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Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells

(cyclosporin A/FK506/nuclear translocation/DNA binding)

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ABSTRACT **The immunosuppressive drugs cyclosporin A and FK506 interfere with the inducible transcription of cytokine genes in T cells and in other immune cells, in part by preventing the activation of NF-AT (nuclear factor of activated T cells). We show that transcription factor NFAT1 in T cells is rapidly dephosphorylated on stimulation, that dephosphorylation occurs before translocation of NFAT1 into the cell nucleus, and that dephosphorylation increases the affinity of NFAT1 for its specific sites in DNA. Cyclosporin A prevents the dephosphorylation and the nuclear translocation of NFAT1 in T cells, B cells, macrophages, and mast cells, delineating at least one mechanism that contributes to the profound immunosuppressive effects of this compound.**

The nuclear factor of activated T cells (NF-AT), a multisubunit protein, is thought to regulate transcriptional induction of the interleukin 2 (IL-2) gene and other cytokine genes in antigen-stimulated T cells (1–3). An NF-AT binding activity is detected in cytosolic extracts of resting T cells and in nuclear extracts of cells that have been stimulated with T-cell-receptor ligands or with ionomycin (4–7). The immunosuppressive drugs cyclosporin A (CsA) and FK506 block the appearance of this DNA-binding activity in nuclear extracts (8, 9) and inhibit the transcription of several cytokine genes in activated T cells (10–16). The identification of the protein phosphatase calcineurin as the immediate target of the immunosuppressive drugs (17–21) led to the proposal that calcineurin is central in the signal transduction pathway leading to transcription of the IL-2 gene and other cytokine genes in T cells and that CsA and FK506 exert a major portion of their immunosuppressive effect by preventing the nuclear translocation of a cytosolic subunit of NF-AT (3, 22–24). NF-AT may participate in the inducible transcription of cytokine genes in cells other than T cells (1, 2), since CsA and FK506 inhibit cytokine gene induction in B cells, mast cells, natural killer (NK) cells, and eosinophils (25–29), and since an NF-AT binding activity has been demonstrated in extracts of B cells, mast cells, and NK cells (8, 29–34).

A protein (NFAT1/NFATp) that meets the defining criteria of the preexisting cyclosporin-sensitive subunit of NF-AT has been purified from cytosolic extracts of a murine T-cell clone (35), and cDNAs encoding three protein isoforms related by alternative splicing have been isolated (36). Additional family members (NFATc, NFATx/NFAT4, NFAT3) encoded by separate genes have been identified (37–39). The presence of the mRNAs for NFAT1, NFATc, and NFATx/NFAT4 in T cells or thymus (36–39) indicates that these proteins could be involved in controlling the expression of the IL-2 gene and other cytokine genes in T cells. NFAT1 is expressed in certain cells of the immune system in addition to T cells (40) and could contribute to cytokine gene expression in these other cell types.

Here we have used specific antisera against NFAT1 to examine the early steps in its activation, which include changes in its phosphorylation state, its subcellular localization, and its DNA-binding activity.

MATERIALS AND METHODS

Cells. Splenic and peritoneal cells were collected from CB6F1 mice. T-cell blasts (>98% $CD3^+$) were obtained as described (41). Purified splenic B cells (>96% $B220^+$) were obtained by adherence to Petri dishes coated with $(Fab')_2$ fragments of goat anti-mouse IgM (Cappel). Enriched splenic T cells (>77% $CD3^+$ $B220^-$, ≈11% $CD3^-$ $B220^+$, and ≈10% $CD3^-$ $B220^-$) were recovered as cells nonadherent to the goat anti-mouse IgM-coated plates. Mast cells (42) from M. Castells (Dana–Farber Cancer Institute) and NK cells (43) from M. Robertson (Harvard Medical School) were judged to be >95% pure by the laboratories supplying the cells. Ar-5 T cells were maintained in culture as described (44).

