Instituto Juan March de Estudios e Investigaciones

158 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

The Calcium/Calcineurin/NFAT Pathway: Regulation and Function

Organized by

E. N. Olson and J. M. Redondo

G. R. Crabtree
K. W. Cunningham
M. S. Cyert
M. Fresno
J. Heitman
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Introduction
Juan Miguel Redondo and Eric N. Olson

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The calcium-regulated phosphatase Calcineurin is implicated in many programs of gene activation, differentiation and development in eukaryotes. The discovery that this protein is a principal target of the immunosuppressive drugs Cyclosporin A and FK-506 has accelerated our understanding of Ca^{2+} signal transduction pathways. In particular, NFAT transcription factors are major substrates of Calcineurin and have been extensively analysed. NFAT proteins couple extracellular signals to the transcriptional activation of genes induced in response to stimuli that mobilise extracellular Ca^{2+}. In the last few years, there has been a substantial expansion in our understanding of the molecular mechanisms that underlie the regulation and function of the Calcineurin/NFAT pathway. This pathway plays critical roles in a diverse array of important biological processes that include the activation and development of the immune system, the development and function of the nervous system, patterning of the vasculature, morphogenesis of the heart valves, and muscle development. Two further important areas where this signalling pathway has been implicated are cardiac and skeletal muscle hypertrophy, and angiogenesis. Indeed, the effectiveness of immunosuppressive drugs in blocking cardiac hypertrophy and angiogenesis in several animal models, together with the major role that NFAT proteins play in immune regulation, provide strong evidence of the critical role of these transcription factors in pathological conditions.

This workshop was held so that scientists working on different aspects of the Calcium/Calcineurin/NFAT pathway could exchange information about the state of the field in different biological systems, including yeast, *Drosophila*, mouse, and humans. Presentations and discussions covered the regulation and function of calcineurins, the signal transduction components and regulation of the pathway in different cell systems, and the distinct roles of the different NFAT members during development. There were also a number of contributions concerning the role of Calcineurin and NFAT in disease, and the identification of components of the pathway as molecular targets in cardiac growth and in muscle regeneration, size and function. Each presentation at the meeting stimulated intense and excellent discussion: creating a unique forum for the exchange and integration of recent findings, and clarifying the critical issues to be addressed in the regulation and function of the Calcium/Calcineurin/NFAT pathway

Juan Miguel Redondo
Eric N Olson

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Session 1: Regulation and function of calcineurin
Chair: Joseph Heitman

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Calcineurin-mediated coupling of Ca^{2+} signaling and oxidative stress

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The Ca^{2+}/calmodulin dependent protein phosphatase, calcineurin, modulates a wide range of cellular processes in response to Ca^{2+}-mobilizing signals. The calcium regulation of calcineurin activity is mediated by two structurally similar but functionally distinct Ca^{2+}-regulated proteins. Ca^{2+} binding to calcineurin B, a regulatory subunit of the enzyme, tightly associated with the catalytic subunit, calcineurin A ($K_d \leq 10^{-13}$ M in the presence of EGTA), is required for enzyme activity, calmodulin activation, and interaction with the immunosuppressive drugs, FK506 and CsA. Ca^{2+} binding to calcineurin B is accompanied by a conformational change of the regulatory domain of calcineurin A resulting in the exposure of the calmodulin- and drug-binding domains. Binding of Ca^{2+}/calmodulin to calcineurin is accompanied by a displacement of an inhibitory domain, exposure of the catalytic center, and a more than 100-fold increase of the V_{max} of the enzyme. The highly cooperative, Ca^{2+}-dependent activation of calcineurin by calmodulin allows its rapid responses to narrow Ca^{2+} transients.

In crude extracts, calcineurin is also subject to a time- and temperature-dependent inactivation upon exposure to calmodulin and concentrations of Ca^{2+} found in stimulated cells (5×10^{-7} M). The enzyme is protected against inactivation by the presence of the autoinhibitory peptide and by superoxide dismutase but not by catalase. The inactivation is readily reversed by treatment with ascorbate. Purified calcineurin that contains 0.9 mol of iron and 0.8 mol of zinc per mol of enzyme with an electron paramagnetic resonance (EPR) spectrum, characteristic of an F^{3+}/Zn^{2+} enzyme, is inactive. Activation to the high specific activity of the crude enzyme requires treatment with ascorbate, under anaerobic conditions, and is accompanied by the disappearance of the characteristic Fe^{3+}/Zn^{2+} EPR signal at g=4.3, suggesting that the presence of one mol of Fe^{2+} at the active site is required for the protein phosphatase activity of calcineurin.

Thus, the displacement of the autoinhibitory domain induced by Ca^{2+}/calmodulin exposes Fe^{2+} in the catalytic center to oxidative damage by the superoxide anion. The involvement of the redox state of iron in the regulation of calcineurin activity provides a mechanism to desensitize the enzyme and to couple Ca^{2+}-dependent protein dephosphorylation to the redox state of the cell. The protection of calcineurin against

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inactivation by superoxide adds a new aspect to the physiological roles of superoxide dismutase, namely the modulation of Ca^{2+}-dependent regulation of cellular processes by protein phosphorylation.

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Ca^{2+}/calcineurin-dependent signal transduction in yeast

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In *Saccharomyces cerevisiae*, the Ca^{2+}/calmodulin regulated protein phosphatase, calcineurin, is activated in response to specific types of environmental stress such as increases in temperature, pH, or salinity. One role of calcineurin during stress is to regulate gene expression. Calcineurin dephosphorylates the transcription factor Crz1p/Tcn1p, causing Crz1p to translocate to the nucleus where it promotes gene transcription. The genes turned on under these conditions encode proteins that participate in many functions including cell wall synthesis, ion homeostasis, membrane trafficking and signal transduction.

Crz1p activation by calcineurin is both rapid and readily reversible, and a critical component of Crz1p negative regulation is its phosphorylation by protein kinases. Using a recently developed assay that employs protein chips[1], we tested 120 yeast kinases and identified several candidate kinases that phosphorylate Crz1p *in vitro*. Hrr25p, a casein kinase I homolog, phosphorylates and interacts with Crz1p both *in vivo* and *in vitro*. Hrr25p antagonizes both Crz1p-dependent transcription and its Ca^{2+}/calcineurin-dependent nuclear accumulation[2].

Genetic evidence indicates that calcineurin performs additional functions in yeast. We have recently identified a novel substrate of calcineurin, Hph1p. Together with the closely related protein, Hph2p, Hph1p promotes calcineurin-dependent growth under conditions of high pH. Hph1p functions independently of Crz1p and does not affect calcineurin/Crz1p-dependent transcription. Hph1p and Hph2p are integral membrane proteins associated with the ER, and Hph1 distribution within the ER is regulated in a Ca^{2+}/calcineurin-dependent manner. The localization of these proteins suggests that they affect trafficking of membrane/ cell wall components through the secretory pathway. Thus, in response to environmental stress, the calcineurin phosphatase may promote remodeling of the yeast cell surface through both transcriptional and post-transcriptional mechanisms.

These studies were supported by NIH grant GM-48728.

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Molecular determinants of fungal virulence as therapeutic targets

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The incidence of fungal infection is increasing and yet available antifungal drugs are limited, some are toxic, and drug resistant strains are emerging. We have elucidated a conserved signal transduction cascade that controls virulence of *Cryptococcus neoformans,* the leading cause of fungal meningitis. The central element of this virulence pathway is the calcium-calmodulin activated protein phosphatase calcineurin, which is the molecular target of the immunosuppressive antifungal drugs cyclosporin A and FK506. *C. neoformans* mutants lacking either the catalytic A or the regulatory B subunit of calcineurin are inviable at 37°C and other stress conditions and, as a consequence, are avirulent in animal models. In recent studies we identified: 1) the calcineurin B regulatory subunit and calmodulin, 2) the calcineurin binding protein (Cbp1) that is a conserved regulator or effector and the founding member of a protein family conserved from fungi to humans, and 3) the novel C2 domain protein Cts1 that functions coordinately with the calcineurin signaling pathway to promote cell wall biogenesis and growth at 37°C. In parallel we discovered that calcineurin is required for virulence of *Candida albicans*, the most common human fungal pathogen. *C. albicans cnb1/cnb1* mutants lacking the calcineurin B regulatory subunit are severely attenuated in animal models. Yet, in contrast to *C. neoformans* calcineurin is not required for growth of *C. albicans* at 37°C. Instead, calcineurin is necessary for *C. albicans* to survive and proliferate in serum. These studies illustrate how a conserved signaling cascade has been co-opted to control virulence of two divergent fungal pathogens by unique molecular mechanisms. Importantly, this pathway can be targeted for therapeutic intervention using non-immunosuppressive calcineurin inhibitors that retain antifungal activity and synergistic drug combinations.

