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NFAT Signaling: Choreographing the Social Lives of Cells

Review

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Calcium signaling activates the phosphatase calcineurin and induces movement of NFATc proteins into the nucleus, where they cooperate with other proteins to form complexes on DNA. Nuclear import is opposed by kinases such as GSK3, thereby rendering transcription continuously responsive to receptor occupancy. Disruptions of the genes involved in NFAT signaling are implicating this pathway as a regulator of developmental cell–cell interactions.

General Features of NFAT Signaling

Probably no signaling molecule is so broad in its distribution yet so specific in its functions as Ca^{2+}. Regulated influx or release of Ca^{2+} is critical to events as seemingly unrelated as fertilization, memory, and muscle contraction. Indeed, if the reader remembers anything of this review, it is likely due to Ca^{2+}-dependent patterns of transcription in specific neurons. This paradox of specificity is a major question in modern biology and one that has eluded much effort (Berridge et al., 1998). One of the roles of Ca^{2+} is to regulate calcineurin (Klee et al., 1998), which in turn dephosphorylates and induces the nuclear localization of the cytoplasmic components (NFATc proteins) of NFAT transcription complexes (Figure 1). In the nucleus, NFAT transcription complexes assemble on DNA to activate genes primarily dedicated to cell–cell interactions. Although this signaling pathway was one of the first to be defined (Clipstone and Crabtree, 1992; Emmel et al., 1989; Flanagan et al., 1991; Liu et al., 1991; Shaw et al., 1988), genetic redundancy has made it difficult to study by conventional means. In addition, homologs of NFATc family members are not present in invertebrates. Thus, an understanding of the functions of NFAT signaling has had to await the production of mice containing null mutations in more than one of the genes encoding its apparently redundant components. The molecular components shown in blue in Figure 1 constitute the core NFAT signaling cassette that functions in many cell types in vertebrates.

Specialized Ca^{2+} Channels and "Inside-Out" Signaling Are Necessary to Maintain NFATc Proteins in the Nucleus

Ligand binding of many receptors results in the activation of PLC, release of IP_3, and a transient release of Ca^{2+} from intracellular stores through IP_3 receptors. This initial release of Ca^{2+} is not sufficient to activate NFAT target genes in a number of cell types. Rather an influx of Ca^{2+} through specialized Ca^{2+} release activated Ca^{2+} channels, or CRAC channels, is required (Figure 2). This "inside-out" pathway was first recognized by Putney and colleagues, who found that depletion of intracellular stores by activation of IP_3-gated release from the endoplasmic reticulum resulted in the rapid influx of Ca^{2+} (Putney and Bird, 1993). Remarkably little is known of the nature of the signals that pass from the ER to the cell membrane, and several models are presently being investigated. The requirement for inside-out signaling for lymphocyte activation and NFAT translocation was first demonstrated in mutant lymphocytes selected using an NFAT-directed diptheria toxin construct (Serafini et al., 1995). Surviving cells were unable to activate NFAT-dependent transcription and were also defective in the activation of genes such as those encoding IL-2, IL-4, and CD40 ligand. The defect in the mutant cell lines was shown to be a failure to promote an influx of Ca^{2+} through CRAC channels, resulting in only the rapid transient release of Ca^{2+} from internal stores through IP_3 receptors (Figure 2B). In these mutant cells, NFATc proteins do not stay in the nucleus but rather rapidly return to the cytoplasm. Nuclear localization of NFATc1 overcomes the defect in these cell lines, indicating that the CRAC channel provides the persistent Ca^{2+} signal necessary to maintain NFATc proteins in the nucleus (Timmerman et al., 1996). Similar defects in CRAC channel activation have been found in some patients with severe combined immunodeficiency (SCID) (Feske et al., 2001; Le Deist et al., 1995). These individuals have normal expression of the various signaling components used by the antigen receptor but fail to activate genes such as IL-2, IL-4, and CD40 ligand that are cyclosporin A (CsA)-sensitive and largely dedicated to cellular interactions. The importance of this channel is demonstrated by studies from Rao and colleagues, who showed that it is essential for the activation of most TCR-dependent genes in T cells (Feske et al., 2001). Studies of this inside-out signaling pathway have been hampered by the inability to identify the gene(s) encoding the CRAC channel. Recently, the CaT1 gene was shown to have the expected electrophysiologic characteristics of the CRAC channel (Yue et al., 2001). This channel has seven membrane spanning domains, four ankyrin repeats, and a domain similar to the *Drosophila* retinal Ca^{2+} channel Trp and the *C. elegans* OSM-9 protein involved in olfaction and mechanosensation. As yet it is not known if mutations of the CaT1 gene account for the CRAC channel defects in the SCID patients or the CRAC mutant Jurkat cells. Nevertheless, the mechanism of CRAC channel activation is one of the major unknown steps in lymphocyte signaling.

An additional example of the importance of specialized Ca^{2+} channels in regulating NFATc function is seen in perinatal hippocampal neurons where L-type channels activate NFATc4 translocation and function (Graef et al., 1999). L-type channels are one of many different

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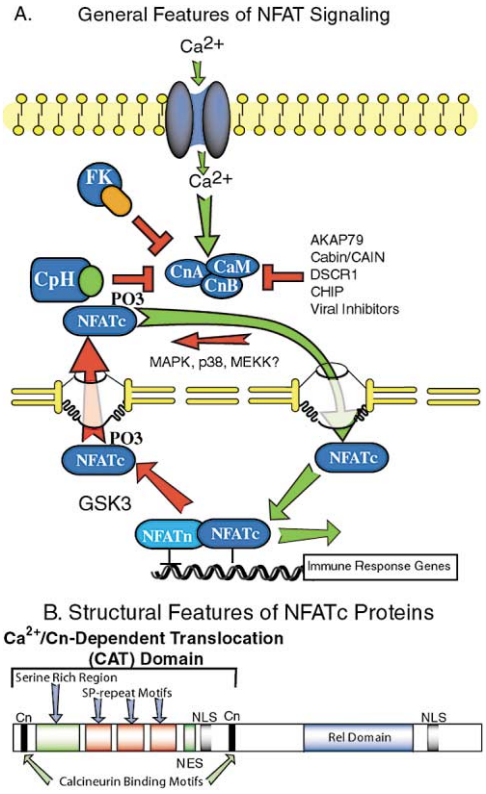

Figure 1. Multiple Levels of Control of NFAT Signaling

(A) General features of NFAT signaling. The components shown in blue appear to modulate NFAT signaling in many different cell types. Orange and green ovals represent FK506 and cyclosporin, respectively. Red arrows denote inhibitors and green arrows denote positive regulators of NFAT signaling. A simplified nuclear pore complex (NPC) is shown; ATP hydrolysis is omitted for simplicity.