Treatment with Ionomycin. Cells, in suspension ($2–10 \times 10^6$ cells per ml) or adhering to glass coverslips coated with poly(D-lysine) [or, for mast cells, with vitronectin (GIBCO)], were equilibrated at 37°C and then treated for 20 s to 20 min with ionomycin. Where indicated, cells were pretreated for 5–20 min with 1 μM CsA or 100 nM FK506 or ethanol, as vehicle control. To allow rigorous control of temperature, pH, and timing in the short incubations of Ar-5 cells, the incubations were carried out in T-cell medium (44) buffered with 10 mM Hepes (pH 7.4) rather than with bicarbonate in a 37°C warmroom. For immunocytochemistry, the incubation was stopped by flooding the coverslip with 3% (wt/vol) paraformaldehyde at 37°C or with ice-cold methanol. For protein samples, cells were collected by brief centrifugation in a microcentrifuge, and the incubation was stopped precisely at the indicated time by addition to the cell pellet of 80 μl of 5% (wt/vol) SDS/30 mM sodium pyrophosphate/5 mM EDTA/2 mM phenylmethylsulfonyl fluoride, and in some cases also containing 250 μM leupeptin, aprotinin (100 μg/ml), and 5 mM iodoacetamide.

Immunocytochemistry. NFAT1 was stained with affinity-purified anti-67.1 antiserum (45) followed by Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch). The primary antiserum recognizes a peptide located near the N terminus of NFAT1 that is absent from NFATc, NFATx/NFAT4, and NFAT3 (37–39). Other staining reagents were fluorescein-anti-CD3ε (clone 145-2C11; PharMingen) and fluorescein-anti-CD45R/B220 (clone RA3-6B2; PharMingen).

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Abbreviations: NF-AT, nuclear factor of activated T cells; IL-2, interleukin 2; CsA, cyclosporin A; NK, natural killer.

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Western Blot Analysis. NFAT1 in cell lysates, immunoprecipitates, and extracts was detected by Western blot analysis with the affinity-purified anti-67.1 antiserum (40, 45).

^{32}P Labeling. Ar-5 T cells were incubated overnight at 37°C with [^{32}P]orthophosphate at 0.25–0.5 mCi/ml (1 Ci = 37 GBq) in phosphate-free medium. The cells were then washed twice, stimulated as indicated, and lysed (4×10^6 cells per ml) in RIPA buffer (50 mM Tris·HCl, pH 8.0/150 mM NaCl/1% Triton X-100/1% deoxycholate/0.1% SDS/5 mM EDTA) with protease inhibitors [2 mM phenylmethylsulfonyl fluoride/10 mM iodoacetamide/aprotinin (100 μg/ml)/25 μM leupeptin] and phosphatase inhibitors (50 mM NaF/1 mM Na orthovanadate/30 mM sodium pyrophosphate). DNA was sheared by repeated passage through a 23-gauge needle, the lysate was centrifuged, and the supernatant was recovered and precleared with protein A-Sepharose. NFAT1 was immunoprecipitated for 3 h at 0°C with affinity-purified anti-67.1 antiserum and protein A-Sepharose. The precipitate was washed four times with RIPA buffer, and immunoprecipitated proteins were solubilized by boiling in 5% SDS.

Electrophoretic Mobility Shift Assay. Ar-5 T cells (250×10^6 cells) were incubated with medium alone or with 3 μM ionomycin for 5 min at 37°C. Extracts were prepared by centrifuging the cell suspension for 5 min at 1500 rpm in a TH-4 rotor at 6°C; resuspending the pellet in 600 μl of RSB (10 mM NaCl/3 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4) with 2 mM dithiothreitol, 5 mM EGTA, protease inhibitors [80 μM leupeptin/aprotinin (40 μg/ml)/8 mM phenylmethylsulfonyl

