Nicotinamide mononucleotide adenylyltransferase (NMNAT) is the central enzyme of the NAD biosynthetic pathway. Three human NMNAT isoforms have recently been identified, but isoform-specific functions are presently unknown, although a tissue-specific role has been suggested. Analyses of the subcellular localization confirmed NMNAT1 to be a nuclear protein, whereas NMNAT2 and -3 were localized to the Golgi complex and the mitochondria, respectively. This differential subcellular localization points to an organelle-specific, nonredundant function of each of the three proteins. Comparison of the kinetic properties showed that particularly NMNAT3 exhibits a high tolerance toward substrate modifications. Moreover, as opposed to preferred NAD⁺ synthesis by NMNAT1, the other two isoforms could also form NADH directly from the reduced nicotinamide mononucleotide, supporting a hitherto unknown pathway of NAD generation. A variety of physiological intermediates was tested and exerted only minor influence on the catalytic activities of the NMNATs. However, gallotannin was found to be a potent inhibitor, thereby compromising its use as a specific inhibitor of poly-ADP-ribose glycohydrolase. The presence of substrate-specific and independent nuclear, mitochondrial, and Golgi-specific NAD biosynthetic pathways is opposed to the assumption of a general cellular NAD pool. Their existence appears to be consistent with important compartment-specific functions rather than to reflect simple functional redundancy.

For many years the pyridine nucleotides have been recognized as vital to all organisms because of their essential role in energy transduction. They participate as coenzymes in a multitude of metabolic hydrogen transfer reactions. Recently, however, an entirely unexpected role of NAD and NADP has been discovered that is now the subject of intense investigations; these molecules represent important factors in a variety of signaling pathways (1). On the one hand, NAD⁺ is a substrate for covalent protein modifications such as mono-ADP-ribosylation, poly-ADP-ribosylation, and protein deacetylation. These processes participate in the regulation of critical cellular events such as replication, telomere maintenance, apoptosis, and transcription as well as gene silencing (1–5). On the other hand, NAD(P)⁺ is the direct precursor of two calcium-mobilizing messengers, cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP). These molecules are involved in a variety of signaling pathways including the regulation of intracellular insulin levels and its secretion, T-cell activation, or catecholamine secretion (6–9). The discovery of NAD-dependent regulatory mechanisms has also led to the realization that NAD(P) biosynthesis has to be an ongoing process to fuel these pathways. That is, although the metabolic redox reactions are not accompanied by any net consumption of pyridine nucleotides, their participation in signaling pathways requires a steady resynthesis to maintain a stable cellular concentration.

Nicotinamide mononucleotide adenylyl-transferase (NMNAT) is a central enzyme in NAD biosynthesis (10). It catalyzes the reversible reaction $\text{NMN} + \text{ATP} \rightleftharpoons \text{NAD}^+ + \text{PP}_i$. The first primary structures of a human NMNAT (NMNAT1) and its counterparts from other species were only recently established (11–17). Nevertheless, several studies over the past few years have already revealed important regulatory functions of this enzyme. For example, overexpression of NMNAT extends the life span of yeast cells (18). This effect has been attributed to the activation of NAD-dependent protein deacetylation catalyzed by the family of silent information regulator 2 (Sir2) proteins whose pharmacological activation by resveratrol or overexpression caused life span extension in a variety of organisms (19–21). Furthermore, a spontaneous mutation in mice has been discovered that leads to the overexpression of a chimeric protein composed of the full-length NMNAT1 and the N terminus of the ubiquitin assembly protein, Ufd2a (22). Mutant mice show a slowed Wallerian degeneration (Wlds). This axonal degeneration is a self-destructive process observed after an axonal injury or during neurodegenerative diseases such as Parkinson or Alzheimer disease (23). The axons of the Wlds-mice are protected from degeneration because of the enhanced biosynthesis of NAD by NMNAT1. It has now been demonstrated that the ensuing silencing of genes involved in the degeneration process is the reason for the delayed axon degeneration and that it is mediated via the activation of SIRT1, the mammalian ortholog of the yeast Sir2 protein (24).

