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Smith–Magenis syndrome: a mechanistic perspective

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Abstract

Clinical features of Smith–Magenis syndrome (SMS) often correlate with mutations in the gene that encodes RAI1. However, despite numerous advances, little is known about the mechanisms and pathways through which RAI1 regulates transcription. We applied tools in Bioinformatics to identify potential protein-networks, signaling pathways, and sequence features that might contribute to RAI1-mediated gene expression. Through searches of the BioGRID database, we identified two proteins reported to interact with RAI1: tescalcin (TESC) and PIN1. In alignments of human RAI1, human TCF20, and a related protein from zebrafish, we localized several highly conserved domains. In one domain, we identified several closely-spaced and highly conserved Ser/ThrPro (S/TP) motifs and thus obtained clues into how PIN1 and TESC might regulate RAI-1 mediated gene expression. The results predict that the activity of RAI1 is controlled by regulatory kinases and phosphatases, through pathways controlled by Ca^{2+} signaling systems. The results predict that in cytoplasm of cells, phosphorylated S/TP motifs in RAI1 interact with a complex consisting of TESC and calcineurin A. In nucleus of cells, the phosphorylated motifs would provide sites for interactions of RAI1 with PIN1. Furthermore, we found evidence hinting at a potential role for the NHE1 ion transporter in the regulation of RAI1 activity in response to periodic flux in Ca^{2+} concentration in pericytes in the central nervous system.

Introduction

SMS is a multisystem disorder characterized by developmental delay and mental retardation, a distinctive behavioral phenotype, and sleep disturbance (1). The disorder is caused by haploinsufficiency of the gene that encodes RAI1, retinoic acid-induced 1, reviewed in (2). Functional assays have identified collection of genes that are either upregulated or downregulated as a consequence of RAI1 haploinsufficiency (3). In mice, RAI1 deficiency causes learning impairment and motor dysfunctions (4). In addition to SMS, RAI1 has been implicated in spinocerebellar ataxia type 2, as a potential modifier of age at the onset of the disease (5).

It seems likely that RAI1 regulates transcription (2). Its amino acid sequence includes a bipartite nuclear localization signal, polyglutamine and polyserine tracts, and a C-terminal plant homeodomain (PHD)/zinc-finger domain (2,6-9). The sequence of RAI1 shows similarity to transcription factor 20 (TCF20), also known as SPBP (8,10). However, despite genetic and biochemical advances, little is known about pathways through which RAI1 would exert control on transcription.

In this report, we use tools in Bioinformatics to obtain clues into pathways of RAI1-mediated gene expression. We provide evidence implying that in cells, the activity of RAI1 might be regulated via Ca^{2+} dependent pathways. We provide evidence suggesting that RAI1 activation might resemble the NFAT-signaling pathway, regulated by Ca^{2+} and calmodulin (11). We also show that as the NFAT family of transcription factors, the amino sequence of RAI1 includes conserved sequence-motifs for recognition by serine- and threonine-specific kinases and phosphatases. Furthermore, we examine evidence suggesting a potential role for NHE1 ion transporter in the regulation of RAI1 activity in response to periodic flux in Ca^{2+} concentration in pericytes in the central nervous system.

Results

RAI1 interacts withPIN1. Studies of protein-protein interactions are becoming increasingly important to understanding mechanisms of human diseases on a system-wide level (12). Development of databases has facilitated accessing the results of high-throughput methods for analysis and interpretation. To identify proteins that interact with RAI1, we searched biological databases that include collections of protein and genetic interactions from major model organism species (13,14). The BioGRID database contains over 198,000 interactions from six different species, as derived from both high-throughput studies and conventional focused studies (13). In searches of BioGRID, we discovered that RAI1 was reported to interact with PIN1: a phosphorylation-dependent, peptidylprolyl *cis*/*trans* isomerase (15). PIN1 is expressed in most neurons at unusually high levels and its expression is induced during neuron differentiation (16). PIN1 regulates mitosis and plays unexpected roles in diverse cellular processes including regulation of transcription (16,17).

