Direct Demonstration of NFAT<sub>p</sub> Dephosphorylation and Nuclear Localization in Activated HT-2 Cells Using a Specific NFAT<sub>p</sub> Polyclonal Antibody*

Valerie A. Ruff and Karen L. Leach‡
From the Department of Cell Biology and Inflammation Research, The Upjohn Company, Kalamazoo, Michigan 49001.

Nuclear factor of activated T cells (NFAT) regulates transcription of a number of cytokine genes, and NFAT DNA binding activity is stimulated following T cell activation. Several lines of evidence have suggested that NFAT is a substrate for calcineurin, a serine/threonine phosphatase. Using a polyclonal antibody to murine NFAT is a substrate for calcineurin, a serine/threonine phosphatase. Western blot analysis of various mouse tissues demonstrated that the 110–130-kDa NFAT<sub>p</sub> protein was highly expressed in thymus and spleen. Treatment of immunoprecipitated NFAT<sub>p</sub> from untreated HT-2 cells with calcineurin resulted in the dephosphorylation of NFAT<sub>p</sub>, demonstrating that NFAT<sub>p</sub> is an in vitro substrate for calcineurin. NFAT<sub>p</sub> immunoprecipitated from <sup>32</sup>P-labeled HT-2 cells migrated as an approximately 120-kDa protein that was localized to the cytosol of the cells. Treatment of the cells with ionomycin resulted in a decrease in the molecular weight of NFAT<sub>p</sub> and a loss of <sup>32</sup>P, consistent with NFAT<sub>p</sub> dephosphorylation. The dephosphorylation of NFAT<sub>p</sub> was accompanied by localization of the protein to the nuclear fraction. Both of these events were blocked by preincubation of the cells with FK506, a calcineurin inhibitor, consistent with the hypothesis that NFAT<sub>p</sub> is a calcineurin substrate in cells.

The immunosuppressant drugs cyclosporin A (CsA) and FK506 inhibit the early steps of antigen-stimulated T cell activation. These drugs prevent activation of a number of cytokine genes, including IL-2, granulocyte-macrophage colony-stimulating factor, IL-4, and tumor necrosis factor α (for review, see Refs. 1 and 2). IL-2 plays a key role in controlling T cell proliferation, and consequently, numerous studies have focused on understanding IL-2 gene regulation. The results of these studies demonstrated that transcriptional activation requires nuclear factor of activated T cells (NFAT), a DNA-binding protein, which binds to specific sites on the regulatory regions of the cytokine genes (3–6).

Molecular cloning and biochemical studies have provided insights into the actions of NFAT. The approximately 120-kDa NFAT is a member of a gene family whose members appear to contain a Rel homology region, which is important for DNA binding activity. Two members were identified: NFAT<sub>c</sub> was cloned from a human T cell library (7), while NFAT<sub>p</sub> was purified and cloned from murine T cells (8). In addition, several alternatively spliced forms of NFAT<sub>p</sub> were identified (2). NFAT<sub>c</sub> and NFAT<sub>p</sub> are approximately 73% identical in the Rel homology region, although they share little similarity outside that domain. In unactivated T cells, NFAT DNA binding activity is localized primarily in the cytosol, and following T cell activation, activity is detected in the nucleus. Nuclear NFAT forms a complex with Fos and Jun, and mutations in NFAT that inhibit binding to these proteins eliminate NFAT-mediated gene transcription. Furthermore, treatment of cells with either CsA or FK506 inhibits the appearance of NFAT binding activity in the nucleus (3, 9–11).

An understanding of the link between the drugs FK506 and CsA and NFAT has recently started to emerge with the identification of calcineurin (CaN) as a cellular target of the drugs. FK506 and CsA bind to their respective immunophilins, FKBP12 and cyclophilin A, and the drug-immunophilin complexes bind to and inhibit the activity of CaN, a calmodulin-dependent phosphatase (12, 13). Overexpression of a constitutively active form of CaN in T cells renders the cells more resistant to the effects of the drugs, and the ability of a number of FK506 and CsA analogues to inhibit the phosphatase activity of CaN correlates with their ability to inhibit T cell activation (14).

