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T-Cell Receptor Stimulation Elicits an Early Phase of Activation and a Later Phase of Deactivation of the Transcription Factor NFAT1

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We show here that NFAT1 is rapidly activated, then slowly deactivated, by stimulation of T cells through their antigen receptor. Within minutes of T-cell receptor stimulation, NFAT1 is dephosphorylated, translocates from the cytoplasm into the nucleus, and shows an increase in its ability to bind to DNA. These changes are dependent on calcium mobilization and calcineurin activation, since they are also elicited by ionomycin and are blocked by the immunosuppressive drug cyclosporin A. After several hours of T-cell receptor stimulation, the majority of the NFAT1 in the cell reverts to its original phosphorylated form, reappears in the cytoplasm, and again displays a low affinity for DNA. Deactivation of NFAT1 is facilitated by phorbol 12-myristate 13-acetate and inhibitors of capacitative calcium entry and most likely reflects the slow return of intracellular free calcium concentrations towards resting levels. Our results suggest that calcineurin-dependent signalling pathways mediate the early activation of NFAT1, while phorbol 12-myristate 13-acetate-dependent feedback pathways contribute to the late deactivation. Persistent NFAT-dependent cytokine gene transcription in activated T cells may be mediated by other NFAT family proteins in addition to NFAT1 during the immune response.

The transcription factor NFAT (nuclear factor of activated T cells) is essential for cytokine production during the immune response (reviewed in references 11, 20, and 46). The promoter-enhancer regions of several activation-associated genes possess binding sites for NFAT family proteins, including those encoding the cytokines interleukin-2 (IL-2), IL-3, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor alpha (8, 10, 15, 24, 32, 33, 44, 49, 54, 56) and the cell surface receptor CD40L (53). Four NFAT family proteins, NFAT1 (formerly NFATp), NFATc, NFATx/NFAT4/NFATc3, and NFAT3, which bind DNA with similar but not identical specificities, have been described elsewhere (17, 18, 29, 31, 34, 41). NFAT1 (29, 34) and NFATx/NFAT4/NFATc3 (17, 18, 31) are expressed as several alternatively spliced isoforms differing at their C-terminal ends. The DNA-binding domains of NFAT family proteins are highly homologous to one another (17, 18, 31, 41) and are distantly related to the DNA-binding domains of the Rel family proteins (19, 40, 42). A region of 300 amino acids N terminal to the DNA-binding domain also shows significant sequence similarities among the four proteins and may constitute a regulatory domain (17, 18, 29, 41).

The cell and tissue distributions of the mRNAs encoding the four NFAT-family proteins are different, suggesting that the proteins have different functional roles. Although NFAT1 mRNA is detected in the spleen, the thymus, and peripheral blood lymphocytes and in a variety of nonlymphoid tissues (17, 18, 31, 41), Western (immunoblot) analysis using specific antibodies against N- and C-terminal peptides and the DNA-binding domains indicates that the T-cell isoforms of NFAT1 are not ubiquitously expressed (59). Rather, NFAT1 is expressed in a limited number of cell types, including T cells, B cells, mast cells, NK cells, and a subset of macrophages (55, 59); in some endothelial cell lines (59); in the olfactory epithelium (16); and in a restricted subset of cells elsewhere in the nervous system (44a). NFATc mRNA is expressed in lymphoid tissues, in skeletal muscle, and in activated T cells and NK cells (2, 18, 31, 41). NFAT4 mRNA is expressed at much higher levels in the thymus than in peripheral immune cells, while the converse is true of NFAT1 and NFATc (17, 18, 31, 41). Thus, NFAT4 may regulate T-cell development, while NFAT1 and NFATc may be more important for the peripheral immune response. NFAT3 mRNA is predominantly expressed outside the immune system (18).

NFAT proteins are the cytosolic components of an inducible multisubunit transcription factor complex (13; reviewed in references 11, 20, and 46). The regulation of NFAT1 has been studied in some detail (43, 51, 55). A major mechanism for regulating NFAT1 activity is its compartmentalization in the cytoplasm of resting cells, thus preventing its access to DNA (29, 55). Treatment of murine T cells with the calcium ionophore ionomycin results in a rapid calcineurin-dependent dephosphorylation of NFAT1 that is apparent as a change in its mobility on sodium dodecyl sulfate (SDS) gels (51, 55) and precedes its nuclear translocation (55). Dephosphorylation is accompanied by an increase in the DNA-binding activity of NFAT1 (51, 55). The immunosuppressive drugs cyclosporin A and FK506, which bind to intracellular immunophilin receptors and inhibit the phosphatase activity of calcineurin (reviewed in references 4, 27, and 52), block the nuclear appearance of NFAT DNA-binding activity (6, 13, 35) and the nuclear translocation and increase in DNA-binding activity of NFAT1 (55). Indeed, NFAT1 is a substrate for calcineurin in vitro (21, 35, 43) and binds to calcineurin directly (28, 62), consistent with the hypothesis that calcineurin dephosphorylates NFAT1 in activated T cells and thereby regulates its function (27, 52). Physiological stimulation of T cells via their antigen receptors

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results not only in calcium mobilization but also in the activation of protein kinase C (PKC), Ras, and the various Ras-dependent pathways (61). We examined the contribution of these additional pathways to NFAT1 activation in T cells triggered through the T-cell receptor (TCR).

A second mechanism for regulating NFAT activity is the interaction of NFAT family proteins with other transcription factors in the nucleus (reviewed in references 11, 20, and 46). The best-characterized interactions involve the cooperative binding of NFAT family proteins with Fos-Jun and Jun-Jun dimers to the IL-2 promoter NFAT site (5, 7, 21, 22, 63), as well as to NFAT sites in the IL-4 promoter and the IL-3–granulocyte-macrophage colony-stimulating factor intergenic enhancer (8, 50). NFAT-dependent transactivation at the IL-2 and IL-4 promoter sites is markedly augmented by Fos and Jun family proteins (5, 22, 42, 50). The optimal transcriptional activity of NFAT at a given regulatory site may depend on the precise combination of NFAT, Fos, and Jun family proteins that assembles at the site.

