Identification and Functional Characterization of a Novel, Tissue-specific NAD\(^+\)-dependent Isocitrate Dehydrogenase β Subunit Isoform*

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To understand the interactions and functional role of each of the three mitochondrial NAD\(^+\)-dependent isocitrate dehydrogenase (IDH) subunits (α, β, and γ), we have characterized human cDNAs encoding two β isoforms (β\(_1\) and β\(_2\)) and the γ subunit. Analysis of deduced amino acid sequences revealed that β\(_1\) and β\(_2\) encode 349 and 354 amino acids, respectively, and the two isoforms only differ in the most carboxyl 28 amino acids. The γ cDNA encodes 354 amino acids and is almost identical to monkey IDH\(_γ\). Northern analyses revealed that the smaller β\(_2\) transcript (1.3 kilobases) is primarily expressed in heart and skeletal muscle, whereas the larger β\(_1\) mRNA (1.6 kilobases) is prevalent in nonmuscle tissues. Sequence analysis of the IDHβ gene indicates that the difference in the C-terminal 28 amino acids between β\(_1\) and β\(_2\) results from alternative splicing of a single transcript. Among the various combinations of human IDH subunits co-expressed in bacteria, αβγ, αβ\(_γ\), and αγ combinations exhibited significant amounts of IDH activity, whereas subunits produced alone and β\(_γ\) showed no detectable activity. These data suggest that the α is the catalytic subunit and that at least one of the other two subunits plays an essential supporting role for activity. Substitution of β\(_1\) with β\(_2\) in the co-expression system lowered the pH optimum for IDH activity from 8.0 to 7.5. This difference in optimal pH was analogous to what was observed in mouse kidney and brain (β\(_1\), prevalent; optimal pH 8.0) versus heart (β\(_2\), prevalent; pH 7.6) mitochondria. Experiments with a specially designed splicing reporter construct stably transfected into HT1080 cells indicate that acidic conditions favor a splicing pattern responsible for the muscle- and heart-specific β\(_2\) isoform. Taken together, these data indicate a regulatory role of IDHβ isoforms in determining the pH optimum for IDH activity through the tissue-specific alternative splicing.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s)U49283, AF023265, and U40272 (for human IDH\(_α\), IDH\(_β\), and IDH\(_γ\) cDNA, respectively). The partial genomic nucleotide sequence for the human IDH\(_β\) gene was deposited to the GenBankTM/EMBL database under the accession number AF046929.

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Isocitrate dehydrogenases (ICDHs); EC 1.1.1.41 and EC 1.1.1.42 catalyze the oxidative decarboxylation of isocitrate into α-ketoglutarate, producing either NADH or NADPH (1). In mammals, three classes of ICDH isoenzymes exist: mitochondrial NAD\(^+\)-dependent ICDH (IDH), mitochondrial NADP\(^+\)-dependent ICDH, and cytosolic NADP\(^+\)-dependent ICDH (2–4). In addition, recent data suggest that a peroxisomal NADP\(^+\)-dependent ICDH exists as the fourth ICDH isozyme in yeast. This putative protein probably provides NADPH, which is required for the peroxisomal oxidation of unsaturated fatty acids (5, 6). In contrast to eukaryotics, only one type of ICDH (NADP\(^+\)-dependent enzyme) is present in Escherichia coli (7).

Among the eukaryotic ICDH isoenzymes, IDH has been assumed to play a major role in the oxidative decarboxylation of isocitrate in the tricarboxylic acid cycle (8, 9). Its key role is underscored by the fact that its activity is regulated by numerous allosteric regulators. For example, it is positively regulated by ADP in mammals and AMP in yeast, yet inhibited by ATP, NADH, or NADPH. Calcium ions have also been known to enhance IDH activity in the presence of isocitrate and adenine nucleotide (10).