Evidence suggests that CaN is not just involved in mediating the actions of FK506 and CsA but that the enzyme plays a role in the physiological pathway of T cell activation. O'Keefe, et al. (15) demonstrated that in cells transfected with CaN, PMA- and ionophore-stimulated IL-2-driven gene expression was stimulated approximately 2-fold. Furthermore, in cells overexpressing a constitutively active form of CaN, IL-2-driven gene expression was stimulated approximately 150-fold in the presence of PMA alone, indicating that active CaN can replace the usual requirement for ionophore. Similar results were obtained by Clipstone and Crabtree (16), who demonstrated reporter gene activity in CaN-transfected cells at suboptimal concentrations of ionomycin that elicited little or no activity in control cells. Taken together, these results demonstrate that CaN overexpression augments T cell activation resulting from both calcium and PKC stimulation and suggest that CaN plays a key role in this process.

The studies with CsA and FK506 led to the suggestion that NFAT is a CaN substrate, either direct or indirect, in T cells. First, indirect evidence indicates that NFAT<sub>c</sub> is a phosphoprotein. Fractionation of Jurkat cell cytosol demonstrated that NFAT DNA binding activity is detected with proteins in a molecular mass range of 94–116 kDa, suggesting heterogeneity in the size of the NFAT protein (17). To determine whether...
NFAT phosphorylation could explain the heterogeneity, McCaffrey, et al. (18) treated cell extracts enriched for NFAT with alkaline phosphatase. This enzymatic treatment resulted in a shift in NFAT binding activity from the 127–143-kDa molecular weight fraction to a 101–113-kDa fraction, which is consistent with the hypothesis that NFAT is a phosphoprotein. Second, treatment of purified NFAT with purified bovine brain CaN also resulted in a shift in the molecular weight of NFAT, suggesting that NFAT is in vitro substrate for CaN (19). Whether NFAT is a direct CaN substrate in intact cells has not yet been determined, however.

In the majority of reported NFAT studies, NFAT DNA binding activity has been the end point usually measured, since relatively little is known about the NFAT protein. However, the recent cloning and expression of NFAT genes has yielded valuable information about the proteins, and direct protein analyses should now be possible. Towards that end, antibodies to NFAT will be important reagents to develop for probing protein structure and function. In the studies reported here, we developed a peptide antibody to NFATp and used it to characterize NFATp in cells. We demonstrate directly in 32P labeling experiments that NFATp is a phosphoprotein and that T cell activation results in NFATp dephosphorylation that can be blocked by pretreatment with FK506. Furthermore, we show that dephosphorylation is accompanied by translocation of NFATp protein from the cytosol to the nucleus of cells.

**EXPERIMENTAL PROCEDURES**

Materials—FK506 and rapamycin were prepared at the Upjohn Company from fermentation broths of Streptomyces tsukubaensis (Upjohn Culture Collection 11052) and Streptomyces hygroscopicus (Upjohn Collection 9931), respectively. CaN and calmodulin were obtained from Sigma, and PP2a was from UBI. [32P]Orthophosphate was obtained from DuPont NEN. Protein A-Sepharose CL-4B beads were from Pharmacia Biotech Inc. [35S]Methionine and ECL reagents were obtained from Amersham Corp. Tyrosine phosphatase PTP1 was a gift from Dr. John Bleasdale (The Upjohn Company). CaN autoinhibitory peptide (22) was synthesized at The Upjohn Company.

Cell Culture—The human T lymphoblastoid cell lines J SD9 and HSB and the murine T lymphoma cell line EL-4 were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. The murine helper T cell line HT-2 was grown in the medium just described with the addition of 12% rat T-STAT with concanavalin A as a source of IL-2 (Collaborative Biomedical Products). 50 μM β-mercaptoethanol, and 10 mM HEPES, pH 7.4. Human peripheral blood lymphocytes were prepared from leukophoresis packs. Cells were centrifuged over Ficol, and cells at the interface were collected, diluted with RPMI, and pelleted. The cell pellet was suspended in RPMI containing 10% fetal calf serum and incubated for 1 h at 37 °C. Nonadherent cells were collected, pelleted, and stored at −70 °C.

Antibody Production—The amino acids CCG were added to the amino terminus of the sequence LSPGAYPTTVIQQOTTAPSQR corresponding to peptide 25 of NFATp (8). The cysteine residue was coupled to maleimide-activated KLH using the Imject Immungun Conjugation Kit (Pierce), and rabbits were injected. Sera was tested against purified peptide, and bleeds determined to have the highest titer were affinity-coupled in the Fig. 3 interface were collected, diluted with RPMI, and pelleted. The cell pellet was suspended in RPMI containing 10% fetal calf serum and incubated for 1 h at 37 °C. Nonadherent cells were collected, pelleted, and stored at −70 °C.

