Dynamic equilibrium between calcineurin and kinase activities regulates the phosphorylation state and localization of the nuclear factor of activated T-cells

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The nuclear factor of activated T-cells (NFAT_p) is a phosphorylated transcription factor that resides in the cytoplasm of unactivated T-cells. T-cell activation results in the activation of the phosphatase calcineurin (CaN), which leads to the dephosphorylation and subsequent nuclear localization of NFAT_p. We have investigated the role of kinases in the phosphorylation state and subcellular localization of NFAT_p. The phosphorylation state and nuclear/cytoplasmic location of NFAT_p were determined in unstimulated murine HT-2 cells treated with a panel of kinase inhibitors. Two of the seven kinase inhibitors, staurosporine (St) and bisindolylmaleimide I (BI), resulted in the dephosphorylation and nuclear localization of NFAT_p. These St-induced effects were inhibited by pretreatment with FK506,

indicating that CaN activity was required for the observed effects on NFAT_p. Treatment of cells with ionomycin resulted in NFAT_p dephosphorylation and nuclear localization. Removal of ionomycin from the cells resulted in the reappearance of phosphorylated NFAT_p in the cytosol. St and BI also inhibited the re-accumulation of NFAT_p in the cytoplasm and its re-phosphorylation after ionomycin removal. The re-accumulation of NFAT_p in the cytosol after ionomycin withdrawal was shown to be energy- and temperature-dependent. Taken together, these results suggest that in unstimulated cells NFAT_p is actively maintained in the cytoplasm by kinases acting in opposition to basal CaN activity.

INTRODUCTION

Antigen-induced T-cell activation results in the transcriptional activation of a variety of cytokine genes, including interleukin 2, interleukin 4 and colony-stimulating factor. Activation of these genes is mediated in part by nuclear factor of activated T-cells (NFAT), a DNA-binding protein that binds to specific sites on the regulatory regions of these genes (reviewed in [1,2]). Molecular cloning studies have demonstrated that at least four different NFAT genes exist, including NFAT_p, NFAT_c, NFAT3 and NFAT_x, all of which contain a Rel homology region that is important for DNA binding [3–6]. In addition, a serine/proline repeat region of unknown function is present in each of the family members, as well as a putative nuclear localization sequence. The exact biological function of each of the NFAT proteins is not known, although differences in cell and tissue expression, as well as DNA-binding activity, have been observed [3,7,8].

Studies with the immunosuppressant drugs cyclosporin A (CsA) and FK506, which inhibit T-cell activation, have provided insight into the molecular mechanism of NFAT action. These drugs, when bound to their respective immunophilins, cyclophilin, and FKBP-12, inhibit the activity of calcineurin (CaN), a calmodulin-dependent phosphatase [9,10]. The ability of a number of FK506 and CsA analogues to inhibit CaN phosphatase activity is correlated with their ability to inhibit T-cell activation, consistent with the hypothesis that CaN is a cellular target of the drugs. In addition, overexpression of a constitutively active form of CaN augments T-cell activation resulting from stimulation by both Ca²⁺ and protein kinase C (PKC), suggesting that CaN has a key role in the physiological pathway of T-cell activation [11–13].

The link between CaN and NFAT_p (also called NFATC2 [6] and NFAT1 [14]) is provided by evidence demonstrating that NFAT_p is a CaN substrate. Using immunocomplex assays we have shown that NFAT_p is a direct CaN substrate *in vitro* [15]. In addition, a direct association between CaN and NFAT_p has been demonstrated with immobilized CaN bound to calmodulin–Sepharose, and the site of interaction on NFAT_p has been mapped to the N-terminal 415 amino acid residues [14,16]. In intact HT-2 cells we demonstrated directly, by using ³²P-labelling experiments, that NFAT_p is present in a phosphorylated state in the cytosol of unactivated T-cells. Furthermore, the activation of cells after treatment with ionomycin results in dephosphorylation of NFAT_p that is inhibited by pretreatment with either CsA or FK506 [15,17].

