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Concerted Dephosphorylation of the Transcription Factor NFAT1 Induces a Conformational Switch that Regulates Transcriptional Activity

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Summary

NFAT transcription factors are highly phosphorylated proteins that are regulated by the calcium-dependent phosphatase calcineurin. We show by mass spectrometry that NFAT1 is phosphorylated on fourteen conserved phosphoserine residues in its regulatory domain, thirteen of which are dephosphorylated upon stimulation. Dephosphorylation of all thirteen residues is required to mask a nuclear export signal (NES), cause full exposure of a nuclear localization signal (NLS), and promote transcriptional activity. An inducible phosphorylation site in the transactivation domain contributes to transcriptional activity. Our data suggest that dephosphorylation promotes NFAT1 activation by increasing the probability of an active conformation, in a manner analogous to that by which depolarization increases the open probability of voltage-gated ion channels. This conformational switch paradigm may explain modification-induced functional changes in other heavily phosphorylated proteins.

Introduction

Protein modifications provide a means to induce rapid and profound changes in the function of a protein in response to extracellular signals (reviewed in Karin and Hunter, 1995; Berger, 1999). In a large number of well-documented instances, modification controls protein–protein interactions by creating or eliminating docking sites for the recruitment of specific binding partners (Shoelson, 1997; Yaffe et al., 1997; Dhalluin et al., 1999; Komeili and O'Shea, 1999; Winston and Allis, 1999). In other cases, modifications alter local conformations in the vicinity of a binding region or an enzyme (e.g., kinase) active site, thereby modulating binding affinity or enzyme activity (Cobb and Goldsmith, 2000). A third scenario, in which modification alters protein activity by inducing global conformational changes in protein structure (Sicheri and Kuriyan, 1997; Xu et al., 1999), may be especially pertinent to proteins which contain large numbers of phosphorylated residues (Ko and Prives, 1996; Morrison and Cutler, 1997; Harbour et al., 1999). However, this has been difficult to document largely because of the technical difficulties involved in identifying multiple modification sites and dissecting their functional relevance.

Recent developments in analyzing protein phosphorylation by mass spectrometry have made it feasible to identify in vivo phosphorylation sites without radiolabeling or mutagenesis (Zhang et al., 1998). Phosphopeptides in an enzymatic digest of the phosphoprotein are identified by their characteristic mass change upon dephosphorylation by a nonspecific phosphatase in vitro (80 Da per phosphate removed). Nonphosphorylated peptides do not show a mass change. Peptide mass is measured by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry with a mass accuracy of ~10 parts per million, thus peptide peaks can be assigned to the sequence of the protein unambiguously if an enzyme such as trypsin or Asp-N with defined cleavage sites is used. The precise sites of phosphorylation are then determined by sequencing of individual phosphopeptides using capillary liquid chromatography-electrospray-tandem mass spectrometry. The method is highly suited to analysis of proteins that are phosphorylated at multiple sites.

In this work, we have used a combination of mass spectrometry and systematic mutational analysis to examine how phosphorylation controls localization and function of the heavily phosphorylated transcription factor NFAT1, one of four calcium-regulated members of the NFAT family of DNA binding proteins (reviewed in Rao et al., 1997; Crabtree 1999; Kiani et al., 2000). NFAT proteins are located in the cytoplasm of resting cells; upon activation they are dephosphorylated by the calmodulin-dependent phosphatase calcineurin and transported into the nucleus. Although this basic phenomenon has been well-established, the mechanism involved remains unclear (reviewed by Kiani et al., 2000). Calcineurin directly binds NFAT, but the residues that are dephosphorylated by calcineurin have not yet been identified; dephosphorylation of a limited number of residues has been postulated to unmask a conserved nuclear localization signal (NLS), but there is no agreement as to which phosphorylated residues are involved. Nuclear export of NFAT has been demonstrated, but the location of the nuclear export sequence (NES) and whether its exposure is regulated is controversial, and there is no consensus on whether dephosphorylation is required solely for nuclear localization or whether it also regulates the transcriptional function of NFAT. Ideally, a consensus model of NFAT activation would take into

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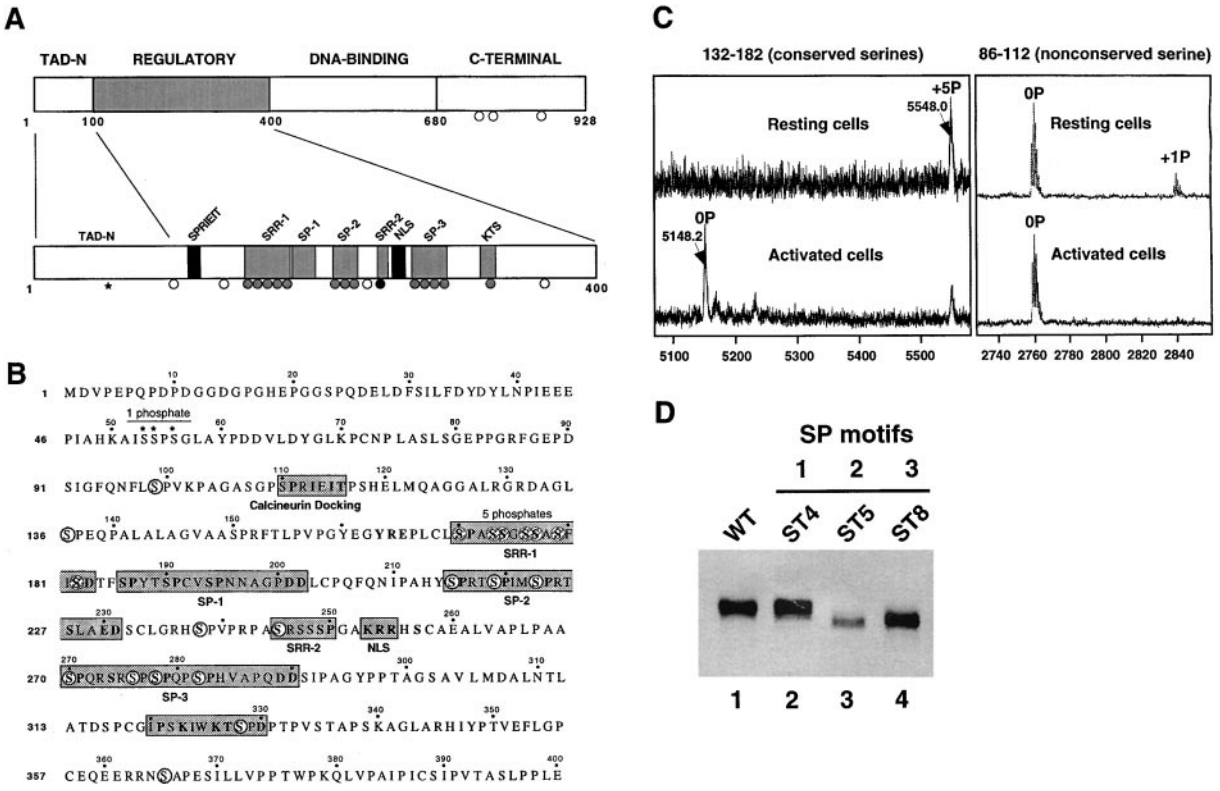

Figure 1. Analysis of NFAT1 Phosphorylation

(A) Diagram of the functional domains of NFAT1. The N-terminal transactivation domain (TAD-N), the regulatory domain, the DNA binding domain, and the C-terminal domain are indicated. The N-terminal region is enlarged to depict the conserved sequence motifs (shaded) and the relative positions of phosphoserine residues (circles) identified by mass spectrometry. Conserved phosphoserines in the regulatory domain that become dephosphorylated upon stimulation are indicated in gray, and nonconserved phosphoserines in the regulatory and C-terminal regions are indicated in white. The constitutively phosphorylated Ser-245 residue N-terminal to the NLS is indicated in black. The single serine residue in the transactivation domain that is phosphorylated only upon PMA/ionomycin stimulation is indicated by an asterisk.

(B) Phosphoserine residues in the regulatory domain. Conserved motifs discussed in the text are boxed in gray and known phosphoserines are circled. The seven serine residues in the SRR-1 region are indicated by hatched circles, five of these are phosphorylated. The three serine residues that are candidates for phosphorylation following ionomycin/PMA stimulation are marked with asterisks.

