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Nucleocytoplasmic shuttling and the control of NF-AT signaling

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Abstract. The nuclear factors of activated T cells (NF-ATs) constitute a family of transcription factors that transduce calcium signals in the immune, cardiac, muscular and nervous systems. Like their distant relatives of the Rel family, including NF-κB, NF-ATs are cytoplasmic in resting cells and activated by means of induced nuclear import. Unlike NF-κB, however, NF-ATs show highly dynamic nuclear shuttling properties that have important implications for graded signaling by these molecules. This review focuses on recent advances in deciphering mechanisms by which calcium signaling regulates the nucleo-cytoplasmic shuttling and therefore transactivation functions of the NF-ATs. These discoveries highlight the interplay between nuclear import and export signals on NF-ATs, and the roles of the calcium-activated phosphatase calcineurin and NF-AT kinases in controlling the activity of these signals. They also reveal that NF-ATs, as well as other transcription factors controlled at the level of nuclear import, face the very real prospect of futile cycling across the nuclear envelope as a consequence of conflicting nuclear import and export signals. We discuss the molecular mechanisms by which calcineurin suppresses futile cycling, as well as the major challenges to our understanding of NF-AT signaling in diverse biological systems.

Key words. NF-AT; nuclear factor of activated T cells; transcription factor; calcium; calcineurin; CRM1; casein kinase I; NLS; nuclear localization signal; NES; nuclear export signal.

NF-ATs: multigene family with diverse functions

A key to understanding T-cell activation was the discovery that nuclear extracts of antigen-stimulated cells contained a factor or factors which recognized the antigen response element of the interleukin 2 promoter [1]. Significantly, these nuclear factors of activated T cells (NF-ATs) were absent from the nuclei of resting cells or of cells activated in the presence of the immunosuppressant cyclosporin A [2, 3]. Molecular cloning of the first NF-AT, termed NF-AT1/p, revealed a C-terminal transactivation and DNA binding domain distantly related to that of the Rel family of transcription factors, which are also regulated at the level of nuclear import [4]. To date, four separate genes encoding NF-ATs have been cloned and are generally denoted NF-AT1/p, NF-AT2/c, NF-AT3 and NF-AT4. All four genes encode proteins with conserved C-terminal, Rel-like DNA binding domains and are able to activate transcription from the interleukin-2 (IL-2) promoter in vitro [5–7]. The N-terminal half of the NF-ATs shows no homology with other proteins and only limited homology amongst family members. However, functional experiments have revealed that this N-terminal domain harbors all of the regulatory mechanisms governing the nuclear import response of many of the NF-AT isotypes to calcium signaling (fig. 1) [8]. Despite their structural and functional similarities, it is now apparent that the NF-ATs show tissue distribution patterns far beyond their eponymous borders. For instance, NF-AT1/p is widely distributed in lymphoid and non-lymphoid tissues, although peripheral blood lymphocytes (PBLs) and placenta show highest levels of this NF-AT isotype [5]. NF-AT2/c is most highly expressed in cardiac and skeletal muscle, and NF-AT3 is found in most tissues but, curiously, is virtually absent from spleen and thymus [5]. Finally, NF-AT4 is most highly expressed in CD4+, CD8+ double-positive (DP) T cells [5]. Given the complex and sometimes

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intersecting expression patterns of the NF-AT isotypes, it is likely that one or more of these transcription factors mediate calcium signals in a wide range of signaling events. In agreement with the intricate expression patterns of the NF-ATs, an increasingly large number of cytokine genes have been shown to contain NF-AT sites in their upstream regulatory sites (URS), including IL-3, IL-4, granulocyte macrophage colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNFα), interferon-γ and the Fas ligand, all of which are responsive to NF-ATs [9, 10].

To distinguish the individual functions of the NF-AT isotypes, several laboratories have employed targeted gene knockouts in mice. NF-AT1/p($-$ / $-$) mice are viable but develop splenomegaly as a consequence of hyperproliferation of B and T cells. Analysis of primary and secondary immune responses of splenocytes in vitro revealed contradictory defects in TH2 lymphokine production, marked by early deficits and late excesses, especially in IL-4 and immunoglobulin E (IgE) [11–14].

As might have been expected from its expression in cardiac and skeletal muscle, the targeted disruption of

