Characterization of recombinant human nicotinamide mononucleotide adenylyl transferase (NMNAT), a nuclear enzyme essential for NAD synthesis

Manfred Schweiger, Klaus Hennig, Felicitas Lerner, Marc Niere, Monica Hirsch-Kauffmann, Thomas Specht, Christoph Weise, Shiao Li Oei, Mathias Ziegler*

Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 14195 Berlin, Germany

Received 1 November 2000; revised 19 January 2001; accepted 25 January 2001

First published online 21 February 2001

Edited by Takashi Gojobori

Abstract Nicotinamide mononucleotide adenylyl transferase (NMNAT) is an essential enzyme in all organisms, because it catalyzes a key step of NAD synthesis. However, little is known about the structure and regulation of this enzyme. In this study we established the primary structure of human NMNAT. The human sequence represents the first report of the primary structure of this enzyme for an organism higher than yeast. The enzyme was purified from human placenta and internal peptide sequences determined. Analysis of human DNA sequence data then permitted the cloning of a cDNA encoding this enzyme. Recombinant NMNAT exhibited catalytic properties similar to the originally purified enzyme. Human NMNAT (molecular weight 31 932) consists of 279 amino acids and exhibits substantial structural differences to the enzymes from lower organisms. A putative nuclear localization signal was confirmed by immunofluorescence studies. NMNAT strongly inhibited recombinant human poly(ADP-ribose) polymerase 1, however, NMNAT was not modified by poly(ADP-ribose). NMNAT appears to be a substrate of nuclear kinases and contains at least three potential phosphorylation sites. Endogenous and recombinant NMNAT were phosphorylated in nuclear extracts in the presence of [γ-^{32}P]ATP. We propose that NMNAT's activity or interaction with nuclear proteins are likely to be modulated by phosphorylation.

Key words: Nicotinamide mononucleotide adenylyl transferase; NMNAT; NAD synthesis

1. Introduction

The role of NAD as a coenzyme in cellular redox reactions has long been recognized. In this function NAD represents an essential component of metabolic pathways in all living cells. Numerous recent studies have demonstrated the importance of NAD as both metabolite and signaling molecule. For example, high levels of NAD are required for life-span extension in yeast mediated by the transcriptional silencing protein Sir2p [1,2]. It has become apparent that NAD also participates in a number of other signaling pathways. These reactions include monoADP-ribosylation, poly(ADP-ribosyl)ation, and the synthesis of calcium-mobilizing molecules such as cyclic ADP-ribose and nicotinate adenine dinucleotide phosphate (NAADP) which require NAD as a precursor (reviewed in [3]). NAD-dependent signaling routes have been detected in several compartments including the cell surface. However, poly(ADP-ribosyl)ation appears to be restricted to the nucleus. The best characterized enzyme catalyzing this process, poly(ADP-ribose) polymerase (PARP1), is involved in a number of nuclear processes and has been established to be a component of the DNA base excision repair complex [4].

It is clear that the synthesis of NAD and its regulation have significant impact on both metabolism and signaling events within the cell. The principal pathways of de novo and salvage syntheses of NAD are well known. For example, essential reactions in both the de novo and the salvage pathways are catalyzed by nicotinamide mononucleotide adenylyl transferase (NMNAT) (EC 2.7.7.1). This enzyme transfers the adenylyl moiety of ATP to NMN resulting in the formation of NAD and the release of pyrophosphate. As this reaction is reversible, the enzyme may in principle be used to form ATP (and NMN) from NAD and pyrophosphate [5]. However, it is surprising that so little is known about the structure and regulation of several enzymes that are crucial for pyridine nucleotide synthesis (e.g. NMNAT, NAD kinase).

NMNAT appears to be localized to the nucleus [6] and its activity has been reported to correlate with DNA synthesis during the cell cycle [7]. The enzyme could be a potential target for therapeutical applications, because its activity is rather low in tumor cells [8,9]. The protein has been proposed to be associated with PARP1 [10,11]. This suggestion may point to an important mechanism regulating NAD supply during genotoxic challenges. Under such conditions PARP1 may consume a large fraction of the cellular NAD to form poly(ADP-ribose) [12,13], a reaction considered most important for efficient DNA repair [14,15]. Since in these situations the ATP concentration is also lowered, the resynthesis of NAD is affected. Therefore, inhibition of PARP1 by NMNAT would counteract the loss of NAD.