(C) Dephosphorylation of NFAT1 peptides assessed by mass spectrometry. Left panel, an Asp-N peptide (residues 132–182) spanning the SRR-1 region contained five phosphates when NFAT1 was obtained from resting cells (top trace); ionomycin stimulation resulted in loss of all five phosphates (bottom trace). A fraction of the peptide retained all five phosphates, presumably due to incomplete stimulation of some cells in the population. A shorter peptide (residues 132–164) containing the sequence N-terminal to the SRR-1 motif was not phosphorylated in either sample (data not shown). Right panel, a tryptic peptide (residues 86–112) was partially phosphorylated on a single serine residue (identified by sequencing as Ser-99) in resting cells (top trace); ionomycin stimulation resulted in dephosphorylation (bottom trace).

(D) The SP-1 region of NFAT1 is unlikely to be phosphorylated in resting cells. The ST5 and ST8 mutant HA-NFAT1 proteins containing Ser-to-Ala substitutions in the SP-2 and SP-3 regions show increased mobility on SDS gels relative to wild-type HA-NFAT1 when transiently expressed in Cl.7W2 T cells. The ST4 mutant protein with similar substitutions in the SP-1 region migrates identically to the wild-type protein.

account the similar regulation of all four NFAT proteins by calcineurin and the striking conservation of structure in the NFAT regulatory domain (Masuda et al., 1997; Crabtree, 1999; Kiani et al., 2000).

Here we have examined these issues for NFAT1. We show by mass spectrometry that NFAT1 is phosphorylated on at least 21 serine residues in resting cells. Eighteen of these residues are located in the regulatory domain, fourteen of them in sequence motifs conserved in the NFAT family; thirteen are dephosphorylated upon stimulation. Full dephosphorylation of all thirteen residues appears to be required to cause full exposure of an identified NLS in the regulatory domain, mask an NES associated with the regulatory domain, and enable maximal transcriptional activation. We also identify an inducible phosphorylation site in the N-terminal transactivation domain that is important for transcriptional activity. Our data are consistent with a conformational switch mechanism in which fully phosphorylated NFAT1 can exist in both active (NLS exposed/NES masked) and inactive (NLS masked/NES exposed) conformations; the probability of the active state is low in the fully phosphorylated protein but is progressively increased by dephosphorylation. This mechanism is conceptually distinct from one in which individual modified residues serve as docking sites for individual effector/adaptor proteins and provides a novel paradigm for how modification might effect global functional changes in other heavily phosphorylated proteins.

Results

Mapping of NFAT1 Phosphorylation Sites

Traditionally, determination of phosphorylation sites has involved separation and identification of radiolabeled

peptides derived from the protein of interest (Boyle et al., 1991). This approach was not successful with NFAT1, a transcription factor that is heavily phosphorylated on serine residues in resting cells. Definitive identification of phosphorylation sites was hindered by the complexity of the NFAT1 phosphopeptide map, incomplete proteolytic cleavage of the phosphorylated protein, and poor recovery of many phosphorylated peptides.

We therefore turned to mass spectrometry to examine the phosphorylation state of NFAT1. Endogenous NFAT1 was purified from the untransformed IL-2-dependent murine T cell clone D5 and its v-fos transformed derivative Cl.7W2 under stringent conditions designed to prevent in vitro dephosphorylation. NFAT1 purified from resting D5 and Cl.7W2 T cells contained at least 21 phosphoserine residues (Figures 1A and 1B). Eighteen of these were located in the conserved N-terminal regulatory domain (residues 99–398). No phosphorylated residues were found within the N-terminal transactivation domain (residues 1–98) or the DNA binding domain (residues 399–680), while three phosphoserines were identified within the C-terminal domain.

We focused our attention on the conserved regulatory domain, which governs the subcellular localization of NFAT1 and its interaction with calcineurin (Luo et al., 1996b). This domain binds calcineurin via the conserved sequence PxIxIT (SPRIEIT in NFAT1) (Aramburu et al., 1998, 1999). It also contains a conserved nuclear localization sequence (NLS) whose core is the basic sequence ^{253}KRR^{255}, a serine-rich region (SRR-1, ^{170}SPASS GSSASFISD^{183}), and three serine-proline repeat motifs (SPxx-repeat motifs) with the characteristic sequence [SPxx]SPxxSPxxSPxxxxx[D/E][D/E] (Table 1; reviewed in Kiani et al., 2000).

Not surprisingly, fourteen of the eighteen phosphoserine residues in the regulatory domain of NFAT1 were located within these conserved sequence motifs (Figure 1B; Table 1). The striking feature was the selectivity of the phosphorylation. The SRR-1 region was phosphorylated on only five of its seven conserved serines, a second serine-rich region (SRR-2, ^{245}SRSSS^{249}) contained only a single phosphate, the first SPxx-repeat motif (SP-1) did not appear to be phosphorylated (see below), and the second and third SPxx-repeat motifs (SP-2 and SP-3) were phosphorylated on three (of four) and four (of five) serines, respectively (Figure 1B; Table 1). Notably, the conserved phosphoserines in the regulatory domain were all stoichiometrically phosphorylated in resting cells (shown for SRR-1 in Figure 1C, left panel), in contrast, the nonconserved phosphoserine residues were partially and variably phosphorylated (shown for S99 in Figure 1C, right panel).

Only one serine-containing peptide of the regulatory domain, residues 183–201, remained unaccounted for in the mass spectrometry data. This peptide contains three serine residues and encompasses the first SPxx-repeat motif (SP-1). Phosphorylation of serine residues in the sequence -SP- generally results in altered mobility of a protein in SDS-polyacrylamide gels; however, substitution of alanines for the conserved serine residues in SP-1 had no detectable effect on the mobility of the mutant protein (Figure 1D, compare lane 2 with lane 1). In contrast, similar substitutions in the SP-2 or SP-3 regions resulted in a marked increase in mobility of the mutant proteins relative to wild-type NFAT1 (Figure 1D, compare lanes 3 and 4 with lane 1), consistent with the mass spectrometry results demonstrating that the serine residues of the SP-2 and SP-3 regions are phosphorylated. We infer that the SP-1 region of NFAT1 is unlikely to be phosphorylated in resting cells.

To determine which residues of NFAT1 are dephosphorylated upon stimulation, NFAT1 was isolated from ionomycin-treated Cl.7W2 cells and subjected to analysis by mass spectrometry. With the exception of the phosphoserine at position 245, which is located immediately N-terminal to the NLS in the moderately conserved SRR-2 region, all phosphoserines in the amino-terminal conserved motifs as well as the C-terminal phosphoserines were dephosphorylated upon stimulation (shown for SRR-1 in Figure 1C). The nonconserved phosphoserines in the regulatory domain were more variably dephosphorylated under the same conditions.

There was no evidence for de novo phosphorylation of either the regulatory domain or the DNA binding domain in stimulated cells. Specifically, the SPRIEIT calcineurin docking motif and its immediate flanking regions were not phosphorylated in cells stimulated with ionomycin (10 min) or PMA plus ionomycin (1 hr), ruling out the possibility that kinases activated under these conditions influence NFAT-calcineurin binding by directly phosphorylating the SPRIEIT-containing region. Similarly, the DNA binding domain was not phosphorylated under these conditions, ruling out the possibility

Table 1. Alignment of Conserved Sequence Motifs in NFAT Family Members

		——*****——
SRR-1	m1	GYREPLC------L--SPAS-SGSSASFISD
	h1	GYREPLC------L--SPAS-SGSSASFISD
	h2	AYRDPSC------L--SPAS-SLSSRSCNSEASS
	h3	GYREAGAQGGGAFFSPSPGSSSLSSWSFFSDASD
	h4	SYRE-SS------LSPSPAS-SISSRSWFSDASS
SP-1	m1	SPYTSPCVSPNNAGPDD
	h1	SPYTSPCVSPNNGGPDD
	h2	SPQTSPWQSPCVSPKTTDPEE
	h3	SPLPSPRASPRPWTPED
	h4	SPLTSPGGSPGGCPGEE
		* * *
SP-2	m1	SPRTSPIMSPRTSLAEDSCLG
	h1	SPRTSPIMSPRTSLAEDSCLG
	h2	SPRHSPSTSPRASVTEESWLG
	h3	SPGGRGPEDSWLL
	h4	SPRQSPCHSPRSSVTDENWLS
		*
SRR-2/NLS	m1	RPAS-RSSSPGAKRRHS
	h1	RPAS-RSSSPGAKRRHS
	h2	R-SS-RPASPCNKRKYS
	h3	TPASPRPASPCGKRRYS
	h4	RPASGPSS-RPTSPCGKRRHS
		* * * *
SP-3	m1	SPQRSRSPSPQPSPHVAPQDD
	h1	SPQRSRSPSPQPSSHVAPQDH
	h2	SPHHSPTPSPHGSPRVSVTDDSWLG
	h3	SPALSRRGSLGEE
	h4	SPHHSPVPSPGHSPRGSVTEDTWLN
		*
KTS	m1	IPSKIWKTSPD
	h1	IPPKMWKTSPD
	h2	VPVKSRKTTLE
	h3	IPQKTRRTSSE
	h4	IPLKTRKTSED

Phosphorylation sites in murine NFAT1 are indicated by asterisks. m1, murine NFAT1; h1, human NFAT1; h2, human NFAT2; h3, human NFAT3; h4, human NFAT4.

