Discoveries of Nicotinamide Riboside as a Nutrient and Conserved *NRK* Genes Establish a Preiss-Handler Independent Route to NAD$^+$ in Fungi and Humans

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Summary

NAD$^+$ is essential for life in all organisms, both as a coenzyme for oxidoreductases and as a source of ADPribosyl groups used in various reactions, including those that retard aging in experimental systems. Nicotinic acid and nicotinamide were defined as the vitamin precursors of NAD$^+$ in Elvehjem's classic discoveries of the 1930s. The accepted view of eukaryotic NAD$^+$ biosynthesis, that all anabolism flows through nicotinic acid mononucleotide, was challenged experimentally and revealed that nicotinamide riboside is an unanticipated NAD$^+$ precursor in yeast. Nicotinamide riboside kinases from yeast and humans essential for this pathway were identified and found to be highly specific for phosphorylation of nicotinamide riboside and the cancer drug tiazofurin. Nicotinamide riboside was discovered as a nutrient in milk, suggesting that nicotinamide riboside is a useful compound for elevation of NAD$^+$ levels in humans.

Introduction

In 1938, the pioneering vitamin-hunter Conrad Elvehjem and his coworkers put dogs on a synthetic diet supplemented with only the known B vitamins. When the dogs were near death and exhibited pellagra-like black tongue symptoms, the investigators fed the animals small-molecule fractions derived from liver. In this manner, nicotinic acid and nicotinamide, now collectively termed niacin, were identified as the "anti-black tongue factor" with essential nutritional activity (Elvehjem et al., 1938). Because niacins are the vitamin forms of nicotinamide adenine dinucleotide (NAD$^+$), and eukaryotes also synthesize NAD$^+$ de novo via the kynurenine pathway from tryptophan (Krehl et al., 1945; Schutz and Feigelson, 1972), niacin supplementation prevents the pellagra that can occur in populations with a tryptophan-poor diet. In 1958, Jack Preiss and Philip Handler determined that nicotinic acid is phosphoribosylated to nicotinic acid mononucleotide (NaMN), which is then adenylylated to form nicotinic acid adenine dinucleotide (NaAD), which in turn is amidated to form NAD$^+$ (Preiss and Handler, 1958a, 1958b).

NAD$^+$ was initially characterized as a coenzyme for oxidoreductases. Though conversions between NAD$^+$, NADH, NADP, and NADPH would not be accompanied by a loss of total coenzyme, it was discovered that NAD$^+$ is also turned over in cells for unknown purposes (Maayan, 1964). In 2000, it became clear that Sir2 and Sir2-related enzymes termed Sirtuins deacetylate lysine residues with consumption of an equivalent of NAD$^+$ and that this activity is required for Sir2 function as a transcriptional silencer (Imai et al., 2000). It was also demonstrated that the Preiss-Handler pathway is required for normal silencing activity in vivo (Smith et al., 2000). NAD$^+$-dependent deacetylation reactions are required not only for alterations in gene expression but also for repression of ribosomal DNA recombination and extension of lifespan in response to calorie restriction (Lin et al., 2000, 2002). NAD$^+$ is consumed by Sir2 to produce a mixture of 2'- and 3' O-acetylated ADPribose plus nicotinamide and the deacetylated polypeptide (Sauve et al., 2001). Additional enzymes, including poly (ADPribose) polymerases and cADPribose synthases are also NAD$^+$-dependent and produce nicotinamide and ADPribosyl products (Ziegler, 2000; Burkle, 2001).

Interest in the noncoenzymatic properties of NAD$^+$ has rekindled interest in NAD$^+$ biosynthesis. In the last two years, at least five publications have schematized NAD$^+$ biosynthesis in yeast as shown in Figure 1 (Panozzo et al., 2002; Sandmeier et al., 2002; Bitterman et al., 2002; Anderson et al., 2003; Gallo et al., 2004). This scheme depicts a convergence of the flux to NAD$^+$ from de novo synthesis, nicotinic acid import, and nicotinamide salvage at NaMN. This is somewhat surprising because biochemists from 1950 to the present day have been characterizing the Nma1 and Nma2 gene products from yeast and their human homologs as NMN adenylyltransferases (Kornberg, 1950; Emanuelli et al., 1999, 2003; Garavaglia et al., 2002) or as dual specificity enzymes that will use either NaMN or NMN as a substrate (Zhou et al., 2002).

