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cDNA cloning and characterization of mouse *nifS*-like protein, m-Nfs1: mitochondrial localization of eukaryotic NifS-like proteins

Yumi Nakai, Yoshihiro Yoshihara, Hideyuki Hayashi, Hiroyuki Kagamiyama*

Department of Biochemistry, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki, Osaka 569-8686, Japan

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Abstract We have isolated a mouse cDNA which shows significant sequence similarity to the yeast *nifS*-like gene (y-*NFS1*), and termed it m-*Nfs1*. The deduced protein sequence (459 amino acids long) has several characteristic features common to those of bacterial NifS proteins, but distinct from them by its amino-terminal extension which contains a typical mitochondrial targeting presequence. m-Nfs1 was found to be a soluble 47-kDa protein in the matrix fraction of mouse liver mitochondria. The m-*Nfs1* gene was ubiquitously expressed in most tissues, suggesting its housekeeping function in vivo. We also found that the y-NFS1 protein was localized in the mitochondrial matrix in yeast cells. These results suggest that both eukaryotic NifS-like proteins may play some roles in mitochondrial functions.

Key words: *NifS*-like protein; Eukaryote; Mitochondrion

1. Introduction

The *nifS* gene was first identified as a member of the family of nitrogen fixation genes (*nif* genes) in some diazotrophic bacteria [1], and, to date, many *nifS*-like genes have also been cloned from a number of non-diazotrophic prokaryotes [2–7]. *Azotobacter vinelandii* NifS protein was shown to possess a cysteine desulfurase activity that produces L-alanine and elemental sulfur from L-cysteine [8], and its physiological function has been considered to be the supply of an elemental sulfur to the iron-sulfur ([Fe-S]) clusters of the active site of nitrogenase [9]. The *Escherichia coli* genome contains three *nifS*-like genes, and one of them has been shown to participate in the synthesis of the [Fe-S] cluster of dihydroxy acid dehydratase in a manner similar to the catalytic property of *A. vinelandii* NifS protein [10,11].

In contrast to various descriptions of prokaryotic *nifS* (and *nifS*-like) genes, few studies have been devoted to the eukaryotic NifS-like proteins. The best-characterized *nifS*-like gene in a eukaryote is *Saccharomyces cerevisiae NFS1* (y-*NFS1* in the present study, alternatively named *spl1*), which may be involved in tRNA splicing [12]. However, the gene product has not been identified or characterized. Thus, its intracellular location and physiological functions remain unclear. The *nifS*-like gene has also been found in *Caenorhabditis elegans* through its genome sequencing project [13]. In addition, more than 10 expressed sequence tag (EST) clones prepared from a human and mouse, which show significant sequence similarities to the y-*NFS1* gene, have been deposited in the EMBL/GenBank databases, although further investigations of these eukaryotic *nifS*-like genes have not been reported.

In the present study, we have cloned the mouse *nifS*-like cDNA (named m-*Nfs1*) and identified its product, the m-Nfs1 protein, in various tissues. Mitochondrial localization of both the m-Nfs1 protein and its yeast counterpart has also been demonstrated. This is the first report of the identification and characterization of eukaryotic NifS-like proteins.

2. Materials and methods

2.1. Isolation of the mouse NifS-like gene from cDNA library

Two degenerate oligonucleotide primers which correspond to the conserved amino acid sequences between y-NFS1 and a human nfs-like EST clone (ITT(Q/R)TEHKC and YFHTDAAQ) were used for the amplification of a partial cDNA fragment of m-*Nfs1* by polymerase chain reaction (PCR) with mouse brain cDNA as a template. The amplified DNA fragment of expected size (211 bp) was subcloned and used as a probe to obtain the full-length m-*Nfs1* gene from an unamplified mouse olfactory epithelium λgt10 cDNA library (5×10^5 recombinant phages). Positive clones were detected by the chemiluminescence method (ECL Direct Labeling System, Amersham, UK). One positive clone was detected and its cDNA insert was excised, subcloned into pUC118, and sequenced for both strands. 5′-RACE (rapid amplification of cDNA ends) was carried out with total RNA prepared from mouse brain (ddY, 5 weeks) using a 5′-RACE Kit (Life Technologies) according to the manufacturer's instructions.