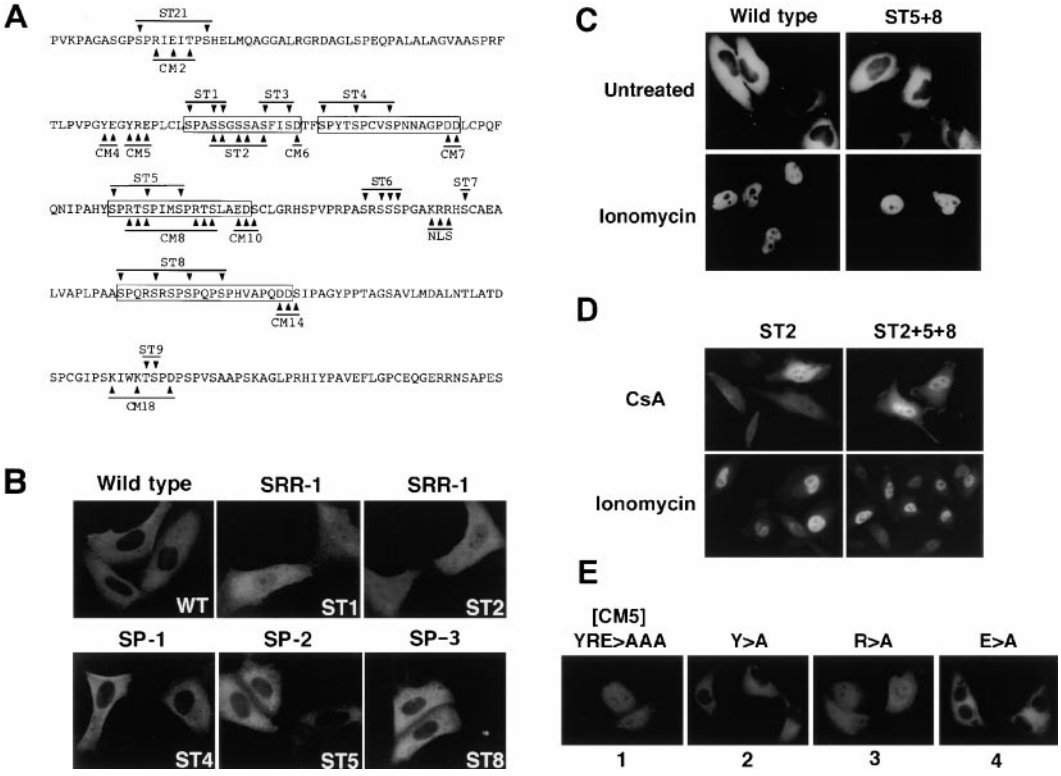

Figure 2. A Short Conserved Sequence ([163]YREPLCLSPASSGSS[177]) Spanning Part of the SRR-1 Region Regulates the Cytoplasmic Localization of NFAT1 in Resting Cells

(A) The ST and CM series of mutant proteins. The conserved serine/threonine motifs are boxed, and the residues substituted with alanine in each mutant protein are indicated by arrowheads.

(B) Subcellular localization of wild-type NFAT1 and ST mutant proteins in resting cells. Full-length proteins were transiently expressed in HeLa cells and visualized by immunocytochemistry using an antibody to the HA epitope tag. Only the ST1 and ST2 mutant proteins bearing mutations in the SRR-1 region showed significant nuclear localization. Identical localization was observed in resting Cl.7W2 T cells.

(C) The subcellular localization of the combined (ST5+8) mutant NFAT1 bearing serine-to-alanine substitutions in the SP-2 and SP-3 regions is identical to that of wild-type NFAT1. Wild-type or (ST5+8) mutant NFAT1(1–460)-GFP fusion proteins were transiently expressed in HeLa cells and visualized by GFP fluorescence without stimulation or after stimulation with ionomycin.

(D) The (ST2+5+8) mutant NFAT1 is more nuclear in resting cells than the ST2 mutant protein. Full nuclear localization requires ionomycin stimulation. Experiments were performed as in Figure 2C.

(E) Subcellular localization of the CM5 ([163]YRE[165]>AAA), Y163A, R164A, and E165A mutant NFAT1(1–460)-GFP proteins in unstimulated HeLa cells.

that the increase in NFAT1 DNA binding observed upon stimulation (Shaw et al., 1995) is directly mediated by phosphorylation of the DNA binding domain.

Identification of a Short Conserved Sequence that Regulates NFAT1 Localization

To identify the functional phosphoserine residues in the regulatory domain, we constructed the ST (serine/threonine) series of mutant proteins, which contain clustered serine-to-alanine substitutions in each of the conserved sequence motifs (Figure 2A). Full-length wild-type NFAT1 and ST mutant proteins were transiently expressed in HeLa cells, and their subcellular distribution was visualized by immunocytochemistry (Figure 2B). Identical results were obtained when the same epitope-tagged proteins were transiently expressed in Cl.7W2 T cells (data not shown).

Of the ten ST series mutants tested, only the ST1 and ST2 mutants in the SRR-1 region exhibited significant nuclear localization in resting cells (Figure 2B). Replacement of conserved serines in the SP-1, SP-2, or SP-3 regions did not cause nuclear localization (Figure 2B, mutants ST4, ST5, and ST8). Even a mutant protein in which the seven SP-2 and SP-3 region mutations were combined was identical to wild-type NFAT1 in being fully cytoplasmic in resting cells and translocating fully to the nucleus upon ionomycin stimulation (Figure 2C, mutant ST5+8). A mutant (ST2+5+8) with twelve serine-to-alanine substitutions, mimicking an almost completely dephosphorylated form of NFAT1, showed more striking nuclear localization than the ST2 mutant in both resting and CsA-treated cells (Figure 2D, top panels); however, it was still responsive to further stimulation, achieving complete nuclear localization only in ionomycin-treated cells (Figure 2D, bottom right panel).

Nuclear localization of the SRR-1 mutant proteins was not a consequence of dephosphorylation at other sites by basally active calcineurin. Pretreatment with CsA for 30 min or overnight did not alter the subcellular distribution of the ST1 or ST2 mutant proteins in resting cells (Figure 2D, top left panel; and data not shown). Moreover, two-dimensional phosphopeptide mapping

showed that mutations in the SRR-1 region did not alter the phosphorylation pattern of NFAT1 at other sites in the regulatory and C-terminal domains (data not shown).

To delineate further the region controlling NFAT1 localization, we generated the CM (conserved motif) series of mutant proteins (Figure 2A). Only the CM5 mutation (^{163}YRE^{165}>AAA) resulted in nuclear localization of NFAT1 in resting cells (Figure 2E, panel 1). The arginine residue in this sequence was critical for maintaining cytoplasmic localization, while mutation of the adjacent tyrosine or glutamate residues had little or no effect (Figure 2E, panels 2–4).

Together these results define a short conserved region encompassing the sequence ^{163}YREPLCLSPAS SGSS^{177}, which has a direct role in maintaining the cytoplasmic localization of NFAT1. Focal mutations within this region, altering either a single positively charged residue (Arg-164) or serine residues that are subject to phosphorylation and calcineurin-mediated dephosphorylation, lead to constitutive but partial nuclear localization of NFAT1 in resting cells. Importantly, however, complete nuclear localization requires further calcineurin-mediated dephosphorylation. Mutations that mimic dephosphorylation of the phosphorylated SP-2 and SP-3 motifs have no apparent effect in the absence of SRR-1 alteration, however, when SRR-1 is mutated, mutations of SP-2 and SP-3 further enhance nuclear localization.