The level of NFAT_p phosphorylation in cells is dependent on CaN activity but, in addition, the phosphorylation state of NFAT_p is a determinant of its localization within the cell. In our previous studies we demonstrated that ionomycin treatment of HT-2 cells results in the dephosphorylation and nuclear localization of NFAT_p; after the removal of ionomycin, NFAT_p reappears in the cytosol in a phosphorylated form. Pretreatment of the cells with FK506 inhibits the ionomycin-induced NFAT_p nuclear localization, and under these conditions NFAT_p remains in the cytosol in a phosphorylated form [15]. Loh et al. [14] demonstrated similar results in immunofluorescence experiments, and in addition they showed that repeated cycles of ionomycin treatment followed by washout result in the successive phosphorylation and dephosphorylation of NFAT_p.

In the studies reported here we have examined the conditions regulating NFAT_p localization. We show that in unstimulated cells, treatment with the kinase inhibitors staurosporine (St) and

Abbreviations used: BI, bisindolylmaleimide I; CaN, calcineurin; CsA, cyclosporin A; NES, nuclear export sequence; NLS, nuclear localization sequence; NFAT_p, nuclear factor of activated T-cells; PKC, protein kinase C; PKI, protein heat-stable inhibitor of cAMP-dependent kinase; St, staurosporine.

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bisindolylmaleimide I (BI) results in NFAT_p dephosphorylation and nuclear localization. Furthermore, the reaccumulation of NFAT_p into the cytosol, which normally occurs after ionomycin withdrawal, is also inhibited by treatment with St and BI. These results suggest a model in which a dynamic equilibrium exists between kinase(s) and CaN that determines the net phosphorylation state and subcellular location of NFAT_p in resting as well as activated T-cells.

MATERIALS AND METHODS

Materials

FK506 was prepared at Pharmacia & Upjohn from fermentation broths of *Streptomyces tsukubaensis* (Upjohn Culture Collection 11052). Ionomycin, PMA and kinase inhibitors were obtained from Calbiochem. Deoxyglucose was obtained from Sigma. Enhanced chemiluminescence reagents were obtained from Amersham. The PKC isoform-specific monoclonal antibodies were from Transduction Laboratories. The PKC β and PKC δ antibodies were a gift from Dr. Susan Jaken (W. Alton Jones Cell Science Center, Lake Placid, NY, U.S.A.).

Cell culture

The murine helper T-cell line HT-2 was grown exactly as described [15].

Cellular fractionation

Treatment of HT-2 cells (10^6 per condition) was performed as described in the Figure legends. Cells were washed in PBS and lysed in buffer (10 mM Tris/HCl, pH 7.5, containing 10 mM NaCl, 3 mM MgCl₂, 1 mM PMSF, 0.5 mM dithiothreitol, 0.1 mM EGTA, 2 μ M leupeptin, 1 μ g/ml aprotinin and 0.05 % Nonidet P40) as described [15]. Lysed cells were centrifuged at 650 *g* to pellet the nuclei; the supernatant was removed. The nuclear pellet was washed in lysis buffer without detergent, and the final pellet was resuspended in Laemmli buffer. The low-speed supernatant containing the cytosol and non-nuclear membranes (referred to as cytosol in the Figures) was re-centrifuged to remove any residual nuclei. Equal cell equivalents (4×10^5 cells) of soluble and nuclear fractions were subjected to electrophoresis on SDS/6 % (w/v) polyacrylamide gels, and Western blotting was performed as described below.