(B) Structural features of a typical NFATc protein. The domains shown are from NFAT-C1, but the location of each domain appears to be well conserved, except for the NES, which is shown for C1 (Klemm et al., 1997). The CAT (Ca^{2+}/calcineurin-dependent translocation) domain is shown for C1, but it is similar for all four NFATc family members.

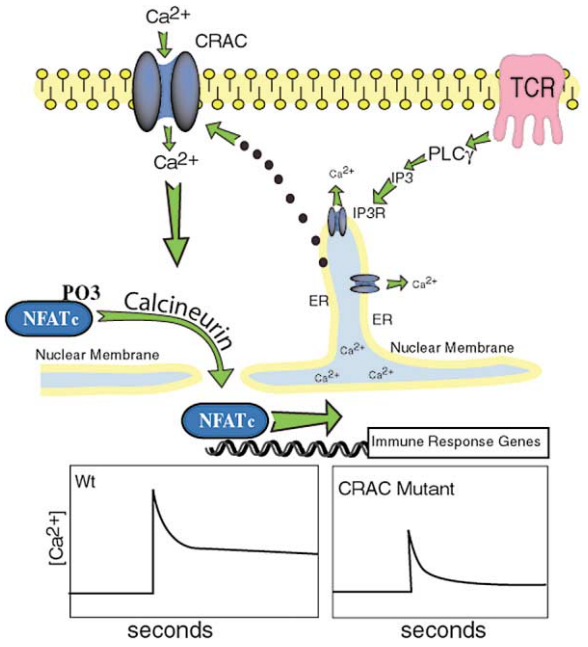

Figure 2. "Inside-Out" Signaling by the CRAC Channel is Essential to Maintain NFATc Proteins in the Nucleus

The lower two panels show the short duration of the Ca^{2+} fluxes in CRAC mutants.

types of Ca^{2+} channels in neurons, but the reason that L-type channels are most effective at inducing translocation of NFATc4 is not clear. NFATc4 appears to directly regulate the neuronal-specific IP_3 receptor (IP_3R1), which is expressed during the first week of life. Since IP_3R1 is necessary for release of intracellular stores of Ca^{2+}, it is possible that L-type channels evoke a positive feedback loop leading to nuclear translocation of NFATc4 and the activation of the IP_3R1 gene with subsequent enhancement of Ca^{2+} release. Such a positive feedback loop could be important in the refinement of synaptic connections that occur during the critical period of mammalian neural development; however, this speculation remains untested.

Modulators of NFAT Import. The above studies called attention to the fine balance between import and export of NFATc proteins. Several different regulators of NFATc import and export have been reported (Figure 1). Nuclear import of NFATc proteins is controlled by the Ca^{2+}- and calcineurin-dependent dephosphorylation of serines within the N terminus of the NFATc proteins located in the SP-repeats and the serine-rich region (Beals et al., 1997a, 1997b; Zhu et al., 1998) (Figure 1B). Phosphoserines within these regions appear to mask nuclear localization sequences (NLS). Dephosphorylation exposes the NLS and leads to rapid nuclear import. Surprisingly, the N-terminal Ca^{2+}/Cn-dependent translocation (CAT) domain (Figure 1B) of NFATc1 was found not to be structured in solution, suggesting that it requires a second protein to mask the NLS and the NES sequences (Park et al., 2000). An alternative possibility is that the N-terminal CAT domain makes contact with other regions of the protein in the C terminus such as the C-terminal NLS. Since NFATc proteins are involved in several therapeutically important processes, structural studies of the full-length phosphorylated and dephospho-protein are likely to be critical for design of inhibitors of specific NFATc family members.

The recent observation that CnB mutant mice phenocopy NFATc3/c4 (c3/c4) mutant mice indicated that calcineurin was somehow dedicated to NFAT signaling (Graef et al., 2001a). The basis of this enzyme-substrate specificity is likely related to a high affinity interaction between calcineurin complexes and NFATc proteins. The N terminus of C1 is an effective dominant negative (Northrop et al., 1994), an action that arises from an interaction between calcineurin and two conserved sequences within the N termini of NFATc proteins that interact with calcineurin (Aramburu et al., 1999; Park et al., 2000). Preexisiting Cn-NFATc complexes may account for the speed of dephosphorylation and translocation of NFATc after a calcium stimulus, as well as the specificity of calcineurin's phosphatase activity on NFATc proteins. Indeed, transfected CnA will cotranslocate with transfected NFATc3 (Zhu and McKeon, 1999);

however, it is not clear that the affinity between these proteins is high enough to induce cotranslocation of the endogenous proteins at physiologic concentrations.

To date, four cellular inhibitors of calcineurin phosphatase complexes have been identified, all of which are able to block nuclear translocation of NFATc proteins. These include the AKAP79 scoffold protein that binds calcineurin and appears to prevent calcineurin's access to substrates (Klauck et al., 1996). Interestingly, AKAP also binds protein kinase A, which is an NFAT kinase that opposes NFATc1 nuclear localization. A second calcineurin inhibitor is the CAIN or CABIN protein, which directly blocks calcineurin activity (Lai et al., 1998; Sun et al., 1998). A third inhibitor is the calcineurin B homolog, CHP, which is thought to bind to CnA but is not able to induce its activation (Lin et al., 1999). Finally, the Down Syndrome Critical Region 1 gene has provided an intriguing connection with Down's syndrome (Fuentes et al., 1995; Rothermel et al., 2000). There are three DSCR1-related genes that are also called MCIP1, 2, and 3. Only one of them is located at the Down's syndrome critical region, but they all apparently have the ability to block NFATc phosphorylation and translocation. Their role in the pathogenesis of Down's syndrome is only speculative at this time.

Pharmacologic inhibitors of NFATc translocation include FK506 and CsA. These agents revolutionized transplant therapy because of their ability to prevent the immune response to transplanted tissue. These chemically different microbial products bind to two different intracellular proteins, FKBP and Cyclophilin. The drug–protein composite surface then binds to the interface of the calcineurin A/B complex, blocking its phosphatase activity by preventing substrate access (Griffith et al., 1995; Kissinger et al., 1995) (Figure 1). Although a number of CsA targets other than calcineurin have been implicated from biochemical studies, new results indicate that these inhibitors are remarkably specific for calcineurin and, in fact, NFATc proteins (Graef et al., 2001a). This conclusion derives from the observation that the phenotype of CnB mutant mice is nearly identical to that of NFATc3/c4 double mutant mice, which in turn is identical to that seen in embryos of mothers given CsA during early embryonic development. These studies establish the specificity of CsA for calcineurin in mice and indicate that at least in the E10.5 mouse, calcineurin is largely dedicated to the control of NFATc proteins. These studies indicate that the drugs CsA and FK506 can be used for rapid and reversible inactivation of calcineurin in embryos.