Here we have investigated the early steps in the activation of NFAT1 in T cells stimulated through the TCR. We show that stimulation with antigen or anti-CD3 resembles ionomycin stimulation (55) in that it results in a rapid calcineurin-dependent dephosphorylation and nuclear translocation of NFAT1 and an increase in its affinity for DNA. However, unlike ionomycin-mediated NFAT1 activation, which persists for several hours, TCR-mediated NFAT1 activation is relatively short-lived. Within 5 h of TCR stimulation, the bulk of the cellular NFAT1 reverts to a phosphorylated, cytoplasmic form with low affinity for DNA. This process is accelerated by phorbol 12-myristate 13-acetate (PMA) stimulation and by inhibition of capacitative calcium entry and may reflect the slow return of intracellular free calcium concentrations $[Ca^{2+}]_i$ towards resting levels, as a result of feedback mechanisms triggered optimally by TCR ligands and less effectively by ionomycin. Nevertheless, NFAT activity persists at low levels in the nucleus, and nuclear extracts from cells stimulated with anti-CD3 for long times contain both NFAT1 and NFATc. Our results suggest that the early phase of cytokine gene transcription in physiologically stimulated T cells is mediated through a relatively transient, posttranslational activation of NFAT1. In contrast, the later phases of cytokine gene transcription may involve a partial replacement of NFAT1 by newly synthesized NFATc or other NFAT family members.

MATERIALS AND METHODS

Cells and reagents. The Ar-5 murine T-cell clone, responsive to arsonate-conjugated ovalbumin (Ars-OVA) in the context of the murine major histocompatibility complex class II molecule IA^d, was cultured and grown as previously described (47). Chinese hamster ovary cells expressing IA^d and mouse B7.1 (CHO-IA^dB7) were kindly donated by Gordon Freeman (48). Ars-OVA and sulfonate-conjugated ovalbumin (S-OVA) were made as previously described (38). Anti-67.1, a peptide rabbit polyclonal antibody to a peptide in the N-terminal region of NFAT1 (16), was affinity purified on a peptide-bound Affi-Gel 10 (Bio-Rad) column. Anti-NFATc (7A6), ascitic fluid raised against recombinant NFATc, was a kind gift from G. Crabtree (41). This antibody does not cross-react with NFAT1. Hybridoma cells producing 145-2C11, a hamster monoclonal antibody to the murine CD3 ε chain, were kindly provided by J. Bluestone (25). Cyclosporin A, FK506, and rapamycin were kindly donated by Sandoz, Fujisawa, and Wyeth Ayerst, respectively. Ionomycin, PMA, and SKF 96365 were purchased from Calbiochem.

Immunofluorescence. Coverslips were coated with 1 mg of poly-D-lysine per ml in borate-buffered saline for 4 to 24 h and washed with phosphate-buffered saline (PBS). CHO-IA^dB7 cells were plated onto these poly-D-lysine-coated coverslips at 2.5×10^6 cells per ml with 100 μg of Ars-OVA or S-OVA per ml for 4 h at 37°C. The medium was removed, and 100 μl of fresh medium containing 10^6 Ar-5 T cells was added to the antigen-pulsed CHO-IA^dB7 cell monolayer on the coverslips and incubated for 15 min at 37°C. For unstimulated or ionomycin-stimulated samples, Ar-5 T cells were added to the poly-D-lysine-coated coverslips and allowed to attach by incubation at 37°C. For anti-CD3 stimulation, 100 μl of a 3-mg/ml solution of rabbit anti-hamster immunoglobulin G (Organon Teknika/Cappel) was used to coat the coverslips at room temperature for 2 h. The coverslips were washed to remove excess antibody. Ar-5 T cells were then added to the coverslips with 1 μl of anti-CD3 antibody and incubated at 37°C. Cells were fixed in 3% paraformaldehyde in PBS for 15 min and then stained with 0.01 μg of anti-67.1 antibody per ml and 3 μg of Cy3-conjugated donkey anti-rabbit (Jackson Labs) secondary antibody per ml. To assess specific staining, the antibody (0.01 μg) was preincubated for 30 min at 4°C with 5 μg of 67.1 peptide in 100 μl and then diluted and used as usual. Cells were photographed with a Zeiss MC80 microscope and Kodak Tri-X Pan ASA400 film.

SDS extracts and detection of NFAT1 by Western blotting. Ar-5 T cells in suspension (1×10^6 cells per sample) were left unstimulated or stimulated in microcentrifuge tubes with 1 μM ionomycin (in some experiments, 10 nM PMA alone or 10 nM PMA plus 1 μM ionomycin) or 0.5×10^6 CHO-IA^dB7 cells that had been previously pulsed with Ars-OVA or S-OVA for 4 h at 37°C. PMA, PMA plus ionomycin, and ionomycin stimulations at time points up to an hour were carried out in microcentrifuge tubes incubated in a 37°C water bath with the Ar-5 T cells maintained in suspension by occasional shaking. After stimulation, cells were centrifuged for 30 s in a microcentrifuge and all of the medium was removed. The cells were then resuspended in 30 μl of a buffer containing 40 mM Tris (pH 8.0), 60 mM sodium PP_i, and 10 mM EDTA and lysed upon addition of an equal volume of 10% SDS and boiling for 20 min with occasional vigorous mixing by vortex. Antigen stimulation as shown in Fig. 2 was performed in microcentrifuge tubes by centrifuging a the mixture of Ar-5 T cells and antigen-pulsed CHO-IA^dB7 cells for 10 s to form a loose cell pellet and then incubating the mixture for 1 min in a 37°C water bath. The cells were then lysed as described above for ionomycin stimulation. For stimulation under physiological conditions, as shown in Fig. 3A, CHO-IA^dB7 cells were first plated in a 48-well tissue culture plate and pulsed with antigen for 4 h at 37°C. The medium was then removed, and the Ar-5 T cells were added in a 100-μl volume to each well and incubated at 37°C in a 10% CO_2 incubator for the desired times. The medium was removed, and 50 μl of 40 mM Tris (pH 8.0)–60 mM sodium PP_i–10 mM EDTA was added to each well; this was followed by the addition of an equal volume of 10% SDS and incubation for 20 min in a 90°C water bath. For stimulation with immobilized anti-CD3 ε antibody, Ar-5 T cells (10^6 cells per well) were added a 48-well tissue culture plate that had been precoated for 2 h with 100 μl of a 3-mg/ml solution of rabbit anti-hamster antibody. Anti-CD3 ε antibody (1:100 dilution of supernatant) was added to the wells, and the cells were incubated at 37°C in a 10% CO_2 incubator for the desired times. Ionomycin stimulation for times greater than 1 h was also carried out in a 48-well tissue culture plate at 37°C in a 10% CO_2 incubator; the cells were lysed as described above for anti-CD3 stimulation. Cell extracts were analyzed by electrophoresis on SDS–6% polyacrylamide gels. The separated proteins were transferred to nitrocellulose and incubated with anti-67.1 antibody; this was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) and visualization by enhanced chemiluminescence (Amersham).

Inhibitors. Before stimulation with ionomycin or antigen-pulsed CHO-IA^dB7 cells, Ar-5 T cells were pretreated with 1 μM cyclosporin A, FK506, or rapamycin for 15 min at 37°C and then used in the immunofluorescence staining or Western blotting assays described above. Ar-5 T cells were incubated in medium containing 200 nM PMA for 48 h at 37°C to downmodulate the classical isozymes of PKC (58) and then used in the Western blotting assays described above.

