Calmodulin-driven Nuclear Entry: Trigger for Sex Determination and Terminal Differentiation*

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We originally proposed that Ca\textsuperscript{2+}-calmodulin mediates a novel nuclear entry pathway distinct from the canonic Ran-dependent pathway (Sweitzer, T. D., and Hanover, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14574–14579). Although seemingly redundant, Ca\textsuperscript{2+}-calmodulin-driven nuclear entry is now known to facilitate nuclear delivery of architectural transcription factors to chromatin. Intriguingly, defects in calmodulin-driven nuclear import of the transcription factors SRY and SOX9 in Sertoli cells lead to human sex reversal diseases with altered male gonad development. Calmodulin-triggered nuclear entry is an evolutionarily ancient feature of eukaryotes observed from yeast to man. Ca\textsuperscript{2+}-calmodulin-triggered nuclear entry of key architectural transcription factors is a potentially key epigenetic regulator of terminal differentiation in response to cell signaling.

We previously identified a Ran-independent nuclear import pathway mediated by calmodulin, the ubiquitous calcium sensor (1). This evolutionarily ancient, calcium-dependent molecular switch was initially proposed to facilitate uptake of a distinct subset of nuclear proteins during cell activation (1). Our recent findings suggest that calmodulin-mediated transport is conserved throughout eukaryotic evolution. Although functionally redundant with the canonic Ran-dependent pathway, the import of nuclear proteins by calmodulin is unique; it is subject to independent regulation by intracellular Ca\textsuperscript{2+} mobilization. Perhaps because of this redundancy, abnormalities in either calmodulin- or Ran-dependent import can result in diseases leading to sex reversal (2–5). These sex reversal syndromes represent the first direct examples of a defect in a nuclear import pathway leading to human disease. Furthermore, these findings have solidified the physiological significance of the Ca\textsuperscript{2+}-calmodulin-regulated transport pathway (2–5). A similar autosomal sex reversal phenotype occurs when three insulin-related receptors are ablated in mice, suggesting that intracellular signaling cascades may impact sexual differentiation (4, 5). The calmodulin-triggered pathway may also regulate the SOX family of proteins, involved in many cellular differentiation events. In this minireview, we will summarize the evidence for this alternate nuclear entry pathway, highlighting its potential significance in triggering terminal differentiation.

Initial Discovery of Calmodulin-driven Nuclear Entry

Studies in the yeast Saccharomyces cerevisiae (6–9) and biochemical studies utilizing digitonin-permeabilized mammalian cells (1, 10, 10–16) first led to the identification of a number of key nuclear import factors. Two families of proteins (now termed importins α and β or karyopherins) that recognized nuclear localization sequences were identified. In addition, the GTPase Ran was shown to be essential for the nuclear import of a wide variety of cargo with importins. The importin families now contain at least 20 members. One factor identified that did not fit into a model for Ran-dependent import was the molecular switch calmodulin. In initial studies, we showed that calmodulin supported the import of a fluorescent protein to which the SV40 T-antigen NLS\textsuperscript{2} (a basic NLS) was covalently attached (1). We noted that the import occurred with peptides containing stretches of basic amino acids like the SV40 T-antigen sequence (PKKKRKV) or sequences from small HMG proteins (PKRK . . . (X\text{30}) . . . KGKKG) but was distinct in many ways from the canonic pathway (Fig. 1A). Unlike Ran-dependent nuclear entry, this import was independent of GTP and dependent upon Ca\textsuperscript{2+}. Calmodulin inhibitors (such as calmidazolium chloride) blocked import in cytosolic extracts. In addition, recombinant calmodulin was sufficient to reconstitute import in permeabilized cells. Besides calmodulin, other factors (such as transport receptors bound to nuclear pores) were thought to be present in the permeabilized cell preparations, facilitating the role of calmodulin in import. We also found that recombinant calmodulin could mediate uptake of a reporter protein in nuclei isolated from Xenopus laevis oocytes. Import occurred in the absence of other exogenously added import factors (data not shown). These findings led us to propose the model shown in Fig. 1A, in which calmodulin would respond to elevation of cytoplasmic calcium levels to mediate nuclear import during cell activation. Ran and known importins would carry out “housekeeping” nuclear import functions. Calmodulin had been shown to have unusual nuclear import characteristics (17), and it was therefore plausible that it could serve as a molecular switch in a novel nuclear import pathway. However, some complications of our proposed model at the time were that 1) it was unclear which proteins used each pathway and 2) the identity of the import receptors was unknown. The importin (karyopherin) family is now known to be large and diverse (18–20), yet these receptors all seem to be capable of using a variant of Ran-dependent association and dissociation. Our findings suggested that the calmodulin-dependent entry pathway was independent of the Ran pathway (Fig. 1, A and B). This led to attempts to identify the relevant nuclear localization sequences recognized by the calmodulin-driven import pathway.