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Stimulation and inhibition of calcineurin by RCNs

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RCNs are a conserved family of proteins in all fungi and animals that directly bind and regulate calcineurin. Recombinant RCNs inhibit the phosphopeptide phosphatase activity of calcineurin *in vitro* and overexpressed RCNs can inhibit calcineurin signaling in yeast and mammalian cells. In contrast to these early studies, gene knockout experiments in yeast and mouse indicated that RCNs can also stimulate calcineurin signaling. To investigate the mechanism by which RCNs stimulate calcineurin, we isolated mutations in other genes that mimmick the *rcn1* mutant phenotype of yeast. All the mutations mapped to the *MCK1* gene, which encodes a homolog of GSK-3 kinases conserved in all eukaryotes. The calcineurin-deficient phenotypes of *mck1* mutants and *rcn1* mutants were not worsened in *mck1 rcn1* double mutants, suggesting yeast Mck1p and Rcn1p function in the same pathway to stimulate calcineurin signaling. Purified Mck1p phosphorylated Rcn1p at a GSK-3 consensus site that is 100% conserved among all fungal and animal RCNs. As is typical for GSK-3 kinases, phosphorylation of Rcn1p by Mck1p required prior phosphorylation at a nearby site by a priming kinase. Mutation of the either the Mck1p phosphorylation site or the priming site of Rcn1p to non-phosphorylatable residues abolished the stimulation of calcineurin *in vivo* but stimulated its ability to inhibit calcineurin signaling when overexpressed *in vivo*. These findings suggest that Mck1p and an unknown priming kinase together phosphorylate Rcn1p and convert it from an inhibitor to a stimulator of calcineurin signaling. The mechanism of calcineurin stimulation by phospho-RCNs appears to be universal because hRCN1 of humans (also termed DSCR1 and MCIP1) undergoes a similar priming-dependent phosphorylation by GSK-3 *in vitro* and specifically loses its ability to stimulate yeast calcineurin *in vivo* when these sites are mutated to non-phosphorylatable residues. A similar mechanism has been described for regulation of type-1 protein phosphatase (PP1) by a protein known as Inhibitor-2 (Inh2) or Glc8p in yeast. Phosphorylation of Inh2/Glc8p by GSK-3 kinases converts it from an inhibitor to a stimulator of PP1 without dissociation of the complex. Thus, Inh2/Glc8p appears to function as an allosteric regulator of PP1 that is sensitive to conformational changes in response to phosphorylation and dephosphorylation. Although phospho-RCNs have not yet been shown to stimulate calcineurin phosphatase activity *in vitro*, computational modeling of RCN-calcineurin interactions based upon the Inh2-PP1 mechanisms indicated that low concentrations of RCNs may stimulate calcineurin signaling and high concentrations of calcineurin may inhibit calcineurin signaling *in vivo*. Calcineurin induces expression of Rcn1p and hRCN1, and thus RCNs may exist in both positive and negative feedback loops with calcineurin. This regulatory circuit may also help to explain the complex phenotypes of knockout mice that lack the ortholog of hRCN1. Positive feedback may explain the large overexpression of hRCN1/DSCR1 in brain of Down syndrome individuals who carry one extra copy of this gene due to Trisomy 21.

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Session 2: Signal transduction by Ca/ calcineurin and NFAT
Chair: Gerald R. Crabtree

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The calcineurin-NFAT signalling pathway in lymphocytes

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The NFAT family of transcription factors encompasses five proteins evolutionarily related to the Rel/NFκB family[1]. The primordial family member is NFAT5, the only NFAT-related protein represented in the Drosophila genome; it is identical to TonEBP, a transcription factor that regulates the response to osmotic stress[2]. The remaining four NFAT proteins (NFAT1-4, also known as NFATc1-c4) are regulated by the Ca^{2+}/ calmodulin-dependent serine phosphatase calcineurin[3]. NFAT proteins are phosphorylated and reside in the cytoplasm in resting cells; upon stimulation, they are dephosphorylated by calcineurin, translocate to the nucleus and become transcriptionally active, thus providing a direct link between intracellular Ca^{2+} signalling and gene expression[4]. NFAT activity is further modulated by additional inputs from diverse signalling pathways, which affect NFAT kinases and nuclear partner proteins. Recent structural data emphasise the remarkable versatility of NFAT binding to DNA[5-8]. The primordial family member, NFAT5/ TonEBP, binds as a dimer similar to NFκB[6]. At composite NFAT:AP-1 elements found in the regulatory regions of many target genes, NFAT proteins bind cooperatively with an unrelated transcription factor, AP-1 (Fos-Jun)[5]. At DNA elements which resemble NFκB sites, NFAT proteins bind DNA as dimers[7,8]. In cooperation with Fos-Jun and other classes of DNA-binding partners, NFAT activates transcription of a large number of genes during an effective immune response[9,10]. There is evidence that NFAT and NFAT-Fos-Jun complexes activate distinct subsets of target genes in lymphocytes[10]. Another novel aspect of gene regulation by NFAT is that this transcription factor participates in an early phase of chromatin remodelling that occurs at specific genetic loci in differentiating T cells[11].

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Insights on NF-AT signaling from endogenous calcineurin inhibitors

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The NF-ATs transcription factors are represented by four gene products that share nominally similar regulatory dynamics based on calcium-triggered nuclear import (Crabtree, 1999; Hogan et al., 2003). Each has a conserved, C-terminal Rel-like DNA domain and an N-terminal domain that is thought to control cytoplasmic-nuclear translocation in a calcineurin-dependent manner. This calcium-dependent translocation event is blocked by cyclosporin A, an immunosuppressant that suppresses cytokine transactivation by inhibiting calcineurin. The four NF-AT genes were likely to have different functions, a notion confirmed by mouse knockout models (Ho and Glimcher, 2003). Intriguingly, mice lacking NF-ATc2 showed enhanced lymphoproliferation, hyperactivation, and enhanced Th2 responses suggesting an enhanced sensitivity to activation rather than the expected decrease. Similarly, NF-ATc3-deficient mice appeared hyperresponsive to stimuli suggesting it too encoded an inhibitor or at least a modifier of the cytokine transcriptional response. The combined loss of NF-ATc2 and NF-ATc3 leads to massive lymphoproliferation and hyperactivation of the Th2 response, suggesting an unleashed immune response. To date only the loss of NF-ATc1, in an Rag2-null background, gives a T cell phenotype suggesting an activation deficit marked by reduced proliferation and decreased IL-4 production. NF-ATc4 has been even more enigmatic as it is not expressed in T cells and the null mouse is without a defined phenotype. Thus an overriding question is how are the NF-ATs coordinated to yield the cytokine transcriptional profiles observed during T cell activation? Our concern for this question came by the indirect route of seaching for additional calcineurin interacting proteins in the brain (Ryeom et al., 2003). We generated a two-hybrid library from mouse hippocampal cDNAs and screened it for calcineurin interactions. Two small, related cDNAs, DSCR1 and Zaki4 were isolated and found to be homologous with a third gene, DSCR1L. Further analysis showed that the expression of any of these cDNAs blocked calcium-activated NF-AT translocation by directly interacting with calcineurin, and we therefore termed them the calcipressins. Our subsequent generation of a mouse deficient in Csp1 (please see Ryeom poster) yielded a hypersensitive Th1 population that underwent apoptosis upon primary activation, a phenomenon we attributed to premature Fas ligand expression. Upon detailed analysis of cytokine gene expression, we noted that the loss of Csp1 was associated with a generalized shift in the activation thresholds of these genes, such that each was triggered at nominally lower T cell receptor stimulation. Additionally, each gene appeared to have a peak of expression at a unique level of TCR signaling, above which it was depressed. Thus in the simplest model where the NF-ATs were solely responsible for these activation profiles, complex interactions would be necessary. It is possible that inherent in the structure of the different NF-ATs is the molecular basis of signal-dependent activation, as we observe distinct profiles of calcium

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dependent activation for each of the NF-ATs. Our long term goal of this work is to determine how the molecular features of the NF-ATs and other transcription factors, together with calcium and calcineurin activity, translate into exquisitely controlled cytokine genetic programs, and how to intervene in this process to address human disease.

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Interaction of the transcription factor NFAT5 with calcineurin

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NFAT5 differs from NFATc proteins in that it lacks their characteristic N-terminal domain that contains the calcineurin docking site PxIxIT and clusters of regulatory phosphoserines. However, NFAT5 displays a DNA binding specificity highly similar to that of NFATc and is induced in activated T cells in a calcineurin-dependent manner, suggesting that it might participate in regulatory pathways that engage calcineurin and NFATc proteins. We have investigated whether an interaction exists between NFAT5 and the calcineurin-NFATc pathway. Our results show that calcineurin can bind to the DNA binding domain of NFAT5. This interaction is not compited by peptides comprising the PxIxIT calcineurin docking site of NFATc proteins. Conversely, the DNA binding domain of NFAT5 does not inhibit the binding and dephosphorylation of NFAT1 by calcineurin nor the enzyme's phosphatase activity. Expression of an NFAT5 construct lacking its C-terminal domain potentiates the calcineurin-mediated dephosphorylation of NFAT1 and its transcriptional activity. These results indicate that NFAT5, independently of its own transcriptional function, might cooperate with NFAT1 by facilitating its activation by calcineurin.

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Germline mutations that regulate calcium flux in B cells. Effects on B cell development and signalling

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Signal transduction by membrane immunoglobulin (Ig), the B-cell receptor (BCR), regulates the proliferation, differentiation, and survival of B-lymphocytes at several major developmental checkpoints. We have been interested in signalling in immature B cells that have newly formed functional membrane Ig on their surface. These immature B cells can either be negatively selected to die following encounter of self-antigen or positively selected to become mature B cells by unknown mechanisms which are BCR dependent. Signal transduction by the BCR is mediated by kinases that associate with the non-polymorphic subunits of membrane Ig (CD79).