Although several mammalian NMNATs have been purified [5], to date the primary structures have only been reported for enzymes from microorganisms such as *Saccharomyces cerevi-*

*Corresponding author. Fax: (49)-30-838 56509.
E-mail: mziegler@chemie.fu-berlin.de

Abbreviations: MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; NMNAT, nicotinamide mononucleotide adenylyl transferase; PARP1, poly(ADP-ribose) polymerase 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

0014-5793/01/$20.00
PII: S0014-5793(01)02180-9

siae [16], *Methanococcus jannaschii* [17], *Synechocystis* sp. [18], and *Escherichia coli* [19].

In this study the primary structure of the human NMNAT has been established. The identity of the enzyme was verified by expression of the recombinant protein in *E. coli* and the nuclear localization of the protein was demonstrated in human cell lines. Recombinant NMNAT exerted a strong inhibitory effect on recombinant human PARP1. Moreover, NMNAT was demonstrated to be phosphorylated in nuclear extracts and therefore, besides its function as an enzyme, NMNAT can be implicated in regulatory processes as well.

2. Materials and methods

2.1. Purification of human NMNAT

Purification of NMNAT was performed according to the procedure described by Magni et al. [20]. Briefly, the isolation of the enzyme from human placenta involved a pH precipitation (at pH 5) and chromatography using adsorption and affinity resins including phenyl-Sepharose, matrix gel green A and hydroxyapatite. The final purification step was carried out using a TSKgel phenyl 5PW column (TosoHaas) on an FPLC system (BioLogic, Bio-Rad). The pooled fractions were concentrated and stored at 4°C in a buffer containing 50 mM potassium phosphate, pH 7.4, 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM EDTA, and 300 mM KCl (NMNAT buffer).

All purification steps were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration was estimated using the BCA assay (Pierce) with bovine serum albumin (BSA) as standard.

2.2. Enzyme assay

Activity of NMNAT (synthesis of NAD) was measured in a coupled enzyme assay by monitoring the increase in absorbance at 340 nm caused by the reduction of NAD to NADH. The reaction was performed in 16 mM semicarbacide–HCl, 0.625% (v/v) ethanol, 30 mM HEPES buffer (pH 7.4), 12.25 mM $MgCl_2$, 1.17 mM ATP, 15 U alcohol dehydrogenase (Sigma), and started by adding NMN to a final concentration of 0.625 mM.

The reverse reaction (using NAD and pyrophosphate as substrates) and the ability of NMNAT to utilize substrate analogs were determined by metabolite analysis using an anion exchange column (MonoQ, Pharmacia). For quantification, [^{32}P]NAD or [α-^{32}P]ATP were used. Metabolites were separated by thin layer chromatography and quantified with the aid of a phosphoimager.

2.3. Peptide sequencing

The isolated protein was run on SDS–PAGE to separate it from residual contaminating proteins. Following staining with Coomassie blue the band corresponding to an apparent molecular weight of 33 000 was excised and subjected to 'in gel' digestion by trypsin [21]. The resulting peptides were separated by reverse-phase high performance liquid chromatography (HPLC) on a Vydac C_{18} column (4.6×250 mm) using a Waters 626 liquid chromatography HPLC system (Waters, Milford, MA, USA). The peptides were eluted in a gradient from 0.1% (v/v) trifluoroacetic acid in water to 0.085% (v/v) trifluoroacetic acid in acetonitrile.

Amino acid sequences were obtained from purified peptide fractions by Edman degradation or by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Reflex mass spectrometer) via analysis of fragment ions generated by post-source decay [22], using the FAST™ method (Bruker).

2.4. Reverse transcription PCR

Cytoplasmic RNA from lymphoblastoid cells was isolated as described in [23]. Reverse transcription of 3 μg of RNA was performed in RT buffer (Promega) containing 250 μM dNTP, 40 U RNase inhibitor (Life Technologies), and 140 U M-MLV reverse transcriptase (Promega) in a total volume of 30 μl at 37°C for 1 h. Following cDNA synthesis RNA was digested by adding 40 U RNase H (Takara). cDNA amplification was carried out in PCR buffer (Perkin-Elmer) containing 125 μM dNTP, 1.5 mM $MgCl_2$, 20 pmol of each primer, and 1.5 U Taq Polymerase (Perkin-Elmer). The primers for amplification of NMNAT cDNA were 5′-CTT TGG ATC CGA AAA TTC CGA GAA GAC TGA-3′ and 5′-CCA CAA GCT TCT ATG TCT TAG CTT CTG CAG-3′.

