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Molecular Cloning of the cDNA of Mouse Mitochondrial NADP-Dependent Isocitrate Dehydrogenase and the Expression of the Gene During Lymphocyte Activation

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Abstract The current report documents the molecular cloning of the mouse mitochondrial NADP-dependent isocitrate dehydronegase (mNADP-IDH) cDNA. The cDNA was 1,863 bp in length and contained one open reading frame encoding a 523-residue polypeptide with a predicted molecular weight of 58 kDa. The cDNA and the deduced amino acid (AA) sequence of the mouse mNADP-IDH had a high degree of homology with those of porcine, bovine, alfalfa, and yeast. The recombinant mNADP-IDH expressed in *Escherichia coli* had active enzymatic function, as well as an expected molecular weight. The heart had the highest constitutive expression of the steady-state mNADP-IDH mRNA, followed by the kidney, while the expression of the gene in other tissues was low. The enzymatic activity of different tissues was in agreement with their mNADP-IDH mRNA levels. The resting lymphocytes had low constitutive expression of the gene, but the steady-state mRNA could be induced 48 h after mitogen stimulation. At the protein level, the resting lymphocytes had low enzymatic activity of mNADP-IDH, but the activity was augmented fivefold after mitogen stimulation. The cytosolic NADP-IDH, on the contrary, remained low or undetectable before and after the mitogen stimulation. Based on our current findings as well as the known roles of the mNADP-IDH in anabolism and in the isocitrate shuttle, it is conceivable that the mNADP-IDH is necessary for optimizing proliferation in lymphocytes.

Key words: NADP, isocitrate dehydrogenase, EC 1.1.1.42, mitochondrion, lymphocyte activation

Cell cycle entry is a tightly controlled event with a major restriction point in the late G_1 phase, and this point is termed START in budding yeast [Hartwell et al., 1974]. In yeast, START is probably controlled by a fairly small and specific family of regulatory proteins, mainly kinases and phosphatases [Hunt and Kirschner, 1993]. The passage of G_1 control point is necessary, but not sufficient for the initiation of chromosome replication, as demonstrated in the case of yeast *Saccharomyces cerevisiae* [Hunt and Kirschner, 1993]. Additional gene products must be present to sustain and optimize the cascade of molecular events leading to cell proliferation. The gene products in this category include, among other things, enzymes for energy supply and for synthesis of metabolites in order to meet the increasing requirement during cell replication [Johnson, 1992].

After appropriate activation, resting lymphocytes in the G_0 phase will traverse through G_1, enter the S phase, and undergo proliferation. In lymphocytes as well as in other mammalian cells, the entry control and optimization of cell cycling involve more complicated regulation compared to yeast, and de novo mRNA transcription and protein synthesis are essential [Adolph et al., 1993]. At the present time, our knowledge about the molecular events of the cascade from lymphocyte activation to proliferation is limited. We elect to identify mitogen-inducible genes in the lymphocytes with a view to dissecting the cascade. In so doing, we have cloned a previously undocumented mitochondrial NADP-dependent isocitrate dehydrogenase (mNADP-IDH) of the mouse. The expression of this gene at the mRNA and protein levels augmented after lymphocyte

Abbreviations used: cNADP-IDH, cytosolic NADP-dependent IHD; IDH, isocitrate dehydrogenase; mNADP-IDH, mitochondrial NADP-dependent IDH; NAD-IDH, NAD-dependent IDH.

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activation in the late G_1 phase. The mNADP-IDH cDNA and its deduced amino acid (AA) sequence had a high degree of homology with the porcine, bovine, alfalfa, and yeast. The recombinant protein of the cDNA expressed in *Escherichia coli* had the predicted molecular weight of mNADP-IDH with an enzymatic activity. These results confirm that the cDNA encodes NADP-IDH.

MATERIALS AND METHODS

Reagents

Staphylococcus aureus Cowan I (SAC) was purchased from Calbiochem (La Jolla, CA), and Lymphoprep from NYCOMED (Oslo, Norway). RPMI 1640, fetal calf serum (FCS), penicillin-streptomycin, and L-glutamine were ordered from GIBCO/BRL (Gaithersburg, MD). The random primer labelling kit was from Pharmacia-LKB (Baie d'Urfe, Québec), [α-^{32}p]-dCTP was from ICN (Mississauga, Ontario), and [α-^{35}S]-dATP was from Amersham (Arlington Heights, IL). The Librarian I cDNA library construction system and the Subtractor I kit were purchased from Invitrogen (San Diego, CA). The Sequenase 2.0 kit was purchased from U.S. Biochemical Corp. (Cleveland, OH).

Cell Culture

Human tonsillar T and B cells were prepared as described before [Luo et al., 1992]. The T cells were obtained by one-cycle sheep red blood cell (SRBC) rosetting, and such preparations contained about 90–93% $CD3^+$ cells. The T cell depleted fraction was referred to as B cells, which contained more than 90% $CD20^+$ cells. In some experiments, these B cells were further fractionated by Percoll density gradients, and the high density B cells were isolated from the interface of 65 and 55% percoll. Balb/c mouse spleen cells were prepared with Lympholyte-mouse (Cederlane, Hornby, Ontario). All the cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics.

Construction of cDNA Library of Activated B Cells

The high density tonsillar B cells were activated by SAC (1:10,000 dilution) for 16 h, and the total RNA was prepared by the guanidine/CsCl method [Ausubel et al., 1991]. Poly-A^+ RNA (twice selected on oligo-dT columns) mRNA was reverse-transcribed for the first strand cDNA using oligo-dT as primers. After second strand synthesis, the cDNA was blunt-ended, ligated with non-palindromic BstXI linkers, and cloned into the BstXI site of the eukaryotic expression vector pcDNA I. The detailed procedure for the library construction was described in the manufacturer's instruction (Invitrogen). Approximately 0.3×10^6 independent clones were obtained.