Recent functional studies on the yeast ICDH isoenzymes revealed the catalytic and regulatory roles of IDH5 and IDH1, respectively (11–13). In addition to its catalytic role in the tricarboxylic acid cycle, the yeast IDH protein was shown to specifically bind to the 5′-untranslated region of mRNAs of the mitochondrial cytochrome c oxidase subunits I, II, and III as well as cytochrome b, thus suggesting another important regulatory role of the IDH protein in controlling mitochondrial biogenesis and energy metabolism (14).

In mammals, the IDH enzyme exists as a heterotetramer consisting of 2α, 1β, and 1γ subunits. All the subunits have comparable molecular masses (39–41 kDa) and highly similar amino acid sequences (15, 16). Despite numerous biochemical and kinetic studies of IDH isolated from animal tissues, the precise function of each subunit and the nature of their interactions in the catalytically active IDH protein have not been elucidated in mammals. The nucleotide and deduced protein sequences of a cDNA clone encoding the human IDH\(_α\) were previously reported, and the possibility that IDH\(_α\) serves as a catalytic subunit was suggested (17).

Accordingly, to further elucidate the functional roles of the
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three IDH subunits, human cDNA clones for both IDHβ and IDHγ were isolated and characterized and then co-expressed with IDHα in E. coli using a co-expression system that we developed. We demonstrate that IDHα is critical for the catalytic activity of IDH, whereas the IDHβ or -γ subunit has a supporting role in constituting the IDH activity. We also report, for the first time, the tissue-specific expression of an IDHβ isoform (IDHβ9), which is probably derived from a pH-dependent exon-splitting alternative splicing from a single IDH gene caused by an internal acceptor within an intron (18). The substitution of IDHβ2 (non-muscle-specific) with IDHβ2 (muscle-specific) in the recombinant IDHαβγ protein lowered the optimal pH values for IDH activity from 8.0 to 7.6.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4 DNA ligase were purchased from POSCOCHEM (Sungnam, Korea). GeneScreen membrane, [α-32P]dCTP (specific radioactivity 3000 Ci/mmol), and [α-35S]dATP (specific radioactivity 500 Ci/mmol) were from NEN Life Science Products. Bovine and human heart αgt11 cDNA libraries were from CLONTECH (Palo Alto, CA). Nitrocellulose membrane was obtained from Schleicher & Schuell. Terminal deoxyribonucleotide transferase and random primer labeling kit were the products of Roche Molecular Biochemicals. Sephacryl S-300HR, expression vector pT7–7, and protein random primer labeling kit were the products of Roche Molecular Biochemicals. Nitrocellulose membrane was obtained from Bio-Rad. γ-32P-3-Isocitrate, EGTA, and other chemicals were purchased from Sigma.

Oligonucleotide Probes for Screening IDH Clones by Plaque Hybridization—Based on the amino acid sequences of the internal tryptic peptides of pig heart IDHβ and IDHγ (16), oligonucleotide probes were synthesized using the best codon usage (19) with a DNA synthesizer (Applied Biosystems, Foster City, CA). oligonucleotide probe 1 (sense direction for IDHβ), 5′-GTGCCGGTCAGGGCGCCCATCCCTGAGCAACGACC-3′, corresponding to the peptide sequence NH2-VRVEGAFVTML-COOH; oligonucleotide probe 2 (sense direction for IDHγ), 5′-GTGCAGTTGAGGCGGCCTTCCAAGCTACATGCT-3′, corresponding to the peptide sequence NH2-YANVIHC-COOH; and oligonucleotide probe 3 (antisense direction for IDHγ), 5′-CTTGGGTGTCAGCTGACCATGCT-3′, corresponding to peptide sequence NH2-CMMLDHLK-COOH. Oligonucleotides were radiolabeled at the 3′-end with [α-32P]dCTP using terminal deoxyribonucleotide transferase as described by Rosenberg et al. (20) and then used as probes for plaque hybridization.

