The nuclear import of nuclear factor of activated T cells (NFAT) transcription factors is critical for regulating NFAT activity. Here we demonstrate that the sumoylation of NFAT1 defines a novel mechanism of nuclear anchorage and transcriptional activation downstream from the known mechanism of calcineurin-mediated dephosphorylation and nuclear import. We show that Lys\(^{684}\) and Lys\(^{897}\) of NFAT1 can be sumoylated. The sumoylation at Lys\(^{684}\) is required for NFAT1 transcriptional activity and subsequent sumoylation of Lys\(^{897}\), whereas the sumoylation of Lys\(^{897}\) is only required for nuclear anchorage. Because Lys\(^{897}\) of NFAT1 is not conserved among other members of the NFAT family, we propose that sumoylation of Lys\(^{897}\) may provide a mechanism for NFAT1 isotype-specific regulation of nuclear anchorage and transcriptional activation. Furthermore, we found that treatment with both ionomycin and phorbol 12-myristate 13-acetate ensured efficient nuclear anchorage with the recruitment of NFAT1 into the SUMO-1 bodies, whereas treatment with ionomycin alone induced nuclear translocation of NFAT1 but not recruitment into the SUMO-1 bodies. Our results suggest that the recruitment of NFAT1 into SUMO-1 bodies may be required for the progressive transcriptional activity of NFAT1 upon co-stimulation with ionomycin and phorbol 12-myristate 13-acetate, whereas anergic transcription stimulated by ionomycin alone may occur without recruitment into the SUMO-1 bodies.

The nuclear factor of activated T cells (NFAT)\(^1\) defines a family of Ca\(^{2+}\)-inducible transcriptional factors that play important roles in regulating the transcription of cytokine genes (1). Four canonical members of NFAT family are known: NFAT1 (NFATp/NFATc2) (2), NFAT2 (NFATc/NFATc1) (3), NFAT3 (NFATc3), and NFAT4 (NFATc3/NFATx) (4, 5). Different members of the NFAT family are expressed in a distinct but overlapping set of cell types. Mice deficient in different NFAT family members exhibit markedly different phenotypes (6–9), although the mechanisms that determine the regulatory and functional specificities of different NFATs are not fully elucidated.

The N termini of NFAT family members share a conserved sequence about ~300 amino acids in length known as the NFAT homologous region (NFAT-h). The NFAT-h is known to regulate nuclear/cytoplasmic trafficking of NFATs in response to changes in intracellular Ca\(^{2+}\) concentrations. In resting T cells, NFATs are retained in the cytoplasm, and the NFAT-h is heavily phosphorylated. Engagement of the T-cell antigen receptor or treatment of cells with Ca\(^{2+}\)-ionophores activates calcineurin, a Ca\(^{2+}\)/calmodulin-dependent Ser/Thr phosphatase, which dephosphorylates the NFAT-h, resulting in rapid translocation of the proteins to the nucleus (10). The nuclear translocation of NFATs is critically important for regulating NFAT activity. Immunosuppressive drugs FK506 and cyclosporin A block this translocation by inhibiting calcineurin activity in T cells as well as in nonhemopoietic cells, which leads to undesired side effects (11, 12). Considerable efforts are being made to identify additional mechanisms that regulate the nuclear translocation of NFATs with the hope of elucidating isoform-specific or cell type-specific mechanisms that would allow the development of new drugs without unpleasant side effects.

Protein posttranslational modifications by a variety of mechanisms play crucial roles in regulating protein function. Ubiquitination represents a particular case where ubiquitin, itself a small polypeptide, is covalently linked to lysine residues in a protein to target it for proteasomal degradation or other signaling pathways. Recently, several proteins that share similarity with ubiquitin have been identified. One member of this ubiquitin-like protein family is SUMO-1 (also known as sentrin, GMP1, UBL1, and PIC1), a polypeptide of 101 amino acids that can be attached covalently to proteins in a process that is mechanistically analogous to ubiquitination (13). The SUMO-1 activating enzyme catalyzes the ATP-dependent activation of SUMO-1, the first step in the conjugation pathway (14–16), and transfers the activated SUMO-1 to UbC9, the E2-conjugating enzyme involved in this process (17–20). To date, the known substrates of SUMO-1 include RanGAP1 (21, 22), PML (23, 24), Sp100 (25), and IксA (26, 27). The observation that RanGAP1 and PML are targeted to distinct subcellular structures upon conjugation to SUMO-1 suggests that modification by SUMO-1 might play an important role in regulating the subcellular localization of proteins. Modification by SUMO has been shown to play critical roles in both nuclear and cytoplasmic processes, such as nuclear transport, transcription, and subnuclear targeting, which ultimately contribute to regulation of the cell cycle, cell growth, and apoptosis.