2.2. Northern blot analysis

Total RNA was isolated from 11 tissues (brain, heart, lung, thymus, liver, pancreas, stomach, spleen, kidney, skeletal muscle, and testis) of the mouse (ddY, 5 weeks, male), and equal amounts of RNA (10 μg) were separated in 1.2% agarose gel containing formaldehyde and blotted on a nylon membrane (Hybond-N^+, Amersham, UK). An 820 bp *Spe*I/*Kpn*I DNA fragment of the m-*Nfs1* gene was radiolabeled with ^{32}P by the random-prime labeling method (BcaBEST DNA Labeling Kit, TaKaRa, Japan). The blots were hybridized with the radiolabeled probe, heated at 68°C for 16 h, washed for 1 h at 68°C in 2×SSC, then dried and autoradiographed.

2.3. Expression of the mouse Nfs1 protein in E. coli and production of the antisera

The entire coding region of the m-*Nfs1* cDNA was inserted into pET21b (Novagen) to make a translational fusion protein of m-Nfs1 with amino-terminal 12 amino acid residues (T7 tag) and the carboxy-terminal hexahistidine (His6 tag). The fusion protein (T7-m-Nfs1-His6 protein) was expressed in *E. coli* BL21(DE3), purified, and then used for the generation of polyclonal antibodies against the m-Nfs1 protein in rabbits.

2.4. Preparation of cell extracts and subfractions from mouse tissues

Eleven tissues (brain, heart, lung, thymus, liver, pancreas, stomach, spleen, kidney, skeletal muscle, and testis) from the mouse (ddY, 5 weeks, male) were homogenized and suspended in phosphate-buffered saline to a final concentration of 1 mg protein/ml, and 30 μg of each was used for Western analysis. Subcellular fractionation of mouse liver cells was performed as follows: mouse liver cells (1.5 g) were suspended in 15 ml of ice-cold 0.25 M sucrose solution containing 1 mM phenylmethylsulfonyl fluoride and homogenized, and were centrifuged for 10 min at $600\times g$. The pellets were washed with the same buffer and used for the isolation of the nuclear fraction. The supernatants were centrifuged for 10 min at $10\,000\times g$ to obtain crude

*Corresponding author. Fax: (81) (726) 84-6516.