RAI1 interacts with TESC. Searches of BioGRID further revealed that in addition to PIN1, RAI1 was reported to interact with TESC (also known as TSC and CHP3). TESC is abundant in several mouse tissues including brain (18). Remarkably, TESC shows sequence similarity to calcineurin B (18), the regulatory subunit of calcineurin (19). The other subunit (calcineurin A) is an S/TP specific phosphatase (19).

The NFAT family of transcription factors represents the most studied and the best known example of Ca^{+2} signaling pathways regulated by calcineurin and calmodulin (11). NFAT proteins exit in cytoplasm, as an inactive form, in association with calcineurin (11). Increases in Ca^{2+} concentration leads to activation of calmodulin, followed by activation of calcineurin B, and dephosphorylation of NFAT by calcineurin A (11). Following this step, the nuclear localization signal in NFAT becomes exposed, leading to NFAT nuclear import to regulate transcription (11).

TESC interacts with NHE1. To obtain additional mechanistic clues, we searched BioGRID to identify proteins reported to interact with TESC. The retrieved list includes SLC9A1, best known as NHE1. NHE1 is the best studied member of a family of proteins that regulate ion exchange across the cellular membrane (20). NHE1 exists on the plasma membrane of all mammalian cells (20). In addition to its well-known biochemical function (Na^+/H^+ exchange), NHE1 performs important physiological roles. Examples include control of cell volume, shifts in intracellular pH, and changes in cell growth or functional states (20). More recent studies implicate NHE1 in playing a key role in calcium signaling in pericytes in the human central nervous system (21). Furthermore, we noted that in addition to TESC, NHE1 was reported to bind calmodulin (20). Therefore, it seemed reasonable to deduce that the association of TESC with NHE1 (22,23) might play a role in mechanisms leading to RAI1 activation.

The amino acid sequence of RAI1 includes S/TP motifs. Among peptidylprolyl *cis*/*trans* isomerases, PIN1 is the only enzyme known to bind sequences that contain phosphorylated S/TP (15,17,24,25). Furthermore, the formation of calcineurin-NFAT complexes involves several closely-spaced and conserved SP motifs (11). Inspection of the amino-acid sequence of RAI1 revealed potential sites for phosphorylation by S/T specific kinases. Therefore, we asked whether RAI1 includes S/TP motifs and whether such motifs were conserved in cross-species sequence-alignment.

To address these questions, we used the BLAST engine at NCBI (26). In results of BLASTs, we noted that extensive amino acid sequence similarity in higher vertebrates obscured clear delineation of highly conserved domains. We noted that zebrafish included a single gene whose product showed sequence similarity to both human RAI1 and human TCF20 (Figure 1). We did not find a similar sequence in *Ciona intestinalis*. Thus, the genes encoding RAI1 and TCF20 are vertebrate-specific.

In alignments of human RAI1, human TCF20, and the zebrafish protein, we localized several conserved domains (Figure 1). Conserved domain 1 (CDI) is at the amino terminus of the aligned proteins (Figure 1A). CDI is within a relatively long transactivation domain at the amino terminal segment in mouse RAI1and TCF20 (8,27). CDII includes several conserved SP and TP motifs (marked as SP/TP box in Figure 1B). Phosphorylated forms of these motifs provide good candidates for interaction of RAI1 with PIN1 and with complexes that contain TESC. Furthermore, RAI1 includes a cluster of TP and SP motifs and a cluster of SP motifs that do not appear to be conserved and hence might be RAI1-specific (for example see the TP/SP box and SP box in RAI1, in Figure 1).

Another conserved domain (CDIII) is within a mapped transactivation domain in mouse RAI1 (27). CDIV is detected in alignment of RAI1 and TCF20 but does not fully appear in the sequence of zebrafish (Figure 1). CDV is conserved in all species.

