Glycogen Synthase Kinase-3 Inhibits the DNA Binding Activity of NFATc*

The NFAT family of transcription factors is required for the expression of numerous immunologically important genes and plays a pivotal role in both the initiation and coordination of the immune response. NFAT family members appear to be regulated primarily at the level of their subcellular localization. Here we show that NFATc is additionally regulated at the level of its DNA binding activity. Using gel mobility shift assays, we demonstrate that the intrinsic DNA binding activity of NFATc is negatively regulated by phosphorylation. We found that activation of calcineurin activity in cells and dephosphorylation of NFATc in vitro enhanced NFATc DNA binding activity, whereas phosphorylation of NFATc in vitro inhibited its ability to bind DNA. Through the analysis of NFATc mutants, we identified the conserved Ser-Pro repeat motifs as critical quantitative determinants of NFATc DNA binding activity. In addition, we provide several lines of evidence to suggest that the phosphorylation of the Ser-Pro repeats by glycogen synthase kinase-3 inhibits the ability of NFATc to bind DNA. Taken together, these studies afford new insights into the regulation of NFATc and underscore the potential role of glycogen synthase kinase-3 in the regulation of NFAT-dependent gene expression.

Physiological engagement of the T cell antigen receptor initiates a complex series of intracellular signaling events that ultimately activate the expression of a panel of genes involved in both initiating and coordinating the immune response (1, 2). The NFAT\(^1\) family of transcription factors is known to play a pivotal role in the regulation of these events (3, 4). All members of the NFAT family share a conserved region of 270 amino acid residues that is related to the Rel domain and is involved in sequence-specific DNA binding (5–9). NFAT proteins bind to DNA as monomers (7), although, in many cases, they have been shown to bind cooperatively together with a nuclear binding partner, such as members of the AP-1 family of transcription factors (4, 10–13). NFAT family members are involved in the transcription of many immunologically important genes, including the cytokines IL-2, IL-3, IL-4, IL-5, granulocyte/macrophage colony-simulating factor, and tumor necrosis factor-α, and several cell-surface molecules such as CD40L and FasL (3, 4). Three members of the NFAT family are expressed in T cells: NFATp (NFAT1/NFATc2), NFATc (NFAT2/NFATc1), and NFAT4 (NFATx/NFATc3) (5–9). Analysis of mice deficient in individual NFAT family members has revealed that these proteins play distinct and largely non-overlapping functions in T cell biology (14–19). In addition to their effects in lymphocytes, NFAT family members have also been shown to play a variety of roles in other non-lymphoid tissues (20–23).

NFAT proteins appear to be regulated primarily at the level of their subcellular localization (3, 4, 24–26). They are normally located in the cytoplasm of resting cells in a latent form, but are induced to enter the nucleus in response to an elevation in the intracellular calcium concentration and the subsequent action of the calcium-regulated serine/threonine phosphatase calcineurin (3, 4, 24–26). Once activated, calcineurin directly dephosphorylates a number of highly conserved serine residues located in the NFAT N-terminal regulatory domain, causing NFAT proteins to translocate rapidly into the nuclear compartment, bind their DNA target sequences, and activate gene transcription (4, 25, 26). The calcineurin-mediated dephosphorylation and nuclear localization of NFAT are directly counteracted by a number of protein kinases (27–32). These kinases can either antagonize NFAT nuclear translocation or promote the nuclear export of NFAT proteins. As a result, efficient initiation of NFAT-dependent gene expression requires a sustained increase in the intracellular calcium concentration (33–35), presumably to maintain calcineurin in an active state capable of overcoming the inhibitory effects of the NFAT kinases. In addition, sustained signaling through the calcium/calmodulin pathway is required to maintain NFAT-dependent transcription since the inhibition of calcineurin is known to attenuate ongoing NFAT-dependent gene expression (28). Furthermore, the efficiency of NFAT-dependent gene expression has been shown to be extremely sensitive to the frequency of intracellular calcium oscillations (35). Accordingly, NFAT-dependent transcription is highly dynamic and exquisitely sensitive to both quantitative and qualitative changes in the calcium/calmodulin signaling pathway.