Considering the fundamental role of NMNAT in NAD biosynthesis and as a regulator in signaling processes, it was an exciting discovery that there is more than one protein possessing NMNAT activity. Two other human NMNATs (NMNAT2 and NMNAT3) have now been cloned and partially characterized (25–27). However, the roles of these new isoforms in NAD biosynthesis and NAD-dependent signal transduction pathways have remained unclear. The proteins appear to differ in their tissue-specific expression and, possibly, in their subcellular

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3 The abbreviations used are: NAADP, nicotinic acid adenine dinucleotide phosphate; NHD, nicotinamide hypoxanthine dinucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; GFP, green fluorescent protein; NMNH, reduced nicotinamide mononucleotide; Sir2, silent information regulator 2; Wlds, slowed wallerian degeneration; EGCG, epigallocatechin gallate.
localization (11, 26, 27). Although endogenous NMNAT1 was localized exclusively to the nucleus (12), NMNAT2- and NMNAT3-GFP fusion proteins were targeted to the cytosol, although NMNAT3 was also partially found within the mitochondria (27).

Surprisingly, the oligomeric state of the three human NMNAT isoforms appears to differ considerably. As deduced from the crystal structures NMNAT1 is a homohexamer (28–30), whereas NMNAT3 is a homotetramer (27). Recombinantly expressed NMNAT2 has been suggested to be a homodimer (25). Only NMNAT1 has been extensively studied with regard to physical and kinetic properties (31, 32). The kinetic properties of NMNAT2 were reported for the generation of NAD$^+$ (25), whereas no functional studies of NMNAT3 have been conducted, except for the fact that it does synthesize NAD$^+$ from NMN and ATP (27). Given the rather different tertiary structures of the proteins, their functional characteristics could also differ substantially. We aimed our study, therefore, at comparing the catalytic properties of the individual human NMNAT isozymes. To this end, the three proteins were cloned and overexpressed in *Escherichia coli*. Moreover, to understand their specific physiological functions, we established their subcellular localization. The results revealed both a strict compartment-specific expression of NMNAT2 and -3 in the Golgi complex and mitochondria, respectively, and substantial differences in the substrate specificities of the three isoforms. Analysis in the presence of mRNA in two human cell lines also found that all three isoforms can be expressed simultaneously. Therefore, the existence of three NMNAT isoforms appears to be consistent with important compartment-specific functions rather than to reflect simple functional redundancy.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning for Prokaryotic Expression and Purification of Recombinant Human NMNATs**—Human NMNAT1 was cloned into the pQE30 prokaryotic expression vector (Qiagen) as described previously (12). Human NMNAT2-cDNA and NMNAT3-cDNA were amplified from cDNA prepared from human brain and human heart mRNAs (kindly provided by Antje Ludwig), respectively. The primers for amplification were 5′-ATG ACC GAG ACC ACC AAG AC-3′ and 5′-CTA GCC GGA GGC ATT GAT GT-3′ for human NMNAT2 and 5′-ATG AAG AGC CGA ATA CTT GTG-3′ and 5′-CTA GCT TGT CTT GGC CTC AG-3′ for human NMNAT3. PCR was carried out for 35 cycles with an annealing temperature of 58 °C. The PCR products were cloned into the pCR-2.1-TOPO vector (Invitrogen) and transformed into *E. coli* according to the manufacturer’s protocol. The cDNAs encoding human NMNAT2 and NMNAT3 were subsequently cloned into the pQE30 vector using the KpnI (sense) and HindIII (antisense) recognition sites for NMNAT3. Thereby, the recombinant proteins were extended by an N-terminal His$^+$ tag.

**Prokaryotic Expression and Purification of Recombinant Human NMNATs**—His-tagged human recombinant NMNAT1 was expressed and purified by Ni$^{2+}$-NTA affinity chromatography as described previously (12). For overexpression, the NMNAT2 and NMNAT3 vector constructs were transformed into *E. coli* (M15) cells. Overnight cultures were transferred to 350 ml of LB medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin. Overexpression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 or 1 mM for expression of NMNAT2 or NMNAT3, respectively, at an optical density of 0.6 (600 nm) of the cells. Protein expression was carried out for 12 h at 30 °C or 4 h at 37 °C for NMNAT2 and NMNAT3, respectively. The cells were then harvested and broken up using a French press (SLM Instruments) in lysis buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 10 mM MgCl$_2$, 1× complete-mini protease inhibitor-mix (Roche Applied Science), 10 μg/ml lysozyme, and 5 mM diithiothreitol for NMNAT2 and 50 mM sodium phosphate, pH 8, 300 mM NaCl for NMNAT3). The overexpressed proteins were purified using Ni$^{2+}$-affinity chromatography according to the manufacturer’s protocol (Qiagen). The purified proteins were stored at 4 °C, except NMNAT2, which was rather unstable and, therefore, stored at −80 °C and thawed shortly before use.

