Mechanisms of Transactivation by Nuclear Factor of Activated T Cells-1

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Summary

Nuclear factor of activated T cells-family proteins (NFAT1/NFATp, NFATc, NFAT3, and NFAT4/NFATx/NFATc3) play a key role in the transcription of cytokine genes and other genes during the immune response. We have defined the mechanisms of transactivation by NFAT1. NFAT1 possesses two transactivation domains whose sequences are not conserved in the other NFAT-family proteins, and a conserved DNA-binding domain that mediates the recruitment of cooperating nuclear transcription factors even when it is expressed in the absence of other regions of the protein. The activity of the NH2-terminal transactivation domain is modulated by an adjacent regulatory region that contains several conserved sequence motifs represented only in the NFAT family. Our results emphasize the multiple levels at which NFAT-dependent transactivation is regulated, and predict significant differences in the architecture of cooperative transcription complexes containing different NFAT-family proteins.

The nuclear factor of activated T cells (NFAT) is a calcium-regulated transcription factor that is a major target for the immunosuppressive drugs cyclosporin A (CsA) and FK506 (1–3). The DNA-binding specificity of NFAT is conferred by a novel family of transcription factors, of which four members (NFAT1/NFATp, NFATc, NFAT3, and NFATx/NFAT4/NFATc3, each containing several isoforms) have so far been described (4–9). NFAT1 protein is expressed in several classes of immune system cells (10–12), consistent with the detection of nuclear NFAT DNA-binding activity in activated T, B, mast, and NK cells (13–19). The distribution of the other NFAT-family proteins has not yet been assessed at the protein level; however the mRNAs encoding these proteins differ in their cell and tissue distributions and their representation in resting versus activated cells (5–7), suggesting that each protein may subserve a different function. NFATc mRNA is expressed at high levels in skeletal muscle and in activated lymphoid cells, NFATx/NFAT4 mRNA is predominantly expressed in the thymus, and NFAT3 mRNA is most strongly expressed outside the immune system (5–7).

An important component of transactivation by NFAT-family proteins is their cooperation in the nucleus with Fos and Jun (20, 21). This cooperation is mediated by interaction of the highly conserved DNA-binding domains (DBDs) of NFAT-family proteins with Fos-Jun and Jun-Jun dimers (6, 7, 22); the bZIP (basic region-leucine zipper) regions of Fos and Jun suffice for this interaction (21), although an acidic region of Fos has also been implicated (23). Overexpression of Fos- and Jun-family proteins in T cells greatly augments transcription driven by tandem copies of the IL-2 and IL-4 promoter NFAT sites in activated T cells (20, 24–26). It has also been suggested that NFAT-family proteins are able to act independently or cooperate with transcription factors of the GATA family (18, 27). We asked whether NFAT-family proteins contain intrinsic transactivation domains (TADs), or whether they function by recruiting other transcription factors.

Here we define the mechanisms of transactivation by NFAT1. We show that NFAT1 contains two intrinsic TADs at its NH2- and COOH-terminal ends that are not represented in the other NFAT-family proteins. The activity of the NH2-terminal TAD is regulated by the adjacent NFAT homology region (NHR), a serine- and proline-rich region that contains several sequence motifs conserved within the NFAT family. We also show that the conserved DBD of NFAT1 contributes potently to transactivation at conserved NFAT sites, since it is capable of recruiting cooperating transcription factors even when expressed independently of other regions of NFAT1. Our results suggest that NFAT-dependent transactivation involves a common mechanism mediated by the conserved DBD, as well as unique mechanisms involving the nonconserved TADs.
Materials and Methods

Expression Plasmids: The pEFTAG, pLGP-mNFAT1-C, and pEFTAG-mNFAT1-C expression plasmids have been described (8). The pBLCAT5/NFAT(M3)3X and pBLCAT5/NFAT(M4)3X reporter plasmids were made by replacing the trimer of NFAT sites in pBLCAT5/NFAT3X with trimers of NFAT(M3) and NFAT(M4) (22), respectively. The NFAT3X-Luc and 5'IL2-Luc plasmids were kind gifts from Dr. David McKeon (Mayo Clinic, Rochester, MN).