Isolation of cDNA Clones Encoding IDHβ and IDHγ—Bovine heart αgt11 cDNA library was screened with 32P-labeled oligonucleotide probes (oligo 1 and 2 for IDHβ and oligo 3 and 4 for IDHγ). Conditions for hybridization and washing were as described (20). The positive cDNA clones from plaque hybridization were subcloned into plasmid pGEM7(+), and their nucleotide sequences were determined. The cDNA inserts containing a deduced amino acid sequence matching the amino acid sequences of the partial tryptic peptide sequences (16) of the pig heart IDHβ and IDHγ were isolated, labeled with [α-32P]dCTP by random primer labeling, and then used to screen a αgt11 cDNA library from human heart. Conditions for hybridization and washing were as described previously (21). The cDNA inserts for human IDHβ2, IDHγβ, and IDHγ were isolated and subcloned into plasmid pGEM7(+) for subsequent characterization as described below.

Isolation of Genomic Clones for Human IDHβ Gene—Genomic clones for human IDHβ gene were isolated from a JEMBL3 human lymphocyte genomic DNA library (Dr. Frank J. Gonzalez, NCI, National Institutes of Health) by plaque hybridization with [α-32P]dCTP-labeled IDHβ1 cDNA as a probe. The conditions for labeling, hybridization, and washing were the same as described above.

Nucleotide Sequence Analyses—Complete nucleotide sequences of the largest cDNA clones encoding human IDHβ2, IDHγβ, and IDHγ were determined by the dideoxynucleotide chain termination method (22) with a Sequenase version 2.0 kit (U.S. Biochemical Corp.). To accomplish double-stranded DNA sequencing, cDNA subclones were gradually deleted from both ends using a kit (Erase-a-Base from Promega (Madison, WI)) with the protocol recommended by the manufacturer. Nucleotide sequence data were assembled and analyzed using the computer software PC/GENE (IntelliGenetics, Mountain View, CA).
**FIG. 2.** Nucleotide and deduced amino acid sequences of human heart IDHβ cDNA clones. The entire nucleotide and deduced amino acid sequences of human IDHβ (1.6 kb) and IDHβa (1.3 kb) cDNA inserts are shown. Nucleotide numbers are indicated on the right, and amino acid numbers are shown on the left. The canonical polyadenylation signal, AATAAA, is double-underlined. The vertical arrow indicates the cleavage site of the mitochondrial leader sequence. The shaded region indicates the intron-like cassette sequence (317 bp) absent in the human IDHβα cDNA sequence. Deduced amino acid positions are shown in parenthesis. The C-terminal amino acid sequence of the human IDHβα homologous to that of IDHβa is denoted by boldface letters and its amino acid positions are shown in parenthesis. The C-terminal amino acid sequence of IDHβa, XhoI and NeoI restriction enzyme sites used to generate human IDHβ, mRNA-specific cDNA probe are indicated by the underlines.

The nucleotide sequence analysis for genomic DNA was also performed as described above.

Northern Blot Analyses—A membrane for human multiple tissue Northern (MTN) blot (CLONTECH, Palo Alto, CA) was successively probed with 32P-labeled IDHβ and IDHγ cDNAs. Total RNAs from various mouse (C57BL6/NCR) tissues were prepared using RNAzol B (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. Polyadenylated RNA (4 μg/lane) from different mouse tissues was separated by electrophoresis on 0.66 M formamide, 1% agarose gels, and transferred to GeneScreen membranes, and hybridized with 32P-labeled human cDNA probes for IDHα, IDHβ, and IDHγ, respectively. The C-terminal amino acid sequence of the human IDHβα homologous to that of IDHβa is denoted by boldface letters, and its amino acid positions are shown in parenthesis. The C-terminal amino acid sequence of IDHβa, XhoI and NeoI restriction enzyme sites used to generate human IDHβ, mRNA-specific cDNA probe are indicated by the underlines.