Although much attention has been paid to the regulation of NFAT subcellular localization, less is known about the regulation of the intrinsic DNA binding activity of NFAT. However, given the dynamic nature of NFAT-dependent gene expression, it is likely that the interaction of NFAT with DNA is also tightly regulated. In fact, previous work has suggested that the DNA binding activity of NFATp is influenced by its state of phosphorylation, although the mechanisms involved remain unclear (36–38). In this study, we investigated the regulation of NFATc DNA binding activity. We demonstrate that the intrinsic DNA binding activity of NFATc is negatively regulated by phosphorylation. Furthermore, we identify the three highly conserved Ser-Pro (SP) repeat motifs within the NFAT
homology domain as key quantitative determinants of NFATc DNA binding affinity and provide evidence that phosphorylation of these residues by the protein kinase GSK-3 inhibits the ability of NFATc to bind DNA. Taken together, our studies afford new insights into the regulation of NFATc and underscore the potential role of GSK-3 in the regulation of NFAT-dependent gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—COS and Jurkat TAg cells were maintained at 37 °C in 7.5% CO2 in growth medium (RPMI 1640 from Bio-Rad Laboratories, Inc., supplemented with 10% fetal calf serum (HyClone Laboratories, Inc.). 100 units/ml penicillin G, and 100 μg/ml streptomycin). For COS cell transfections, cells (105) were suspended in 0.4 ml of growth medium and placed in an electroporation cuvette (0.4-cm gap; Bio-Rad) together with the indicated amount of plasmid DNA (3 μg for NFATc expression vectors and 10 μg for GSK-3β-S9A). The amount of plasmid DNA was held constant by addition of the pcDNA3 vector control. After incubation at room temperature for 5 min, cells were exposed to an electric field of 230 V at a capacitance of 960 microfarads (Gene Pulser II, Bio-Rad) and, after a 5-min recovery period, were replated in growth medium and placed at 37 °C. Jurkat TAg cell transfections were similar, except that an electric field of 250 V and 10 μg of plasmid DNA was held constant with expression vectors with no restriction.

Expression Constructs and Reconstituent Proteins—The FLAG-tagged wild-type and mutant NFATc expression constructs in the mammalian expression vector pBbJ5 have been described previously (39). The NFATc mutants comprise the indicated serine-to-alanine substitutions: NFATc-SR5 (S172A, S175A, S176A, S179A, S181A, S184A, S187A, S188A, S191A, and S194A), NFATc-mSpX (S199A, S203A, S207A, and S211A), NFATc-mSp6 (S190A, S203A, S207A, and S211A) and, NFATc-mSpXp (S199A, S203A, S207A, and S211A, S233A and S241A) and S278A, S282A, S286A, and S290A). The NFATc-N414 deletion mutant encodes NFATc amino acids 415-718 in the pBbJ5 mammalian expression vector. For in vitro translation constructs, wild-type and mutant NFATc constructs without the FLAG tag were subcloned into pcITE-4b (Novagen). GSK-3β-S9A in pcDNA3 was a generous gift of E. G. Krebs (40).

Whole Cell Extracts (WCE) and Electrophoretic Mobility Shift Assay (EMSA)—40 h post-transfection, COS cells were treated for 30 min with 3 μM ionomycin (Calbiochem) plus 10 mM CaCl2 in the presence or absence of FK506 (5 μM). Where indicated, 20 μM LiCl was also added to cells. Cells were washed twice with cold phosphate-buffered saline (137 mM NaCl, 2.5 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4, final pH 7.4), scrapped off plates, and briefly centrifuged at room temperature at 15,000 × g. Cell pellets were vigorously resuspended in 0.15 ml of WCE buffer (20 mM HEPES-KOH, pH 7.6, 25% glycerol, 1 mM dithiothreitol, 1 mM EGTA, 0.5 mM MgCl2, and 5 mM MgCl2, supplemented with the anti-FLAG M2 antibody (1 μg/ml; Sigma) for 1 h, washed three times with cold phosphate-buffered saline, and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed Laboratories Inc.) for 1 h. The blots were washed again and visualized using ECL (Amersham Pharmacia Biotech) and Kodak XAR film. In vitro translated samples labeled with [35S]methionine (Amersham Pharmacia Biotech) were resolved on an 8% SDS-polyacrylamide gel, dried, and exposed to Kodak XAR film overnight.

In Vitro Translation—In vitro transcription and translation of wild-type and mutant pCITE-4 constructs were performed using the Single Tube Protein System 2 (Novagen) as recommended by the manufacturer. WCE were resolved by electrophoresis on a 4% native polyacrylamide gel, dried, and exposed to autoradiography as described above. An additional control included the use of unlabeled methionine for SDS-PAGE analysis or unlabeled methionine for EMSA.

GSK-3 in Vitro Phosphorylation Reactions—To determine the effects on NFATc of GSK-3 in vitro phosphorylation, 1.5 μl of either the wild-type or mutant NFATc in vitro translation products were incubated at 30 °C for 20 min in kinase buffer (20 mM Tris-CI, pH 7.5, 10 mM MgCl2, and 5 mM dithiothreitol) supplemented with 0.5 mM ATP, 1.5 μg of poly(dI-dC), and 5% glycerol in either the presence or absence of 5 units of purified GSK-3β (New England Biolabs Inc.). Subsequently, 0.3 ng of radioactive probe was added; the reaction was incubated at room temperature for 20 min; and protein-DNA complexes were separated by native gel electrophoresis. To determine the effects of GSK-3 phosphorylation on preformed NFATc-DNA complexes, either the wild-type or mutant NFATc in vitro translation products were incubated in kinase buffer supplemented with 0.5 mM ATP, 1.5 μg of poly(dI-dC), 5% glycerol, and radiolabeled NFATc probe for 20 min at room temperature to allow for efficient NFATc-DNA binding. Reactions were then incubated in either the presence or absence of 5 units of purified GSK-3β at 30 °C for the indicated times, and protein-DNA complexes were separated by native gel electrophoresis.