Results and Discussion

We assessed the intrinsic transactivation potential of the NH2- and COOH-terminal regions of NFAT1, that flank the centrally located DBD, by testing chimeric proteins in which amino acids 1-415 and 727-927 of NFAT1 were fused to the COOH terminus of the heterologous DBD of GAL4. Both regions have features resembling TADs: the NH2-terminal region is acidic and proline rich, whereas the COOH-terminal region is glutamine and proline rich (8). When expressed in Jurkat T cells, both chimeric proteins were capable of transactivation, provided that the reporter plasmid contained GAL4 binding sites (Fig. 1 A, compare lane 2 with lane 3 and lane 4 with lane 5). The GAL4 DBD alone was inactive (lane 1). The difference in the activities of GAL4-NFAT1(1-415) and GAL4-NFAT1(727-927) may be a consequence of differences in the level of expression of the two GAL4 fusion proteins, or may reflect an intrinsic difference in their ability to transactivate in this context.

When cells expressing the GAL4-NFAT1(1-415) chimeric protein were stimulated with the calcium ionophore, ionomycin, GAL4-dependent transactivation was reproducibly enhanced (Fig. 1 B, lanes 1 and 2; 2.0 ± 0.4-fold, n = 8). Stimulation with PMA alone had no effect (lane 3; 0.8 ± 0.1-fold, n = 3), and stimulation with PMA plus ionomycin did not increase the level of CAT activity over that observed with ionomycin alone (lane 4; 2.5 ± 0.9-fold, n = 3). Notably, CsA completely blocked the ionomycin-induced increase in CAT activity mediated by the GAL4-NFAT1(1-415) chimeric protein (Fig. 1 B; lanes 5–8). CsA did not block the basal level of transactivation mediated by this protein in unstimulated Jurkat cells, indicating that CsA does not suppress the transcriptional function of the chimera per se. Transactivation by the GAL4-NFAT1(727-927) chimeric protein was not affected by ionomycin, PMA, or CsA (data not shown), indicating that although both the NH2- and COOH-terminal regions of NFAT1 possessed transactivating function, only the activity of the NH2-terminal region of NFAT1 was capable of being regulated in a calcium- and calcineurin-dependent fashion. The lack of effect of PMA on the transactivation activities of these chimeras suggests that the intrinsic transactivation activity of NFAT1 is not regulated by PMA-stimulated signaling pathways.