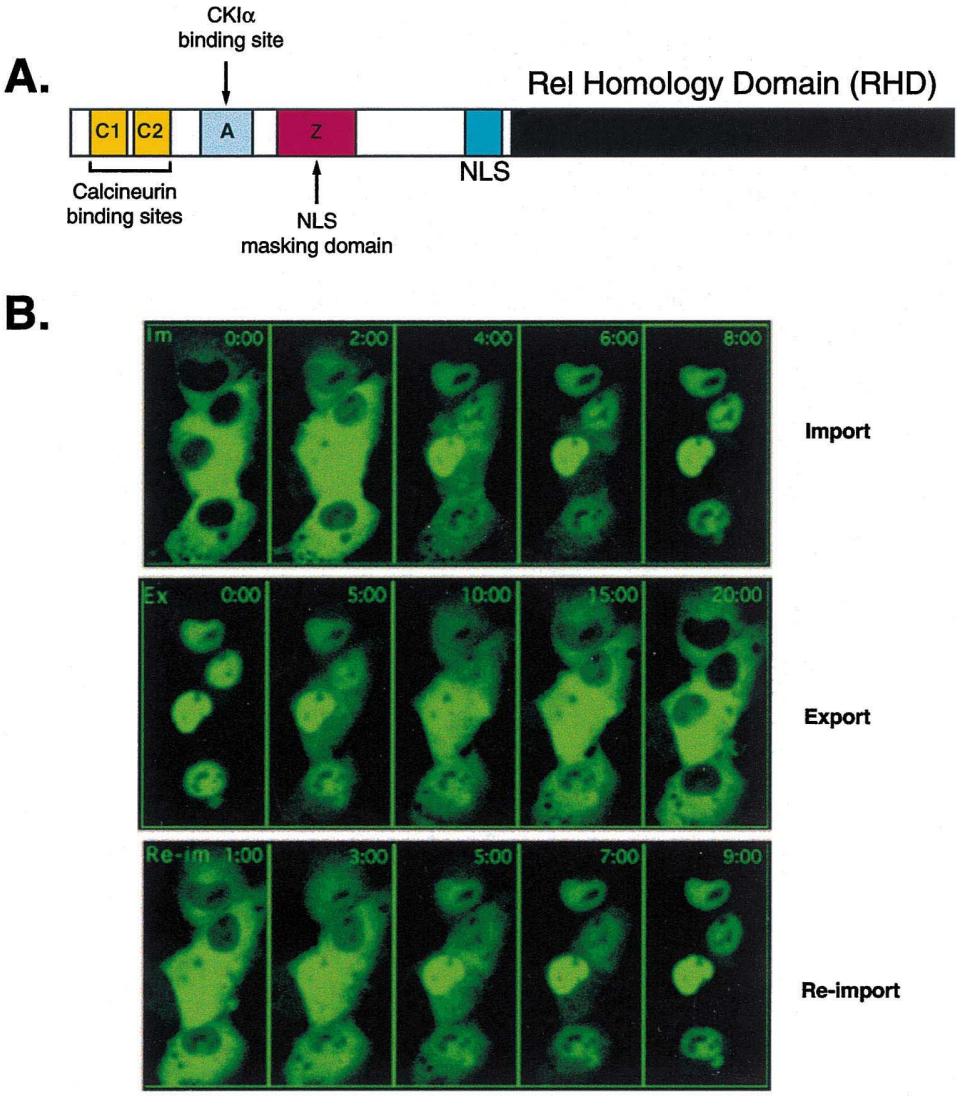

Figure 1. (*A*) A schematic representation of the functional domains on NF-AT. The C-terminal RHD (Rel homology domain) contains DNA binding/transactivation domains that are distantly related to those of NF-κB/c-Rel family. The N-terminal regulatory region determines calcium-dependent NF-AT nuclear translocation. Within this region, there are two calcineurin binding sites, C1 (low-affinity binding site) and C2 (high-affinity binding site). In addition, domain A serves as an NF-AT kinase, CKIα docking site. The NLS masking sequence is denoted as Z domain, which when hyperphosphorylated, directly interacts with the downstream NLS. (*B*) Video images of GFP-NF-AT4 undergoing nucleo-cytoplasmic shuttling in response to calcium signaling.

the NF-AT2/c in mice led to profound developmental defects [15, 16]. NF-AT2/c $-/-$ mice die of congestive heart failure at embryonic day 13.5–17.5 and show a striking absence of aortic and pulmonary heart valves. To examine NF-AT2/c function in lymphocytes, RAG-2-deficient blastocyts were reconstituted with NF-AT2/c $-/-$ embryonic stem (ES) cells to generate viable chimeric mice lacking NF-AT2/c in lymphocytes [17]. Whereas in most respects these NF-AT2/c-deficient lymphocytes showed normal immune responses, certain defects were noted. For instance, repopulation of thymocytes and peripheral lymphocytes in adoptive transfer experiments was considerably slower in the NF-AT2/c-deficient mice, and T cell activation and IL-4 production also appeared suboptimal. In contrast to NF-AT1/p or NF-AT2/c, NF-AT4 is preferentially expressed in DP thymocytes. NF-AT4 $-/-$ mice show increased apoptosis of DP cells and impaired development of CD4 + and CD8 + single positive lymphocytes and peripheral T cells. In addition, the peripheral T cells that are formed display hyperactive responses to antigen challenge [18]. Thus, NF-AT4 may play a pivotal role in the generation and survival of T cells at critical stages of T-cell selection. Finally, transgenic mice overexpressing an activated NF-AT3 develop a cardiomyopathy similar to that resulting from the overexpression of calcineurin in these animals [19]. NF-AT3 was shown to interact with the cardiac transcription factor GATA4, resulting in the activation of fetal cardiac genes. The NF-AT gene knockout models in mice provide strong evidence for the function of these transcription factors in immune system regulation as well as calcium signaling in heart and skeletal muscle development.