In this study, we show that nicotinamide riboside, which was known to be an NAD$^+$ precursor in bacteria such as *Haemophilus influenza* (Gingrich and Schlenk, 1944; Leder and Handler, 1951; Shifrine and Biberstein, 1960) that lack the enzymes of the de novo and Preiss-Handler pathways (Fleischmann et al., 1995), is an NAD$^+$ precursor in a previously unknown but apparently conserved eukaryotic NAD$^+$ biosynthetic pathway. We identify yeast nicotinamide riboside kinase, Nrk1, and both human Nrk enzymes and demonstrate their specific functions in NAD$^+$ metabolism biochemically and genetically. Their specificity suggests additionally that they are the long-sought tiazofurin kinases that perform the first step in converting cancer drugs such as tiazofurin and benzamide riboside into toxic NAD$^+$ analogs (Cooney et al., 1983). Finally, we utilize yeast mutants of defined genotype to hunt for vitamins in a pathway-specific manner and show that milk is a source of nicotinamide riboside.

Results

Recently, we characterized the *S. cerevisiae QNS1* gene encoding glutamine-dependent NAD$^+$ synthetase and showed that mutation of either the glutaminase active
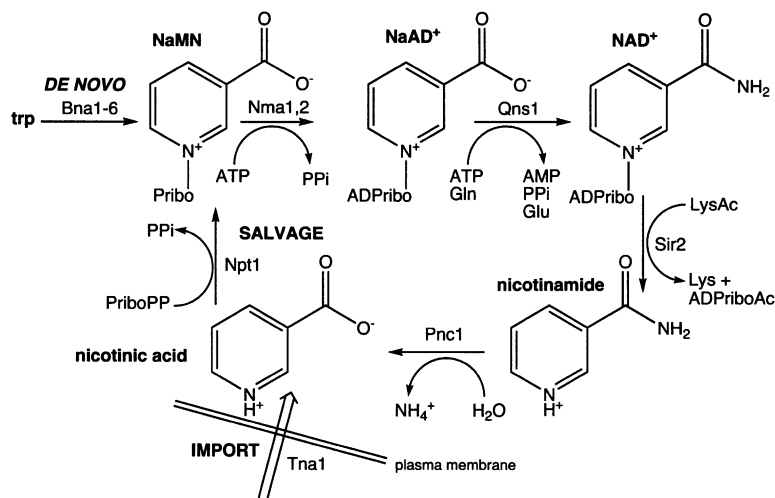
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Figure 1. Three Known Biosynthetic Routes to NAD$^+$ in *S. cerevisiae*

NAD$^+$ is synthesized from the de novo kynurenine pathway that originates with tryptophan using the Bna1-Bna6 gene products, an import pathway that originates with nicotinic acid, and a salvage pathway that utilizes nicotinamide produced as a function of Sir2-related lysine deacetylases. According to this scheme, nicotinic acid mononucleotide (NaMN) is common to all three pathways and Qns1, the glutamine-dependent NAD$^+$ synthetase that converts nicotinic acid adenine dinucleotide (NaAD) to nicotinamide adenine dinucleotide (NAD$^+$), is required for all pathways (Panozzo et al., 2002; Sandmeier et al., 2002; Bitterman et al., 2002; Anderson et al., 2003; Gallo et al., 2004). Note that Nma1 and Nma2 are schematized as NaMN adenylyltransferases, though they were initially characterized as nicotinamide mononucleotide (NMN) adenylyltransferases (Kornberg, 1950; Emanuelli et al., 1999, 2003).

site or the NAD$^+$ synthetase active site resulted in inviable cells (Bieganowski et al., 2003). Possession of strains containing the *qns1* deletion and a plasmid-borne *QNS1* gene allowed us to test whether the canonical de novo, import, and salvage pathways for NAD$^+$ as depicted in Figure 1 (Panozzo et al., 2002; Sandmeier et al., 2002; Bitterman et al., 2002; Anderson et al., 2003; Gallo et al., 2004) are a complete representation of the metabolic pathways to NAD$^+$ in *S. cerevisiae*. This scheme makes two specific predictions. First, because nicotinamide is deamidated to nicotinic acid before the pyridine ring is salvaged to make more NAD$^+$, supplementation with nicotinamide should not rescue *qns1* mutants by shunting nicotinamide-containing precursors through the pathway. Second, because *QNS1* is common to the three pathways, there should be no NAD$^+$ precursor that rescues *qns1* mutants. As shown in Figure 2A, consistent with the scheme's implicit assumptions about nicotinamide metabolism, nicotinamide does not rescue *qns1* mutants even at 1 or 10 mM. However, apart from any intermediate or enzymatic transformation depicted in the scheme, nicotinamide riboside, which was characterized as an NAD$^+$ precursor in bacteria such as *Haemophilus influenza* (Gingrich and Schlenk, 1944; Leder and Handler, 1951; Shifrine and Biberstein, 1960), functions as a vitamin form of NAD$^+$ at 10 μM.