Preparation of a Subtracted cDNA Probe

mRNA was prepared from the SAC-activated (16 h) high density B cells, and reverse-transcribed using random primers and [α-^{32}P]dCTP. The radiolabelled cDNA probe was subtracted with tenfold excess of mRNA from high density B cells using a Subtractor I kit from Invitrogen according to the manufacturer's instruction.

Screening of cDNA Libraries

The cDNA library of human activated B cells was screened with subtracted cDNA probes prepared as described above. λgt10 mouse heart cDNA library (Clontech, Palo Alto, CA) was screened with a 0.8 Kb human mNADP-IDH cDNA probe, which was ^{32}P-labelled with random primers. The detailed screening prototols were described by Ausubel et al. [1991].

DNA Sequencing

cDNA was sequenced with the chain termination method on double-stranded plasmid DNA using Sequenase 2.0 and [α-^{35}S]dATP according to the manufacturer's instruction.

Northern Blot Analysis

Total lymphocyte RNA and tissue RNA were electrophoresed on 0.85% agarose/formaldehyde gels, and RNA loading (5–15 μg/lane, consistent within a given experiment) was monitored by the intensity of 18S and 28S bands of ribosomal RNA after ethidium bromide staining. The RNA was transfered onto Biotrans nylon membranes (ICN, St-Laurent, Quebec) and crosslinked with UV irradiation. The membranes were hybridized at 42°C overnight with the mNADP-IDH cDNA probe. The final wash of the membranes was at 56°C for 30 min with

0.1 × SSC buffer (15 mM NaCl, 1.5 mM Na_3 citrate) containing 0.1% SDS.

Expression of Recombinant mNADP-IDH in *E. coli*

The full-length mouse mNADP-IDH cDNA from λgt10 was first cloned into the EcoRI site of plasmid pGEM4, and the plasmid was designated as pGEM-mIDH. From pGEM-mIDH, a PstI/SmaI fragment was directionally cloned into the PstI/EcoRV sites of Bluescript SK^+. This fragment started from the triplet encoding the 84th AA residue of the open reading frame and extended to the stop codon followed by a 206 bp 3′ untranslated (UT) region, and it was placed in-frame 35 AA downstream of the start codon of the β-galactosidase in the Bluescript SK^+. The resulting plasmid was named pBLSK-mIDH. *E. coli* DH5α was transformed with the plasmid, and the expression of the fusion protein was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 2–3 h as described elsewhere [Ausubel et al., 1991]. After sonication of the cells, the lysate and insoluble inclusion bodies were analyzed by SDS-PAGE. The lysate and renatured protein from the inclusion bodies were also assayed for their IDH activity.

SDS-PAGE

SDS-PAGE was carried out in 12% polyacrylamide gels as described in detail by Laemmli [Laemmli, 1973].

Assays for IDH Activity

The NADP-IDH activity was assayed as described by Bergmeyer et al. [1983]. The organ mitochondrial fraction was prepared according to Guerra [1974]. Briefly, the tissues were first homogenized with Dounce homogenizer, and were centrifuged at 800*g* to remove cell debris. The homogenate was then centrifuged at 13,000*g* for 30 min to pellet the mitochondrial fraction, while the supernatant represented the cytosolic fraction. For lymphocyte mitochondrial preparation, the cell membrane was solubilized with 2 mM digitonin [Zuurendonk et al., 1979], and the fraction of mitochondria, the membrane of which is resistant to digitonin, was pelleted by centrifugation at 13,000*g* for 5 min. The supernatant represented the cytosolic fraction. The mitochondrial protein was corrected for cytosolic protein contamination, and vice versa, according to lactate dehydrogenase (LDH) and glutamate dehydrogenase (GLDH) activities [Guerra, 1974; Ragan, 1983]. The correction formular is as follows:

$$\begin{cases} \text{mIDH} \times \text{mProtein} = (\text{C} \times \text{a}) + [\text{M} \times (\text{mProtein} - \text{a})] \\ \text{cIDH} \times \text{cProtein} = (\text{M} \times \text{b}) + [\text{C} \times (\text{cProtein} - \text{b})] \end{cases}$$

where:

$$\text{a} = \frac{\text{mLDH}}{\text{cLDH}} \times \text{mProtein}$$

$$\text{b} = \frac{\text{cGLDH}}{\text{mGLDH}} \times \text{cProtein}$$

mIDH, mLDH, and mGLDH stand for measured IDH, LDH, and GLDH in the mitochondrial fraction, respectively, while cIDH, cLDH, and cGLDH stand for those in the cytosolic fraction. mProtein and cProtein represent protein concentration in the mitochondrial and cytosolic preparations, respectively. C and M are the corrected activity of mitochondrial and cytosolic IDH, respectively. The contamination of mitochondrial protein to the cytosolic protein was normally below 15%, while the cytosolic to the mitochondrial was below 35%.

RESULTS

cDNA Library Screening

In order to identify genes which were induced in late G_1 phase of lymphocyte activation, a plasmid cDNA library of activated B cells was screened with a probe made from cDNA of activated B cell subtracted with mRNA of resting B cells. About 10% of the clones screened were positive, and 150 of the positive clones were partially sequenced. After excluding those clones with inserts derived from ribosomal RNA, repetitive elements, poly-A tails, and other known genes, 15 clones were selected. The inserts of these clones were used as probes in Northern blot analysis of the total RNA of resting or mitogen-activated T cells in order to identify activation-related genes common to both T and B cells. Nine of the 15 genes had detectable expression. Among the nine, the expression of clone 256 along with the other four was upregulated after the mitogen stimulation. We elected to further characterize clone 256.