A number of viruses mitigate immune responses by inhibiting calcineurin/NFAT signaling. Most notable is the A238L protein of the African swine fever virus (Miskin et al., 1998). This protein binds directly to calcineurin and inhibits NFATc translocation in much the same way as CsA and FK506. The repeated targeting of calcineurin by microorganisms as distinct as Trichoderma, Streptomyces, and leukemia viruses indicates that calcineurin is likely to be the "Achilles heel" in the immune response. Supporting evidence for this view comes from data indicating that clinical immunosuppression of transplant rejection is achieved with only 50% inhibition of calcineurin phosphatase activity (Batiuk et al., 1995)

Modulators and Mechanism of NFATc Nuclear Export

The realization that nuclear export (or prevention of import) of NFATc1 was critical to its Ca^{2+} channel selectivity lent interest to the mechanism of NFATc export. Identification of kinases able to promote export came about by first identifying phosphoserines in the N terminus of NFATc1 that were critical to nuclear export (Beals et al., 1997a). Purification of the kinases necessary to phosphorylate these sites, but not other nonfunctional sites, gave GSK3 (Figure 1) (Beals et al., 1997b). An NES was identified in NFATc1 that is essential for export, leading to the view that export was controlled by the exposure of the NES, possibly by release from DNA (Klemm et al., 1997; Neal and Clipstone, 2001), a speculation that will require structural studies for verification. Recently, GSK3 has been shown to be a negative regulator of lymphocyte activation and myocardial hypertrophy in mice, as expected for an NFATc export kinase (Antos et al., 2002; Haq et al., 2000; Ohteki et al., 2000).

Other kinases that oppose NFATc translocation have been suggested by a candidate gene approach and include p38, MAP kinase, and MEKK-1. These proteins will phosphorylate NFATc1 or c3 in vitro. In many cell types, Ras and MAP kinase signals facilitate NFAT-dependent transcription rather than blocking it, as would be expected if these kinases opposed NFATc translocation in vivo. In addition, casein kinase 1 has been purified using an immobilized c3 column and will phosphorylate c3 in vitro (Zhu et al., 1998).

Coincidence Detection and Signal Integration by NFAT Transcription Complexes

NFATc proteins probably do not bind DNA in vivo without assistance, since a Ca^{2+} signal by itself activates few target genes. This observation indicates that NFATc genes are probably not "master" genes that wear camouflage clothes and live in caves, but rather that they function within the community of proteins in a cell when a Ca^{2+} signal is given. This observation was the basis of the reconstitution experiments used to define the components of NFAT complexes (Flanagan et al., 1991). The structural basis of cooperative binding is rooted in the contact residues between NFATc and DNA (Wolfe et al., 1997). These structural and biochemical data indicate that NFAT transcription complexes function as signal integrators and concidence detectors. One signal must be Ca^{2+}/calcineurin (Crabtree, 1989), while the second can be developmental, as with the GATA4 and MEF2 transcription factors in muscle cells (see below) or via the Ras-MAP kinase pathway in lymphocytes (Woodrow et al., 1993). Ras/MAP kinase signaling activates AP-1 and perhaps other nuclear partners that cooperate with NFATc1 and c2 to bind to DNA (Chen et al., 1998; Zhou et al., 1998). Recent evidence indicates that certain NFAT complexes might include transcriptional repressors.

Feedback Control NFAT Signaling

Recently, several levels of feedback regulation over NFAT signaling have become evident. In muscle cells, the calcineurin inhibitor DSCR1/MCIP-1 is activated by calcineurin and NFATc (Yang et al., 2000), creating negative feedback control (Figure 3). Positive feedback control is exerted in at least two ways. The NFATc1 gene is activated by antigen receptor signaling in T cells, with a requirement for calcineurin, NFATc1(Northrop et al.,

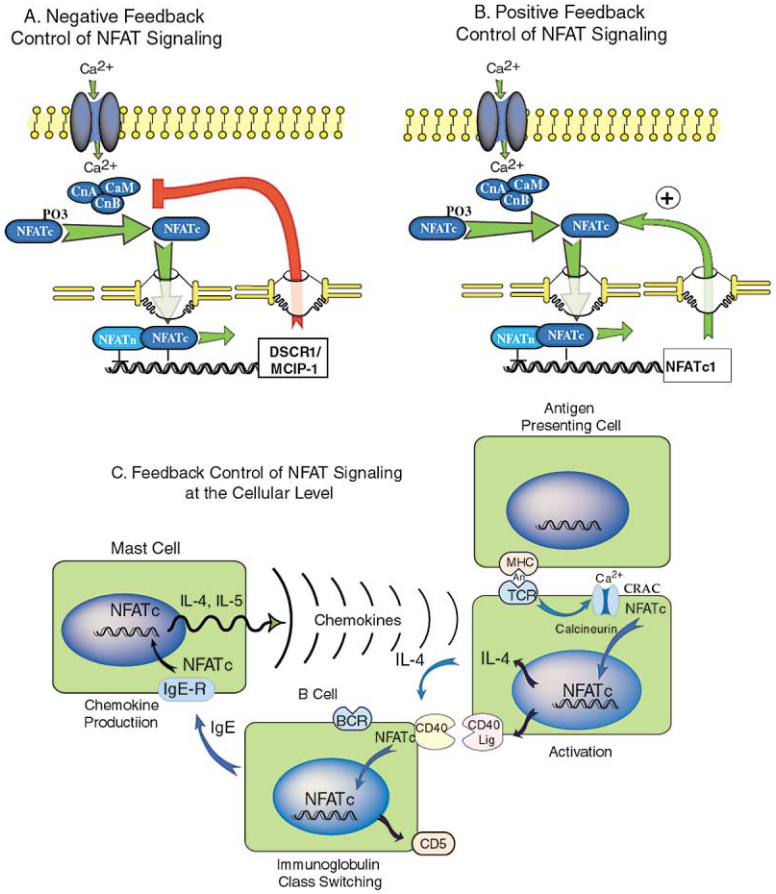

Figure 3. Feedback Control of NFAT Signaling

1994), and a NFAT site within the NFATc1 promoter/enhancer (Zhou et al., 2002). This positive feedback loop could reinforce the cellular commitment to lymphocyte activation (Figure 3B) and may have a role in focusing NFATc1 expression to endocardial precursors of heart valves. A third level of positive feedback arises from the observation that the IP_3R1 gene, which is a critical component needed for NFAT activation, is a target of calcineurin/NFAT signaling during the first week of life in hippocampal and cortical neurons (Genazzani et al., 1999; Graef et al., 1999). In neurons, positive feedback might have a role in synaptic refinement.