2.5. Expression cloning

PCR products were ligated into the TOPO 2.1 vector (Invitrogen) and transfected into *E. coli* according to the manufacturer's protocol. NMNAT cDNA was subcloned into the pQE30 vector (Qiagen) via the *Bam*HI and *Hin*dIII sites and the vector construct was transfected into *E. coli* (NM 522) cells. Recognition sites for the restriction enzymes were included into the PCR primer sequences. The resulting recombinant protein carried a '6×His'-tag at the N-terminus for subsequent purification.

2.6. Expression and purification of recombinant human NMNAT

An overnight culture was transferred into 1 l of TY medium supplemented with 100 μg/ml ampicillin. At an optical density of 0.6 at 600 nm of the bacteria culture expression was induced by adding isopropyl-β-thiogalactopyranoside to a final concentration of 2 mM.

Following 4 h of expression the bacteria were harvested by centrifugation. Cells were sonicated and the lysate was applied to a nickel-nitrilotriacetic acid column. The column was washed and eluted with imidazole according to the manufacturer's protocol (Qiagen). The purification yielded almost homogenous (< 5% contaminating protein) recombinant NMNAT.

2.7. Cell culture and immunofluorescence analyses

Purified recombinant human NMNAT was used as antigen for the generation of a rabbit antiserum. NMNAT-specific antibodies were extracted from the serum as follows. Recombinant NMNAT was coupled to CNBr-Sepharose (Sigma). The antiserum was incubated with the resin for 1 h. After several washes the antibodies were eluted with 300 mM glycine–HCl, pH 2.8 and 300 mM NaCl. The pH of the eluate was immediately adjusted with Tris to pH 7–7.5 and glycerol was added to 20% (v/v).

Human cell lines (HeLa, HepG2, primary and transformed fibroblast cell lines) were grown on cover slips and fixed with paraformaldehyde. Following successive incubation with the NMNAT-specific antibodies and fluorescein-labeled anti-rabbit IgG the presence of NMNAT in the cells was visualized using a Leica fluorescence microscope. For staining of the transcription factor YY1 a monoclonal antibody was used (H-10, Santa Cruz). The second antibody was an anti-mouse IgG from goat coupled with rhodamine.

2.8. Poly(ADP-ribosyl)ation assay

Human recombinant PARP1 (300 ng) was incubated in the absence or presence of recombinant NMNAT (300 ng) in a buffer containing 10 mM Tris–HCl, pH 8.0, 7 mM $MgCl_2$, 50 mM $ZnCl_2$, 1% BSA, 10 μg/ml sonicated salmon sperm DNA, and 5 nM [^{32}P]NAD. After 10 min aliquots were withdrawn and precipitated with trichloroacetic acid (TCA, 10% w/v). To the remaining sample 10 μM of unlabeled NAD was added and the reaction continued for 10 min. Thereafter, the samples were stopped by TCA precipitation. Proteins were separated by 10% SDS–PAGE and poly(ADP-ribosyl)ation visualized by autoradiography. In parallel experiments the incorporation of poly(ADP-ribose) was quantified by Cerenkov counting.

2.9. Protein phosphorylation assay

Nuclear extracts were prepared from human fibroblasts according to [24]. The extracts (20 μg of protein) were incubated at 30°C with 100 μM [γ-^{32}P]ATP (NEN DuPont) in the presence or absence of 10 μg of recombinant NMNAT in a buffer consisting of 10 mM HEPES-KOH, pH 7.9, 7 mM $MgCl_2$, 50 mM KCl, 100 mM NaCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, and 10% (v/v) glycerol. Following incubation for 30 min the reaction was stopped and the samples subjected to SDS–PAGE. If no recombinant NMNAT was added to the incubations, after 30 min immunoprecipitation of the endogenous NMNAT was conducted using the polyclonal antibodies and protein A Sepharose. Phosphorylated proteins were detected by autoradiography following SDS–PAGE.