Electrophoretic mobility shift assays (EMSAs). Ar-5 T cells (25×10^6) were left unstimulated or stimulated as described above for SDS lysates, harvested, and washed once in 1× PBS–0.1% bovine serum albumin. Whole-cell high-salt extracts were made by resuspending cells in 50 μl of RSB buffer [10 mM Tris (pH 7.4), 120 mM sodium PP_i, 20 mM EDTA, and 0.4 μM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), supplemented with 80 μM leupeptin, 40 μg of aprotinin per ml, 100 μg of soybean trypsin inhibitor per ml, 8 μM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol), which was followed by addition of 50 μl of 5× gel shift buffer (1 M KCl, 5 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 5 mM EGTA, 20% glycerol) and incubation on ice for 30 min. The extracts were centrifuged at 4°C in a microcentrifuge. Supernatants were transferred to fresh microcentrifuge tubes and diluted on ice with 100 μl of water. Extracts were analyzed by SDS–6% polyacrylamide gel electrophoresis followed by Western blotting with anti-NFAT1 antibody to assess the amount of NFAT1 present in the extracts. Different volumes of extracts normalized for amounts of NFAT1 were incubated with 200 μg of poly(dI)-poly(dC) per ml as a nonspecific competitor and 10,000 cpm (0.1 to 1 ng) of end-labelled oligonucleotide 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 reaction mixtures were incubated at room temperature for 20 min. The resulting DNA-protein complexes were separated by electrophoresis on a 4% nondenaturing gel. The oligonucleotide used was as follows: 5′ gatcGCCCAAAGAG GAAAATTTGTTTCATACAG 3′ (corresponding to the distal NFAT site of the murine IL-2 promoter).

Small-scale nuclear extracts were made as previously described (22). Briefly, cells were resuspended in a buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5 mM dithiothreitol, supplemented with 0.1 mM EGTA, 2 μM leupeptin, 1 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride. The supernatant was removed to a separate tube,

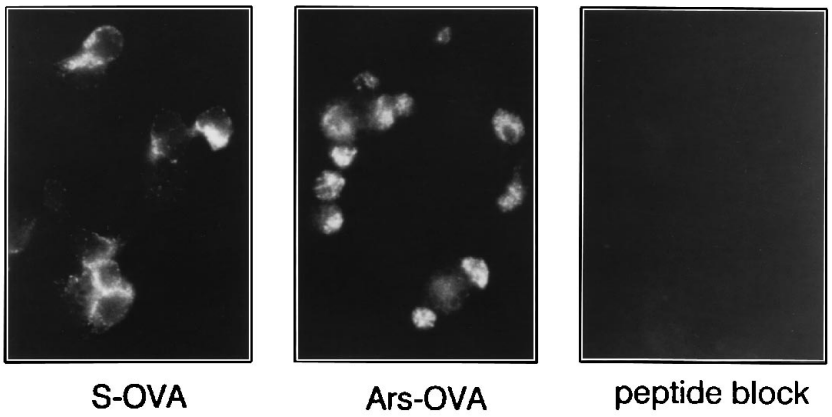

FIG. 1. NFAT1 translocates to the nucleus in antigen-stimulated T cells. Ar-5 T cells were stimulated with CHO-IA^{d}B7 cells pulsed with the nonstimulatory antigen S-OVA (100 μg/ml) (left) or the stimulatory antigen Ars-OVA (100 μg/ml) (middle), fixed, and stained with anti-NFAT1 antibody and fluorescent second antibody. (Right) As a control for nonspecific staining, Ars-OVA-stimulated Ar-5 T cells were stained with anti-NFAT1 antibody that had been preincubated with 67.1 peptide. The cytoplasmic location of NFAT1 in the S-OVA-treated cells is especially evident in the elongated cell at the top of the left panel and in the clump of four cells at the bottom. The nuclear location of NFAT1 in the Ars-OVA-treated cells is apparent in the two cells at the bottom of the middle panel, which are separated by a CHO cell that shows weak nonspecific staining.

and the nuclear pellet was extracted in 60 μl of buffer C containing 20 mM HEPES (pH 7.4), 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol, supplemented with the protease inhibitors described above. The extract was diluted with an equal volume of buffer D containing 20 mM HEPES (pH 7.4), 50 mM KCl, 0.2 mM EDTA, and 20% glycerol. Bradford protein assays (Bio-Rad) performed on extracts to determine total protein content showed that the efficiencies of extraction of total nuclear proteins were similar in resting and stimulated cells. Extracts (0.5 to 10 μg of total protein) were incubated in the binding reaction mixtures described above for the high-salt extracts.

RESULTS

Stimulation of T cells with antigen results in rapid calcineurin-dependent activation of NFAT1. The physiological stimulus for T cells is a combination of major histocompatibility complex-bound antigen and a costimulatory molecule, such as a member of the B7 family of proteins (reviewed in reference 1). We showed that antigen stimulation of T cells resulted in translocation of NFAT1 from the cytoplasm to the nucleus. Chinese hamster ovary (CHO) cells that stably expressed the murine major histocompatibility complex class II molecule, IA^d, and the costimulatory molecule, B7.1 (48), were used to present antigen to Ar-5 murine T cells (47). Ars-OVA was used as the stimulatory antigen, and S-OVA, a structurally related antigen that is 3 orders of magnitude less efficient at stimulating the Ar-5 T cells (38), was used as a control at nonstimulatory concentrations. Ar-5 T cells exposed to S-OVA-pulsed CHO-IA^{d}B7 cells displayed fully cytoplasmic localization of NFAT1 (Fig. 1, left panel), whereas cells exposed to the stimulatory antigen, Ars-OVA, showed nuclear localization (center panel). The nuclear translocation occurred within 10 min, and NFAT1 persisted in the nucleus for at least 1 h (data not shown). The staining was specific, as preincubation of the anti-NFAT1 antibody with the corresponding peptide reduced staining to background levels (right panel). Nuclear translocation of NFAT1 required TCR engagement but did not require the CD28-B7 interaction (data not shown). Likewise, the nuclear translocation of NFAT1 required calcineurin, as it was blocked by cyclosporin A and FK506 but not by rapamycin, a potent immunosuppressant that is structurally related to FK506 and binds FKBP12 but does not inhibit calcineurin (not shown).