Tissue Homogenates—Homogenates were prepared from organs dissected from BALB/C mice as described in Ref. 20 using a Polytron homogenizer. The tissues were homogenized at 0.3 g wet weight/ml in 10 mM Tris, pH 7.5, containing 150 mM NaCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 100 μg/ml aprotinin and soybean trypsin inhibitor, 250 μg leupeptin, 10 μg iodoacetamide, and 2 μg phenylmethylsulfonlfuoride. SDS was added to bring the final concentration to 2%. The sample was heated at 100 °C for 10 min and then centrifuged for 10 min in a microfuge. The supernatant was removed, and protein was determined by the microassay method of Bradford (23) using reagents from Bio-Rad.

Cellular Fractionation—Treatment of HT-2 cells (1 × 10⁶ cells/condition) was carried out as described in the figure legends. The cells were then washed in ice-cold PBS and resuspended (2.5 × 10⁶ cells/ml) in buffer (10 mM Tris-Cl, pH 7.5, containing 10 mM NaCl, 3 mM MgCl₂, 1 mM phenylmethylsulfonlfuoride, 0.5 mM dithiothreitol, 0.1 mM EDTA, 2 μg leupeptin, 1 μg/ml aprotinin, 0.05% Nonidet P-40). Cells were centrifuged at 650 × g for 10 min and then resuspended in 40 mM Tris, pH 7.5, containing 10 mM EDTA, and 60 mM sodium pyrophosphate. SDS was added to bring the final concentration to 5%. The sample was boiled for 10 min and then centrifuged at 10,000 × g for 15 min. The supernatant was washed in ice-cold PBS, and the final pellet was resuspended in 1% SDS, 5% glycerol, 50 μg/ml aprotinin, 0.05% Nonidet P-40 and incubated at 37 °C for 30 min. The sample was resolved in Laemmli buffer (100 mM NaCl, 6.25% SDS, followed by a final wash in radioimmune precipitation buffer containing 0.3% SDS, followed by a final wash in radioimmune precipitation buffer. The sample was resuspended in Laemmli buffer and electrophoresed on a 6% SDS-polyacrylamide gel followed by autoradiography. The autoradiogram was digitized on a Molecular Dynamics PhosphorImager using ImageQuant Software.

Immunocomplex Assays—Unlabeled cells (1.5 × 10⁶ cells/ml) were untreated or stimulated for 10 min with 2 μg/ml ionomycin, immunoprecipitated as described above, and washed five times in assay buffer (100 mM Tris, pH 7.5, containing 500 mM NaCl, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 mM MgCl₂, and 0.5 mM MnCl₂). The immunoprecipitate from untreated cells was incubated in assay buffer containing the enzymes and inhibitors indicated in the Fig. 3B legend in a final volume of 100 μl for 30 min at 30 °C. 50 μl of Laemmli buffer was added, the sample was boiled, and the Sepharose beads were pelleted. The supernatant was electrophoresed on a 6% SDS-polyacrylamide gel, and Western blotting was carried out as above.

Phosphatase Assays—The phosphatase activity of CaN and PP2A was measured in a total volume of 100 μl containing 40 mM Tris, pH 8.6, 100 mM NaCl, 0.5 mM CaCl₂, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 10 mM MgCl₂, 600 μM 4-methylumbelliferyl phosphate and 1 mM EDTA containing 50 or 0.1 units of CaN or NAT-1. The reaction was started by the addition of 0.0015 of CaN. To measure CaN activity, assays also included 100 mM calmodulin and the presence or absence of 100 μM inhibitory peptide (22). The mixture was incubated for 1 h at 37 °C following the addition of 50 μl of stop buffer (300 mM glycine, pH 11.2, containing 15 mM EDTA), and the fluorescence was read at 365/405 nm.

[35S]Methionine Pulse-Chase—Cells were resuspended at 3 × 10⁵ cells/ml in growth medium containing 2% of the usual methionine plus 10% dialyzed fetal calf serum, in the presence or absence of 2 μg/ml ionomycin. [35S]Methionine was added at 175 μCi/ml.
for 5 min, and 1.5 x 10^6 cells were removed for the zero time point. The remainder of the cells were washed in PBS and resuspended in normal growth medium in the presence or absence of 2 μM ionomycin. At the indicated times, aliquots (1.5 x 10^6 cells) were removed, lysed, and immunoprecipitated with the NFATp antibody as described above. The autoradiograph was quantitated as described above.