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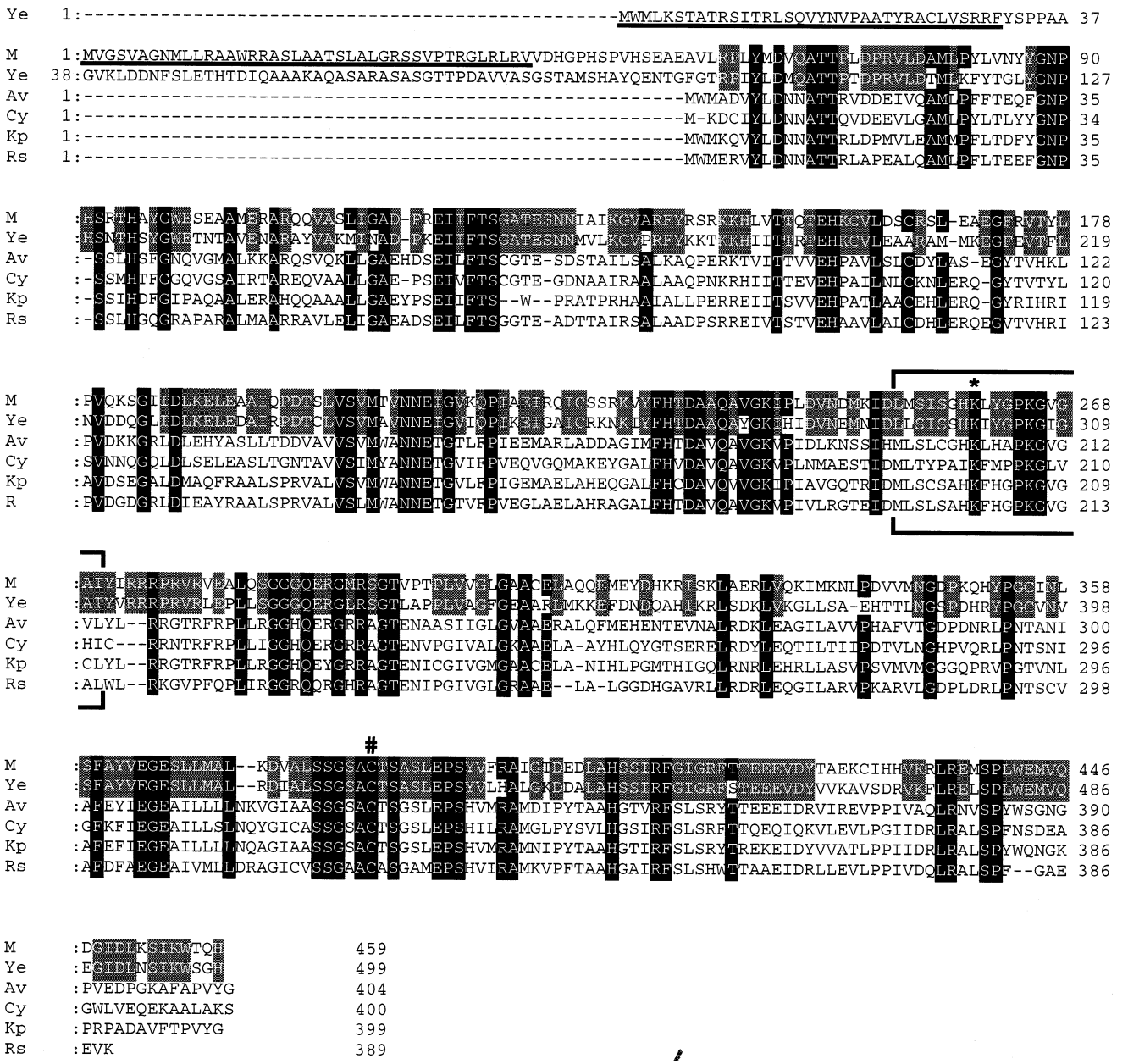

Fig. 1. Amino acid sequence alignment of mouse, yeast, and diazotrophic bacterial NifS proteins. The deduced amino acid sequence of the mouse Nfs1 protein is compared to that of *S. cerevisiae* NFS1 protein and those of the representative NifS proteins in several diazotrophs. M, mouse Nfs1 (m-Nfs1); Ye, *S. cerevisiae* NFS1 (y-NFS1); Av, *A. vinelandii* NifS; Cy, *Cyanothece* PCC8801 NifS; Kp, *Klebsiella pneumoniae* NifS; Rs, *Rhodobacter sphaeroides* NifS. Residues conserved between m-Nfs1 and y-NFS1 are halftone-inverted and those conserved among m-Nfs1 and bacterial NifS proteins shown in this diagram are fulltone-inverted. A putative PLP binding motif found in all sequences shown is covered with blankets, which includes a lysine residue for PLP binding (marked with an asterisk). The cysteine residue which is thought to be involved in the cysteine desulfuration activity of *A. vinelandii* NifS protein is conserved among all members of these proteins, and is marked with a sharp. The putative mitochondrial sorting signal sequences found in m-Nfs1 and y-NFS1 proteins are underlined.

mitochondrial pellets and post-mitochondrial supernatants. The crude mitochondrial pellets were washed once and resuspended in 3 ml of the same buffer. The mitochondrial fraction was used for further analysis through sucrose-density gradient centrifugation and also used for submitochondrial fractionation. The post-mitochondrial supernatants were re-centrifuged for 60 min at 100 000 × *g* to obtain microsomal fraction and soluble cytosolic fraction. Submitochondrial fractionation was performed as previously described [14].