### Construction of Plasmids for Co-production of Human IDH Proteins in E. coli

The recombinant plasmids for co-production of IDH proteins were constructed by the strategy outlined in Fig. 1. For co-production of IDHα and IDHγ, a Scal/BglIII fragment containing the IDHγ coding region was isolated from the plasmid pHIDHγ and treated with Klenow enzyme to produce a blunt end and then ligated to the Scal-cleaved plasmid pHIDHα to generate plasmid pHIDHαβγ and pHIDHγ (Fig. 1).

### Preparation of Plasmids for Co-production of Human IDH Proteins in E. coli

Preparation of 3' and 5' untranslated regions of human IDHα, IDHβ, and IDHγ cDNA inserts. The cloning site was confirmed by DNA sequencing. The modified DNA fragments were excised with NdeI/BamHI enzymes and inserted into the NdeI/BamHI site of the expression vector pTE7-7 (Amersham Pharmacia Biotech) to generate the plasmids pHIDHα, pHIDHβ, pHIDHαβ, and pHIDHγ (Fig. 1).
sity). To introduce T7 RNA polymerase gene into the chromosome of E. coli EB106, the recombinant phase ΔDE3 was infected to E. coli EB106 by ΔDE3 Lysogenization kit (Novagen, Madison, WI) according to the manufacturer’s instructions. The ΔDE3 lysogen of E. coli EB106 was designated as E. coli EB106 (DE3) and used as a host to overproduce the recombinant IDH proteins of various combinations, as described above.

Preparation of the Recombinant IDH Proteins in E. coli—The recombinant plasmids were transferred to E. coli EB106 (DE3) by the CaCl₂ method (24), and bacterial colonies with positive inserts were grown overnight at 37 °C in LB media supplemented with ampicillin (50 µg/ml). The overnight cultures of transformants were diluted 100-fold into 1 liter of the same media and allowed to grow at 37 °C until the cell density reached 0.6. The cultures containing transformants were cooled in an ice-water bath and treated with 0.4 mm isopropyl β-D-thiogalactopyranoside to induce gene expression. During the induction period, bacterial cells were grown at 25 °C for 24 h with poor aeration. E. coli cells were harvested by centrifugation, washed with 1× phosphate-buffered saline, and resuspended in a lysis buffer (10 ml of phosphate-buffered saline containing 0.1% Triton X-100 and 1 mM phenylmethane-sulfonyl fluoride). Resuspended cells were disrupted by sonication with five 20-s treatments at half-maximum power of a sonicator (Bronzon model 350) on ice. The soluble lysates obtained by centrifugation at 9000 x g for 20 min were used to measure the IDH activity. All procedures for the soluble lysate preparation were conducted at 4 °C.

Microscopic Observation and Measurement of GFP Fluorescence—GFP fluorescence in HT1080 cells was observed by an inverted microscope (Olympus BX50) with a GFP filter. To measure the relative GFP fluorescence intensity, Hoechst 33342 was used. Different solutions of each sample were made under different pH values were homogenized with 70% ethanol and spun down at 15,000 x g for 20 min at 4 °C, and protein concentration was determined by the Bradford method (26) with bovine serum albumin as standard. The intensity of GFP fluorescence was determined for each cell layer grown on (0.3 mg of protein in 3 ml) at least three times per sample by a fluorescence spectrophotometer (Kontron SFM25) with excitation and emission at 488 and 510 nm, respectively.

RESULTS

Isolation and Partial Characterization of cDNA Clones for Bovine IDHβ and IDHγ—Mixed oligonucleotide probes as described under “Experimental Procedures” were used to screen a bovine heart λgt11 cDNA library in order to isolate cDNA clones encoding IDHβ and IDHγ subunits. Six and five positive cDNA clones for IDHβ and IDHγ, respectively, were isolated from about 2 million plaques screened. The size of DNA inserts ranged from 0.7 to 1.6 kb. Independent cDNA inserts for IDHβ and IDHγ were purified. The entire nucleotide sequences of the clones encoding IDHβ and IDHγ, respectively, were isolated and sequenced (28).