α[35S]Methionine Degradation—Cells were resuspended at 6 x 10^6 cells/ml in low methionine-containing medium plus [α-35S]methionine (50 μCi/ml) and incubated for 18 h. The cells were washed in PBS and resuspended at 3 x 10^6 cells/ml in normal growth medium containing Me2SO (control), 2 μM ionomycin, or 100 nM FK506 plus 2 μM ionomycin. Cells were pretreated with FK506 5 min prior to the addition of ionomycin. Cells (1.5 x 10^6) were removed immediately (zero time point) or at the indicated times, and the lysates were immunoprecipitated as described above. Autoradiographs were quantitated as described above and analyzed by linear regression to determine the NFATp half-life.

RESULTS

A polyclonal antibody was developed against a peptide from the COOH-terminal domain of NFATp. This peptide is outside the Rel homology domain and is from a unique region of NFATp that is not present in NFATc (7). Therefore, this antibody is a specific reagent for characterizing the NFATp protein.

Western blot analysis was carried out in order to determine the expression of NFATp in various mouse tissues (Fig. 1A). The highest level of NFATp expression was observed in spleen and thymus tissue. In thymus, the antibody strongly hybridized with proteins in the 110–130-kDa molecular mass range, suggesting protein heterogeneity, while in spleen tissue, reactivity was predominantly against a 110-kDa protein. In both these tissues, reactivity with smaller proteins of 72, 66, and 59 kDa was observed. It is unknown whether these proteins are proteolytic fragments of the larger NFATp protein. However, in competition experiments in which the NFATp peptide was included in the antibody incubation, no reactivity was observed, indicating the specificity of the interactions (lanes 8 and 9). In kidney and liver, there was little or no antibody reactivity against proteins in the 120-kDa range. Cross-reactivity against proteins of 66 and 72 kDa was shared among kidney, spleen, and thymus, although interestingly, in spleen and thymus, reactivity against the 66-kDa protein was predominant, while in kidney, antibody reactivity was greater against the 72-kDa protein, compared with the 66-kDa protein. Expression of NFATc was very low in brain, while in heart no NFATp was detected.

The high level of NFATp expression in spleen and thymus is consistent with previous reports demonstrating NFAT DNA binding activity in T cells. Various T cell lines were profiled to investigate whether NFATp levels differ among T cells (Fig. 1B). Peripheral blood lymphocytes had the lowest level of NFATp expression (lane 4). These results could not simply be explained by a lack of reactivity of the murine peptide-derived antibody against human protein, since human T cell Jurkat cells showed significant reactivity with the antibody (lane 1). HSB cells, a human T cell line, also expressed the approximately 140-kDa NFATp (lane 2). In EL-4 cells, a mouse thymoma cell line, NFATp was expressed, but the protein was more heterogeneous, since proteins from 120 to 140 kDa acted with the antibody (lane 3). HT-2 cells, an IL-2-dependent mouse T cell line, had the highest level of NFATp expression compared with the other cell lines (lane 5). In the experiment shown in Fig. 1B, lysate from 2.5 x 10^6 HT-2 cells resulted in a comparable or greater signal, compared with the other cell lines, in which 4-fold more cell equivalents (1 x 10^6) were used. In all of the cell lines, cross-reactivity with a 66-kDa protein was seen, suggesting, as in the tissue samples, that other NFATp protein forms may be expressed. Competition experiments using NFATp peptide and EL-4 or HT-2 cell extracts (lanes 6 and 7) demonstrated that all the reactivity could be competed by excess peptide. No NFATp protein was detected in MOLT-4 cells, a human T cell leukemia line or in NIH 3T3 cell fibroblasts (data not shown).