2.5. Western blot analysis with purified anti-m-Nfs1 antibody

Proteins in various cell homogenates or subcellular fractions were separated by electrophoresis on a 12.5% polyacrylamide-SDS gel and transferred to polyvinylidene difluoride (PVDF) membranes, then subjected to Western analysis with the purified anti-m-Nfs1 antibodies. The immunoreactive proteins were detected with ECL Western Detection Kit and Hyper ECL film (Amersham, UK).

2.6. Expression and detection of c-Myc-tagged NFS1 protein in S. cerevisiae

The *S. cerevisiae* genomic DNA fragment containing the *NFS1* gene and its 1.0 kb 5′-upstream region was obtained by the PCR amplification using the yeast genomic DNA prepared from strain D273-10B (*MAT*α) as a template. The amplified fragment and a yeast-*E. coli* shuttle vector pYEUra3 (Clontech) were used to construct a yeast expression plasmid pYE-y-NFS1/myc which encodes a modified y-NFS1 protein containing the carboxy-terminal c-Myc epitope tag of

11 amino acids (EQKLISEEDLN). The pYE-y-NFS1/myc was transformed into yeast strain W303-1B (*MATα ade2-2 his3-11,15 ura3-1 leu2-3,112 trp1-1 can1-100*). Subcellular and submitochondrial fractionations of yeast cells were performed essentially according to the published procedure [14]. The anti-c-Myc peptide tag monoclonal antibody (9E10, Boehringer Mannheim) was used to detect the c-Myc-tagged y-NFS1 protein.

3. Results

To clone cDNA encoding the mouse *NifS*-like protein, we designed a set of degenerate oligonucleotides corresponding to the amino acid stretches conserved between *S. cerevisiae NFS1* and a putative human NifS-like protein found in an EST clone. Using these oligonucleotides as primers, the 211 bp cDNA fragment which appeared to encode a part of the mouse *NifS*-like gene was obtained by PCR amplification of the mouse brain cDNA. With this cDNA fragment, the full-length clone of the mouse *Nfs1* cDNA was isolated by screening 5×10^5 phages of a mouse olfactory epithelium cDNA library. One positive plaque isolated contained the cDNA insert of 2022 nucleotides. We also carried out 5′-RACE to obtain the sequence at the 5′ end, and assembled it into the full-length m-*Nfs1* cDNA. The complete cDNA of 2051 nucleotides contains an open reading frame (ORF) of 1377 nucleotides (the sequence has been submitted to the EMBL Data Library at Hinxton in UK under accession number AJ222660). If the first ATG in this ORF is referred to as the initiation codon, the m-*Nfs1* cDNA potentially encodes a protein of 459 amino acid residues with a calculated molecular mass of 50 716 Da.

A comparison of the deduced amino acid sequence to entries in the NCBI (National Center for Biotechnology Information at NIH, USA) database by the BLAST program [15] revealed that the ORF shows the highest degree of homology to that of the *S. cerevisiae* NFS1 protein (68.5% identity) and also exhibits significant similarity to those of diazotrophic bacterial NifS proteins (39.7–43.9% identity). The deduced amino acid sequence of the ORF was aligned with those of representative members of diazotrophic bacterial NifS proteins and *S. cerevisiae* NFS1 protein (Fig. 1). MOTIF-Search analysis (DBGET integrated database retrieval systems, GenomeNet) identified a putative pyridoxal phosphate (PLP) binding motif, which is also found in the *S. cerevisiae* NFS1 sequence and in those of other diazotrophic bacterial NifS proteins (Fig. 1). It contains a lysine (marked with an asterisk in Fig. 1) which is believed to form a Schiff base with PLP. The cysteine residue essential for the cysteine desulfuration activity of *A. vinelandii* NifS protein [8,16] and conserved among all the members of NifS and NifS-like proteins is also found in the m-Nfs1 protein (Cys-383; marked with a sharp in Fig. 1). Based on these sequence characteristics, we considered that the ORF encodes a mouse NifS-like protein, and thus named it m-Nfs1.