**Measurements of NMNAT Activity**—The enzymatic activities of the three human NMNATs for the forward reaction were measured and quantified by a photometric assay as described previously (12). To determine substrate specificities and kinetic data for the reverse reaction, the activity was calculated from product quantification of reverse phase HPLC separations of the reaction mixture. The purified human recombinant NMNATs were incubated with the indicated substrates in reaction buffer (50 mM Tris/HCl, pH 8, 5 mM MgCl$_2$, 1× complete-mini protease inhibitor-mix) for 1 h at 37 °C. The reaction was allowed to run for the indicated time at room temperature. After the reaction was stopped by diluting the sample 1:10 with ice-cold water immediately followed by centrifugation through a Centricon microfiltration device (Millipore, size exclusion 5 kDa). The filtrate was analyzed by reverse-phase HPLC as described previously (33). Nucleotides were quantified by peak integration. The equilibrium of the NMNAT reaction was determined by incubating the enzymes with varying amounts of ATP and NMIN or NAD$^+$ and pyrophosphate. After various time intervals the concentrations of the nucleotides were measured by HPLC, whereas the change of the pyrophosphate concentration was assumed to be identical with that of NAD$^+$. The amount of enzyme used was sufficient to establish the equilibrium within 15 min, as detected by additional measurements after 60 min which indicated no further changes in the concentrations of the reactants. The equilibrium constant was calculated as $K_{eq} = \frac{[NAD][PP]/[ATP][NMN]}{[NAD][PP]/[ATP][NMN]}$ using the values measured at 60 min. To measure NADH synthesis from NMNH and ATP, a solution of NADH was incubated in the presence of phos...
Human NMNAT Isoforms

**TABLE ONE**

*Kinetic parameters of NMNAT isozymes*

The *Km* values (in μM) of human recombinant NMNAT1, NMNAT2, and NMNAT3 were determined by the photometric assay when using NMN and ATP as substrates and by the HPLC assay with NAD⁺ (or NADH) and PPi, as the substrates. *Vmax* values are given as μmol × (min × mg⁻¹).

|            | *Km* NMN (μM) | *Km* ATP (μM) | *Km* PPi (μM) | *Km* NAD⁺ (μM) | *Vmax* NAD synthesis (μmol·mg⁻¹·min⁻¹) | *Vmax* NAD⁺ cleavage (μmol·mg⁻¹·min⁻¹) | *Vmax* NADH cleavage (μmol·mg⁻¹·min⁻¹) |
|------------|---------------|---------------|---------------|----------------|--------------------------------------|--------------------------------------|--------------------------------------|
| NMNAT1     | 34            | 40            | 937           | 59             | 25                                   | 60.5                                 | 8.5                                  |
| NMNAT2     | 32            | 204           | 1119          | 70             | 1.1                                  | 3.0                                  | 1.7                                  |
| NMNAT3     | 209           | 29            | 390           | 130            | 3.6                                  | 12.8                                 | 2.9                                  |

**TABLE TWO**

*Substrate specificity of NMNAT isozymes*

Relative substrate specificities of human recombinant NMNATs were measured by incubation for 1 h at room temperature with 1 mM mononucleotide and 2 mM purine nucleotide when measuring the transfer activity or 500 μM dinucleotide and 5 mM pyrophosphate when measuring the dinucleotide cleavage activity. Activities were determined by the HPLC assay and related to those determined for the physiological substrates (100%). ND, no detectable activity. PAR, poly-ADP-ribose; NGD, nicotinamide guanine dinucleotide; ADPR, ADP-ribose; NaMN, nicotinic acid mononucleotide.

|                      | Pyridine mononucleotide transfer activity | Purine nucleotide transfer activity | Pyrophosphorolytic activity |
|----------------------|------------------------------------------|-----------------------------------|----------------------------|
|                      | NMN | NaMN | ATP | ITP | GTP | NAD | NGD | NHD | NAAD | NADP | NAADP | NADH | NADPH | PAR | ADPR |
| NMNAT1               | 100 | 98   | 100 | 6   | 7   | ND  | 26  | 55  | 93  | ND   | ND    | 53   | ND    | ND  | ND   |
| NMNAT2               | 100 | 52   | 100 | 5   | 3   | ND  | 5   | 37  | 98  | 3    | ND    | 98   | ND    | ND  | ND   |
| NMNAT3               | 100 | 100  | 100 | 27  | 19  | ND  | 57  | 98  | 1   | ND   | 99    | 4    | ND    | ND  | ND   |

phodiesterase I from snake venom. The resulting NMNH was purified by HPLC and used as substrate assuming the same molar extinction coefficient at 340 nm as NADH (6200 cm⁻¹ M⁻¹).

