NAD kinase controls animal NADP biosynthesis and is modulated via evolutionarily divergent calmodulin-dependent mechanisms

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Nicotinamide adenine dinucleotide phosphate (NADP) is a critical cofactor during metabolism, calcium signaling, and oxidative defense, yet how animals regulate their NADP pools in vivo and how NADP-synthesizing enzymes are regulated have long remained unknown. Here we show that expression of *Nadk*, an NAD⁺ kinase-encoding gene, governs NADP biosynthesis in vivo and is essential for development in *Xenopus* frog embryos. Unexpectedly, we found that embryonic *Nadk* expression is dynamic, showing cell type-specific up-regulation during both frog and sea urchin embryogenesis. We analyzed the NAD kinases (NADKs) of a variety of deuterostome animals, finding two conserved internal domains forming a catalytic core but a highly divergent N terminus. One type of N terminus (found in basal species such as the sea urchin) mediates direct catalytic activation of NADK by Ca²⁺/calmodulin (CaM), whereas the other (typical for vertebrates) is phosphorylated by a CaM kinase-dependent mechanism. This work indicates that animal NADKs govern NADP biosynthesis in vivo and are regulated by evolutionarily divergent and conserved CaM-dependent mechanisms.

metabolism | NADP | calcium signaling | deuterostome animals | NAD kinase

NADP (including the oxidized form NADP⁺ and reduced form NADPH) is one of the most fundamental metabolic molecules inside cells. NADPH serves essential functions in reductive biosyntheses, detoxification processes (mediated by cytochrome P450), and the regeneration of oxidative defense systems. Moreover, it acts as an electron donor for NADPH oxidases, which generate reactive oxygen species for immune cell respiratory bursts and for redox signaling (1). NADP⁺ can also be converted to NAADP, a potent intracellular calcium messenger (2). However, despite its importance for cellular function, surprisingly little is known about how animals regulate their cellular NADP pools.

Earlier work has shown that animals use vitamin B3 to produce NAD, and in vitro studies have established that NAD can be phosphorylated to yield NADP by NAD kinase (NADK) enzymes using ATP as a phosphoryl donor (3–5) (Fig. 1A). However, there have been no in vivo studies examining the function of animal NADK genes, and thus whether NADK is required for sustaining NADP levels in vivo has remained unknown.

In addition, the mechanisms governing the regulation of the NADK enzyme are not well-understood. Previous studies, however, have demonstrated a species-dependent differential response of NADK activity following exposure to calcium/calmodulin (Ca²⁺/CaM). For instance, NADK activity in plant extracts is increased by the addition of Ca²⁺/CaM (6). However, none of the three recombinantly expressed NADK isoforms from *Arabidopsis thaliana* were responsive to Ca²⁺/CaM (7, 8). In animals, a Ca²⁺/CaM-dependent rise of NADP following sea urchin egg fertilization was observed more than 30 y ago, although the molecular mechanisms underlying this phenomenon have not been elucidated.

We previously identified and characterized the human NADK encoded by the gene *Nadk* (1, 9), finding its enzymatic activity insensitive to Ca²⁺/CaM. Thus, the interaction between the NADK enzyme and Ca²⁺/CaM signaling has remained unresolved.

In this study, we endeavored to determine whether expression of the *Nadk* gene controls NADP generation in vivo as well as its regulatory mechanisms. For this, we examined NADKs during frog (*Xenopus tropicalis*) and sea urchin (*Strongylocentrotus purpuratus*) development, finding that, in animals, generation of NADP requires expression of NAD kinase encoded by *Nadk*. We then demonstrate that the animal NADK enzyme is modified by evolutionarily conserved and divergent calcium signaling-dependent regulatory mechanisms.

Results

Nadk Governs NADP Production During *Xenopus* Embryogenesis. To address the in vivo role of *Nadk*, we chose to use the *X. tropicalis*

Significance

Metabolism relies on a set of molecules that provide the chemical framework for all cellular activities. Among these molecules is NADP, a metabolite synthesized from vitamin B3 that is critical for basic metabolism, calcium signaling, and antiinflammatory processes. Despite NADP's fundamental importance, very little is known about how animal cells regulate their NADP pool. This study shows that the enzyme NAD kinase is required for maintaining NADP levels in animals, is essential for embryonic development, and exhibits conserved regulatory mechanisms among evolutionarily diverse animals such as humans and sea urchins. Together, these results reveal new insights into why vitamin B3 is essential and how it is converted to NADP, and suggests new therapeutic avenues to improve human and animal metabolism.