Three classes of SH-2 domain containing tyrosine kinases propagate BCR signals. The tyrosine kinase Syk has been shown to play an important role, particularly in immature B cells that display an absolute requirement for this kinase in order to develop further. Gene targeting experiments have shown the requirement for Src family kinases Lyn, Fyn and Blk that individually play redundant roles in BCR signal initiation. Bruton's tyrosine kinase (Btk) is also crucial for proper B cell development, its mutation in humans leading to X-linked agammaglobulinemia and in the mouse to X-linked immunodeficiency (*xid*). These kinases are recruited to the BCR through their SH2-domains binding to the phosphorylated immunoreceptor tyrosine based activation motifs (ITAMs) of Ig associated CD79α and β as well as the adapter protein BLNK/SLP-65 (14). This latter protein is thought to serve as a molecular scaffold that recruits further downstream effectors of BCR signalling such as phosphatidylinositol 3-kinase (PI3-K), Btk, Vav proteins, and phospholipase C (PLC)-γ. Together these molecules have been proposed to form a macromolecular signalling machine termed the "signalosome". One feature of this model is the ability of BLNK/SLP-65 to bring Btk into proximity with PLCγ2, where it is thought Btk directly phosphorylates PLCγ2 on specific tyrosine residues thereby increasing its enzymatic activity towards its substrate phosphatidylinositol 4,5 bisphosphate (PIP2). The hydrolysis of PIP2 liberates two further signalling molecules, diacylglycerol (DAG) and inositol-3, 4, 5-trisphosphate (IP3). DAG can contribute to the regulation of protein localisation or activity through direct binding while IP3 binds to specific receptors on the endoplasmic reticulum and triggers the release of calcium into the cytoplasm

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Mice lacking the p110δ catalytic subunit of PI3K (Clayton et. al. 2002) have reduced numbers of B1 and marginal zone B cells, reduced levels of serum immunoglobulins, respond poorly to immunisation with Type II thymus-independent antigen and are defective in their primary and secondary responses to thymus-dependent (TD) antigen. p110$\delta^{-/-}$ B cells proliferate poorly in response to BCR signals *in vitro*, fail to activate protein kinase B and are prone to apoptosis. p110δ function is required for BCR-mediated calcium flux, activation of PLCγ2 and Bruton's tyrosine kinase.

B-lymphocytes from mice lacking the guanine nucleotide exchange factors Vav-1 and Vav-2 have defects in B cell receptor (BCR) mediated calcium mobilisation. These defects are more severe in B cells lacking all three Vav proteins. However there is still some Vav-independent ca flux. B cell responses initiated upon cross-linking of membrane immunoglobulin (mIg) by antigen are modulated by coreceptors such as CD19. Optimal activation of Ca^{2+} flux following CD19 coligation with mIg requires Vav and PI3K function and PI3K activation was Vav dependent. By contrast, when B cells lacking both Vav-1 and Vav-2 are activated by mIg crosslinking through IgM, PI3K is activated normally and the Ca^{2+} flux is partially PI3K and Vav independent. Our results show the p110δ catalytic subunit and the p85α regulatory subunit make an important contribution to the total PI3K activity elicted by IgM and by mIg/CD19 co-ligation. However there importance depends on the nature of the stimulus (antigen).

Mice lacking PLCγ2 have few mature B cells and those that develop express lower levels of the pro-survival protein Bcl-2. Introduction of a *bcl-2* transgene restored the numbers of marginal zone and follicular B cells in PLCγ2-mutant mice. BCR stimulated calcium flux was reduced in PLCγ2 mutant B cells and was completely blocked by a PLC inhibitor indicating a role for PLCγ1 in BCR signalling. PLCγ2 deficient B cells also showed a defect in the activation of ERK and phosphorylation of IκBα. Although the *bcl-2* transgene promoted development of PLCγ2-mutant B cells it could not restore BCR induced Ca^{2+} flux, ERK and IκBα phosphorylation or proliferation. These data suggest PLCγ2 performs a critical role in B cell development through regulation of survival rather than differentiation.

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Session 3: CN/ NFAT dependent transcription and function
Chair: Juan Miguel Redondo

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NFAT in physiological and pathological angiogenesis

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Angiogenesis, or capillary sprouting from pre-existing vasculature, occurs under physiological conditions during embryonic development and is required for wound repair and reproduction in the adult. It is controlled via balance between pro- and anti-angiogenic factors: the slightest disturbances in this balance trigger cell responses required for angiogenesis (1). Although indispensable for physiological processes, angiogenesis is highly regulated and persistence of angiogenesis in adult is tightly linked to human disease, including cancer, chronic inflammatory disease, and diabetic retinopathy (2, 3). Induction of the signaling pathways and molecular mediators involved in angiogenesis is common to all of these. Both cancer cells and cells attracted to the sites of inflammation are able to produce pro-angiogenic factors that cause endothelial cell recruitment and proliferation. Ample evidence shows that angiogenesis blockade often relieves the severity of the disease. It is then not surprising that angiogenesis has become the focus of intensive study aimed to develop highly desirable anti-angiogenic compounds for therapeutic intervention.

Cyclooxygenase (COX) enzymes, the targets of non-steroidal anti-inflammatory drugs (NSAIDs) that directly block their activity, are represented by COX-1 and COX-2. COX-2 is thought to contribute to angiogenesis mainly by inducing the production of prostanoids (prostaglandins and thromboxanes) that act by promoting expression of pro-angiogenic proteins. A number of studies demonstrate that COX-2 inhibition by NSAIDs leads to restricted angiogenesis and down-regulated production of pro-angiogenic factors such as VEGF and bFGF in a range of cell types. All these findings point to COX enzymes as important therapeutic targets in the treatment of pathological angiogenesis. We have determined that VEGF regulates NFAT in endothelial cells (5), and that Cyclosporin A (CsA), an immunosuppressive drug that inhibits the activity of transcription factors of the NFAT (nuclear factor of activated T cells) family, inhibits migration of primary endothelial cells and *in vitro* angiogenesis induced by vascular endothelial growth factor (VEGF); this effect appears to be mediated through the inhibition of COX-2, the transcription of which is activated by VEGF in primary endothelial cells. Consistent with this, we have shown that the induction of Cox-2 gene expression by VEGF requires NFAT activation. In addittion, the *in vivo* corneal angiogenesis induced by VEGF, but not by bFGF, was selectively inhibited in mice treated with CsA systemically (6). We have also determined that COX-2 inhibitors also blocked angiogenesis *in vivo* and this inhibition was similar to that displayed by CsA. Given the role of VEGF and its receptors in ischemic retinopathies we have analyzed the role of COX-2 and NFAT inhibitors in mice models of these diseases. Altogether, our findings point to a role for NFAT in angiogenesis, and may provide a novel mechanism underlying the beneficial effects of CsA in angiogenesis-related diseases.

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NFAT transcription factors in T cell apoptosis and cancerogenesis

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Several lines of evidence indicate that NFAT transcription factors control activation induced cell death (AICD) and cancerogenesis of lymphocytes. When peripheral T lymphocytes are stimulated by plate-bound αCD3 antibodies for 6 h or longer the majority of cells undergoes AICD, as shown by annexin V staining. Only 50% of cells become apoptotic when NFATc2-deficient T cells are stimulated (1), and less than 10% when T cells from mice doubly-deficient for NFATc2 and c3 are used. On the same hand, infection of primary murine T lymphocytes with retroviruses expressing NFATc2 or NFATc1/C, i.e. the long isoform of NFATc1 bearing a C-terminal domain of 250 amino acids, leads to an increase in number of apoptotic cells after αCD3Ab stimulation. The C-terminal domains in NFATc1/C and NFATc2 harbor a transactivation domain (designated TAD-B) which exerts both trans-acting and trans-repressing activities (2). TAD-B appears to be involved in AICD since NFATc1/A, the shortest isoform of NFATc1 which is strongly induced upon activation of peripheral T cells, lacks TAD-B and is unable to induce AICD in T cells (1). However, in BALM-14 Burkitt lymphoma cells, NFATc1/A can suppress AICD upon B cell receptor triggering while NFATc2 – as in T cells – facilitates also AICD in these B tumor cells (Ref. 3 and E. Kondo, personal comm.).

In several human lymphoid tumors, such as in Reed-Sternberg cells of Hodgkin lymphomas and in diffuse large B-cell lymphomas, expression of NFATc1 is suppressed. In Hodgkin lymphomas, NFATc1 repression is associated with hypermethylation of NFATc1 promoter DNA and histone H3 de-acetylation of NFATc1 promoter chromatin. Retroviral tagging experiments indicated the *NFATc1* (and *NFAT5*) genes as frequent targets of proviral insertions and, therefore, as cancer genes (2, 4). Infection of more than 500 mice deficient for NFATc2 and/or NFATc3 with the tumor virus SL3-3 resulted in a more rapid formation of T cell tumors in NFATc3-/- mice than wild type mice (Coll. with F. Pedersen, Aarhus) indicating NFATc3 as a tumor suppressor for T cell tumors generated by SL3-3 infection. These and further experimental results indicate an important role of NFAT factors in the generation of lymphoid tumors.

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Accumulation of NFAT mediates IL-2 expression in memory, but not naïve CD4$^+$ T cells

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During an immune response, naïve CD4$^+$ T cells are activated by specific antigens and differentiate into effector T helper (Th) cells. Effector T cells rapidly perform their function and contribute to the clearance of pathogens. Most effector CD4$^+$ T cells die after pathogen clearance, but a few cells survive and become memory CD4$^+$ T cells. Memory cells are key elements in the maintenance of long term immunological memory. They represent a unique population that shares certain phenotypic and functional characteristics with naïve cells, but also shares specific features of effector cells. How can a given antigen rapidly induce the expression of cytokine genes in memory CD4$^+$ T cells, but not naïve cells? We propose an additional mechanism for the enhanced cytokine gene expression in memory CD4$^+$ T cells. Prior to stimulation, memory cells accumulate high levels of the transcription factors required for the expression of the specific cytokine genes. We have shown that naïve CD4$^+$ T cells contain very low levels of NFAT transcription factors, while higher levels of NFATc1 and NFATc2 are present in memory CD4$^+$ T cells. In correlation, using reporter transgenic mice we demonstrated that NFAT-mediated transcription is more rapidly induced in memory CD4$^+$ T cells than in naïve cells. We have generated transgenic mice expressing a dominant negative mutant of NFAT specifically in T cells. Consistent with the slow activation of NFAT in naïve CD4$^+$ T cells, inhibition of NFAT does not affect IL-2 production in naïve CD4$^+$ T cells. In contrast, expression of IL-2 in memory CD4$^+$ T cells requires NFAT activation. Thus, accumulation of specific transcription factors such as NFAT in memory CD4$^+$ T cells contributes to their rapid cytokine gene expression in response to antigen stimulation. This is the first time that the regulation and function of NFAT in memory CD4$^+$ T cells has been addressed.