To explore the mechanisms and functions of sumoylation in regulating cellular processes, we developed a screening system...
for sumoylated proteins. Using this system, we determined that NFAT1 is sumoylated and that sumoylation of NFAT1 regulates the cytoplasmic-nuclear trafficking, subnuclear localization, and transcription of NFAT1. We show that ionomycin and phorbol 12-myristate 13-acetate (PMA), agents commonly used to activate the transcriptional activity of NFATs and T-cell activation, exhibit distinct effects on the sumoylation of NFAT1. Our study identified sumoylation as a novel nuclear anchorage and transcriptional regulatory mechanism for NFAT1 that may provide isoform-specific regulation.

MATERIALS AND METHODS

Plasmids and Antibodies—pE-GFP-SUMO-1-(1–97), pEGX-6P-SUMO-1-(1–97), and pcDNA4-HisMax-SUMO-1-(1–97) were generated by PCR amplification of SUMO-1 from expressed sequence tag cDNA clone 25786 (Research Genetics, Inc., Huntsville, AL). Myc-tagged NFAT1 expression vector was made by cloning mouse NFAT1 cDNA into XhoI and Apal sites of pcDNA3-myc vector. Point mutants of NFAT1, K684R, K897R, K684R/K897R, and DBD (K532R), were generated using the QuikChange kit (Stratagene). A deletion mutant NFAT1, del C(1–683), was generated by PCR. Anti-NFAT1 antibodies (clones 4G6-G5 and G1-D10), monoclonal anti-myc antibody (9E10), and polyclonal anti-hemagglutinin antibody (Y-11) were purchased from Santa Cruz Biotechnology. Anti-SUMO-1 antibodies (CLC7 and FL101) were purchased from Zymed Laboratories Inc. and Santa Cruz Biotechnology, respectively.

In Vitro Expression Cloning for Sumoylation Substrates—Pools from a mouse spleen cdNA library (28) were in vitro transcribed and translated as [35S]methionine-labeled proteins as described previously. Sumoylation assays were performed in a total volume of 20 μl in reaction buffer (20 mM Hepes-NaOH, pH 7.4, 5 mM MgCl2, 0.05% phosphocreatine) containing 200 μg/ml recombinant human SUMO-1 activating enzyme 1/SUMO-1 activating enzyme 2 as E1 and 50 μg/ml g/ml recombinant human SUMO-1 in the sumoylation reaction mix, and the products were analyzed by SDS-PAGE. After screening of five pools, a positive clone was identified as murine NFAT1, a transcription factor that controls T-cell activation as well as the differentiation of a variety of other cell types (Fig. 1A). This cDNA clone contains the coding region for amino acids 715–1064 of NFAT1.

To determine whether full-length NFAT1 can be sumoylated, we transcribed and translated in vitro a cDNA clone encoding a myc-tagged full-length NFAT1 in the presence of [35S]methionine and performed an in vitro sumolation assay with [35S]methionine-labeled NFAT1. In the presence of recombinant sumoylation enzyme E1 and UBC9, a portion of myc-NFAT1 migrated on SDS-PAGE at a slower rate, consistent with the covalent modification of NFAT1 by glutathione S-transferase-SUMO-1 (37 kDa) (Fig. 1B). Two forms of modified NFAT1 were detected (Fig. 1B); because SUMO-1 is not known to polysumoylate itself (23), this result suggests that NFAT1 has two possible sumoylation sites.