Activation of T cells by treatment with PMA and calcium ionophore stimulates NFAT DNA binding activity (2). The NFATp antibody was used to investigate directly the effect of T cell activation on NFAT protein levels (Fig. 2A). For these and subsequent experiments, HT-2 cells were used, due to their high level of expression of NFATp. NFATp migrated as an approximately 120-kDa protein in untreated cells. Treatment of the cells with either ionomycin alone (lane 2) or the combination of PMA plus ionomycin (data not shown), resulted in a decrease in the molecular weight of NFATp, suggestive of a proteolytic and/or dephosphorylation event. Identical shifts in the NFATp protein were observed when the cell treatments and lysate preparation were carried out in the presence of a panel of protease inhibitors, including leupeptin, phenylmethylsulfonyl fluoride, and 1-chloro-3-tosylamido-7-amino-2-heptanone (data not shown), suggesting that the mobility shift was not the result of proteolysis. The predominant NFATp protein band in the ionomycin-treated cells was approximately 110 kDa, although the protein was heterogeneous in size. Pretreatment of the cells with 500 nM FK506 blocked the ionomycin-induced shift in NFATp, suggesting a role for CaN. As a control, cells were pretreated with rapamycin prior to ionomycin treat-
NFAT<sub>p</sub> Dephosphorylation and Nuclear Localization

**Fig. 2.** Ionomycin treatment of HT-2 cells results in NFAT<sub>p</sub> dephosphorylation. A and B, HT-2 cells were treated and lysates were prepared for Western blotting (A), or NFAT<sub>p</sub> was immunoprecipitated from ³²P-labeled cells (B) as described under "Experimental Procedures." Lane 1, control; lane 2, 2 µM ionomycin for 10 min; lane 3, 500 nM FK506 pretreatment (1 h) followed by 2 µM ionomycin for 10 min; lane 4, 1 µM rapamycin pretreatment (1 h) followed by ionomycin for 10 min.

**Fig. 3.** Immunocomplex phosphatase assay and phosphotyrosine blot. A, immunocomplex phosphatase assay. Immunoprecipitates from untreated cells (lane 1) and ionomycin-treated cells (lane 2) are shown. Immunoprecipitates were prepared from untreated cells as described under "Experimental Procedures" and incubated with the following: 50 nM CaN plus 200 nM calmodulin (lane 3), 50 nM CaN, 200 nM calmodulin and 500 µM autoinhibitory peptide (lane 4), 0.1 units PP2a (lane 5), PP2a plus 500 nM okadaic acid (lane 6), PTP1 (lane 7), and 50 nM CaN and 200 nM calmodulin (lane 8), developed with second antibody alone. The thick arrow points to the phosphorylated form of NFAT<sub>p</sub>, and the thin arrow points to the dephosphorylated form. B, phosphotyrosine blot. Lane 1, HT-2 cell lysate; lane 2, A431 cell lysate. The ~170-kDa epidermal growth factor receptor is shown in lane 2 as a positive control for the phosphotyrosine antibody.

To determine whether the changes in NFAT<sub>p</sub> mobility were the result of ionomycin-induced dephosphorylation, ³²P labeling experiments were carried out (Fig. 2B). Cells were labeled with [³²P]orthophosphate for 4 h followed by treatments similar to those shown in Fig. 2A, and immunoprecipitation was carried out with the NFAT<sub>p</sub> antibody. A similar decrease in molecular weight was observed in the ³²P-labeled NFAT<sub>p</sub> following ionomycin treatment as was observed in the Western blot experiments. Quantitation of the ³²P autoradiograms showed that ionomycin treatment resulted in an approximately 65% loss in ³²P from NFAT<sub>p</sub> (data not shown). Interestingly, ionomycin treatment never resulted in a complete loss of ³²P from NFAT<sub>p</sub>, indicating that complete dephosphorylation did not occur. In addition, FK506 inhibited the loss of ³²P, while rapamycin had no effect. The results of these experiments demonstrate directly that the shift in molecular weight following ionomycin treatment was the result of NFAT<sub>p</sub> dephosphorylation and that FK506 pretreatment blocked the dephosphorylation.