NF-AT activation by nuclear translocation

While NF-AT was initially identified in nuclear extracts of activated T cells, NF-AT is cytoplasmic and therefore functionally inactive in resting cells. The mobilization of NF-AT to the nucleus is strictly dependent on the elevation of intracellular calcium concentration following T-cell antigen receptor (TCR) stimulation. In general, costimulation of the TCR and CD28 on cell surfaces results in tyrosine phosphorylation of the TCR by membrane-associated protein kinases. By virtue of its SH2 domains, phospholipase C-γ (PLCγ) is then recruited to these phosphorylated sites on the TCR, therefore positioning PLCγ in the vicinity of the plasma membrane. Hydrolysis of phospholipids by PLC gives rise to secondary signal molecules (IP3) that trigger the release of calcium from intracellular storage sites, which in turn promotes the transport of cytoplasmic NF-AT to the nucleus [20–25] (fig. 3). A key to understanding how elevated calcium levels could promote the nuclear import of NF-ATs came with the analysis of the major immunosuppressant drugs cyclosporin A and FK506. Both drugs were known to block calcium-dependent signaling in T cells at a step downstream from those mediating the rise in intracellular calcium [26–28]. Elegant affinity chromatography using these drugs complexed with their intracellular receptors, known as the immunophilins, revealed their common binding to calcineurin, a calcium-activated protein phosphatase [26]. Much of the research into the control of NF-AT signaling over the past few years has been directed at uncovering how calcineurin promotes NF-AT nuclear import, and the nature of the kinases that oppose and reverse the actions of this phosphatase.

From a signaling standpoint, the NF-ATs possess numerous advantages over their relatives in the Rel/NF-κB family. These latter transcription factors are driven to the nucleus in an all-or-none fashion by the signaled degradation of associated IκB molecules, which function to mask nuclear location signals on NF-κB. Once in the nucleus, NF-κB cannot exit until new IκB is synthesized, a process that may take hours [29–31]. In contrast, NF-AT4 translocates to the nucleus with a $t_{1/2}$ of five min and, upon a drop in intracellular calcium, exit the nucleus with a $t_{1/2}$ of approximately 12 min [8]. These shuttling dynamics raise the potential for graded signaling by NF-AT4 based on the total time spent in the nucleus, a property of significant potential for positive and negative selection during T-cell development in the thymus. To explore the molecular mechanisms underlying the nuclear shuttling dynamics of NF-AT4, and possibly other NF-AT isotypes, it was important to develop a system for analysis of NF-AT trafficking that would overcome the numerous difficulties presented by the T-cell model. We constructed various epitope- and green fluorescent protein (GFP)-tagged NF-AT4 complementary DNAs (cDNAs) and expressed them in mammalian cell lines. One of the first cell lines tested, baby hamster kidney (BHK) cells, showed a highly discriminating distribution of NF-AT4 to the cytoplasm of resting cells, and yet this NF-AT could be mobilized to the nucleus with the simple addition of a calcium ionophore [8]. Significantly, upon washout of the calcium ionophore, the NF-AT in these cells was efficiently exported from the nucleus. Using the GFP-tagged NF-ATs to monitor multiple rounds of NF-AT cycling to and from the nucleus, we could show that, unlike the NF-κB recycling events, no new protein synthesis was necessary for NF-AT shuttling [8, 32] (fig. 1). Instead, NF-AT4 appeared to be responding only to the level of intracellular calcium levels: at high calcium levels, the NF-ATs move to the nucleus and remain there until calcium levels are brought to resting levels. Another general conclusion from these studies was that the mechanisms which govern the conditional nuclear import and export of the NF-ATs must be largely intrinsic to the molecule, although deciphering these processes was to represent a major challenge.

NF-AT nuclear localization: clash of phosphatases and kinases

Accompanying the dramatic subcellular translocation of NF-AT during calcium signaling are major changes in its phosphorylation status. In resting cells, NF-ATs are cytoplasmic and hyperphosphorylated, whereas translocating and nuclear NF-ATs are dephosphorylated. Upon termination of calcium signaling, NF-ATs are rapidly rephosphorylated and exported from the nucleus [8, 33]. Calcineurin became a prime suspect in NF-AT activation not only because it was the target of cyclosporin A and FK506, immunosuppressants that block T cell activation, but because it is a calcium-activated enzyme [8, 34, 35].

Calcineurin is composed of a catalytic subunit (CnA) and a regulatory subunit (CnB), and is activated by the recruitment of calcium-bound calmodulin [36]. The association of calmodulin with calcineurin alters the structure of a pseudosubstrate domain, thereby allowing access of substrates to the phosphatase active site [37]. A constitutively active, calcium-independent form of calcineurin (ΔCnA) was made by removing the C-terminal calmodulin binding domain and pseudosubstrate sequence of CnA. Coexpression of this calcineurin mutant circumvents the requirement of calcium signaling for the nuclear translocation of NF-AT [8, 38]. More detailed biochemical analyses have demonstrated that calcineurin docks onto two sites located in the N-terminal regulatory domain of NF-AT [32]. As a result of its tight association with NF-AT, activated calcineurin co-translocates with NF-AT into the nucleus.