3. Results

NMNAT was purified from human placenta and the en-

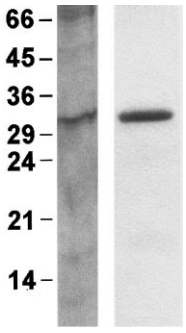

Fig. 1. Gel electrophoretic separation of NMNAT. NMNAT was purified from human placenta (left lane) or from overexpression in *E. coli* (right lane) as described in Section 2. SDS–PAGE was performed using a 12.5% acrylamide gel. Proteins were stained with Coomassie blue. Numbers on the left indicate the mobility of marker proteins.

zyme preparation was analyzed by SDS–PAGE (Fig. 1, left lane). The gel electrophoresis resulted in a prominent protein band of about 33 kDa, well separated from a few minor protein bands. Since it has been reported that the subunit molecular mass of human NMNAT is about 33 kDa [25], this band was excised from the gel and subjected to 'in gel' digestion with trypsin [21]. The sequences of two resulting peptides were established to be SNIHVVNEWIANDISSTK and NAGVILAPLQR using both MALDI-TOF and automated microsequencing. These sequences were compared to protein and nucleic acid sequences deposited in available databases. Coding sequences for both peptides were found in the GenBank database as part of an EST (EST 182517, GenBank accession number AA311657). According to this sequence the two peptides would be located in the protein only 37 amino acids apart from each other. When the NMNAT cDNA sequence was eventually established (see below), it turned out that this EST indeed contains the coding region for the 72 C-terminal amino acids of NMNAT. Moreover, a potential coding region for the first peptide was also found in the sequence of another EST (EST 601141175F1, GenBank accession number BE272001). This EST covers nucleotides 92–759 of the cDNA sequence established in this study. The 5′-terminal sequence of the NMNAT cDNA could be deduced from an EST (EST qh84c06.×1, GenBank accession number AI243824) which contains the coding region of NMNAT up to nucleotide 353 and overlaps with EST 601141175F1 in a stretch of 260 nucleotides. Aligning the three overlapping ESTs an open reading frame was deduced that could potentially encode human NMNAT.

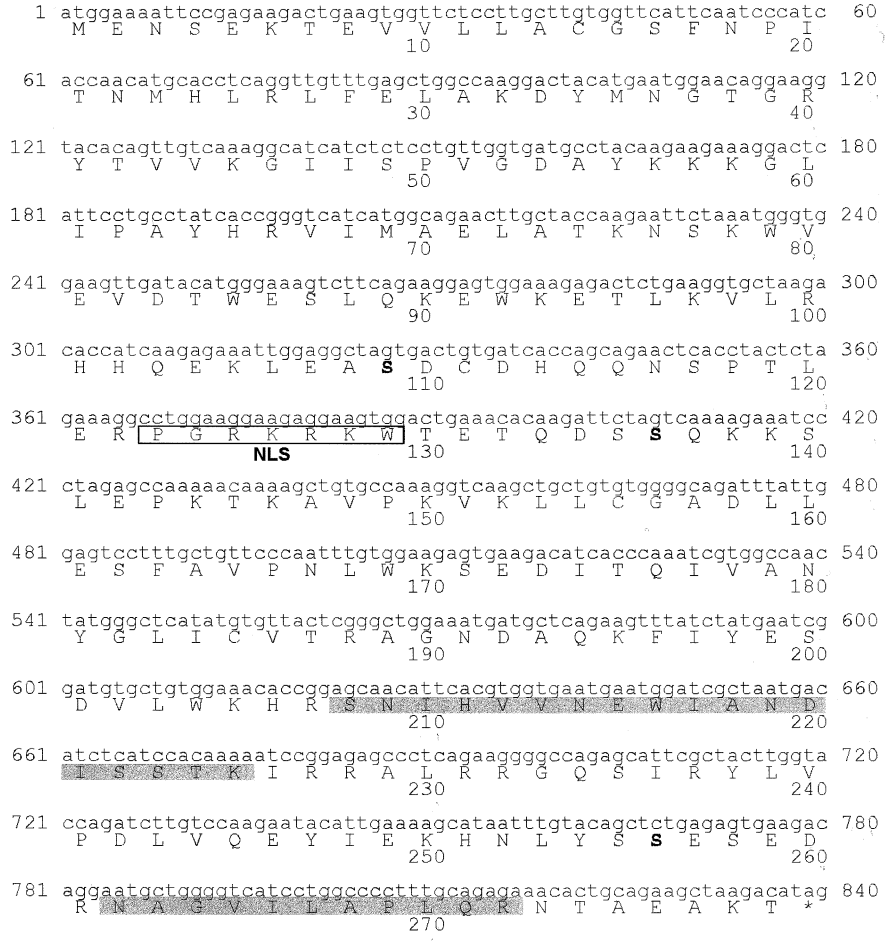

Fig. 2. Complete coding sequence of the human NMNAT cDNA and the deduced amino acid sequence. Gray boxes highlight the two peptides initially determined (amino acids 208–225 and 262–272). A putative nuclear localization signal (NLS) is indicated (amino acids 123–129). Serines 109, 136, and 256 are typed in bold face, because they represent potential phosphorylation sites (probability score >0.98). The GenBank accession number of the sequence is AF314163.