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Phosphorylation dependent regulation of NFAT-transcription

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It is well known that NFAT is located in the cytoplasm in an inactive form. After activation of calcium signals, calcineurin (Cn) is activated and dephosphorylates NFAT allowing its translocation to the nucleus where it activates gene transcription. However, a second level of regulation exists that modulates the intrinsic transactivating activity of NFAT, once it is translocated to the nucleus. Thus, we have found that two independent signal transduction pathways regulate the intrinsic transactivating activity. One is depending on Cn, whereas the other one represents is a new pathway that involves activation of Cot/Tpl-2 and protein kinase C (PK-C)ζ, two serine-threonine kinases. The region of NFAT tranactivation domain responsible for Cn inducing activity maps to N terminal aa 1-144. On the other hand, we have found that activation allows the association of Cot with PK-Cζ resulting in the activation of this kinase. This leads to PK-Cζ interaction and subsequent phosphorylation of NFAT in the transactivation domain (aa 1-57) and a strong increase in their transactivating activity. Other Ser/Thr kinases from the MAPK kinase family are unable to activate this pathway. Moreover, this Cot/PK-Cζ pathway cooperates with that induced by calcium/Cn. This pathway is involved in the activation of several genes important for T cell activation as IL-2 or cyclooxygenase 2.

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NFAT mediation of neurotrophin-induced synaptic plasticity

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Neurotrophins regulate neuronal survival and differentiation during development. Within the adult brain, neurotrophins are also important in neuronal adaptive processes, including activity-dependent plasticity (Huang and Reichardt, Annu Rev Neurosci (24) 2001; Poo, Nat Rev Neurosci (2) 2001). These long-term changes in neuronal excitability and function rely heavily upon alterations in gene expression (Deisseroth et al., Curr Opin Neurobiol (13) 2003). Yet, many of the mechanisms by which neurotrophins influence transcriptional and translational processes remain unknown. Of importance, the highest concentrations of the neurotrophin BDNF are found within the hippocampus (Ayer-LeLievre et al, Science (240) 1988), a brain region where neuronal plasticity underlies learning and memory. Recently, the transcription factor NFATc4 was found expressed within rodent hippocampal neurons (Graef et al., Nature (401) 1999). Interestingly, NFATc4 is particularly sensitive to the second messenger systems activated by BDNF. Thus, we hypothesized that NFAT-dependent transcription may be an important mediator of neurotrophin-induced plasticity.

In cultured rat CA3-CA1 hippocampal neurons, BDNF activated NFAT-dependent transcription via TrkB receptors. Inhibition of calcineurin (CaN) blocked BDNF-induced nuclear translocation of NFATc4, thus preventing transcription. Further, phospholipase C (PLC) was a critical signaling intermediate between BDNF activation of TrkB and the initiation of NFAT-dependent transcription. Both inositol 1,4,5-triphosphate (IP_3)-mediated release of calcium from intracellular stores and activation of protein kinase C (PKC), were required for BDNF-induced NFAT-dependent transcription. Finally, increased expression of IP_3R1 and BDNF following neuronal exposure to BDNF was linked to NFAT-dependent transcription. These results suggest that NFATc4 plays a crucial role in neurotrophin-mediated synaptic plasticity.

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Session 4: CN/ NFAT signalling in development
Chair: Anjana Rao

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Calcium signaling modules regulating chromatin remodeling and transcription

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The second messenger calcium plays a pivotal role in thymocyte apoptosis. Calcium signaling leads to the transcriptional activation of a family of pro-apoptotic genes including Nur77 [1,2]. We have been focusing on how calcium signal is transduced from the cytosol into the nucleus to activate transcription of the Nur77 gene. Earlier work by Winoto and colleagues revealed that the primary calcium-responsive elements on the Nur77 promoter correspond to the binding sites for myocyte enhancer factor (MEF)-2 [3]. Our work in this area began with the identification of an endogenous calcineurin inhibitor named Cabin1/cain [4,5]. As Cabin1 is a large protein and calcineurin-binding domain only occupies a small segment of Cabin1, it was reasoned that Cabin1 must have additional functions through interactions with proteins other than calcineurin. Using the yeast two-hybrid screen, MEF2 was identified as a Cabin1 interacting protein [6]. Subsequent investigation revealed that Cabin1 is a repressor of the transactivation activity of MEF2, silencing the Nur77 promoter in the absence of calcium signaling. The question then became what is the mechanism by which Cabin1 represses MEF2 activity. It was found that Cabin1 represses MEF2 by recruiting histone deacetylases [7]. In order for MEF2 to be activated during calcium signaling, the Cabin1-HDAC complex has to be removed from MEF2. It was found that calmodulin is capable of binding to Cabin1, removing Cabin1 and its associated HDACs from MEF2, providing an unprecedented mechanism of calcium signaling directly impinging on the association of a chromatin remodeling complex with MEF2 [6]. This mechanism of calcium-dependent derepression appears to be true for other MEF2 transcriptional repressors including HDAC4, 5, 7 and 9 [8]. In addition to the MEF2-Cabin1-HDAC signaling module, the calcieurin-NFAT signaling module was also shown to be required for TCR-mediated Nur77 expression, as it is sensitive to inhibition by CsA and FK506. The calcineurin-NFAT signaling module was found to be integrated with MEF2-Cabin1-HDAC signaling module through the interaction between NFAT and MEF2, which synergistically recruit the transcriptional coactivator p300, leading to full activation of Nur77 and thymocyte apoptosis [9].

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"Steering" axons to their targets with NFAT signaling

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Studies of axonal guidance have indicated the rate of advance of the growing axonal tip is a fundamental characteristic of a neuron established at the time of neural determination. However we find that mice lacking NFATc2/c3 and c4 as well as calcineurin B1are unable to respond to axonal guidance cues with an increase in the rate of axonal outgrowth (Greaf et al Cell 2003). As a result critical morphologic features of the vertebrate nervous system such as midline crossing of commissural neurons and axonal splitting at the dorsal funiculus do not occur. We have investigated the mechanism underlying the role of NFAT signaling in controlling the rate of advance of the axonal tip by defining the target genes of NFATc, calcineurin and cyclosporin using transcript arrays. Our studies of both the phenotypes (Graef et al Cell 2001 and Graef et al Cell 2003) and the genes whose expression is modified in mice lacking calcineurin, NFATc2/c3/c4 or treated with cyclosporin indicate that CsA is specific for calcineurin and in turn calcineurin is highly specific for NFATc. We find that NFAT transcription complexes regulate the activation of genes essential for control of the cytoskeleton at the axonal tip. These genes include three categories; those regulating microtubule nucleation in the growth cone, those regulating actin nucleation and dynamics at the tips of the growing axons and those that control the processing of membrane necessary for advance of the growth cone. Our studies suggest a new concept in axonal guidance: that guidance factors, in addition to stimulating the tips of growth cones to induce turning, must also control the rate of advance of the growth cone. By controlling the rate of advance of the axonal tip, NFAT signaling allows the growth cone to meet developmental "windows of opportunity" where it must split, send off collaterals or engage in other morphogenic events. We speculate that this means of control emerged in vertebrates in response to the larger body size and longer paths traversed by axons in vertebrates.

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Requirement of transcription factor NFAT in developing myocardium

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Nuclear factor of activated T cell (NFAT) is a ubiquitous regulator involved in multiple biological processes. We have demonstrated that NFAT is temporally required in the developing atrial myocardium between embryonic day E14 and P0 (birth) [J. Cell Biol. 161, p861]. Inhibition of NFAT activity by conditional expression of dominant-negative NFAT causes thinning of the atrial myocardium. The thin myocardium exhibits severe sarcomere disorganization and reduced expression of cardiac troponin-I and cardiac troponin-T. Promoter analysis indicates that NFAT binds to and regulates transcription of the cardiac troponin-I and the cardiac troponin-T genes. Thus, regulation of cytoskeletal proteins gene expression by NFAT may be important for the structural architecture of the developing atrial myocardium. Recent data indicating that inhibition of NFAT leads to additional heart defects will also be presented.

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Calcineurin-NFAT signaling in the development and function of the nervous system

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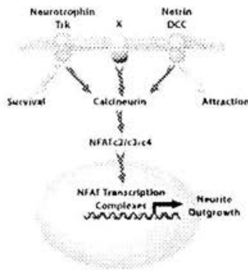
Signaling through calcineurin and NFAT plays critical roles in the function of cortical and hippocampal neurons where synaptic activity results in Ca^{2+} influx through L-type channels and NMDA receptors leading to rapid dephosphorylation and nuclear import of the cytoplasmic subunits (NFATc family) of NFAT transcription complexes (Graef et al Nature 1999;401:703). This role is likely to be critical to short term memory (Cell. 2001; 104: 675, Cell. 2001;107:617) and may play a role in human schizophrenia (PNAS 100, 8993, 2003). To investigate the role of NFAT signaling in neural development we have prepared mice lacking NFATc2, c3 and c4 as well as mice with conditional inactivation of the CnB1 regulatory subunit in the nervous system. We find the calcineurin NFAT signaling is critical for extension of axons in response to many guidance factors including neurotrophins and netrins (Graef et al Cell 113:657 2003). Remarkably, NFAT signaling does not appear to be necessary for the survival functions of neurotrophins indicating a specific role of this pathway in transducing signals from guidance factors. In addition, calcineurin-NFAT signaling was not required for the expression of a variety of differentiation markers in the developing CNS. Studies using the highly specific calcineurin inhibitors FK506 and Cyclosporin A indicate that calcineurin-NFAT signaling controls the rate of axonal extension. Our studies indicate that guidance factors control not only the turning of an axon growth cone, but also use Cn/NFAT to control the rate of extension. This notion of "axonal steering" contrast with the prevailing notion that the rate of axonal extension is an intrinsic characteristic of a neuron.



