT DNA-binding activity in the cell nucleus when T cells and other immune system cells are stimulated (1, 18, 21–26), and blocks NFAT-dependent reporter gene transcription (21, 23, 25). Conversely, overexpression of calcineurin or expression of constitutively active calcineurin partially replaces the calcium requirement for NFAT-dependent transcriptional activation in T cells (17, 27–29).

Detailed analysis of one NFAT-family protein, NFAT1, has provided more insight into the molecular mechanisms by which calcineurin regulates NFAT activity. 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 (30, 55). Early events in the activation of NFAT1 are its rapid dephosphorylation, an increase in its affinity for NFAT sites in DNA, and its nuclear import (30). Each of these three hallmarks of activation is dependent on calcineurin, since each is blocked by CsA or FK506 (30). Here we present evidence suggesting that the close control of NFAT1 activation by the calcium/calcineurin pathway reflects a protein–protein interaction that targets calcineurin to NFAT1.

MATERIALS AND METHODS

cDNA Constructs. The cDNAs encoding murine NFAT1, human NFAT1(1–415), murine NFAT1(399–927), and murine NFAT1(398–694) were subcloned into pEFTAG, a derivative of the expression vector pEF-BOS (31) that has been modified to encode an influenza hemagglutinin (HA) peptide at the N terminus of the expressed proteins. Details of the subcloning steps are available from the authors. Murine NFAT1(398–694) is the DNA-binding domain that has been expressed in bacteria as the hexahistidine-tagged protein NFATpXS(1–297) (11).

Bacterial expression plasmids for the glutathione *S*-transferase (GST) fusion proteins GST-NFAT1(1–415) and GST-NFATc(1–418) were made by subcloning the corresponding regions of human NFAT1 and NFATc cDNAs into pGEX2T.

Site-Directed Mutagenesis. Single-stranded plasmid DNA containing uracil (32) was prepared from a pLGP3 vector (33) with the murine NFAT1 cDNA insert. Site-directed mutagenesis (32) using the oligonucleotides 5′-TCACCCGGTGCCGCTGCAGCTCATTCGTGCGCA-3′ and 5′-GTCATCAACGGAGCTGCAGCCACTAGTCAGCCACAGCA-3′ was carried out to replace the codons for KRR or KRKR, respectively, and plasmids carrying the mutant sequences were identified by DNA sequencing. *Eco*NI–*Bgl*II and *Mlu*I–*Hin*dIII fragments, carrying respectively the region encoding the KRR to AAA substitution and the region encoding the KRKR to AAAT substitution, were used to replace the corresponding fragments of pBluescript–mNFAT1-C (C.L., unpublished work). The *Aat*II–*Sal*I fragments of the resulting plasmids were subcloned into pEFTAG for expression in T cells. The sequence of the entire fragment subcloned from the pLGP3 vector was verified by DNA sequencing by the dideoxy chain-termination method (34).

Immunocytochemical Localization of Recombinant Proteins. Plasmids were introduced into Jurkat cells by electroporation, and into clone 7W2 cells by the DEAE–dextran method. Two days later cells in medium at 37°C were treated

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NFAT, nuclear factor of activated T cells; CsA, cyclosporin A; HA, hemagglutinin; GST, glutathione *S*-transferase; NLS, nuclear localization sequence.

†C.L. and K.T.-Y.S. contributed equally to this work.
‡Present address: Center for Blood Research and Harvard Medical School, Boston, MA 02115.
§Present address: Department of Physiology and Cell Biology, University of Nevada School of Medicine, Reno, NV 89557.
**To whom reprint requests should be addressed.

as indicated, then fixed and stained with the anti-HA tag antibody (12CA5; Boehringer Mannheim) and Cy-3-labeled donkey anti-mouse IgG (Jackson ImmunoResearch). Transient expression of the tagged protein was detectable in 1–5% of human Jurkat T cells and in 0.2–2% of murine clone 7W2 cells. At least three independent experiments were analyzed for each recombinant protein.

***In Vitro* Dephosphorylation.** Unstimulated Ar-5 T cells were lysed in RIPA buffer (30) supplemented with protease inhibitors and phosphatase inhibitors, and NFAT1 was immunoprecipitated. Immunoprecipitates were resuspended in calcineurin assay buffer (50 mM Hepes, pH 7.4/2 mM $MnCl_2$/0.5 mM EDTA/15 mM 2-mercaptoethanol) with 0.1 mg/ml BSA, 20 μM leupeptin, and 10 μg/ml aprotinin, and incubated at 30°C for 10 min with 100 nM calcineurin (35) and 500 nM purified calmodulin. SDS lysates were prepared as described (30). The samples were examined by Western blotting with anti-NFAT1 antiserum (anti-67.1) (30, 36).