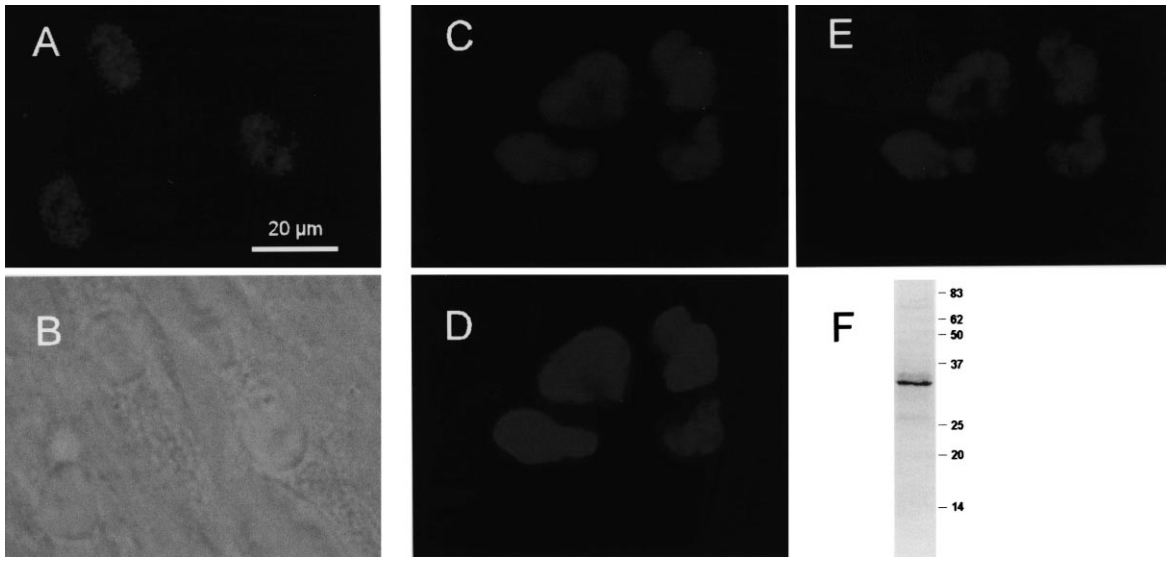

Fig. 3. Immunofluorescent subcellular localization of the human NMNAT. Human fibroblasts (A and B) or HepG2 cells (C–E) were immunostained with a purified polyclonal NMNAT antibody raised in rabbit (A and C). B: Phase contrast image. D: Chromosomal stain with DAPI. E: Immunofluorescence of transcription factor YY1 was used as an additional control for nuclear staining. F shows a Western blot of HepG2 cell lysates developed with the purified NMNAT antibody. Numbers on the right indicate the mobility of prestained marker proteins.

This information was used to clone the cDNA encoding NMNAT. PCR was carried out using primers corresponding to the presumed 5′- and 3′-terminal sequences and a cDNA library prepared from human lymphoblastoid cells. The amplified DNA had the expected size of approximately 850 bp. Sequencing revealed that the amplified cDNA indeed contained two internal sequence stretches that would encode the previously established peptides. Moreover, the sequences of the ESTs were almost identical to the corresponding parts of the amplified cDNA. The sequence of the 5′-upstream region of the cDNA was verified to ensure the correct prediction of the open reading frame. Eventually, the putative coding region was cloned into the pQE vector in order to attach an N-terminal 6×His-tag to the protein.

The recombinant protein was then overexpressed in *E. coli* and purified (Fig. 1, lane 2). The specific activity of the purified recombinant enzyme amounted to about 25 U/mg of protein. The properties of this protein were virtually identical to those found for NMNAT purified from human placenta [25].

In Fig. 2 the complete DNA sequence corresponding to the coding region of the mRNA of human NMNAT is shown along with the deduced amino acid sequence. The two tryptic peptides initially sequenced are indicated. Both peptides matched fully with the deduced amino acid sequence.

The human NMNAT consists of 279 amino acids and has a theoretical molecular mass of 31 932 Da. This molecular mass is in good agreement with that estimated for the purified protein in this (Fig. 1) and previous studies [20,25].