Stimulation of Ar-5 T cells by Ars-OVA-pulsed CHO-IA^{d}B7 cells caused a rapid change in the electrophoretic mobility of NFAT1 on SDS gels (Fig. 2). Ar-5 T cells were mixed with the Ars-OVA-pulsed CHO-IA^{d}B7 cells in a microcentrifuge tube, centrifuged briefly to ensure rapid contact of T cells with the CHO cells, and then incubated at 37°C. Within 1 min of antigen stimulation, a change in apparent molecular mass of NFAT1 from ~135 to ~120 kDa was observed (Fig. 2, lane 2). A similar shift is induced by treatment of cells with ionomycin and has been shown to reflect a calcineurin-dependent, partial dephosphorylation of NFAT1 (51, 55). CHO-IA^{d}B7 cells

FIG. 2. NFAT1 undergoes a rapid dephosphorylation in antigen-stimulated Ar-5 T cells. Ar-5 T cells were stimulated for 1 min at 37°C in microcentrifuge tubes with CHO-IA^{d}B7 cells previously incubated with no antigen, 100 μg of Ars-OVA per ml, or 100 μg of S-OVA per ml. For lane 4, the T cells were stimulated with Ars-OVA-pulsed CHO-IA^{d}B7 cells in the presence of 1 μM cyclosporin A (CsA). SDS lysates of the cells were analyzed by electrophoresis on a 6% polyacrylamide gel, followed by Western blotting with anti-NFAT1 antibody. Lysates of CHO-IA^{d}B7 cells alone do not show any detectable NFAT1 (data not shown).

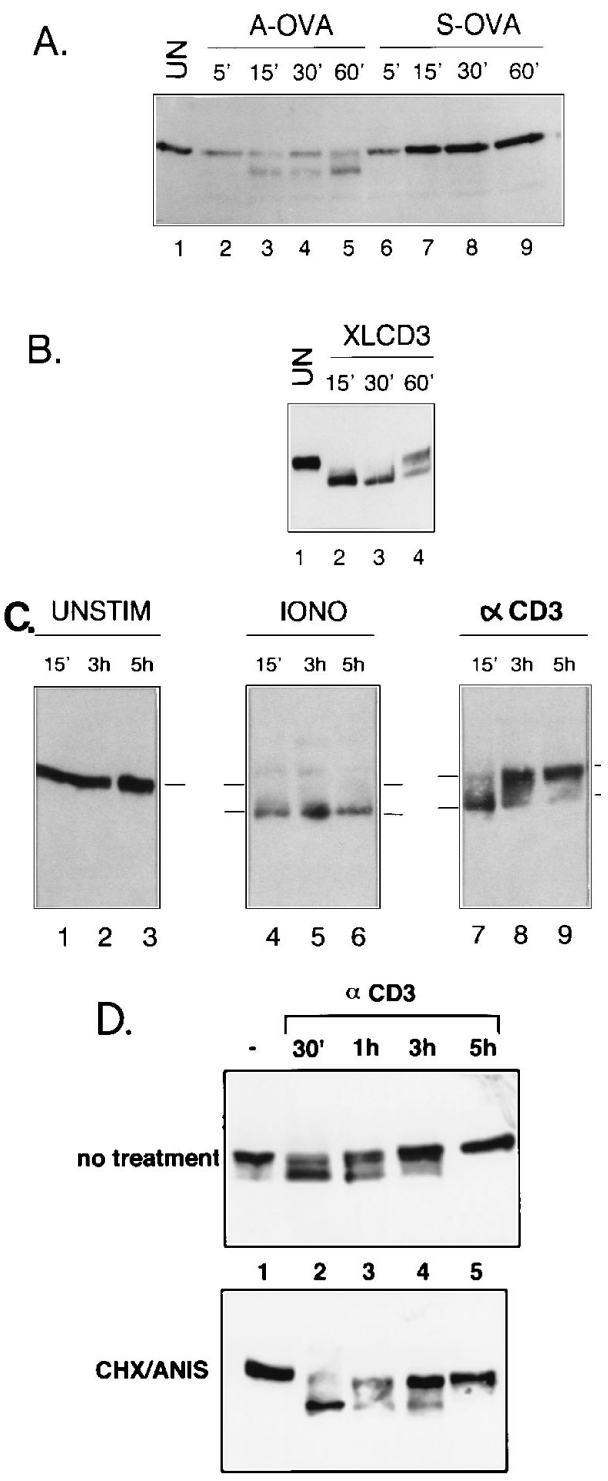

FIG. 3. Effects of antigen or anti-CD3 ε stimulation on NFAT1. (A) Antigen stimulation. CHO-IA^{d}B7 cells were incubated for 4 h at 37°C with 100 μg of Ars-OVA or S-OVA per ml in the wells of a tissue culture plate. Ar-5 T cells were added to the wells; incubated at 37°C for 5, 15, 30, or 60 min as indicated; and then lysed directly in the wells with SDS. The lysates were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody. UN, unstimulated. (B) Anti-CD3 stimulation. Ar-5 T cells were added to wells of a 48-well plate that had been precoated with rabbit anti-hamster immunoglobulin G. The cells were left unstimulated (UN) or stimulated by incubation with anti-CD3 ε antibody for 15, 30, and 60 min at 37°C (XLCD3) and then lysed directly in the wells with SDS. The lysates were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody. (C) Long time course of ionomycin and anti-CD3 stimulation. Ar-5 T cells were added to the wells of a 48-well tissue culture plate and left unstimulated (UNSTIM), stimulated with 1 μM ionomycin (IONO), or stimulated with immobilized anti-CD3 ε antibody (αCD3) at 37°C for the times indicated. The cells were lysed directly in the wells with SDS. The lysates were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody. The lines indicate the positions of the phosphorylated and the dephosphorylated forms of NFAT1 in each panel. (D) Effect of protein synthesis inhibitors. Ar-5 T cells were incubated in standard culture medium (top) or in medium containing 10 μM cycloheximide (CHX) and 40 μM anisomycin (ANIS) (bottom) for 1 h at 37°C. The cells were left unstimulated (lanes 1) or stimulated with immobilized anti-CD3 ε antibody for the indicated times and then lysed in SDS. The SDS lysates were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody. NFAT1 undergoes partial rephosphorylation as early as 1 h following anti-CD3 stimulation (lane 3).

alone, or CHO-IA^{d}B7 cells pulsed with nonstimulatory concentrations of S-OVA, did not induce the dephosphorylation of NFAT1 (lanes 1 and 3). The dephosphorylation induced by Ars-OVA stimulation was inhibited by pretreatment of the T cells with cyclosporin A (lane 4), suggesting that calcineurin was involved.