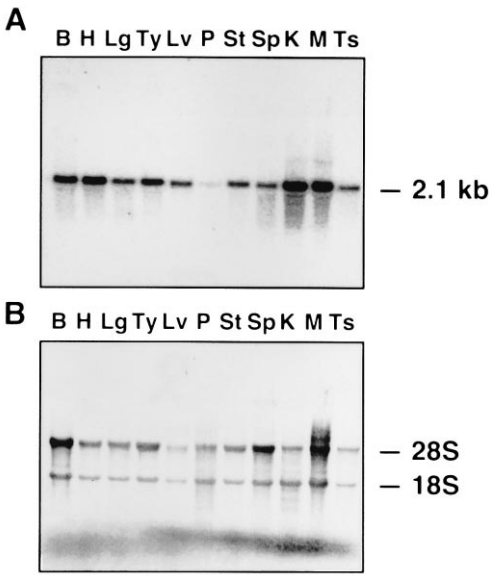

Fig. 2. Northern blot analysis of mouse *Nfs1* mRNA. Total RNA prepared from 11 mouse tissues (B, brain; H, heart; Lg, lung; Ty, thymus; Lv, liver; P, pancreas; St, stomach; Sp, spleen; K, kidney; M, skeletal muscle; Ts, testis) was prepared as described in Section 2, and a 10 μg aliquot from each tissue was analyzed. The upper panel (A) shows radioautograms of the blot after probing with the ^{32}P-labeled m-*Nfs1* cDNA, and the lower (B) shows the same blot stained with methylene blue. The positions of the 2.1 kb RNA transcript and ribosomal RNAs (28S and 18S) are indicated on the right.

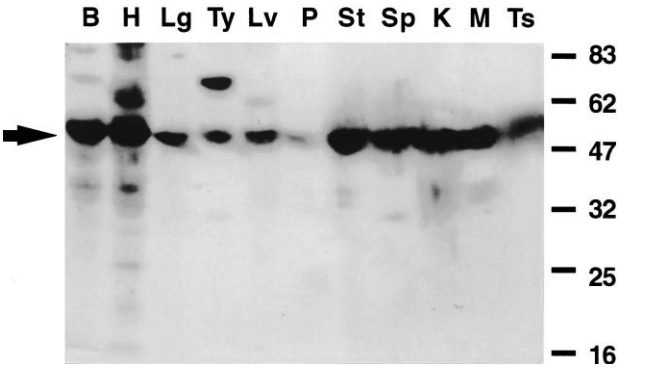

Fig. 3. Western analysis of the m-Nfs1 protein in mouse tissues. Cell extracts from 11 tissues of mouse (B, brain; H, heart; Lg, lung; Ty, thymus; Lv, liver; P, pancreas; St, stomach; Sp, spleen; K, kidney; M, skeletal muscle; Ts, testis) were prepared separately as described in Section 2, and a 30 μg aliquot was separated with 12.5% SDS-PAGE and blotted. The blots were analyzed with polyclonal anti-m-Nfs1 antibody. Numbers indicate the molecular masses (in kDa) of marker proteins. The position of the immunoreactive 47 kDa protein is indicated by an arrow.

Comparison of the m-*Nfs1* cDNA sequence with mouse EST clones which potentially encode NifS-like protein revealed that almost all of them were derived from the m-*Nfs1* mRNA. However, one EST clone (EMBL accession number W64820) shows significantly lower sequence identity (52.2%) to the m-Nfs1 protein, suggesting that another *nifS*-like gene might exist in the mouse.

Both the mouse and yeast NifS-like proteins (m-Nfs1 and y-NFS1, respectively) have an amino-terminal extension more than 50 residues longer than those of bacterial NifS proteins. Although the amino-terminal 58 residues of m-Nfs1 protein have no significant sequence similarities to the corresponding region of y-NFS1, a protein sorting signal searching program (PSORT WWW Server; http://psort.nibb.ac.jp/) detected mitochondrial sorting signals in the amino-terminal extensions of both the m-Nfs1 and y-NFS1 proteins (underlined in Fig. 1). Both amino-terminal extensions are rich in basic and hydroxylated amino acid residues, which are characteristic features of mitochondrial sorting signals. Moreover, the PSORT

program also detected consensus patterns of sequence around the mitochondrial processing peptidase cleavage sites in both amino-terminal extensions (LRVVDH in the m-Nfs1 and RRFYSP in the y-NFS1). Therefore, these amino-terminal extensions are most likely the mitochondrial targeting presequence which are cleaved off upon their import into the mitochondria.