Evidence for Active and Inactive Conformations of NFAT1

To understand why full dephosphorylation was required for nuclear translocation, we asked whether dephosphorylation facilitates NFAT1 translocation by increasing NLS exposure, masking an NES, or both. In the course of these experiments, we unexpectedly found that even fully phosphorylated NFAT1 was capable of entering the nucleus at a very low rate, as judged by its very low level of nuclear accumulation in resting cells treated for 1 hr with the nuclear export inhibitor leptomycin B (LMB) (Wolff et al., 1997) (Figure 3A, top panel). This slow import is not due to dephosphorylation by basally active calcineurin, since it is not blocked by CsA (data not shown); however, it does depend on recognition of the NLS, since it is abolished by the KRR>AAA mutation (Figure 3A, bottom panel). Additional evidence that the basal rate of NFAT1 nuclear import does not depend on dephosphorylation was obtained by analysis of the docking site mutant CM2. This mutant protein is highly resistant to calcineurin-mediated dephosphorylation and does not undergo detectable nuclear translocation in ionomycin-treated cells (Aramburu et al., 1998). Nevertheless, it is very slowly imported into the nucleus in unstimulated cells, as observed when nuclear export was inhibited with LMB (Figure 3A, middle panel). These data prove that fully phosphorylated NFAT1 can assume, with low probability, an active conformation in which the NLS is exposed.

We showed that dephosphorylation greatly accelerates the unidirectional nuclear import of NFAT1. NFAT1 was rapidly and fully localized in the nucleus within 15 min in ionomycin-treated cells (Figure 3A, top right panel), confirming that the unidirectional nuclear import rate of dephosphorylated NFAT1 is many times faster than the slow basal nuclear import of fully phosphorylated NFAT1, which is barely detectable even after 1 hr of treatment with LMB (Figure 3A, top middle panel).

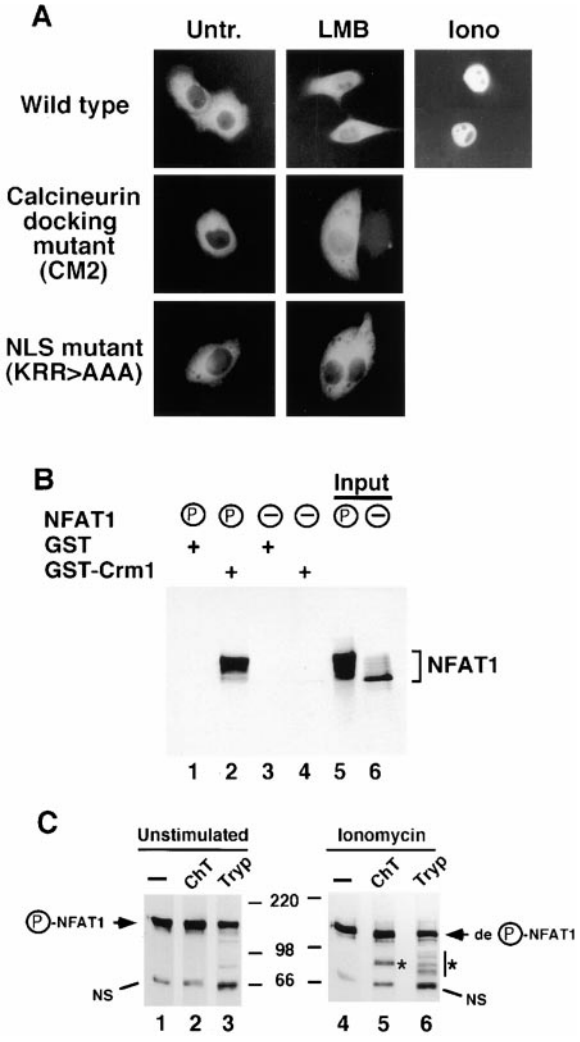

Figure 3. Full Dephosphorylation of NFAT1 Induces an "Active" Conformation with an Exposed NLS, Concealed NES, and Increased Protease Accessibility

(A) The nuclear export inhibitor LMB promotes a very slow nuclear import of NFAT1 that is independent of dephosphorylation but dependent on NLS exposure. Ionomycin-stimulated nuclear import is far more rapid, showing that dephosphorylation accelerates the unidirectional rate of nuclear import. Wild-type and mutant HA-NFAT1(1–460)-GFP fusion proteins were expressed in HeLa cells and visualized by GFP fluorescence.

(B) Phosphorylation-dependent interaction of NFAT1 with Crm1. Lysates containing phosphorylated ("P") or in vitro–dephosphorylated ("—") HA-NFAT1 were incubated with GST or GST-Crm1 bound to glutathione-Sepharose beads. Precipitated NFAT1 was detected by Western blotting with anti-HA antibody.

(C) Dephosphorylation of NFAT1 increases its protease accessibility. Cytosolic lysates from unstimulated or ionomycin-treated D5 cells were incubated with chymotrypsin or trypsin, and proteolytic fragments of NFAT1 were detected by Western blotting with antibody to a C-terminal peptide. "NS" denotes nonspecific bands.

A novel finding was that dephosphorylation also regulates the nuclear export of NFAT1. Because it is not straightforward to compare NES function of phosphorylated and dephosphorylated NFAT1 in cells, we used a direct biochemical measure of NES exposure—the ability to bind Crm1, the LMB-sensitive transport receptor

reported to mediate the nuclear export of NFAT2 and NFAT4 (Kehlenbach et al., 1998; Zhu and McKeon, 1999). Interaction of NFAT1 with Crm1 was markedly dependent on its phosphorylation state (Figure 3B). Phosphorylated NFAT1 bound strongly to GST-Crm1 with the more highly phosphorylated forms showing preferential binding (Figure 3B, lane 2), indicating that phosphorylated NFAT1 possesses an exposed Crm1-binding NES. As expected, this interaction was abolished by LMB (data not shown). In contrast, NFAT1 that had been completely dephosphorylated in vitro by calf intestinal phosphatase (CIP) was retained very poorly by the export factor (Figure 3B, lane 4), indicating that dephosphorylation results in loss or masking of an NES.

We asked whether dephosphorylation induces a conformational change in NFAT1 by comparing the protease susceptibility of fully phosphorylated and fully dephosphorylated NFAT1 (Figure 3C; for the remainder of this article the phrase "conformational change" will encompass changes in protein–protein associations as well). Dephosphorylated NFAT1 yielded a novel chymotryptic fragment that was not observed with phosphorylated NFAT1; likewise, dephosphorylated NFAT1 exhibited increased susceptibility to trypsin cleavage compared to fully phosphorylated NFAT1 (Figure 3C, compare lanes 2 and 3 with lanes 5 and 6). The site of chymotrypsin cleavage maps just N-terminal to SRR-1, while the multiple sites of trypsin cleavage map to the vicinity of the NLS. To rule out the possibility that phosphorylation directly occludes the cleavage sites, cell extracts were boiled in SDS to denature NFAT1 then diluted and digested with chymotrypsin. Under these conditions, the chymotrypsin cleavage site was accessible even in fully phosphorylated NFAT1 (data not shown).

Together these experiments show that dephosphorylation regulates both the nuclear import and the nuclear export of NFAT1 by inducing an active conformation that simultaneously exposes an NLS and masks an NES.

The SRR-1 Region Regulates the Active Conformation of NFAT1

To dissect the contribution of the SRR-1 region to NFAT activation, we studied the behavior of the ST2 mutant protein, which resembles a partially dephosphorylated form of NFAT1. Nuclear import of this protein reflects exposure of its NLS, since replacement of the core sequence of the NLS, ^{253}KRR^{255}, with alanine residues completely abolished its constitutive nuclear localization (Figure 4A). The ST2 protein also has an exposed NES because treatment with LMB even in the presence of CsA significantly increased its nuclear localization in resting cells (Figure 4B, compare panels 3 and 4 with panel 1). The cellular localization assay averages nuclear import and nuclear export over time, and so these findings indicate either that the ST2 protein alternates between two conformations, one presenting the NES and the other presenting the NLS, or that it has a single conformation that simultaneously exposes both the NLS and the NES.