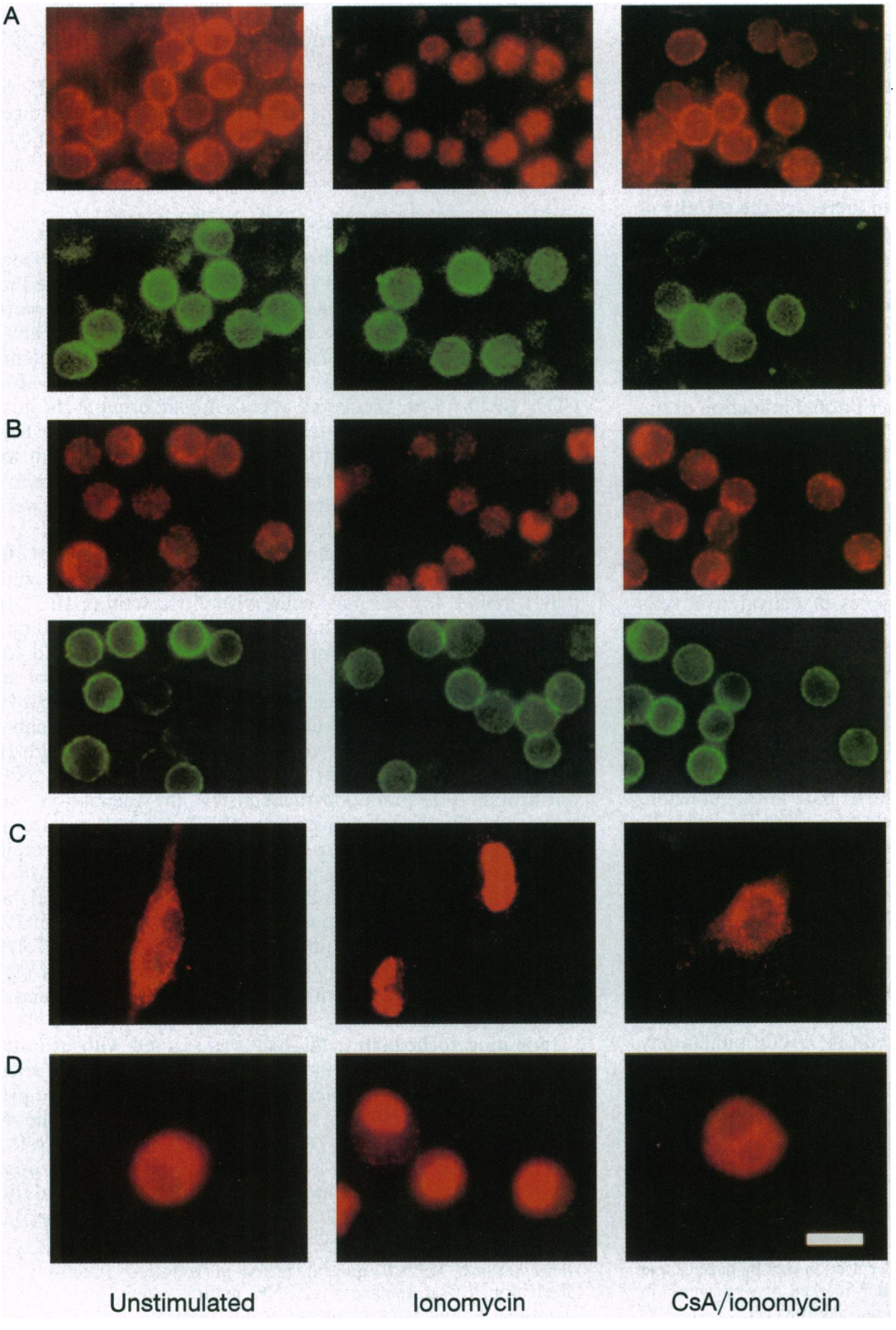

FIG. 1. Immunocytochemical staining of murine T cells, B cells, macrophages, and mast cells for NFAT1 and for other markers as indicated. (*A*) Splenic T cells were stained with anti-NFAT1 (*Upper*) and anti-CD3 (*Lower*). Splenic lymphocyte populations were not purified for this experiment, since separate staining experiments showed that the lymphocytes could be unambiguously classified as $CD3^+$ $B220^-$ or $B220^+$ $CD3^-$. (*B*) Splenic B cells were stained with anti-NFAT1 (*Upper*) and anti-B220 (*Lower*). (*C*) Resident peritoneal macrophages. Only a small fraction of resident peritoneal macrophages, but a majority of splenic macrophages, stained specifically with anti-NFAT1. (*D*) Bone marrow-derived mast cells. Ionomycin concentration was 1 μM for T cells and B cells, 10 μM for macrophages, and 0.3 μM for mast cells. Treatment was for 20 min. When the anti-NFAT1 antiserum was mixed with its corresponding peptide before the immunocytochemical incubation (45), there was little or no staining of the cells. To score specific staining as cytoplasmic or nuclear, the same fields were examined by phase-contrast microscopy. The diffuse orange staining that appears to coincide with the nucleus in some resting cells and that is particularly noticeable in macrophages and mast cells is due to staining of cytoplasm above and below the plane of focus. (Bar = 10 μm.)