NFAT1 reverts to its resting state after long periods of stimulation through the TCR. To determine whether the dephosphorylation of NFAT1 was maintained for longer periods of time after T-cell exposure to antigen, a time course of antigen stimulation was performed (Fig. 3A). To avoid stressing the cells by incubating them in a cell pellet for long periods of time, the antigen stimulation was performed by adding the T cells to an adherent layer of antigen-presenting CHO cells. Under these conditions, the rate-limiting step is the time required for the T cells to settle onto and make contact with the antigen-pulsed CHO-IA^{d}B7 cells; thus, the change in mobility of NFAT1 is not apparent at the 5-min time point but is clearly evident at 15 min and is maintained for at least an hour (lanes 3 to 5). However, antigen stimulation did not result in quantitative conversion of NFAT1 to the form with a lower apparent molecular weight (lanes 3 to 5), perhaps because of inadequate contact by some of the T cells with the CHO antigen-presenting cells.

To ensure quantitative stimulation of all T cells, we used the stronger stimulus provided by cross-linking the TCR with activating anti-CD3 ε antibodies (Fig. 3B). Ar-5 T cells were incubated with immobilized anti-CD3 ε antibodies in the wells of a tissue culture plate. Essentially quantitative dephosphorylation of NFAT1 was observed in Ar-5 T cells stimulated in this manner for 15 and 30 min (lanes 2 and 3). In contrast, in cells stimulated for 1 h, a fraction of the NFAT1 migrated at the position of the phosphorylated form observed in unstimulated cells (lane 4). This observation indicated that longer stimulation times might alter the phosphorylation status of NFAT1. We therefore performed an even longer time course of stimulation using immobilized anti-CD3 ε (Fig. 3C). While NFAT1 in ionomycin-stimulated cells remained in the dephosphorylated form even at 5 h (lanes 4 to 6), NFAT1 in anti-CD3 ε-stimulated cells slowly reverted to a form which comigrated with the NFAT1 from unstimulated cells (lanes 7 to 9). This change was obvious at 3 h and almost complete by 5 h following stimulation with anti-CD3 ε.

The return of NFAT1 to its original electrophoretic mobility after 5 h of anti-CD3 ε stimulation was not due to degradation of nuclear NFAT1 and its replacement by newly synthesized, phosphorylated NFAT1 (Fig. 3D). In cells treated with the protein synthesis inhibitors cycloheximide and anisomycin, NFAT1 reverted to its original slower-mobility form with kinetics similar to those in untreated cells (compare lanes 2 to 5 in the top and bottom panels). Labelling experiments with

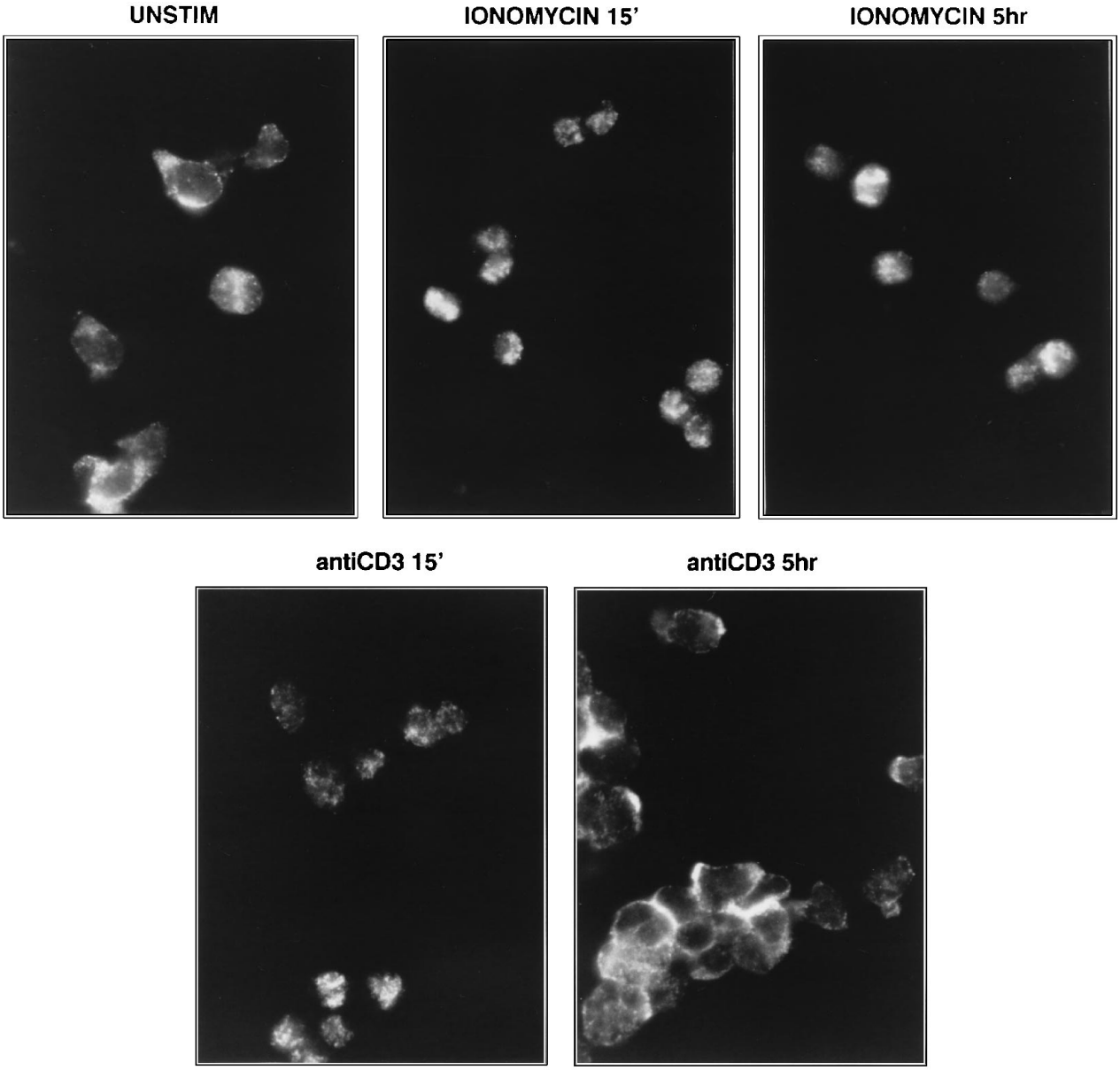

FIG. 4. NFAT1 is detected in the cytoplasm of cells stimulated for 5 h with anti-CD3. Ar-5 T cells were left unstimulated (UNSTIM), stimulated with 1 μM ionomycin for 15 min or 5 h, or stimulated with immobilized anti-CD3 ε antibody for 15 min or 5 h. The cells were fixed and stained with anti-NFAT1 antibody.

[^{35}S]methionine and [^{35}S]cysteine indicated that the inhibitors effectively blocked protein synthesis under these conditions (data not shown). Pulse-chase analysis of metabolically labelled T cells has shown that the half-life of NFAT1 is 19 to 22 h in both stimulated and unstimulated cells (reference 51 and data not shown). The simplest interpretation is that NFAT1 is a long-lived protein which is dephosphorylated early in the course of TCR stimulation and becomes rephosphorylated at later times.