RESULTS

NFATc DNA Binding Activity Is Negatively Regulated by Phosphorylation—Whereas previous studies on NFATc have largely focused on the regulation of its subcellular localization, we have chosen to investigate the regulation of its DNA binding activity. We first wanted to determine the effects of calcineurin activation on the DNA binding activity of NFATc. COS cells were used as a convenient model system for this study since they are known to support calcium-dependent, FK506-sensitive changes in NFATc subcellular localization, yet lack endogenous NFAT proteins (29, 32, 39). Thus, COS cells were transfected with an expression vector encoding FLAG-tagged wild-type NFATc and stimulated with calcium ionophore in either the presence or absence of FK506. Whole cell extracts were subsequently prepared and analyzed by EMSA using a radiolabeled NFAT probe derived from the murine IL-4 promoter (41). Using whole cell extracts, we were able to eliminate differences due to alterations in subcellular localization and instead focus on differences in the intrinsic DNA binding activity of NFATc. COS cells were treated with 2 μM ionomycin for 30 min, and COS cells were stimulated as described above. Where indicated, LiCl was added to a final concentration of 20 mM. Cell extracts were prepared by lysis in cell extract buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 2.5 μg/ml leupeptin, and phosphatase inhibitors).

Immunoblotting and SDS-PAGE—Cell extracts were mixed with an equal volume of 2× SDS gel loading buffer (100 mM Tris, pH 6.8, 4%, SDS, 0.2% bromphenol blue, 20% glycerol, and 2% 2-mercaptoethanol) and heated to 100 °C for 5 min. Samples were resolved on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose blots were washed again and visualized using ECL (Amersham Pharmacia Biotech) and Kodak XAR film. In vitro translated samples labeled with [35S]methionine (Amersham Pharmacia Biotech) were resolved on an 8% SDS-polyacrylamide gel, dried, and exposed to Kodak XAR film overnight.

Phosphorylation—The in vitro phosphorylation of NFATc, NFATc-transfected cells were harvested in WCE buffer lacking phosphate sinkers. WCE were then incubated in phosphate buffer (50 mM Tris-HCl, pH 8.5, and 0.1 mM EDTA) in either the presence or absence of 1 unit of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals) at 37 °C for 30 min. As described previously (41), the effect of endogenous kinases on NFATc DNA binding activity, WCE prepared from ionomycin-stimulated NFATc-transfected cells were incubated at 37 °C in the presence or absence of 1 mM ATP for 1 h and analyzed by EMSA.

For the experiment shown in Fig. 4B, NFATc-transfected Jurkat TAg cells were stimulated with 2 μM ionomycin for 30 min, and COS cells were stimulated as described above. Where indicated, LiCl was added to a final concentration of 20 mM. Cell extracts were prepared by lysis in cell extract buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 2.5 μg/ml leupeptin, and phosphatase inhibitors).
NFATc DNA binding activity was potently inhibited by the presence of the immunosuppressant drug FK506 (Fig. 1, upper panel, lane 4), indicating a role for calcineurin. The specificity of the DNA binding complex was confirmed by its competition with an excess of unlabeled IL-4 probe, but not with an excess of an unrelated oligonucleotide (data not shown). Importantly, the complex was also not competed with a consensus AP-1 probe (data not shown). This observation indicates that the observed gel shift did not result from cooperative binding of NFATc and AP-1, but rather represents NFATc binding to the probe (data not shown). This observation indicates that the observed DNA binding complex was specifically shifted complexes (arrowhead) were determined by scanning densitometry and are indicated below each lane. Lower panel, parallel samples were analyzed by 8% SDS-PAGE, followed by immunoblotting with the anti-FLAG M2 mAb.

Next we performed the converse experiment by incubating extracts prepared from non-stimulated NFATc-transfected COS cells transiently transfected with FLAG-tagged wild-type NFATc were incubated at 37 °C for 1 h in the presence of buffer alone (NS) or were treated with 3 mM ionomycin (Iono) or 3 mM ionomycin plus 5 ng/ml FK506 (Iono + FK506) for 30 min. WCE were prepared, and DNA binding activity was measured by EMSA. The asterisk indicates a nonspecific band observed in all lanes. The relative intensities (R.I.) of the specifically shifted complexes were determined by scanning densitometry and are indicated below each lane. Lower panel, parallel samples were analyzed by 8% SDS-PAGE, followed by immunoblotting with the anti-FLAG M2 mAb.