Western blots

Cells were treated as indicated in the Figure legends; control cells received DMSO as a vehicle control. For control and ionomycin-treated lysates, cells were left untreated or treated with 2 μ M ionomycin for 10 min at 37 °C respectively. After treatment the cells were washed with ice-cold PBS and resuspended in 40 mM Tris/HCl, pH 7.5, containing 10 mM EDTA and 60 mM sodium pyrophosphate. SDS was added to bring the final concentration to 5 % (w/v). Alternatively, the cells were lysed directly into Laemmli buffer. The sample was boiled for 15 min and 4×10^5 cell equivalents were subjected to electrophoresis on an SDS/6 % polyacrylamide gel. Western blot analysis was performed as described by using affinity-purified NFAT_p antibody [15], and detection was by the enhanced chemiluminescence system. For PKC Western blots, cell lysates (2.5×10^5 cell equivalents) or fractions (4×10^5 cell equivalents) were subjected to electrophoresis on SDS/10 % polyacrylamide gels and blotted as above with PKC isoform-specific monoclonal antibodies and sheep anti-mouse IgG conjugated to horseradish peroxidase (Amersham).

Withdrawal of ionomycin

For ionomycin withdrawal experiments, cells were treated in bulk with 2 μ M ionomycin for 10 min at 37 °C, pelleted and then resuspended in ice-cold PBS. The cells were then split into a separate tube for each treatment and pelleted. The cell pellet was then resuspended at 2×10^5 cells/ml in complete growth medium at 37 °C. For experiments involving kinase inhibitors, DMSO (vehicle control) or kinase inhibitor was added to the growth medium before resuspension.

RESULTS

Effects of kinase inhibitors on NFAT_p phosphorylation and localization

In our previous studies with HT-2 cells we demonstrated that the phosphorylation state of NFAT_p is a predictor of its cellular localization: phosphorylated NFAT_p is cytosolic, whereas dephosphorylated NFAT_p is localized to the nucleus [15]. These results suggest that the net activity of kinases and phosphatases determines the location as well as the phosphorylation state of NFAT_p. CaN has been shown to dephosphorylate NFAT_p but the identity of the NFAT_p kinase(s) is not known. To identify potential pharmacological tools useful in characterizing the NFAT_p kinase(s), we treated HT-2 cells with various kinase inhibitors followed by cellular fractionation and Western blotting for NFAT_p. We previously established in ³²P-labelling experiments [15] that phosphorylated and dephosphorylated NFAT_p can be distinguished on the basis of the marked change in mobility under SDS/PAGE that accompanies dephosphorylation (Figure 1, arrows). The kinase inhibitors were chosen to target different enzyme specificities, including tyrosine kinases (B46) and serine/threonine kinases, including calmodulin-dependent kinase (KN93), cAMP-dependent kinase (H-89), cGMP-dependent protein kinase (KT5823), myosin light-chain kinase (KT5926) and protein kinase C (St and BI). Inhibitors were used at 10 μ M, a concentration that has been shown to be effective in other studies on intact cells [18]. As previously observed, in untreated cells NFAT_p is phosphorylated and resides in the cytosol. Surprisingly, treatment of the unactivated HT-2 cells with either St or BI, but with none of the other kinase inhibitors, resulted in the dephosphorylation of NFAT_p; the dephosphorylated NFAT_p was localized to the nucleus (Figure 1A). The effect of St was complete, i.e. no detectable NFAT_p remained in the cytosol after treatment with St. Treatment with BI resulted in nuclear localization of the majority of NFAT_p; however, a small but detectable level of dephosphorylated NFAT_p remained in the cytosol. Staurosporine (St) inhibits serine/threonine kinases and has a limited specificity for PKC. BI is an St analogue that shows specificity for PKC (K_i 0.01 μ M); however, the concentration used here, 10 μ M, is also above the IC₅₀ values for the inhibition of other kinases (IC₅₀ values of 0.7 μ M for phosphorylase kinase, 2.0 μ M for protein kinase A) [19]. Thus these results are consistent with the hypothesis that one or more serine/threonine kinases, possibly PKC, is involved in phosphorylating NFAT_p. Importantly, the results also clearly demonstrate that the kinase(s) is/are critical in maintaining NFAT_p in its phosphorylated, cytosolic form, even in unactivated cells.