Lymphocyte Development and Activation

T lymphocyte activation initiates a highly choreographed series of gene regulations that, based on studies of transcript arrays, activates or inactivates an estimated 4000 genes, or about 15% of the mammalian genome (Diehn et al., 2002). Most of these genes are not directly involved in the immune response but are activated as a result of the transition from a metabolically inactive, nondividing cell to a highly metabolically active, dividing cell. Perhaps the most interesting class are genes dedicated to communication with other cell types (Figure 4). These include genes encoding proteins such as TNFα that are secreted and appear in the plasma and cytokines such as IL-2, 4, and others that produce their effects by autocrine or paracrine mechanisms on nearby cells. A final class of T cell activation genes includes those such as CD40 ligand and Fas ligand that are surface bound and hence regulate only adjacent cells. The four genes encoding the cytoplasmic subunits of NFAT transcription complexes play critical roles in regulating the genes necessary for interactions with cells that do not have antigen receptors. By this means, NFAT signaling conveys the specificity of the T cell receptor to other cell types involved in the immune response.

That NFAT signaling is at least partly dedicated to cell–cell interactions is not surprising in light of the fact that the pathway was defined by a biochemical effort to link the transcriptional activation of the IL-2 gene to the antigen receptor. The IL-2 gene was chosen as a target of TCR signaling because it was critical to coordinate the actions of other cells involved in an immune response. IL-2's regulatory regions turned out to be small (Siebenlist et al., 1986) and, when analyzed, bound NFAT transcription complexes at two sites and perhaps at other critical sites (Shaw et al., 1988). Further analysis indicated that this complex was made up of a heterogenous cytoplasmic, Ca^{2+}- and CsA-sensitive subunit (NFATc) and a nuclear subunit called NFATn (Emmel et al., 1989; Flanagan et al., 1991). Calcineurin was found to be necessary for the translocation of NFATc to the nucleus and the function of NFAT transcription complexes (Clipstone and Crabtree, 1992; Liu et al., 1991), thereby linking Ca^{2+} influx induced by the antigen receptor to the activation of the IL-2 gene (Figure 1).

Targeted disruption of the genes involved in NFAT

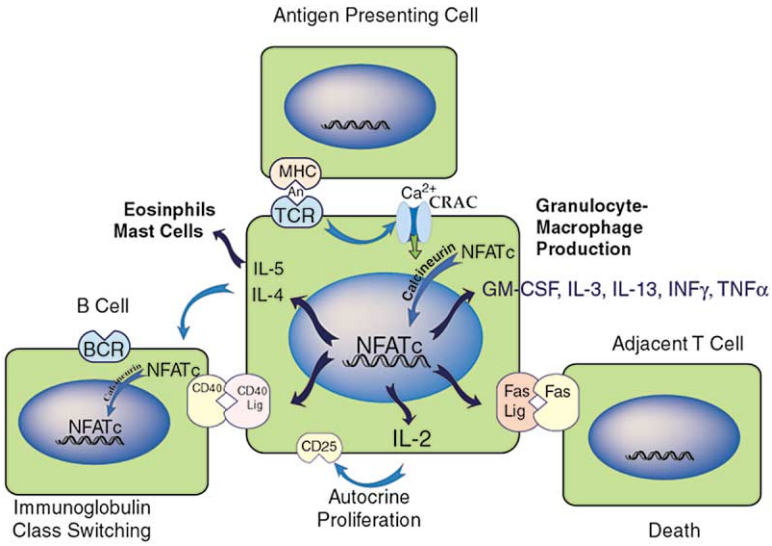

Figure 4. NFAT Signaling and Lymphocyte Cell–Cell Interactions

signaling has demonstrated early roles in the development and function of the cardiovascular, musculoskeleton, and nervous systems that have complicated analysis of immunologic phenotypes (Table 1). Although calcineurin/NFAT signaling is almost certainly essential for T and B cell development, this role has not been directly demonstrated. Nevertheless, certain roles of NFAT signaling are beginning to emerge. Because NFATc1 is essential for morphogenesis of the mammalian heart (see below), thymic development in C1 null mice was examined in Rag1$^{-/-}$ chimeric animals and revealed a moderate reduction in the transition of CD4^{-}/CD8^{-} or double negative (DN) cells to double positive (DP) CD4^{+}/CD8^{+} cells and moderate reduction in the activation of several genes such as IL-2, IL-4, and others (Ranger et al., 1998b; Yoshida et al., 1998). On the other hand, mice with null mutations in NFATc2 survive to adulthood, and their lymphocytes show hyperproliferation in response to antigen receptor signals and eosinophilic infiltration of the lungs, characteristic of severe

Table 1. Phenotypes of Mice with Mutations in NFAT Signaling Components

Gene	Other Names	Null Phenotype	Reference
CRAC	Trp	Failure of NFATc1 translocation and immune response gene activation	(Le Deist et al., 1995; Feske et al., 2001)
Connexin 45		Failure of NFATc1 translocation and cardiac morphogenesis	(Kumai et al., 2000)
CnAα		Moderate reduction in T cell activation	(Zhang et al., 1996)
CnAβ		Not yet reported	
CnAγ		Not available	
CnBα		Vascular patterning abnormality Memory defects in CaMK-Cre conditional deletion	(Graef et al., 2001a)
CnBβ		N.D.	
Cain/Cabin		T cell hyperactivation	(Esau et al., 2001)
DSCR1	MCIP1	N.D.	
CHP		N.D.	
AKAP-79		N.D.	
NFATc1	c1, c	Lethal failure of cardiac morphogenesis Defects in thymic development and T cell activation in Rag-1$^{-/-}$ chimeras	(de la Pompa et al., 1998; Yoshida et al., 1998; Ranger et al., 1998a)
NFATc2	c2, p	Immune hyperactivation and allergic responses Suppression of chrondrogenesis	(Xanthoudakis et al., 1996; Ranger et al., 2000)
NFATc3	c3,4	Defects in thymic development and hyperproliferation of lymphocytes	(Oukka et al., 1998)
NFATc4	c4,3	Normal development, no apparent defects	(Graef et al., 2001a)
c1 + c2		Failure of T cell activation and immune response gene activation	(Peng et al., 2001)
c2 + c3		Spontaneous TH1 differentiation, excessive allergic responses	(Ranger et al., 1998c)
c3 + c4		Lethal vascular patterning defects	(Graef et al., 2001a)
GSK3β		Lethal defects in liver development Transgenic overexpression suppresses lymphocyte development, activation, and cardiac hypertrophy	(Ohteki et al., 2000; Antos et al., 2002; Hoeflich et al., 2000)

allergic responses (Xanthoudakis et al., 1996). At least part of the hyperproliferative syndrome is likely to be due to a failure to induce the Fas ligand, leading to the survival of cells that would normally undergo activation-induced death.