Expression constructs encoding HA-tagged NFAT1 or NFAT1(1–415) were introduced into COS cells using DEAE–dextran combined with adenovirus (37). After 48 h, cytosolic extracts were prepared in lysis buffer [20 mM tris·HCl, pH 7.4/30 mM NaCl/5 mM EDTA/10 mM iodoacetamide/2 mM phenylmethylsulfonyl fluoride (PMSF)/10 μg/ml aprotinin/25 μM leupeptin/5% glycerol/0.05% Nonidet P-40]. Cytosolic extracts (2 μl, from 2×10^4 cells) were incubated at 30°C for 20 min in a total volume of 15 μl of 100 mM NaCl with no additions; or with 2 mM Ca^{2+}, 100 nM calcineurin, and 500 nM calmodulin. Where indicated, 300 nM okadaic acid, 30 mM sodium pyrophosphate, 20 μM CsA, 2 μM FK506, 2 μM rapamycin, 2 μM cyclophilin, or 2 μM FKBP12 were preincubated with the calcium/calmodulin/calcineurin at 4°C for 1 h before addition of the cell extract. The samples were examined by Western blotting with anti-NFAT1 antiserum.

Calcineurin Binding. Unstimulated Ar-5 T cells (10^7 cells) were lysed in 0.5 ml binding buffer (50 mM tris·HCl, pH 7.5/150 mM NaCl/0.5 mM EDTA/10 mM sodium pyrophosphate/10 mM NaF/1% Triton X-100/1 mM sodium orthovanadate/10 mM iodoacetamide/5 mM $MgCl_2$/30 mM 2-mercaptoethanol/100 μg/ml aprotinin/25 μM leupeptin/2 mM PMSF) supplemented with either 1 mM $CaCl_2$ or 5 mM EGTA. (The concentration of free Ca^{2+} in the samples supplemented with $CaCl_2$ has not been determined directly, but it will necessarily be much lower than 1 mM, given the presence of F^- and other anions in the binding buffer.) Cell lysates or purified calcineurin (Sigma, 0.3 μg in 0.5 ml binding buffer, supplemented with 1 mM $CaCl_2$ or 5 mM EGTA) were incubated with 10 μl (in some experiments, 15 μl) glutathione-Sepharose with bound GST-NFAT1(1–415), GST-NFATc(1–418), GST, or GST-LSF (38), or with glutathione-Sepharose or Sepharose alone at 4°C for 1 h or 1.5 h. The resin was washed, and proteins were eluted by boiling in Laemmli reducing buffer and analyzed by Western blotting using anti-calcineurin antiserum. The anti-calcineurin is a rabbit antiserum raised against the peptide YITSFEEAKGLDRINERMPPRRDAMPSD, corresponding to the auto-inhibitory domain of the calcineurin A chain (C.L., unpublished).

RESULTS

The Region NFAT1(1–415) Controls Nuclear Import of NFAT1. To define the region of NFAT1 that renders its cytoplasmic/nuclear localization sensitive to calcineurin, we have expressed NFAT1 and specific truncated NFAT1 proteins in T cells. The DNA-binding domain of NFAT1 has been identified (11), and based on its insensitivity to proteolytic digestion is likely to form a compactly folded domain. Judging that NFAT1 fragments truncated immediately N terminal or C terminal to the DNA-binding domain are likely to be correctly folded, we prepared expression constructs encoding the region N terminal to the DNA-binding domain, human NFAT1(1–415); the region from the beginning of the DNA-binding domain to the C terminus, murine NFAT1(399–927) (here denoted NFAT1ΔN); and the DNA-binding domain itself, murine NFAT1(397–694) [here denoted NFAT1(DBD)]. Each recombinant protein was tagged at its N terminus with an influenza HA peptide.

HA-tagged recombinant NFAT1 shows correctly regulated intracellular localization in human Jurkat T cells. The protein is restricted to the cytoplasm in unstimulated cells, it translocates to the cell nucleus on stimulation with ionomycin, and its translocation is completely prevented by pretreatment with CsA (Fig. 1). In a minority of cells, nuclear translocation in response to ionomycin is only partial, a finding that may reflect the high levels of expression of the recombinant protein in individual cells. However, because recombinant protein is never seen in the nucleus of unstimulated cells or of cells treated with CsA, the incomplete translocation in a few cells does not affect the interpretation of the experiments.

Recombinant NFAT1(1–415) is regulated in the same way as the full-length protein, and its translocation is similarly sensitive to CsA (Fig. 1). In contrast, the truncated proteins NFAT1ΔN and NFAT1(DBD), which lack the N-terminal region of NFAT1, are not restricted to the cytoplasm in unstimulated cells and show no redistribution in response to ionomycin (Fig. 1 and data not shown).

We have confirmed these results for HA-tagged NFAT1, NFAT1(1–415), and NFAT1(DBD) in the murine T cell clone 7W2 (39). As in Jurkat cells, nuclear translocation of NFAT1 or NFAT1(1–415) requires stimulation with ionomycin and is blocked by CsA, whereas the intracellular localization of NFAT1(DBD) is insensitive to ionomycin (data not shown).