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2 The abbreviations used are: NLS, nuclear localization signal; HMG, high mobility group; ESP, eukaryotic signature protein; PAM, point accepted mutation.
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Sex Determination and Nuclear Entry of SRY and SOX9

Mammalian sex determination is a binary switch locking the embryonic gonads into a fate as testes or ovaries. Two architectural transcription factors, SRY and SOX9, are key components of mammalian sex determination pathways, and these appear to be regulated in part by nuclear transport (Fig. 1B). The discovery of the Y chromosome sex-determining gene in 1990 seemed destined to uncover the mechanism of human sex determination. This rapidly evolving gene, SRY, contains an “HMG domain” that induces a 60–85° bend upon binding to target DNA (Fig. 1B) (21). It was also known to have two independently acting nuclear localization sequences bracketing the HMG domain (Figs. 1B and 2A) (22). Most SRY mutations leading to male-to-female sex reversal in man alter the ability of SRY to interact with and bind DNA (21, 23–25). However, defective nuclear localization of SRY has also been linked to sex reversal (3, 26, 27). The N-terminal NLS of SRY is present in a region of the molecule previously shown to be a calmodulin-binding site (Fig. 2A) (28). This prompted an examination of calmodulin as a potential mediator of SRY nuclear localization.

As outlined above, SRY provides the primary trigger for maleness in mammals. Its action is known to be “dosage-dependent”; it must reach a threshold concentration before it can act. Thus, nuclear import may play a critical role in its regulation. Defects in SRY are associated with a disease called Swyer syndrome, a form of pure gonadal dysgenesis (Fig. 1B). Some forms of Swyer syndrome are associated with mutations in either the C-terminal (importin-binding) or N-terminal NLS located at the N and C termini of the HMG box (Fig. 2A). These nuclear import sequences bracket a nuclear export sequence present in the middle of the HMG box of SOX9 and the rest of this SOX family (Fig. 2A). The export signal of SOX9 binds to the export receptor CRM1, and nuclear export of SOX9 can be inhibited with leptomycin B. This drug blocks the interaction between CRM1 and the nuclear export signal (32). The steady-state localization of SOX9 is modulated by the action of the export and import sequences. Of the nuclear import sequences, the best characterized is the C-terminal NLS, a simple basic sequence with similarities to the SV40 large T-antigen NLS. The N-terminal NLS is reminiscent of the bipartite NLS originally identified in nucleoplasmin (Fig. 2A). As observed with SRY, this N-terminal NLS was shown to be a calmodulin-binding region (28). These studies established that the interaction of SOX9 and calmodulin is both calcium-dependent and associated with a conformational change in SOX9. More important, the calmodulin antagonist calmidazolium chloride, a reagent identified to block calmodulin nuclear import in vitro (1), inhibited the calmodulin recognition of SOX9. This calmodulin inhibition led to inhibition of nuclear import and the consequent transcriptional activity of SOX9 in calmidazolium-treated cells. A missense SOX9 mutation (A158T) identified in a patient with campomelic dysplasia bound importin normally but was nuclear import-defective. These studies showed that calmodulin is involved in the nuclear entry of SOX9 in a process likely to involve direct interaction with SOX9 (Figs. 1B and 2A).