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Elucidating calcineurin functions during *Drosophila* development

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Calcineurin subunit genes are conserved among animal species, with the *Drosophila* genome containing three *cna* and two *cnb* genes that are highly homologous to their vertebrate relatives (1). Given these strong sequence identities, it made sense to investigate the possible conservation of the calcineurin/NFAT pathway in *Drosophila* and determine what functions this signaling cassette might assume during fly development and life processes. NFAT proteins contain a DNA-binding domain of the rel class and comparative evolutionary studies, based on the sequencing of numerous animal genomes, has led to the categorization of Rel family proteins (2). Surprisingly, while the fly genome encodes proteins like Dorsal, Dif, and Relish that are related to the vertebrate transforming protein Rel and NFκB family, calcium-responsive Rel proteins such as the NFAT group are present only in vertebrates. That is, while certain *Drosophila* genes may encode the conserved DNA-binding domain, they do not possess sequences encoding a calcium/calcineurin-responsive domain that defines the calcium-dependent NFAT proteins. Thus given the strong conservation of calcineurin genes, and the absence of NFATc proteins from the fly, challenging questions arise as to the developmental functions and effectors of calcineurin signaling in *Drosophila*.

We are investigating the requirements for calcineurin function in two developmental contexts in *Drosophila*, blood cell production during embryogenesis and indirect flight muscle (IFM) formation in the adult. Relevant to the former, hematopoiesis is a complex developmental process that involves stem cell generation, followed by the commitment of multipotent progenitors and the differentiation of mature blood cells within distinct lineages. Seminal to these events is the coordinated regulation of hematopoietic transcription factors that function combinatorially to direct lineage-specific gene expression. In vertebrates, many of these factors have been identified based on their role as trans-regulators of blood cell expressed genes or their characterization as loci that are chromosomally rearranged in specific leukemias. A hematopoietic system exists in *Drosophila* and recent studies have demonstrated that genes essential for blood cell development in the fly represent functional homologues of certain hematopoietic factors found in higher eukaryotes. Concerning transcriptional regulators, the utilization of fly genetics has allowed for the functional analysis of the GATA factor Serpent, Friend of GATA factor U-shaped, and Runx-related protein Lozenge in blood cell specification and differentiation (3-6). We are characterizing the specialized roles of Serpent, U-shaped, and Lozenge in the distinct cellular events of embryonic and larval hematopoiesis, and conducting a genome-wide screen for genes that function in crystal cell specification and differentiation. Our screen has uncovered three interesting phenotypes thus far: vast overproduction of blood cells, strong diminution of hemocyte populations, and abnormally located blood cells. Mutations in calcineurin pathway genes result in the latter phenotype and we plan to elucidate the specific functions of these genes in crystal cell formation and behavior.

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We are also deciphering calcineurin's function in adult IFM formation. An analysis of strong mutations in the *canB2* subunit gene showed the gene is essential for normal *Drosophila* development, with mutants dying at a late larval/early pupal stage (1). However, genetic combinations involving weaker alleles result in adult escapers that are flightless with wings positioned at abnormal angles (7). This phenotype was suggestive of irregularities among IFM groups located in the thorax. The dorsal longitudinal muscle (DLM) subgroup develops from three pairs of larval oblique muscles that fail to histolyze during metamorphosis within the pupal period. These persistent muscles split into six pairs that serve as a framework for DLM formation, with muscle growth occurring due to the ordered fusion of Twist-expressing adult myoblasts onto the templates. When formed, the six central DLM pairs and seven flanking pairs of dorsal ventral indirect flight muscles (DVM) fill the adult thorax. The analysis of IFM structure in *canB2* mutant pupae and adults revealed two reproducible phenotypes (7). In animals that were able to eclose as adults, abnormalities were observed in the DLM pattern with most muscle pairs absent from anterior thoracic sections and disorganized muscle masses present in posterior sections. It was concluded this phenotype was not a result of the lack of larval muscle templates or initial muscle formation, but due to the displacement of most DLM to the posterior of the thorax. Presumably, sufficient IFM integrity exists in these animals so as to facilitate movement out of the pupal case. A more severe phenotype was observed when IFM formation was followed in living pupae using a sensitive *MHC-GFP* transgene muscle marker. In animals that progressed only to the pharate adult stage, a complete retraction of all IFM to a posterior thoracic position was observed. It is likely such animals are unable to eclose due to thoracic compression and/or insufficient IFM contraction, resulting in lethality. IFM retraction in *canB2* animals is reminiscent of the hypercontracted phenotype found in certain *myosin heavy chain* and *troponin I* gene mutants (8, 9). It is possible that calcineurin activity is required for the modification of a myogenic transcription factor, whose function is needed for the regulation of specific IFM contractile protein genes. Focused genetic screens are ongoing to identify the transcriptional regulator that serves as the target of calcineurin phosphatase activity and the muscle structural genes controlled by such a factor. Consistent with the muscle phenotypes observed in mutant pupae and adults, *canB2* and the *cna* gene *Pp2B-14D* are expressed in forming IFM, as well as in the central nervous system (7). In contrast, calcineurin subunit genes are not expressed in developing embryonic muscles, indicating a clear specificity in calcineurin's myogenic function during *Drosophila* development.

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Session 5: The calcineurin/ NFAT pathway in cardiac growth and muscle function
Chair: Eric N. Olson

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Control of cardiac growth and remodeling by calcium-dependent transcription

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The adult heart responds to a stress signals by hypertrophic growth, which is accompanied by reprogramming of cardiomyocyte gene expression and activation of fetal cardiac genes. We have shown that calcineurin and CaM kinase transduce hypertrophic stimuli to the MEF2 transcription factor, which regulates stress-responsive and fetal cardiac genes. The activity of MEF2 is governed by class II histone deacetylases (HDACs), which act as transcriptional repressors. Signaling by calcineurin, CaMK, and other stress-responsive kinases leads to phosphorylation of two conserved serines in class II HDACs. Phosphorylation of these sites triggers the dissociation of HDACs from MEF2 and their export from the nucleus to the cytoplasm, with resulting activation of MEF2-dependent genes. Signal-resistant HDAC mutants lacking these sites are refractory to hypertrophic signaling and act as dominant suppressors of cardiomyocyte hypertrophy. Conversely, mutant mice lacking HDACs 5 or 9 are supersensitive to hypertrophic signals and develop cardiomegaly in response to stress. These findings identify class II HDACs as nuclear integrators of hypertrophic signaling cascades and signal-responsive suppressors of cardiac hypertrophy.

The actions of calcineurin on cardiac muscle cells are dependent on an array of effector proteins that influence its enzymatic activity, subcellular distribution, and stability. Additional proteins transmit calcineurin-dependent signals to the nucleus with consequent changes in gene transcription. While many of its effectors are ubiquitous, others are restricted to cardiac (and skeletal) muscle, providing muscle-specificity to calcineurin signaling. The diversity of calcineurin effectors provides entry points into the signaling pathways that govern cardiac growth and function and provides opportunities for pharmacological and genetic modification of these processes. Strategies for modulating the activity of calcineurin and its effectors in the setting of heart disease will be discussed.

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Role of calcineurin isoforms in muscle regeneration

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The remarkable potential of certain mammalian tissues to rebuild themselves has been linked to mounting evidence for populations of adult mammalian progenitor cells, perhaps set aside during gestation. These regenerator cells can contribute to multiple tissue types under appropriate experimental conditions and in response to damage. Lack of a sufficiently robust regenerator cell population may explain why some organs do not regenerate as well as others, and may underlie the decreased regenerative capacity of aging tissues. Studies on regeneration in lower vertebrates provide an alternate mechanism whereby the rebuilding of damaged tissue could couple acute response to injury with activation of cell dedifferentiation and plasticity in surrounding tissues, providing a local source of regenerator cell pools.

We previously reported that regeneration was enhanced in transgenic mouse muscle expressing a local isoform of Insulin-like Growth Factor 1 (mIGF-1), which induces hypertrophy and maintains tissue integrity during aging [1,2,] enhances healing following injury [2] or exercise [3], and counters muscle decline in degenerative disease [4]. In more recent studies we have documented an increase in regenerator cell markers in injured mIGF-1 muscles. Elevated recruitment of proliferating bone marrow cells to sites of muscle regeneration in mIGF-1 transgenic mice was accompanied by increased bone marrow stem cell production, revealing an unexpected humoral response to distal trauma. Damaged mIGF-1 transgenic muscles also activated novel genes implicated in urodele amphibian regeneration, suggesting that enhanced regenerative capacity of these animals may involve local responses as well.

To further explore the molecular basis of muscle regeneration, we examined the role of calcineurin-mediated signalling, which in skeletal muscle cultures is activated by mIGF-1 in association with NFATc1 and GATA-2 [5]. The calcineurin family includes multiple isoforms of the CnA and CnB subunits. Differential expression patterns of calcineurin A and B isoforms in muscle during aging, neurodegenerative disease, and injury included a dramatic isoform switch in CnAbeta isoforms during the regeneration process, enhanced by mIGF-1. Over-expression of native CnA genes in skeletal muscle cultures revealed surprising differences in isoform activity. Preliminary analysis of mice over-expressing a muscle-specific, full-length CnAalpha transgene revealed novel physiological effects distinct from those of the truncated, active variant of CnAalpha, in resting, exercising or regenerating muscles. These observations implicate diverse calcineurin signalling pathways in normal and

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pathological skeletal muscle functions, and reveal potential roles for specific calcineurin isoforms in both distal and local regenerative responses.