Comparison to the amino acid sequence of the yeast enzyme [16] revealed that the human form lacks the region of the first 197 N-terminal amino acids, similar to the putative NMNATs from *Caenorhabditis elegans* [16]. This is in accordance with the difference in subunit molecular masses (48 kDa and 32 kDa for the yeast and human enzymes, respectively). The C-terminal part of the yeast protein (corresponding to the complete human sequence) displays about 38% identity to the human enzyme.

Analysis of the primary structure of the human NMNAT by the PSORT II program revealed the presence of a putative nuclear localization signal (PGRKRKW, amino acids 123–129, cf. Fig. 2). The nuclear localization of this enzyme was clearly demonstrated by immunofluorescence (Fig. 3). An affinity-purified rabbit antibody raised against the recombinant NMNAT was highly specific for NMNAT as revealed by Western blot analysis conducted with lysates of HepG2 (Fig. 3F) and other cells (HeLa, fibroblasts, not shown). Im-

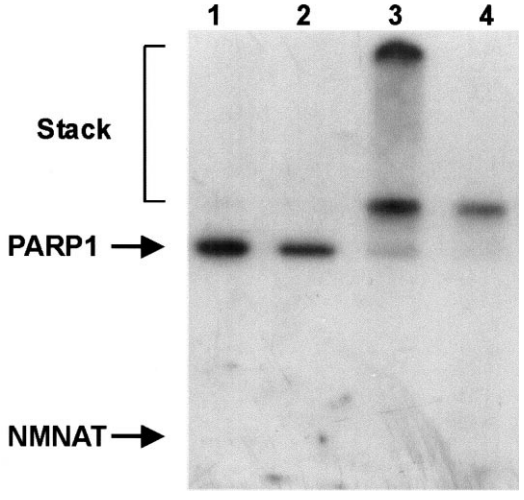

Fig. 4. Influence of recombinant NMNAT on the activity of PARP1. Recombinant human PARP1 was incubated with [^{32}P]NAD in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of recombinant human NMNAT under the conditions described in Section 2. At low concentrations of [^{32}P]NAD (5 nM, lanes 1 and 2) only short ADP-ribose oligomers are formed (1–7 units). Addition of 10 μM of unlabeled NAD to the samples presented in lanes 1 and 2 led to the formation of long ADP-ribose polymers and a concomitant shift of PARP1's apparent molecular mass (lanes 3 and 4). A long stacking gel was used to demonstrate the inability of a significant fraction of PARP1 to enter the gel indicating the formation of branched polymers (lane 3) which was not observed in the presence of NMNAT (lane 4). The autoradiogram of a representative gel is shown.

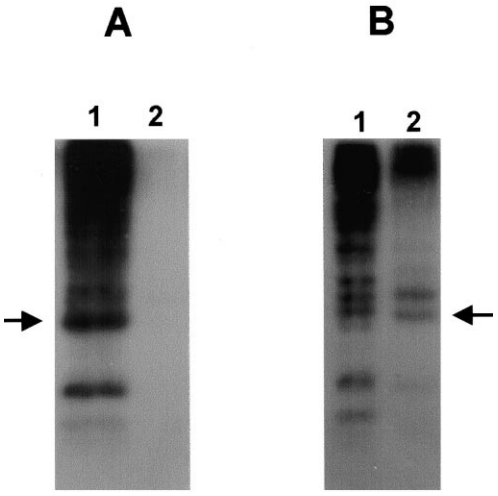

Fig. 5. Phosphorylation of endogenous and recombinant NMNAT in nuclear extracts. Nuclear extracts were prepared from human fibroblasts (cf. Fig. 3) and incubated for 30 min at 30°C with [γ-^{32}P]ATP in the presence (A) or absence (B) of recombinant NMNAT as described in Section 2. A: Following incubation an aliquot was withdrawn (lane 1). The remainder was supplemented with 2 U of alkaline phosphatase and incubated for another 30 min (lane 2). B: Following incubation, immunoprecipitation of endogenous NMNAT was conducted using the purified antibody and protein A Sepharose (see Section 2). Unbound protein (lane 1) was removed, the Sepharose washed thoroughly and specifically bound proteins (lane 2) were eluted. Samples were separated in a 12% SDS–polyacrylamide gel and phosphorylated proteins visualized by autoradiography. The arrows indicate the positions of recombinant or endogenous NMNAT, respectively.

munostaining of primary human fibroblasts (Fig. 3A,B) and HepG2 cells (Fig. 3C–E) demonstrated the presence of NMNAT in the nuclei. The assignment of the nucleus was based on costaining of the transcription factor YY1 (Fig. 3D) and staining of the chromosomes by 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 3E). Nuclear localization of NMNAT was also observed in HeLa cells (not shown).