(29). Calmidazolium chloride blocks nuclear import of wild-type SRY in vitro but has no effect upon SRY bearing mutations in the N-terminal (calmodulin-binding) NLS. The mutations appear to alter the conformation of the calmodulin-SRY complexes. These findings suggest that calmodulin is important for maintaining the proper nuclear concentrations of SRY (29).

Another SRY-related factor required for male sex determination is SOX9 (Fig. 1B) (23, 30). This architectural transcription factor plays a key role in regulating genes involved in both chondrogenesis and testis formation. Mutations in the HMG box of SOX9 result in a disease known as campomelic dysplasia (Fig. 1B) (2, 30, 31). This is a severe bone malformation disease in which most XY individuals also show male-to-female sex reversal. During early mammalian embryogenesis, SOX9 is found in the cytoplasm of Sertoli cells in both genders, but with SRY expression, SOX9 moves into the nucleus in male embryos. SOX9 also contains two NLSs
SOX Family of Architectural Transcription Factors and Calmodulin Interactions

SRY is the founding member of a family of proteins termed the SOX family (SRY-related HMG box), which includes SOX9. During evolution, the SOX family arose with the advent of multicellularity in mammals; this family of transcription factors triggers numerous key developmental programs in addition to sex determination (33–35). These developmental programs include central nervous system neurogenesis, oligodendrocyte development, chondrogenesis, and neural crest cell development (34). The SOX family members all contain a highly conserved HMG box, like SOX9 (Fig. 2A) (35). It is therefore likely that all SOX family members interact with calmodulin at the N-terminal NLS of the HMG box. Obviously, this may have important implications for the regulation of cellular differentiat-

Nuclear Transport via a Molecular Switchboard: Ran and Calmodulin

Accumulation of proteins on either side of the nuclear envelope can be generated by a spatially and temporally organized cycle of interactions between the Ran GTPase, specific carriers, and their cargos and nucleoporins (36). This cycle of interactions alters the affinity of the carriers for their cargo and allows cargo to slip through the diffusion barrier offered by the nuclear pore. The nuclear pores are large macromolecular complexes with a central channel lined by nucleoporins with multiple FG repeats (37–40). The carriers are spring-like proteins made up of either Armadillo repeats (importin α) or HEAT repeats (importin β). The HEAT repeats form helicoids making at least three points of contact with nucleotide-bound Ran (36). The nucleotide state of Ran is maintained by the action of a cytoplasmic Ran GTPase-activating protein, which facilitates Ran-mediated GTP hydrolysis, and a nuclear Ran guanine nucleotide exchange factor (RCC1), which catalyzes nucleotide exchange. The net result of the action of these spatially separated enzymes is the formation of a Ran gradient with high concentrations of Ran-GTP in the cytoplasm and high concentrations of Ran-GDP in the nucleus. This gradient modulates the affinity of the various importins for their cargo and confers directionality to nuclear transport. The low concentration of Ran-GTP in the cytoplasm allows stable import complexes to form; nuclear Ran-GTP destabilizes import complexes and is part of a trimolecular export complex with transporter and cargo. Therefore, Ran serves as a molecular switch facilitating the directional movement of proteins across the nuclear pore complex.

In principle, any other molecular switch could provide directionality and specificity to nuclear transport. Calmodulin is the prototypical molecular switch because of its ability to dramatically alter its conformation in response to Ca^{2+} binding (41). Unlike the Ran gradient, which is established following each cell division and maintained constantly, Ca^{2+}-calmodulin complexes are formed and act transiently. Calcium entry from the plasma membrane and from stores in the endoplasmic reticulum and mitochondria is associated with intracellular sig-