Like all members of the NFAT family, NFAT1 is normally present in the cytoplasm of resting cells; when T cells are activated, NFAT1 is dephosphorylated by the Ca2+/calmodulin-dependent phosphatase calcineurin, translocates to the nucleus, and becomes transcriptionally active (1, 30, 31). Nuclear localization of NFAT1 can be induced by low, sustained levels of elevated intracellular free Ca2+, which can be achieved by stimulation of self-antigen, low-affinity peptide-major histo-compatibility complex complexes, or most conveniently, low concentrations of Ca2+ ionophore ionomycin (32). To examine the sumylation of NFAT1 in vivo and a possible effect of intracellular free Ca2+ on sumylation, BHK cells were transfected with the expression constructs of myc-tagged NFAT1 and GFP-SUMO-1. 24 h later, the cells were treated with control, ionomycin, or ionomycin plus PMA. The results were analyzed by Western blots and immunoprecipitation (Fig. 1, C and D). Consistent with previously published reports (30), when myc-NFAT1 was transfected alone without additional treatment, NFAT1 was expressed as a single protein species that was known to be phosphorylated. Treatment with ionomycin, with or without co-treatment with PMA, induced dephosphorylation of NFAT1 as indicated by a downshift on SDS-
Interestingly, co-transfection of myc-NFAT1 with GFP-SUMO-1 significantly elevated the myc-NFAT1 levels (Fig. 1C); furthermore, a portion of myc-NFAT1 now migrated as two slower-migrating species consistent with singly (177 kDa) and doubly (214 kDa) sumoylated forms (Fig. 1C). These two bands were confirmed as NFAT1 by anti-

**Fig. 1. Identification of sumoylation substrates by small pool expression screening.** A, clone 18-3 showed an upshift in mobility consistent with sumoylation. Pools of cDNA clones were screened by in vitro sumoylation assay and subdivided to identify positive candidates. Clone 18-3 was found to encode a partial cDNA for NFAT1. B, in vitro sumoylation of full-length NFAT1. C, modification of NFAT1 by SUMO-1 in BHK cells. BHK cells were transfected with myc-tagged NFAT1 and GFP-SUMO-1 or GFP, and 48 h later, cells were treated with or without ionomycin or ionomycin plus PMA for 1 h. Cell lysates were analyzed by Western blotting using anti-myc antibody. D, confirmation of sumoylation of NFAT1 by immunoprecipitation and Western blotting. An asterisk indicates the nonspecific band. BHK cell lysate as described in C was immunoprecipitated by anti-myc antibody and Western blotted using anti-GFP for GFP-SUMO-1. E, sumoylation of endogenous NFAT1. Jurkat cells were incubated with or without ionomycin or ionomycin plus PMA in the presence or absence of cyclosporin A for 1 h. The cell lysate was analyzed by Western blotting using anti-NFAT1 antibody (left panel). For detection of sumoylated NFAT1 bands, Jurkat cells were transfected with a His-tagged sumo-1 construct by electroporation and, 24 h later, treated with or without ionomycin or ionomycin plus PMA in the presence or absence of cyclosporin A for 1 h. Cells were lysed directly with 6 M guanidine, and then His-tagged sumo-1-conjugated proteins were purified by Ni-column. Sumoylated NFAT1 was detected by Western blotting with anti-NFAT1 antibody (right panel).
NFAT1 antibody (data not shown). Treatment with ionomycin with or without PMA induced dephosphorylation of myc-NFAT1 and increased the levels of sumoylated NFAT1 (Fig. 1C).

To confirm that the two slower-migrating myc-NFAT1 forms are indeed sumoylated, total myc-tagged NFAT1 protein was immunoprecipitated with anti-myc antibody and then analyzed by Western blotting with anti-GFP antibody to detect GFP-SUMO-1-conjugated proteins (Fig. 1D). This analysis confirmed that both slower-migrating bands are indeed SUMO-1-modified myc-NFAT1. These results suggest that overexpression of SUMO-1 can increase the level of NFAT1 and induce the sumoylation of NFAT1, which can be further stimulated by treatment with ionomycin.