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Calcineurin/NFAT signaling sustains long-term pathologic, but not physiologic cardiac hypertrophy

Benjamin J. Wilkins, Yan-Shan Dai, Orlando F. Bueno, Stephanie A. Parsons, David M. Plank, Fred Jones, Thomas R. Kimball, and Jeffery D. Molkentin

Calcineurin (PP2B) is a calcium-calmodulin activated, serine-threonine phosphatase that transmits signals to the nucleus through the dephosphorylation and translocation of nuclear factor of activated T cell (NFAT) transcription factors. While calcineurin-NFAT signaling has been implicated in regulating the hypertrophic growth of the myocardium, considerable controversy persists as to its role in maintaining versus initiating hypertrophy, its role in pathologic versus physiologic hypertrophy, and its role in heart failure. To address these issues we report the generation and characterization of NFAT-luciferase reporter transgenic mice. These mice show robust and calcineurin-specific activation in the heart that is inhibited with cyclosporine A. In the adult heart, NFAT-luciferase activity is upregulated in a delayed, but sustained manner throughout eight weeks of pathologic cardiac hypertrophy induced by pressure-overload, or more dramatically following myocardial infarction-induced heart failure. In contrast, physiologic hypertrophy as produced in two separate models of exercise training failed to show calcineurin-NFAT signaling in the heart at multiple time points, despite measurable increases in heart-to-body-weight ratios. Moreover, stimulation of hypertrophy with growth hormone-insulin-like growth factor-1 (GH-IGF-1) failed to activate calcineurin-NFAT signaling in the heart or in a culture-based model, despite hypertrophy, activation of Akt, and activation of p70 S6K. Lastly, exercise- or GH-IGF-1-induced cardiac growth failed to show induction of hypertrophic marker gene expression compared with pressure-overloaded animals. These results suggest that separable signaling pathways regulate pathological versus physiological hypertrophic growth of the myocardium, with calcineurin-NFAT functioning as a sustained mediator of maladaptive hypertrophy and heart failure.

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NFAT is a nerve activity sensor and controls the fast/slow phenotype in skeletal muscle

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We are interested in identifying the signal transduction pathways that mediate the effect of nerve activity on the muscle phenotype. Using pharmacological approaches and *in vivo* transfection (by intramuscular plasmid injection) with DNAs coding for constitutively active or inhibitory factors, we have previously shown that calcineurin controls nerve activity-dependent fiber type specification but not muscle growth (Serrano et al., 2001). The transcription factor NFAT is a well-known target of calcineurin in different cell systems. However, the role of NFAT as a calcineurin downstream effector in skeletal muscle is controversial. We have used multiple approaches to determine the role of NFAT in skeletal muscle *in vivo*.

1. First we used a constitutively active form of NFAT and showed that it has a constitutive nuclear localization and activates a co-transfected NFAT-dependent reporter following transfection *in vivo*. Constitutively active NFAT was found to induce the expression of slow myosin heavy chain (MyHC) in denervated regenerating rat soleus muscle (that normally shows a default fast phenotype), thus reproducing the effect of slow motor neuron activity.
2. To block selectively the activation of NFAT by calcineurin, we used the NFAT peptide inhibitor VIVIT linked to GFP. VIVIT-GFP blocks the expression of MyHC-slow but not the increase in fiber size in regenerating innervated soleus muscle. In the adult soleus muscle, VIVIT-GFP inhibits the expression of MyHC-slow transcripts and the activity of a MyHC-slow promoter.
3. Two NFAT-dependent reporters, derived from the DSCR1/MCIP1 and IL-4 promoter, were used to monitor NFAT transcriptional activity *in vivo*. NFAT activity is higher in muscles expressing a slow phenotype compared to muscles with a fast phenotype and is blocked by VIVIT-GFP and by the calcineurin inhibitor cain/cabin1. Furthermore, NFAT activity is decreased by denervation in adult slow muscles and is increased by electrostimulation of denervated muscles with a low frequency impulse pattern, mimicking the firing pattern of slow motor neurons, but not with a high frequency pattern typical of fast motor neurons.
4. Transfection *in vivo* with NFATc1-GFP was used to follow the intracellular localization of NFAT in response to electrostimulation with specific activity patterns. In resting muscles, as well as muscles stimulated with high frequency trains of impulses (fast-like pattern), NFATc1-GFP has a cytoplasmic localization. In contrast, stimulation with low frequency trains of impulses (slow-like pattern) rapidly induces nuclear translocation of NFATc1-GFP.

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These results demonstrate that NFAT is a nerve activity sensor in skeletal muscle *in vivo* and controls electrical activity-dependent fibre type specification.

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Cell fusion in skeletal muscle: Central role of NFATC2 in regulating muscle cell size

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Skeletal muscle formation and growth require the fusion of myoblasts to form multinucleated myofibers or myotubes. Studies of the calcium activated transcription factor NFATC2 demonstrate that cell fusion during myogenesis occurs in two distinctly regulated phases. NFATC2 controls myoblast fusion after the initial formation of a myotube and is necessary for further cell growth. Recently we have shown that following myotube formation, myotubes recruit myoblast fusion by secretion of IL-4 and prostaglandin F2a. Molecules that control muscle cell fusion are of great interest from a therapeutic standpoint to enhance growth of muscle after injury or to alleviate the loss of muscle mass found in disease or aging.

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POSTERS

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Characterization of the calcium/calcineurin-mediated response in yeast under alkaline pH stress

Raquel Serrano, Laia Viladevall, Amparo Ruiz, Anna Barceló and Joaquin Ariño

Exposure of the yeast *Saccharomyces cerevisiae* to an alkaline environment represents a situation of stress. In response to such situation, these cells modulate their transcriptional program, which result in induction or repression of a number of genes (1,2). We have observed that, in some cases, the transcriptional response derived from exposure to alkali depends, in full or in part, of the integrity of the calcineurin pathway (2). This suggest that exposure to alkali could trigger a burst of intracellular calcium as primary response. Our current efforts are focused in the characterization of the nature of this calcium burst. In addition, by using DNA microarray technology we seek to evaluate at the genome level the extent of the calcium-mediated transcriptional response to alkaline stress.

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Phosphorylation at the FLISPP motif of calcipressin 1 affects calcineurin inhibition and calcipressin 1 stabilization

Lali GENESCÀ, Anna AUBAREDA, Juan J. FUENTES, Xavier ESTIVILL, Susana DE LA LUNA, and Mercè PÉREZ-RIBA

Calcipressin 1 is a member of the calcipressin family, which are endogenous inhibitors of calcineurin. Calcipressin 1 is encoded by the human chromosome 21 DSCR1 gene. It consists of seven exons, exons 1 to 4 being alternative first exons in isoforms 1 to 4. We demonstrate that the N-terminal region of the isoform 1 is longer than the one previously described generating a new polypeptide of 252 amino acids. This polypeptide can interact in vivo with calcineurin A and B and inhibits NF-AT-mediated transcriptional activation. Calcipressin 1 is a phosphoprotein and phosphorylation takes place at the two serine residues of the FLISPP motif, which is the signature of the calcipressin family, and at least at one site outside this motif, visible upon two-dimensional gel electrophoresis analysis, also indicating that the site outside the motif is independent of the FLISPP phosphorylation state. The functional implications of the phosphorylation at the FLISPP motif are an increase in the capacity to inhibit calcineurin and proteasome-dependent degradation of calcipressin 1. We suggest that phosphorylation of calcipressin 1 is involved in the regulation of the phosphatase activity of calcineurin and therefore has the capacity to modulate calcineurin dependent cellular pathways.

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Calsarcin-1, a sarcomeric protein that modulates calcineurin activity

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Calsarcins represent a novel protein family that are localized to the sarcomere and interact with several other Z-disc-proteins including alpha-actinin, gamma-filamin, telethonin and ZASP/Cypher/oracle. Interestingly, calsarcins also interact with the calcium-/calmodulin dependent phosphatase calcineurin, suggesting that they may serve as a site of integration for sarcomeric and signaling proteins. Previously, calcineurin has been implicated in the development of cardiac hypertrophy as well as the control of the fiber type specification in skeletal muscle.

In the adult, calsarcin-1 is specifically expressed in cardiac and slow-twitch skeletal muscle, while calsarcin-2 and 3 are only expressed in fast-twitch skeletal muscle. *In vitro*, calsarcin-1 acts as a modulator of calcineurin activity by slightly activating calcineurin at low concentrations, while inhibiting calcineurin activity at higher concentrations. In order to study the function of calsarcins *in vivo*, we generated mice with targeted ablation of the calsarcin-1 gene. Calsarcin-1-deficient mice are viable and display no overt defects in cardiac or skeletal muscle. Careful analysis, however, revealed that skeletal muscle of calsarcin-1 null mice shows an increase in the number of slow-twitch muscle fibers. Moreover, cardiomyocytes of calsarcin-1-/- hearts are smaller than those from wild type hearts. Calsarcin-1-/- hearts also exhibit an increased expression of hypertrophic markers, such as Atrial Natriuretic Factor, beta-Myosin Heavy Chain and Brain Natriuretic Peptide. Electron microscopy of the hearts of calsarcin-1-/- mice shows wider and "fuzzier" Z bands compared to wild type hearts. We performed thoracic aortic banding on the calsarcin-1-/- mice to subject them to increased biomechanical stress. Interestingly, we observed an exaggerated hypertrophic response in calsarcin-1-/- mice compared to wild type mice. Similarly, when calsarcin-1-/- mice were crossed with transgenic mice that expressed activated calcineurin in the heart (Cna-tg), those animals displayed massive hypertrophy and died before four weeks of age. Morphometric analysis revealed an increase in cardiomyocyte size in the calsarcin-1-/-/Cna-tg transgenic mice compared to calcineurin transgenic mice alone.

Taken together, our data show that the absence of calsarcin-1 sensitizes the heart to pathological signals leading to hypertrophy. These findings suggest that calsarcin-1 negatively regulates cardiac hypertrophy *in vivo* and that this effect might be mediated by modulation of calcineurin activity.

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Normal B-1a cell development requires B cell-intrinsic NFAT2 activity

Robert Berland and Henry H. Wortis

B-1a cells, an anatomically, phenotypically, and functionally distinct subset of B cells that produce the bulk of natural serum IgM and much of gut-associated IgA, are an important component of the early response to pathogens (reviewed in 1). Because the induced expression of CD5, a hallmark of B-1a cells, requires an NFAT-dependent enhancer (2), we examined the role of NFAT transcription factors in B-1a development.