Since it is well established that calcineurin promotes nuclear translocation of NFATc through dephosphorylation of regulatory serine residues (4, 25, 26), we next chose to test whether the phosphorylation status of NFATc plays a direct role in regulating its DNA binding activity. Accordingly, whole cell extracts prepared from non-stimulated NFATc-transfected cells were incubated in vitro with calf intestinal alkaline phosphatase. As shown in Fig. 2A, this treatment resulted in a dramatic increase in NFATc-dependent DNA binding activity, an effect that was inhibited by the presence of phosphatase inhibitors. Thus, it appears that dephosphorylation of NFATc in vitro is able to directly increase its ability to bind DNA, which is consistent with previous findings for NFATp (37). Next we performed the converse experiment by incubating extracts that contained a high level of NFATc DNA binding activity in the presence of ATP. As shown in Fig. 2B, NFATc DNA binding activity was greatly diminished in extracts incubated with ATP compared with control extracts incubated without ATP. Presumably, the endogenous protein kinases present in the extracts are able to rephosphorylate NFATc and to inhibit its ability to bind DNA. These results strongly suggest that the phosphorylation status of NFATc plays an important role in regulating its ability to bind DNA.

Identification of the NFATc Ser-Pro Repeats as Critical Determinants of DNA Binding Activity—Having implicated the phosphorylation state of NFATc as an important determinant of its ability to bind DNA, we next wanted to identify the specific regulatory amino acid residues that mediate this effect. To identify the region of NFATc responsible for the phosphorylation-dependent regulation of DNA binding, we first examined the effects of ionomycin stimulation on the DNA binding activity of an NFATc deletion mutant (NFATc-NA415) composed of only the C-terminal Rel homology domain. As shown in Fig. 3B, the C-terminal fragment of NFATc bound DNA constitutively and was unaffected by ionomycin stimulation. This constitutive DNA binding activity observed following removal of the NFATc N-terminal 415 amino acids suggests that the N-terminal region normally acts to inhibit the activity of the C-terminal DNA binding domain. Furthermore, the lack of ionomycin responsiveness suggests that the N-terminal region, not the C-terminal DNA binding domain, is likely to contain the regulatory phosphoamino acid residues targeted by calcineurin.

The N terminus of NFATc is known to be highly phosphorylated and to contain several functionally important conserved sequence motifs rich in serine residues (4, 25, 26), including the serine-rich domain (SRD) and three Ser-Pro repeat motifs (Fig. 3A). Both of these sequence motifs have previously been implicated in the phosphorylation-dependent control of NFATc subcellular localization (29, 39). To investigate the potential role of each of these motifs in the regulation of phosphorylation-dependent NFATc DNA binding activity, we examined the DNA binding activity of NFATc mutants containing substitutions of serine to non-phosphorylatable alanine residues in each of these conserved sequences. The NFATc-mSRD in which all three SP repeat motifs were alanine-substituted, was found to constitutively bind DNA even in the absence of ionomycin stimulation (Fig. 3C, upper panel, lane 3). In fact, ionomycin stimulation did not further increase the level of DNA binding of this NFATc mutant (see Fig. 4B, compare lanes 5 and 6). In contrast, the alanine-substituted NFATc-mSRD mutant, which is known to be constitutively localized to the nu-
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Conserved SP repeat motifs are critical determinants of NFATc DNA binding activity. A, shown is a schematic of the FLAG-tagged NFATc mutants used in this study. The various serine-to-alanine substitutions in the conserved SRD and SP repeat motifs are indicated. B, shown are the results from EMSA analysis of WCE prepared from COS cells transiently transfected (TF) with either control plasmid (mock) or NFATc-NA415 and either left non-stimulated (NS) or treated with 3 μM ionomycin (iono) for 30 min. The mobility of the specifically shifted complex is indicated by the arrowhead, and the nonspecific band is indicated by the asterisk. C, WCE were prepared from COS cells transiently transfected with plasmids encoding the indicated FLAG-tagged NFATc molecules and analyzed for DNA binding activity by EMSA (upper panel). The relative intensities (R.L.) of the specifically shifted complexes were determined by scanning densitometry and are indicated below each lane. Parallel samples were analyzed by 8% SDS-PAGE, followed by immunoblotting with the anti-FLAG M2 mAb (lower panel).