To determine the subcellular localization of the rephosphorylated form of NFAT1, immunocytochemical staining was performed (Fig. 4). NFAT1 staining localized to the nucleus of cells stimulated for 15 min with anti-CD3 or with ionomycin, with little or no staining detected in the cytoplasm (second and fourth panels). However, whereas NFAT1 persisted in the nucleus following 5 h of ionomycin stimulation (third panel), NFAT1 was present in the cytoplasm of cells stimulated for 5 h with immobilized anti-CD3 (fifth panel). This analysis indicates that the bulk of the nuclear NFAT1 returns to the cytoplasm after several hours of TCR stimulation.

We assessed the DNA-binding function of the rephosphorylated form of NFAT1 in anti-CD3 ε-stimulated cells (Fig. 5). Whole-cell extracts were made by high-salt extraction of T cells stimulated for 15 min or 5 h with ionomycin or anti-CD3 and analyzed for their content of NFAT1 by Western blot analysis (Fig. 5A) and for their NFAT DNA-binding activity by EMSA (Fig. 5B). The majority of the NFAT binding activity in these extracts is attributable to NFAT1, as an anti-NFAT1 antibody recognizes and completely supershifts the NFAT DNA-protein complexes formed (data not shown). Anti-CD3 stimulation resulted in an increase in the DNA-binding activity of NFAT1 within 15 min (Fig. 5B, lanes 13 to 15) that was comparable to

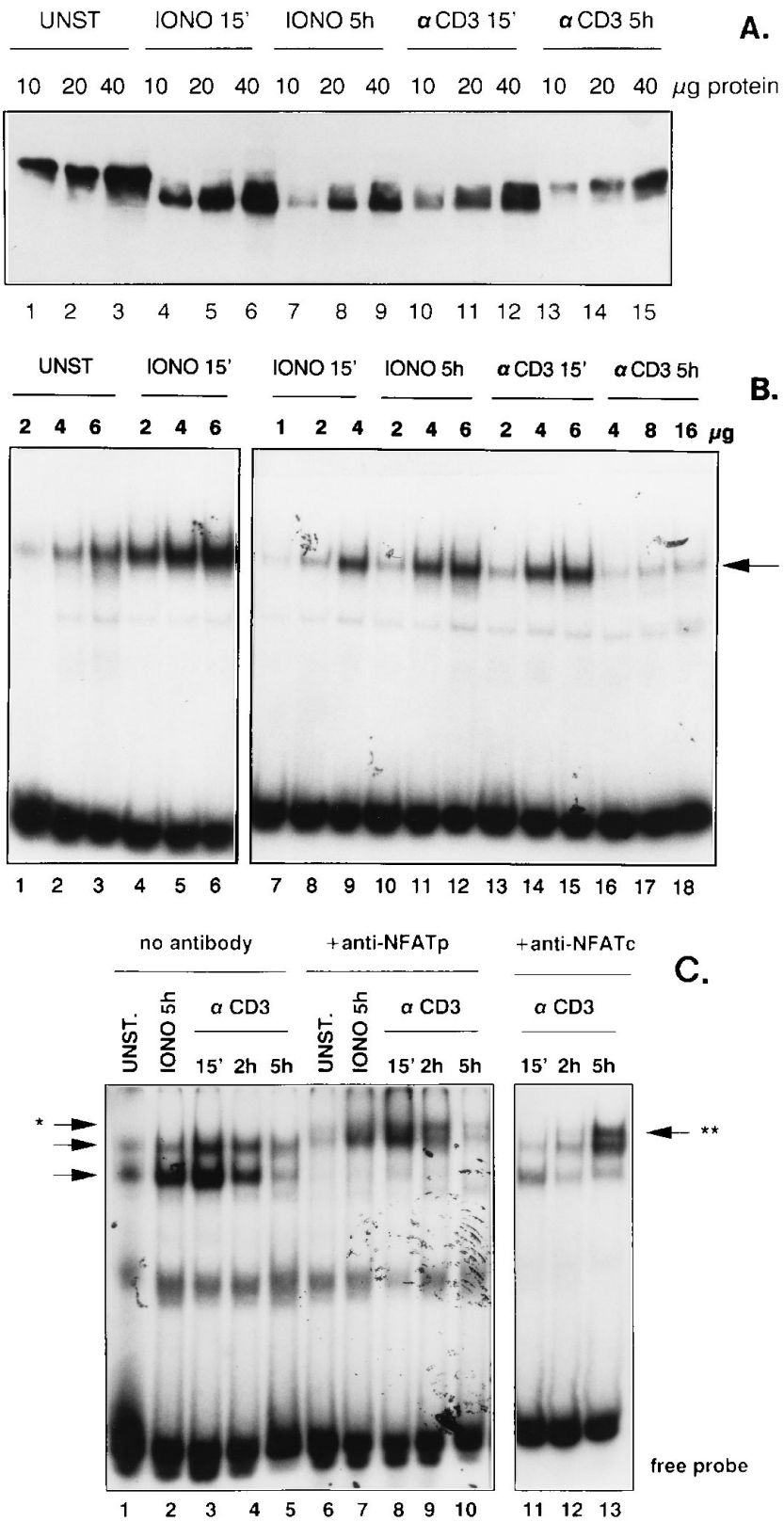

FIG. 5. The DNA-binding activity of NFAT1 decreases after 5 h of stimulation with immobilized anti-CD3 ε. (A) Western blot analysis for NFAT1 content. Ar-5 T cells in tissue culture flasks were left unstimulated (UNST) or stimulated for 15 min or 5 h with 1 μM ionomycin (IONO) or immobilized anti-CD3 ε (αCD3). The cells were harvested, and whole-cell extracts were prepared as described in Materials and Methods. The total protein, as determined by the Bradford assay, loaded in each lane is indicated. The extracts were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody. (B) DNA-binding activity in whole-cell extracts. Extracts, normalized for equivalent amounts of NFAT1 based on the titration in panel A, were analyzed for DNA-binding activity by EMSA. The end-labelled oligonucleotide probe corresponds to the distal NFAT site of the murine IL-2 promoter. The total protein used in the binding reactions is indicated at the top of each