Further, just as observed for wild-type NFAT1 (Figure 3A), unidirectional nuclear import of the ST2 mutant protein is greatly accelerated by dephosphorylation. The protein was fully localized to the nucleus within 15 min of ionomycin stimulation (Figure 4B, panel 5). In contrast, it was only partially nuclear in cells treated for 1 hr with LMB to block nuclear export and thereby demonstrate the basal rate of nuclear import (Figure 4B, panels 3 and 4). We conclude that mimicking dephosphorylation of the SRR-1 region produces only a partially active form of NFAT1 and that more complete dephosphorylation is needed for full exposure of the NLS and concealment of the NES.

An intriguing feature distinguishing the ST2 mutant from phosphorylated wild-type NFAT1 is that the mutant protein is at least 10-fold more sensitive to dephosphorylation by calcineurin at sites outside the SRR-1 region (Figure 4C). Treatment with 660 nM ionomycin was required to achieve ~90% dephosphorylation of wild-type NFAT1 (Figure 4C, top panel, lane 4), while the same level of dephosphorylation of the ST2 mutant protein was achieved in cells treated with 66 nM ionomycin (bottom panel, lane 2). Mass spectrometric analysis further suggested that at low stimulus strengths the SRR-1 region was more susceptible to dephosphorylation than the SPxx-repeat motifs (Figures 4D–4F). A simple interpretation is that the SRR-1 region is an early target for calcineurin-mediated dephosphorylation in NFAT1, and that dephosphorylation of the SRR-1 region promotes the further dephosphorylation needed for full activation. However, currently available technology does not allow us to establish whether there is an obligatory order of dephosphorylation that is the same for all molecules of NFAT1.

Extensive Dephosphorylation Is Needed for Transcriptional Activation of NFAT1

If dephosphorylation were necessary only for maintaining complete nuclear localization, fully phosphorylated NFAT1 would show maximal transcriptional activity when artifically localized to the nucleus. To test this hypothesis, we generated the constitutively nuclear protein NFAT1-NLS and examined its ability to activate an AP-1-independent reporter plasmid driven by the IL-13 promoter (Macián et al., 2000). NFAT1-NLS was fully phosphorylated in resting cells and was fully dephosphorylated in a calcineurin-dependent manner upon stimulation (data not shown), thus allowing a clear test of the separate contributions of nuclear localization and dephosphorylation in promoting the transcriptional function of NFAT1.

Surprisingly, these experiments showed that NFAT1-NLS was only marginally more active than wild-type NFAT1 in unstimulated Jurkat T cells (Figure 5A, NFAT1-NLS [WT], open bar). As observed for endogenous NFAT and overexpressed NFAT1, full activation of NFAT1-NLS required combined PMA/ionomycin stimulation (black bar) and was calcineurin-dependent since it was blocked by CsA (hatched bar) to the level observed with PMA stimulation alone (gray bar). Thus, maximal NFAT1-mediated transcription cannot be achieved merely by localizing NFAT1 constitutively to the nucleus, rather, calcineurin and a PMA-stimulated process continue to be required for full transcriptional activity.

We used two independent approaches to show that full dephosphorylation of NFAT1 by calcineurin is required for transcriptional activity. First, we showed that a specific inhibitor of the NFAT–calcineurin interaction, GFP-VIVIT (Aramburu et al., 1999), dramatically reduced the transcriptional activity of NFAT1-NLS (Figure 5A), implying that calcineurin binding (and calcineurin-mediated dephosphorylation) is required to activate the transcriptional function of this constitutively nuclear protein.

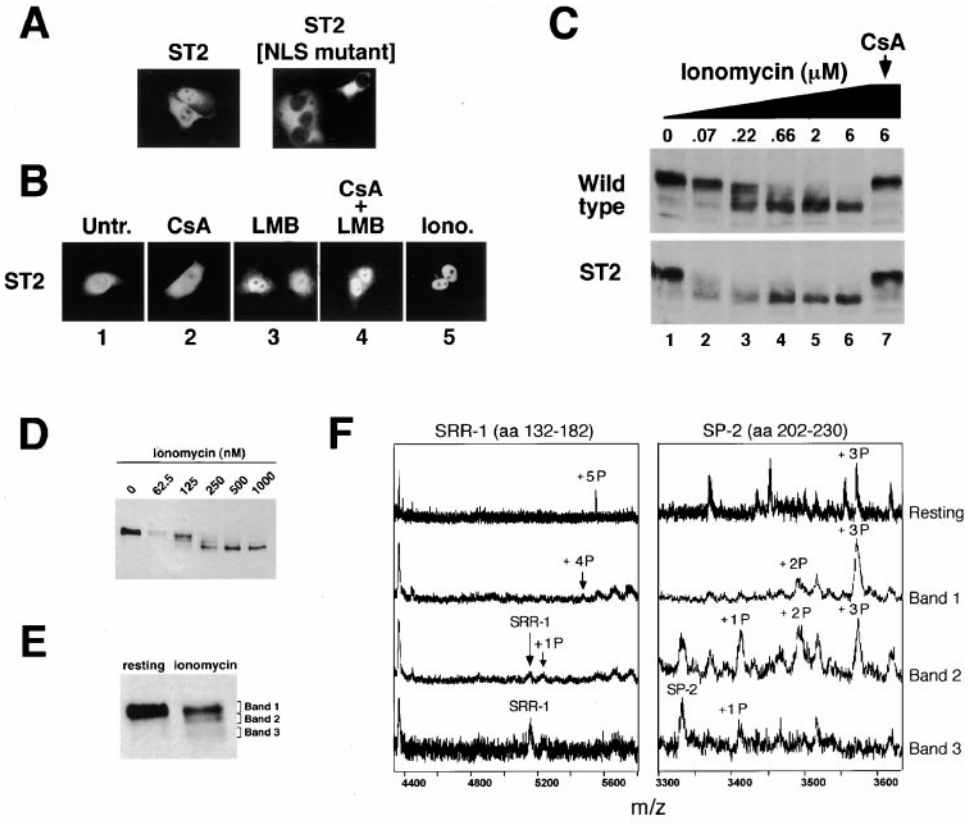

Figure 4. The SRR-1 Region Regulates the Active Conformation of NFAT1

(A) Nuclear import of the ST2 mutant protein reflects exposure of its NLS. HeLa cells transiently expressing ST2 NFAT1(1–460)-GFP or the same protein into which the NLS mutation (KRR>AAA) had been introduced were analyzed by GFP fluorescence. Only the ST2 protein with an intact NLS shows nuclear localization in resting cells.

(B) The ST2 mutant protein has an exposed NES. Treatment with LMB even in the presence of CsA significantly increases the nuclear localization of this protein in resting cells. Ionomycin-stimulated nuclear import is much more rapid, showing that dephosphorylation accelerates the unidirectional nuclear import of the ST2 mutant protein.

(C) Increased sensitivity of the ST2 mutant to calcineurin-mediated dephosphorylation. Cl.7W2 cells transiently expressing wild-type or ST2 mutant NFAT1(1–460)-GFP were stimulated with the indicated concentrations of ionomycin. Dephosphorylation of NFAT1 was analyzed in cell lysates by Western blotting with anti-HA antibody.

(D) Cl.7W2 T cells were treated with increasing concentrations of ionomycin to determine the minimum stimulus required to initiate dephosphorylation of endogenous NFAT1. Dephosphorylation was terminated by lysing cells in buffer containing 8 M urea. Lysates were diluted into RIPA buffer and immunoprecipitated with antibodies against NFAT1. Western blotting was performed using anti-NFAT1.

(E) Cl.7W2 cells were treated with 110 nM ionomycin for 10 min, and endogenous NFAT1 was isolated by immunoprecipitation. Separation on SDS gels resulted in the detection of three distinct bands corresponding to partial dephosphorylation states of NFAT1 that were excised for analysis by mass spectrometry.

(F) Mass spectrometric analysis of dephosphorylation in the SRR-1 and SP-2 regions. The combined data from all the bands suggest that the SRR-1 region is more susceptible to dephosphorylation than the SPxx repeat motifs at low stimulus strengths.