Evidence of a Role for GSK-3 in the Regulation of NFATc DNA Binding Activity in COS Cells—Previous work has shown that GSK-3 can phosphorylate the SP repeat motifs of NFATc and is thought to promote NFATc nuclear export (29). Since the SP repeat motifs also appear to regulate DNA binding activity, GSK-3 seemed a likely candidate as a potential regulator of NFATc DNA binding activity. We therefore investigated whether manipulation of GSK-3 activity in cells could affect the DNA binding activity of NFATc. Previous work has demonstrated that LiCl can directly inhibit the enzymatic activity of GSK-3 in cells (42, 43); therefore, we tested the effect of LiCl treatment on NFATc DNA binding activity. As shown in Fig. 4A (upper panel, lane 4), treatment of NFATc-transfected COS cells with LiCl induced NFATc to bind DNA at a level comparable to that of ionomycin stimulation, whereas treatment of cells with both ionomycin and LiCl resulted in an additive increase in DNA binding activity. Immunoblot analysis revealed equivalent levels of protein expression and that stimulation with both ionomycin and LiCl resulted in a marked increase in the migration of NFATc as detected by SDS-PAGE (Fig. 4A, lower panel). This increase in migration of NFATc likely indicates the generation of highly dephosphorylated forms of NFATc, presumably caused by the combined effects of calcineurin-mediated NFATc dephosphorylation and the attenuation of NFATc rephosphorylation by the LiCl-mediated inhibition of GSK-3. Taken together, these results are consistent with the notion that GSK-3-mediated phosphorylation acts to negatively regulate the DNA binding activity of NFATc.

To further investigate the potential role of GSK-3 in the regulation of NFATc, we examined the consequences of GSK-3 overexpression on the ionomycin-induced increase in NFATc DNA binding activity. The GSK-3 protein kinase is normally constitutively active in resting cells, but is inhibited when phosphorylated by a number of mitogen-activated protein kinases (44–46). Consequently, to ensure that GSK-3 remained active in our assays, we used a GSK-3β mutant (GSK-3β-S9A) that contains an alanine substitution at Ser9, the principal regulatory site of phosphorylation, and is therefore resistant to mitogen-induced inhibition (40). As shown in Fig. 4B (upper panel, compare lanes 2 and 4), overexpression of GSK-3β-S9A attenuated the increase in NFATc DNA binding activity following stimulation with ionomycin. Similar results were obtained.
with wild-type GSK-3β (data not shown). This effect appears to be mediated by the conserved SP repeats, as overexpression of GSK-3β-S9A did not have an effect on the DNA binding activity of the mutant NFATc-mSPx3 (Fig. 4B, upper panel, lanes 5–8).

Next we examined how the extent of phosphorylation and dephosphorylation of NFATc that we have observed in COS cells relates to that in T cells. Thus, we compared the migration of FLAG-tagged NFATc expressed in COS cells with that expressed in the Jurkat T cell line. As shown in Fig. 4C, NFATc isolated from either COS or Jurkat cells under non-stimulated conditions exhibited a similar migration on SDS-PAGE, indicating that, under resting conditions, NFATc is likely to be phosphorylated to a similar extent in the two different cell types (Fig. 4C, upper panel, compare lanes 1 and 4). Whereas treatment with ionomycin resulted in a modest increase in the migration of NFATc in both cell types, a more marked increase in the migration of NFATc was observed following stimulation with both ionomycin and LiCl. Initial inspection of the immunoblot indicated that these increases in NFATc migration were more profound in COS cells than in the Jurkat cell line. However, it is important to note that the fastest migrating forms of NFATc observed in ionomycin/LiCl-treated Jurkat cells appeared to comigrate with the fastest migrating NFATc forms observed in ionomycin/LiCl-treated COS cells. This observation was more evident upon examination of a longer exposure of the Jurkat samples (Fig. 4C, upper panel, compare lanes 3 and 6; and lower panel, lane 3). Thus, it appears that the absolute degree of NFATc dephosphorylation, as measured by mobility shift, is similar in both Jurkat and COS cells, although the efficiency of dephosphorylation in the two cell types may differ.

Moreover, these results indicate that LiCl markedly affects the migration of NFATc in both cell types, presumably by preventing GSK-3 from phosphorylating NFATc. Finally, it should be noted that the NFATc-mSPx3 mutant exhibited a similar migration to the most rapidly migrating forms of NFATc detected in ionomycin/LiCl-treated Jurkat and COS cells. This observation is consistent with the notion that the NFATc-mSPx3 mutant does indeed mimic a highly dephosphorylated form of NFATc.