The insert in clone 256 was about 0.8 Kb, and had a high degree of homology with cDNA of porcine mNADP-IDH (89.6% indentity in 625

A

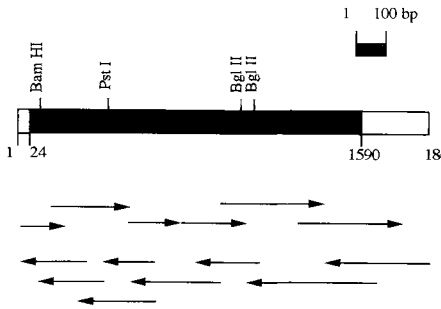

Fig. 1. **A:** Sequencing strategy for, and selected restriction sites of, mouse mNADP-IDH cDNA. The shaded area represents the coding region. The *arrows* indicate the orientation and length of the sequencing performed on each strand, using either nested primers or plasmid subclones. **B:** The cDNA and AA sequences of the mouse mNADP-IDH.

B

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                    20                  40                  60                  80                 100                 120
agtctctggccgagataacactgatgcagaaattggtctatatgcaactctggatccagctcggatcagagcgggggtgcgggcgagcttggccaggcgaacatctttcttcctggcggc
                      M  Q  K  L  V  Y  M  Q  L  W  I  Q  L  G  S  E  R  G  C  G  R  A  W  P  G  E  H  L  S  S  W  R  R

                   140                 160                 180                 200                 220                 240
gcggcgtggatctaggtgacaggcgacgtgggctcctctccccgcttcctctctcccgaggcggcggcagtggcggcggcagaggtagaagcagcggcgtgctcggacctcgcgtgctcgg
  G  V  D  L  G  D  R  R  R  G  L  L  S  R  F  L  S  P  E  A  A  A  V  A  A  A  E  V  E  A  A  A  C  S  D  L  A  C  S  E

                   260                 280                 300                 320                 340                 360
aatggccggctactgcgggctgtgagctcctctgcagagcctcgggctcagcgcggacctgggcaccggcagcactgactgtccccagctggccggagcagccgcggcgccactatgctg
  W  P  A  T  A  G  C  E  L  L  C  R  A  S  G  S  A  R  T  W  A  P  A  A  L  T  V  P  S  W  P  E  Q  P  R  R  H  Y  A  E

                   380                 400                 420                 440                 460                 480
agaagaggatcaaggtggagaagccggtagtggagatggacggtgacgagatgacccggatcatctggcagttcatcaaggagaagctcatcctgcctcacgtggatgttcagctcaagt
  K  R  I  K  V  E  K  P  V  V  E  M  D  G  D  E  M  T  R  I  I  W  Q  F  I  K  E  K  L  I  L  P  H  V  D  V  Q  L  K  Y

                   500                 520                 540                 560                 580                 600
attttgaccttgggcttccaaaccgtgaccagaccaatgaccaggtcaccattgactctgctctggccgcccagaagtacagtatggctgtcaagtgtgccacaatcacccctgatgagg
  F  D  L  G  L  P  N  R  D  Q  T  N  D  Q  V  T  I  D  S  A  L  A  A  Q  K  Y  S  M  A  V  K  C  A  T  I  T  P  D  E  A

                   620                 640                 660                 680                 700                 720
cccgtgtggaagagttcaagctgaagaaaatgtggaagagcctaacggaacgatccggaacatcttgggggaaccgtcttcagagagccaagcatctgcaaaaacatccccgccttg
  R  V  E  E  F  K  L  K  K  M  W  K  S  P  N  G  T  I  R  N  I  L  G  G  T  V  F  R  E  P  S  I  C  K  N  I  P  R  L  V

                   740                 760                 780                 800                 820                 840
tccctgggtggaccaagcccatcaccattggcaggcacgcccatggcgaccagtacaaggccacagattttgtggtagatcgagctggcacgttcaagttggtcttcaccccaaagaatg
  P  G  W  T  K  P  I  T  I  G  R  H  A  H  G  D  Q  Y  K  A  T  D  F  V  V  D  R  A  G  T  F  K  L  V  F  T  P  K  N  G

                   860                 880                 900                 920                 940                 960
gcagcagtgccaaggagtgggaggtgtataacttccctggcggaggcgttgggatggggatgtacaacaccgacgagtccatttcgggcttcgcgcacagctgcttccagtactctatcc
  S  S  A  K  E  W  E  V  Y  N  F  P  G  G  G  V  G  M  G  M  Y  N  T  D  E  S  I  S  G  F  A  H  S  C  F  Q  Y  S  I  Q

                   980                1000                1020                1040                1060                1080
agaagaaatggccgctctacttgagcaccaagaacaccattctgaaggcctatgacgggcgtttcaaggacatcttccaggagatctttgacaagcactataagactgacttcgacagga
  K  K  W  P  L  Y  L  S  T  K  N  T  I  L  K  A  Y  D  G  R  F  K  D  I  F  Q  E  I  F  D  K  H  Y  K  T  D  F  D  R  N

                  1100                1120                1140                1160                1180                1200
ataagatctggtatgaacatcggctcatcgacgacatggtggcccaggtgctcaagtcttccggtggctttgtgtgggcttgcaaaaactatgatggagacgtgcagtctgacatcctgg
  K  I  W  Y  E  H  R  L  I  D  D  M  V  A  Q  V  L  K  S  S  G  G  F  V  W  A  C  K  N  Y  D  G  D  V  Q  S  D  I  L  A

                  1220                1240                1260                1280                1300                1320
cctcaaggtttggctccctcggcctgatgacatctgtgctggtctgccctgatgggaagacaattgaggctgaggctgctcatgggacagtcacccgccattaccgagaacaccagaagg
  S  R  F  G  S  L  G  L  M  T  S  V  L  V  C  P  D  G  K  T  I  E  A  E  A  A  H  G  T  V  T  R  H  Y  R  E  H  Q  K  G