Since NFATc1 and c2 have similar DNA binding specificity (Ho et al., 1995), it was expected that NFATc1 and c2 would be redundant. This was confirmed when a double knockout was made by reconstituting $Rag1^{-/-}$ mice with fetal liver from C1/C2 double knockout mice (Peng et al., 2001). Lymphocytes from C1/C2 null mice made essentially no IL-2, IL-4, and TNFα and reduced levels of IL-5. CD69, which is activated with only a stress or Ras/PKC signal and is CsA insensitive and calcineurin independent, was induced normally in these double knockout cells, indicating that C1 and C2 are not involved in stress responses but are essential for activation of cytokine genes.

Mice with a mutation of the NFATc3 gene have defects in thymic development characterized by a loss of DP cells through programmed cell death (Oukka et al., 1998). This loss of DP cells is likely related to the failure to induce Bcl-2 during thymic development. No defects in positive selection were noted and excessive activation of the Fas ligand in the periphery produced excessive activation-induced cell death. The possibility that the c3-deficient cells had compensated by control of the DSCR1, CAIN, or other calcineurin/NFAT inhibitors (Figure 3) was not investigated.

Mice with mutations in both NFATc2 and c3 show spontaneous differentiation into TH2 (T helper 2) cells, the highest levels of IgE yet recorded in mice, as well as severe allergic responses (Ranger et al., 1998c). T cells from these mice also show spontaneous hyperproliferation and are independent of CD28 stimulation for activation. The paradox of hyperproliferation, enlarged lymphoid tissue and excessive cytokine production is likely related to defective activation of Fas ligand and hence an inability to undergo activation-induced cell death. This difference between the C3 and the C2 + C3 mouse may be in part related to the fact that C2 + C3 mice have spontaneous nuclear accumulation of NFATc1, indicating that compensation for the lack of NFATc2 and c3 involves nuclear import of NFATc1 by an as yet unknown mechanism.

NFAT signaling in other cell types in the immune system appears to produce positive feedback regulation that may be important for allergic responses (Figure 3C). T cell activation leads to the induction of CD40L through NFAT signaling. One role of CD40 ligand in conjunction with B cell receptor signals and IL-4 is to activate NFAT signaling in B cells leading to immunoglobulin class switching and the production of IgE by some B cells (Verweij et al., 1990; Choi et al., 1994). IgE activates NFATc1 translocation and function in mast cells, leading to the production of an array of cytokines and chemokines that in turn influence T cell function (Figure 3C) (Weiss and Brown, 2001). Signaling in eosinophils also uses NFAT transcription complexes to activate cytokines and cell surface molecules (Handen and Rosenberg, 1997). These observations imply that NFAT signaling regulates the actions of cells needed for allergic responses.

HIV replication in T cells requires T cell receptor signaling, an effect that appears to be in part mediated by NF-κB and NFAT. Although NFATc and NF-κB proteins both bind to DNA through Rel domains, they do not share regulatory mechanisms. Nolan and colleagues have recently shown that NFAT complexes contribute about 50% of the DNA binding activity found at the NF-κB binding sites in the HIV LTR (Kinoshita et al., 1997). Consistent with the binding data, both NFAT and NF-κB complexes make significant contributions to the replication of the HIV virus in primary T cells (Kinoshita et al., 1998).

Calcineurin's role in lymphocyte activation is underscored by the success of CsA and FK506 as clinically effective immunosuppressants. However, mutation of the calcineurin Aα catalytic subunits produces minor T cell activation and developmental defects (Zhang et al., 1996), presumably due to redundancy with Aβ and perhaps the Aγ chains. Mutation of the regulatory Bα subunit leads to death due to angiogenic patterning abnormalities at E10.5 (see below), and hence immune function can not be examined. Previous work has shown that CsA blocks positive selection as well as negative selection in the thymus and prevents lymphocyte activation. CsA or FK506 completely prevents NFATc dephosphorylation and translocation to the nucleus, but does not compromise the in vitro transcriptional activity of NFAT complexes (Flanagan et al., 1991). Most of the effects of CsA are thought to be due to its ability to block nuclear translocation of NFATc proteins, since nuclear expression of NFATc but not rel/NF-κB proteins or Fos-Jun family members renders Jurkat cells CsA resistant (Timmerman et al., 1996).

Roles for Calcineurin-NFAT Signaling in Musculoskeletal Development

Calcineurin/NFAT signaling also regulates several aspects of skeletal muscle gene expression and remodeling (Figure 5). Calcineurin signaling has been implicated in myoblast differentiation and in the mechanism whereby insulin-like growth factor-1 induces myocyte hypertrophy based on the inhibition of these processes by CsA. Ectopic expression of activated calcineurin also stimulates differentiation and hypertrophy in cultured myocytes (Musaro et al., 1999; Semsarian et al., 1999), whereas NFATc3 mutant mice exhibit skeletal muscle hypoplasia that apparently reflects impaired embryonic muscle development (Kegley et al., 2001). The finding that hypertrophy of skeletal myocytes is accompanied by nuclear translocation of NFATc1 and its association with GATA2 suggests that elements of the pathway for hypertrophic signaling are similar in cardiac and skeletal muscle cells (Molkentin et al., 1998; Musaro et al., 1999). Calcineurin signaling has also been implicated in skeletal muscle hypertrophy in vivo based on the ability of CsA to prevent myofiber growth in response to chronic load (Dunn et al., 1999). CsA also prevents muscle regeneration in response to injury (Abbott et al., 1998). The target genes of the calcineurin pathway that mediate these muscle-remodeling responses remain unknown.

Adult skeletal muscle fibers can be generally classified as fast or slow twitch, based on their contractile and metabolic properties and associated gene expression profiles (Olson and Williams, 2000). These properties