Effects of GSK-3 Treatment on the DNA Binding Activity of NFATc in Vitro—Next we wanted to test whether purified GSK-3 could directly inhibit NFATc DNA binding activity in vitro. For these experiments, in vitro translated wild-type NFATc was incubated in either the presence or absence of purified GSK-3β and then examined for DNA binding activity by EMSA. As shown in Fig. 5A, the ability of the in vitro translated wild-type NFATc to bind DNA was potently inhibited by incubation with purified GSK-3β. This effect appeared to be dependent upon the SP repeat motifs, as the NFATc-mSPx3 mutant was largely resistant to the inhibitory effects of GSK-3β (Fig. 5A, lanes 3 and 4). Concomitant with its effects on NFATc-DNA binding, GSK-3β treatment had a significant effect on the mobility of wild-type NFATc as detected by SDS-PAGE (Fig. 5B). This difference in mobility presumably reflects the GSK-3-mediated phosphorylation of the SP repeat motifs since the NFATc-mSPx3 mutant was unaffected by GSK-3 treatment. Thus, it appears that treatment with GSK-3β potently inhibits the DNA binding activity of NFATc, presumably via phosphorylation of the SP repeat motifs. It should be noted that, in many cases, the phosphorylation of a protein substrate by GSK-3 is dependent upon the prior activity of a priming kinase (47). We believe it is therefore possible that GSK-3 may not act alone to phosphorylate NFATc, but might require the prior action of a priming kinase present in the in vitro transcription/translation extracts used in these experiments.

Finally, we wanted to test whether treatment with GSK-3β could affect preformed NFATc-DNA complexes. Hence, in vitro translated NFATc was first incubated with radiolabeled probe and allowed to form NFATc-DNA complexes. The complexes were then incubated at 30 °C in the presence or absence of purified GSK-3β for various periods of time and analyzed by EMSA. As shown in Fig. 5 (C and D), treatment with GSK-3β promoted a rapid reduction in the level of DNA-bound wild-type NFATc compared with mock-treated samples. In contrast,
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FIG. 5. Treatment with GSK-3β inhibits the DNA binding activity of NFATc in vitro. A, in vitro translated (IVT) wild-type NFATc or NFATc-mSPx3 was incubated in the presence or absence of purified GSK-3β at 30 °C for 20 min and DNA binding activity was analyzed by EMSA. B, 35S-labeled in vitro translated wild-type NFATc or NFATc-mSPx3 was incubated in the presence or absence of purified GSK-3β for 20 min at 30 °C and analyzed by 8% SDS-PAGE, followed by autoradiography. C–E, in vitro translated wild-type NFATc (C and D) or NFATc-mSPx3 (E) was allowed to form a complex with a 32P-labeled DNA probe at room temperature. Samples were then incubated at 30 °C for the indicated time periods with either GSK-3β (C and E) or buffer alone (D), and the level of NFATc remaining bound to the probe was assessed by EMSA. The relative intensities (R.I.) of the specifically shifted complexes were determined by scanning densitometry and are indicated below each lane.

GSK-3β treatment had little effect on the DNA binding activity of the NFATc-mSPx3 mutant (Fig. 5E). Thus, GSK-3 treatment leads to a rapid reduction in the level of NFATc DNA binding activity, an effect that is dependent upon the integrity of the SP repeat motifs. Presumably, GSK-3 is able to shift the equilibrium in favor of unbound NFATc, either by phosphorylating DNA-bound NFATc and inducing its dissociation or by phosphorylating dissociated NFATc and precluding its reassociation with DNA.

DISCUSSION

In this study, we provide evidence that the intrinsic DNA binding activity of NFATc is negatively regulated by phosphorylation. We show that dephosphorylation of NFATc in vitro and activation of calcineurin in cells lead to enhanced NFATc-DNA binding, whereas phosphorylation of NFATc in vitro potently inhibits its ability to bind DNA. We believe that this phosphorylation-sensitive regulation of DNA binding is likely to be a shared general property of the NFAT family since both the in vitro and in vivo dephosphorylation of NFATp have also previously been shown to enhance its DNA binding activity (36–38). Importantly in this study, we identify the conserved SP repeat motifs as critical quantitative determinants of NFATc DNA binding activity. Finally, we demonstrate that the GSK-3 protein kinase can act to inhibit NFATc DNA binding activity, an effect that appears to be mediated by phosphorylation of the SP repeats. Taken together, these studies afford new insights into the regulation of NFATc and highlight a potential role for the GSK-3 protein kinase in the regulation of NFATc-dependent gene expression.