**Eukaryotic Expression of Recombinant Human NMNATs**—The cDNAs encoding human NMNAT1 and NMNAT2 were cloned into the pFLAG-CMV-4™ eukaryotic expression vector (Sigma) providing the recombinant proteins with an N-terminal FLAG tag. NMNAT3 was cloned into the pFLAG-CMV-5™ eukaryotic expression vector (Sigma) providing the recombinant protein with a C-terminal FLAG tag. The HindIII (sense) and BamHI (antisense) recognition sites were used for NMNAT3-cDNA cloning. The HindIII recognition site was used for NMNAT2-cDNA cloning, and the EcoRI (sense) and KpnI (antisense) recognition sites were used for NMNAT1-cDNA cloning. For eukaryotic expression of the proteins, cells were transiently transfected with the pFLAG-NMNAT constructs by either calcium phosphate precipitation or using Lipofectamine™ 2000 (Invitrogen). Where indicated, cotransfection with plasmids encoding an enhanced yellow fluorescent protein targeted to the Golgi complex (Clontech) or GFP targeted to the mitochondria (Invitrogen) was carried out. Cells were analyzed by immunostaining after 24–48 h of transfection.

To visualize NMNAT1 and NMNAT3, cells were fixed for 45 min at 4 °C with 4% formaldehyde and permeabilized with 0.5% Triton X-100 for 15 min at room temperature. To visualize NMNAT2 cells were fixed for 20 min at −20 °C with methanol. The samples were then blocked with 10% fetal calf serum and immunostained with the monoclonal ANTI-FLAG® M2 antibody (Sigma). Mitochondria were visualized by Mitotracker (Molecular Probes/Invitrogen) according to the manufacturer’s protocol.

**RESULTS**

To conduct a detailed functional characterization of the three different human NMNAT isozymes, their cDNAs were cloned into the pQE30 prokaryotic expression vector, and the respective proteins were overexpressed in *E. coli*. As shown in Fig. 1, all three proteins were overexpressed, and their molecular weights were in accordance with the predicted sizes. The pQE30 vector endows proteins with an N-terminal His₆ tag enabling purification by Ni²⁺ affinity chromatography. NMNAT1 and -3 were purified to homogeneity in one step. The NMNAT2 preparations, however, contained impurities (Fig. 1). Nevertheless, the major protein in these preparations was NMNAT2, and nucleotide converting activities other than those expected for this type of enzyme were not detected (not shown).

To determine and compare the affinities (*Km* values) of human NMNAT1, -2, and -3 for their substrates, NMN and ATP, the coupled photometric assay was used. NMNAT1 exhibited the highest affinity for these substrates (see TABLE ONE) as well as the highest specific activity. The values for NMNAT1 (11) and 2 (25) are in accordance with those previously reported. NMNAT3 has not been kinetically characterized so far. Both NMNAT1 and -3 showed no preference with regard to the pyridine mononucleotides (NMN and nicotinic acid mononucleotide (NaMN), TABLE TWO), whereas NMNAT2 preferred NMN over NaMN.
NMNAT1 is known to also catalyze the reverse reaction. That is, it generates ATP (and NMN) from NAD$^+$ and pyrophosphate. Using the HPLC-based assay, this capacity was also tested for NMNAT2 and -3. These two isozymes, too, catalyze the pyrophosphorolytic cleavage of NAD$^+$ with high efficiency (TABLE ONE). Using NMNAT1 or NMNAT3, we estimated the equilibrium constant for the reaction (see “Experimental Procedures”). As expected, it was similar for both enzymes and amounted to about 0.3. This result is somewhat surprising, because it indicates that the equilibrium is actually in favor of the pyrophosphorolytic cleavage of NAD$^+$. Presumably, under physiological conditions the pyrophosphate concentration limits the reverse reaction.