NFAT1(1–415) Contains a Candidate Nuclear Localization Sequence (NLS). The fact that NFAT1(1–415) shows regulated intracellular localization independently of other domains of the protein raises the question whether this region of

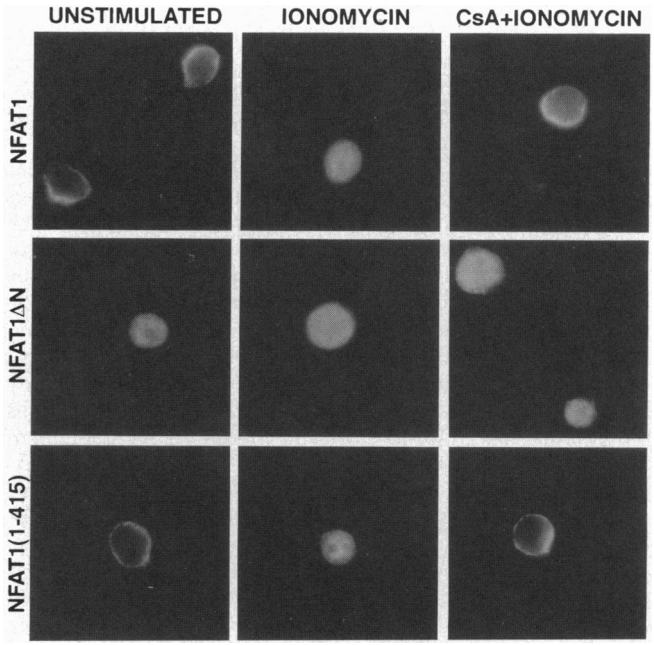

FIG. 1. Intracellular localization of HA-tagged full-length and truncated NFAT1 in Jurkat T cells. Jurkat cells transiently expressing full-length NFAT1, NFAT1ΔN, or NFAT1(1–415) were left unstimulated, were treated with 3 μM ionomycin for 15 min, or were pretreated with 1 μM CsA for 15 min and then treated with ionomycin and CsA for an additional 15 min. Recombinant proteins were visualized by immunocytochemical staining with antibody recognizing the epitope tag. Only the cells expressing recombinant NFAT1 or its fragments are visible with anti-HA staining.

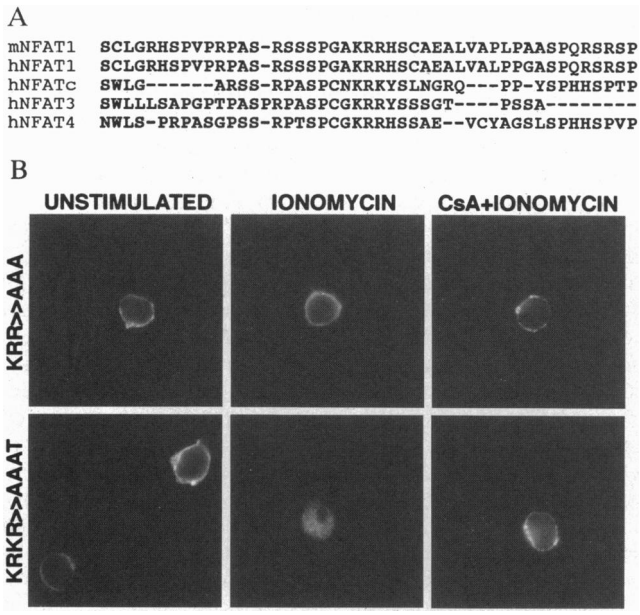

FIG. 2. Identification of a candidate NLS in NFAT1. (*A*) Sequence alignment of murine NFAT1(232–277) with human NFAT1(230–275), NFATc(250–285), NFAT3(246–280), and NFAT4(253–295). (*B*) The substitutions KRR > AAA (at residues 253–255) or KRKR > AAAT (at residues 666–669) were introduced into murine NFAT1. Jurkat cells expressing either the KRR > AAA or the KRKR > AAAT mutant of NFAT1 were treated as in Fig. 1.

NFAT1 contains a functional NLS. There is a relatively basic region extending from residues 236–275 in murine NFAT1, with the sequence KRR (residues 253–255) at its core, that could serve as an NLS. A similar KRR (or KRK) sequence is present in the other NFAT proteins (Fig. 2*A*). The mutation KRR > AAA in NFAT1 prevents nuclear translocation of the full-length recombinant protein in response to stimulation with ionomycin (Fig. 2*B*). Another basic motif conserved in NFAT-family proteins, the sequence KRKR located at residues 666–669 in the Rel similarity domain of murine NFAT1, has been proposed as an NLS (8, 10, 40, 41). Replacing KRKR with the sequence AAAT in NFAT1 has no effect on the cytoplasmic localization of the full-length protein in unstimulated cells, or on its nuclear localization in stimulated cells (Fig. 2*B*).