In ionomycin- and ionomycin + PMA-treated but not control or ionomycin + cyclosporin A-treated Jurkat cells, our Western blots using anti-NFAT1 detected two additional species with a larger apparent molecular mass than that of phosphorylated and unphosphorylated NFAT1 (Fig. 1E). As commonly found in sumoylation studies, the presence of possible efficient de-sumoylation mechanisms in lysed cells prevented us from immunoprecipitating these two larger forms of NFAT1 efficiently. To circumvent this problem and determine whether endogenous NFAT1 can be sumoylated, we electroporated Jurkat cells with His-tagged SUMO-1 expression construct. Twenty-four h after electroporation, the cells were treated with ionomycin, ionomycin + PMA, or ionomycin + cyclosporin A or left untreated for 1 h, and then the cells were lysed directly in 6 M guanidine. The cell lysate was applied to a Ni-column, and the proteins eluted from the Ni-column were Western blotted using anti-NFAT1 (Fig. 1E). Consistent with our above-mentioned data regarding transfected NFAT1, the two slower-migrating species of endogenous NFAT1 can only be detected in cells treated with ionomycin or ionomycin + PMA. Therefore, our data indicated that endogenous NFAT1 can be sumoylated in response to ionomycin and ionomycin + PMA stimulation.

**Determination of Sumoylation Sites of NFAT1**—Sumoylation is known to occur on the lysine residue with a consensus se-
To confirm that Lys<sup>684</sup> and Lys<sup>897</sup> are sumoylated in vivo, wild type, K684R, K897R, or K684R/K897R mutants were co-expressed in BHK cells together with GFP-SUMO-1, and the results were analyzed by SDS-PAGE (Fig. 2, C and D). The sumoylation of slower-migrating species of NFAT1 was again confirmed by immunoprecipitation and Western blotting (Fig. 2, C and D). Consistent with Fig. 1, the predominant sumoylated form of wild-type NFAT1 in the absence of ionomycin treatment is the 177-kDa form (Fig. 2C), which is eliminated by the K684R mutation but not by K897R mutation, suggesting that the 177-kDa NFAT1 is sumoylated at Lys<sup>684</sup>. On the other hand, the K897R mutation had no effect on the appearance of 177-kDa NFAT1. Treatment with ionomycin induced dephosphorylation of wild-type and mutant NFAT1 and promoted both sumoylated NFAT1 forms (177 and 214 kDa). Consistent with in vitro sumoylation analysis, 214-kDa NFAT1 is eliminated by both the K684R single mutation and K684R/K897R double mutation. These results suggest that 214-kDa NFAT1 is di-sumoylated at both Lys<sup>684</sup> and Lys<sup>897</sup> and that the sumoylation of Lys<sup>684</sup> occurs first and is required for the sumoylation of Lys<sup>897</sup>. Any combination of mutations in these lysines, however, had no effect on the calcineurin-induced dephosphorylation induced by ionomycin, suggesting that the sumoylation of NFAT1 occurs in a step downstream from dephosphorylation by calcineurin.

A Functional Role of Sumoylation at Lys<sup>897</sup> in the Nuclear Retention of NFAT1—Sumoylation has been shown to promote...
Because NFAT1 is known to shuttle between the cytoplasm and the nucleus, we determined whether sumoylation of NFAT1 plays a role in the nuclear translocation of NFAT1. As reported, wild-type NFAT1 expressed in BHK cells is predominantly cytoplasmic without additional treatment; the addition of ionomycin induced NFAT1 nuclear translocation efficiently (Fig. 3A). Treatment with LMB alone increased the nuclear presence of NFAT1, suggesting that NFAT1 shuttles between the cytoplasm and nucleus in the unstimulated cells. The expressed K684R, K897R, and K684R/K897R NFAT1 in unstimulated BHK cells were indistinguishable from those of wild-type NFAT1 (Fig. 3A, top row; Fig. 3B, top left panel). In contrast to wild-type NFAT1, however, a considerable portion of NFAT1 mutants in ionomycin-treated BHK cells remained cytoplasmic (Fig. 3A, second row). A quantitation of staining such as that in Fig. 3A showed that wild-type NFAT1 was nuclear in 75% of cells after ionomycin treatment, whereas the proportion of cells with nuclear NFAT1 in cells expressing K684R, K897R, and K684R/K897R was 30.1%, 28.3%, and 30.3%, respectively (Fig. 3B, top right panel). An additional portion of cells showed mutant NFAT1 in both the cytoplasm and nucleus (Fig. 3B, top right panel). These results suggest that mutations in these three lysine residues had a significant impact on either the nuclear transport or retention of NFAT1.