Two independent lines of evidence suggest that a pathway to NAD$^+$ from nicotinamide riboside may exist in fungi and other eukaryotes. First, though the recent literature (Panozzo et al., 2002; Sandmeier et al., 2002; Bitterman et al., 2002; Anderson et al., 2003; Gallo et al., 2004) depicts all of the metabolic flux through NaMN, the enzymes placed on this scheme as NaMN adenylyltransferases were initially classified as NMN adenylyltransferases (Kornberg, 1950; Emanuelli et al., 1999, 2003; Garavaglia et al., 2002) or as dual specificity NMN/NaMN adenylyltransferases (Zhou et al., 2002). Though it is possible that eukaryotic adenylyltransferases were misclassified, it is reasonable to ask whether eukaryotes have a pathway that produces NMN to feed into these enzymes.
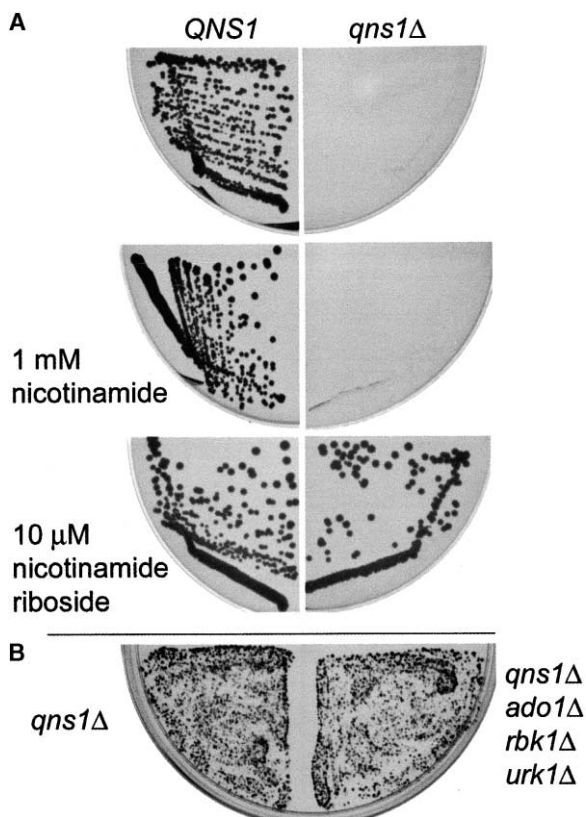

Figure 2. Nicotinamide Riboside Allows NAD$^+$ Synthetase-Independent Growth Via a Novel Pathway

(A) *qns1* cells transformed with a plasmid carrying the *QNS1* and *URA3* genes were tested for growth on synthetic dextrose complete media and 5-fluoroorotic acid (5-FOA). NAD$^+$ synthetase mutant *qns1* is inviable without supplements or with 1 mM nicotinamide but is rescued by 10 μM nicotinamide riboside.

(B) Strains with indicated mutations were plated on medium supplemented with 5-FOA and 10 μM nicotinamide riboside. Deletion of genes for uridine/cytosine kinase, adenosine kinase, and ribokinase do not alter the ability of yeast cells to utilize nicotinamide riboside.
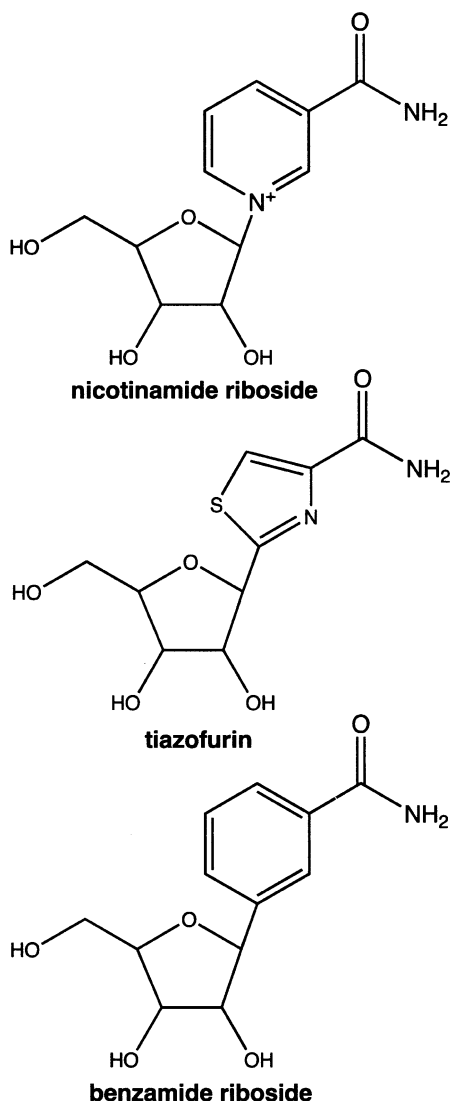
Figure 3. Nicotinamide Riboside and its Analogs, Tiazofurin and Benzamide Riboside

Nicotinamide riboside, first reported as the lowest molecular weight "V factor" for *Haemophilus influenza* (Gingrich and Schlenk, 1944), is shown alongside the IMP dehydrogenase prodrugs, tiazofurin (Cooney et al., 1983), and benzamide riboside (Krohn et al., 1992). NMN and its analogs are produced via phosphorylation of the 5′ hydroxyl groups.