                  1340                1360                1380                1400                1420                1440
gccggcccaccagtaccaaagggtattgccagcatctttgcctggacacgggtctggagcatcgtgggaagctggatgggaaccaggaccttatcaggtttgcacagacgcgggagaagg
  R  P  T  S  T  K  G  I  A  S  I  F  A  W  T  R  G  L  E  H  R  G  K  L  D  G  N  Q  D  L  I  R  F  A  Q  T  R  E  K  V

                  1460                1480                1500                1520                1540                1560
tgtgcgtgcagactgtggagggagcgatgaccaaggacctggctggctgtatccatggcctcagcaatgtgaagctgaacgagcacttcctgaacaccacagacttcctggacaccatta
  C  V  Q  T  V  E  G  A  M  T  K  D  L  A  G  C  I  H  G  L  S  N  V  K  L  N  E  H  F  L  N  T  T  D  F  L  D  T  I  K

                  1580                1600                1620                1640                1660                1680
agagcaacctggacagagctctgggcaagcagtaggggtgacactactcagcaccagtggtactctgtacatggtagagggtgcctcacagcccctctctggtggcctttctagggtca
  S  N  L  D  R  A  L  G  K  Q

                  1700                1720                1740                1760                1780                1800
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                  1820                1840                1860
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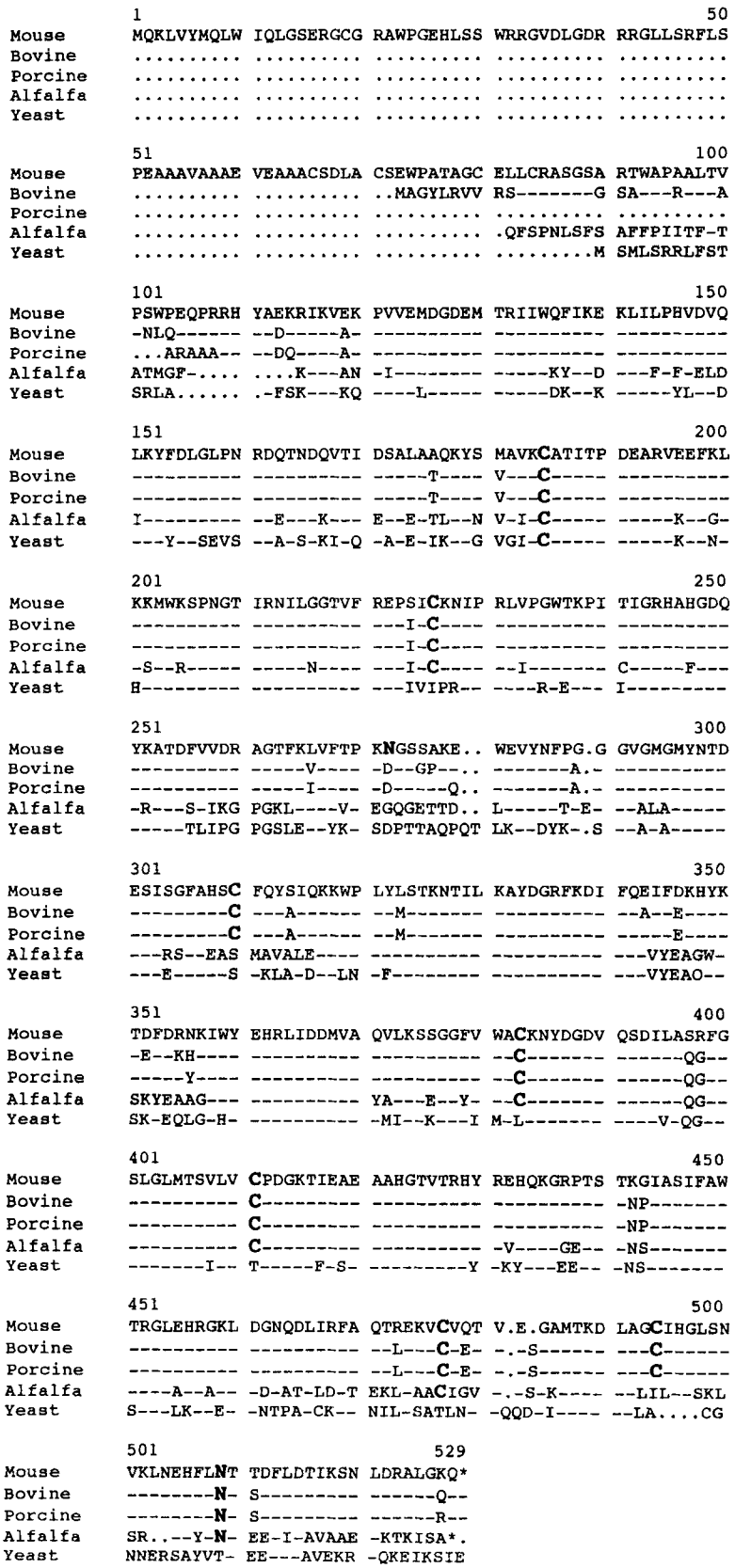

Fig. 2. Sequence homology of mNADP-IDH of different species. **C** and **N** represent cysteines and N-linked glycosylation sites, respectively.