Inhibition by FK506 of St-induced NFAT_p dephosphorylation and localization

In the presence of St or BI, NFAT_p was dephosphorylated, suggesting that a basal level of cellular phosphatase activity

Figure 1 Treatment of HT-2 cells with BI and St results in the dephosphorylation and nuclear localization of NFAT_p

HT-2 cells were treated, and low-speed supernatant (referred to as cytosol) and nuclear fractions were prepared and Western blotted as described in the Materials and methods section. Western blots of cytosol (upper panels) and nuclei (lower panels) are shown. Lanes 1 and 2 contained cell lysates of untreated and ionomycin-treated cells respectively as controls. The heavy arrow indicates the phosphorylated form of NFAT_p; the light arrow indicates the dephosphorylated form. The kinase inhibitors were used at 10 μ M, FK506 at 500 nM and ionomycin at 2 μ M. (A) Cells were untreated (lane 3) or treated with DMSO (lane 4), BI (lane 5), H-89 (lane 6), KT5823 (lane 7), KT5926 (lane 8), KN93 (lane 9), St (lane 10) or B46 (lane 11). (B) Cells were pretreated for 15 min and then treated for 15 min with the indicated compounds: FK506 pretreatment only (lane 3), ionomycin (I) treatment only (lane 4), FK506 then ionomycin (lane 5), ionomycin then FK506 (lane 6), St treatment only (lane 7), FK506 then St (lane 8) or St then FK506 (lane 9).

exists in opposition to kinase activity. To determine whether CaN could account for the phosphatase activity in unactivated cells, HT-2 cells were treated with a combination of FK506,

Figure 2 Inhibition by BI and St of cytosolic accumulation of NFAT_p after withdrawal of ionomycin

HT-2 cells were treated with ionomycin, washed with PBS and resuspended in growth medium containing DMSO (lanes 3 and 4) or 10 μ M BI (lanes 5 and 6), H-89 (lanes 7 and 8), KT5823 (lanes 9 and 10), KT5926 (lanes 13 and 14), KN93 (lanes 15 and 16), St (lanes 17 and 18) or B46 (lanes 19 and 20). Cells were removed at the indicated time points, fractionated into cytosol and nuclei and then Western blotted as described in the Materials and methods section. Lanes 1 and 2 contained cell lysates of untreated and ionomycin-treated cells respectively as controls. The heavy arrow indicates the phosphorylated form of NFAT_p; the light arrow indicates the dephosphorylated form. Cytosol (A) and nuclei (B) are shown from cells incubated with DMSO (control) and the indicated kinase inhibitor for either 0 min or 45 min after withdrawal of ionomycin, as indicated.

ionomycin or St (Figure 1B). Ionomycin treatment results in NFAT_p dephosphorylation by activating CaN; pretreatment with FK506, which inhibits CaN, blocked dephosphorylation and nuclear localization (Figure 1B, lane 5). Treatment with FK506 after treatment with ionomycin also reversed ionomycin-stimulated NFAT_p dephosphorylation and nuclear localization (Figure 1B, lane 6), although treatment of cells with FK506 alone did not alter NFAT_p phosphorylation or localization (Figure 1B, lane 3). In cells pretreated with FK506 for 15 min before treatment with St, the St-induced dephosphorylation and nuclear localization of NFAT_p were blocked (Figure 1B, lane 8). Unlike the reversal of ionomycin effects by FK506, treatment with FK506 after treatment with St did not reverse the St-induced nuclear localization (Figure 1B, lane 9). This result demonstrates that St does not act by activating CaN indirectly; rather, St acts directly to inhibit NFAT_p phosphorylation. FK506 pretreatment reversed the effect of St, indicating that CaN is active in resting HT-2 cells. Taken together, these results demonstrate that even in unactivated T-cells the basal activities of kinases and of the phosphatase CaN contribute to the phosphorylation state of NFAT_p and thus to its localization within the cell.