Recent studies have revealed a phosphorylation-dependent intramolecular NLS masking mechanism that switches on and off the nuclear localization signal of NF-ATs [38]. NF-AT4 contains an NLS in the N-terminal regulatory domain that controls NF-AT cellular localization in a calcium-dependent manner. Curiously, the region encompassing the NLS is not phosphorylated, suggesting that the NLS is not regulated by direct actions of kinases and phosphatases [38]. However, the deletion of an upstream masking domain that contains phosphorylation sites results in the constitutive activation of the NLS. Considerable evidence exists to suggest that the NLS of NF-AT4 directly binds phosphorylated residues in the masking domain, thereby preventing recognition of the NLS by members of the importin NLS receptors [38, 39]. Calcineurin removes these phosphate residues from the mask, thereby exposing the NLS and promoting NF-AT nuclear import (fig. 2).

Despite major advances in understanding the role of calcineurin in NF-AT activation, we know considerably less about kinases that oppose or reverse the actions of calcineurin. However, as the default localization of NF-AT is cytoplasmic in the absence of calcium signaling, it should be assumed that such kinases would be constitutively active. In this regard, many candidate NF-AT kinases have been proposed. One of these, GSK-3, was examined because its substrate specificity appeared similar to some of the phosphorylated sites on NF-AT [40]. While constitutively active in some circumstances, GSK-3 does not directly phosphorylate NF-AT2/c unless NF-AT2/c is prephosphorylated by PKA in vitro [40]. In agreement with the in vitro data, overexpression of GSK-3 or PKA alone has no apparent effect on NF-AT2/c nuclear translocation initiated by calcium ionophore. Coexpression of both PKA and GSK-3 was shown to accelerate the nuclear export of NF-AT2/c, possibly due to enhanced phosphorylation of NF-AT2/c [40]. Another protein kinase, JNK1, was identified by its interaction with NF-AT in a yeast two-hybrid system. JNK can phosphorylate NF-AT in vitro and in vivo. Overexpression of one upstream activator of JNK1, MKK-8, blocks calcium ionophore-induced NF-AT nuclear import. Based on these observations, it was hypothesized that phosphorylation by JNK1 may have adverse effects on NF-AT nuclear translocation [41]. However, several issues remain for this hypothesis. First, in resting cells, JNK is inactive, and therefore it is unlikely that the hyperphosphorylation of cytoplasmic NF-AT is due to JNK kinase activity. Second, JNK directly activates c-Jun, a component of AP-1 complex that binds NF-AT and results in NF-AT transcriptional activation [4]. It has been established that during T-cell activation, simultaneous activation of AP-1 (Jun/Fos) and NF-AT is absolutely essential for cytokine production. In the absence of AP-1, NF-AT itself does not induce the transcription of cytokine genes [10]. Thus, a scheme in which JNK activates AP-1 but simultaneously inhibits NF-AT nuclear translocation seems unfeasible. In addition, the immediate upstream activator of JNK, SEK1, shows no effect on NF-AT nuclear translocation [38]. While the physiological significance of NF-AT phosphorylation by JNK remains to be determined, JNK may function as an NF-AT regulator under certain physiological circumstances.

More recently, affinity chromatography techniques using the N-terminal regulatory domain of NF-AT have yielded yet an additional candidate NF-AT kinase [38]. This protein, casein kinase Iα (CKIα), is interesting because it is known to be constitutively active and phosphorylates the NLS masking domain of NF-AT4 without the requirement of additional kinases. A serine-rich region in the N-terminal half of NF-AT, known to be phosphorylated in vivo, mediates the interaction with CKIα. CKIα subsequently phosphorylates a contiguous domain that directly masks the NLS of NF-AT in a phosphorylation-dependent manner. Support for CKIα's role as a functionally relevant NF-AT kinase