To distinguish whether mutations in these three lysine residues affect the nuclear import or export of NFAT1, we treated cells expressing wild-type or mutant NFAT1 with the nuclear export inhibitor LMB. The response of NFAT1 mutants to LMB treatment alone was not significantly different from that of the wild type: each showed an increase in the nuclear presence of NFAT1, with the majority of cells exhibiting both cytoplasmic and nuclear NFAT1 in the presence of LMB (Fig. 3A, third row; Fig. 3B, bottom left panel). This result suggests that the basal cytoplasmic and nuclear shuttling of NFAT1 was not affected by the lysine mutations and that inhibition of nuclear export is sufficient to increase the nuclear presence of these NFAT1 mutants to an extent similar to that of wild type. Interestingly, whereas treatment with ionomycin alone was sufficient to induce efficient nuclear translocation of the wild type but not of the NFAT1 lysine mutants (Fig. 3A, second row; Fig. 3B, top right panel), the addition of LMB in the presence of ionomycin resulted in an exclusively nuclear presence of NFAT1 in ~80% of cells expressing K684R, K897R, or K684R/K897R mutant as well as wild-type NFAT1 (WT) (Fig. 3A, last row; and Fig. 3B, bottom right panel). These results suggest that sumoylation is
required not for nuclear import but for the nuclear retention of NFAT1 in control as well as in ionomycin-treated cells. Because the K897R mutation minimally affects the appearance of 214-kDa sumoylated NFAT1, these results suggest that sumoylation of Lys$^{897}$ is critically needed for the nuclear retention of NFAT1.

A Functional Role of Sumoylation at Lys$^{684}$ in the Transcriptional Activity of NFAT1—To assess the role of sumoylation on NFAT1 function, we determined the impact of sumoylation on the transcriptional activity of NFAT1 using two known NFAT1-targeting promoters, a FasL promoter (34) and a NFAT/AP-1 promoter, in promoter-driven luciferase expression assays (Fig. 4A, left panels). FasL mutant promoter- and nuclear factor-κB promoter-driven luciferase assays were used as negative controls (Fig. 4A, right panels). As reported previously (30), wild-type NFAT1 (WT) showed a high level of transcriptional activity after ionomycin plus PMA stimulation (Fig. 4A, left panels). The expression of the K684R, K897R, and K684R/K897R NFAT1 mutants showed a low level of transcriptional activity that did not respond to ionomycin plus PMA stimulation, consistent with their defects in nuclear retention. Because, as shown in Fig. 3, treatment with LMB and ionomycin promoted the nuclear presence of the wild-type, K684R, K897R, and K684R/K897R mutants, we examined whether treatment with LMB and ionomycin plus PMA could enhance the transcriptional activity of the wild-type, K684R, K897R, and K684R/K897R mutants (Fig. 4A, left panels). Treatment with LMB enhanced the transcriptional activity of wild-type NFAT1 (WT) slightly, in proportion to the slight increase in its nuclear localization (Fig. 4A, left panels). Surprisingly, treatment with LMB in the presence of ionomycin and PMA enhanced the transcription of the K897R mutant, but not that of the K684R and K684R/K897R mutants (Fig. 4A, left panels). This result suggests that the sumoylation at Lys$^{684}$ of NFAT1 is important for transcriptional activity of NFAT1; whereas sumoylation at Lys$^{897}$ is only required for nuclear retention of NFAT1.

PMA Promoted the Recruitment of NFAT1 into Nuclear SUMO-1 Bodies—PMA and ionomycin are often used conjunctively to promote the transcriptional activity of NFAT family and T-cell activation (10). Because treatment with ionomycin alone is sufficient to increase the sumoylation of NFAT1 (Fig. 1C), we determined whether PMA has a discernable effect on the sumoylation of NFAT1 by immunocytochemistry. We co-transfected BHK cells with myc-NFAT1 and GFP-SUMO-1; in unstimulated cells, the majority of SUMO-1 is cytoplasmic, whereas the majority of SUMO-1 is nuclear (Fig. 5A, top row). Treatment with ionomycin induced nuclear translocation of NFAT1, but myc-NFAT1 is diffusely distributed throughout the nucleus (Fig. 5A, second row). Treatment with PMA alone resulted in an increase in the number of SUMO-1 bodies in the nucleus and a possible increase in nuclear membrane-associated NFAT1 but was not sufficient to induce nuclear translocation of myc-NFAT1 (Fig. 5A, fourth row); however, treatment with both ionomycin and PMA resulted in the recruitment of NFAT1 into the SUMO-1 nuclear bodies (Fig. 5A, third row; Table I). These results suggest that the powerful effect of PMA on T-cell activation in the presence of ionomycin may be exerted by promoting the recruitment of NFAT1 into the nuclear SUMO-1 bodies. Furthermore, the recruitment of NFAT1 into the nuclear SUMO-1 bodies by PMA and ionomycin suggests that the SUMO-1 bodies are nuclear transcriptional hot spots.