FIG. 3. Comparison of the amino acid sequences and predicted secondary structures of the C-terminal amino acid sequences of IDHβ isoforms. A, the partial tryptic amino acid sequence for pig heart IDHβ subunit (P-IDHβXIII) (16), monkey IDHβ (M-IDHβ) (29), H-IDHβγ, and H-IDHβα are compared. B, the secondary structures of the C-terminal regions of H-IDHβγ and H-IDHβα are predicted by theGOR method (31). h, helix structure; e, extended or β-sheet; c, coil structure. Amino acid positions are designated on the right, and the identical amino acid residues are shadowed. Helix-rich region in H-IDHβγ (h), and extended (e) or β-sheet-rich region in H-IDHβα are marked by the respective boxes.
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**FIG. 4.** Nucleotide and deduced amino acid sequences of a human IDHα cDNA clone. The entire nucleotide and deduced amino acid sequences for the largest cDNA clone encoding human IDHα (1.5 kb) are shown. Nucleotide and deduced amino acid numbers are indicated on the right, and amino acid numbers are shown on the left. The potential polyadenylation signal, AATAAA, is underlined. The vertical arrow indicates the cleavage site of the mitochondrial leader peptide.

for the entire protein coding region of IDHβ, starting with the initiation codon, ATG, and ending with the termination codon, TAG. One site for potential polyadenylation signal (AATAAA) was found in the 3′-untranslated region. The N terminus of the deduced amino acid sequence of the human IDHβ precursor protein (Ala1–Ala29) is identical to the N-terminal 33-amino acid sequence of pig IDHβ (16) except for the 14th and 31st amino acids. Therefore, the precursor human protein contains 385 amino acids (42,211 Da), and the mature protein consists of 351 amino acids (38,794 Da) most likely representing a human counterpart of the previously reported IDHβ1 isoform, as characterized in this report, is a newly identified IDHβ isoform. It is possible that the alteration of the C-terminal 28 amino acid residues (from Val325 to Ser351) found in the H-IDHβ1 protein sequence (Figs. 2 and 3) might result in significant changes in protein secondary structures as predicted by Garnier et al. (31). The C-terminal 28 amino acid residues of IDHβ1 consisted of extended or β-sheet structure, whereas that of IDHβ2 was predicted to contain helix structure, thus suggesting a potential role of C-terminal amino acids of IDHβ in regulating the IDH activity (Fig. 3B).

For the characterization of the recombinant IDH subunit interactions and functions, we also isolated human cDNA-
clones coding IDH\textsubscript{y} subunit (Fig. 4). Our cDNA clone (1.5 kb) for IDH\textsubscript{y} (H-IDH\textsubscript{y}) contained 181 bases of 5' untranslated region followed by 1,179 bases of an open reading frame and 108 bases of 3' untranslated region prior to a poly(A) tail. The structural analysis of H-IDH\textsubscript{y} cDNA revealed that it possesses the entire IDH\textsubscript{y} protein coding region, comprising the leader sequence for the precursor protein and the mature protein coding sequences. The canonical polyadenylation signal AATAAA in the 3'-untranslated region was observed 15 base pairs upstream of the poly(A) tail. The N terminus of the deduced human IDH\textsubscript{y} protein sequence (Phe\textsuperscript{1}-Val\textsuperscript{104}) is identical with the N terminus 34-amino acid sequence of the pig IDH\textsubscript{y} (16). Therefore, the precursor protein for human IDH\textsubscript{y} contains 393 amino acids (42,794 Da) with the 39 amino acids as a mitochondrial signal peptide. Thus, the mature protein consists of 354 amino acids (38,814 Da). The deduced H-IDH\textsubscript{y} protein sequence shares less amino acid homology (53\%) with that of H-IDH\textsubscript{b} (44%).