fluoride], and phosphatase inhibitors (120 mM sodium pyrophosphate/20 mM EDTA); adding 600 μl of high salt extraction buffer [1 M KCl/25 mM $MgCl_2$/100 mM Hepes·KOH, pH 7.4/20% (vol/vol) glycerol/5 mM EGTA]; and incubating 30 min at 0°C. The extract was diluted with 1.2 ml of distilled deionized water at 0°C, centrifuged 6 min at 1500 rpm in a TH-4 rotor at 6°C, divided into aliquots, and rapidly frozen for storage at −80°C. Extracts were examined in an electrophoretic mobility shift assay with a ^{32}P-labeled oligonucleotide corresponding to the distal NF-AT site of the murine IL-2 promoter (7). The ability of specific antiserum to recognize the protein–DNA complexes was tested by addition of anti-67.1 antiserum (40, 45) or anti-72 antiserum (36) to the incubation as described (36, 45).

RESULTS AND DISCUSSION

We examined the presence and subcellular localization of NFAT1, in cells prepared from murine spleen, peritoneal cavity, and bone marrow, by immunocytochemical staining. The cells were freshly isolated or were maintained for only a short period in culture, thus avoiding the potential problem of inappropriate expression, phosphorylation, or regulation of NFAT1 in transformed cell lines. NFAT1 resides in the cytoplasm in unstimulated T cells and B cells from murine spleen and in splenic and resident peritoneal macrophages (Fig. 1). NFAT1 is also cytoplasmic in the vast majority of bone marrow-derived mast cells, although nuclear staining that could represent activation of the cells on attachment to a substrate was observed in a few percent of the cells. Stimulation with ionomycin resulted in the translocation of NFAT1 into the cell nucleus in all the cells examined, and this translocation was prevented by treatment of the cells with physiologically active concentrations of CsA.

Treatment of the cells with ionomycin led to an early biochemical change in NFAT1 detectable by Western blot analysis. NFAT1 in extracts from resting immune cells migrated as a single protein band with an apparent molecular mass of ≈140 kDa on an SDS/polyacrylamide gel, while NFAT1 in extracts from ionomycin-stimulated cells migrated as a distinct band or a doublet of increased mobility corresponding to an apparent molecular mass of ≈120 kDa (Fig. 2*A*). The ionomycin-induced change in the electrophoretic mobility of NFAT1 was apparent within a few minutes of the addition of ionomycin and was similar in murine T-cell blasts, purified murine splenic T cells, purified murine splenic B cells, purified murine splenic adherent macrophages, murine bone-marrow-derived mast cells, and human NK cells (Fig. 2*A* and data not shown). In each case this protein modification was prevented by pretreatment of the cells with 1 μM CsA.

The murine T-cell clone Ar-5 (44) was used for a more detailed examination of the timing and nature of this early biochemical effect. Fig. 2*B* shows that the change in mobility of NFAT1 was well under way by 1 min (*Left*, lane 2), was complete by 2 min (lane 3), and persisted in the continuing presence of ionomycin (lanes 4 and 5). The effect produced by ionomycin appeared to be a reversible protein modification, since the electrophoretic mobility of NFAT1 reverted to that characteristic of resting cells within a few minutes when the cells were returned to medium lacking ionomycin (data not shown). The change in electrophoretic mobility was blocked by treatment of the cells with 1 μM CsA or with 100 nM FK506 (Fig. 2*B*). Concentration-dependence experiments indicated that the change in mobility was largely blocked at concentrations of CsA and FK506 (100 nM and 1 nM, respectively) similar to those required for inhibition of cytokine production in these cells, while it was not inhibited by rapamycin, an analogue of FK506 that binds to FKBP12 but that does not inhibit calcineurin (17, 18), at concentrations up to 1 μM (data not shown).

The protein modification observed in ionomycin-treated cells is a dephosphorylation. Ar-5 T cells were labeled with [^{32}P]orthophosphate, NFAT1 was immunoprecipitated from whole-cell extracts, and the resulting precipitate was analyzed after SDS/polyacrylamide gel electrophoresis and transfer to nitrocellulose. Recovery of NFAT1 was assessed by using the anti-peptide antiserum (Fig. 2*C Left*), and radioactivity in the NFAT1 bands was detected by PhosphorImager scanning (Fig. 2*C Right*). Similar amounts of immunoreactive protein were present in the immunoprecipitates from untreated cells, from