Figure 3 Titration of St concentration in ionomycin withdrawal experiment

HT-2 cells were treated with ionomycin, washed with PBS and resuspended in growth medium containing DMSO (lanes 3 and 4) or St at 10 μ M (lanes 5 and 6), 1 μ M (lanes 7 and 8), 0.1 μ M (lanes 9 and 10) or 0.01 μ M (lanes 11 and 12). Cells were removed at the indicated time points, fractionated into cytosol and nuclei and Western blotted as described in the Materials and methods section. Lanes 1 and 2 contained cell lysates of untreated and ionomycin-treated cells respectively as controls. Cytosol (upper panel) and nuclei (lower panel) are shown from cells incubated with DMSO (control) or St at the indicated concentrations for either 0 min or 45 min after ionomycin withdrawal, as indicated.

Effects of kinase inhibitors on the cytoplasmic re-localization of NFAT_p

The inhibitor experiments demonstrated that kinase activity regulates NFAT_p translocation to the nucleus from the cytosol. A potential role of kinase activity in regulating NFAT_p transit from the nucleus back into the cytosol was investigated. Cells were treated with ionomycin, which causes the nuclear localization of NFAT_p, followed by washout and resuspension in complete medium containing the same kinase inhibitors used in the experiment of Figure 1 (Figure 2). After incubation for 45 min, the cellular localization of NFAT_p was measured. Removal of ionomycin and resuspension of the cells in medium alone results in the reappearance of NFAT_p in the cytosol (Figure 2, lane 4) [15]. Furthermore, the cytosolic NFAT_p was exclusively in the phosphorylated form. In cells treated with either 10 μ M BI or St, NFAT_p cytosolic accumulation was blocked and NFAT_p remained in the nucleus in a dephosphorylated state. Complete inhibition was observed with 10 μ M St, whereas 1.0 μ M St partly inhibited, and 0.1 and 0.01 μ M St did not block nuclear export (Figure 3). BI inhibited significantly, but not completely, because in the BI-treated cells some dephosphorylated NFAT_p was also detected in the cytosol at 45 min. As with NFAT_p localization to the nucleus, none of the other kinase inhibitors had any effect on NFAT_p reaccumulation in the cytosol after ionomycin removal. These results demonstrate that the reaccumulation in the cytosol and rephosphorylation of NFAT_p are clearly inhibited by St and BI. Furthermore, they establish that kinase activity regulates NFAT_p when it is in both the cytosolic and nuclear compartments.

Expression and distribution of PKC isoforms

Our experiments indicate that one or more serine/threonine kinase(s) are involved in NFAT_p phosphorylation, but little

Figure 4 PMA treatment of HT-2 cells does not inhibit NFAT_p nuclear export

(A) HT-2 cells were treated with DMSO [control (C)] (lanes 1 and 3) or ionomycin (I) (lanes 2 and 4), washed with PBS and fractionated into non-nuclear (cyt) (lanes 1 and 2) or nuclear (nuc) (lanes 3 and 4) fractions. Aliquots were Western blotted with isoenzyme-specific PKC antibodies. (B) Cells were treated with 500 nM PMA for 72 h (P, lane 2) or left untreated (C, lane 1). Cells were washed with PBS and lysed, and aliquots were Western blotted with isoenzyme-specific PKC antibodies. PKC α , ϵ and θ levels also were down-regulated (results not shown). (C) HT-2 cells were treated with 500 nM PMA for 72 h or left untreated (control). Cells were then treated with ionomycin, washed with PBS and resuspended in growth medium. Cells were removed at the indicated time points, fractionated into a non-nuclear low-speed fraction (cytosol) and nuclei, and then Western blotted as described in the Materials and methods section. Lanes 1 and 2 contained cell lysates of untreated and ionomycin-treated cells respectively as controls.