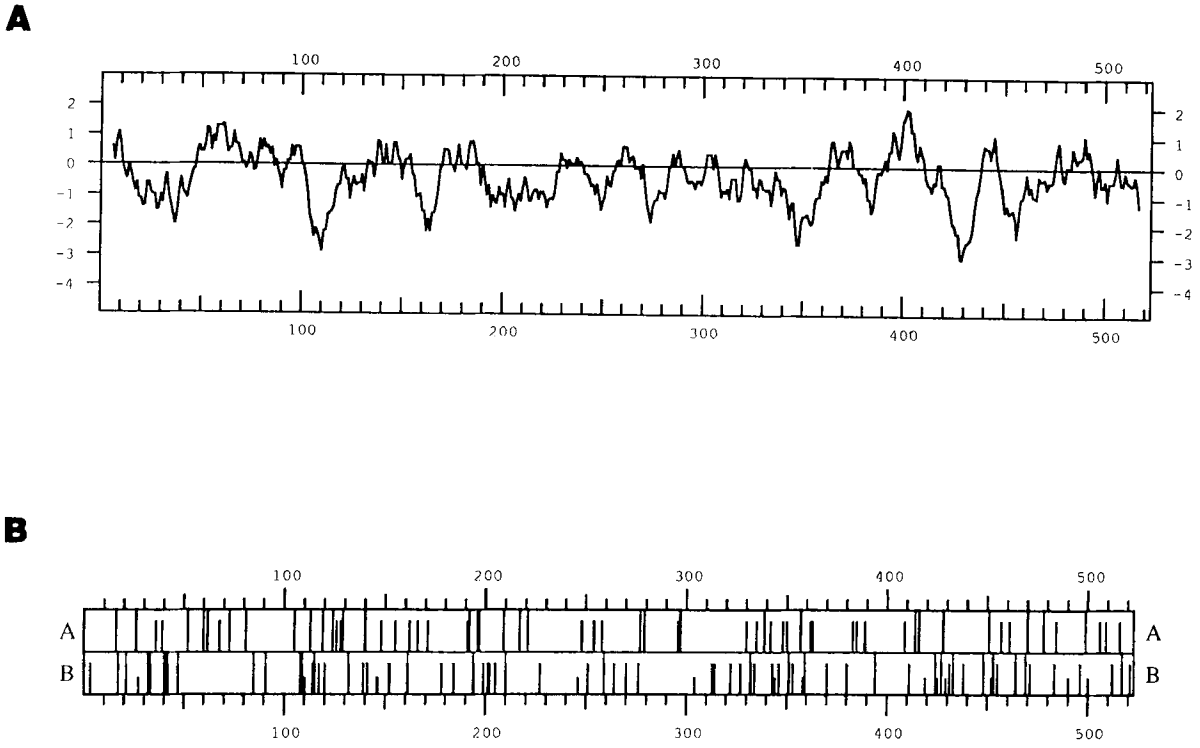

Fig. 3. A: The Kyte-Doolittle hydropathic plot of the mouse mNADP-IDH cDNA. B: The acidic and basic AA plot of the mouse mNADP-IDH. A: acidic; B: basic.

bp overlap). This insert was then used as a probe to screen a λgt10 mouse heart cDNA library, since the heart had the highest expression of the mNADP-IDH mRNA (refer to following sections). About 80 positive clones were identified and the longest insert was cloned into the EcoRI site of pGEM4.

DNA Sequence Analysis of the Putative Mouse mNADp-IDH

The cDNA of the putative mouse mNADP-IDH was sequenced on both strands, and the sequencing strategy was depicted in Figure 1A. Some ambiguity around positions 636 and 704 was verified by sequencing the PCR (polymerase chain reaction) product (from positions 567 to 1558) containing this region. The same mouse heart cDNA library was used as the PCR templet. The DNA sequence (submitted to Genbank and EMBL) and deduced AA sequence were analyzed with programs of Genetics Computer Group. The sequence databanks from GenBank (version 85, October 1994), EMBL (version 40, September 1994), Swissport (version 30, October 1994), and PIR protein (version 42, September 1993) were employed. The cDNA was 1,863 bp in length and it contained a single open reading frame encoding a 523-residue polypeptide with a predicted molecular weight of 58 KDa (Fig. 1B). The coding sequence of the cDNA had a high degree of homology with the porcine (87.5% in 1,274 bp overlap) [Haselbeck et al., 1992], bovine (85.3% in 1,384 bp overlap) [Hul et al., 1993], alfalfa (67% in 1,224 bp overlap) [Shorrosh and Dixon, 1992] and yeast (61.7% in 1,152 bp overlap) [Haselbeck et al., 1991] cDNAs of mNADP-IDH.

Since only very limited information of the 5′ UT region of mNADP-IDH cDNA of other species was available, sequence comparison for this region was not performed. As is the case for the coding region, the mouse 270 bp 3′UT of mNADP-IDH cDNA shared a similarly high degree of homology with the corresponding regions of mNADP-IDH of other species, i.e., 86.3% in 270 bp overlap with the porcine, 85.9% in 270 bp overlap with the bovine, 73.3% in 270 bp overlap with that of alfalfa, and 65.8% in 263 bp overlap with that of yeast. The deduced AA sequence of the putative mNADP-IDH protein was also highly conserved when compared with the porcine (86.8% homology in 416 AA overlap), the bovine (85.3% homology in 442 AA overlap), as well as that of alfalfa (59.8% homol-

ogy in 410 AA overlap) and yeast (55.2% homology in 411 AA overlap) (Fig. 2).

The mouse protein had all 7 conserved cysteine residues (Fig. 2). One of the cysteine-containing regions, DLAGCIHGLSNVK (AA residues 490 to 502, Fig. 2), was 100% conserved among the mouse, bovine, and porcine, and this region has been implicated in the formation of NADP-binding pocket according to affinity labelling of the peptide with a reactive NADP analogue [Bailey and Colman, 1987]. In *E. coli* mNADP-IDH, seven AA residues (i.e., Arg 119, Arg 153, Tyr 160, Lys 230, Asp 307, Asp 311, and Glu 336) are implicated in binding of isocitrate plus Mg^{++} according to crystallographic analysis [Hurley et al., 1991]. In the mouse protein, all the seven corresponding residues, i.e., Arg 212, Arg 244, Tyr 251, Lys 326, Asp 389, Asp 393, and Glu 418 or Glu 420, were conserved (Fig. 2). The mouse protein contained two N-linked glycosylation sites on residues Asn 272 and Asn 509, and the latter was conserved among the mouse, bovine, and porcine (Fig. 2).