A role for CaN in the ionomycin-stimulated dephosphorylation of NFAT<sub>p</sub> was shown directly in immune complex assays (Fig. 3). NFAT<sub>p</sub> was immunoprecipitated from untreated HT-2 cells, and the immunoprecipitates were washed and incubated with CaN. CaN dephosphorylated NFAT<sub>p</sub> in vitro, as shown by a decrease in the NFAT<sub>p</sub> molecular weight, which was comparable to the molecular weight shift resulting from treatment of cells with ionomycin [compare lanes 2 and 3]. CaN contains an autoinhibitory domain in its COOH-terminal domain, and a peptide from this region inhibits CaN dephosphorylation of a cAMP-dependent protein kinase peptide in vitro (Ref. 22 and data not shown). Addition of the autoinhibitory peptide to the immune complex assay blocked the dephosphorylation of NFAT<sub>p</sub> by CaN (lane 4). In contrast, treatment of the precipitated NFAT<sub>p</sub> with another serine/threonine phosphatase, PP2A, resulted in little or no dephosphorylation of NFAT<sub>p</sub> (lane 5), although the enzyme readily dephosphorylated 4-methylumbelliferyl phosphate (data not shown). The tyrosine phosphatase PTP1 also did not dephosphorylate NFAT<sub>p</sub> (lane 7), suggesting that NFAT<sub>p</sub> may not contain phosphorylated tyrosine residues. Consistent with this hypothesis was the observation that there was no reactivity on Western blots of antiphosphotyrosine antibodies with NFAT<sub>p</sub> (Fig. 3B). Taken together, these results indicate that NFAT<sub>p</sub> readily serves as an in vitro substrate for CaN.

Previous studies demonstrated that activation of T cells is accompanied by an increase in nuclear NFAT DNA binding activity. Using the NFAT<sub>p</sub> antibody, we showed directly the presence of the NFAT<sub>p</sub> protein in the nucleus of stimulated cells (Fig. 4A). In untreated cells, all of the NFAT<sub>p</sub> protein was detected in the low speed supernatant (lane 1). Following a 10-min ionomycin treatment, however, the lower molecular weight, dephosphorylated form of NFAT<sub>p</sub> was predominantly localized to the nuclear fraction (lane 5). Pretreatment of cells with FK506 prior to ionomycin stimulation blocked the dephosphorylation, as previously observed, and also blocked the translocation of NFAT<sub>p</sub> to the nucleus (compare lanes 3 and 6).

The time course for NFAT<sub>p</sub> dephosphorylation and nuclear localization was examined in more detail (Fig. 4B). Fractionation of cells immediately following the addition of ionomycin indicated that NFAT<sub>p</sub> dephosphorylation was rapid (lane 4), as shown by the ladder of protein bands. After ionomycin treatment for 10 min, the dephosphorylated NFAT<sub>p</sub> was present in both the low speed supernatant and the nuclear fractions (lane 5), suggesting that NFAT<sub>p</sub> is dephosphorylated in the cytosol, followed by localization of the dephosphorylated form to the nucleus. With increasing time of incubation with ionomycin, dephosphorylated NFAT<sub>p</sub> was present almost exclusively in the nuclear fraction, although a small level of dephosphorylated NFAT<sub>p</sub> was detected in the supernatant throughout the 4-h time course. These results suggest that NFAT<sub>p</sub> is rapidly dephosphorylated and localized to the nucleus following ionomycin treatment and that the dephosphorylated NFAT<sub>p</sub> remains in the nucleus in the continuing presence of ionomycin. A similar localization experiment was carried out with cells pretreated with ionomycin, followed by removal of the ionomycin (Fig. 4C). At the start of the experiment, following a 10 min treatment with ionomycin, essentially all of the NFAT<sub>p</sub> was localized to the nucleus, and the nuclear NFAT<sub>p</sub> was in the...
Migration from the nucleus was accompanied by rapid rephosphorylation of the higher molecular weight, phosphorylated form, suggesting that following washout of the ionomycin, NFATp was still in the lower molecular weight, dephosphorylated form. After 10 min following washout time course. For the experiment shown in panel C, cells were stimulated with 2 μM ionomycin, and the zero time aliquot (1 × 10^6 cells) was removed immediately after the addition of ionomycin. For the experiment shown in panel B, cells were stimulated with 2 μM ionomycin for 10 min. The cells were washed in PBS and resuspended at 2 × 10^6 cells/ml in normal growth medium, and an aliquot (1 × 10^6 cells) was removed immediately for the zero time point. Lanes 1 and 2 contain lysate prepared from 2.5 × 10^6 cells from untreated or ionomycin-treated cells, respectively, as controls. Supernatant (upper part) and nuclei (lower part) are shown from untreated cells (lane 3) and cells treated with ionomycin for the indicated times: 0 time (lane 4), 10 min (lane 5), 30 min (lane 6), 1 h (lane 7), 2 h (lane 8), 3 h (lane 9), and 4 h (lane 10).