Here we show that the B-1a compartment is normal in mice lacking NFAT1 but essentially absent in mice lacking NFAT2. Loss of NFAT2 affects both peritoneal and splenic B-1a cells. Since there is a loss of B-1 cells defined by markers other than CD5, NFAT2 is not required simply for CD5 expression on B-1a cells. Using mixed allotype chimeras and retroviral-mediated gene transduction we show that the requirement for NFAT2 is B cell intrinsic. We also demonstrate that NFAT2 protein expression is elevated about five fold in B-1a cells compared to B-2 cells. This is the first definitive demonstration of a B cell intrinsic function for an NFAT family transcription factor.

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The role of *Candida albicans* calcineurin for virulence and survival in serum

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The protein phosphatase calcineurin, which controls the nuclear import of the transcription factor NF-AT (1, 6) in humans and Crz1 (7) in the fungus *Saccharomyces cerevisiae*, is important for stress response (temperature, pH, and cations) in fungi (3-5, 8). Calcineurin is essential for the virulence of the human fungal pathogen *Cryptococcus neoformans* because it is required for growth at temperatures higher than 37° C (4, 5). Calcineurin also enables the human fungal pathogen *Candida albicans* to survive in the host environment. *C. albicans* is the most common cause of invasive fungal infection in humans, affecting both immunocompetent and immunocompromised patients. Interestingly, calcineurin is not important for survival during temperature stress in this fungus but is essential for survival in serum(2). This survival defect of the calcineurin mutant strains in the bloodstream prevents the establishment of a lethal systemic infection in the murine tail-vein injection model of candidiasis. Biochemical and genetic studies are in progress to establish calcineurin's role in protecting *C. albicans* in serum. Biochemical analysis of serum has revealed a small molecular weight (<3 kD) and proteinase K insensitive component is toxic to calcineurin mutant strains. Genetic studies have revealed spontaneous mutants of calcineurin mutant strains containing suppressors that partially restore survival in serum, and genomic analysis has revealed potential targets for calcineurin activity under serum stress. This study has the potential to reveal novel aspects of innate immunity as well as gain a greater understanding of calcineurin signalling pathways in fungi.

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Inhibition of NFAT dependent transcription. A new pharmacological effect of salicylates

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The anti-inflammatory effects of salicylates, originally attributed to inhibition of cyclooxygenase activity, are now associated to other mechanisms. In this study we have investigated the possible involvement of NFAT-mediated transcription in those drugs effects. Transactivation experiments performed with different reporters showed that trifluoromethylated salicylate derivatives strongly inhibited NFAT-dependent transcription at therapeutically relevant concentrations, whereas sodium salicylate and aspirin required concentrations as high as 2-5 mM. RNase protection assays (RPA) showed that salicylate derivatives inhibited mRNA expression of several NFAT-containing and cyclosporin A (CsA)-sensitive genes in a dose-dependent manner. In Jurkat cells, inhibition of IL-3 and GM-CSF and partial inhibition of TNF-a, TGF-b1, IL-2, lymphotactin (Ltn), macrophage inflammatory protein (MIP)-1a, MIP-1b and IL-8 were observed. In monocytic cell lines, partial inhibition of M-CSF, G-CSF, stem cell factor, IFN-g, TGF-b1, lymphotoxin-b1, MIP-1a, MIP-1b, and IL-8 was observed. Sodium salicylate and aspirin only showed significant effect above therapeutical concentrations. When a T-cell specific enhancer in the IL-3 locus, which contains NFAT/Oct sites, and a GM-CSF full promoter were used, inhibition of transcriptional activity by salicylate and derivatives was also observed. In summary, these data provide the first evidence of inhibition of NFAT-dependent transcription as a new pharmacological property of salicylates.

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Regulation of NFATs by multiple phosphorylation sites

Thomas Höfer, Carlos Salazar

NFAT transcription factors are activated through dephosphorylation by the phosphatase calcineurin. Experimental data show that thirteen conserved phosphorylation sites conspire to control the transition between an active and an inactive conformation. We propose a quantitative model of the underlying molecular mechanisms that may generally apply to multiply phosphorylated proteins. Mathematical analysis shows that multiple phosphorylation sites can create a threshold for protein activation. Properties that favor such a threshold are a multi-hit mechanism of phosphate processing and cooperativity between the individual sites, and the modeling suggests experimental approaches to probe for these features. The sharpness of the threshold increases with the number of phosphorylation sites, thus providing a rationale for the involvement of the large number of serines in NFAT activation. An extension of the model including the nuclear import and export processes reproduces experimental data on the NFAT activation kinetics by different types of calcium signals. Studying the impact of NFAT kinases on activation, we find that the effect of nuclear kinases is generally larger than that of cytoplasmic kinases.

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Selective inhibition of calcineurin-NFAT signalling by small nonpeptide inhibitors

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The calcineurin-NFAT pathway is a critical link between the T cell receptor and cytokine gene expression, and participates in the genetic programs of muscle fiber-type specialization, osteoclast differentiation, and myocardial hypertrophy. This pathway is of major clinical interest, since the effectiveness of the immunosuppressive compounds cyclosporin A (CsA) and FK506 is correlated with their inhibition of calcineurin enzymatic activity. The toxicity of these calcineurin enzyme inhibitors is due in part to the fact that calcineurin controls many downstream effectors including transcription factors, enzymes, ion transporters, transmembrane ion channels, and proteins involved in apoptosis. Inhibition of calcineurin-NFAT signalling would be an attractive method of treating asthma, inflammatory and autoimmune diseases, and conceivably pathological cardiac hypertrophy, if the serious side effects of CsA and FK506 could be minimized.

The existence of diverse mechanisms for targeting calcineurin to its cellular substrates raises the possibility of designing substrate-selective inhibitors of calcineurin that do not block enzyme activity *per se*, but rather interfere with recognition of one or more of its substrates. We previously showed that the efficiency and specificity of calcineurin-NFAT signalling depends on docking of NFAT-family proteins to a recognition site in calcineurin, and that calcineurin-NFAT signalling in cells can be selectively inhibited by intracellular delivery of a peptide that competes at the recognition site. We have extended this approach by screening a library of small organic molecules to identify compounds that inhibit calcineurin-NFAT docking. We selected a few compounds for more detailed characterization, and showed that they block NFAT dephosphorylation *in vitro*, and prevent activation of NFAT and induction of NFAT-dependent cytokine mRNAs in T cells. These findings highlight the promising new strategy of blocking intracellular signalling pathways by interfering with specific protein-protein interactions.

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Regulation of cyclooxygenase-2 expression in human T lymphocytes by glucocorticoids: An essential role of the nuclear factor of activated T cells

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Cyclooxygenase (COX) is the enzyme responsible for the conversion of arachidonic acid to prostaglandin (PG)-H2, the main step in the prostanoids synthesis pathway. Two isoforms of this enzyme, named COX-1 and COX-2, have been shown to be expressed in mammalian tissues. COX-2 isoform is induced by several stimuli including cytokines and mitogens and its induction is inhibited by glucocorticoids. It is thought to be the responsible for the increased production of PGs in pathologic processes (reviewed in 1). We have previously reported that transcriptional induction of COX-2 occurs early after T cell receptor triggering, suggesting functional implications of COX-2 activity in this process (2). Here, we show that glucocorticoids block COX-2 induction and prostaglandin synthesis in T cells activated by anti-CD3 plus anti-CD28 agonist antibodies or by phorbol ester plus calcium ionophore. COX-2 inhibition by glucocorticoids occurs mainly at the transcriptional level, since this drug inhibited COX-2 promoter induction. We have recently identified two NFAT sites in the COX-2 promoter required for glucocorticoid–mediated inhibition (3). Glucocorticoid treatment did not inhibit NFAT translocation to the nucleus but diminished its binding to DNA. It also prevented induction of the transactivation function of Gal4-NFAT deletion constructs containing the N-terminal transactivation domain of human NFATc2 (4). These effects are dependent on the presence of the glucocorticoid receptor. Evenmore, an activation-deficient glucocorticoid receptor mutant is as effective as the wild-type receptor in repression of NFAT transactivation (5). These results could explain some of the anti-inflammatory properties of glucocorticoids through their ability to interfere with the signal transduction pathways leading to activation of NFAT transactivation and induction of proinflammatory genes as COX-2 in activated human T cells.

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Role of peroxynitrite in endothelial damage mediated by Cyclosporine A

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The calcineurin inhibitor Cyclosporine A (CsA) is a potent immunosuppresant widely employed in the clinical setting. Its use is not devoid of side effects, among which vascular injury and nephrotoxicity are significant. These effects have been related in part to the pro-oxidative action of CsA. We have been studying the basis of endothelial dysfunction associated to CsA. While endothelial cells (EC) exposed to CsA for long periods of time produce more nitric oxide (NO) due to the transcriptional activation of eNOS, EC incubated for 2 h with CsA generate reactive oxygen and nitrogen intermediates (ROI and RNI). With the use of several fluorescent probes (dihydroethidium-DHE, diaminofluorescein/diacetate-DAF-2/DA, dihydrorhodamine 123-DHR 123), electron spin resonance with the spin trap DMPO, ozone chemilumiscence and nitrotyrosine immunocytochemistry we were able to show that a) CsA promotes the generation of peroxynitrite (OONO-) b) this generation leads to an increased tyrosine nitration which is N-acetylcysteine sensitive and is reflected in the nitration of proteins crucial in the balance of the redox state such as Mn-SOD and c) superoxide anion (O2-) is the limiting factor in the formation of ONOO-. Mice treated with CsA for 2 h showed an increased presence of nitrotyrosine in the vascular wall as developed by immunocytochemistry staining of aortic rings with antinitrotyrosine antibodies. Thus, it is possible that ONOO- and nitrotyrosine formation in EC may represent underlying pathophysiological mechanisms for CsA-associated vascular injury.