Second, anticancer agents such as tiazofurin (Cooney et al., 1983) and benzamide riboside (Krohn et al., 1992) have been shown to be metabolized intracellularly to NAD$^+$ analogs tiazofurin adenine dinucleotide and benzamide adenine dinucleotide, which inhibit IMP dehydrogenase, the rate-limiting enzyme for guanine nucleotide biosynthesis. As these compounds can be considered analogs of nicotinamide riboside (Figure 3), generation of NAD$^+$ analogs would necessarily involve phosphorylation of the 5′ hydroxyl group and subsequent adenylylation of these intermediates. Though an NMN/NaMN adenylyltransferase is thought to be the enzyme that converts the mononucleotide intermediates to NAD$^+$ analogs and the structural basis for this has

been established (Zhou et al., 2002), several different enzymes including adenosine kinase, 5′ nucleotidase (Fridland et al., 1986; Saunders et al., 1990) and a specific nicotinamide riboside kinase (Saunders et al., 1990) have been proposed to be responsible for tiazofurin phosphorylation in vivo. Indeed, a putative nicotinamide riboside kinase (Nrk) activity was reportedly purified but no amino acid sequence information was obtained and, as a consequence, no genetic test was ever performed to assess its function in nutrient or drug metabolism (Sasiak and Saunders, 1996).

To test whether any of the nucleoside kinases proposed to phosphorylate tiazofurin are uniquely or collectively responsible for utilization of nicotinamide riboside, we prepared a *qns1* deletion strain that was additionally deleted for all of the candidate genes for which yeast homologs exist, namely adenosine kinase *ado1* (Lecoq et al., 2001), uridine/cytidine kinase *urk1* (Kern, 1990; Kurtz et al., 1999), and ribokinase *rbk1* (Thierry et al., 1990). As shown in Figure 2B, despite these deletions, the strain retained the ability to utilize nicotinamide riboside in an anabolic pathway independent of NAD$^+$ synthetase. Given that mammalian pharmacology provided no useful clue to the identity of a putative fungal Nrk, we considered whether the gene might have been conserved with the Nrk of *H. influenza*. The Nrk domain of *H. influenza* is encoded by amino acids 225 to 421 of the NadR gene product (the amino terminus of which is NMN adenylyltransferase). Though this domain is structurally similar to yeast thymidylate kinase (Singh et al., 2002), sensitive sequence searches (not shown) revealed that bacterial Nrk has no ortholog in yeast. Indeed, genomic searches with the Nrk domain of *H. influenza* NadR have identified a growing list of bacterial genomes predicted to utilize nicotinamide riboside as an NAD$^+$ precursor (Kurnasov et al., 2002). Thus, had fungi possessed NadR Nrk-homologous domains, comparative genomics would have already predicted that yeast can salvage nicotinamide riboside.