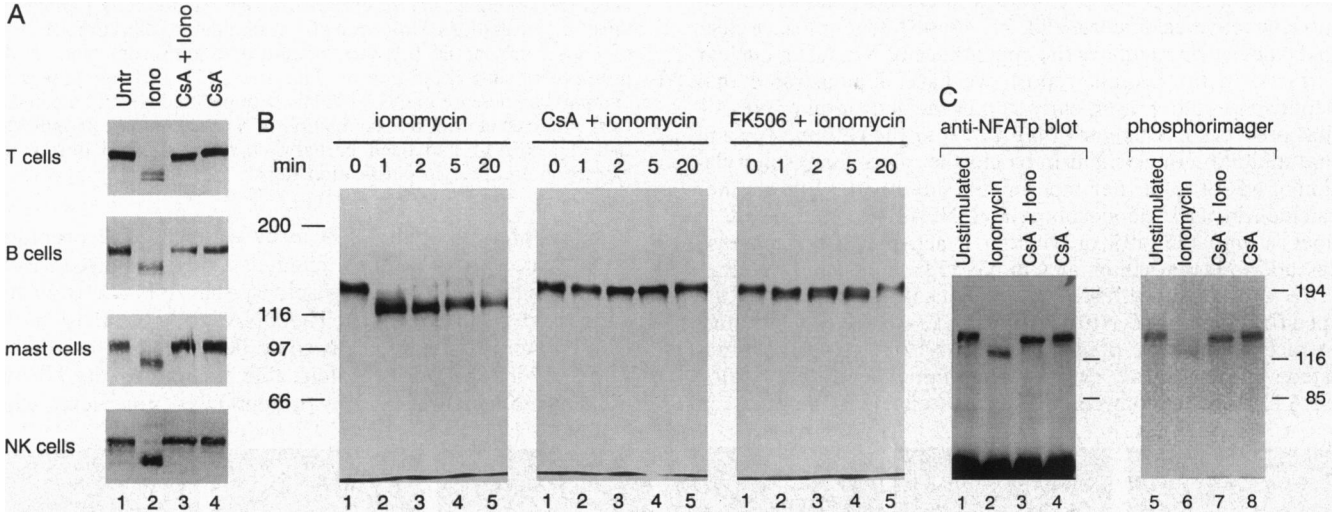

FIG. 2. SDS/polyacrylamide gel electrophoresis of NFAT1 in lysates of unstimulated and stimulated cells. (*A*) Western blot analysis of cell lysates from splenic T cells, splenic B cells, bone marrow-derived mast cells, and NK cells, using an antiserum against NFAT1. The samples were from untreated (Untr) cells (lanes 1) and from cells treated for 5 min with 1 μM ionomycin (lanes 2), with 1 μM ionomycin in the presence of 1 μM CsA (lanes 3), or with 1 μM CsA alone (lanes 4). (*B*) Western blot analysis of NFAT1 in lysates of Ar-5 T cells after various periods of treatment with ionomycin. Cells were preincubated with medium alone (*Left*), with 1 μM CsA (*Middle*), or with 100 nM FK506 (*Right*), and then lysed in SDS without further incubation (lanes 1) or after 1 min, 2 min, 5 min, or 20 min of incubation with 1 μM ionomycin (lanes 2–5, respectively). (*C*) Analysis of NFAT1 immunoprecipitated from extracts of ^{32}P-labeled Ar-5 T cells. The T cells were preincubated with or without 1 μM CsA and then left unstimulated or treated with 1 μM ionomycin for 5 min. Each lane contains the protein immunoprecipitated from 10^7 cells. (*Left*) Probed with anti-NFAT1 antiserum. (*Right*) PhosphorImager scan of the same membrane.

cells treated with CsA prior to ionomycin stimulation, and from cells treated with CsA alone (Fig. 2*C*, lanes 1, 3, and 4), and the radioactivity in NFAT1 was comparable in these lanes (Fig. 2*C*, lanes 5, 7, and 8). In general somewhat less immunoreactive protein was recovered from the same number of cells after ionomycin treatment (Fig. 2*C*, lane 2), ranging in different experiments from ≈60% to 100% of the amount extracted from unstimulated cells, probably due to less efficient extraction of NFAT1 from the cell nucleus. However, treatment with ionomycin invariably resulted in a decrease in ^{32}P label in NFAT1 that could not be accounted for solely by the modest reduction in the amount of protein (Fig. 2*C*, lane 6). CsA prevented the mobility change and the loss of radiolabel in cells treated with ionomycin (Fig. 2*C*, lane 7). In all experiments there was detectable radiolabel in the ≈120-kDa NFAT1 band from stimulated cells, indicating that the physiological dephosphorylation of NFAT1 is a partial dephosphorylation.