NMNAT1 has been known to accept a variety of dinucleotides as substrates (31, 32). Using a number of commercially available dinucleotides we compared the substrate specificities of the three NMNATs for the reverse reaction. NMNAT3 proved to be the most flexible of the three isozymes and converted NAAD, nicotinamide guanine dinucleotide (NGD), and NADH to the same extent as NAD$^+$. Moreover, it even cleaved NGD rather efficiently (TABLE TWO). Although NMNAT2 also accepted NHD as substrate, it did so to lower extent. NMNAT1 displayed the highest selectivity and strongly preferred NAD$^+$ and NAAD, the presumed physiological substrates. It degraded NADH and NHD far less effectively and NGD only by about 26% under the conditions of the assay. It is interesting to note that the sensitivity toward nucleobase modifications in the dinucleotide is similarly reflected in the preference for the substrates of the forward reaction. For example, compared with the other two isoforms, NMNAT3 cleaved NGD with the highest efficiency and also exhibited the highest activity to synthesize this dinucleotide from GTP (TABLE TWO). The same is valid for cleavage of NHD and its synthesis from ITP (TABLE TWO). All three NMNAT isoforms essentially failed to cleave the phosphorylated dinucleotides NADP$^+$, NADPH, and NAADP$^+$ (TABLE TWO).
Human NMNAT Isoforms

**TABLE THREE**

| NA  | NAm | ADP | AMP | GTP | NADP | NAADP | ADPR | PAR | 3ABA | GT | EGCG |
|-----|-----|-----|-----|-----|------|-------|------|-----|------|----|------|
| NMNAT1 | 99 | 100 | 82 | 102 | 98 | 69 | 95 | 95 | 100 | ND | 110 |
| NMNAT2 | 102 | 106 | 68 | 67 | 98 | 83 | 103 | 103 | 102 | ND | 228 |
| NMNAT3 | 107 | 105 | 101 | 96 | 98 | 102 | 102 | 102 | 94 | ND | 142 |

A rather unexpected observation was the highly efficient cleavage of NADH by NMNAT2 and 3 compared with their NAD synthesis activities (TABLE ONE and Fig. 2). In this reaction the hitherto uncharacterized metabolite NMNH should be formed. Indeed, ATP was observed as a product along with a metabolite that retained the absorbance at 340 nm, typical for the reduced form of NAD (Fig. 2). As opposed to NADH, it was hardly retained on the HPLC column, as would be expected for NMNH in the method used for nucleotide separation. To verify the ability of NMNATs to synthesize NADH from NMNH and ATP, the following experiment was conducted. NADH was cleaved to NMNH and AMP by phosphodiesterase I from snake venom. NMNH was then purified by HPLC (Fig. 3). As can be inferred from Fig. 3, in the presence of NMNATs NADH was generated from NMNH and ATP. Therefore, these enzymes can use both the oxidized (NMN) or reduced (NMNH) forms of nicotinamide mononucleotide for NAD synthesis, respectively. At the concentrations used (125 mM NMN or NMN and 0.5 mM ATP), NMNATs 1 and 2 generated the corresponding dinucleotide at similar rates, whereas NMNAT3 exhibited a preference toward NMNH as substrate (Fig. 3D).

Although it has been realized that the synthesis as well as the concentration of NAD have critical impacts on a multitude of regulatory processes, mechanisms that regulate NAD synthesis are still unknown. We tested the influence of a variety of physiological intermediates (nicotinic acid, nicotinamide, ADP, AMP, GTP, NADP, NADPH, ADPR, AMP-ribose, poly-ADP-ribose) on the activity of the NMNAT isoforms (TABLE THREE). Although ADP-ribose was previously reported to exert an inhibitory effect on NMNAT1 (32), we did not detect any significant influence, even at concentrations as high as 5 mM. ADP and NADP had a slight effect of 20–30% inhibition.

Furthermore, we also examined the potential influence of compounds previously used to inhibit other enzyme activities related to NAD metabolism. For examples, 3-aminobenzamide inhibits poly-ADP-ribose-sylation, and gallotannin inhibits polymer degradation by poly-ADP-ribose glycohydrolase. Gallotannin caused a strong inhibition of NMNAT1, and no detectable activity was left at 100 mM (TABLE THREE). The two other isoforms were also tested, and NMNAT3
proven to be most sensitive against gallotannin. At 2 μM its activity was reduced to 50%, whereas for NMNAT1 10 μM and for NMNAT2 55 μM gallotannin were required to achieve the same inhibition (TABLE FOUR). On the other hand, epigallocatechin gallate (EGCG), a condensed tannin that inhibits poly-ADP-ribose glycohydrolase to a very low extent, activated the enzymatic activity of NMNATs. At 50 μM EGCG, the activity of NMNAT2 was more than doubled. NMNAT3 was activated by 42%, whereas the effect on the activity of NMNAT1 was rather small.