To identify the Nrk of *S. cerevisiae*, we established an HPLC assay for the enzymatic activity and utilized a biochemical genomics approach to screen for the gene encoding this activity (Martzen et al., 1999). Sixty-four pools of 90–96 *S. cerevisiae* open reading frames fused to glutathione *S*-transferase (GST), expressed in *S. cerevisiae*, were purified as GST fusions and screened for the ability to convert nicotinamide riboside plus ATP to NMN plus ADP. As shown in Figure 4A, whereas most pools contained activities that consumed some of the input ATP, only pool 37 consumed nicotinamide riboside and produced NMN. Examination of the 94 open reading frames that were used to generate pool 37 revealed that YNL129W encodes a predicted 240 amino acid polypeptide with a 187 amino acid segment containing 23% identity with the 501 amino acid yeast uridine/cytidine kinase Urk1 and remote similarity with a segment of *E. coli* pantothenate kinase panK (Yun et al., 2000) (Figure 4B). Cloning of YNL129W into a bacterial expression vector allowed us to test the hypothesis that this homolog of metabolite kinases is the eukaryotic Nrk. Strikingly, the specific activity of purified YNL129W was ~100 times that of pool 37, consistent with the idea that all the Nrk activity of pool 37 was encoded by this open reading frame. To test genetically whether this gene
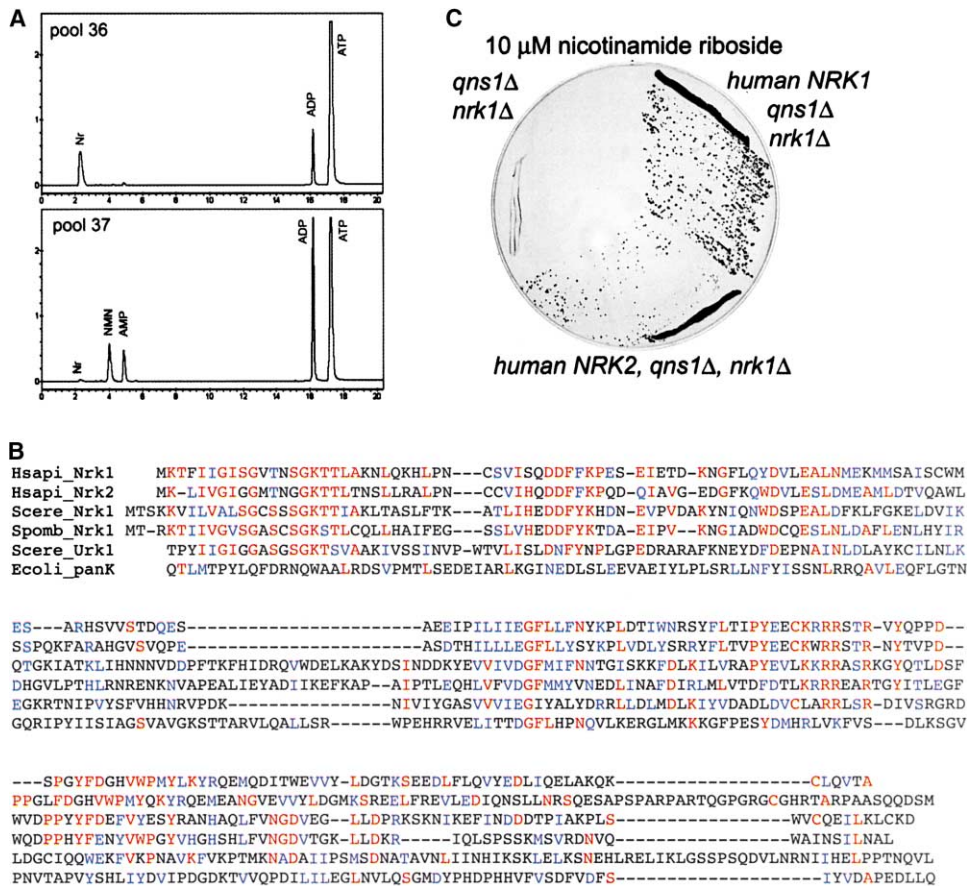
Figure 4. Cloning and Genetic Validation of Yeast and Human Nicotinamide Riboside Kinases

(A) HPLC traces of a negative pool (36) and positive pool (37) of GST-ORF fusions. In pool 36, some ATP was converted to ADP and production of AMP occurred in several pools including 37. In pool 37, approximately half of the 1 mM ATP was converted to ADP and the 500 μM Nr peak was almost entirely converted to NMN.

(B) Amino acid sequence alignment of human Nrk1, human Nrk2, *S. cerevisiae* Nrk1, *S. pombe* nrk1, and portions of *S. cerevisiae* uridine/cytidine kinase Urk1 and *E. coli* pantothenate kinase.

(C) *nrk1* deletion introduced into a *qns1* deletion strain blocks the ability to form colonies in the presence of nicotinamide riboside. Expression of human *NRK1* or human *NRK2* cDNAs restores growth to *qns1 nrk1* deletion strains, indicating that human Nrk1 and Nrk2 are authentic nicotinamide riboside kinases in vivo.

product phosphorylates nicotinamide riboside in vivo, we created a deletion of YNL129W in the *qns1* background and found that nicotinamide riboside rescue of the *qns1* deletion strain is entirely dependent on this gene product (Figure 4C). Having shown biochemically and genetically that YNL129W encodes an authentic Nrk activity, we named this gene *NRK1*.

We ran PSI-BLAST (Altschul et al., 1997) on the predicted *S. cerevisiae* Nrk1 polypeptide and discovered the apparent orthologous human protein Nrk1 (locus NP_060351) encoded at 9q21.31, encoding a polypeptide of 199 amino acids annotated as an uncharacterized protein of the uridine kinase family. In addition, we found

a second human gene product Nrk2 (locus NP_733778) that is 57% identical to human Nrk1. Nrk2 is a 230 amino acid splice form of what was described as a 186 amino acid muscle integrin β1 binding protein (ITGB1BP3) encoded at 19p13.3 (Li et al., 1999, 2003). Amino acid conservation between *S. cerevisiae*, *S. pombe*, and human Nrk homologs and similarity with fragments of *S. cerevisiae* Urk1 and *E. coli* panK is shown in Figure 4B. As shown in Figure 4C, complementation of the failure of *qns1 nrk1* to grow on nicotinamide riboside-supplemented media was provided by human *NRK1* and human *NRK2* cDNAs expressed from the yeast *GAL1* promoter.