The (1-415) region of NFAT1 contains a unique region (amino acids 1-100) not represented in the other NFAT-family proteins, as well as an adjacent ~300-amino acid NHR (amino acids ~100-~380) that shows a moderate level of sequence conservation within the NFAT family.
Figure 1. The transactivation and regulatory domains of NFAT1. (A) NFAT1 has two independent transactivation domains. Jurkat cells were transfected with 5 μg of RSV-GAL4DBD (lane 1), GAL4-NFAT1(1-415) (lanes 2 and 3), or GAL4-NFAT1(727-927) (lanes 4 and 5) together with 5 μg of reporter plasmid of GAL4-CAT (lanes 1, 2, and 4) or BG-CAT (lanes 3 and 5) and assayed for CAT activity 2 d later, as described in Materials and Methods. (B) The transactivation activity of the GAL4-hNFAT1(1-415) chimera protein is induced by ionomycin stimulation and is sensitive to CsA. Jurkat cells were transfected with 5 μg of GAL4-hNFAT1(1-415) and 5 μg of GAL4-CAT plasmids and cultured in eight aliquots. Cells were left unstimulated (lanes 1 and 5), or stimulated overnight with 1 μM ionomycin (lanes 2 and 6), 10 nM PMA (lanes 3 and 7), or both (lanes 4 and 8), in the absence (lanes 1-4) or presence (lanes 5-8) of 1 μM CsA. Shown is a CAT assay representative of three independent experiments. (C) Relative transactivation activity of GAL4 fusion proteins with various NFAT1 NH2-terminal fragments. The GAL4 fusion proteins were expressed in Jurkat cells without (−) or with (+) ionomycin (iono) stimulation. Transfection efficiencies were normalized by measuring hGH expression from a cotransfected RSV-hGH plasmid. Relative CAT activities (mean ± SD, n = 3) shown are CAT activities normalized to that of unstimulated cells transfected with GAL4-hNFAT1 (1-415). Fold induction is shown where there is significant difference between CAT activities of unstimulated and ionomycin stimulated cells (P < 0.02).
Detailed inspection of the NHR reveals the presence of several strongly conserved sequence motifs (8), including three repeats of the SP motifs (6) (consensus sequence, SPxxSPxxSPxxxx[D/E][D/E]). To localize the regions within the GAL4-NFAT1(1-415) protein required for transactivation and regulation by ionomycin, we tested GAL4 chimeric proteins containing NH2- or COOH-terminally deleted derivatives of the NFAT1(1-415) fragment (Fig. 1 C). These proteins were expressed in Jurkat cells with the GAL4-CAT reporter plasmid. Deletion of as few as 57 residues from the NH2 terminus of NFAT1 abolished transactivation (Fig. 1 C, row 3), and further NH2-terminal deletions did not restore function (rows 4 and 5). In contrast, chimeric proteins containing deletions of up to 185 residues from the COOH terminus of the 1-415 region (Fig. 1 C, rows 6-10) retained the ability to mediate transactivation. The low basal activities of proteins containing the NH2-terminal half of the NHR (Fig. 1 C, rows 6-8) were likely due to their low level of expression, since increasing the amount of plasmid DNA in transfections led to higher basal activities (data not shown); however their transactivation activities were clearly augmented in ionomycin-stimulated cells (row 6-8). Deletions of most of the NHR (Fig. 1 C, rows 9 and 10), or the NH2-terminal half of the NHR (row 11), resulted in high basal activities that were not further increased by stimulation with ionomycin, even when the amount of plasmid DNA used in the transfection was titrated to yield a lower basal level of CAT activity (data not shown).

These results indicated that the NH2-terminal region of NFAT1 contains a strong TAD encoded within amino acids 1-144. However, the activity of this TAD is fully revealed only in the absence of amino acids 145-387, which constitute essentially all of the NHR. In the presence of the NHR, moreover, the activity of the NH2-terminal TAD of NFAT1 was regulated in a calcium/calcineurin-dependent manner. Indeed, we found that the NHR was capable of conferring a small degree of ionomycin responsiveness on an unrelated protein, since transactivation by a chimeric protein (GAL4-BB-E2F), in which amino acids 57-318 of NFAT1 were introduced between the GAL4 DBD and the E2F TAD, was significantly stimulated by ionomycin treatment of Jurkat cells (1.65 ± 0.1-fold induction, n = 6, P < 0.001). CsA pretreatment reproducibly blocked the ionomycin-mediated stimulation (1.02 ± 0.32-fold induction, n = 6), but did not affect the basal level of transactivation. The level of transactivation by the parent GAL4-E2F chimera was unaffected by ionomycin stimulation (0.96 ± 0.2-fold induction, n = 3).

We compared the transactivation functions of full-length NFAT1 with those of NFAT1AN (939-927) (here termed NFAT1ΔN), which lacks the NH2-terminal TAD and NHR. Expression of either protein in Jurkat T cells resulted in increased basal transactivation of a CAT reporter plasmid containing three copies of the NFAT site of the murine IL-2 promoter. As expected, further induction of CAT activity by full-length recombinant NFAT1 required stimulation with both ionomycin and PMA, and was completely inhibited by pretreatment with CsA (Fig. 2, left). In contrast, transactivation by NFAT1ΔN was stimulated by PMA alone and was not inhibited by pretreatment with CsA (Fig. 2, right).