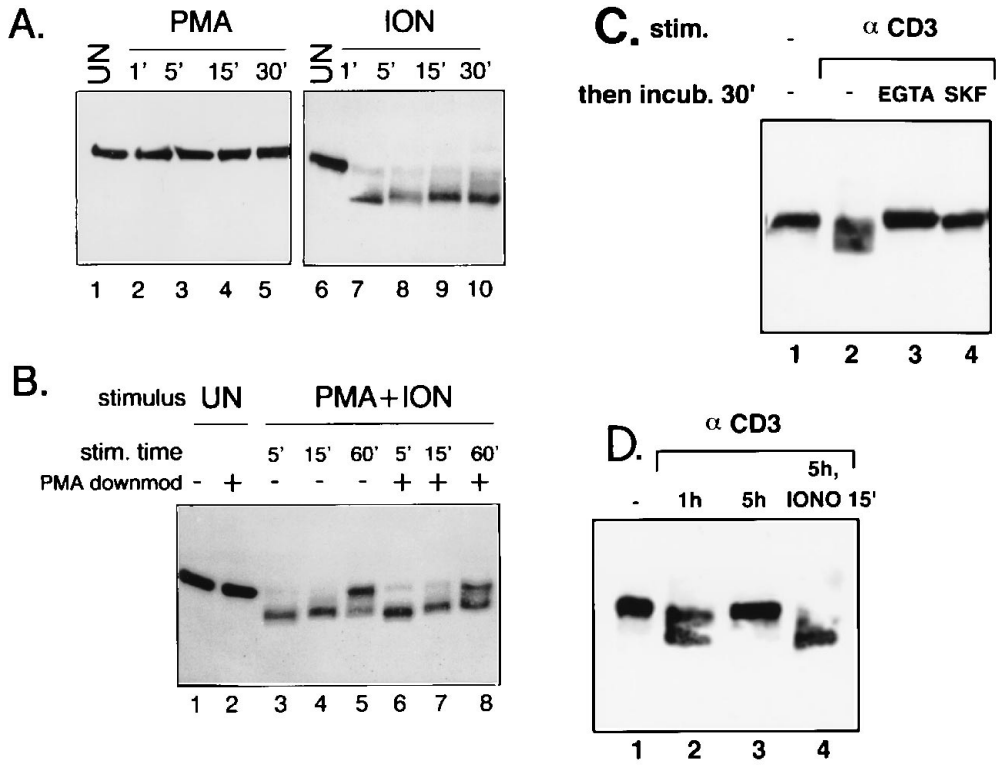

FIG. 6. Deactivation of NFAT1 is facilitated by PMA and inhibitors of capacitative calcium entry. (A) PMA stimulation. Ar-5 T cells were left unstimulated (UN) or stimulated with 10 nM PMA alone or 1 μM ionomycin alone (ION) for the times indicated before lysis in SDS. The lysates were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody. (B) Downmodulation of classical PKC isozymes. Ar-5 T cells were incubated in standard culture medium (−) or medium containing 200 nM PMA for 48 h at 37°C (+). The cells were left unstimulated or stimulated with 10 nM PMA plus 1 mM ionomycin for the times indicated before lysis in SDS. The lysates were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody. Stimulation of the PMA-treated cells with PMA plus ionomycin did not result in IL-2 production (data not shown), indicating effective downmodulation of the classical PKC isozymes. (C) Ar-5 T cells were left unstimulated (−) or stimulated with immobilized anti-CD3 for 30 min at 37°C. In lanes 3 and 4, EGTA (2 mM) or SKF 96365 (100 μM) was added and the cells were allowed to incubate at 37°C for a further 30 min before lysis in SDS. The lysates were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody. (D) Ar-5 T cells were left unstimulated (−) or stimulated with 1 μM ionomycin (IONO) or immobilized anti-CD3 for 1 or 5 h. Ionomycin at 1 μM was added to cells that had been stimulated for 5 h (lane 4), and the cells were allowed to incubate for a further 15 min at 37°C before lysis in SDS. The lysates were analyzed by SDS–6% PAGE followed by Western blotting with anti-NFAT1 antibody.

the increase seen with ionomycin stimulation (lanes 1 to 6 and 7 to 12). In contrast, NFAT1 from cells stimulated with anti-CD3 for 5 h showed a return to the low levels of DNA binding observed in resting cells (lanes 16 to 18), indicating that the DNA-binding activity of NFAT1 in whole-cell extracts correlated with its phosphorylation status in cells at different stages of activation.

We evaluated the level of nuclear NFAT activity by EMSA in cells stimulated with anti-CD3 for different times (Fig. 5C). Nuclear NFAT and NFAT/AP-1 protein-DNA complexes were markedly increased in cells stimulated with anti-CD3 for short times (lanes 3 and 4) but decreased to a level only slightly higher than that in unstimulated cells in cells stimulated for 5 h with anti-CD3 (lane 5). Anti-NFAT1 antibody supershifted a major fraction of both complexes at each time tested (lanes 6 to 9), suggesting that NFAT1 accounted for the bulk of the nuclear NFAT DNA-binding activity. However, the use of an anti-NFATc antibody revealed that NFATc accounted for a fraction of the nuclear NFAT DNA-binding activity in cells stimulated with anti-CD3 for 2 and 5 h (lanes 12 and 13) but not in extracts of cells stimulated with anti-CD3 for only 15 min (lane 11). NFATc DNA-binding activity was not detected in nuclear extracts of ionomycin-stimulated cells (data not shown). These data are consistent with a previous report that NFATc mRNA expression is upregulated within 3 h of stimulation of Jurkat T cells with PMA and ionomycin (41).

Deactivation of NFAT1 is facilitated by PMA and inhibitors of capacitative calcium entry. As TCR stimulation can be mimicked by treatment of T cells with both ionomycin and PMA, which activates PKC (57), we asked whether PMA treatment influenced either the early activation or the late deactivation of NFAT1 in response to ionomycin stimulation. Treatment of Ar-5 T cells with 10 nM PMA did not result in any discernible change in the electrophoretic mobility or subcellular localization of NFAT1 (Fig. 6A, lanes 2 to 5, and data not shown) and did not affect the rapid dephosphorylation of NFAT1 observed

lane. The arrow indicates the specific NFAT1-DNA complex. The faint band migrating below the specific NFAT1-DNA complex is nonspecific. (C) DNA-binding activity in nuclear extracts. Nuclear extracts from Ar-5 T cells were analyzed by EMSA in the absence or presence of 0.2 μg of anti-NFAT1 antibody (anti-NFATp) or 1 μl of a 1:10 dilution of ascitic fluid containing anti-NFATc antibody. In lane 13, a slightly larger amount of nuclear extract (1.5×) was used in the binding reaction to visualize the supershift more clearly. The bottom two arrows on the left indicate the two specific nuclear complexes containing NFAT proteins (lower) and NFAT/AP-1 proteins (upper). The arrow with one asterisk indicates the supershift induced by anti-NFAT1; the arrow with two asterisks indicates the supershift induced by NFATc. Neither antibody bound to or shifted the probe (data not shown).

with ionomycin alone (compare Fig. 6A, lane 8, with Fig. 6B, lane 3). Stimulation with 10 nM PMA and 1 μM ionomycin did, however, mediate a slow rephosphorylation of NFAT1 that was not generally observed with 1 μM ionomycin alone (Fig. 6B, lane 5). This effect did not require the classical PKC isozymes (α, β, and γ), which are downmodulated by treatment of cells with 200 nM PMA for 48 h (58) (compare lanes 5 and 8).