additional information is known about the kinase(s). Because St and BI both inhibit PKC, and PKC is involved in T-cell activation, we investigated a possible role for PKC in regulating the NFAT_p phosphorylation state. PKC isoenzyme expression was characterized in HT-2 cells by using Western blot analysis and isoenzyme-specific antibodies (Figure 4A). Localization of the isoforms was also examined, because differences exist in their cellular locales [20–24]. Seven different PKC isoforms were expressed, including α , δ , ϵ , ζ , θ , ι and μ . All of the isoenzymes, with the exception of ζ , were localized to the non-nuclear fraction;

Figure 5 Temperature dependence of cytosolic accumulation of NFAT_p after withdrawal of ionomycin

HT-2 cells were treated with ionomycin, washed with PBS, resuspended in growth medium and incubated at 37 or 4 °C. Cells were removed at the indicated time points, fractionated into non-nuclear and nuclear fractions and Western blotted as described in the Materials and methods section. Lanes 1 and 2 contained cell lysates of untreated and ionomycin-treated cells respectively as controls. The heavy arrow indicates the phosphorylated form of NFAT_p; the light arrow indicates the dephosphorylated form. Cytosol (upper panel) and nuclei (lower panel) are shown from untreated cells (lane 3) and cells withdrawn from ionomycin for the indicated times: 0 min (lane 4), 10 min (lanes 5 and 8), 30 min (lanes 6 and 9), 60 min (lane 7 and 10) and 120 min (lane 11).

treatment of the cells with ionomycin did not alter this localization (Figure 4A). In contrast, PKC ζ was expressed in both the non-nuclear and nuclear fraction of the cells under both untreated and ionomycin-treated conditions.

Effect of down-regulation of PKC by PMA on NFAT_p localization

To investigate a possible role for PKC in NFAT_p phosphorylation, PKC levels were down-regulated by PMA treatment. Cells were treated with 500 nM PMA for 72 h, which resulted in the disappearance of the PMA-sensitive PKC isoforms α , δ , ϵ and θ from the cells, as shown by Western blot analysis (Figure 4B, and results not shown). No change in the level of the three PMA-insensitive PKC isoforms ζ , ι and μ was detected, as expected. No change in the cytosolic accumulation of phosphorylated NFAT_p after ionomycin washout was observed between control and cells down-regulated by PMA (Figure 4C). Although these results do not rule out the involvement of one of the PMA-insensitive PKC isoforms in nuclear export and rephosphorylation, they suggest that a PMA-sensitive isoform of PKC is not involved in these processes.

Effect of low temperature on the cytosolic reaccumulation of NFAT_p

NFAT_p was localized to the nucleus after kinase inhibition (by St) or CaN activation (by ionomycin). Conversely, ionomycin washout, or addition of FK506 after ionomycin treatment, resulted in the reaccumulation of NFAT_p in the cytosol. The 120 kDa NFAT_p is above the limit for diffusion through nuclear pores, suggesting an active export mechanism [25,26]. To determine whether NFAT_p reaccumulation in the cytosol is an active process, temperature experiments were performed. Ionomycin-treated cells were washed and resuspended in complete growth medium at either 37 or 4 °C (Figure 5). NFAT_p remained in the nucleus in the chilled cells throughout the 120 min time course, whereas at 37 °C export was complete within 30–60 min. Throughout the temperature experiments the viability of the

cells, as measured by Trypan Blue exclusion, was not affected (results not shown). These results demonstrate that the reaccumulation of NFAT_p into the cytosol is temperature-dependent, consistent with an active export process.

DISCUSSION

Our results demonstrate that in both activated and unactivated T-cells, the steady-state level of NFAT_p phosphorylation is determined by a dynamic equilibrium between CaN and cellular kinases. In resting cells, inhibiting kinase activity with St resulted in unopposed CaN phosphatase activity, leading to a net dephosphorylation of NFAT_p. Addition of FK506 to inhibit CaN blocked the St effect, demonstrating the regulatory role of CaN. In activated cells CaN is activated directly by ionomycin treatment, also resulting in a shift in the kinase–CaN equilibrium. These results are consistent with a model in which NFAT_p is constantly undergoing dephosphorylation and rephosphorylation with the net effect, in resting cells, of a phosphorylated, cytoplasmic NFAT_p. The concept that the level of phosphorylation is the net result of kinase and phosphatase activities is not novel. However, our results focus on the role of kinases in regulating NFAT_p; our results also emphasize that there is a basal level of CaN and kinase activity in resting cells.