In order to determine the tissue distribution of m-*Nfs1* mRNA, Northern blot analysis was performed with total RNAs prepared from various mouse tissues. A single 2.1 kb transcript was detected in all examined tissues (Fig. 2). The length of this transcript was in good agreement with the size of m-*Nfs1* cDNA of 2051 bp. A rather weak hybridization signal was seen for total pancreas RNA (Fig. 2), but prolonged exposure clearly showed the presence of the transcript in this tissue (data not shown). This result suggests that the m-*Nfs1* is ubiquitously expressed in various tissues of the mouse.

To obtain antisera against the m-Nfs1 protein, a translational fusion protein, T7-m-Nfs1-His6, was expressed in *E. coli*. The recombinant m-Nfs1 fusion protein, whose calculated molecular mass is 53 052 Da, accumulated as the inclusion body in the host *E. coli* cells and was detected as a 53 kDa band by SDS-PAGE (data not shown). Polyclonal antibodies were raised against this recombinant T7-m-Nfs1-His6 protein, purified, and used for immunological detection in cell extracts prepared from various tissues of the mouse. Anti-m-Nfs1 antibodies detected an immunoreactive protein of 47 kDa in all cell extracts examined (Fig. 3). We then further investigated its intracellular localization in mouse tissues.

To determine the intracellular localization of the m-Nfs1 protein, mouse liver was homogenized and fractionated into mitochondrial, microsomal, and cytosolic fractions, and each fraction was examined by Western analyses with anti-m-Nfs1 antibodies. The 47 kDa immunoreactive protein was detected mainly in the mitochondrial fraction (Fig. 4A) as was the mitochondrial marker protein F1-β (the β subunit of mitochondrial F1-ATPase). To confirm that the 47 kDa protein exists in mitochondria but not in any contaminated other cellular membrane fractions, the mitochondrial fraction was further purified by centrifugation through the sucrose density gradient. Distribution of the 47 kDa protein in the gradient was essentially identical to that of the mitochondrial F1-β (Fig. 4B). We further examined the submitochondrial localization of the 47 kDa protein. As shown in Fig. 4C, the protein was found mainly in the matrix fraction of mitochondria. These results suggest that the m-Nfs1 protein localizes in the mitochondrial matrix as a soluble protein.

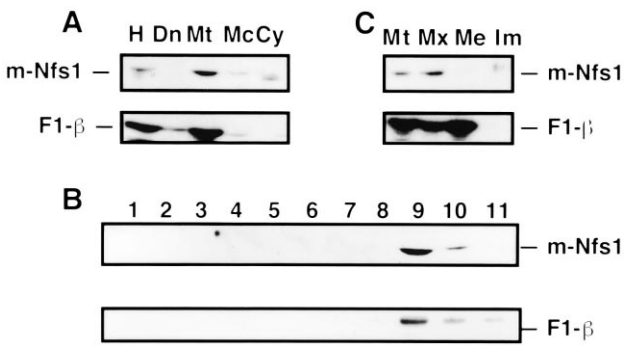

Fig. 4. Intracellular localization of mouse liver Nfs1 protein. 20 mg of protein in each subcellular (A, B) or submitochondrial (C) fraction of mouse liver cells was separated with 12.5% polyacrylamide gels and analyzed by Western blotting. In each panel, the upper column shows the immuno-blots of the m-Nfs1 protein, and the lower shows that of the mitochondrial marker protein F1-β (F1-ATPase β subunit, a peripheral protein associated with another subunit anchored the mitochondrial inner membrane). In panel B, 11 aliquots of mitochondria fractions separated by sucrose density gradient, which are sequential toward dense fractions, were used. H, cell homogenates; Dn, the nuclear fraction precipitated by low-spin centrifugation of the cell homogenates which also contain cell debris; Mt, the mitochondrial fraction; Mc, the microsomal fractions; Cy, the soluble cytosolic fraction; Mx, the mitochondrial matrix fraction; Me, the mitochondrial membrane fraction; Im, the mitochondrial inner membrane space fraction.