The timing of nuclear translocation in the murine T-cell clone Ar-5 was examined under conditions of stimulation identical to those used in the biochemical experiments. In resting Ar-5 T cells, immune staining of NFAT1 was restricted to the cytoplasm (Fig. 3). NFAT1 was also localized in the cytoplasm of Ar-5 T cells at 20 s or 1 min of stimulation with 1 μM ionomycin, while little or no NFAT1 was detected in the nucleus of most cells. At 1 min, occasional scattered cells were found with weak to moderate nuclear staining in addition to the cytoplasmic staining. Comparison with Fig. 2*B* (*Left*, lane 2) shows that there is substantial dephosphorylation at a time when the protein is still located in the cytoplasm. Cultures fixed at 2 min showed variability in staining, with relatively little nuclear staining in some experiments (as in Fig. 3) and with a majority of cells having predominantly nuclear staining in other experiments. All detectable NFAT1 was localized to the nucleus at 5 min and remained in the nucleus for at least 60 min in the continuing presence of ionomycin (Fig. 3 and data not shown). Like the dephosphorylation, the nuclear translocation was blocked by treatment of the cells with 1 μM CsA or 100 nM FK506 but was not affected by treatment with 1 μM rapamycin (data not shown).

Studies of the mechanism of action of the immunosuppressive drugs (4–6, 8–19) and studies of cells expressing constitutively active calcineurin (20, 21, 46–48) have made it clear that calcineurin regulates the appearance of NF-AT in nuclear extracts. In the present report, we have demonstrated that dephosphorylation is an early step in the activation of NF-AT that precedes movement of NFAT1 into the cell nucleus and that inhibition of calcineurin blocks the rapid dephosphorylation of NFAT1. Further experiments should establish whether calcineurin itself dephosphorylates NFAT1 in the cell, as it does *in vitro* (35, 40), or whether it activates a phosphatase cascade (49) that culminates in NFAT1 dephosphorylation.

To explore the effect of physiological stimulation on the specific binding of NFAT1 to DNA, we prepared native extracts containing phosphorylated NFAT1 from unstimulated Ar-5 cells and extracts containing dephosphorylated NFAT1 from ionomycin-stimulated cells (Fig. 4*A*). In an electrophoretic mobility shift assay with the IL-2 promoter NF-AT site, NFAT1 from stimulated T cells displayed a 5- to 15-fold higher DNA-binding activity than NFAT1 from unstimulated T cells (Fig. 4*B*). The DNA-binding activity in the extracts from stimulated cells could be attributed to NFAT1, rather than to another protein able to bind to the NF-AT oligonucleotide, because the protein–DNA complexes were