**Tissue-specific Expression of IDH Transcripts in Human and Mouse Tissues**—The expression patterns of the IDH\textsubscript{b} and IDH\textsubscript{y} transcripts in human and mouse tissues were analyzed by mRNA Northern blot analyses. As shown in Fig. 5A (top), more than two species of IDH\textsubscript{b} mRNA transcripts exist in various human tissues. The smallest mRNA transcript (1.3 kb) is predominantly expressed in heart and skeletal muscle, whereas a larger transcript (1.6 kb), at a lower level, is present in all nonmuscle tissues examined. A larger transcript (2.4 kb), detected in human heart, skeletal muscle, and pancreas may be the unprocessed IDH\textsubscript{b} mRNA. To further characterize the two major IDH\textsubscript{b} mRNA transcripts (1.3 and 1.6 kb), we performed Northern blot analysis with a H-IDH\textsubscript{b}\textsuperscript{-specific probe (175 bp of XhoI–NcoI fragment from the 317-bp intron-like sequence in H-IDH\textsubscript{b} but absent in H-IDH\textsubscript{b}\textsubscript{2}, as in Fig. 2). The H-IDH\textsubscript{b}\textsuperscript{-specific probe (H-IDH\textsubscript{b}XN) only recognized the larger mRNA transcript (1.6 kb) (Fig. 5A, middle). These data indicate that the smaller transcript (1.3 kb) present in heart and skeletal muscle represents the H-IDH\textsubscript{b}\textsubscript{2} transcript. A similar pattern of expression of the two IDH\textsubscript{b} transcripts seen in human tissues was also observed in mouse tissues. The smaller IDH\textsubscript{b} mRNA transcript (1.2 kb) is only detected in heart and skeletal muscle, whereas the larger IDH\textsubscript{b} mRNA transcript (1.5 kb) is expressed in all other tissues examined (Fig. 5B, top). In contrast, only a single species of IDH\textsubscript{y} mRNA transcript was detected in the various human (1.5 kb, Fig. 5A, bottom) and mouse tissues studied (1.3 kb, Fig. 5B, middle). The pattern and level of expression of the major IDH\textsubscript{y} transcript are very similar in both human and mouse tissues.

**Alternative Splicing of H-IDH\textsubscript{b}\textsubscript{1} and H-IDH\textsubscript{b}\textsubscript{2} mRNA Transcripts from a Single Human IDH\textsubscript{b} Gene**—In order to determine whether the two species of H-IDH\textsubscript{b} mRNAs were transcribed from two distinct but structurally similar genes or from a single gene and then produced by an alternative splicing mechanism, the region of the IDH\textsubscript{b} gene coding for the amino terminus of IDH\textsubscript{b}\textsubscript{1} was isolated. Several genomic clones (average size about 20 kb) for H-IDH\textsubscript{b} were isolated from a λEMBL3 human lymphocyte genomic DNA library by plaque hybridization (data not shown). A single DNA fragment (1.9 kb) was amplified from these genomic clones by PCR analyses using a sense primer C (5'-CTGCAGTGCTGTGAGGAAGTT-3') and an antisense primer B. The amplified DNA fragment was subcloned into plasmid pGEM7(+), and its entire nucleotide sequence was determined. The amplified genomic DNA verified that the 317-bp intron-like sequence, present in H-IDH\textsubscript{b}\textsubscript{1}, and absent in H-IDH\textsubscript{b}\textsubscript{2}, exists in the genomic sequence (Fig. 6A). Therefore, the two human IDH\textsubscript{b} mRNA transcripts corresponding to H-IDH\textsubscript{b}\textsubscript{1} and H-IDH\textsubscript{b}\textsubscript{2} are most likely produced by an alternative splicing from a single IDH\textsubscript{b} gene (Fig. 6B). Thus, the GT dinucleotide (nucleotide positions 819 and 820 in Fig. 6A) is used as the common 5'-splicing site in all tissues, while two alternative AG dinucleotides (nucleotide positions 1503 and 1504 for H-IDH\textsubscript{b}\textsubscript{1} or 1820 and 1821 for H-IDH\textsubscript{b}\textsubscript{2} in Fig. 6A) are utilized as the 3'-splicing sites, depending on the tissues (Fig. 6B). This type of alternative splicing pattern has been characterized previously in the fibronectin, G\alpha\textsubscript{s} signal transducer, histocompatibility antigen H2K, and prolactin transcripts (18).