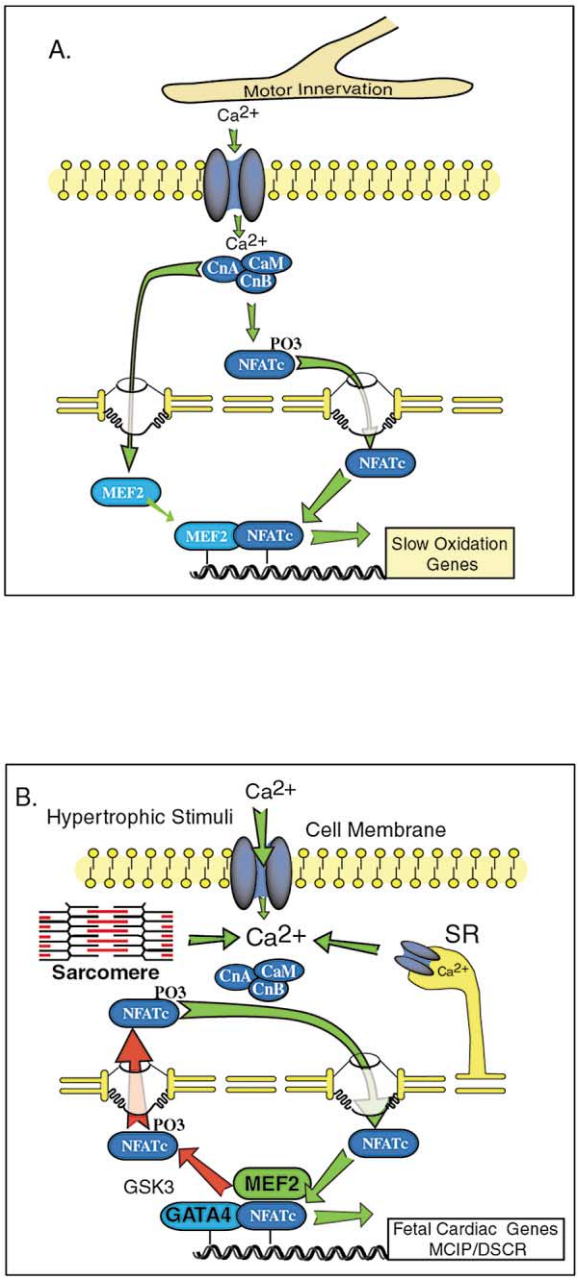

Figure 5. NFAT Signaling in Cardiac and Skeletonal Muscle Development and Function

(A) NFAT and MEF2 cooperate in muscle fiber type specification.
(B) NFAT signaling in cardiac hypertrophy.

are largely dependent on the pattern of motor nerve innervation and contractile activity. Slow twitch myofibers, which are involved in chronic contractile events, exhibit an oxidative metabolism and maintain relatively high intracellular calcium levels (100–300 nM). In contrast, fast twitch fibers, which are involved in rapid bursts of contraction, are glycolytic and are characterized by brief, high amplitude calcium transients and lower calcium levels (<50 nM).

Recent studies have revealed a key role for calcineurin signaling in activation of muscle-specific genes associated with the slow twitch phenotype. Treatment of rodents with CsA induces a reduction in slow skeletal muscle fibers and an increase in fast fibers, though this transformation is incomplete (Chin et al., 1998; Serrano et al., 2001). Conversely, expression of activated calcineurin under control of a skeletal muscle-specific promoter induces an increase in slow fibers and a decrease in fast fibers in transgenic mice (Naya et al., 2000). However, in contrast to cardiac muscle, activated calcineurin alone does not induce hypertrophy of skeletal myofibers in vivo, suggesting that its hypertrophic effects in cultured cardiomyocytes may require additional signaling inputs.

A slow fiber-specific element of the slow troponin I gene contains a canonical NFAT site that is required for maximal expression in slow muscle fibers and for maximal responsiveness to calcineurin (Calvo et al., 1999; Wu et al., 2000). An adjacent binding site for the MEF2 transcription factor cooperates with the NFAT site to confer calcineurin sensitivity. Indeed, transgenic mice that harbor a lacZ transgene linked to a multimerized MEF2 site show slow fiber-specific expression of the transgene, which can be inhibited by CsA or by muscle-specific expression of exogenous MCIP1 (Wu et al., 2001). These findings confirm results obtained in other cell types in which calcineurin signaling has been shown to stimulate the transcriptional activity of preexisting MEF2 protein (Liu et al., 1997). The physical interaction between NFAT and MEF2 proteins provides a mechanism for linking calcineurin signaling to MEF2 activation (Blaeser et al., 2000) (Figure 5).

An additional, potentially therapeutically significant role of NFAT-dependent gene expression comes from the observation that NFATc2 null mice show continued growth of cartilage after it would normally undergo senescence near puberty (Ranger et al., 2000). Remarkably, these null mice heal cartilaginous wounds more effectively, indicating that NFATc2 signaling blocks cartilaginous differentiation. Mesenchymal stem cell lines created from C2 null mice preferentially differentiate into cartilage, while overexpression of C2 in these cells blocked differentiation to cartilage, revealing a role in the differentiation of mesenchymal stem cells into cartilage. As yet the ligand-receptor pair controlling C2 function in chrondrocytes has not been identified, but represents an important challenge for therapeutic advances in this area. A straightforward approach would be to screen for ligands extracted from immature cartilage that could induce C2 dephosphorylation or translocation in chrondrocytes.

NFAT Signaling in the Development and Function of the Cardiovascular System

The Morphogenesis of the Heart and Cardiac Valves—A Gap Junction-Dependent Morphogenic Gradient?

Our moment-to-moment existence is dependent on the delicate leaflets that allow effective pumping of blood by the heart. Malformations of the heart and valves are the most common congenital abnormality in humans and occur in about 1 in 100 infants. Despite their remarkable importance, relatively little is known about their formation. Studies with mice deficient in calcineurin,

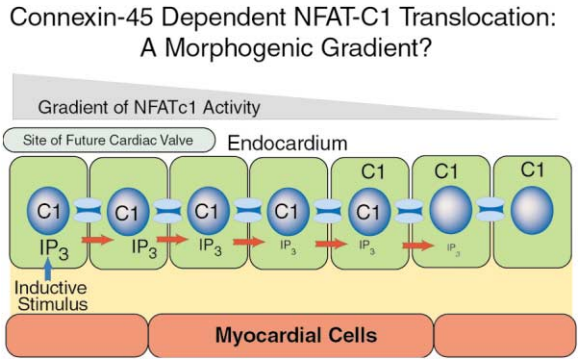

Figure 6. A Putative Gap Junction-Dependent Morphogenic Gradient Essential for Heart Development

The model proposes a localized stimulus to an endothelial layer connected by gap junctions (blue), which then allows a graded distribution of IP_3 leading to a gradation of Ca^{2+} and hence C1 activity.

connexin 45, and NFATc1 have suggested that the formation of cardiac valves involves a gap junction-dependent morphogenic event, speculatively an intracellular morphogeneic gradient (Figure 6).

At about embryonic day E8.5, NFATc1 is expressed in a highly specific pattern only in endocardial cells that are destined to make up the lining of the heart and valves (de la Pompa et al., 1998; Ranger et al., 1998a). By the following day, C1 is restricted to cells that will undergo an epithelial-to-mescenchymal transformation and make up the endocardial cushion, which is the precursor to both the outflow valves and the septal structures. C1-deficient mice fail to form the valves in the outflow tract and fail to completely close the septum separating the two atria (de la Pompa et al., 1998; Ranger et al., 1998a). These defects are lethal by E13.5 due to cardiac failure. A graded distribution of C1 nuclear localization is seen in endothelial cells surrounding the presumptive site of valve formation. When embryos are treated with CsA, it prevents C1 nuclear localization and phenocopies the defect in valve development. Mice with mutations of CnBα also show failure of C1 nuclear localization in the endocardium (Graef et al., 2001a). Thus, calcineurin/NFAT signaling is necessary for valve formation, raising the question of the nature of the upstream regulator.