Evidence from previous work by us and others [14,15,17], as well as the results shown here, indicate that NFAT_p cellular localization depends on its phosphorylation state. Dephosphorylation of NFAT_p by treatment with either St or ionomycin results in nuclear localization of NFAT_p. As recently suggested by Shibasaki et al. [27], CaN might also co-localize with NFAT to the nucleus. In cells washed free of ionomycin, NFAT_p is rephosphorylated and relocalized in the cytoplasm. The mechanism by which dephosphorylation results in nuclear localization is not known. However, at least one putative nuclear localization sequence (NLS) has been identified in NFAT_p within the C-terminal region of the Rel homology domain [7]. Dephosphorylation of sites at or near the NLS, either by kinase inhibition or CaN activation, might unmask the NLS, allowing nuclear import of NFAT_p. Similar mechanisms have been identified for other transcription factors, such as v-Jun, in which phosphorylation of a serine adjacent to the NLS negatively regulates nuclear import [28,29].

Energy depletion and decreased temperature are known to block both nuclear import and export [30–34]. Our results indicate that the accumulation of NFAT_p from the nucleus into the cytosol also was blocked under low-temperature conditions, consistent with the nuclear export of NFAT_p as an active process. However, our results also demonstrate that St and BI block NFAT_p transit from the nucleus. Kinase activity also might be inhibited under the lower-temperature conditions. Thus the low temperature might be blocking the export process directly, as well as the NFAT_p kinase, resulting in the net effect of blocking the reaccumulation of NFAT_p in the cytosol.

Protein import into the nucleus has been studied in permeabilized cells by using model proteins, allowing the characterization both of factors required for import and of the nuclear pore complex itself [25,26,34,35]. Less is known about protein nuclear export, although it utilizes the same pore complex [36]. Active export of several proteins has been characterized, including the protein heat-stable inhibitor (PKI) of cAMP-dependent kinase [26,37,38]. PKI contains a nuclear export sequence (NES) that stimulates rapid export of the PKI–protein kinase A complex [20]. Comparison of the PKI NES with the NES of HIV-1 Rev and TFIIA has suggested a loose consensus sequence for nuclear export that is rich in leucine and other hydrophobic

Scheme 1 Dynamic equilibrium model of NFAT_p phosphorylation state and nuclear/cytoplasmic cycling

In resting and activated cells NFAT_p is constantly being dephosphorylated by CaN and re-phosphorylated by one or more kinases. In resting cells the equilibrium is shifted in favour of phosphorylated NFAT_p; in activated cells the CaN activity is increased, shifting the equilibrium to dephosphorylated NFAT_p, which is imported into the nucleus. Dephosphorylated NFAT_p in the nucleus is constitutively exported from the nucleus. In the continuing presence of ionomycin (or antigen), CaN activity is maintained and NFAT_p is kept dephosphorylated and imported back into the nucleus. If ionomycin or antigen is withdrawn, CaN activity returns to its basal activity level and kinase activity results in the net re-phosphorylation of NFAT_p, which is retained in the cytoplasm.

residues with conserved spacing between hydrophobic residues [37]. A similar export sequence might exist in NFAT_p. In fact, NFAT_p contains the sequence LKLRNADIEL, corresponding to residues 521–530, which is homologous with the PKI NES, LALKLAGLDI, if homologous substitutions are allowed. The underlined amino acids in the PKI NES indicate residues that have been identified by mutagenesis as particularly important for nuclear export [20]. By homology comparison between different NESs, it seems that I and L can be substituted for each other at important sites. Of course, any role that this sequence might have in NFAT_p nuclear export will need to be experimentally determined.