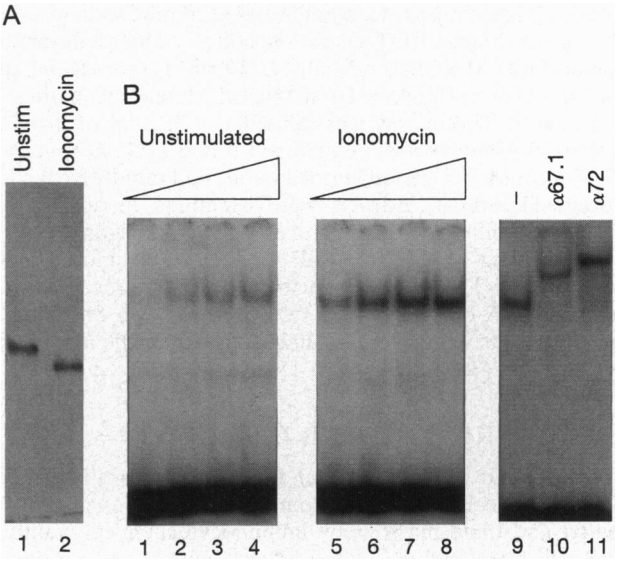

FIG. 4. DNA-binding activity of NFAT1 in extracts from unstimulated and stimulated cells. (*A*) Western blot analysis of NFAT1 in extracts from unstimulated Ar-5 T cells (lane 1) or from cells treated with 3 μM ionomycin (lane 2). The NFAT1 bands in the extracts aligned with the bands in corresponding SDS lysates prepared as in Fig. 2*B* (data not shown), indicating that the phosphorylation state of the protein was preserved during extraction. Separate titrations by Western blot analysis had indicated that NFAT1 was 3-fold more concentrated in the extract from unstimulated cells in this experiment, and to give comparable band intensities in this gel, 72 μg of protein was loaded in lane 1 and 200 μg of protein was loaded in lane 2. (*B*) Electrophoretic mobility shift assay of the extracts in *A*. Lanes: 1–4, incubations with 2 μl, 3 μl, 4 μl, and 5 μl of extract from unstimulated cells, respectively; 5–8, incubations with the same amount of extract from stimulated cells. Total extract protein added was 36 μg (lane 4) and 33 μg (lane 8), and hence ≈3-fold more NFAT1 was present in corresponding lanes in the extract from unstimulated cells (see *A*). The binding of extract from stimulated cells in this experiment, determined from the PhosphorImager scan as the fraction of probe migrating in the NFAT1–DNA complex, ranged from 3.1 to 4.3 times the binding in the corresponding lane of extract from unstimulated cells, indicating an ≈10-fold difference in DNA-binding activity after adjusting for the lower yield of NFAT1 in the extracts from stimulated cells. Lanes 9–11 illustrate incubations with extract from stimulated cells in the absence of antiserum, in the presence of anti-67.1 antiserum, and in the presence of anti-72 antiserum. Jain *et al.* (35) did not observe a comparable increase in DNA binding with purified NFAT1 treated *in vitro* with calcineurin. Reexamination of figure 1a in ref. 35 indicates that the NFAT1 had been partially dephosphorylated during the extraction and protein purification steps.

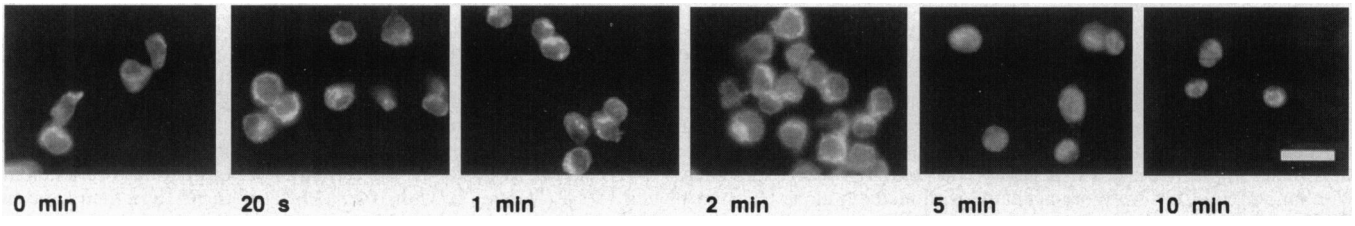

FIG. 3. Immunocytochemical staining of Ar-5 T cells with an antiserum against NFAT1. Cells were untreated (0 min) or stimulated with 1 μM ionomycin for the periods indicated. In the experiment illustrated, fixation was with 3% paraformaldehyde, but identical results were obtained after fixation with ice-cold methanol. (Bar = 15 μm.)

recognized by two specific anti-NFAT1 antisera (Fig. 4*B*, lanes 10 and 11). Treatment of the cells with CsA did not change the binding in extracts from unstimulated cells but did prevent the increase in binding on stimulation with ionomycin (data not shown). These data complement the demonstration that *in vitro* dephosphorylation increases the DNA-binding activity of NFAT1 extracted from CsA-treated cells (50). We conclude that the physiological dephosphorylation of NFAT1 or of a protein associated with NFAT1 in the extracts contributes to activation by increasing the binding of NFAT1 to DNA.

These experiments establish that the cyclosporin-sensitive transcription factor NFAT1 is rapidly dephosphorylated in response to stimulation in T cells and in other immune cells. Several observations suggest that this dephosphorylation could have an essential role in the activation of NFAT1. (*i*) The dephosphorylation is prevented by immunosuppressive drugs. (*ii*) In T cells the partial dephosphorylation of NFAT1 precedes nuclear import, consistent with the hypothesis (22–24) that dephosphorylation of NFAT1 is necessary for nuclear translocation. (*iii*) Dephosphorylation of NFAT1 or an associated protein increases the specific binding of NFAT1 to DNA. In extending this work, it will be important to determine whether the subcellular localization and the DNA binding of NFAT1 are controlled mainly by phosphorylation of certain residues in NFAT1, by complex formation with another protein, or by both mechanisms.

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