The Kyte-Doolittle hydropathic plot [Kyte and Doolittle, 1982] of the AA sequence is shown in Figure 3A, and the acidic-basic AA map is shown in Figure 3B. The first 50 AA of the N-terminal sequence had high percentage of leucine (16%), serine (10%), and arginine (16%) residues, compared to those in the whole 522 AA polypeptide sequence (8.4, 7.7, and 5.7%, respectively). Another feature of this region, especially from AA 17 to 50, was its rich content of basic AA (26.4 vs. 15.3% in the whole 522 AA sequence). These are the common characteristics of the leader peptides targeting for mitochondria [von Heijne, 1986].

Expression of mNADP-IDH in Different Tissues

The full-length mouse cDNA was used as a probe in Northern blot analysis for the mouse lymphocyte and tissue samples. In mouse spleen cells stimulated with concanavalin A (Con A) and phorbol myristate acetate (PMA), the expression of the steady-state mRNA was induced after 48 h (Fig. 4A). In different mouse tissues examined (Fig. 4B), the heart had the highest mRNA expression, followed by the kidney. For the other tissues examined, i.e., the thymus, liver, brain, lung, muscle, and spleen, the expression of the mRNA was low or undetectable.

The mNADP-IDH enzymatic activity in mitochondrial preparations of several tissues was also investigated, and is shown in Figure 5A. The highest constitutive enzymatic activities were detected in the heart followed by the kidney, in agreement with the mRNA expression in

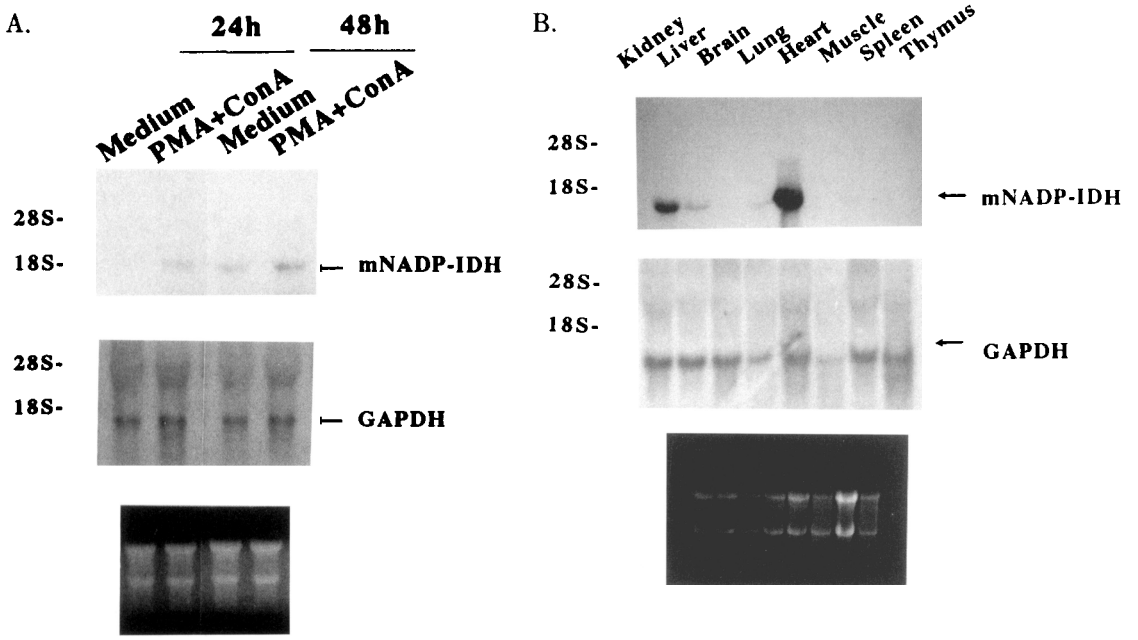

Fig. 4. Northern blot analysis of mNADP-IDH expression. Loading of the total RNA is shown in the lower panels by ethidium bromide staining of the 18S and 28S ribosomal RNA. After the signal of mNADP-IDH mRNA decayed, the membrane was rehybridized with the GAPDH (glyceraldehyde phosphate dehydrogenase) probe to further verify the RNA loading. **A:** Total RNA from Balb/c spleen cells cultured in the presence or absence of PMA (1 ng/ml) and Con A (2 μg/ml) for 24, 48, and 72 h. **B:** Total RNA from different Balb/c mouse tissues.