Table 1. Specific Activity (nmole mg^{-1} min^{-1}) of human Nrk1, Nrk2, and yeast Nrk1 for Phosphorylation of Nucleoside Substrates

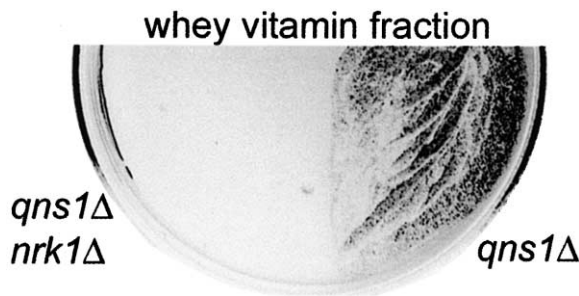|	Nicotinamide riboside	Tiazofurin	Uridine	Cytidine
Human Nrk1	275 ± 177	538 ± 277	19.3 ± 1.77	35.5 ± 6.44
Human Nrk2	2320 ± 200	2150 ± 2100	2220 ± 170	222 ± 88
Yeast Nrk1	535 ± 600	1129 ± 1344	15.2 ± 3.44	82.9 ± 4.44

Figure 5. Nicotinamide Riboside Is Present in an Acid Whey Vitamin Fraction of Cow's Milk

Yeast *qns1* mutants grow when supplemented with whey in an *NRK1*-dependent manner, indicating that whey is a source of nicotinamide riboside.

Purification of yeast Nrk1 and human Nrk1 and Nrk2 revealed high specificity for phosphorylation of nicotinamide riboside and tiazofurin (Table 1). In the cases of yeast and human Nrk1, the enzymes actually prefer tiazofurin to the natural substrate nicotinamide riboside by a factor of two and both enzymes retain less than 7% of their maximal specific activity on uridine and cytidine. In the case of human Nrk2, the 186 amino acid integrin β1 binding protein form is devoid of enzymatic activity (data not shown). On the other hand, the 230 amino acid form is essentially equally active on nicotinamide riboside, tiazofurin, and uridine with less than 10% of corresponding activity on cytidine. Thus, though Nrk2 may contribute additionally to formation of uridylate in the tissues in which it is expressed, these data demonstrate that fungi and mammals possess specific Nrks that function to synthesize NAD$^+$ through NMN in addition to the well-known pathways through NaMN. Identification of Nrk enzymatic activities thus accounts for the dual specificity of fungal and mammalian NaMN/NMN adenylyltransferases.

We used the yeast *qns1* mutant to screen for natural sources of nicotinamide riboside and, as shown in Figure 5, we found it in a vitamin fraction of cow's milk. Unlike the original screen for vitamins in protein-depleted extracts of liver for reversal of black tongue in starving dogs (Elvehjem et al., 1938), this assay is pathway-specific in identifying NAD$^+$ precursors. As shown in Figures 1 and 2, because of the *qns1* deletion, nicotinic acid and nicotinamide do not score positively in this assay. Because the factor from milk requires nicotinamide ribo-

side kinase for growth, the nutrient is clearly nicotinamide riboside and not NMN or NAD$^+$.

Discussion

A revised metabolic scheme for NAD$^+$, incorporating Nrk1 homologs and the nicotinamide riboside salvage pathway is shown in Figure 6. A little appreciated difference between humans and yeasts concerns the organisms' uses of nicotinamide and nicotinic acid, the compounds coidentified as anti black tongue factor (Elvehjem et al., 1938). Humans encode a homolog of the *Haemophilus ducreyi nadV* gene, termed pre-B-cell colony enhancing factor, that may convert nicotinamide to NMN (Rongvaux et al., 2002), which is highly induced during lymphocyte activation (Samal et al., 1994). In contrast, *S. cerevisiae* lacks a homolog of *nadV* and instead has a homolog of the *E. coli pncA* gene, termed *PNC1*, that converts nicotinamide to nicotinic acid for entry into the Preiss-Handler pathway (Ghislain et al., 2002; Sandmeier et al., 2002). Though the Preiss-Handler pathway is frequently considered a salvage pathway from nicotinamide, it technically refers to the steps from nicotinic acid to NAD$^+$ (Preiss and Handler, 1958a, 1958b). Reports that nicotinamidase had been purified from mammalian liver in the 1960s (Petrack et al., 1965) may have contributed to the sense that fungal and animal NAD$^+$ biosynthesis is entirely conserved. However, animal genes for nicotinamidase have not been identified and there is no compelling evidence that nicotinamide and nicotinic acid are utilized as NAD$^+$ precursors through the same route in mammals. The persistence of "niacin" as a mixture of nicotinamide and nicotinic acid may attest to the utility of utilizing multiple pathways to generate NAD$^+$ and suggests that supplementation with nicotinamide riboside as third importable NAD$^+$ precursor may be beneficial for certain conditions.