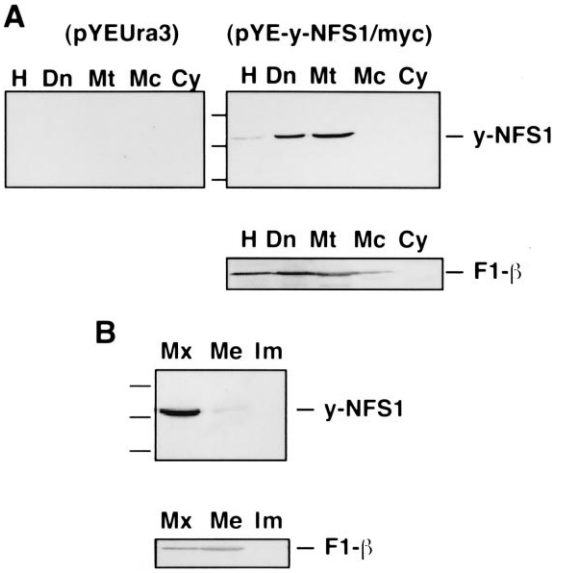

Fig. 5. Intracellular localization of c-Myc-tagged yeast NFS1 protein. Intracellular localization of the c-Myc-tagged y-NFS1 protein from *S. cerevisiae* was analyzed by Western blotting. (A) The intracellular localization of the c-Myc-tagged y-NFS1 protein. Immunoblot with yeast cells including the vector pYEUra3 is shown in the left panel, and the blot with yeast cells containing pYE-NFS1/myc is shown in the right panel. (B) The sub-mitochondrial localization of the protein. The fraction of each lane is indicated with the same mark as in Fig. 4. Bars indicate the positions of molecular weight marker proteins: 62, 47, and 32 kDa (from top to bottom).

As previously mentioned, the m-Nfs1 shows a relatively high degree of sequence homology to the y-NFS1 protein, and both proteins have potential mitochondrial sorting signals at their amino-terminal regions. To clarify the intracellular localization of the y-NFS1 protein, a c-Myc peptide-tagged y-NFS1 protein was expressed in yeast cells, and its cellular distribution was analyzed by Western blotting using anti-c-Myc peptide antibody. As shown in Fig. 5A, the immunoreactive 50 kDa protein was associated mainly with the mitochondrial fraction. A small fraction of the 50 kDa protein was also detected in the cell debris fraction which contains unbroken cells and a nucleus (Fig. 5A). This distribution of the 50 kDa protein in these cell fractions was similar to that to the mitochondrial marker protein F1-β (Fig. 5A, lower panel). Upon submitochondrial fractionation, the 50 kDa protein was predominantly recovered in the matrix fraction (Fig. 5B), thus suggesting that the y-NFS1 protein also exists in the mitochondrial matrix.

4. Discussion

In the present study, we have cloned the cDNA for the mouse NifS-like protein, m-Nfs1, and immunologically identified the 47 kDa soluble protein in the mitochondrial matrix. This is the first report of the identification and the characterization of the mammalian NifS-like protein. We also showed the mitochondrial localization of *S. cerevisiae* NFS1, another eukaryotic NifS-like protein whose gene had been analyzed [12]. The deduced amino acid sequence of the m-Nfs1 shows a relatively high sequence identity of 68.5% with that of the y-NFS1. Thus, we propose that the eukaryotic NifS-like protein is a mitochondrial protein that is highly conserved between lower eukaryotes and higher mammals.