Second, we showed that the mutant protein (ST2+5+8)-NLS, which mimics a largely dephosphorylated and constitutively nuclear form of NFAT1, is considerably more active than either wild-type NFAT1-NLS or ST2-NLS in cells stimulated with PMA alone (Figure 5B, gray bars; and data not shown). We conclude that transcriptional activation of NFAT1 requires not only nuclear localization but also the extensive dephosphorylation of the SRR-1, SP-2, and SP-3 regions that is mimicked by the (ST2+5+8) mutant protein.

PMA/Ionomycin Stimulation Induces Phosphorylation at a Novel Site Important for Transcriptional Activity

It was striking that even using an AP-1-independent promoter and the (ST2+5+8)-NLS protein, combined PMA/ionomycin stimulation was needed to achieve maximal NFAT1-dependent transcription (Figure 5B). To test whether PMA/ionomycin stimulation directly activated nuclear NFAT1, we purified endogenous NFAT1 from Cl.7W2 cells treated for 1 hr with PMA and ionomycin and subjected it to mass spectrometric analysis. These experiments identified a novel, singly phosphorylated AspN peptide (residues 45–61) derived from the N-terminal transactivation domain, which was not observed in NFAT1 isolated from resting or ionomycin-stimulated cells.

To determine whether this activation-induced phosphorylation plays a role in NFAT1-mediated transcription, we analyzed the ^{53}SSPS^{56} site in the context of a GAL4 fusion protein containing the isolated NFAT1 transactivation domain (GAL4-NFAT1[1–144]). This construct avoided any contributions from the C-terminal

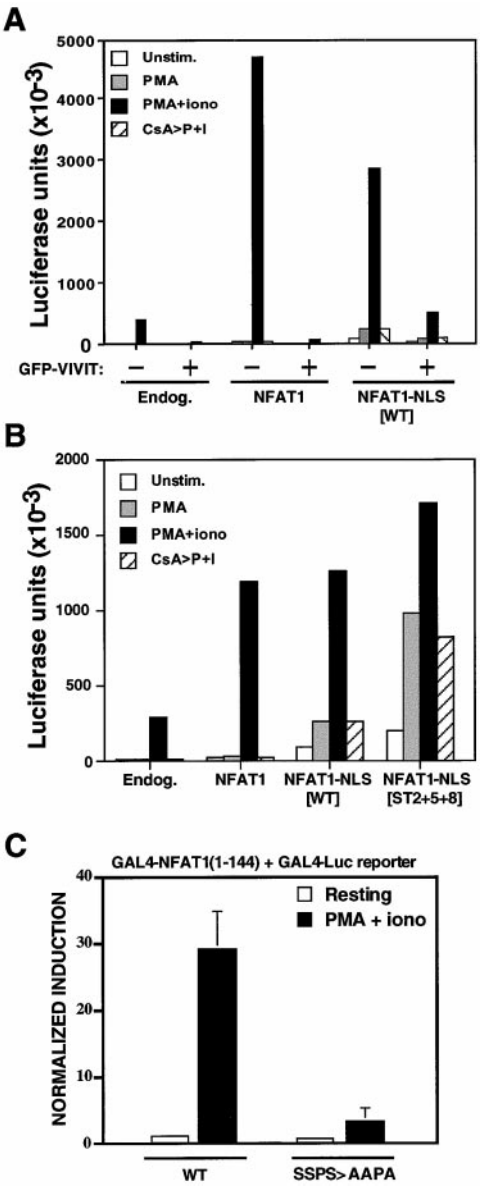

Figure 5. Transcriptional Activation of NFAT1 Requires Extensive Dephosphorylation and De Novo Phosphorylation of the N-Terminal Transactivation Domain

(A) Calcineurin is required for transcriptional activity of constitutively-nuclear NFAT1-NLS. NFAT1 and NFAT1-NLS induced IL-13 promoter-luciferase activity only in Jurkat T cells stimulated with both PMA and ionomycin; induction was inhibited by the calcineurin inhibitor CsA and by the peptide inhibitor of the NFAT–calcineurin docking interaction, GFP-VIVIT. "Endog." denotes endogenous NFAT.

(B) The NFAT1-NLS (ST2+5+8) mutant protein, which mimics an extensively dephosphorylated form of NFAT1, shows substantial transcriptional activity in PMA-stimulated cells. Jurkat T cells were cotransfected with the IL-13 promoter-luciferase plasmid and expression plasmids encoding NFAT1, NFAT1-NLS (WT), or NFAT1-NLS (ST2+5+8). Results are representative of five similar experiments.

(C) Activity of the N-terminal transactivation domain of NFAT1 requires the SSPS sequence that is subject to de novo phosphorylation in PMA/ionomycin–stimulated cells. Expression plasmids encoding wild-type or SSPS>AAPA mutant GAL4-NFAT1(1–144) were transfected into Jurkat T cells along with the GAL4-luciferase reporter. Results (mean ± SD of four experiments) are expressed relative to the activity of wild-type GAL4-NFAT1(1–144) in resting cells (normalized to 1).

transactivation domain of NFAT1 or from the DNA binding domain, which is capable of recruiting other transcription factors into cooperative complexes on DNA (Luo et al., 1996a). Since the exact phosphorylated residue was not known, all three potential phosphoserine sites were mutated to alanine and the effect on transactivation was examined. The wild-type GAL4-NFAT1 fusion protein was inactive in resting Jurkat T cells as well as in cells treated with PMA alone or ionomycin alone; it became highly active upon stimulation with both PMA and ionomycin, thus recapitulating the behavior of full-length NFAT1 (Figure 5C). Mutation of the three serine residues in the NFAT1 TAD resulted in a dramatic (88%) impairment of the transcriptional response to PMA/ionomycin stimulation (Figure 5C). The results strongly implicate these serine residues in the function of the NFAT1 N-terminal transactivation domain and suggest that combined PMA and ionomycin stimulation may potentiate NFAT1 transcriptional activity by promoting phosphorylation of this region in stimulated cells.

Discussion

A Conformational Switch Model of NFAT Activation

We show here that NFAT1 can exist in at least two alternative conformational states, an active conformation in which the NLS is exposed and the NES is masked, and the reciprocal inactive conformation in which the NLS is masked and the NES exposed (Figure 6). Progression of inactive NFAT1 to the fully active conformation is initiated not by dephosphorylation at one or a few sites but rather by extensive dephosphorylation at multiple sites. These data are consistent with two models of NFAT activation, the conformational switch model depicted in Figure 6A and the more conventional model of progressive conformational change depicted in Figure 6B.

In the conformational switch model, there are two functionally distinct conformations. For fully phosphorylated NFAT1, a large fraction of molecules are in the inactive conformation at any given time, but a few are in the active conformation (Figure 6A, top). Complete dephosphorylation reverses this conformational preference (Figure 6A, bottom), while dephosphorylation to intermediate levels progressively increases the probability that an individual molecule will assume the active conformation. The best experimental precedents for proteins that switch between two conformational states are provided by voltage-gated and ligand-gated ion channels, in which distinct closed and open states have been documented, and the total ionic current carried by a population of channels can be modulated physiologically by controlling the number of channels open at a given time (Sakmann and Neher, 1984; Armstrong and Hille, 1998).

In the alternative model of progressive conformational changes, a single preferred conformation of NFAT1 is determined by each phosphorylation state. The fully phosphorylated and fully dephosphorylated proteins exist only in the inactive and active conformations, respectively, and intermediate levels of dephosphorylation

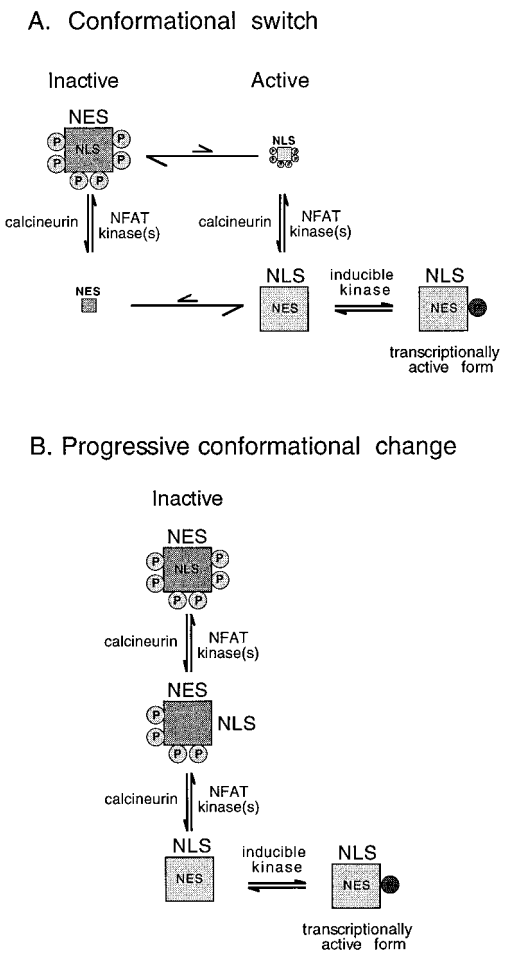

Figure 6. Models for NFAT1 Activation

(A) Dephosphorylation increases the probability of a conformational switch in NFAT1. Both phosphorylated and dephosphorylated NFAT1 can assume the "active" (NLS exposed/NES masked) and reciprocal "inactive" (NLS masked/NES exposed) conformations but with significantly different probabilities. Dephosphorylation of SRR-1 and other phosphorylated regions progressively increases the probability that an individual molecule of NFAT1 will be found in the active state.