One of the principal findings of our study is that the phosphorylation of the conserved SP repeat motifs appears to play an important role in negatively regulating the DNA binding activity of NFATc. In support of this notion, we have shown that alanine substitution of the conserved serine residues in these motifs leads to a marked increase in the level of NFATc DNA binding activity. In addition, we have shown that the SP repeat motifs are critically required for the inhibitory effects of the GSK-3 protein kinase on NFATc-DNA binding. Furthermore, through the analysis of NFATc mutants with increasing numbers of alanine-substituted SP repeats, we have provided evidence that the state of phosphorylation of the SP repeats motifs is inversely related to the degree of NFATc-DNA binding. This latter observation suggests that the phosphorylation state of these motifs does not appear to act as a simple binary switch to regulate NFATc-DNA binding, but rather implies that the differential phosphorylation of the SP repeats might act to quantitatively regulate the DNA binding activity of NFATc. Thus, we propose that the phosphorylation status of the NFATc Ser-Pro repeat motifs is likely to act as a form of molecular rheostat by integrating the relative activities of both the calcium/calcineurin signaling motif and the opposing NFATc kinase(s), thereby determining the ultimate degree of NFATc DNA binding activity.

Our identification of the conserved SP repeat motifs as critical quantitative determinants of NFATc DNA binding activity suggests that the kinases that directly phosphorylate these residues are likely to have an important influence on the regulation of NFATc-dependent gene expression. In particular, by opposing the action of calcineurin and acting to attenuate the DNA binding activity of NFATc, they are likely to make an important contribution to the exquisite calcium sensitivity and dynamic regulation of NFATc-dependent gene expression observed in vivo (25, 28, 33–35). Identification of the kinases involved in the regulation of NFATc DNA binding activity is therefore of considerable interest. In fact, our results provide several independent lines of evidence to suggest a potential role for GSK-3 in the phosphorylation-dependent regulation of NFATc DNA binding activity. First, LiCl, which is known to be an inhibitor of GSK-3 activity, acted together with ionomycin to enhance NFATc DNA binding activity in transiently transfected COS cells. Second, overexpression of a GSK-3 mutant (GSK-3β-S9A) attenuated the ionomycin-induced DNA binding activity of wild-type NFATc, but had no effect on the NFATc-mSPx3 mutant. Third, phosphorylation of wild-type NFATc in vitro with purified GSK-3 strongly inhibited NFATc DNA binding activity, an effect that appeared to be mediated via phosphorylation of the conserved SP repeat motifs. Although our studies have implicated a potential role for GSK-3 in the regulation of NFATc DNA binding activity, it is important to note that we cannot rule out the possibility that other cellular protein kinases may be involved.

Interestingly, in addition to the effects of GSK-3 on NFATc DNA binding activity that we have described here, several other independent studies also support a potential role for GSK-3 in the regulation of the NFAT signaling pathway. First, Crabtree and co-workers (29, 48) have proposed a role for GSK-3 in the regulation of NFATc nuclear export. In those studies, GSK-3 was identified by biochemical purification and immunodepletion experiments as the principal cellular kinase involved in the regulation of NFATc DNA binding activity. One of the principal findings of our study is that the phosphorylation of the conserved SP repeat motifs appears to play an important role in negatively regulating the DNA binding activity of NFATc. In support of this notion, we have shown that alanine substitution of the conserved serine residues in these motifs leads to a marked increase in the level of NFATc DNA binding activity. In addition, we have shown that the SP repeat motifs are critically required for the inhibitory effects of the GSK-3 protein kinase on NFATc-DNA binding. Furthermore, through the analysis of NFATc mutants with increasing numbers of alanine-substituted SP repeats, we have provided evidence that the state of phosphorylation of the SP repeats motifs is inversely related to the degree of NFATc-DNA binding. This latter observation suggests that the phosphorylation state of these motifs does not appear to act as a simple binary switch to regulate NFATc-DNA binding, but rather implies that the differential phosphorylation of the SP repeats might act to quantitatively regulate the DNA binding activity of NFATc. Thus, we propose that the phosphorylation status of the NFATc Ser-Pro repeat motifs is likely to act as a form of molecular rheostat by integrating the relative activities of both the calcium/calcineurin signaling motif and the opposing NFATc kinase(s), thereby determining the ultimate degree of NFATc DNA binding activity.

Our identification of the conserved SP repeat motifs as critical quantitative determinants of NFATc DNA binding activity suggests that the kinases that directly phosphorylate these residues are likely to have an important influence on the regulation of NFATc-dependent gene expression. In particular, by opposing the action of calcineurin and acting to attenuate the DNA binding activity of NFATc, they are likely to make an important contribution to the exquisite calcium sensitivity and dynamic regulation of NFATc-dependent gene expression observed in vivo (25, 28, 33–35). Identification of the kinases involved in the regulation of NFATc DNA binding activity is therefore of considerable interest. In fact, our results provide several independent lines of evidence to suggest a potential role for GSK-3 in the phosphorylation-dependent regulation of NFATc DNA binding activity. First, LiCl, which is known to be an inhibitor of GSK-3 activity, acted together with ionomycin to enhance NFATc DNA binding activity in transiently transfected COS cells. Second, overexpression of a GSK-3 mutant (GSK-3β-S9A) attenuated the ionomycin-induced DNA binding activity of wild-type NFATc, but had no effect on the NFATc-mSPx3 mutant. Third, phosphorylation of wild-type NFATc in vitro with purified GSK-3 strongly inhibited NFATc DNA binding activity, an effect that appeared to be mediated via phosphorylation of the conserved SP repeat motifs. Although our studies have implicated a potential role for GSK-3 in the regulation of NFATc DNA binding activity, it is important to note that we cannot rule out the possibility that other cellular protein kinases may be involved.