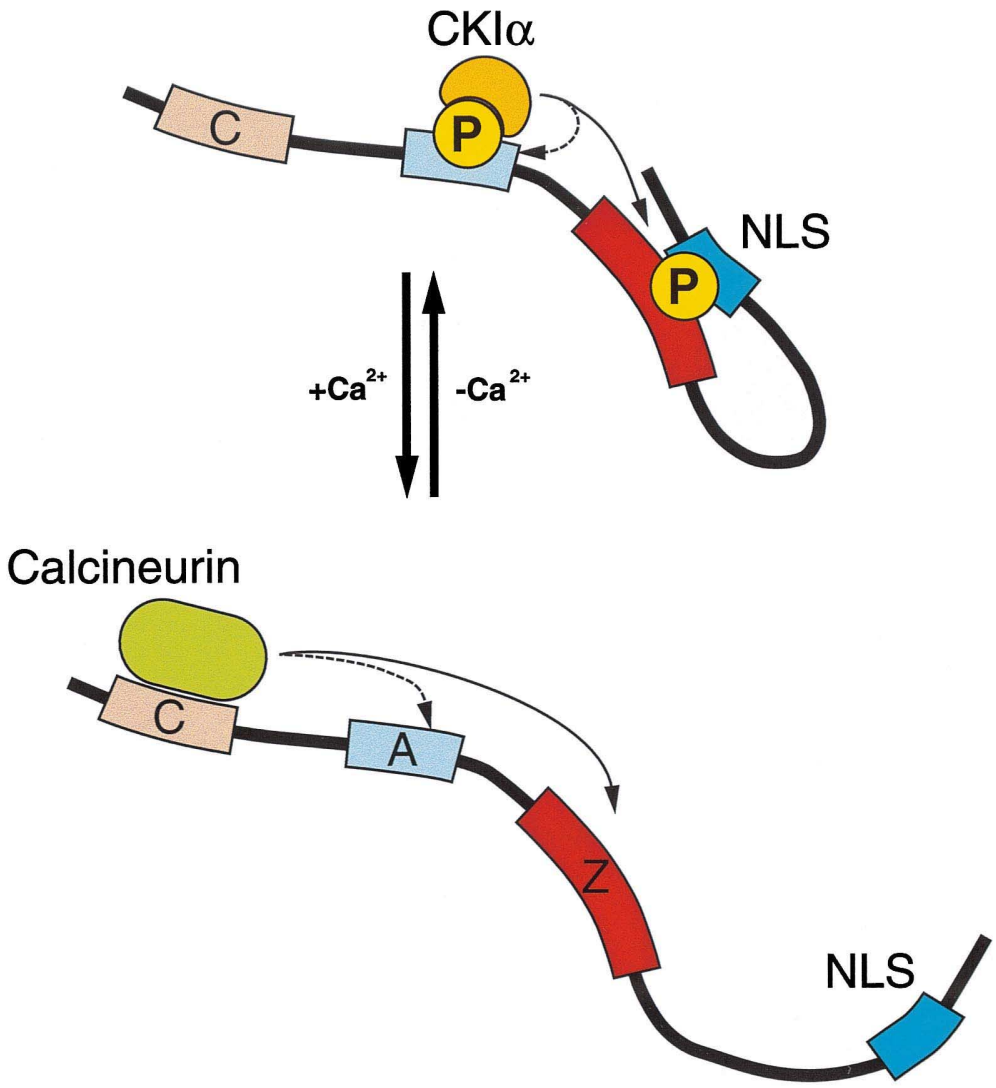

Figure 2. CKIα directly phosphorylates and binds to the A domain of NF-AT, and subsequently phosphorylates the Z domain. The phosphorylated Z domain then assumes a conformation which blocks the recognition of the NLS sequence by cytoplasmic nuclear translocation factors. Upon calcium influx, activated calcineurin binds the C domain of NF-AT4 and dephosphorylates both A and Z domains, resulting in the disruption of CKIα/NF-AT association and exposure of the NLS, respectively.

came from the observation that a dominant-negative CKIα mutant blocks NF-AT phosphorylation and causes NF-AT nuclear translocation in the absence of calcium influx. Interestingly, CKIα is known to prefer previously phosphorylated substrates, and the interaction between CKIα and NF-AT is phosphorylation-dependent [38]. CKIα directly phosphorylates its binding site in NF-AT, thereby establishing its NF-AT association and facilitating subsequent phosphorylation of the NLS masking domain on NF-AT. The CKIα binding site also functions as a platform on which other kinases can regulate the interaction between CKIα and NF-AT. One such kinase, MEKK1, indirectly induces sustained phosphorylation of the CKIα binding site on NF-AT and consequently the tight association between CKIα and NF-AT. As a result of stabilizing the CKIα/NF-AT interaction, MEKK1 completely blocks NF-AT nuclear import initiated by calcium ionophore. While it is likely that constitutively active kinases such as CKIα play the primary role in maintaining NF-AT in its dormant, cytoplasmic location, other kinases may act to fine-tune these interactions to affect NF-AT signaling (fig. 3).

Nuclear export and futile cycling of NF-ATs

An essential feature of NF-AT signaling is the presence of a constitutive nuclear export mechanism superimposed

onto a regulated import control. When the intracellular calcium concentration drops back below 150 nM, NF-ATs are exported from the nucleus with a $t_{1/2} \sim 12$ min [8]. Nuclear export is now known to be an active process involving nuclear export signal (NES) sequences recognized by NES receptors such as CRM1 [42, 43]. Nuclear export of NF-AT is blocked by leptomycin B, a natural product known to inhibit the CRM1 NES receptor [32, 44]. Further, the overexpression of CRM1 prevents the calcium-induced nuclear localization of NF-AT. Using these tools, together with NF-AT mutants, two functionally sufficient NES sequences were defined in the N-terminus of NF-AT4, and both were shown to bind CRM1. These observations raised