To conclusively define the subcellular localization of NMNAT2 and -3, we first cloned their cDNAs into the pFLAG-CMV-4TM or pFLAG-CMV-5TM eukaryotic expression vector, respectively, and transfected HeLa and HEK293 cells with the generated constructs. The overexpressed proteins were immunostained with the monoclonal FLAG antibody. Similar to the endogenous protein, the FLAG-tagged NMNAT1 was located exclusively within the nucleus (Fig. 4). Unlike observations with a GFP fusion construct (27), NMNAT2 did not appear to be cytosolic but, rather, displayed a distinct perinuclear staining typically observed for proteins of the Golgi complex. Indeed, cotransfection with an enhanced yellow fluorescent protein construct targeted to the Golgi permitted colocalization of the two proteins, thereby establishing a quite unexpected subcellular location of NMNAT2 (Fig. 4). The predominant localization of NMNAT2 to the Golgi complex was independent of the presence of the FLAG tag at the N or C terminus of the protein (Fig. 4). We noted, however, that immunostaining of the C-terminal-tagged protein resulted also in some punctual staining in the cytosol, most likely the endoplasmic reticulum. This could indicate that part of the protein has not been folded properly and was, therefore, retained within the endoplasmic reticulum. To further verify the association of NMNAT2 with the Golgi complex, overexpressing cells were treated with brefeldin A. Under these conditions the Golgi complex disassembles, and proteins of this compartment acquire an apparent cytosolic localization. As shown in Fig. 4, NMNAT2 behaved exactly as predicted for a protein of the Golgi complex.

NMNAT3 C-terminally fused to GFP had previously been shown to be located in the cytosol but also partially colocalized with Mitotracker (27). When endowed with a C-terminal FLAG tag the protein largely colocalized with Mitotracker (Fig. 3). Interestingly, in several cells it was noted that Mitotracker staining was significantly weaker when the NMNAT3-FLAG construct was overexpressed. Most likely, this effect has caused the only partial colocalization obtained in the merged image. Eventually, when co-transfected with GFP targeted to the mitochondrial matrix, the signals from GFP and the immunodecorated FLAG tag almost perfectly colocalized, indicating that NMNAT3 is a mitochondrial protein (Fig. 4). Furthermore, using an antiserum raised against recombinantly expressed NMNAT3 (cf. Fig. 1) cells exhibited mitochondrial staining. These experiments demonstrate that the overexpressed, FLAG-tagged NMNAT3 had been directed to its native compartment, the mitochondria.

Finally, it was of interest whether cells might rely on a specific subset of NMNAT isoforms or rather express all three known NMNATs. As shown in Fig. 5, the cDNAs generated from total mRNA isolated from HEK293 cells contained sequences that could be amplified by primers specific for the cDNAs encoding the individual NMNAT isoforms. Similar results were obtained using total mRNA from HeLa cells, except that NMNAT3 appeared to be far more weakly expressed in these cells. This observation is in accordance with the immunocytochemical analyses, which indicated a rather weak expression of NMNAT3 in HeLa cells compared with HEK293 cells. The results confirm previous analyses that indicated tissue-specific expression of NMNAT2 and -3 but a rather ubiquitous expression of NMNAT1 (TABLE FIVE). Nevertheless, at least HeLa and HEK293 cells express the genes of all three NMNAT isoforms.

**DISCUSSION**

The observations of the present study document the existence of independently operating, specifically localized NMNAT isoforms in human cells, suggesting nonredundant, organelle-specific functions of the three proteins. Remarkably, because of the absence of any of the NMNAT isoforms the cytosol appears to be devoid of NAD generation. The unexpected observation that NMNATs readily synthesized NADH from NMNH and ATP in fact establishes a novel pathway of NAD biosynthesis. This alternative to generating NAD⁺ could be of significance with regard to regulation or precursor availability in the specific subcellular compartments.