FIGURE 2. Conserved nuclear trafficking of the HMG box family of transcription factors from yeast to man. A, the HMG box family of transcription factors contains a conserved calmodulin-binding domain (gray), a nuclear export sequence (NES; blue), and an importin-binding domain (pink). The HMG box protein found in yeast, Nhp6Ap, also contains a calmodulin-binding domain (gray). LEF (lymphoid enhancer-binding factor 1) and HMG are also shown for comparison. HMG box proteins are characterized by three helices (green). Below each helix is the NMR structure of SRY bound to DNA (62). The yellow regions correspond to the calmodulin-binding NLS, the nuclear export signal, and the C-terminal NLS, respectively. B, a molecularly detailed model for the calmodulin-dependent, Ran-independent nuclear import of Nhp6Ap is shown. The model is based on the free and DNA-bound forms of Nhp6Ap (63). The calmodulin (CaM)-binding region is highlighted in yellow. Step 1 of calmodulin-driven import requires binding of calmodulin-Ca^{2+} to the first 36 amino acids (yellow) of Nhp6Ap. An importin β-family member (importin?) likely contributes to the nuclear import efficiency. Step 2 is translocation across the nuclear pore complex. Step 3 involves DNA-dependent dissociation of the transport complex. The high affinity of Nhp6Ap for DNA disrupts the import complex and releases calmodulin and associated factors. The export of these factors may depend on the Ran gradient. Cyto, cytoplasm; Nuc, nucleus.
naling events (1, 17, 41). The binding of four calcium ions to calmodulin alters its conformation and promotes its interaction with a number of other proteins, including several classes of protein kinases. Calmodulin also activates phosphatases such as calcineurin. Thus, elevation of intracellular Ca\(^{2+}\) stores leads to coordinate activation of a number of signal transduction cascades (41). As outlined above, one of these events may be a transient increase in the nuclear concentration of transcription factors during cell activation. A rapid and transient increase in the Ca\(^{2+}\) levels accompanying cell activation could both trigger nuclear accumulation of the architectural transcription factors and modulate the loading of these factors onto their specific targets on chromatin. This is in contrast to the way in which a stable gradient of Ran is established by differentially localized GTPase-activating proteins and guanine nucleotide exchange factors. In one sense, then, Ran-dependent nuclear transport functions as a housekeeping nuclear import pathway. In contrast, the calmodulin-dependent pathway functions as a Ca\(^{2+}\)-inducible “gatekeeper,” subject to independent regulation by cell signaling cascades mobilizing Ca\(^{2+}\) stores.

**Other Import Cargo**

Although the SOX family of architectural transcription factors is large (~20 members), it may not be the only group of proteins whose nuclear import is influenced by calmodulin. Other transcription factors may also bind to calmodulin. The best characterized of these are the basic helix-loop-helix transcription factors, which have a calmodulin-binding domain (42–50). Here, calmodulin binding may interfere with nuclear import of the transcription factor in response to Ca\(^{2+}\). Sequence similarities suggest that the c-Rel-related transcription factors also have this recognition motif. c-Rel and RelA bind to calmodulin in a Ca\(^{2+}\)-dependent fashion, and binding of calmodulin appears to inhibit nuclear transport (42). Structural examination of the modes of interaction between calmodulin and its transcription factor targets will help solve this puzzle. These findings could allow us to distinguish between the interaction of calmodulin with targets whose nuclear import may be triggered (SRY and the SOX family) and those that use this interaction for other purposes (c-Rel).