These findings are consistent with a role for the N-terminal region of NFAT1 in controlling the intracellular localization of the protein. The simplest interpretation is that the KRR sequence at residues 253–255 forms part of a functional NLS, and that activation leads to unmasking of the NLS through a conformational change or through dissociation of a protein–protein complex. In contrast to the requirement for the KRR sequence in the N-terminal region, the KRKR sequence at residues 666–669 in the Rel similarity region of NFAT1 is not necessary and is not by itself sufficient for nuclear import of full-length NFAT1. Because estimates of nuclear pore size (42, 43) and data for bovine serum albumin (44) indicate that proteins smaller than about 70 kDa can enter the nucleus by passive diffusion through nuclear pores, the presence of NFAT1ΔN and NFAT1(DBD) in the nucleus does not necessarily imply that they possess an NLS or associate with an NLS-containing protein. On the other hand, the exclusion of

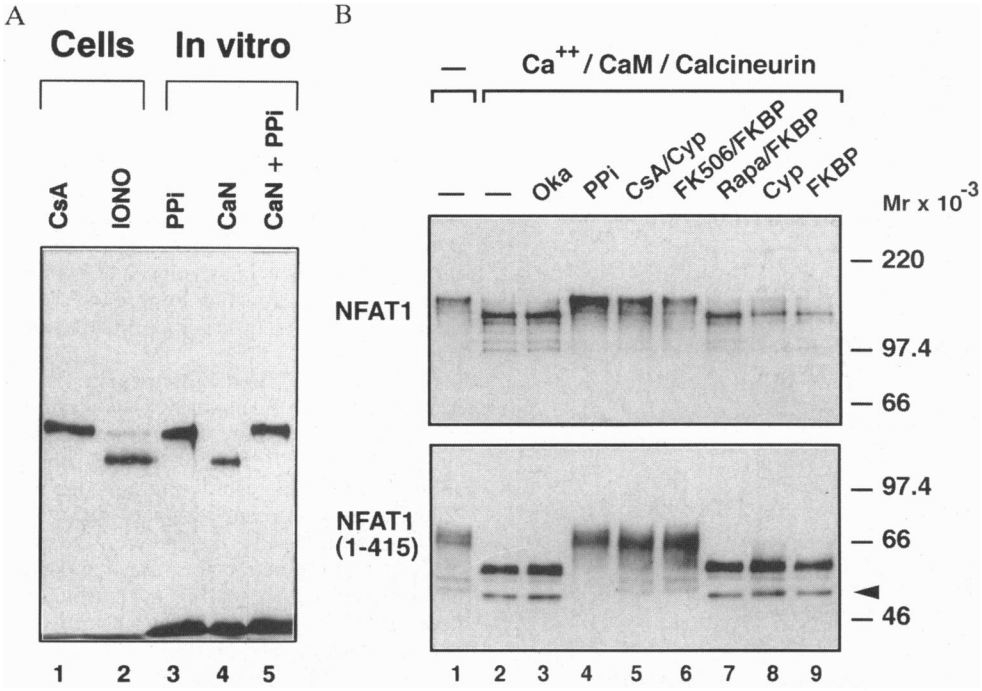

FIG. 3. *In vitro* dephosphorylation of NFAT1 and NFAT1(1–415) by calcineurin. (*A*) NFAT1 immunoprecipitated from unstimulated Ar-5 T cells was incubated with 30 mM sodium pyrophosphate (lane 3), with 100 nM calcineurin (CaN) and 500 nM calmodulin (CaM) (lane 4), or with calcineurin and calmodulin in the presence of 30 mM sodium pyrophosphate (lane 5), then examined by Western blotting with anti-NFAT1 antiserum. For comparison, protein samples from Ar-5 T cells treated with 1 μM CsA (lane 1) or with 1 μM ionomycin (lane 2) and then lysed in SDS were included on the same gel. The mobility of NFAT1 from T cells treated with CsA does not differ from that of NFAT1 from resting cells (30). (*B*) Cytosolic extracts from COS cells expressing full-length NFAT1 (*upper*) or NFAT1(1–415) (*lower*) were incubated with no additions (lane 1), or with 2 mM Ca^{2+}, 100 nM calcineurin, and 500 nM calmodulin (lanes 2–9). For lanes 3–9, the phosphatase inhibitors okadaic acid (lane 3) and sodium pyrophosphate (lane 4), the specific calcineurin inhibitors CsA/cyclophilin complex (lane 5) and FK506/FKBP12 complex (lane 6), or the control additions rapamycin/FKBP12 complex (lane 7), cyclophilin alone (lane 8), or FKBP12 alone (lane 9) were preincubated with the Ca^{2+}/calmodulin/calcineurin mixture before it was added to the cell extract. After incubation, the samples were analyzed by Western blotting with anti-NFAT1. NFAT1 was not detected in control COS cells not transformed with NFAT1 expression plasmids. The positions of protein molecular weight standards and of unphosphorylated NFAT1(1–415) purified from bacteria (arrowhead) are indicated at right.