Loss of NFATp from the nuclear fraction could result from NFATp degradation. To investigate this possibility, [35S]methionine labeling experiments were carried out to measure the half-life of the NFATp protein. Cells were labeled overnight with [35S]methionine and then chased in medium containing unlabeled methionine. The rate of loss of immunoprecipitated NFATp was determined for untreated, ionomycin-treated, and FK506- and ionomycin-treated cells, and is shown as a semi-log plot (Fig. 5A). In untreated cells the t1/2 for NFATp was 16.9 ± 0.86 h (mean ± S.E., n = 3), which was similar to the value obtained from cells treated with the combination of FK506 and ionomycin (19.2 ± 3.9 h). Treatment with ionomycin, however, caused a slight decrease in the time required for loss of 50% of the prelabeled NFATp, to 11.9 ± 2.7 h. These results indicate that the loss of NFATp from the nucleus was not the result of degradation of the protein and support the hypothesis that nuclear NFATp reappears in the cytosol following ionomycin removal.

During short labeling times, the major NFATp was in the dephosphorylated form (Fig. 5B). However, this form was rapidly chased into the higher molecular weight, phosphorylated form of NFATp, suggesting that phosphorylation of NFATp occurs very rapidly following synthesis.

**DISCUSSION**

Much of the available information concerning NFAT is the result of studies of NFAT DNA binding activity (1–3, 9, 11). More recently, several forms of NFAT have been cloned, and Northern analysis demonstrated that NFAT mRNA levels vary among different tissues and under stimulus conditions (7). Investigations with a specific NFATp antibody allowed expansion of these studies to include direct analysis of the NFATp protein. The peptide sequence used to generate the antibody described here is derived from the COOH-terminal region of NFATp, which is not present in NFATc, and thus the antibody
differentiates between the two NFATp forms. The NFATp protein was highly expressed in thymus and spleen tissue, which is consistent with mRNA analysis (7). However, Northrup et al. (7) reported that NFATp mRNA levels were similar in brain, heart, thymus, and spleen, while our immunoblotting results (Fig. 1) showed that no NFATp protein was detected in heart tissue, and only very low levels in brain. These results suggest that the presence of NFATp mRNA may not predict the expression of the protein in all tissues or cells. In addition, lower molecular weight proteins reacted with the NFATp antibody, suggesting that other forms of NFATp may not predict the expression of the protein in all tissues or cells. The results of the localization experiments suggest that the phosphorylation state of NFATp may be a determinant of its localization within the cell. In untreated cells, NFATp was found in the cytosolic and nuclear fractions, while at later time points the phosphorylated form was exclusively localized to the nucleus. As long as ionomycin was present, NFATp remained both phosphorylated and dephosphorylated in the nucleus. Previous studies demonstrated that NFATp DNA binding activity is localized to the cytosol of untreated T cells, and to the nuclear fraction in stimulated cells (3). The results shown here demonstrate directly that the shift in DNA binding activity seen by others reflects a change in the cellular location of the NFATp protein.

Following ionomycin removal, NFATp reappeared in the cell supernatant fraction, in the higher molecular weight, phosphorylated form. The [35S]methionine labeling experiments demonstrated that NFATp is a relatively stable protein, with a half-life of approximately 17 h in untreated cells and approximately 12 h in ionomycin-treated cells. Thus, the loss of signal from the nucleus following ionomycin removal cannot be explained by degradation of NFATp. These results suggest that NFATp may shuttle in and out of the nucleus, depending on the activation state of the cell as well as the phosphorylation state of the protein. A potential nuclear localization sequence has been identified within the Rel homology region of NFATp (23). There are a number of potential phosphorylation sites in NFATp, including one within the nuclear localization sequence. One possibility is that activation-induced dephosphorylation of NFATp results in an unmasking of the nuclear localization sequence, leading to nuclear localization of the protein. The immunocomplex assay demonstrated directly that NFATp is a CaN substrate in vitro. Furthermore, the CaN-induced molecular weight shift in NFATp was similar to that resulting from ionomycin treatment of cells. Treatment of intact cells with FK506, a CaN inhibitor, blocks the ionomycin-induced dephosphorylation, which further supports but does not prove the hypothesis that NFATp is a CaN substrate in intact cells.