CDVI is conserved in higher vertebrates, but only a short segment of this domain appears in the zebrafish sequence (Figure 1). CDVI corresponds to a PHD domain that also appears in the MLL family of regulators of transcription (8,9). Remarkably, MLL-like PHD sequences might interact with methylated histone H3 (28). Therefore, mechanisms of RAI1-mediated transcription might include reading epigenetic marks in chromatin.

In the studies, we also examined alignments of RAI1 with its orthologous sequences in primates. In these alignments, we identified three human-specific residues: PTQQ**G**LQGRPAF (198), LSEP**P**SSTP (505), QSLHG**S**LPLDSFS (567). The numbering follows the system shown in Figure 1 (shown in following page).

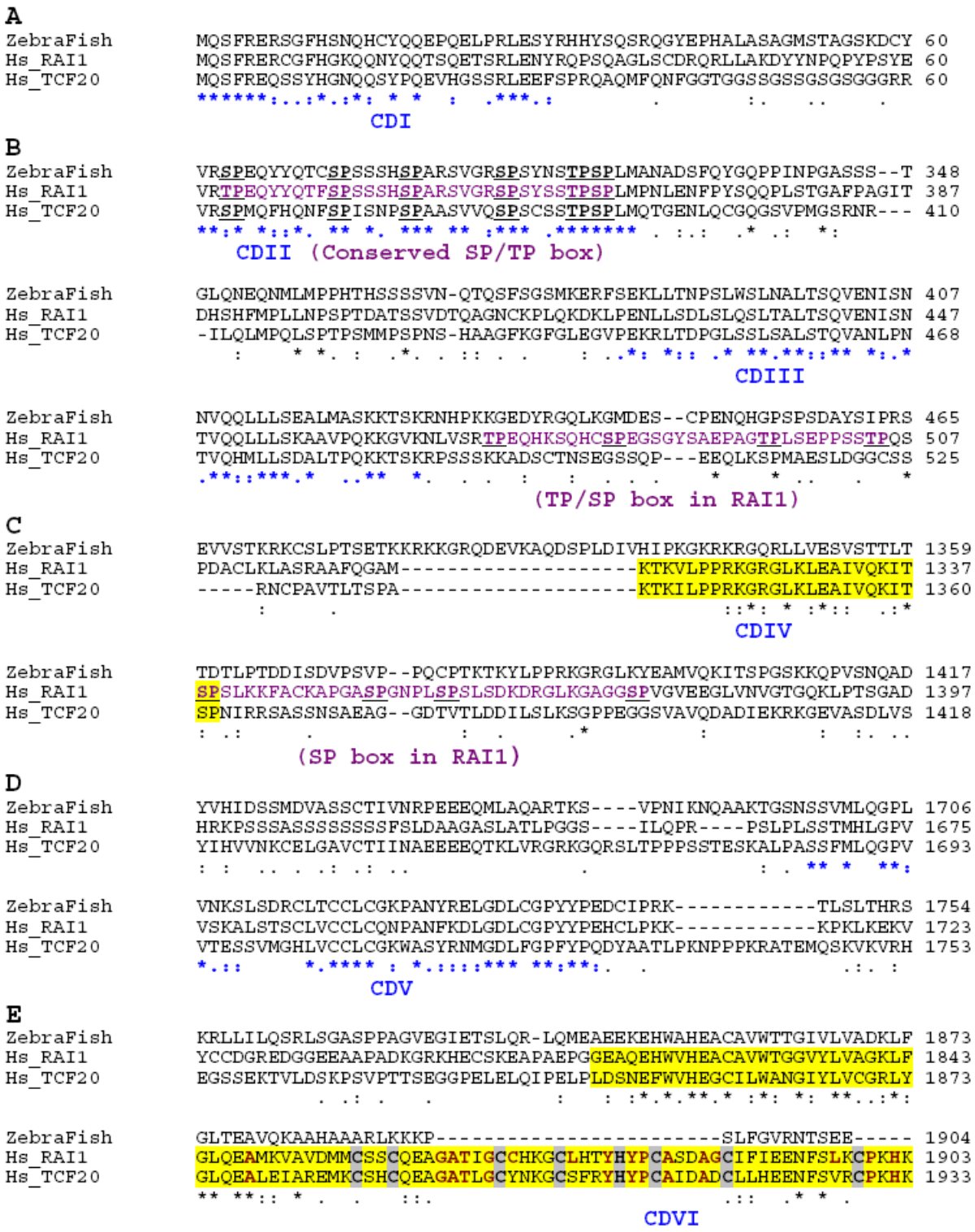

Figure 1

Localization of conserved domains. The sequences correspond to human RAI1 (accession number NP_109590), human TCF20 (accession number NP_852469), and a similar sequence in zebrafish (accession number XP_001333161). A to E include the sections that contain conserved regions. Highly conserved domains correspond to CDI, CDII, CDIII, CDIV, and CDV. CDIV and CDVI (highlighted in yellow) were only observed in alignments of sequences from higher vertebrates. In the PHD domain (CDVI), gray boxes mark residues implicated in Zn coordination. Brown letters highlight identity to residues 250-330 in human MLL3 (accession number NP_733751.2).

Discussion

We have identified two proteins (PIN1 and TESC) that interact with RAI1. PIN1 is a Phosphorylation-dependent peptidylprolyl *cis*/*trans* isomerase (15) that plays unexpected roles in diverse cellular processes including regulation of transcription (16,17). Remarkably, PIN1 targets include several transcription factors that function in responses controlled by signaling systems (16,17,29). Examples include RNA polymerase II, p53, SPT5, c-JUN, c-FOS, RARα, c-MYC, p65 subunit of NF-κB, and the NFAT family of regulators of transcription (16,17,29,30). PIN1 regulates transcription via three mechanisms (29): stabilization of transcription factors that are constitutively or rapidly turned over (i.e. p53); stimulation of degradation of transcription factors (i.e. RARα); influence on phosphorylation status and conformation of a transcription factor, thereby controlling its association with other regulators of gene expression (i.e. the CTD of RNA polymerase II).