We have previously shown that full-length NFAT1 is cytoplasmic in resting T cells and translocates into the nucleus upon ionomycin stimulation (8), whereas NFAT1AN is diffusely distributed throughout the nucleus and the cytoplasm in both resting and stimulated cells (29). Thus, the results shown in Fig. 2 indicate that nuclearly localized NFAT1 and NFAT1AN require PMA for optimal transactivation, whereas ionomycin is required only for translocation of full-length NFAT1 into the nucleus. Given that PMA does not stimulate the activity of the intrinsic TADs of NFAT1 (see above), we postulated that the PMA requirement for transactivation most likely reflected the requirement for cooperation of NFAT1 with PMA-stimulated transcription factors such as Fos and Jun (1).

To evaluate the contribution of cooperating nuclear proteins to NFAT1-dependent transactivation, we tested whether the DBD of NFAT1 (which is sufficient for cooperation with Fos and Jun proteins; 22) could independently mediate transactivation. Expression of NFAT1DBD (amino acids 398-694 of murine NFAT1) caused transactivation of a luciferase reporter plasmid containing three tandem NFAT sites (NFAT3X-Luc) in unstimulated Jurkat T cells, and stimulation with PMA plus ionomycin greatly increased the level of transactivation (Fig. 3 A, left). This effect is probably not due to an intrinsic transactivation function possessed by the DBD, since a longer fragment of NFAT1 containing the NFAT1DBD does not show transactivation activity in vitro (4). The effect is striking because isolated DNA-binding fragments of other transcription factors usually inhibit transactivation when overexpressed, most likely by competing for endogenous dimerization partners and/or blocking the relevant sites on DNA (30, 31).
NFAT1DBD contains the region corresponding to both the NH2-terminal specificity subdomain and the COOH-terminal dimerization domain of p50 NF-kB (32, 33). However, the smallest fragment of NFAT1 capable of DNA binding and cooperative interaction with Fos and Jun is a shorter 187-amino acid fragment (NFAT1DBDn, amino acids 398-584 of murine NFAT1) that corresponds to the NH2-terminal specificity subdomain of p50 NF-kB (32, 33). Strikingly, NFAT1DBDn was also able to mediate the inducible transcription of NFAT3X-Luc in Jurkat T cells (Fig. 3 A, left). Moreover, both NFAT1DBD and NFAT1DBDn increased the transcription of a luciferase reporter gene controlled by the IL-2 promoter (Fig. 3 A, right). In this case, the increased transcription was apparent in stimulated but not in unstimulated Jurkat T cells, presumably because other inducible transcription factors such as NF-kB are required for IL-2 promoter induction (3).

The binding of NFAT1DBD and NFAT1DBDn to the composite NFAT site of the IL-2 promoter is greatly stabilized in the presence of Fos and Jun (20). To test whether the transcriptional activation observed to be mediated by these fragments was due to AP-1 proteins recruited to the composite site, we used CAT reporter plasmids containing mutated NFAT sites. The M3 mutation of the NFAT site of the IL-2 promoter abrogates the binding of NFAT proteins and their recruitment of AP-1 proteins, whereas the M4 mutation permits the binding of NFAT proteins but prevents the formation of the NFAT–Fos-Jun complex (22, 34). Both the M3 and M4 mutations severely compromised transactivation by NFAT1DBD and NFAT1DBDn (Fig. 3 B). Western analysis of the epitope-tagged NFAT1DBD confirmed that it was expressed equivalently in each transfection (data not shown). These results indicated that transactivation by NFAT1DBD and NFAT1DBDn required DNA binding by the NFAT1 fragments and recruitment of AP-1 proteins or other cooperating transcription factors to the adjacent AP-1 site, and was unlikely to involve other mechanisms such as squelching or titrating a repressor.