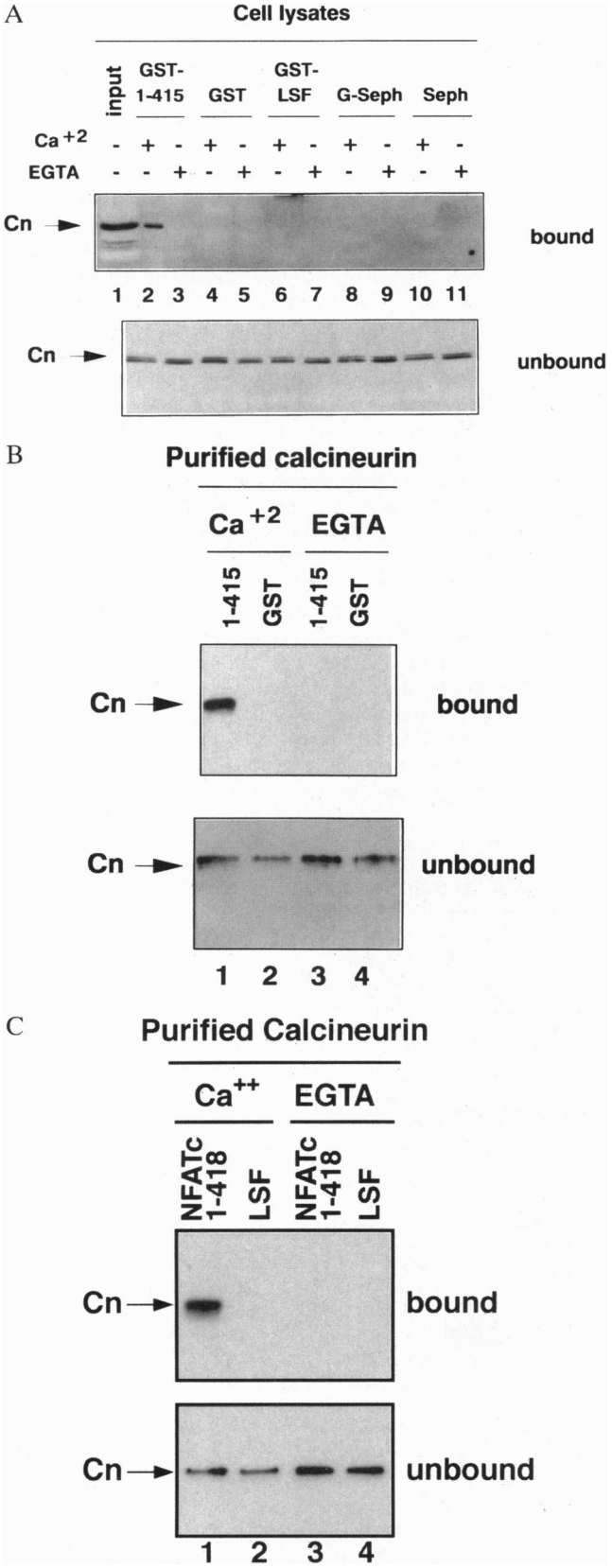

FIG. 4. Binding of calcineurin to GST-NFAT1(1–415) and to GST-NFATc(1–418). (*A*) Lysates from unstimulated Ar-5 T cells were incubated with GST-NFAT1(1–415), GST, or GST-LSF bound to glutathione-Sepharose, or with glutathione-Sepharose or Sepharose alone. The binding buffer was supplemented either with 1 mM $CaCl_2$ or with 5 mM EGTA as indicated. Bound protein (*upper*) and one-fifth of the protein remaining unbound (*lower*) were analyzed by Western blotting with anti-calcineurin antiserum. The sample in lane 1 is total protein from Ar-5 T cells. (*B*) Purified calcineurin was incubated with GST-NFAT1(1–415) or GST bound to glutathione-Sepharose in buffer containing either 1 mM $CaCl_2$ or 5 mM EGTA. Bound protein (*upper*) and unbound protein (*lower*) were analyzed as in *A*. (*C*) Purified calcineurin was incubated with GST-NFATc(1–418) or GST-LSF bound to glutathione-Sepharose. Bound protein (*upper*) and one-tenth of the protein remaining unbound (*lower*) were analyzed as in *A*.

the small NFAT1(1–415) fragment from the nucleus of unstimulated cells suggests that the N-terminal region of NFAT1 is part of a larger protein complex in the cytoplasm of resting cells.

NFAT1(1–415) Is a Substrate for Calcineurin. The nuclear translocation of NFAT1 is preceded by dephosphorylation that is apparent as a mobility change on SDS/polyacrylamide gels (30). In cells this dephosphorylation is calcineurin-dependent since it is blocked by CsA and FK506. Previous work (1, 2, 41, 45) indicated that purified NFAT1, and NFAT1 in cell lysates and immunoprecipitates, is dephosphorylated *in vitro* by calcineurin, but in those studies the *in vitro* dephosphorylated protein bands either were not compared with or did not comigrate precisely with the physiologically dephosphorylated protein. We have therefore examined dephosphorylation of T cell NFAT1 under carefully defined conditions. *In vitro* dephosphorylation of T cell NFAT1 with calcineurin in cell extracts or in immunoprecipitates causes NFAT1 to migrate at a position very similar to that of NFAT1 from ionomycin-stimulated T cells (Fig. 3*A*, lanes 2 and 4, and data not shown). The change in mobility results from dephosphorylation, and not simply from proteolytic cleavage, because it is prevented by inclusion of the general phosphatase inhibitor sodium pyrophosphate in the incubation (lane 5). From these results it is plausible that the same subset of sites is dephosphorylated *in vitro* by calcineurin and in cells, although identification of the individual sites will be required to establish this point.