TESC has been implicated in playing a key role in Ca^{2+} signaling pathways resembling those described for calcineurin (18). Intracellular calcium signaling plays central roles in development of the central nervous system including proliferation, migration, and maturation of neurons, leading to the widespread formation of connections between neurons and the establishment of rudimentary neuronal circuits (31). Downstream effectors of calcium signaling events include transcription factors that regulate the expression of genes required for various aspects of neuronal functions (31). Examples include: the transcription factor CREB (often used as the prototype for calcium-dependent regulators of transcription); and the NFAT family of transcription factors, known to regulate neuronal survival and axonal outgrowth (31).

The results of our analysis predict that the activity of RAI1 might be controlled through signaling pathways resembling those reported for the NFAT family of regulators of gene expression (Figure 2). NFAT signaling involves responses regulated by Ca^{2+}, calcineurin, and calmodulin (11,19). Calcineurin is a major player in regulation of processes controlled by calcium signaling systems (19). Striatal and hippocampal neurons are particularly rich in calcineurin (19). In hippocampal neurons activation of calcineurin results in inhibition of the release of the neurotransmitters, glutamate and γ-aminobutyric acid (19). The activation pathway is also involved in the desensitization of the postsynaptic NMDA receptor-coupled Ca^{2+} channels (19).

Calcineurin is a calcium/calmodulin-dependent S/T specific phosphatase and plays a critical role in coupling Ca^{2+} mediated signals to cellular responses (19). Calcineurin contains two subunits: calcineurin A catalyzes the dephosphorylation reaction; calcineurin B controls the phosphatase activity of calcineurin A (11,19).