Taken together, these results indicate that transactivation by NFAT-family proteins is regulated at multiple levels. The strongly conserved DBDs operate even in the absence of intrinsic TADs to mediate transcription via cooperating factors recruited to composite sites. The minimal 187-amino acid DBD suffices for this effect, emphasizing the remarkable specialization of NFAT-family proteins for cooperative interactions on DNA. The DBDs of GATA and MyoD1 are sufficient to induce cell differentiation (35, 36), suggesting that the use of DBDs of transcription factors as docking sites for recruiting other transactivating factors may be a general mechanism of transactivation. NFAT1 also contains two intrinsic TADs that are not conserved within the NFAT family: indeed, the region corresponding to the COOH-terminal TAD of NFAT1 is missing in NFATc and in the shorter isoforms of NFAT4 (5–9). Finally, the conserved NHR appears to repress the activity of the NH2-terminal TAD, since its removal greatly enhances transactivation mediated by the unique NH2-terminal region.

Our results are consistent with the hypothesis that the NHR of NFAT1 controls the subcellular localization of this protein, which is known to be regulated by a calcium- and calcineurin-dependent pathway (10). Removal of the NHR or the entire NH2-terminal region from NFAT1 abrogates its regulated subcellular localization in resting and stimulated T cells (29), and transactivation by the truncated protein NFAT1ΔN is no longer sensitive to ionomycin or CsA. Despite the presence of a strong nuclear localization signal in the GAL4 DBD, GAL4 chimeras containing the NHR also show significant responsiveness to ionomycin and CsA. One hypothesis is that the presence of the NHR alters the nuclear-to-cytoplasmic ratio of these GAL4 chimeric proteins in a calcineurin-dependent manner. Alternatively, calcineurin may regulate the ability of the NHR to mask the transactivation properties of the strong NH2-terminal TAD of NFAT1. In either case, the NHR is likely to be a major target for calcineurin within NFAT1, and in fact we have shown that fragments containing the NHRs

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**Figure 3.** The DBD of NFAT1 causes transcription from NFAT sites and the IL-2 promoter. (A) Jurkat cells were transfected with 10 μg of pEFTAG (endo), pEFTAGNFAT1-C (FL), pEFTAGNFAT1DBD (DBD), or pEFTAGNFAT1DBDn (DBDn) together with 1 μg of NFAT–Luc (NFAT, left) or 2 μg of 5′IL2-Luc (IL-2, right) reporter plasmids. Luciferase activity (in light units) was assayed as described in Materials and Methods. (B) Jurkat cells were transfected with 5 μg of the indicated expression plasmids and 1 μg of pBLCAT5/NFAT3X (WT), pBLCAT5/NFAT(M3)3X (M3), or pBLCAT5/NFAT(M4)3X (M4). Cells were left unstimulated (Unstim) or were stimulated with PMA and ionomycin (P + I) 1 d after transfection. CAT activity was assayed 2 d after transfection. The data shown are representative of at least three independent experiments.
of both NFAT1 and NFATc bind calcineurin directly (29, 37). The conserved sequences of the NHRs of NFAT-family proteins suggest that NHR may contribute to the Ca2+-sensitive response to ionomycin stimulation of all NFAT-family members, and the variation in NHRs of NFAT-family proteins could account for their different behavior in resting cells.

Although there is no clearcut conservation of sequence, the NH2-terminal ~100 residues of NFAT1, NFAT3, and NFAT4 are each rich in acidic residues and proline (5–8), and contain acid hydrophobic patches characteristic of acidic TADs (8, 38, 39). In contrast, the NH2-terminal region of NFATc (which is also capable of transactivation; Luo, C., unpublished data) is proline rich but not acidic. Thus transactivation by the different NFAT-family proteins may be mediated by distinct interactions of the different TADs with coactivators, TATA, binding protein-associated factors (TAFs), and other components of the basal transcription machinery (40). In conjunction with the distinct site preferences of NFAT-family proteins (7, 9) and the diverse sequences and arrangements of NFAT sites in inducible promoter/enhancer regions (1), such interactions may determine the cell-specific expression of cytokine genes.

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