Whereas nicotinamide metabolism is not conserved between vertebrates and fungi, presence of *NRK* genes suggests that the nicotinamide riboside kinase pathway is conserved more broadly in eukaryotes, though it is likely to be time and tissue-restricted in animals.

First reported in 1955, high doses of nicotinic acid are effective at reducing cholesterol levels (Altschul et al., 1955). Since the initial report, many controlled clinical studies have shown that nicotinic acid preparations, alone and in combination with HMG co-A reductase inhibitors, are effective in controlling low-density lipo-
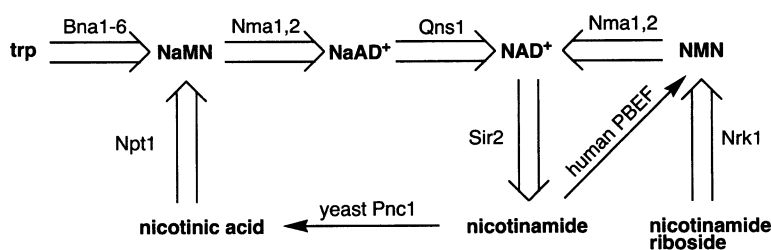

Figure 6. NAD$^+$ Metabolism in Humans and Yeast

A revised scheme for NAD$^+$ metabolism in which double arrows depict steps common to yeast and humans (with yeast gene names). Single arrows depict steps unique to humans (PBEF, nicotinamide phosphoribosyltransferase) and yeast (Pnc1, nicotinamidase). Additional gene products are responsible for some of the steps. For example, there are multiple Sir2-related lysine deacetylases in humans and yeast and additional NAD$^+$ glycohydrolases in humans along the arrow marked Sir2, and there are two Nrk enzymes in humans along the arrow marked Nrk1. The scheme remains incomplete in that there are enzyme and E.C. names for some additional possible steps in NAD$^+$ biosynthesis, particularly for breakdown of NAD$^+$ to nicotinamide riboside (Magni et al., 2004), that have not been genetically validated.

protein cholesterol, increasing high-density lipoprotein cholesterol, and reducing triglyceride and lipoprotein a levels in humans (Pasternak et al., 1996). Though nicotinic acid treatment effects all of the key lipids in the desirable direction and has been shown to reduce mortality in target populations (Pasternak et al., 1996), its use is limited because of a side effect of heat and redness termed "flushing," which is significantly effected by the nature of formulation (Capuzzi et al., 2000). Thus, it will be important to test whether nicotinamide riboside supplementation is a preferred route to improve lipid profiles in humans. Additionally, study of the expression and regulation of NAD⁺ biosynthetic enzymes is expected to reveal approaches to sensitize tumors to compounds such as tiazofurin, to protect normal tissues from the toxicity of compounds such as tiazofurin adenine dinucleotide, and to stratify patients for the most judicious use of tiazofurin chemotherapy.

Experimental Procedures

S. cerevisiae Strains
Yeast diploid strain BY165, heterozygous for *qns1* deletion and haploid BY165-1d carrying a chromosomal deletion of *qns1* gene, transformed with plasmid pB175 containing *QNS1* and *URA3* were described previously (Bieganowski et al., 2003). Genetic deletions were introduced by direct transformation with PCR products (Brachmann et al., 1998) generated from primers listed in Supplemental Data available at http://www.cell.com/cgi/content/full/117/4/495/DC1. Plating on media containing 5-fluoroorotic acid (Boeke et al., 1987) was after growth for 24 hr on complete media. The *ado1* disruption cassette was constructed by PCR with primers 7041 and 7044 and plasmid pRS413 as a template. Yeast strain BY165 was transformed with this PCR product, and homologous recombination in histidine prototrophic transformants was confirmed by PCR with primers 7042 and 7043. This strain was transformed with plasmid pB175 and subjected to sporulation and tetrad dissection. One of the resulting haploids carrying *qns1* and *ado1* deletions and plasmid was selected for further experiments and named BY237. The *urk1* deletion was introduced into strain BY237 by transformation with the product of the PCR amplification that used pRS415 as a template and primers 7051 and 7052. Disruption was confirmed by PCR with primers 7053 and 7054, and the resulting strain was named BY247. The *rbk1* disruption cassette was constructed by PCR with primers 7063 and 7065 and plasmid pRS411 as a template. Disruption was introduced into strain BY242 by transformation with the product of this reaction and confirmed by PCR with primers 7062 and 7064. The resulting strain, carrying deletions of *qns1*, *ado1*, *urk1*, and *rbk1* genes was named BY252. A *qns1* yeast strain carrying disruption of the *nrk1* locus was made by transformation of strain BY165-1d with an *nrk1Δ::HIS3* cassette generated by PCR with primers 4750 and 4751 and plasmid pRS413 as template. Correct integration of the *HIS3* marker into the *nrk1* locus was confirmed by PCR with primers 4752 and 4753.