Calcineurin B, CHP, CHP2, and TESC are thought to perform related functions (18). Since calcineurin A also interacts with TESC (18), it seems plausible that a complex of calcineurin A and TESC might have regulatory properties similar to those ascribed to calcineurin. In that context, interaction of RAI1 with TESC appears significant.

Specifically, the interaction of RAI1 with TESC and the interaction of TESC with calcineurin A imply that the activity of RAI1 might be controlled through mechanisms resembling the NFAT signaling pathway (Figure 2). That is: RAI might exit

in cytoplasm, as a phosphorylated inactive form, in association with a heterodimer of TESC and calcineurin A. In that complex, as calcineurin B, TESC would play a regulatory role. As in NFAT signaling pathway, increases in Ca^{2+} concentration would lead to the activation of calmodulin, followed by activation of the complex of TESC and calcineurin A, dephosphorylation of RAI1, and import of dephosphorylated RAI1 to the nucleus (Figure 2). Consistent with this model is a report showing that as calcineurin B, TESC can inhibit the phosphatase activity of calcineurin A (18).

Conserved residues in NFAT proteins include SP motifs involved in phosphorylation and dephosphorylation reactions (11). The results of our analysis predict that the S/TP boxes in RAI1 (Figure 1) are strong candidates for dephosphorylation by S/TP specific phosphatases. The tau protein implicated in Alzheimer also includes several phosphorylated S/TP sites that are candidate for dephosphorylation by calcineurin (32).

Our results further predict that RAI1 might be subject to phosphorylation by S/T specific kinases. As the NFAT proteins (11), phosphorylation of RAI1 in the cell nucleus might tag RAI1 for export to the cytoplasm. Our results predict that in the nucleus, phosphorylated RAI1 might associate with PIN1 (Figure 2). Association of PIN1 with transcription factors can lead to their degradation or may lead to fine-tuning of transcription (29).

The results of our analysis also predict a role for NHE1 in signaling pathways for RAI1 activation (Figure 3). The scheme in Figure 3 draws from two lines of evidence: (i) the finding that the cytoplasmic domain of NHE1 interacts with both TESC and calmodulin (33); and (ii) a report showing important roles for NHE1 in Ca^{2+} signaling in pericytes in the human central nervous system (21).

In the brain, pericytes are in active communication with the cells of the neurovascular unit and make fine-tuned regulatory adjustments in response to stress stimuli (34). Significantly, in the human central nervous system, NHE1 functions in coupling pH and Ca^{+2} signaling, as well as responses to periodic flux in Ca^{2+} concentration (21). We imagine that in the brain pericytes, the ternary complex (consisting of RAI1, TESC, and calcineurin A) might associate with the cytoplasmic domain of NHE1 (Figure 3). This arrangement might facilitate fast, calmodulin-mediated, responses to oscillation and periodic changes in Ca^{2+} concentration (Figure 3).

A potential role for pericytes in Smith-Magenis syndrome appears plausible. Pericytes are important components of blood brain barrier (34,35). A number of neurological disorders has been ascribed to dysfunctions in blood-neural barriers (35). Examples of barriers include blood-brain, -cerebrospinal, -spinal cord, -ear-labyrinth, -retinal, and -nerve barrier (35). Furthermore, pericytes also function in cell-cell-signaling under physiological and pathological conditions (35). Therefore, abnormalities in RAI1-mediated regulation in pericytes might contribute to manifestation of a number of clinical features ascribed to Smith-Magenis syndrome.