(B) Dephosphorylation induces progressive conformational changes in NFAT1, promoting the transition from the inactive to the active form.

In both models, the active state of NFAT1 is characterized by full exposure of the NLS, full concealment of the NES, and full acquisition of DNA binding (not depicted) and transcriptional activity. In (A), dephosphorylation of the SRR-1 region is the major determinant of the conformational switch, but extensive dephosphorylation of the regulatory domain is required to tilt the equilibrium fully in the direction of the active form. In (B), dephosphorylation of the SRR-1 region causes a intermediate conformational change that partially exposes the NLS, while further dephosphorylation results in additional exposure of the NLS and complete masking of the NES. Phosphorylation of the N-terminal transactivation domain by an inducible kinase is required for full transcriptional activity.

specify a series of intermediate conformations on the path to the fully active form (Figure 6B). Experimentally, the two models could best be distinguished at the single-molecule level (Moerner and Orrit, 1999; Weiss, 1999) by asking whether individual NFAT1 molecules in a partially dephosphorylated preparation alternate between active and inactive states or assume a stable conformation that is intermediate between these states.

Even for proteins with many phosphorylation sites that undergo complex conformational changes, the available biochemical data have been interpreted in terms of sequential conformational changes. In $p47^{phox}$, a signaling subunit of the phagocyte NADPH oxidase, phosphorylation of about nine serines in a short region of the protein disrupts an intramolecular interaction of that region with the tandem SH3 domains, activating superoxide production by increasing the ability of arachidonic acid to induce a further conformational change in the protein and by facilitating assembly of $p47^{phox}$ into a multiprotein signaling complex at the plasma membrane (Shiose and Sumimoto, 2000). In the tumor suppressor Rb, which contains at least 16 consensus sites for cyclin-dependent kinase (Cdk) phosphorylation, successive phosphorylation by two different Cdks during the cell cycle triggers a sequence of intramolecular conformational changes that abrogate first the ability of Rb to recruit histone deacetylase, and subsequently the ability to bind to and inactivate E2F (Harbour et al., 1999). While it is certain that protein modification can produce sequential changes in the function of these and other proteins, the reason for utilization of multiple phosphorylation/dephosphorylation sites is not obvious at present. As appropriately sensitive methods are developed, it will be of considerable interest to look more closely at heavily phosphorylated proteins to determine whether phosphorylation at each single site specifies a distinct conformation or merely alters the probability of switching between two endpoint conformations of the protein.

The Role of the YRE/SRR-1 Region

It is clear from our mutational analysis that the YRE/SRR-1 region has at least two crucial roles in regulating NFAT1 function. First, dephosphorylation of SRR-1 is likely to partially expose the NLS, as also suggested for NFAT2 (Beals et al., 1997). However, masking by SRR-1 phosphoserines cannot be the only determinant of NLS exposure, since further dephosphorylation of the SRR-1 mutant proteins significantly increases their rate of nuclear import. Second, dephosphorylation of SRR-1 is likely to increase the susceptibility of NFAT1 to calcineurin-mediated dephosphorylation at the SPxx-repeat motifs, as judged by the increased calcineurin sensitivity of our ST2 mutant protein. This function may be shared by the SRR-1 region of NFAT4 (Chow et al., 1997; Zhu et al., 1998).

In NFAT2, the SRR-1 region was additionally reported to participate in an interdomain interaction with a conserved basic segment in the DNA binding domain (Beals et al., 1997). This basic region of NFAT proteins contacts the phosphate backbone of DNA in the NFAT1-DNA complex (Chen et al., 1998). If the interdomain interaction is conserved in the NFAT family and requires phosphorylation of the SRR-1 region as suggested, its disruption could account for the increase in DNA binding affinity that is observed upon dephosphorylation of NFAT1 and NFAT2 (Shaw et al., 1995; Park et al., 1995; Loh et al., 1996; Kehlenbach et al., 1998). Alternatively, the DNA binding affinity of NFAT proteins could be controlled by the phosphorylation status of the SPxx-repeat motifs.

For NFAT1, mutations in the SPxx-repeat motifs do

not affect subcellular localization in resting cells. In contrast, clustered serine-to-alanine mutations in the SP-1 regions of NFAT2 or NFAT4 promote their nuclear accumulation (Beals et al., 1997; Zhu et al., 1998), indicating that the SP-1 region and perhaps the other SPxx-repeat motifs make a more pronounced contribution to activation of these two NFAT family members. Curiously, however, we found no indication that the SP-1 region is phosphorylated in resting NFAT1. An interpretation that preserves a single mechanism of NFAT activation is that individual SPxx-repeat motifs are used interchangeably in different NFAT proteins, especially if, as is plausible, the region encompassing these motifs folds as a quasi-symmetrical structure.

Functional Implications

We have shown that PMA/ionomycin stimulation results in inducible phosphorylation of a novel site in the N-terminal transactivation domain of NFAT1 that is important for transcriptional activity (Figure 5C). There are many ways in which phosphorylation of the transactivation domain might facilitate the assembly of an active transcription complex. For instance, it might extend the lifetime of the active conformation of NFAT1 and/or promote its interactions with coactivator proteins in the nucleus. Given the sequence diversity of NFAT transactivation domains and the diverse spectrum of genes activated by NFAT proteins in different tissues (Crabtree 1999; Kiani et al., 2000), it is possible that distinct kinases are responsible for the inducible phosphorylation of different NFAT transactivation domains in different cell types. Such differential phosphorylation may provide a means of recruiting cell-type specific cofactors to the regulatory regions of distinct sets of NFAT target genes.

What advantages are offered by this complex mechanism of NFAT1 regulation involving cooperative and potentially ordered dephosphorylation at so many different sites? An analogy is provided by the ζ subunit of the T cell receptor (TCR), which upon TCR engagement undergoes an ordered series of tyrosine phosphorylations at three ITAM motifs (Kersh et al., 1998). The steady-state pattern of ζ phosphorylation reflects the nature and intensity of TCR stimulation; complete phosphorylation of all six tyrosine residues is associated with productive signaling in response to agonist peptides, while the incomplete phosphorylation elicited by weak agonists (altered peptide ligands) is associated with incomplete signaling or an anergic state. It is similarly conceivable for NFAT1 that the requirement for multiple dephosphorylations imposes a needed threshold for transcriptional activation. While basal or transient activation of calcineurin or nonspecific phosphatases might result in partial dephosphorylation of the SRR-1 region or the SPxx-repeat motifs, this would not be sufficient in the face of rephosphorylation by constitutively active NFAT kinases to cause nuclear accumulation of NFAT1 or maintain it in an active conformation in the nucleus.

Experimental Procedures

Mapping of NFAT1 Phosphorylation Sites

Endogenous NFAT1 was purified from the murine T cell clone D5 (Ar-5, Rao et al., 1984) or its transformed derivative Cl.7W2 (Valge-Archer et al., 1990). Cells were treated with CsA (1 μM/20 min), ionomycin (1 μM/10 min), or PMA and ionomycin (10 nM PMA, 1 μM ionomycin/1 h) at 37°C in DMEM + 2 mM $CaCl_2$ and lysed by resuspending in 20 mM Tris-HCl (pH 8.0), 8M urea, 100 mM NaCl, 5 mM EGTA, 10 mM iodoacetamide. The lysate was diluted 10-fold into RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 2 mM EGTA, 20 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium *o*-vanadate, 10 μM leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM DTT) and immunoprecipitated with antibodies to N- or C-terminal peptides of NFAT1 (Ho et al., 1994; Wang et al., 1995). Immunoprecipitated NFAT1 was separated by SDS–PAGE, digested with trypsin or Asp-N protease, and analyzed by mass spectrometry (Zhang et al., 1998).