This question was in part answered when valve abnormalities similar to those in the C1 null mice were noted in connexin 45 null mice (Kumai et al., 2000). Remarkably, C1 was cytoplasmic in the endocardial cells of knockout mice, indicating that connexin 45 was upstream of calcineurin and NFATc1. Connexins are complex molecular assemblies that form gap junctions connecting one cell to its neighbors (Kelsell et al., 2001). Gap junctions allow transfer of certain ions, IP_3, and electrical impulses from one cell to another. These observations suggest a speculative model for the role of connexin-45 in heart valve development shown in Figure 6. This model involves a localized inducing stimulus leading to PLC activation and the release of IP_3. IP_3 could then be transmitted though GAP junctions to adjacent cells, leading to Ca^{2+} release and the opening of extracellular CRAC channels, influx of Ca^{2+}, activation of calcineurin, and nuclear import of C1. The transcriptional response would be expected to be topographically graded by virtue of the limited distribution of IP_3 and the anticipated morphologic result would be a ring-like swelling similar to that of the endocardial cushions. Additional work needs to be done to confirm this novel mechanism for the formation of a morphogenic gradient. However, delineation of the developmental steps in heart valve formation will likely be critical to future therapeutic efforts to grow valves in situ by recapitulating development. Since nearly 5% of the population of developing countries will eventually suffer valvular insufficiency, this is a worthwhile, if futuristic, goal.

Patterning of the Vasculature Requires NFAT Signaling to Mediate Endothelial-Mesenchymal Interactions

Development of the vascular system produces a remarkably intricate and stereotyped pattern of vessels that meet and probably anticipate the oxygenation needs of the organism. A critical aspect of vascular development is the cross-talk that must occur between endothelial cells and the surrounding embryonic mesenchyme that provides the cues needed for correct patterning (Yancopoulos et al., 2000). Vascular patterning is more complex than simply supplying oxygenation, since vessels must take specific courses dictated by issues such as protection from injury and compatibility with movement. Mice bearing deletions in NFATc3 and c4 die at about E10.5 with vasculature patterning defects (Graef et al., 2001a). c3/c4 null mice have no defect in the initial ability of endothelial cells to differentiate, but rather these differentiated cells fail to respond to and give signals essential for the assembly of vessels along specific pathways. A similar but more severe defect is observed in mice bearing a mutation of the CnBα gene that prevents the Ca^{2+}-dependent interaction of CnB with CnA and the subsequent dephosphorylation of NFATc4 (Graef et al., 2001a). Close inspection of these embryos revealed that vessels often grew into inappropriate sites (the somites and neural tube) normally avoided by developing vessels. Studies using transcript arrays identified several c3/c4 target genes including VEGF and other vascular growth factors. VEGF was found to be overexpressed about 3- to 4-fold higher in the somites, which are avoided by early vessels, indicating that NFAT signaling suppresses genes that would normally promote the growth of vessels. CsA administration to the mothers reproduced the CnB mutant phenotype indicating that CsA was specific for calcineurin at this time of development. Furthermore, since the phenotype of mice treated with CsA was similar to the c3/c4 null phenotype, CsA was selective for NFAT signaling at this time of development (Graef et al., 2001a). By giving short pulses of drug, calcineurin was shown to be essential between E7.5 and E8.5. At this time, NFAT was found to be expressed and localized to the nucleus in mesenchymal cells. Lower levels of c4 were seen in endothelial cells. The simplest explanation of these results is that NFAT signaling suppresses expression of vascular attractants or activates the expression of vascular inhibitors at or near sites where vessel growth must be limited.

An endothelial role for NFAT signaling in vascular development and function was demonstrated by the studies of Redondo and colleagues who found that VEGF activates NFATc translocation and transcription in en-

dothelial cells (Hernandez et al., 2001). CsA prevented NFAT-dependent transcription and endothelial migration as well as the activation of specific endothelial genes such as Cyclooxygenase-2 (Cox-2). Blocking NFAT translocation in adult animals with CsA prevented the growth of vessels into tumors in the corneal micropocket assay. These studies strongly indicate that while the role of mesenchymal NFAT signaling is to provide angiogenic factors, the role of endothelial NFAT signaling is to mediate the response to factors such as VEGF. These studies have three important therapeutic implications: (1) inhibitors of NFAT signaling might be effective modulators of tumor angiogenesis, (2) the downstream genes (secreted cytokines) induced by NFAT signaling could be useful as inhibitors of tumor angiogenesis, and (3) CsA's beneficial effects on diseases such as rheumatoid arthritis might be related to the NFATc regulation of Cox-2 production by endothelial cells.

NFAT Signaling in Cardiac Growth and Function

In addition to its role in formation of the embryonic cardiovascular system, the calcineurin-NFATc pathway is required for function of the adult heart. Calcium is a key regulator of cardiac contractility, growth, and gene expression. Remarkably, however, the first indication of a specific role for calcineurin in the heart came from the unexpected finding that NFATc4 associated with the cardiac zinc finger transcription factor GATA4, which is involved in cardiac development and hypertrophy (Molkentin et al., 1998).

The adult myocardium responds to a variety of stimuli by a hypertrophic growth response, which is associated with an increase in size of cardiomyocytes, the assembly of sarcomeres, and the activation of a fetal program of cardiac gene expression. Cardiomyocyte hypertrophy is triggered by a variety of calcium-dependent signaling pathways that are activated by extracellular agonists, altered contractile activity, and volume or pressure overload on the heart (Frey et al., 2000a). Numerous fetal cardiac genes that are induced during hypertrophy are controlled by GATA4, but the mechanism that linked this transcription factor to calcium signaling was unclear. In a two-hybrid screen, it was discovered that GATA4 interacted with NFATc4, providing a potential link between calcineurin signaling and the activation of fetal cardiac genes during hypertrophy. This interaction is mediated by the second zinc finger of GATA4 and the C-terminal portion of the Rel homology domain of NFATc4 (Molkentin et al., 1998).

Subsequent studies showed that expression of constitutively activate calcineurin in the hearts of transgenic mice is sufficient to induce massive myocardial hypertrophy that progresses to dilated cardiomyopathy and sudden death, mimicking the pathologic sequelae associated with heart disease in humans. Evidence that NFAT dephosphorylation is sufficient for hypertrophy has also been provided by the observation that an N-terminal deletion mutant of NFATc4 that lacks the sites for calcineurin dephosphorylation, and is therefore constitutively localized to the nucleus, can induce cardiac growth when overexpressed in vivo. However, the extent of hypertrophy that results from overexpression of calcineurin is more severe than from activated NFATc4. Whether this reflects the existence of calcineurin substrates in addition to c4 or variations in expression levels of the ectopically expressed proteins remains to be resolved. The observation that about 2%–10% of NFATc3/c4 double mutant mice live for a few weeks after birth and appear to die of cardiac insufficiency (Graef et al., 2001a) indicates that c3 and c4 may play redundant roles for cardiac development. It should also be pointed out that initial conclusions about the sufficiency of calcineurin and NFATc4 to induce cardiac hypertrophy were based on overexpression of constitutively active proteins under control of the α-*myosin heavy chain (MHC)* promoter, which directs superphysiological levels of expression.