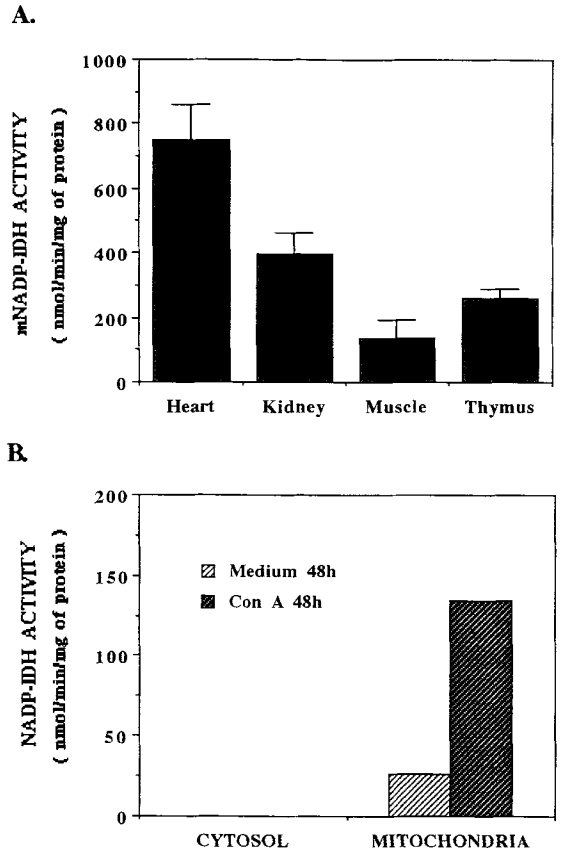

Fig. 5. A: mNADP-IDH activity in mitochondria of different organs of Balb/c mice. The plot represents the results (mean ± 1SD) of two experiments and the data have been corrected for cross contamination between cytosolic and mitochondrial proteins. B: mNADP-IDH activity in mitochondria of resting and activated lymphocytes. The spleen cells of CD1 mice were activated by Con A (2 μg/ml) for 48 h, and the cytosolic and the mitochondrial fractions were assayed for NADP-IDH activity. The cross contamination between the cytosolic and mitochondrial proteins was corrected. The result of a representative experiment is shown and similar results were obtained in two other experiments.

these tissues, while relatively low activities were detected in the thymus and muscle. In lymphocytes, the mNADP-IDH enzymatic activity increased after the Con A stimulation, and at 48 h it was fivefold higher than that of the resting lymphocytes (Fig. 5B). It was interesting to note that the cytosolic NADP-IDH activity remained below the detectable level before and after mitogen-stimulation, and was at least tenfold lower than that of the mNADP-IDH.

Expression of Recombinant Mouse mNADP-IDH in *E. coli*

A truncated mouse mNADP-IDH cDNA was cloned in-frame into the expression vector Bluescript SK$^+$ and the resulting plasmid pBLSK-mIDH was expected to express a β-galactosidase-mNADP-IDH fusion protein of 474 AA residues in length. The fusion protein contained a truncated 439 AA mNADP-IDH polypeptide sequence which lacked the first 84 AA residues according to the full-length open reading frame. This truncated mNADP-IDH polypeptide was supposed to be enzymatically active, since the AA sequences of the mature mNADP-IDH polypeptides of the porcine and yeast start from a place 28 and 29 AA residues, respectively, further downstream [Haselbeck et al., 1991, 1992] after the cleavage of the leader sequences for mitochondrial targeting. As shown in Figure 6B, the lysate supernatant of the *E. coli* transformed with pBLSK-mIDH had 4-fold higher level of NADP-IDH activity compared to that of the *E. coli* transformed with the vector Bluescript SK$^+$ (pBLSK). A large amount of insoluble inclusion bodies was present in the *E. coli* transformed with pBLSK-mIDH, but not in that transformed with pBLSK. SDS-PAGE analysis showed (Fig. 6A) that the inclusion bodies contained an overexpressed protein of 58 kDa, which was the expected molecular weight of the fusion protein. However, the protein in the inclusion bodies was not able to be renatured and solubilized after various attempts, and consequently the NADP-IDH enzymatic activity of the pellet containing the inclusion bodies was not detectable. Probably due to that, only a small amount of fusion protein was soluble, and no distinct overexpressed 581 KDa band was detected in the lysate supernatant of pBLSK-mIDH-transformed *E. coli*.

DISCUSSION

The significance of this study lies in: (1) the cloning and sequencing of the NADP-IDH of the mouse is an essential step to further investigate the role of the enzyme in lymphocyte activation and cell biology using gene knock-out approach in mice; (2) to our knowledge, it is the first time a recombinant functional mNADN-IDH has been produced, confirming the function of the gene; and (3) the elevation of the expression of the gene in the activated lymphocyte is a new finding, and this contributes to our knowledge in lymphocyte physiology.

There are three isoforms of IDH in mammalian cells. The NAD-dependent IDH (NAD-IDH, EC.1.1.1.41) is present only intramitochondrially. The other two are NADP-dependent