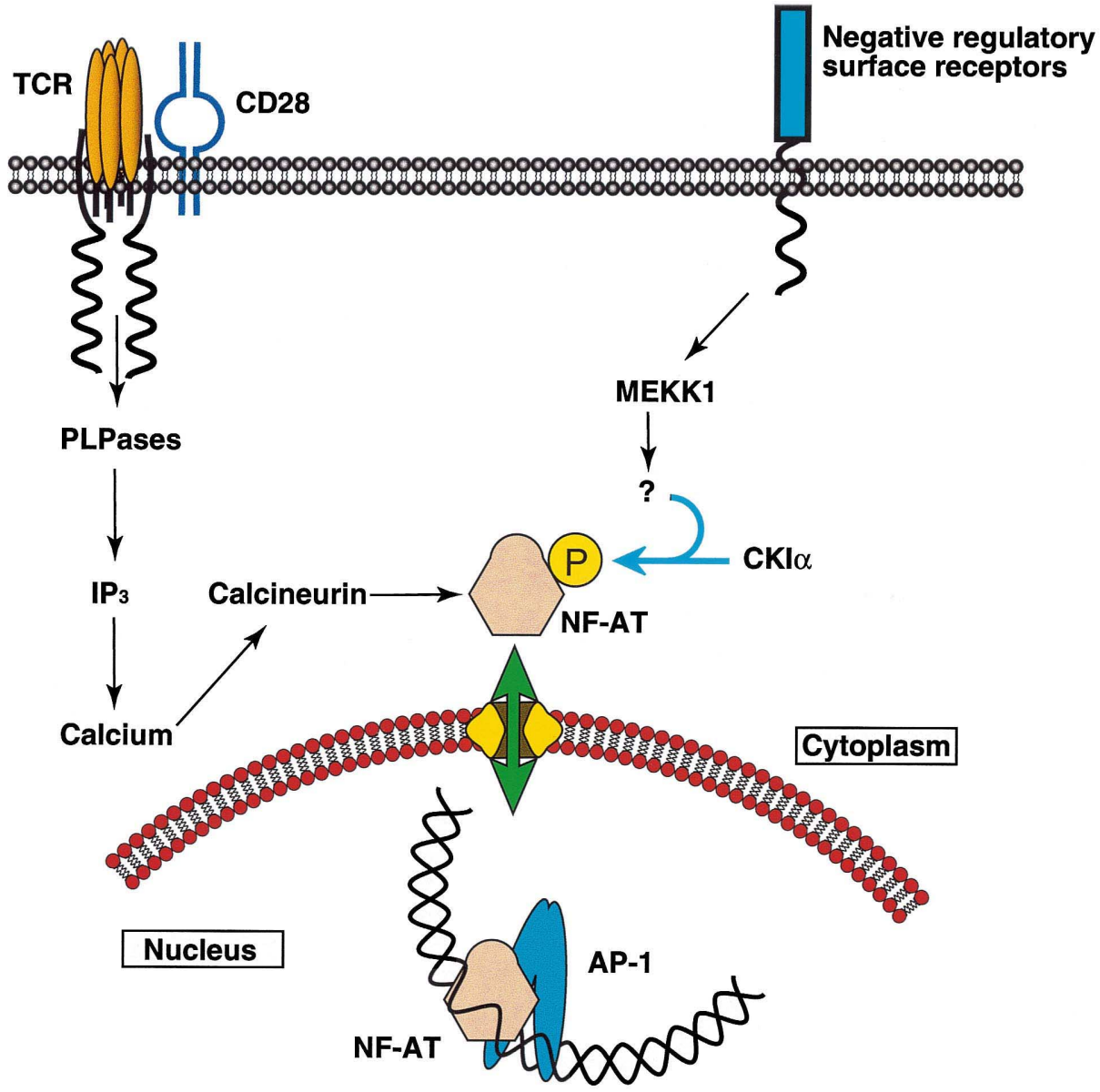

Figure 3. Regulation of NF-AT signaling by cell surface receptors through opposing actions of calcineurin and NF-AT kinases. Coligation TCR and CD28 by antigen-presenting cells induces hyperphosphorylation of the TCR zeta chain by membrane-associated protein tyrosine kinases. PLPγ is then recruited to the plasma membrane through its SH2 domain binding to the phosphotyrosine residues on the TCR zeta chain. PLPγ catalyzes the hydrolysis of phospholipids, giving rise to a secondary signal molecule, IP3, which releases calcium from intracellular stores. Elevated intracellular calcium concentration finally leads to the activation of calcineurin, prompting NF-AT translocation to the nucleus. The primary negative impetus on NF-AT signaling is from the constitutively active NF-AT kinase, CKIα. CKIα constantly opposes calcineurin-mediated NF-AT dephosphorylation. Other kinases, such as MEKK1, may provide a link that enables negative regulatory surface receptors to thwart NF-AT signaling during the activation of calcineurin by functionally enhancing the action of CKIα.