Further evidence for the involvement of calcineurin-NFAT signaling in cardiac hypertrophy has been provided by the observations that CsA and FK506 can prevent myocyte hypertrophy in vitro and can block cardiac hypertrophy in vivo in response to numerous stimuli including pressure overload (Shimoyama et al., 1999), myocardial infarction (Deng et al., 2001), hypertension (Sakata et al., 2000; Shimoyama et al., 2000), and some (but not all) abnormalities in contractile proteins (Sussman et al., 1998). However, there has been considerable variability in the responses of rodent models to calcineurin-inhibitory drugs, with some studies reporting complete rescue from hypertrophy and others reporting partial or no effects (reviewed in Leinwand, 2001). It has also been reported that hypertrophy is exacerbated by CsA treatment of mice harboring a mutation in the α-*MHC* gene that mimics a common mutation in humans (Fatkin et al., 2000). Additional approaches will be required to resolve these discrepancies and to define the specific pathologic stimuli that result in calcineurin activation with resulting cardiac enlargement and heart failure.

Several genetic approaches involving overexpression of calcineurin-inhibitory proteins in the heart or ablation of calcineurin genes have also provided strong support for the involvement of calcineurin in cardiac hypertrophy, without the complications associated with secondary effects of CsA or FK506 on noncardiac tissues. Overexpression of MCIP-1 (Figure 1) in the heart under control of a cardiac-specific promoter severely inhibits hypertrophy in response to activated calcineurin, pressure overload, and exercise (Rothermel et al., 2001). Intriguingly, MCIP-1 is upregulated in the heart in response to activated calcineurin, which may provide an endogenous feedback loop to suppress calcineurin signaling. The upregulation of MCIP-1 expression in cardiomyocytes is mediated by a cluster of 15 tandem NFAT binding sites in the MCIP-1 gene promoter (Yang et al., 2000).

Overexpression of Cabin/Cain and a dominant negative calcineurin B subunit in the heart also prevents hypertrophy in response to a variety of stimuli (De Windt et al., 2002; Zou et al., 2001). Recently, knockout mice lacking the calcineurin Aβ subunit gene were also shown to be resistant to hypertrophy in response to pressure overload (De Windt et al. 2002), adding further support to the role of calcineurin in hypertrophy. Consistent with the signaling pathway outlined in Figure 5B, cardiac-specific expression of a constitutively active mutant of GSK-3 in the heart substantially diminishes hypertrophy in response to calcineurin activation and pressure overload, with reduced nuclear localization of NFAT (Antos

et al., 2002). However, residual hypertrophy is still observed in the presence of constitutively active GSK-3. The basis for this resistance to GSK-3 signaling remains to be resolved.

Together, the weight of evidence argues strongly that calcineurin activation is critical for myocardial growth. The calcineurin-NFAT pathway therefore represents an auspicious target for therapy. However, it should also be pointed out that the level of calcineurin expression in the heart is approximately 10-fold higher than in the immune system, making it unlikely that CsA or FK506 could be used chronically at sufficient doses to treat cardiac disease without immune suppression and secondary renal toxicity. An alternative approach would be to exploit various endogenous suppressors of the calcineurin-NFAT pathway to interfere with hypertrophic signaling.

While dephosphorylated NFATc4 is able to induce hypertrophy when overexpressed in the heart, there is currently no evidence that hypertrophic growth requires the direct binding of NFATc4 to specific target genes that mediate the hypertrophic program. However, since GATA and MEF2 factors regulate numerous hypertrophic responsive genes, and since they both associate with NFATc4 (Molkentin et al., 1998; Blaeser et al., 2000) as well as each other, it is likely that combinatorial interactions among these factors mediate the activation of downstream genes in the hypertrophic signaling pathway, without a requirement for NFAT to bind directly to every calcineurin-responsive target gene. It is also likely that calcineurin has targets in addition to transcription factors that mediate its effects on cardiac muscle. For example, calcineurin directly dephosphorylates specific cytoskeletal proteins in muscle and influences other signaling pathways. The association of calcineurin with calcium release channels is also likely to have profound influences on calcium homeostasis with resulting effects on gene expression in cardiac muscle.

An important issue that has not yet been satisfactorily explained is how calcineurin activation can control sustained responses in the heart and be insulated from the sharp fluctuations in calcium that occur with each cycle of contraction and relaxation. Undoubtedly, calcineurin must respond to a specific pool of intracellular calcium, but how and where this occurs within the myocyte is unclear. One possibility is that calcineurin is localized at specific intracellular sites through association with binding proteins. In this regard, a family of muscle-specific calcineurin binding proteins, calsarcins, has been identified (Frey et al., 2000b). Calsarcins are localized to the sarcomere where they bind calcineurin. The association of calcineurin with calsarcins provides a potential mechanism for localizing the enzyme to the contractile apparatus where it may respond to alterations in calcium signaling resulting from contractile abnormalities.

The Evolution of NFAT Signaling and the Origin of Vertebrates

A surprise of the last decade of research on signal transduction is that only about a dozen biochemical pathways appear to transmit all signals from receptors into the nucleus and that virtually all of these pathways are present in both invertebrates and vertebrates. In contrast, NFAT signaling appears to have arisen in vertebrates since there are no invertebrate homologs of NFATc family members (Graef et al., 2001b). These observations raise the possibility that the NFATc family arose to suit the specialized needs of vertebrates. The early results from knockout animals indicate that this speculation is likely to be correct. To date, NFAT signaling appears necessary for the formation of a complex vascular system, a recombinational immune system, certain aspects of the nervous system, and complex adaptive responses characteristic of vertebrates.

A plausible mechanism for the origin of the NFATc gene family involves translocation of a Rel domain into a precursor gene that contained a calcineurin-sensitive Ca^{2+} sensor, at once creating a new gene and destroying the precursor. Gene duplication could then give rise to the four existing NFATc genes (Graef et al., 2001b). This primitive translocation could provide, at the dawning of vertebrate life, a new pathway linking Ca^{2+} signals to the nucleus, useful for the formation of several new systems needed to deal with a larger body and complex adaptive responses. The evolutionary creation of a pathway operating at the informationally rich interface of Ca^{2+} and tyrosine kinase signaling, dedicated to cell–cell interactions and structured to cooperate with the other pathways existing 500 million years ago, could have been helpful for making the transition to vertebrate life.

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