We have extended this approach to examine the dephosphorylation of NFAT1(1–415) by calcineurin, using recombinant NFAT1(1–415) expressed in COS cells. Control experiments established that full-length recombinant NFAT1 from COS cells migrates slightly slower than T cell NFAT1 on SDS/polyacrylamide gels, consistent with the presence of the HA-tag peptide, and undergoes an increase in mobility similar to that of T cell NFAT1 on dephosphorylation by calcineurin *in vitro* (Fig. 3*B* and data not shown). NFAT1(1–415) expressed and phosphorylated in COS cells is also dephosphorylated by calcineurin (Fig. 3*B*). The dephosphorylation is an effect of calcineurin, because it does not occur in extracts incubated without addition of calcineurin, and it is blocked by CsA–cyclophilin and by FK506–FKBP12, specific inhibitors of calcineurin, and by the general phosphatase inhibitor sodium pyrophosphate. It is not blocked by the protein phosphatase inhibitor okadaic acid, which is ineffective against calcineurin. The results indicate that some or all of the sites in NFAT1 dephosphorylated by calcineurin are within the N-terminal region NFAT1(1–415).

NFAT1(1–415) Binds Calcineurin. Because NFAT1(1–415) is a substrate for calcineurin *in vitro*, and because kinases and phosphatases in many cases interact detectably with their substrates or are tethered to their substrates by a targeting subunit, we have tested whether the N-terminal region of NFAT1 could interact with the calcineurin in cell lysates. A GST-NFAT1(1–415) fusion protein specifically binds calcineurin present in T cell extracts, and causes its retention on glutathione-Sepharose (Fig. 4*A*). Binding is inhibited in the presence of EGTA, indicating a requirement for Ca^{2+}. There is no binding of calcineurin to GST itself, to a fusion protein of GST with LSF [an unrelated DNA-binding protein (38)], or to glutathione-Sepharose or Sepharose alone, even though equal amounts of calcineurin are present in each case (Fig. 4*A*, *lower*). In further experiments, GST-NFAT1(1–415), but not

GST, specifically bound purified calcineurin (Fig. 4*B*), demonstrating that the interaction of calcineurin with GST-NFAT1(1–415) is a direct protein–protein interaction, and does not require other proteins present in cell lysates. As the NFAT1 protein expressed in bacteria is not likely to be phosphorylated, the calcineurin–NFAT1 interaction apparently involves determinants other than simple recognition of a phosphopeptide substrate.

The other NFAT-family protein prominent in mature immune system cells is NFATc. We have expressed the N-terminal region of NFATc in bacteria as a GST-NFATc(1–418) fusion protein, and demonstrated its ability to bind calcineurin in the same assay (Fig. 4*C*). NFAT1 and NFATc differ substantially in the N-terminal ≈100 residues, but have common sequence motifs in the region represented by human NFAT1(110–415), suggesting that the interaction of NFAT1 with calcineurin involves this portion of the protein.

DISCUSSION

We have identified a calcineurin-responsive regulatory region in NFAT1 that controls the subcellular localization of NFAT1 in resting and activated T cells. This regulatory region is distinct from the DNA-binding domain of NFAT1, but includes the NFAT homology region of ≈300 residues immediately N terminal to the DNA-binding domain. Within the NFAT homology region, the KRR (or KRK) candidate NLS and certain other protein motifs are conserved in all NFAT proteins, raising the possibility that the NFAT homology region is a conserved regulatory domain that controls the intracellular localization of NFAT-family proteins in response to the calcium/calcineurin signaling pathway or other signaling pathways.

The binding of calcineurin to the N-terminal portion of NFAT1 and NFATc, and evidence from a different experimental approach that calcineurin can form a complex with phosphorylated NFAT1 extracted from T cells (46, 47), is reminiscent of known targeting interactions involving protein phosphatases. The preferential localization of calcineurin and other serine/threonine phosphatases at specific intracellular locations has been documented (48–50), and in a few cases the protein–protein interactions that target the phosphatases have been identified. For example, the catalytic subunit of protein phosphatase 1, which shows relatively little substrate specificity *in vitro*, is directed toward one set of substrates, the enzymes involved in glycogen metabolism, by a glycogen-binding targeting subunit (49). Targeting subunits directing protein phosphatase 1 to myofibrils and to sarcoplasmic reticulum have also been identified (49). A protein phosphatase 1–retinoblastoma protein interaction has been detected, and may serve either to direct the phosphatase to retinoblastoma protein as a physiological substrate or to target protein phosphatase 1 to specific nuclear sites (51). Calcineurin itself is directed to the IP3 receptor and to the ryanodine receptor by an interaction with FKBP12 (52). And, in neurons, the A-kinase anchor protein AKAP-79 binds both calcineurin and protein kinase A (53), conferring subsynaptic localization and positioning calcineurin to act on its substrate the PKA subunit RII and on other phosphoprotein substrates generated by activation of protein kinase A.