It has been proposed [10,11] that NMNAT may interact with the nuclear protein PARP1. Using both recombinant human enzymes the influence of NMNAT on PARP1 activity was tested. As shown in Fig. 4 NMNAT substantially reduced the automodification of PARP1. Moreover, NMNAT did not serve as acceptor protein of the modification (lane 2). Quantification of automodification of PARP1 established that the presence of NMNAT led to a decrease to about 35%. NMNAT appeared to completely prevent the formation of branched ADP-ribose polymers.

According to a previous report [25] the enzyme isolated from human placenta exhibited several acidic isoelectric points (pH 4.7, 5.7, and 6.6). However, if calculated from the amino acid composition found here, the isoelectric point would be at pH 8.98. This discrepancy could be, at least in part, due to post-translational modifications such as phosphorylation. Indeed, an analysis using the NetPhos 2.0 program strongly indicated the presence of potential phosphorylation sites (Fig. 2). The probability score for three serine residues (amino acids 109, 136, and 256, cf. Fig. 2) exceeded 0.98. It was tested, therefore, whether NMNAT serves as substrate for nuclear kinases. Incubation of the recombinant NMNAT with nuclear extracts in the presence of [γ-^{32}P]ATP led to substantial labeling of the enzyme (Fig. 5A). The label was removed by incubation with alkaline phosphatase supporting the conclusion that NMNAT is a substrate for phosphorylation. This notion was further substantiated by incubating nuclear extracts in the presence of [γ-^{32}P]ATP and subsequent immunoprecipitation of endogenous NMNAT using a purified polyclonal antibody generated against the recombinant enzyme (cf. Fig. 3F). As shown in Fig. 5B, the majority of phosphorylated proteins were not precipitated (lane 1), whereas a precipitated radiolabeled protein migrated corresponding to NMNAT (lane 2). An unidentified phosphorylated protein of approximately 36 kDa appeared to specifically coprecipitate with NMNAT (Fig. 5B, lane 2). It cannot be ruled out, however, that this band represents a hyperphosphorylated form of NMNAT.

4. Discussion

The identification of the primary structure of human NMNAT represents a major step towards the understanding of pyridine nucleotide biosynthesis. It is the first established sequence of NMNAT from an organism higher than yeast. All known pathways leading to the synthesis of NAD require NMNAT. Therefore, this enzyme is of crucial importance for metabolism, energy supply and signal transduction. Although the enzyme would be expected to be highly conserved, because it must be present in all organisms, there are substantial differences to the counterparts in lower organisms. For example, the yeast enzyme contains an amino-terminal stretch of 179 amino acids which is completely absent from the human enzyme. Nevertheless, the catalytic properties of the two forms are very similar. Possibly, variations in the primary structure may be related to mechanisms regulating the catalytic activity or interactions with other proteins. Comparing the sequence of the human NMNAT (279 amino acids) to that of *M. jannaschii* (168 amino acids) revealed only very weak similarities in short sequence stretches.

Investigations over the past years have added further weight to the essential role of the pyridine coenzymes. The role of NAD as precursor of signaling molecules and as substrate for protein modification underlines the importance of the regulation of NAD synthesis. In this regard it is intriguing that NMNAT is located within the nucleus as clearly demonstrated in this study. It is also an important finding that NMNAT appears to be a substrate for nuclear kinase(s). The immunoprecipitation conducted in this study indicated that phosphorylated NMNAT is in a complex with at least one other phosphorylated protein. A specific interaction between recombinant human NMNAT and PARP1 has been found in this study by demonstrating a significant inhibition of automodification of PARP1 in the presence of NMNAT (Fig. 4). An essential role has been ascribed to NMNAT in the cellular response to genotoxic damage. Under these conditions PARP1 may consume most of the cellular NAD. The decrease of the ATP concentration has been proposed to be a result of the resynthesis of NAD which requires ATP [26]. Consequently, inhibition of PARP1 by NMNAT may be directed to prevent NAD and ATP depletion.

In addition to its essential role in NAD synthesis, NMNAT could also be important for the synthesis of the NADP derivative, NAADP. This nucleotide has been demonstrated to be a highly potent calcium-mobilizing agent (reviewed in [27]). A potential synthetic pathway could include the synthesis of

NAAD from nicotinic acid mononucleotide and ATP by NMNAT followed by phosphorylation by NAD kinase or a similar enzyme. In conclusion, the present study suggests that NMNAT is likely to be more than a simple metabolic enzyme, because it is located exclusively within the nucleus, exhibits specific interactions with PARP1 and appears to be regulated by phosphorylation.