**Calmodulin-dependent Nuclear Entry Is Conserved from Yeast to Man**

The canonical Ran-dependent pathway of nuclear protein import was discovered by *in vitro* analysis of the components involved, augmented by genetic analysis in the yeast *S. cerevisiae* (51–54). Until recently, it was unclear whether a calmodulin-dependent pathway described in mammals (1) existed in yeast. Genetic studies had focused on Ran-dependent processes, although some provocative findings suggested that Ran-independent nuclear transport might occur (55). Recently, we found that the architectural transcription factor Nhp6Ap requires calmodulin, but not Ran, for its nuclear entry (Fig. 2B) (56). The calmodulin-binding motif of Nhp6Ap has similarities to those in SRY and the SOX family proteins, and when mutated, nuclear entry of Nhp6Ap is blocked. In addition, temperature-sensitive alleles of calmodulin block nuclear entry of Nhp6Ap. The calmodulin-binding NLS of Nhp6Ap is sufficient to direct nuclear entry of proteins larger than 50 kDa, such as pyruvate kinase-green fluorescent protein. Binding is dependent upon Ca\(^{2+}\) and is mutually exclusive, *i.e.* in the presence of DNA, the Nhp6Ap-Ca\(^{2+}\)-calmodulin complex is disrupted. These findings led us to propose the model shown in Fig. 2B. It is also not yet clear how widespread this pathway is in yeast or how many other proteins may utilize this pathway. Because Nhp6Ap nuclear entry is solely dependent upon calmodulin, but not Ran, it is somewhat different from known mammalian proteins where the Ran and calmodulin pathways cooperate to regulate nuclear entry of proteins triggering sex determination (*e.g.* SRY). Intriguingly, loss of *NHP6A/NHP6B* in yeast results in defects in yeast mating-type switching, hinting at an evolutionarily conserved function for Ca\(^{2+}\)-triggered nuclear entry. These features make yeast a good model for identifying other components of the calmodulin-driven import pathway. These genetic and biochemical analyses are under way in our laboratories.

**Evolutionary Issues**

Calmodulin is one of the most evolutionarily ancient eukaryotic proteins and also one of the most slowly evolving. One measure of evolutionary change is the “family-specific rate,” which for calmodulin is ~1 PAM/billion years (1 unit of evolutionary matrix change/billion years) (57). Ran is slightly more rapidly evolving (6 PAM/billion years) compared with the mean protein family change of ~50 PAM/billion years. These quantitative measures of rates of evolutionary change are consistent with theories concerning the origins of the eukaryotic cell (58). One of the most provocative of these theories suggests that a group of proteins termed ESPs is conserved throughout eukaryotic evolution but largely absent in archaea or bacteria; calmodulin and Ran are among the 108 ESPs associated with signaling systems (58). From analysis of these ESPs, a cell called the “chronocyte” is envisioned to be the precursor to the eukaryotic cell. The chronocyte contained a cytoskeleton, internal membranes, inositol phosphate lipids, and a complex internal Ca\(^{2+}\) signaling system. In this model, the eukaryotic nucleus was formed when archaea and/or bacteria were engulfed by the chronocyte in a process coordinated by the cytoskeleton and Ca\(^{2+}\)-calmodulin. Thus, Ca\(^{2+}\)-calmodulin may have been the first molecular switch adapted to facilitate nucleocytoplasmic exchange and may have preceded and subsequently coevolved with the Ran-dependent pathway. Calmodulin and Ran are not the only evolutionarily ancient factors that act to stabilize and destabilize nuclear transport complexes. Recently, the inositol polyphosphates have been suggested to play key roles in the regulation of mRNA export and the control of eukaryotic gene expression (59).

**Summary**

The calmodulin- and Ran-triggered pathways can now be viewed as evolutionarily ancient, tightly interwoven systems acting in consort to facilitate the nuclear entry of SOX family architectural transcription factors and other import cargo. These proteins are critical determinants of development and differentiation. A number of recent studies strongly suggest that nuclear transport plays a key role in regulating the pro-
yses of terminal differentiation from stem and progenitor cell populations (see Ref. 60). The yeast protein Nhp6p, which uses calmodulin-dependent entry (see above), is a known component of the yeast FACT complex, a highly conserved complex acting as a nucleosome chaperone and mediating chromatin reorganization in processes as diverse as transcription and DNA replication and repair (61). Similar HMG domain proteins are present in the FACT complex found in all metazoans. Ca\(^{2+}\) mobilization in response to signaling is a conserved and universal mechanism for cell activation terminating in cellular differentiation. Thus, the Ca\(^{2+}\)-calmodulin-triggered nuclear import pathway may provide a means of both recruiting architectural transcription factors and initiating the formation of complexes mediating epigenetic alterations in chromatin. This gatekeeping function allows the Ca\(^{2+}\)-calmodulin pathway to initiate the chromatin-remodeling steps associated with stem cell maintenance and terminal differentiation.

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