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Fig. 1. *Nadk* controls NADP biosynthesis in vivo. (A) Schematic diagram showing the generation of NADP⁺ from NAD⁺ via NAD kinase. (B) HPLC chromatograms showing the generation of NADP⁺ from NAD⁺ and ATP in the presence of purified recombinant XtnADK versus BSA as control. mAU, milliabsorbance units. (C) Schematic diagram of 6xhis-*nadk* overexpression experiments and immunoblot detection of 6xhis-XtnADK in NF St. 26 embryo extracts. (D, Left) Representative bright-field and fluorescent images of 6xhis-Xtnadk- and eyfp-expressing embryos. 6xhis-Xtnadk overexpressing embryos showed no overt morphological phenotypes versus controls. (Right) Bar graph showing NADP measurements from 6xhis-Xtnadk- or eyfp-overexpressing embryos ($n = 3$). (E) Validation of morpholino-mediated loss-of-*nadk* function experiments. (Top) Western blot analysis showing how the *nadk* atg morpholino (ATG MO) results in a marked reduction but not complete loss of *nadk-ha* expression following coinjection. (Bottom) Bar graph showing RT-qPCR results verifying the loss of the endogenous wild-type *nadk* mRNA transcript following injection of the *nadk* splice morpholino ($n = 3$). cMO, control morpholino. (F) Bar graph showing NADP measurements from control or *nadk* morpholino-injected embryos at NF St. 26 ($n = 4$). (G) Bright-field and fluorescent images of embryos injected with control or *nadk*-suppressing FITC-labeled morpholinos. Embryos injected with *Nadk*-suppressing morpholinos showed a shorter body axis at NF St. 35. Error bars indicate SD. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$ (t test).

frog, a vertebrate embryo model amenable to both metabolomic analyses (10) and genetic manipulation via the microinjection of nucleic acids into fertilized eggs (reviewed in ref. 11). We cloned the putative *X. tropicalis* (*Xt*) NADK gene and verified that the encoded protein possessed NAD⁺ kinase activity in vitro (Fig. 1B, Table 1, and Fig. S14). Next, we injected in vitro transcribed mRNA encoding a 6xHis-tagged NADK (6xhis-Xtnadk) or control (eyfp) mRNA into fertilized eggs. Translation of 6xhis-Xtnadk mRNA was confirmed using Western blot analysis at the early tailbud stage [Nieuwkoop and Faber (NF) stage (St.) 26] (12) (Fig. 1C and D). Notably, a comparison of NADP levels showed that embryos injected with 6xhis-Xtnadk mRNA possessed nearly four times more NADP than eyfp mRNA-injected embryos (Fig. 1D).

We then asked whether *nadk* expression was required for maintaining NADP levels. To perform loss-of-function experiments, we generated antisense oligonucleotide morpholinos that targeted the

ATG start codon (ATG MO) or the intron–exon splice junction border following the first coding exon (splice MO) and confirmed the knockdown efficacy of these reagents using Western blot and RT-quantitative (q)PCR (Fig. 1E). We detected a profound reduction but not complete loss of translation of an *nadk-ha* mRNA encoding a C-terminally HA epitope-tagged XtnADK when coinjected with the ATG MO. This result suggested that most, but not all, of the maternally deposited *Nadk* mRNA transcripts were suppressed using this morpholino.

Injection of *nadk*-targeting morpholinos into fertilized eggs resulted in NF St. 35 embryos with ~50% less total NADP than control morpholino-injected embryos (Fig. 1F). In addition, *nadk* morphant embryos displayed perturbed development into the tadpole stage, as evidenced by shortened anterior–posterior axis lengths (Fig. 1G). To address the specificity of this phenotype, we generated a morpholino-resistant version of *nadk* (Δ *nadk*). We confirmed the NAD⁺ kinase activity of the corresponding Δ NADK protein in vitro (Fig. S1B) and found that coinjection of the Δ *nadk* rescue construct with *nadk*-targeting morpholinos largely restored the reduced anterior–posterior axis phenotype (Fig. 1G and Fig. S1C and D). These data demonstrated that, in the embryonic context, *nadk* expression is required for proper NADP generation in vivo and is necessary for full embryonic growth.

***Nadk* Exhibits Dynamic Expression During Frog and Sea Urchin Embryogenesis.** The lethality of *nadk* morphant frog embryos led us to wonder about the expression pattern of *nadk* during

Table 1. Kinetic parameters of NAD kinases from *H. sapiens*, *X. tropicalis*, and *S. purpuratus*

Protein	K_M (NAD ⁺), μ M	K_M (ATP), μ M	V_{max} , μ mol·(min·mg) ^{−1}
HsNADK*	540	3,300	6.70
XtnADK	756	1,586	2.66
SpNADK-1	540	397	2.27
SpNADK-2	212	263	1.38

*Values for the human NADK were reported earlier (9).