Acknowledgements: This study was supported by the Deutsche Forschungsgemeinschaft (SCHW 532/8-1). We thank Dr. Peter Franke for the mass spectrometric analyses.

References

[1] Lin, S.-J., Defossez, P.-A. and Guarente, L. (2000) Science 289, 2126–2128.
[2] Smith, J.S., Brachmann, C.B., Celic, I., Kenna, M.A., Muhammad, S., Starai, V.J., Avalos, J.L., Escalante-Semerena, J.C., Grubmeyer, C., Wolberger, C. and Boeke, J.D. (2000) Proc. Natl. Acad. Sci. USA 97, 6658–6663.
[3] Ziegler, M. (2000) Eur. J. Biochem. 267, 1550–1564.
[4] Lindahl, T. and Wood, R.D. (1999) Science 286, 1897–1905.
[5] Magni, G., Amici, A., Emanuelli, M., Raffaelli, N. and Ruggieri, S. (1999) Adv. Enzymol. Relat. Areas Mol. Biol. 73, 135–182.
[6] Balducci, E., Emanuelli, M., Magni, G., Raffaelli, N., Ruggieri, S., Vita, A. and Natalini, P. (1992) Biochem. Biophys. Res. Commun. 189, 1275–1279.
[7] Solao, P.B. and Shall, S. (1971) Exp. Cell Res. 69, 295–300.
[8] Emanuelli, M., Raffaelli, N., Amici, A., Balducci, E., Natalini, P., Ruggieri, S. and Magni, G. (1995) Biochem. Pharmacol. 49, 575–579.
[9] Jayaram, H.N., Cooney, D.A. and Grusch, M. (1999) Curr. Med. Chem. 6, 561–574.
[10] Uhr, M.L. and Smulson, M. (1982) Eur. J. Biochem. 128, 435–443.
[11] Ruggieri, S., Gregori, L., Natalini, P., Vita, A., Emanuelli, M., Raffaelli, N. and Magni, G. (1990) Biochemistry 29, 2501–2506.
[12] Sims, J.L., Berger, S.J. and Berger, N.A. (1983) Biochemistry 22, 5188–5194.
[13] Das, S.K. and Berger, N.A. (1986) Biochem. Biophys. Res. Commun. 137, 1153–1158.
[14] Oei, S.L., Griesenbeck, J. and Schweiger, M. (1997) Rev. Physiol. Biochem. Pharmacol. 131, 127–174.
[15] D'Amours, D., Desnoyers, S., D'Silva, I. and Poirier, G.G. (1999) Biochem. J. 342, 249–268.
[16] Emanuelli, M., Carnevali, F., Lorenzi, M., Raffaelli, N., Amici, A., Ruggieri, S. and Magni, G. (1999) FEBS Lett. 455, 13–17.
[17] Raffaelli, N., Pisani, F.M., Lorenzi, T., Emanuelli, M., Amici, A., Ruggieri, S. and Magni, G. (1997) J. Bacteriol. 179, 7718–7723.
[18] Raffaelli, N., Lorenzi, T., Amici, A., Emanuelli, M., Ruggieri, S. and Magni, G. (1999) FEBS Lett. 444, 222–226.
[19] Mehl, R.A., Kinsland, C. and Begley, T.P. (2000) J. Bacteriol. 182, 4372–4374.
[20] Magni, G., Emanuelli, M., Amici, A., Raffaelli, N. and Ruggieri, S. (1997) Methods Enzymol. 280, 241–247.
[21] Eckerskorn, C. and Lottspeich, F. (1989) Chromatographia 28, 92–94.
[22] Chaurand, P., Luetzenkirchen, F. and Spengler, B. (1999) J. Am. Soc. Mass Spectrom. 10, 91–103.
[23] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Coning – A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press.
[24] Schreiber, E., Matthias, P., Muller, M.M. and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419.
[25] Emanuelli, M., Natalini, P., Raffaelli, N., Ruggieri, S., Vita, A. and Magni, G. (1992) Arch. Biochem. Biophys. 298, 29–34.
[26] Pieper, A.A., Verma, A., Zhang, J. and Snyder, S.H. (1999) Trends Pharmacol. Sci. 20, 171–181.
[27] Lee, H.C. (2000) J. Membr. Biol. 173, 1–8.