After treatment with ionomycin, ~30% of cells expressing NFAT mutants K684R, K897R, and K684R/K897R showed nuclear localization of mutant proteins (Fig. 3B). To determine whether mutations in the sumoylation sites affect the recruitment of NFAT1 mutants into the SUMO-1 bodies, we determined the subnuclear distribution of NFAT1 mutants with nuclear localization by immunostaining and confocal microscopy. K897R (Fig. 5C), but not K684R (Fig. 5B) and K684R/K897R NFAT1 mutants (Fig. 5D; Table I), was recruited into SUMO-1 bodies after treatment with ionomycin plus PMA. From these results, we conclude that Lys$^{684}$, but not Lys$^{897}$, of NFAT1 is required for the recruitment into the nuclear SUMO-1 bodies.

FIG. 5. The distinct effects of ionomycin and PMA on the nuclear translocation and subnuclear localization of NFAT1. BHK cells were transfected with NFAT1 wild type (A), K684R (B), K897R (C), or K684R/K897R (D) mutants; 24 h later, the cells were treated with or without ionomycin, ionomycin plus PMA, or PMA alone for 1 h. The cells were fixed and immunostained with anti-myc antibody followed by Texas Red-conjugated anti-mouse secondary antibody. The data were analyzed by laser scanning confocal microscopy.

DISCUSSION

Our study identified a novel mechanism regulating the nuclear presence and transcriptional activity of NFAT1. The sumoylation of Lys$^{684}$ and Lys$^{897}$ may occur in a sequential or simultaneous fashion because mutation of Lys$^{684}$ blocks the sumoylation of Lys$^{897}$. Furthermore, the K684R mutation blocks both transcriptional activity and recruitment into the nuclear SUMO-1 bodies, implying a possible mechanistic link between these two sumoylation events. On the other hand, the K897R mutation blocks only nuclear anchorage, which can be partially substituted by treatment with LMB. Furthermore, because mutations in neither Lys$^{684}$ nor Lys$^{897}$ affect the dephosphorylation of NFAT1 by calcineurin, our results suggest that sumoylation of NFAT1 regulates the nuclear anchorage and transcription of NFAT1 downstream from phosphorylation.

Because Lys$^{897}$ is localized to the C terminus of NFAT1 and distal to the NFAT-h, which is known to regulate nuclear translocation by interacting with calcineurin, our study identified a new mechanism that contributes to the regulation of the nuclear presence of NFAT1. Our study identified a previously unexpected role of Lys$^{897}$, located in the C terminus of NFAT1, in the nuclear retention of NFAT1 and suggests the
possibility that the C terminus of NFAT1 may interact with the nuclear export signal localized in the N terminus of the protein. The C-terminal tails of the NFAT family, where Lys^{687} is localized in NFAT1, are highly divergent, and a similar KXE sequence cannot be found in the other members of NFAT family. Although it is possible that sumoylation at Lys residues less conforming to the KXE consensus sequence occur in the C termini of other NFAT family members, our study suggests that sumoylation may provide one of the regulatory mechanisms differentiating the nuclear presence of different NFAT family members.