Calcineurin–NFAT1 and calcineurin–NFATc interactions could position calcineurin to dephosphorylate sites on NFAT1, on NFATc, or on associated proteins. One possibility is that the efficient activation of NFAT is ensured by the presence of a preformed calcineurin–NFAT complex in the resting cell. Alternatively, even a transient calcineurin–NFAT interaction could serve to enhance the rate of dephosphorylation, to stabilize the enzyme–substrate complex sufficiently to allow dephosphorylation of NFAT1 at multiple sites, or to ensure that NFAT1 is not rephosphorylated before its interaction with NLS-binding proteins. Whether there is in fact a preformed calcineurin–NFAT1 complex will be determined by the K_d for the calcineurin–NFAT1 binding reaction at resting cytoplasmic Ca^{2+} concentrations, by the intracellular concentration of free calcineurin, and conceivably by the presence of other cellular proteins that stabilize the complex.

There are practical grounds for more detailed study of the calcineurin–NFAT interaction. The nephrotoxic and neurotoxic effects of current immunosuppressive drugs correlate with their ability to inhibit calcineurin, but it is plausible that substrates other than NFAT are the principal physiological targets of calcineurin in kidney and brain. In that case, compounds that interfere selectively with the calcineurin–NFAT1 and calcineurin–NFATc interactions, but that do not inhibit the enzymatic activity of calcineurin against other substrates, would lack the toxic effects of CsA and FK506. Such compounds would be useful not only in the management of transplant rejection, but also in the treatment of asthma, allergy, inflammation, and autoimmune diseases where the toxicity of CsA and FK506 precludes their routine use.

We thank M. Tremblay and D. A. Cantrell for plasmids. This work was supported by National Institutes of Health Grants CA42471 and GM46227 (to A.R.) and GM41292 (to B.A.P. and T. R. Soderling). C.L. and J.A. were supported by Lady Tata Memorial Trust postdoctoral fellowships, K.T.-Y.S. is a postdoctoral fellow of the Medical Research Council of Canada, and F.G.-C. is supported by a fellowship from the Spanish Ministry of Science and Education.

1. McCaffrey, P. G., Perrino, B. A., Soderling, T. R. & Rao, A. (1993) *J. Biol. Chem.* **268,** 3747–3752.
2. Jain, J., McCaffrey, P. G., Miner, Z., Kerppola, T. K., Lambert, J. N., Verdine, G. L., Curran, T. & Rao, A. (1993) *Nature (London)* **365,** 352–355.
3. McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain, J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. L., Curran, T., Verdine, G. L., Rao, A. & Hogan, P. G. (1993) *Science* **262,** 750–754.
4. Rao, A. (1994) *Immunol. Today* **15,** 274–281.
5. Crabtree, G. R. & Clipstone, N. A. (1994) *Annu. Rev. Biochem.* **63,** 1045–1083.
6. Jain, J., Loh, C. & Rao, A. (1995) *Curr. Opin. Immunol.* **7,** 333–342.
7. Northrop, J. P., Ho, S. N., Thomas, D. J., Chen, L., Timmerman, L., Nolan, G. P., Admon, A. & Crabtree, G. R. (1994) *Nature (London)* **369,** 497–502.
8. Masuda, E. S., Naito, Y., Tokumitsu, H., Campbell, D., Saito, F., Hannum, C., Arai, K.-I. & Arai, N. (1995) *Mol. Cell. Biol.* **15,** 2697–2706.
9. Hoey, T., Sun, Y.-L., Williamson, K. & Xu, X. (1995) *Immunity* **2,** 461–472.
10. Ho, S. N., Thomas, D. J., Timmerman, L. A., Li, X., Francke, U. & Crabtree, G. R. (1995) *J. Biol. Chem.* **270,** 19898–19907.
11. Jain, J., Burgeon, E., Badalian, T. M., Hogan, P. G. & Rao, A. (1995) *J. Biol. Chem.* **270,** 4138–4145.
12. Nolan, G. P. (1994) *Cell* **77,** 795–798.
13. Schreiber, S. L. & Crabtree, G. R. (1992) *Immunol. Today* **13,** 136–142.
14. Liu, J. (1993) *Immunol. Today* **14,** 290–295.
15. Tocci, M. J., Matkovitch, D. A., Collier, K. A., Kwok, P., Dumont, F., Lin, S., DeGudicibus, S., Siekierka, J. J., Chin, J. & Hutchinson, N. I. (1989) *J. Immunol.* **143,** 718–726.
16. Kaye, R. E., Fruman, D. A., Bierer, B. E., Albers, M. W., Zydowsky, L. D., Ho, S. I., Jin, Y.-J., Castells, M. C., Schreiber, S. L., Walsh, C. T., Burakoff, S. J., Austen, K. F. & Katz, H. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8542–8546.
17. Goldfeld, A. E., Tsai, E., Kincaid, R., Belshaw, P. J., Schreiber, S. L., Strominger, J. L. & Rao, A. (1994) *J. Exp. Med.* **180,** 763–768.
18. Aramburu, J., Azzoni, L., Rao, A. & Perussia, B. (1995) *J. Exp. Med.* **182,** 801–810.
19. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I. & Schreiber, S. L. (1991) *Cell* **66,** 807–815.

20. Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Alberg, D. G., Belshaw, P. J., Cohen, P., MacKintosh, C., Klee, C. B. & Schreiber, S. L. (1992) *Biochemistry* **31,** 3896–3901.
21. Mattila, P. S., Ullman, K. S., Fiering, S., Emmel, E. A., McCutcheon, M., Crabtree, G. R. & Herzenberg, L. A. (1990) *EMBO J.* **9,** 4425–4433.
22. Flanagan, W. M., Corthesy, B., Bram, R. J. & Crabtree, G. R. (1991) *Nature (London)* **352,** 803–807.
23. Brabletz, T., Pietrowski, I. & Serfling, E. (1991) *Nucleic Acids Res.* **19,** 61–67.
24. Choi, M. S. K., Brines, R. D., Holman, M. J. & Klaus, G. G. B. (1994) *Immunity* **1,** 179–187.
25. Venkataraman, L., Francis, D. A., Wang, Z., Liu, J., Rothstein, T. L. & Sen, R. (1994) *Immunity* **1,** 189–196.
26. Hutchinson, L. E. & McCloskey, M. A. (1995) *J. Biol. Chem.* **270,** 16333–16338.
27. O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J. & O'Neill, E. A. (1992) *Nature (London)* **357,** 692–694.
28. Clipstone, N. A. & Crabtree, G. R. (1992) *Nature (London)* **357,** 695–697.
29. Fruman, D. A., Pai, S.-Y., Burakoff, S. J. & Bierer, B. E. (1995) *Mol. Cell. Biol.* **15,** 3857–3863.
30. Shaw, K. T.-Y., Ho, A. M., Raghavan, A., Kim, J., Jain, J., Park, J., Sharma, S., Rao, A. & Hogan, P. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11205–11209.
31. Mizushima, S. & Nagata, S. (1990) *Nucleic Acids Res.* **18,** 5322.
32. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382.
33. Nardone, J., Gerald, C., Rimawi, L., Song, L. & Hogan, P. G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4412–4416.
34. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
35. Perrino, B. A., Ng, L. Y. & Soderling, T. R. (1995) *J. Biol. Chem.* **270,** 340–346.
36. Ho, A. M., Jain, J., Rao, A. & Hogan, P. G. (1994) *J. Biol. Chem.* **269,** 28181–28186.
37. Forsayeth, J. R. & Garcia, P. D. (1994) *BioTechniques* **17,** 354–359.
38. Shirra, M. K., Zhu, Q., Huang, H.-C., Pallas, D. & Hansen, U. (1994) *Mol. Cell. Biol.* **14,** 5076–5087.
39. Valge-Archer, V. E., de Villiers, J., Sinskey, A. J. & Rao, A. (1990) *J. Immunol.* **145,** 4355–4364.
40. Li, X., Ho, S. N., Luna, J., Giacalone, J., Thomas, D. J., Timmerman, L. A., Crabtree, G. R. & Francke, U. (1995) *Cytogenet. Cell Genet.* **68,** 185–191.
41. Ruff, V. A. & Leach, K. L. (1995) *J. Biol. Chem.* **270,** 22602–22607.
42. Paine, P. L., Moore, L. C. & Horowitz, S. B. (1975) *Nature (London)* **254,** 109–114.
43. Peters, R. (1986) *Biochim. Biophys. Acta* **864,** 305–359.
44. Bonner, W. M. (1975) *J. Cell Biol.* **64,** 421–430.
45. Park, J., Yaseen, N. R., Hogan, P. G., Rao, A. & Sharma, S. (1995) *J. Biol. Chem.* **270,** 20653–20659.
46. Wesselborg, S., Fruman, D. A., Sagoo, J. K., Bierer, B. E. & Burakoff, S. J. (1996) *J. Biol. Chem.* **271,** 1274–1277.
47. Loh, C., Shaw, K. T.-Y., Carew, J., Viola, J. P. B., Luo, C. & Rao, A. (1996) *J. Biol. Chem.* **271,** 10884–10891.
48. Klee, C. B., Draetta, G. F. & Hubbard, M. J. (1988) *Adv. Enzymol.* **61,** 149–200.
49. Hubbard, M. J. & Cohen, P. (1993) *Trends Biochem. Sci.* **18,** 172–177.
50. Sontag, E., Nunbhakdi-Craig, V., Bloom, G. S. & Mumby, M. C. (1995) *J. Cell Biol.* **128,** 1131–1144.
51. Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H. & Elledge, S. J. (1993) *Genes Dev.* **7,** 555–569.
52. Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V. & Snyder, S. H. (1995) *Cell* **83,** 463–472.
53. Coghlan, V. M., Perrino, B. A., Howard, M., Langeberg, L. K., Hicks, J. B., Gallatin, W. M. & Scott, J. D. (1995) *Science* **267,** 108–111.
54. Luo, C., Burgeon, E., Carew, J. A., McCaffrey, P. G., Badalian, T. M., Lane, W. S., Hogan, P. G. & Rao, A. (1996) *Mol. Cell. Biol.* **16,** 3955–3966.
55. Loh, C., Carew, J. A., Kim, J., Hogan, P. G. & Rao, A. (1996) *Mol. Cell. Biol.* **16,** 3945–3954.