Fig. 3. Evolutionarily divergent and conserved Ca^{2+} /calmodulin-dependent modulation at the N terminus of NAD kinase. (A) Graphical representation of the amino acid (a.a.) conservation of NADK among the deuterostomes listed in B. The y axis shows conservation (greater height indicates greater conservation). The relatively low conservation of the N termini is highlighted in red. (B) Phylogenetic relationship between the deuterostome species examined. The presence of predicted CaM binding sites (Fig. S3A) is indicated. (C) Effect of Ca^{2+} /CaM exposure on the NAD^{+} kinase activity of SpNADK-1 and SpNADK-2 proteins. Error bars indicate SD. * $P < 0.05$; *** $P < 0.001$ (t test). (D) Proposed feedback loop underlying the increase in NADP following sea urchin fertilization [NADP $^{+}$ -derived NAADP and 2'-phospho-cyclic ADP-ribose (2'-P-cADPr) are potent intracellular activators of Ca^{2+} release (1, 2)]. (E) Autoradiograph demonstrating in vitro phosphorylation of SpNADK proteins using ^{32}P -labeled ATP and HeLa cell extracts. The 6xHis immunoblot confirms equal loading. (F) Ca^{2+} /CaM-mediated phosphorylation of SpNADK-1 wild type and N-terminally truncated mutants. (G, Left) Autoradiograph and immunoblot of in vitro phosphorylated recombinant SpNADK-1 using extracts from HeLa cells overexpressing HA-CaMKII. (Right) Autoradiograph of purified SpNADK-1 incubated with Ca^{2+} /CaM and extracts from HeLa control cells (MOCK) or cells overexpressing the CaMKII autoinhibitory peptide (AIP). (H) Ca^{2+} /CaM-dependent in vitro phosphorylation of HsNADK using HeLa cell extract. (I) In vitro phosphorylation of HsNADK by extracts from HeLa cells expressing HA-CaMKII (Left) or autoinhibitory peptide (Right). Closed red arrowheads indicate the phosphorylated HsNADK (autoradiographs). The open red arrowhead indicates the Coomassie-stained HsNADK. (J) In vitro phosphorylation as in H of HsNADK WT and N-terminally truncated mutants.

N termini in the deuterostome species, namely those with a putative N-terminal CaM-binding domain (*Ciona intestinalis*, *Ciona savynyi*, and *S. purpuratus* NADK-2) and those without (vertebrate species) (Fig. 3B and Fig. S3A).

To test whether Ca^{2+} /CaM could directly alter NADK activity, we first examined the NADKs of *S. purpuratus*, because this species possessed both CaM binding site-containing and -lacking NADKs (Fig. 3B). We produced bacterial purifications of SpNADK-1 and SpNADK-2 (Fig. S2C) and found that the in vitro NADK activity of SpNADK-2 (but less so for SpNADK-1) was increased to over 300% following addition of Ca^{2+} and CaM (Fig. 3C). Neither CaM nor Ca^{2+} [a CaM activator (17)] alone had an effect on activity. We confirmed that CaM sensitivity was conferred by the isoform-specific N-terminal 91 amino acids of SpNADK-2 by generating an N-terminally truncated but enzymatically active SpNADK (comprising the sequence common to both isoforms), which was insensitive to Ca^{2+} /CaM (1.17 ± 0.07 vs. 1.07 ± 0.06 U/mg in the absence and presence of Ca^{2+} /CaM, respectively).

These data suggest that Ca^{2+} /CaM activates SpNADK-2 directly via its N-terminal region. Given that SpNADK-2 expression is high in the egg (Fig. 2B) and that NADP $^{+}$ can be directly converted to

the potent Ca^{2+} activators NAADP and cyclic phospho-ADP-ribose (1, 2, 18), we posit the existence of an amplification loop that ensures increased NADP biosynthesis following sea urchin egg fertilization (Fig. 3D). Such a mechanism would provide a rationale for the role of Ca^{2+} signaling in the increase of NAD kinase activity previously witnessed in sea urchin eggs (13).