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T cell activation-dependent induction of an NFAT reporter gene. Second, Ohashi and co-workers (49) have recently shown that the retroviral expression of the GSK-3β-S9A mutant in murine primary T cells strongly inhibits antigen-induced IL-2 production and T cell proliferation, whereas inhibition of GSK-3 activity with LiCl enhances T cell responses. Third, overexpression of the Akt/protein kinase B protein kinase, which is known to negatively regulate the activity of GSK-3, has recently been shown to synergize with Fce-receptor I stimulation to enhance both NFAT activity and NFAT-dependent cytokine gene expression in murine bone marrow-derived mast cells (50). Based upon these collective observations, it is tempting to speculate that GSK-3 may play an important role in the regulation of NFATc-dependent gene expression. However, the formal demonstration of a role for GSK-3β in the regulation of NFATc activity in vivo will require either the generation of more specific GSK-3 inhibitors or the analysis of cells rendered genetically deficient in GSK-3 protein kinase activity.

Overall, our results suggest that the DNA binding activity of NFATc is regulated in a phosphorylation-dependent manner by the differential phosphorylation of the conserved SP repeat motifs. What is the underlying molecular mechanism that accounts for this observation? Substantial analysis over the years has indicated that phosphorylation can influence the DNA binding activity of transcription factors by two major mechanisms (51). First, phosphorylation of residues within or close to the DNA binding site itself can directly affect DNA binding (52–55). Second, phosphorylation can lead to a conformational change resulting in an indirect modulation of DNA binding activity (56–58). In the case of NFATc, we have identified the conserved SP repeat motifs as key phosphorylation-sensitive determinants of NFATc DNA binding activity. Since these motifs are located in the NFATc N terminus and are geographically distinct from the C-terminal NFATc DNA binding domain, a simple model in which phosphorylation directly inhibits DNA binding appears to be excluded. Rather, our data support an indirect model of regulation. We have demonstrated that either the removal of the entire NFATc N terminus or alanine substitution of the conserved SP repeat motifs results in constitutive DNA binding activity that is unaffected by the action of calcineurin. This suggests that the phosphorylated NFATc N terminus is likely to exert an intramolecular inhibitory influence on the activity of the NFATc C-terminal DNA binding domain.

There are several potential mechanisms that could explain this negative regulatory influence. First, the phosphorylated N terminus could engage in a direct intramolecular interaction with the C-terminal DNA binding domain and simply occlude its interaction with DNA. In fact, biochemical evidence supporting a direct intramolecular interaction between the NFATc N- and C-terminal domains has been provided (39). This interaction appears to be mediated, at least in part, by the phosphorylated C-terminal DNA binding domain and simply occlude this negative regulatory influence. First, the phosphorylated N terminus is likely to exert an intramolecular inhibitory influence on the activity of the NFATc C-terminal DNA binding domain.

Second, the phosphorylation of residues within or close to the DNA binding site itself can directly affect DNA binding (52–55). This interaction appears to be mediated, at least in part, by the phosphorylation of the SP repeat motifs. In fact, biochemical evidence supporting a direct interaction between the phosphorylated SP repeat motifs, although whether this inhibition involves a direct interaction with the phosphorylated SP repeats remains to be seen. A second related potential mechanism to explain the negative regulatory influence of the N terminus on the DNA binding activity of NFATc is that the phosphorylation of the SP repeat motifs could allosterically favor an NFATc conformation that is incompatible with DNA binding. In fact, such a mechanism has recently been described for the phosphorylation-dependent regulation of Ets-1 DNA binding activity (57). A final possibility is that the phosphorylated N terminus may bind a specific partner protein that acts to sterically hinder the association of NFATc with DNA. If that is the case, such a partner protein would have to be relatively ubiquitous since the phosphorylation-sensitive regulation of NFATc DNA binding activity was observed in both COS cells and reticulocyte lysate in vitro translation extracts. Further insight into the precise molecular mechanisms involved in the phosphorylation-sensitive regulation of NFATc DNA binding activity will have to await the detailed structural analysis of the differentially phosphorylated forms of the full-length NFATc molecule.

Acknowledgments—We thank Dr. E. G. Krebs for the GSK-3-S9A plasmid construct and P. S. Riggins, C. M. Porter, and M. A. Havens for critically reading the manuscript.

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