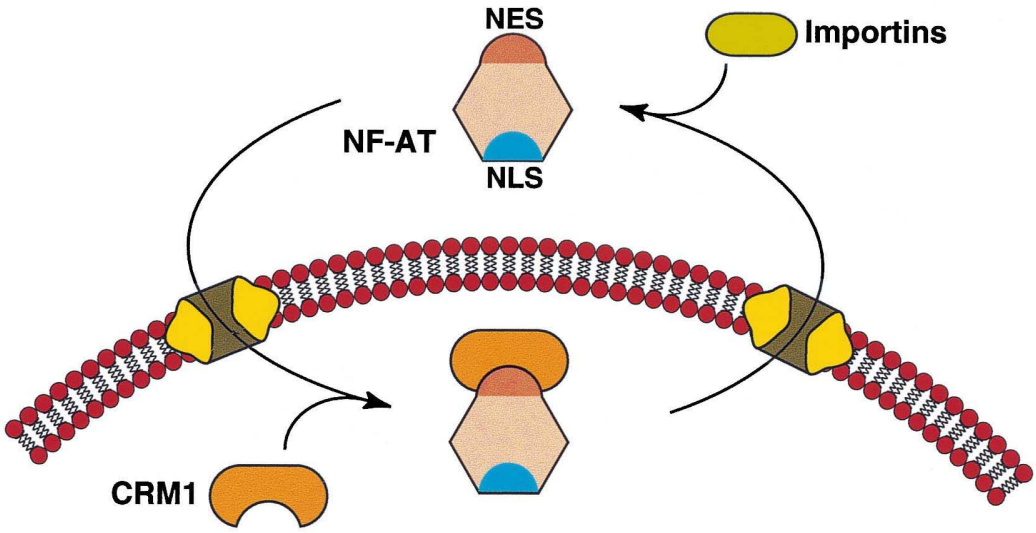

Figure 4. A phenomenon of futile cycling across the nuclear envelope by a molecule with simultaneously active NLS and NES. Having both NLS and NES exposed, the NF-AT molecule is recognized by both importins and exportins. Since nuclear import is kinetically favored over nuclear export, NF-AT shows predominantly nuclear localization. However, due to the engagement of exportin, NF-AT molecules are constantly routed back into the cytosol soon after they enter the nucleus. Under such circumstances, the ability of NF-AT to form transcriptional complexes with its nuclear cofactors to target cytokine gene promoters is severely impaired, and consequently no transcriptional signal is generated.

several important problems for signaling via NF-AT, as well as for other transcription factors possessing both NLS and NES sequences. How, for instance, can NF-AT avoid futile cycling as a consequence of these conflicting subcellular localization signals? Do mechanisms exist that simultaneously coordinate the activation of NLSs and the suppression of NESs to achieve import, and the reverse to achieve export? The critical experiments to demonstrate futile cycling, as well as the means by which NF-AT4 avoids this fate, were performed in mammalian cells using NF-AT and calcineurin mutants [32]. In particular, these experiments revealed that exposed nuclear export signals on NF-AT block its transcriptional activation functions, even under conditions where NF-AT was predominantly localized to the nucleus. NF-AT mutants lacking the NLS masking domain were found to be constitutively nuclear even in the absence of calcium signaling, and yet showed no ability to transactivate NF-AT reporter genes. One possible explanation for this paradoxical result was that an NF-AT with exposed NLSs and NESs would undergo futile cycling across the nuclear envelope, and is therefore unavailable to participate in transactivation complexes (fig. 4). Hence, nuclear translocation of a transcription factor does not represent an endpoint guaranteeing target gene expression, but instead requires an additional layer of regulation involving suppression of nuclear export mechanisms. Interestingly, either calcium ionophores or activated calcineurin expression was able to convert this constitutively nuclear but nontransactivating NF-AT to a transactivator, despite the fact that neither calcium nor calcineurin was required for its nuclear import. Additional experiments showed that expression of even a catalytically deficient calcineurin could impart transactivation function to this cycling NF-AT mutant. In sum, these experiments hinted at an elegant solution to the problem of futile cycling in NF-AT signaling, whereby calcineurin both unmasks nuclear import signals and simultaneously masks export signals. Moreover, these experiments showed that the unmasking of the NLS required calcineurin's phosphatase activity, whereas the masking of NF-AT's export signals was a noncatalytic process brought about by the physical binding of calcineurin to NF-AT. The functional significance of this calcineurin/NF-AT interaction was made apparent by the discovery that previously described calcineurin binding sites overlapped the functionally defined NES sequences on NF-AT. Thus, during calcium signaling, calcineurin binds NF-AT, dephosphorylates the NLS masking domain and is cotransported with NF-AT to the nucleus. In the nucleus, calcineurin competes with CRM1 for NF-AT binding, thereby preventing Crm1-dependent export for the duration of calcium signaling. Upon dissipation of calcium levels, calcineurin/NF-AT interactions weaken and allow CRM1 binding and export of NF-AT from the nucleus (fig. 5).