Using cell fractionation, NMNAT activity was originally found to be
be noted, however, that the identified targets of ADP-ribosylation by drolase (46–49), an enzyme that degrades ADP-ribose polymers. It represents a quite important finding because gallotannin has been used in a variety of studies to specifically inhibit poly-ADP-ribose glycohydrolase (46–49). However, mitochondrial NMNAT3 is more strongly expressed in tissues from which NMNAT2 is nearly absent, i.e. kidney, lung, and spleen. As demonstrated in a previous study of NMNATs 1 and 2 in the pancreas (26), the distribution of NMNAT isoforms may vary considerably within a tissue. For example, using immunohistochemistry, NMNAT2 was found to be strongly expressed in the islets of Langerhans, whereas it was hardly detectable in acinar cells (26). Therefore, although the information retrieved from Northern blot analyses provides some first hints as to the specific functions of the individual NMNAT isoforms, careful studies determining the protein rather than the mRNA level need to be conducted to establish their presence and function in specific cell types.

The variability among the NMNAT isoforms regarding substrate specificity may also have important implications. Of particular interest is the observation that at least NMNAT2 and -3 readily accepted NADH in the reverse reaction. Moreover, all isoforms synthesized NADH using NMNH and ATP as substrate. So far, NAD biosynthesis has been described as a pathway generating the oxidized form, NADH (10), which can then be reduced in a dehydrogenase reaction. Probably, this assumption is at least in part based upon the original detection of NMNAT1. This isoform appears to be the most abundant one and also exhibits the highest specific activity for NADH synthesis and pyrophosphorylisation (TABLES ONE and FIVE). NMNATs 2 and 3 readily cleaved NADH. Moreover, NMNAT3 actually preferred NMNH, a hitherto uncharacterized intermediate, over NMN as substrate for the generation of the pyridine nucleotide. It is quite feasible that NMNH serves as the in vivo substrate of NMNATs because NMNH appears to be a physiological intermediate. For example, nudix hydrolases have been identified that specifically cleave NADH to NMNH and AMP (44). Therefore, at least for the salvage of NMNH into NADH, the ability of the NMNATs to use the reduced substrate might be of importance.

In principle, one or more isoforms could physiologically cleave rather than synthesize NADH or NADPH, although no obvious function can be suggested for such a process. It is at least interesting that a product of this reverse reaction is ATP, which may be more important than NADH under some conditions. Furthermore, it is also possible that at least the less specific NMNAT isoforms might have a function in the synthesis or cleavage of dinucleotides other than NAD. For example, it was shown that poly-ADP-ribose can be cleaved in a reaction similar to the reverse reaction of NMNATs. The ATP thus generated can then be used for DNA ligation during base excision repair (45). However, no such activity could be detected in the present study for any of the NMNATs (TABLE THREE).

Different affinities to substrates and their analogs were also reflected in the different susceptibility to potential inhibitors or activators of the NMNAT isoforms. This divergence may be useful for the development of isomer-specific inhibitors, which would greatly facilitate studies of their individual functions. Most strikingly, all isoforms were rather sensitive to galactannin, with the IC_{50} for NMNAT3 as low as 2 μM. This represents a quite important finding because galactannin has been used in a variety of studies to specifically inhibit poly-ADP-ribose glycohydrolase (46–49), an enzyme that degrades ADP-ribose polymers. Clearly, the interference of galactannin with the regeneration of NAD by NMNATs will have to be considered in interpreting the previously

| Tissues  | NMNAT-1 | NMNAT-2 | NMNAT-3 |
|----------|---------|---------|---------|
| Brain    | (+)     | +       | −       |
| Heart    | +       | +       | (+)     |
| Skeletal muscle | +       | +       | (+)     |
| Liver    | +       | −       | +       |
| Kidney   | +       | −       | +       |
| Lung     | (+)     | −       | +       |
| Spleen   | −       | −       | +       |
| Thymus   | (+)     | +       | (+)     |
| Pancreas | +       | +       | (+)     |
| Colon    | (+)     | −       |          |
| Placenta | +       | (+)     | +       |
| Cell lines |
| HeLa     | +       | +       | (+)     |
| HEK293   | +       | +       | +       |
| K562     | +       | −       | ND      |
| Raji     | +       | −       | (+)     |

The variability among the NMNAT isoforms regarding substrate specificity may also have important implications. Of particular interest is the observation that at least NMNAT2 and -3 readily accepted NADH in the reverse reaction. Moreover, all isoforms synthesized NADH using NMNH and ATP as substrate. So far, NAD biosynthesis has been described as a pathway generating the oxidized form, NADH (10), which can then be reduced in a dehydrogenase reaction. Probably, this assumption is at least in part based upon the original detection of NMNAT1. This isoform appears to be the most abundant one and also exhibits the highest specific activity for NADH synthesis and pyrophosphorylisation (TABLES ONE and FIVE). NMNATs 2 and 3 readily cleaved NADH. Moreover, NMNAT3 actually preferred NMNH, a hitherto uncharacterized intermediate, over NMN as substrate for the generation of the pyridine nucleotide. It is quite feasible that NMNH serves as the in vivo substrate of NMNATs because NMNH appears to be a physiological intermediate. For example, nudix hydrolases have been identified that specifically cleave NADH to NMNH and AMP (44). Therefore, at least for the salvage of NMNH into NADH, the ability of the NMNATs to use the reduced substrate might be of importance.