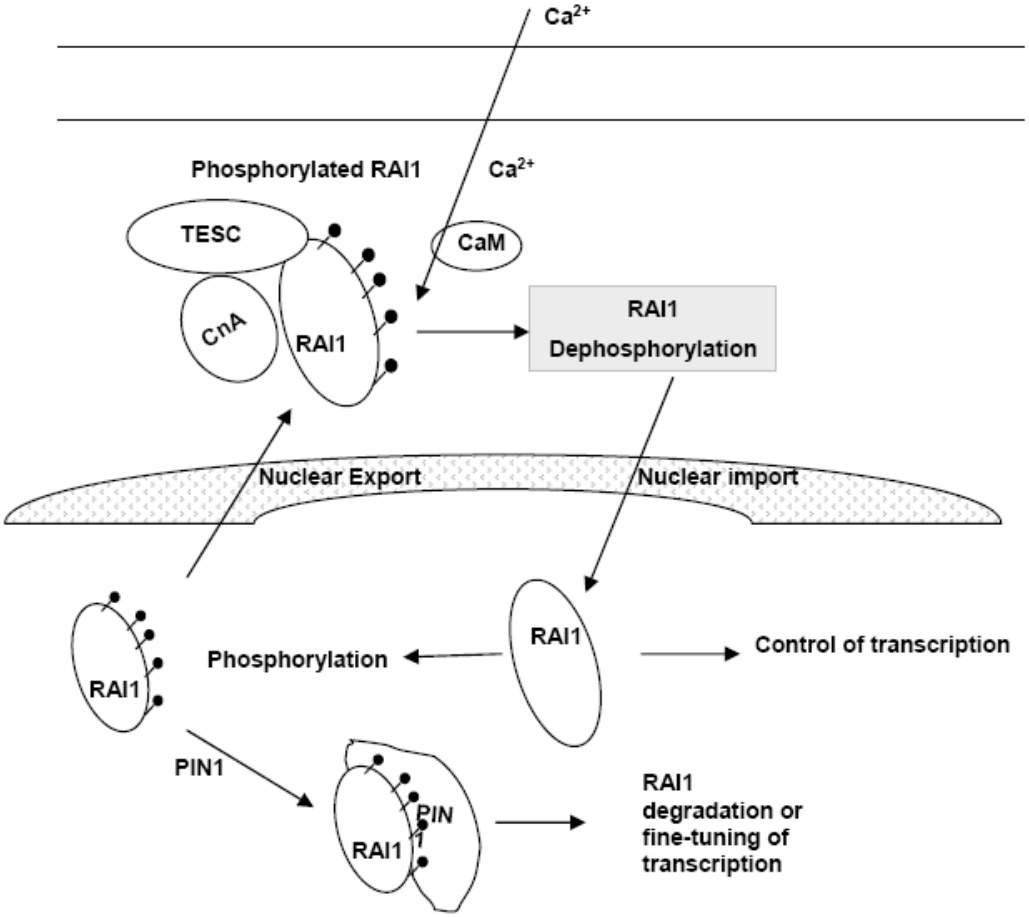

Figure 2

RAI-mediated transcription might resemble the NFAT signaling pathway regulated by calcineurin and calmodulin. In that pathway, a complex of calcineurin A and B is activated by calmodulin in response to elevated Ca^{2+} concentration (11). In the predicted pathway, the complex that is activated by calmodulin (CaM) would consist of a heterodimer of tescalcin (TESC) and calcineurin A (CnA). Activation would cause dephosphorylation of RAI1 and its subsequent nuclear import. Black circles depict phosphorylated residues in RAI1. In the nucleus, RAI1 might be rephosphorylated, tagging the protein for nuclear export or association with PIN1