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Convergence of calcineurin and ERK1/2 signaling on insulin gene transcription

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The insulin promoter is both positively and negatively regulated in response to conditions to which beta-cells are exposed. In the short term, elevated glucose results in an enhancement in the rate of insulin gene transcription. In contrast, chronic exposure of beta-cells to supraphysiological conditions of glucose or free fatty acids results in a desensitization of its glucose-responsiveness. We have previously shown that ERK1/2 activation by glucose is calcineurin-dependent. Here, we describe a mechanism whereby calcineurin influences the insulin gene promoter via NFAT and the ERK1/2-dependent activation of trans-acting factors, MafA and C/EBP-beta. Treatment of INS-1 clonal beta-cells with glucose for 30min resulted in NFAT-MafA cooperative binding to the glucose-responsive A2-C1 element of the insulin gene promoter. This complex was disrupted by C/EBP-beta in cells chronically cultured in high (11mM) glucose conditions. Formation of NFAT-MafA and NFAT-C/EBP-beta complexes was sensitive to MEK1/2 and calcineurin inhibitors. Moreover, exposure of INS-1 cells to glucose for more than 24hrs resulted in the repression of the insulin promoter activity to levels below basal insulin gene transcription. The repression of promoter activity could be restored to basal levels by treatment with MEK1/2 inhibitor, U0126 (10uM). The calcineurin inhibitor, FK506 (100nM), completely blocked insulin gene transcription in all cases. These results indicate that calcineurin can activate the insulin promoter by at least two distinct converging signals: 1) the direct modulation of NFAT and 2) the positive regulation of the ERK1/2 pathway. The ERK1/2 pathway can in turn modulate partners of NFAT which can either stimulate or repress insulin gene transcription during stimulatory and chronic exposure to glucose, respectively.

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The transcription factor NFAT5 facilitates the activation of NFAT1 by calcineurin

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NFAT5 differs from NFATc proteins in that it lacks their characteristic N-terminal domain that contains the calcineurin docking site PxIxIT and regulatory phosphorylation sites. However, NFAT5 displays a DNA binding specificity highly similar to that of NFATc and is induced in activated T cells in a calcineurin-dependent manner, suggesting that it might participate in regulatory pathways that engage calcineurin and NFATc proteins. We have investigated whether an interaction exists between NFAT5 and the calcineurin-NFATc pathway. Our results show that calcineurin can bind to the DNA binding domain of NFAT5. This interaction is not compited by peptides comprising the PxIxIT calcineurin docking site of NFATc nor by the N-terminal domain of NFAT1. Conversely, the DNA binding domain of NFAT5 does not inhibit the binding and dephosphorylation of NFAT1 by calcineurin nor the enzyme's phosphatase activity. In addition, NFAT5 interacts with the phosphorylated form of NFAT1 but not with the dephosphorylated protein. The region of NFAT5 involved in binding to NFAT1 spans its N-terminal and DNA binding domains but not its large C-terminal transactivation domain. Expression of and NFAT5 construct lacking its C-terminal domain potentiates the calcineurin-mediated dephosphorylation of NFAT1 and its transcriptional activity. These results indicate that NFAT5, independently of its own transcriptional function, might cooperate with NFAT1 by facilitating its activation by calcineurin.

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Superoxide targets calcineurin in vascular endothelium

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Vascular endothelium is subjected to oxidative stress under a variety of pathophysiological conditions, such as ischemia/reperfusion, hypercholesteremia or diabetes. Elucidation of molecular targets of reactive oxygen species (ROS) in endothelial cells is critical for understanding the mechanisms leading to endothelial dysfunction in these disease states. Superoxide is the primary ROS formed by univalent reduction of molecular oxygen, and multiple superoxide sources were identified in the endothelium. We have shown previously that low concentrations of superoxide efficiently inhibit phosphatase activity of calcineurin (CaN) by oxidizing Fe^{2+} at the enzyme's active site. In this study we investigated whether CaN and signaling downstream thereof are affected by superoxide in cultured endothelial cells. We used a redox cycling agent, DMNQ, as an intracellular superoxide source. Incubation of bovine aorta endothelial cells (BAEC) or human umbilical vein endothelial cells (HUVEC) with DMNQ led to dose-dependent inhibition of CaN activity in cell lysates. Similar effects had inhibition of intracellular Cu, Zn-superoxide dismutase (SOD1) with diethyldithiocarbamate (DDC).

Pre-incubation with SOD1, in contrast, prevented DMNQ-induced inhibition of CaN activity. We also investigated the effects of DMNQ upon CaN-NFAT pathway in endothelial cells. NFAT is a family of CaN-activated transcription factors, which also participate in the regulation of endothelial gene expression. DMNQ inhibited calcium ionophore-induced NFAT1 dephosphorylation as well as NFAT nuclear translocation in HUVEC. The effect of DMNQ could be prevented by SOD1 or by superoxide scavenger Tiron. In addition, transcriptional activity of NFAT was sensitive to superoxide as judged by luciferase reporter assays. Thus, the calcineurin-NFAT signaling pathway appears to be a critical target of superoxide in vascular endothelium.

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Modulatory calcineurin-interacting proteins (MCIPs): Balancing calcineurin signaling in skeletal muscle and heart

Victoria Copeland, Erik Bush, Christine Mantis, Joseph A. Hill, and Beverly A. Rothermel

Modulatory calcineurin-interacting proteins (MCIPs), also known as the Down syndrome critical region 1 (DSCR1) and DSCR1-like proteins, are a family of small, structurally related proteins that bind to and inhibit calcineurin, a serine/threonine protein phosphatase that is an important transducer of intracellular calcium signals. Transcription of the mammalian MCIP1 gene is induced by calcineurin, suggesting that it functions as an endogenous feedback regulator of calcineurin activity, protecting cells from potentially deleterious effects of unrestrained calcineurin signaling. This transcriptional feedback loop between MCIP and calcineurin is conserved from yeast to humans indicating that it is a fundamental regulatory process. Using an antibody specific for MCIP1, we have verified that the protein is most abundant in brain, heart, and skeletal muscle, tissues that experience frequent fluxuations in calcium levels. In skeletal muscle, MCIP1 is a cytoplasmic protein with three primary isoforms that migrate around 36, 32 and 25 kD on SDS-PAGE. These isoforms differ in their stability and muscle fiber type expression. The 36 kD isoform is abundant in all muscle types and is very stable. The 32 kD isoform is characteristic of fast fiber-rich glycolytic muscles, while the 25 kD calcineurin-induced isoform is specific to highly oxidative muscles that are rich in slow fibers. Numerous cellular stresses, including hypoxia, result in accumulation of the 25 kD protein. The 25 kD protein is turned over rapidly via the proteasome with an apparent T1/2 of less than 5 minutes. Calcineurin activity stabilizes this isoform, substantially slowing the rate of protein turnover. We have examined the role of MCIP1 phosphorylation and calcineurin activity on degradation of this 25 kD species. We propose a model for the calcineurin/MCIP regulatory loop in which equilibrium is established between calcineurin activity and MCIP protein levels. Furthermore, we speculate perturbation of this equilibrium contributes to the multiple pathologies observed in Down syndrome.

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Calcipressin 1 regulates the pattern of calcineurin-dependent gene expression

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Calcineurin links calcium signaling to transcriptional responses in the immune, nervous and cardiovascular systems. To determine the function of the calcipressins (Csps), a family of putative calcineurin inhibitors, the calcineurin-dependent process of T cell activation was assessed in mice engineered to lack the Csp1 gene. We demonstrate that Csp1 regulates calcineurin in vivo, and show that genes triggered in an immune response display unique transactivation thresholds for T cell receptor (TCR) stimulation. In the absence of Csp1, the apparent transactivation thresholds for all of these genes are shifted due to enhanced calcineurin activity. This unregulated calcineurin activity drives Fas ligand expression which normally requires high TCR stimulation and results in the premature death of the T helper 1 cells. Thus, calcipressins modulate the pattern of calcineurin-dependent transcription, and may influence calcineurin activity beyond calcium to integrate a broad array of signals into the cellular response.

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Vav cooperates with calcineurin to activate JNK in T cells

Sandra Kaminski, Amnon Altman and Martín Villalba

Vav, the 95-kDa product of the vav protooncogene, is a member of the Dbl family which is expressed exclusively in hematopoietic cells and becomes phosphorylated on tyrosine in response to antigen receptor ligation. Although Vav was found to act as a Rac-specific guanine nucleotide exchange factor (GEF) and a c-Jun N-terminal kinase (JNK) activator *in vitro* and in ectopic expression systems using non-hematopoietic cells, it is not known whether these activities represent its physiological function in cells that normally express it, e.g., in lymphocytes. Here, we studied the role of Vav in JNK activation in T cells. Although Vav caused significant activation of JNK in non-hematopoietic COS and 293 cells, it barely activated JNK in Jurkat T lymphocytes. In contrast, an active Rac mutant efficiently stimulated JNK under the same conditions. The failure of Vav to activate JNK did not represent a general lack of activity of Vav in T cells since Vav clearly stimulated the activity of a nuclear factor of activated T cells (NFAT) reporter plasmid in the same cells. However, Vav cooperated with calcineurin (Cn) to induce JNK activation (and enhance NFAT activity) in T cells. This cooperation was cell type-specific since it was not observed in COS or 293 cells. Furthermore, Vav did not stimulate the activity of two other mitogen-activated protein (MAP) kinases, i.e., ERK2 and p38, in T cells either alone or in combination with Cn. These findings demonstrate that Vav, which is a poor JNK activator in T cells, cooperates with Cn to activate JNK, and emphasize the importance of studying the physiological functions of Vav in hematopoietic cells

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