In principle, one or more isoforms could physiologically cleave rather than synthesize NADH or NADPH, although no obvious function can be suggested for such a process. It is at least interesting that a product of this reverse reaction is ATP, which may be more important than NADH under some conditions. Furthermore, it is also possible that at least the less specific NMNAT isoforms might have a function in the synthesis or cleavage of dinucleotides other than NAD. For example, it was shown that poly-ADP-ribose can be cleaved in a reaction similar to the reverse reaction of NMNATs. The ATP thus generated can then be used for DNA ligation during base excision repair (45). However, no such activity could be detected in the present study for any of the NMNATs (TABLE THREE).

Different affinities to substrates and their analogs were also reflected in the different susceptibility to potential inhibitors or activators of the NMNAT isoforms. This divergence may be useful for the development of isomer-specific inhibitors, which would greatly facilitate studies of their individual functions. Most strikingly, all isoforms were rather sensitive to galactannin, with the IC_{50} for NMNAT3 as low as 2 μM. This represents a quite important finding because galactannin has been used in a variety of studies to specifically inhibit poly-ADP-ribose glycohydrolase (46–49), an enzyme that degrades ADP-ribose polymers. Clearly, the interference of galactannin with the regeneration of NAD by NMNATs will have to be considered in interpreting the previously associated with the nucleus (34). This observation was confirmed by immunofluorescence studies of endogenous NMNAT1 (12). It was thought that this nuclear enzyme was responsible for the generation of a general "cellular NAD pool," all the more so, since the nuclear pool is supposed to be at equilibrium with the cytosolic pool. On the other hand, the origin of mitochondrial NAD in mammalian cells has so far remained entirely unknown. An uptake or release of NAD does not occur within these organelles except, perhaps, through the permeability transition pore. However, the concentration of mitochondrial NAD is higher than in the cytosol, precluding a simple diffusion mechanism (35). The results of our study suggest that the synthesis of NAD appears to be confined to specific subcellular compartments, the nucleus, the Golgi complex, and the mitochondria. This observation strongly supports the model of an independent pathway of mitochondrial NAD synthesis. In fact, NMNAT activity had been detected in a mitochondrial fraction from rat liver (36) but was subsequently not characterized on a molecular level. The existence of a mitochondrial NAD kinase, which accounts for the NADP generation, in yeast and Arabidopsis (37, 38) adds further weight to the suggestion of an independent pyridine nucleotide metabolism in these organelles. It will be of interest to learn whether one of the two recently discovered human nicotinamide ribose kinases (39) might be mitochondrial. Alternatively, mitochondrial NMN might be produced by the Preiss-Handler pathway (40) provided that the respective enzyme activities are also present within these organelles.
observed phenomena. In fact, the reported affinity of poly-ADP-ribose glycohydrolase to galloantin, \( IC_{50} = 25 \, \mu M \) (50, 51), is lower than that observed here for NMNAT1 and -3, with \( IC_{50} \) values of 10 and 2 \( \mu M \), respectively. On the other hand, NMNATs were activated by EGCG, a polyphenolic constituent of green tea. There are numerous reports on the neuroprotective and cancer-preventive effects of EGCG, but so far little is known about its molecular targets. It appears possible that activation of NMNATs could have a role in these phenomena. For example, activation of NAD synthesis by overexpression of NMNAT1 in yeast and mammalian cells results in activation of sirtuins and thereby causes life span extension or neuroprotection (18, 24).

In conclusion, in human cells at least the final step of NAD biosynthesis is catalyzed by three isozymes of NMNAT in different subcellular compartments. Considering the marked differences in their structures and catalytic properties, these enzymes are likely to be differentially regulated. Therefore, the supply of NAD to specific metabolic or signal-processing processes appears to be compartment-specific and does not necessarily affect the "general pyridine nucleotide pool" as is often implied. Still, because of substrate shuttle systems, the redox state at least between mitochondria and the cytosol/nucleus may be maintained similar throughout the cell.

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