Sumoylation has been shown to have diverse effects on proteins. Sumoylation of RanGAP1 promotes the interaction of RanGAP1 with the Ran-GTP-binding protein RanBP2 at the cytoplasmic face of the nuclear pore complex (21, 35), whereas SUMO-1 modification of the promyelocytic leukemia gene product (PML) targets the protein to PML oncogenic domains (23, 36). Sumoylation of NFAT1 appears to enhance both the nuclear retention of NFAT1 and SUMO-1 body targeting. Although Lys^{687} is localized in the C terminus of NFAT1, in which no homologous Lys residue is found in other members of NFAT family, homologous Lys residues for Lys^{684} NFAT1 that fit the motif KXE can be found at least in NFAT3 and NFAT4, suggesting that NFAT3 and NFAT4 may be similarly sumoylated at this Lys as well. Because Lys^{684} is localized immediately after the known DNA binding domain of NFAT1, our results suggest that sumoylation at this Lys and its interaction with the DNA binding domain may be a shared mechanism for regulation of transcriptional activity in the NFAT family.

How might sumoylation of Lys^{684} regulate the transcriptional activity of NFAT1? Overexpression of GFP-SUMO-1 significantly increased the levels of both sumoylated and unsumoylated NFAT1 (Fig. 2), suggesting that sumoylation of NFAT1 may increase the stability of NFAT1, which may account in part for the transcriptional activation. Furthermore, because K864R NFAT1 is transcriptionally inactive even in the presence of LMB, which prevents its exit from the nucleus, and is unable to be recruited into the nuclear SUMO-1 bodies in the presence of ionomycin and PMA, the sumoylation of Lys^{684} may allow NFAT1 to interact with a key protein complex critical for effective transcription. We propose that the sumoylation of Lys^{684} NFAT1 may provide a license for transcriptional activity of NFAT1.

In lymphocytes, integration of Ca^{2+} and other signaling pathway results in productive activation, whereas unopposed Ca^{2+} signaling leads to tolerance or anergy. Calcium-regulated transcription factor NFAT has an integral role in both aspects of lymphocyte function. Ca^{2+}/calcineurin signaling induces a limited set of anergy-associated genes, distinct from genes induced in the productive immune response. Thus, NFAT1 induces T-cell anergy in the absence of AP-1, and it induces a program of productive activation mediated by the cooperative NFAT-AP-1 complex in the presence of AP-1. Stimulation by ionomycin alone mimics anergy by inducing a limited set of anergy-associated genes, whereas ionomycin + PMA stimulation induces the productive immunoresponsive genes (37). Given the distinct effects of stimulation with ionomycin alone and combined stimulation with both ionomycin and PMA, we speculate that perhaps the activation of anergy-associated genes can occur without association with the SUMO-1 bodies, whereas the activation of a productive immune response requires recruitment of NFAT1 into the nuclear SUMO-1 bodies, where the cooperative NFAT1-AP1 complex may form.

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**REFERENCES**

1. Crabtree, G. R. (1999) *Cell* 96, 611–614
2. McCaffrey, P. G., Luo, C., Kerpola, T. K., Jain, J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A., and Hogan, P. G. (1993) *Science* 262, 750–754
3. Northrop, J. P., Ha, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994) *Nature* 369, 497–502
4. Hoey, T., Sun, Y. L., Williamson, K., and Xu, X. (1995) *Immunity* 2, 461–472
5. Masuda, E. S., Naito, Y., Tomokatu, H., Campbell, D., Saito, F., Hannum, C., Araki, K., and Araki, N. (1995) *Mol. Cell. Biol.* 15, 2697–2706
6. Hodge, M. R., Chun, H. J., Renganarajan, J., Alt, A., Lieberman, R., and Glimcher, L. H. (1996) *Science* 274, 1903–1906
7. Ranger, A. M., Ouleka, M., Renganarajan, J., and Glimcher, L. H. (1998) *Immunity* 9, 627–635
8. Xanthoudakis, S., Viola, J. P., Shaw, R. T., Luo, C., Wallace, J. D., Bozza, P. T., Luo, D. C., Curran, T., and Rao, A. (1996) *Science* 272, 892–895
9. de la Pompa, J. L., Timmerman, L. A., Takimoto, H., Yoshida, H., Elia, A. J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B. L., Crabtree, G. R., and Mak, T. W. (1998) *Nature* 392, 183–188
10. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) *Cell* 66, 871–875
11. Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Fleming, M. A., Caron, P. R., Hsiao, K., and Navia, M. A. (1995) *Cell* 82, 507–522
12. Kissinger, C. R., Purge, H. E., Knighton, D. R., Lewis, C. T., Pelletier, L. A.,...