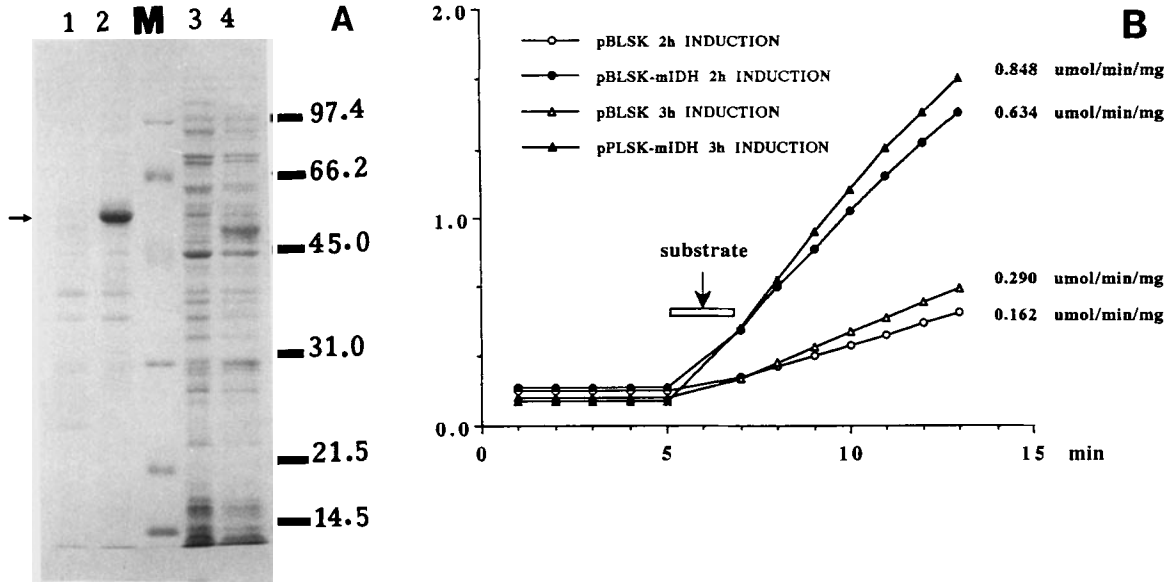

Fig. 6. Expression of the recombinant mNADP-IDH in *E. coli*. *E. coli* DH5α was transformed with the control plasmid Bluescript SK$^+$ (pBLSK) or mNADP-IDH expression plasmid pBLSK-mIDH, and was induced with IPTG for 2 or 3 h. **A:** SDS-PAGE analysis of the fusion mNADP-IDH polypeptide. The lysate sediments (*lanes 1 and 2*) and supernatants (*lanes 3 and 4*) of the bateria transformed with pBLSK (lanes 1 and 3) or pBLSK-mIDH (lanes 2 and 4) were analysed in a 12% gel. M: molecular weight. The size is indicated on the right side of the panel. The arrow indicates the 53 kDa band of the fusion protein. **B:** Enzymatic activity of the recombinant mNADP-IDH. The lysate supernatants of DH5α harboring the pBLSK or pBLSK-mIDH were assayed for their NADP-IDH activity at 37°C. Equal amount of protein (43.7 μg) was present in each sample. Substrate (isocitrate) was added between 5 and 7 min. The $O.D._{340nm}$ is indicated beside each curve, and the enzymatic activity was calculated according to the reaction between 7 and 13 min. The r values of the linear regressions were all more than 0.996. Data of a representative experiment is shown, and similar results were obtained in three other experiments.

(EC.1.1.1.42), one being cytosolic (cNADP-IDH), and the other being in the mitochondrial matrix (mNADP-IDH). It is now known that the two mitochondrial IDH isoforms, i.e., NAD-IDH and mNADP-IDH, are coded by different genes [Cupp and McAlister-Henn, 1991, 1992], and the cytosolic and mitochondrial NADP-dependent IDH, i.e., cNADP-IDH and mNADP-IDH, are immunogically distinct [Jennings et al., 1991]. While the NAD-dependent IDH plays a pivotal role in Kreb's cycle, the relative contribution of NADP-dependent IDH to the anabolic process is less well understood. The cytosolic and mitochondrial NADP-IDHs catalyze the conversion between isocitrate and α-ketoglutarate, and generate NADPH in the process. The two enzymes are also a part of the isocitrate shuttle, the net effect of which is to divert NADPH from the mitochondrion to cytosol. NADPH is an essential element for biosynthesis of lipid, amino acid, and DNA, while α-ketoglutarate is the critical precursor of glutamate. Considering the highly conserved nature of mNADP-IDH in polypeptide and cDNA sequences among different species, the enzyme might play a critical role for the cellular metabolism in general, as well as for the lymphocyte activation.

Unlike many eukaryotic mRNAs, the currently reported cDNA of the mouse mNADP-IDH has no consensus Kozak sequence (GCCGCCAGCC) immediately preceeding the start codon, nor a G residue following the start codon [Kozak, 1989]. However, it is unlikely that the translation starts from somewhere further upstream, because multiple stop codons are present in-frame upstream of the current start codon. Thus, the mNADP-IDH mRNA seems to have a weak translation initiator according to the definition by Kozak. The recombinant fusion mNADP-IDH expressed in *E. coli* had the predicted molecular weight. In addition, the NADP-IDH activity in *E. coli* expressing the fusion protein increased 3–4-fold, when compared with the vector-transformed *E. coli*. The results were consistent and reproducible. These indicate that the putative reading frame is indeed open, and that the cDNA codes for NADP-IDH. It also suggests that glycosylation is not obligatory for the mouse mNADP-IDH enzymatic activity, and that the first 84 AA of the protein is not critical for its

function. Although the cDNAs of mNADP-IDH of several other species have been cloned, to our knowledge, this report documented for the first time a functional recombinant mNADP-IDH. We were unable to renature the overexpressed fusion protein in the inclusion bodies and to reconstitute the enzymatic activity, but this is not uncommon in the *E. coli* expression system. If a large amount of active recombinant mNADP-IDH is required, other expression systems such as yeast or baculovirus could be tried.

In lymphocytes, it has been reported that the NADP/NADPH ratio drops from 1.4 in the resting cells to 0.2 in the cells 4 days after mitogen stimulation [Berger et al., 1982]. The hexose monophosphate shunt is known to be an important source of NADPH in lymphocytes, and the activity of this shunt increases during mitogen stimulation [Sagone et al., 1974]. It has been shown that in lymphocytes, NADP-IDHs (i.e., cytosolic or mitochondrial) are the only other dehydrogenases which are involved in NADP reduction [Klein et al., 1990]. Our results indicate that there is a significant increase of steady-state mRNA of mNADP-IDH during lymphocyte activation. Also, the mNADP-IDH enzymatic activity increases drastically in lymphocytes after the mitogen activation. On the contrary, the cytosolic NADP-IDH remains below the detectable level in both the resting and activated lymphocytes. The differential expression of the cytosolic and mitochondrial NADP-IDH after the mitogen stimulation is an interesting new finding.

It is worth mentioning that NADP/NADPH is the only endogenous nucleotide containing a 2′-phosphoribose moiety, and unlike NADH, NADPH is protected from depletion caused by the ribosylation reactions during DNA damage [Gaal et al., 1987]. A preserved NADPH pool for which mNADP-IDH is a generator, might be essential for the reductive biosynthesis in case of DNA damage.

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