PMA stimulation is known to trigger feedback mechanisms, including TCR internalization and stimulation of the plasma membrane calcium pump, that return $[Ca^{2+}]_i$ to their resting state (reviewed in references 26 and 45). We therefore asked whether the slow rephosphorylation of NFAT1 following anti-CD3 stimulation was accelerated under conditions that lowered $[Ca^{2+}]_i$. Calcium mobilization usually consists of two phases, a rapid spike resulting from emptying of intracellular stores of calcium followed by a prolonged phase of increased, often oscillating $[Ca^{2+}]_i$, mediated by capacitative calcium entry (reviewed in references 3, 9, and 26). This prolonged plateau phase of calcium mobilization, rather than the initial transient spike, has been shown by single-cell measurements of $[Ca^{2+}]_i$ in T hybridoma cells to be required for NFAT-dependent transcription (39).

Capacitative calcium entry is inhibited by EGTA, which chelates extracellular calcium, and by SKF 96365, which is thought to inhibit the plasma membrane I_{CRAC} channel (30, 37). Treatment of cells with these agents mediated a rapid rephosphorylation of NFAT1 in anti-CD3-stimulated cells, even in the presence of ongoing anti-CD3 stimulation (Fig. 6C, lanes 3 and 4). Indo-1 labelling experiments indicated that $[Ca^{2+}]_i$ in cells stimulated for 5 h with anti-CD3 were similar to those in resting cells; moreover, calcium stores were replenished, since ionomycin or thapsigargin treatment again resulted in an increase in $[Ca^{2+}]_i$ (data not shown). Consistent with these results, NFAT1 that had reverted to its original electrophoretic mobility in cells stimulated with anti-CD3 for 5 h (Fig. 6D, lane 3) was again dephosphorylated by the subsequent addition of ionomycin (lane 4). Taken together, these data suggest that a late effect of TCR stimulation is to restore $[Ca^{2+}]_i$ to resting levels and that this mediates the late deactivation of NFAT1.

DISCUSSION

We have shown that stimulation of murine Ar-5 T cells through the TCR results within minutes in an early activation of NFAT1. Activation involves dephosphorylation (manifest as a mobility shift on SDS-polyacrylamide gels), nuclear translocation, and an increase in DNA-binding activity. These hallmarks of NFAT1 activation are calcineurin dependent and resemble those observed after treatment with ionomycin alone (55). At later times, however, TCR stimulation elicits a slow deactivation of NFAT1 that results in its reappearance in the cytoplasm and the return of its DNA-binding activity to a low level. Deactivation involves rephosphorylation of active nuclear NFAT1, rather than degradation of active NFAT1 in the nucleus and its replacement by newly synthesized, inactive NFAT1 in the cytoplasm, and may involve feedback mechanisms that lead to a slow decrease of $[Ca^{2+}]_i$. Similar phases of early activation and late deactivation of NFAT1 are observed in freshly isolated murine spleen cells stimulated with anti-CD3, indicating that these effects are not confined to established T-cell clones.

The combination of PMA and calcium ionophore has classically been used to mimic antigen stimulation (45, 57). However, PMA-induced signalling pathways do not appear to contribute to the early activation of NFAT1. Stimulation of Ar-5 with PMA alone did not result in any change in the phosphorylation status of NFAT1 and did not cause translocation of NFAT1 into the nucleus. Furthermore, the addition of PMA had no detectable influence on the ionomycin-induced dephosphorylation or nuclear translocation of NFAT1. These results are consistent with the fact that at least two NFAT-dependent cytokines, IL-4 and tumor necrosis factor alpha, can be induced by ionomycin stimulation alone (15, 49).

In contrast, our results suggest that PMA-stimulated signalling pathways contribute to the late rephosphorylation and deactivation of NFAT1 and that feedback decreases in $[Ca^{2+}]_i$ are the most plausible mechanism for this effect. While the late deactivation of NFAT1 could conceivably result from direct rephosphorylation of NFAT1 by PKC or other PMA-activated enzymes, the slow kinetics of rephosphorylation suggest an indirect effect. Given the strong dependence of NFAT1 activation on calcium and calcineurin signaling (28, 51, 55, 62), feedback mechanisms that result in lowering of cytoplasmic calcium levels or inhibition of calcineurin activity would be expected to limit the duration of NFAT1 activation in response to TCR stimulation. In fact, PKC is known to cause internalization of the TCR (via serine phosphorylation of CD3 chains) (12, 23, 36), inhibition of phospholipase Cγ (60), inhibition of phosphatidylinositol-4,5-biphosphate hydrolysis (3), and activation of plasma membrane Ca^{2+} pumps (14), all of which are feedback mechanisms which would operate to restore basal $[Ca^{2+}]_i$. Consistent with this hypothesis, both PMA and inhibitors of capacitative calcium entry accelerate the rephosphorylation of NFAT1. Indo-l labelling experiments indicate that $[Ca^{2+}]_i$ are restored to resting levels following 5 h of TCR stimulation; as expected from this result, ionomycin stimulation of cells previously stimulated with anti-CD3 for 5 h results in a second round of NFAT1 dephosphorylation.

Although the bulk of the NFAT1 is found in the cytoplasm of cells stimulated for long times with anti-CD3, some NFAT1 DNA-binding activity is still present in the nucleus at late times. The continuing presence of NFAT1 in the nucleus may be explained by several factors that are not mutually exclusive. A low, maintained level of calcineurin activity in TCR-stimulated cells may suffice to retain a small fraction of NFAT1 in the nucleus; the increased levels of Fos and Jun proteins at later times would act to stabilize the NFAT complex; and additional posttranslational modifications of NFAT1 in the nucleus may increase its affinity for DNA or for other nuclear proteins. Although preexisting NFATc translocates rapidly to the nucleus in ionomycin-stimulated spleen cells (44a), nuclear NFATc DNA-binding activity is detected only after two or more hours of TCR stimulation. This may be due to differential regulation of NFATc and NFAT1, de novo synthesis of NFATc following stimulation (41), or the fact that the binding of NFATc to the IL-2 promoter NFAT site is strongly dependent on the presence of Fos and Jun (18). These results, and those for AP-1 (reviewed in reference 20), suggest that the composition of nuclear NFAT-AP1 complexes changes markedly with time after stimulation. The consequent dynamic differences in the interactions among transcription factors are likely to underlie the precise regulation and cell-type-specific expression of cytokine genes.

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