In general, no consensus sequence for dephosphorylation of substrates by CaN has been identified. However, Donella-Deana et al. (24) have demonstrated in phosphopeptide studies that basic residues on the NH2-terminal side of the phosphoamino acid, especially at the –3 position, are positive determinants for dephosphorylation by CaN, while acidic residues on the COOH-terminal side are negative determinants. Because of a lack of a strong consensus sequence, these results and the results of others (25, 26) have led to the suggestion that more complex protein structural determinants also play a role in defining CaN substrate specificity. Taken together, these results suggest that a number of sites within NFATp may be sites for CaN dephosphorylation. Mapping these sites in vitro and comparing them with the sites of dephosphorylation in intact cells is required to establish whether NFATp is a direct substrate for CaN in intact cells. The results of such experiments will define in more detail the exact role of CaN in T cell activation.

Acknowledgments—We acknowledge Dr. Anjana Rao for the gift of Ar-S extracts, Kay Petruska for secretarial assistance, and Dr. Clark Smith and Carol Bannow for peptide synthesis.

REFERENCES

1. Liu, J. (1993) Immuno. Today 14, 299–305
2. Rao, A. (1994) Immuno. Today 15, 274–281
3. Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E., and Crabtree, G. R. (1989) Science 246, 1617–1620
4. Shaw, J. P., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A., and Crabtree, G. R. (1989) Science 241, 202–205
5. Ullman, K. S., Northrup, J. P., Verweij, C. L., and Crabtree, G. R. (1990) Annu. Rev. Immunol. 8, 421–432
6. Crabtree, G. R., and Clippston, N. A. (1991) Annu. Rev. Biochem. 60, 1045–1083
7. Northrup, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994) Nature 369, 497–502
8. McCaffrey, P. G., Luo, C., Kerpolska, T. K., Jain, J., Badalain, T. M., Ho, A. M., Burghen, E. A., Lane, W. S., Lambert, J. N., Curran, T., Verdin, C. G., Rago, A., and Hogan, P. G. (1993) Science 262, 750–754
9. Flanagan, W. M., Corteshe, B., Bram, R. J., and Crabtree, G. R. (1993) Nature 363, 803–807
10. Jain, J., Miner, Z., and Rao, A. (1993) J. Immunol. 151, 837–846
11. Jain, J., McCaffrey, P. G., Valger-archer, V. E., and Rao, A. (1992) Nature 356, 381–384
12. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J. W., Weissman, I., and Schreiber, S. L. (1991) Cell 66, 807–815
13. Friedman, J., and Weissman, I. (1991) Cell 66, 799–806
14. Liu, J., Albers, M. W., Wandless, T. J., Luam, S., Alberg, D. G., Beilshaw, P. J., Cohen, P., Mackintosh, C., Klee, C. B., and Schreiber, S. L. (1990) Biochemistry 29, 3896–3901
15. O’Keefe, S. J., Tamura, J., Kincard, R. L., Tocci, M. J., and O’Neill, E. A. (1992) Nature 357, 692–697
16. Clippston, N. A., and Crabtree, G. R. (1992) Nature 357, 695–697
17. Northrup, J. P., Ullman, K. S., and Crabtree, G. R. (1993) J. Biol. Chem. 268, 2917–2923
18. McCaffrey, P. G., Perrino, B. A., Soderling, T. R., and Rao, A. (1993) J. Biol. Chem. 268, 3747–3752
19. Jain, J., McCaffrey, P. G., Miner, A., Kerpolska, T. K., Lambert, J. N., Verdin, G. L., Curran, T., and Rao, A. (1993) Nature 365, 352–355
20. Ho, A. M., Jain, J., Rao, A., and Hogan, P. G. (1994) J. Biol. Chem. 269, 28181–28186
21. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
22. Hashimoto, Y., Perrino, B. A., and Soderling, T. R. (1990) J. Biol. Chem. 265, 1924–1927
23. Li, X., Ho, S. N., Luna, J., Giacalone, J., Thomas, D. J., Timmerman, L. A., Crabtree, G. R., and Francke, U. (1995) Cytogenet. Cell Genet. 68, 185–191
24. Donella-Deana, A., Krinks, M. H., Ruzzenze, M., Klee, C. B., and Finn, L. A. (1994) Eur. J. Biochem. 219, 109–117
25. Hemmings, H. C., Jr., Parnell, A. C., Elliott, J. I., and Greengard, P. (1990) J. Biol. Chem. 265, 20369–20376
26. King, M. M., Hsiung, C. Y., Chade, P. B., Nairn, A. C., Hemmings, H. C., Jr., Chan, K. F., and Greengard, P. (1984) J. Biol. Chem. 259, 8080–8083