In contrast to SpNADK-2, we found that Ca^{2+} /CaM had little effect on the catalytic activity of human (9) or SpNADK-1 (Fig. 3C). In the search for potential alternative effects of Ca^{2+} /CaM on SpNADK-1, we noted that a recent large-scale phosphoproteomics study uncovered the phosphorylation of NADK in mouse liver (19). Because protein kinase activity is known to be activated by Ca^{2+} /CaM (20, 21), we wondered whether CaM-dependent kinases might function to phosphorylate NADK. To test this possibility, we incubated SpNADK-1 and SpNADK-2 with HeLa cell extracts and [^{32}P]ATP and found that SpNADK-1 but, interestingly, not Ca^{2+} /CaM-activated SpNADK-2 was phosphorylated in a CaM-dependent manner (Fig. 3E). Using N-terminally truncated SpNADK-1 proteins, we confined the phospho-sensitive region to the first 30 amino acids and, by mutating the serine residues in this region, we found that serine 18

eukaryotic expression vector pFLAG-CMV-5a (Sigma) was achieved via the EcoRI and KpnI restriction sites. Plasmids for prokaryotic and eukaryotic expression of human NADK have been established previously (9, 28). All deletion and point mutations were generated by PCR-based in vitro mutagenesis.

In Situ Hybridization. Digoxigenin (DIG)-labeled probes were created using 10× DIG-labeling nucleotide mix (Roche) and T7 RNA polymerase (Roche), and purification was performed using BioRad Micro Bio-Spin 30 columns. *Xenopus* hybridizations were performed as previously described (29) and sea urchin hybridization as previously described (30).

Protein Alignments. Protein alignments were performed using the MUSCLE algorithm (31). Putative CaM binding sites were identified using the Calmodulin Target Database (calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html).

RT-qPCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the High Capacity cDNA Kit (Applied Biosystems). TaqMan assays were developed to target exon–exon boundaries, and qPCR reactions were run with TaqMan Fast Gene Expression Master Mix (Applied Biosystems) on a StepOnePlus qPCR machine (Applied Biosystems). Further details are described in *SI Methods and Materials*.

NADK Protein Expression and Purification. SpNADK-1, SpNADK-2, and human NADK were expressed in *Escherichia coli* BL21 Codon Plus-RIL cells (Stratagene) transformed with pREP4 (Qiagen). XtNADK was expressed in *E. coli* XL1 Blue cells (Stratagene). Overexpression was achieved by addition of 0.8 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 25 °C overnight. Cells were harvested and sonicated in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 6.5, 10 mM imidazole, 20 mM 2-mercaptoethanol). After centrifugation at 20,400 × g for 30 min at 4 °C, the recombinant proteins were purified from the supernatant using Ni-NTA agarose (Qiagen). Buffer exchange of protein aliquots was done using a desalting spin column (Pierce) equilibrated with 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 10 mM DTT.

NADP Measurements. NADP levels were measured using a commercially available assay according to the provided protocol (Abcam). Twenty embryos were homogenized with a pestle in 500 μL ice-cold extraction buffer and

spun down, and supernatants were passed through 10,000 molecular weight cutoff filters before detection (BioVision).

NADK Activity Assay. NADK activity and substrate specificity were determined by HPLC analysis or spectrophotometrically as described (6). Reactions contained 5 mM ATP and 2.5 mM NAD⁺ in 100 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 1 mM glucose-6-phosphate, and 5 U glucose-6-phosphate dehydrogenase. Kinetic measurements were carried out with varying concentrations of one substrate. Apparent *K_M* and *V_{max}* values were calculated by nonlinear regression using SigmaPlot (Systat Software).

Cell Culture, Transfection, and Preparation of Cell Extracts. HeLa cells were grown in Eagle's MEM with 10% (vol/vol) FCS, penicillin (50 U/mL), streptomycin (50 μg/mL), 2 mM L-glutamine, and 1% Na pyruvate. Cells were transfected using Effectene (Qiagen). For lysates, cells were scraped in ice-cold buffer (10 mM Hepes/KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM Pefabloc), incubated 10 min on ice, and lysed with 0.5% Nonidet P-40. After centrifugation at 16,100 × g for 5 min at 4 °C, the supernatant was used for in vitro phosphorylation assays.

In Vitro Phosphorylation Assay. Phosphorylation of NADK proteins was analyzed in reactions (20-μL) containing 10 mM Hepes/KOH (pH 7.9), 20 mM MgCl₂, 50 mM KCl, 100 mM NaCl, 200 μM EDTA, 1 mM DTT, 100 μM ATP, 1 μCi [γ -³²P]ATP (GE Healthcare), 1.5 μg recombinant NADK, and 6–10 μg cell extract. CaCl₂ (1 mM) and calmodulin (0.2 μg) (from bovine testis; Sigma) were added as indicated. Reactions were incubated at 30 °C and terminated by addition of SDS sample buffer.

Statistics. Statistical analyses were performed with Prism (GraphPad Software). Datasets were first checked for variance, and the appropriate *t* tests were used to generate *P* values.

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