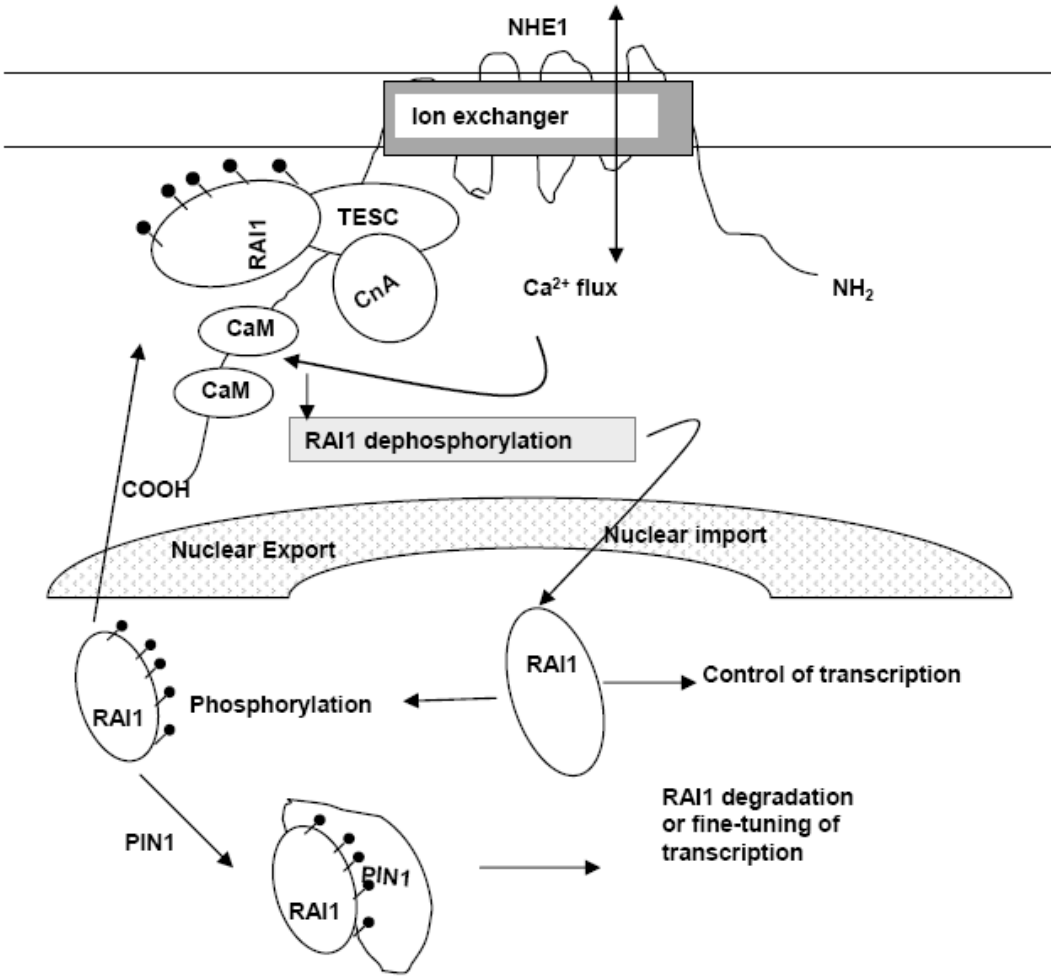

Figure 3

Activation of RAI might be regulated by the NHE1 ion exchanger. In this pathway, the complex consisting of TESC, calcineurin A (CnA) and RAI1 is bond to the carboxyl terminus of the exchanger. The ion exchanger senses changes in Ca^{2+} concentration to transmit a signal to the CaM bound to the carboxyl terminus of the exchanger. The close-proximity of calmodulin to the ternary complex may lead to immediate response to oscillations in Ca^{2+} concentration.

Materials and Methods

Database search. To identify proteins reported to interact with RAI1, we have searched Gene Entrez (26) and BioGRID (13). Via the following links, you can access listing of proteins that interact with

RAI1: http://www.theBioGRID.org/SearchResults/summary/115966

PIN1: http://www.theBioGRID.org/SearchResults/summary/111317

TESC: http://www.theBioGRID.org/SearchResults/summary/120330

NHE1: http://www.theBioGRID.org/SearchResults/summary/112438

A significant number of the reported interactions were discovered in large-scale proteome-mapping of protein-protein interactions (36).

Comparative sequence analysis. We used the BLAST engine at NCBI (26) to identify proteins that showed sequence similarity to human RAI1. The proteins selected for analysis (Figure 1) were aligned using Clustal W (37).

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