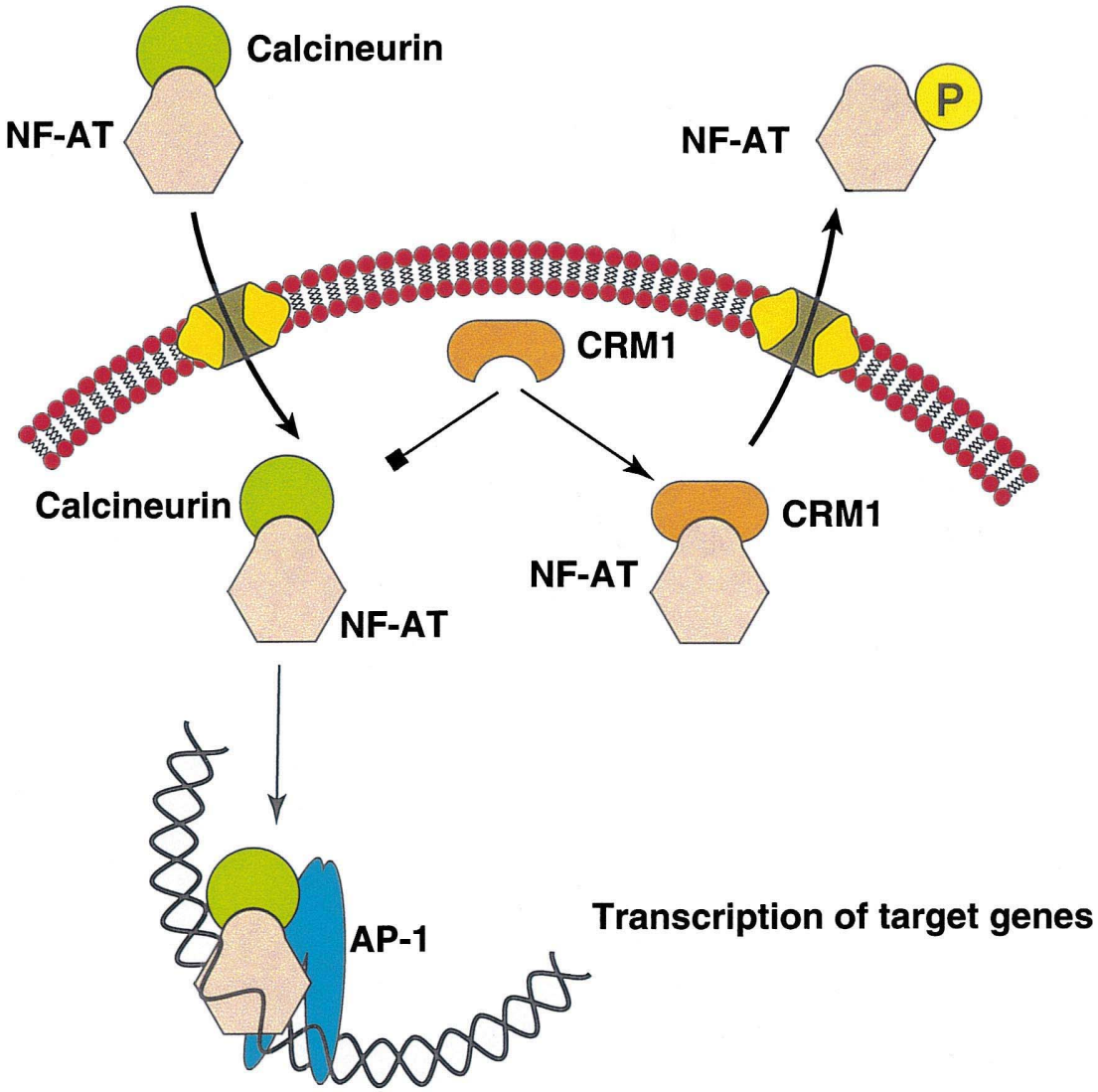

Figure 5. Dual roles of calcineurin in unmasking NLS and preventing nuclear export of NF-AT. The calcium-activated phosphatase calcineurin binds to NF-AT during calcium signaling and dephosphorylates the NLS masking domain, resulting in the nuclear import of NF-AT. Calcineurin is cotransported with NF-AT to the nucleus, where it continues to bind to NF-AT via sites containing NESs. The exportin, CRM1, cannot bind or export NF-AT until calcium signaling ends and calcineurin dissociates from NF-AT. In the absence of calcineurin activity, NF-AT kinases rephosphorylate the NLS mask on NF-AT, further assuring the cytoplasmic disposition of this molecule.

Concluding remarks

The basic regulatory features underlying the remarkable sensitivity of NF-AT4 to calcium signals and their graded transduction mechanisms are now apparent. The design features include a phosphorylation-sensitive intramolecular NLS mask, as well as constitutively active NES sequences within the NF-ATs. These signal sequences are under dual regulation by the calcium-sensitive phosphatase, calcineurin, which can simultaneously unmask import signals while it masks export signals. Despite these advances, we know less about how NF-AT kinases regulate the nuclear import process. Several candidate kinases that appear to oppose calcineurin have been described, with one, CKIα, having the constitutive activity required to maintain NF-AT in the cytoplasm of unstimulated cells. The other candidate kinases may play conditional roles in the regulation of NF-AT import through parallel or integrative pathways of signal transduction, and function downstream of cell

surface receptors known to have positive and negative influences on NF-AT. Whether the molecular mechanism described here for NF-AT4 is functionally conserved in other NF-AT isotypes is under intense investigation.

Finally, the mechanisms described here for NF-AT regulation may be of more general significance to the increasing number of transcription factors regulated at the level of nuclear translocation [45–48]. Each of these factors is likely to contain both nuclear import and export signals whose activities will place these molecules at risk of futile cycling across the nuclear envelope. Understanding the strategies, unique or common, by which these factors avoid futile cycling should yield new insights into their regulatory mechanisms and approaches to their modification.

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