Since dephosphorylation of endogenous NFAT1 in T cells is very rapid (~1 min at 37° C; Shaw et al., 1995), we measured susceptibility of SRR-1 and the SP-2 and SP-3 regions to dephosphorylation at low stimulus strength. Cells were resuspended in DMEM + 2 mM $CaCl_2$, incubated with varying concentrations of ionomycin (37°C/5 min), then immediately placed on ice, centrifuged (4°C/5 min), and lysed in urea. Samples of immunoprecipitated NFAT1 were prepared for mass spectrometry as described above.

Expression Plasmids and Mutagenesis

Expression plasmids HA-NFAT1(1–460)-GFP, GFP-VIVIT, and pEFTAGmNFAT1-C encoding full-length murine NFAT1 isoform C tagged with three copies of the haemagglutinin (HA) epitope have been described (Aramburu et al., 1998, 1999). A pLGP3 expression plasmid (Nardone et al., 1994) encoding HA-tagged NFAT1 was prepared by replacing the SV40 promoter of pLGP3 with the EF1α promoter. ST and CM series mutants in NFAT1 were generated by site-directed mutagenesis (Kunkel et al., 1987) using the (+) strand of the pLGP3 construct as a template. An expression plasmid encoding constitutively nuclear, full-length HA-NFAT1 (NFAT1-NLS) was generated by appending a double-stranded oligonucleotide encoding the SV40 NLS sequence KTPPKKKRKVEDPGRDSRS to the 3′ end of HA-NFAT1 cDNA in the pEGFP.N1 vector (Clontech); the expressed protein was completely and constitutively nuclear regardless of cell stimulation when expressed in HeLa cells. ST and CM series mutations were introduced into HA-NFAT1 (1–460)-GFP and NFAT1-NLS plasmids by standard subcloning techniques. The GAL4-NFAT1(1–144) plasmid was made by subcloning a PCR fragment encompassing codons 1–144 of murine NFAT1 into the pM vector (Clontech) containing the DNA binding domain of GAL4 (residues 1–147). The SSPS>AAPA substitution was introduced by PCR. Plasmids were sequenced to verify the presence of only the desired mutations.

Immunocytochemistry/Subcellular Localization

HeLa cells and Cl.7W2 T cells expressing HA-tagged full-length wild-type or mutant NFAT1 were plated on coverslips and incubated with or without ionomycin. HA-tagged NFAT1 was detected with monoclonal antibody 12CA5 (Boehringer Mannheim) followed by Cy3-conjugated anti-IgG (Jackson Immunoresearch). The ST1, ST2, and CM5 mutant proteins showed partial nuclear localization in resting cells (see Results), the ST6 protein showed barely discernible nuclear localization in a fraction of the cells and was not further investigated, and the CM2 mutation in the calcineurin docking sequence and the KRR>AAA mutation in the NLS both resulted in retention of NFAT1 in the cytoplasm even in activated cells (Luo et al., 1996b; Aramburu et al., 1998). None of the other ST or CM mutations shown in Figure 2A altered NFAT1 localization in resting or activated cells (in particular, note the CM6 mutation at the end of SRR-1 and the CM7, CM8, CM10, and CM14 mutations in the SPxx-repeat motifs).

For studies examining the effect of LMB, HeLa cells expressing NFAT1(1–460)-GFP or the corresponding CM2 or ST2 mutant were treated with LMB (200 nM/1 hr), CsA (1 μM/1 hr; 30 min preincubation), or ionomycin (2 μM/15 min) in DMEM + 2 mM $CaCl_2$. The subcellular localization of NFAT1 was visualized by GFP fluorescence.

Calcineurin Sensitivity

Cl.7W2 cells expressing wild-type or ST2 mutant NFAT1(1–460)-GFP were left unstimulated or stimulated with increasing concentrations of ionomycin (10 min/37°C) in DMEM + 2 mM $CaCl_2$ then lysed by boiling in Laemmli sample buffer supplemented with 20 mM

sodium pyrophosphate and 10 mM EDTA. Samples were separated by SDS–PAGE followed by Western blotting with anti-HA antibody.

Protease Cleavage

D5 T cells were left untreated or treated with ionomycin (1 μM/1 min) then lysed in ice-cold hypotonic buffer (10 mM HEPES [pH 7.4], 0.05% Nonidet P-40, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 10% glycerol, 10 μg/ml aprotinin, 25 μM leupeptin, 2 mM PMSF). Nuclei and cell debris were removed by centrifugation, and the extracts were adjusted to 150 mM NaCl and incubated (5 min/25°C) with 1.6 μM sequencing-grade trypsin or 17 nM chymotrypsin (Boehringer Mannheim). Digestions were terminated by addition of boiling 2× Laemmli sample buffer. Samples were separated by SDS–PAGE followed by Western blotting with a polyclonal antiserum against the C terminus of NFAT1 isoform C (Wang et al., 1995). Protease cleavage sites were estimated from fragment sizes of the proteolytic peptides.

Crm1 Binding Assays

HEK293 cells transiently expressing full-length wild-type NFAT1 were lysed in ice-cold buffer containing 50 mM HEPES (pH 7.5), 0.05% Nonidet P-40, 10 mM KCl, 1 mM GTP, 1 mM EGTA, 10 mM iodoacetamide, 10 μg/ml aprotinin, 25 μM leupeptin, 1 mM PMSF. After centrifugation, the extract was adjusted to 100 mM NaCl, 1 mM DTT. Phosphatase inhibitors (20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM sodium *o*-vanadate, 2 mM EDTA) were added to half the sample to maintain NFAT1 in the fully phosphorylated state; the other half-sample was incubated (30°C/30 min) with calf intestinal phosphatase (50 units, New England Biolabs) and 2 mM $MgCl_2$ to dephosphorylate NFAT1. CIP rather than calcineurin was used to dephosphorylate NFAT1 to eliminate the possibility that calcineurin might compete with Crm1 for binding to the NFAT1 NES, as suggested for NFAT4 (Zhu and McKeon, 1999). Lysates were incubated (30 min/4°C) with GST or GST-Crm1 (~10 μg) previously bound to glutathione-Sepharose beads. Bound NFAT1 was eluted by boiling in Laemmli sample buffer and detected by Western blotting with an antibody against the N terminus of NFAT1. LMB (200 nM) included in the binding reaction almost completely eliminated NFAT1-Crm1 binding, and binding was not enhanced by supplementing lysates with additional Ran-GTP (20 μg/ml). CIP treatment did not alter the level of Crm1 binding to 14-3-3 proteins in the same lysates.

Reporter Assays

Jurkat T cells were electroporated with NFAT1 expression plasmids (0.23 μg/10^6 cells), IL-13 promoter-luciferase plasmid (0.23 μg/10^6 cells), and Renilla luciferase expression plasmid (Promega; 8 ng/10^6 cells) for normalization of transfection efficiency and GFP and GFP-VIVIT expression plasmids (0.46 μg/10^6 cells) (Luo et al., 1996a; Aramburu et al., 1998, 1999). Twenty-four hours later, cells were stimulated for 6 hr with PMA (20 nM) or PMA plus ionomycin (0.5 μM) in the presence or absence of CsA (1 μM, added 30 min before stimulation). Activity of endogenous NFAT was monitored in cells cotransfected with a control GFP plasmid. All NFAT1 proteins were expressed at similar levels, as assessed by Western blotting with anti-HA antibody. For experiments involving the NFAT1 transactivation domain, Jurkat T cells were electroporated with GAL4-NFAT1(1–144) expression plasmid, GAL4-luciferase reporter, and Renilla luciferase expression vector as described above; 24 hr later, cells were stimulated overnight with 1 μM ionomycin and 10 nM PMA. Luciferase units were normalized to Renilla luciferase readings within each transfection and are expressed as relative luciferase units.

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