Nicotinamide Riboside and Whey Preparations
NAD⁺ (Sigma) concentration was determined by conversion to NADH with alcohol dehydrogenase using an absorption coefficient (340 nm) of 6200 cm⁻¹ M⁻¹. The concentration of NMN was determined by converting NAD⁺ to NMN plus AMP with rattlesnake venom NAD⁺ pyrophosphatase (E.C. 3.6.9.1, Sigma). Using 15,400 cm⁻¹ M⁻¹ as the absorption coefficient for AMP at 259 nm, we used relative peak areas to calculate the absorption coefficient (259 nm) of NMN to be 4740 cm⁻¹ M⁻¹. To prepare nicotinamide riboside, 120 μmol NMN (Sigma, concentration corrected by absorption) was treated with 1250 units of calf intestinal alkaline phosphatase (Sigma) for 1 hr at 37°C in 1 ml 100 mM NaCl, 20 mM Tris [pH 8.0], 5 mM MgCl₂. After hydrolysis of NMN to nicotinamide riboside was verified by HPLC, phosphatase was removed by centrifuging the reaction through a 5000 Da filter (Millipore). A whey vitamin fraction

of commercial nonfat cow's milk was prepared by adjusting the pH to 4 with HCl, stirring at 55°C for 10 min, removal of denatured casein by centrifugation, and passage through a 5000 Da filter. In yeast media, nicotinamide riboside was used at 10 μM and whey vitamin fraction at 50% by volume.

Yeast GST-ORF Library
Preparation of the fusion protein library was as described (Martzen et al., 1999; Phizicky et al., 2002) at a 500 ml culture scale for each of the 64 pools of 90–96 protein constructs. 10% of each pool preparation was assayed for Nrk activity in overnight incubations.

Nicotinamide Riboside Phosphorylation Assays
Reactions (0.2 ml), containing 100 mM NaCl, 20 mM Na HEPES [pH 7.2], 5 mM β-mercaptoethanol, 1 mM ATP, 5 mM MgCl₂, and 500 μM nicotinamide riboside or alternate nucleoside were incubated at 30°C and terminated by addition of EDTA to 20 mM and heating for 2 min at 100°C. Specific activity assays, containing 50 ng to 6 μg enzyme depending on the enzyme and substrate, were incubated for 30 min at 30°C to maintain initial rate conditions. Reaction products were analyzed by HPLC on a strong anion exchange column with a 10 mM to 750 mM gradient of KPO₄ [pH 2.6].

NRK Gene and cDNA Cloning and Enzyme Purification
The *S. cerevisiae NRK1* gene was amplified from total yeast DNA with primers 7448 and 7449. The amplified DNA fragment was cloned in vector pSGA04 (Ghosh and Lowenstein, 1997) for *E. coli* expression using restriction sites for NdeI and XhoI included in primer sequences and the resulting plasmid was named pB446. Samples of cDNA made from human lymphocytes and spleen were used as a template for amplification of human *NRK1* performed with primers 4754 and 4755. Plasmid pB449 was created by cloning of the product of this reaction between restriction sites NcoI and BamHI of vector pMR103 (Munson et al., 1994) for *E. coli* expression. Plasmid pB449 was used as a template for PCR reaction with primers 7769 and 7770. The product of this amplification was cloned between BamHI and XhoI sites of vector p425GAL1 (Mumberg et al., 1994) and the resulting plasmid carrying human *NRK1* gene under *GAL1* promoter control was named pB450. Human *NRK2* cDNA was amplified with primers 7777 and 7776. The amplified fragment was digested with NdeI and XhoI enzymes and cloned in plasmid pSGA04 for *E. coli* expression (pB457) and into plasmid p425GAL1 for yeast expression (pB459). His-tagged enzymes were purified by immobilized cobalt affinity chromatography (Becton Dickenson).

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