The identities of the kinases involved in NFAT_p phosphorylation and/or nuclear export are not known. The broad-specificity tyrosine kinase inhibitor B46 had no effect; neither did a number of other serine/threonine kinase inhibitors. However, both St and BI showed activity, and both these compounds inhibit serine/threonine kinases, with particular specificity *in vitro* for PKC.

We investigated a potential role for PKC in NFAT_p phosphorylation. Down-regulation of PKC levels results from prolonged treatment with PMA and is a useful method to investigate a potential role of PKC in cellular responses. Three of the PKC isoforms expressed in HT-2 cells are PMA-sensitive (α , δ and ϵ) and thus are able to be down-regulated, whereas four are PMA-insensitive (ζ , θ , ι and μ) [23,39,40]. In the down-regulated cells, no differences were detected in the cytosolic accumulation and phosphorylation of NFAT_p after ionomycin removal. These results suggest that none of PKC α , δ , or ϵ are involved in NFAT_p phosphorylation, but they do not rule out a potential role for the PMA-insensitive isoforms (ζ , θ , ι and μ). Furthermore, because of the lack of absolute specificity of St and BI, other serine/threonine kinases instead of, or in addition to, PKC might be the NFAT_p kinase. Our fractionation experiments indicate that

NFAT_p phosphorylation occurred in the cytosol and not in the nucleus. Under all of our treatment conditions little or no phosphorylated NFAT_p was detected in the nucleus, suggesting that the NFAT_p kinase(s) reside in the cytosol. Complete identification of the kinase(s) as well as their cellular localization will require further biochemical studies.

Taken together, these results are consistent with the model shown in Scheme 1. NFAT_p is present in the cytosol of the cells in a predominantly phosphorylated state. The net state of phosphorylation results from a dynamic equilibrium between CaN and the NFAT_p kinase(s). Changes in this equilibrium, either through CaN activation or kinase inhibition, result in dephosphorylated NFAT_p. The dephosphorylation of NFAT_p seems to serve as a signal for nuclear import, possibly by unmasking a nuclear localization sequence. Nuclear export of NFAT_p fits the requirements of an active, energy-requiring process that might occur constitutively, possibly through an NES sequence on NFAT_p. In this model, dephosphorylated NFAT_p that is exported back into the cytosol after the withdrawal of ionomycin would be rephosphorylated by the NFAT_p kinase(s). However, as shown in Figure 2, kinase inhibition by St prevents the constitutively exported NFAT_p from being rephosphorylated; it is therefore rapidly re-imported into the nucleus, with the net effect that St seems to block nuclear export.

Constitutive cycling of NFAT_p proposed in this model provides a simple mechanism consistent with the results that in the continuing presence of ionomycin, FK506 induces a complete reversal of the nuclear localization and dephosphorylation of NFAT_p (Figure 1) [14]. This model suggests that in the continuing presence of ionomycin (or antigen stimulation), CaN activity is required for maintaining a net accumulation of dephosphorylated NFAT_p in the nucleus by continually dephosphorylating exported NFAT_p, which would then be re-imported into the nucleus. An inhibitor of CaN activity such as FK506 would block dephosphorylation and would allow exported NFAT_p to be phosphorylated by NFAT_p kinase(s) and therefore retained in the cytoplasm rather than re-imported. Such a simple nuclear/cytoplasmic cycling mechanism would allow the cell to adjust NFAT_p transcriptional activating activity continually to changing CaN activity levels, which in turn reflect changing antigen exposure.

Clearly there are aspects of this model that require further experimental validation. For example, it is not clear from our experiments whether nuclear export itself requires kinase activity. Similarly, whether NFAT_p nuclear export occurs constitutively is not known. However, the results shown here demonstrate clearly that a kinase(s) and the phosphatase CaN act in concert to determine both the phosphorylation state and the cellular localization of NFAT_p.

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