The immunoreactive 47 kDa protein detected with the anti-m-Nfs1 antibody was somewhat smaller than the molecular weight (51 kDa) predicted from the m-*Nfs1* cDNA sequence. Because the amino-terminal extension contains a typical mitochondrial sorting signal, the m-Nfs1 protein is most likely synthesized as a larger precursor of 51 kDa, imported into the mitochondrial matrix, and processed to form a mature polypeptide of 47 kDa. In addition, the c-Myc-tagged y-NFS1 protein was detected as a 50 kDa protein in the mitochondrial matrix, which is smaller than the predicted molecular weight of this fusion protein (56 kDa), suggesting that the yeast NifS-like protein also seems to be proteolytically processed upon import into mitochondria. The existence of a similar amino-terminal extension of the y-NFS1 protein containing a typical mitochondrial targeting sequence supports this idea.

The *S. cerevisiae NFS1* gene has been shown to be essential for cell viability because the *NFS1* disruptant exhibits haploid lethality [12]. We have demonstrated the presence of mouse *Nfs1* transcript and protein in most tissues (Figs. 2 and 3). Moreover, mouse EST clones of the embryonic cDNA, which contain a part of the m-*Nfs1* cDNA, have been deposited in the EMBL database recently, showing that the m-*Nfs1* is expressed in both the adult and embryo. These findings suggest that, as in the case of the y-NFS1 protein, the m-Nfs1 might also possess some important housekeeping functions in vivo.

Kolman and Söll have suggested that the *S. cerevisiae NFS1* gene is involved in tRNA splicing based on the fact that its mutated dominant allele (*NFS1-1*) could complement the splicing deficiency of an inactivated suppressor tRNA gene [12]. Interestingly, the *NFS1-1* could not complement the *nfs1* deficiency itself indicating that this mutant allele lost the full function of the wild-type *NFS1*. Since the sequence and size of the y-*NFS1* gene indicated that it could not encode either the splicing ligase or any other subunits associated with the splicing endonuclease, they speculated that the y-*NFS1* gene might indirectly participate in the process of tRNA splicing; for example, it might influence the NAD^+ requiring step [17] since the *Bacillus subtilis nifS*-like gene has been shown to be involved in NAD^+ biosynthesis [2]. Our findings that the y-NFS1 protein is mainly localized in the mitochondrial matrix (Fig. 5) support this view of indirect involvement of the y-NFS1 in the tRNA splicing because the process is known to occur on the inner surface of the nuclear membrane [18]. However, we could not totally exclude the possibility that a small amount of the y-NFS1 protein is sorted to the nucleus and is actually involved in the tRNA splicing. Such dual localization has been reported in the case of some tRNA modifying enzymes [19,20]. The functional relationship between these enzymes and the y-NFS1 has not yet been clarified.

A. vinelandii NifS possesses a cysteine desulfuration activity [8] and is believed to support [Fe-S] cluster formation in nitrogenase maturation in vivo. However, it can also participate in the construction of [Fe-S] clusters of other iron-sulfur proteins, SoxR [21] and FNR [22], in vitro. NifS-like proteins from other non-nitrogen fixing bacteria such as *E. coli* also have desulfuration activity similar to *A. vinelandii* NifS, and it has been shown to contribute to the formation of the [Fe-S] cluster in dihydroxy acid dehydratase [10]. These findings indicate that the prokaryotic NifS proteins can supply elemental sulfur for the [Fe-S] cluster of iron-sulfur protein(s). The significant sequence homology found between the prokaryotic and eukaryotic NifS (and NifS-like) proteins, including the PLP binding site and the cysteine residue essential for the cysteine desulfuration activity of the *A. vinelandii* NifS protein [8,16], suggests that the eukaryotic counterparts also participate in the formation of the [Fe-S] cluster of some iron-sulfur proteins in mitochondria in vivo. Mitochondria contain a variety of iron-sulfur proteins such as adrenodoxin, aconitase and Rieske protein of the respiratory complex III. We are now attempting to isolate the protein for biochemical analysis in vitro and to ascertain whether the eukaryotic NifS-like protein functions in [Fe-S] cluster formation in vivo.

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