**Production and Characterization of Recombinant IDH Subunit Proteins in E. coli**—Among the subunit combinations produced in E. coli (Fig. 7A), pHIDH\textsubscript{b}\textsubscript{2} showed any detectable IDH activity. The data strongly suggest that H-IDH\textsubscript{b} serves as an essential subunit for the catalytic activity of IDH enzyme and that at least one of the other two subunits plays a necessary supporting role for activity. Furthermore, the catalytically active recombinant H-IDH\textsubscript{a}\textsubscript{β}\textsubscript{γ} protein produced in E. coli
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TABLE I

| Subunit | Primers       | Sequence (5′→3′)       | Nucleotide positions |
|---------|---------------|------------------------|----------------------|
| IDHα    | AM1 (sense)   | AGACATATGGACTGGTGTGGTGTCGAC  | 82–99 b |
|         | AM2 (antisense)| GTGGATCCATTTAGAGTCTGTTAAGCT | 1158–1166 c |
| IDHβ    | BM1 (sense)   | GCCGATATGCGATCGGCGGCCGAG | 103–123 b |
|         | BM2 (antisense)| TATGGATCCGGCTATATGGTTGCGCA | 1491–1474 c |
| IDHγ    | GM1 (sense)   | CGAGGATATGTTTTTCAAGAANAAT | 117–135 c |
|         | GM2 (antisense)| AGGGGATCTAGGGCAGCTTAGGCTCA | 1191–1172 c |

a Nucleotide positions in human IDHα cDNA sequence (17).

b Nucleotide positions in human IDHβ cDNA sequence as shown in Fig. 2.

c Nucleotide positions in human IDHγ cDNA sequence as shown in Fig. 4.

For production of the mature IDH proteins, NdeI sites containing translation initiation codon ATG were introduced to the sense primers.

emerged as a single peak from a Sephacyr S-300HR gel filtration column with an apparent molecular mass of 316 kDa (data not shown). This indicates that the recombinant IDH protein most likely exists as an octamer, 2(2γ), with an molecular mass of the octamer of 301 kDa.

Role of IDHβ Isoforms in IDH Activity—In order to determine any difference in the two IDHβ isoforms’ ability to support IDH activity, we constructed the appropriate expression constructs (IDHβ1γ and IDHβ2γ) (Fig. 1) and produced the respective proteins in E. coli ER106 (DE3). Substitution of IDHβ1 with IDHβ2 in the co-expression system lowered the optimal pH from 8.0 to 7.6 for IDH activity without altering their maximum activities (Fig. 7A). This difference in optimum pH was analogous to what was observed in mouse kidney and brain (β2 prevalent) versus heart (β2 prevalent) mitochondria (Fig. 7B). These results indicate that IDHβ isoforms derived from the tissue-specific alternative splicing could modulate the optimal pH values for IDH activity. The Km values for IDHβ2γ and IDHβ1γ proteins in E. coli lysate were found to be 3.11 ± 0.09 and 1.28 ± 0.02 mM, respectively. Vmax values of IDHβ2γ and IDHβ1γ proteins were calculated to be 263.3 ± 5.5 and 96.4 ± 2.9 milliunits/mg protein, respectively. Thus IDHβ2γ protein exhibited 2.4- and 2.7-fold higher Km and Vmax values than IDHβ1γ protein. The significant changes in the Km and

FIG. 6. Partial nucleotide sequence and organization of a genomic clone for human IDHβ gene. A, a genomic DNA clone for human IDHβ gene was isolated, and its partial sequence (1.9 kb) was determined. Nucleotide sequences corresponding to the exons and introns of H-IDHβ1 gene are indicated by boldface uppercase letters and lowercase letters, respectively. The tentative nucleotide positions and exon names in the partial human IDHβ1 gene are marked on the left and right sides, respectively. The two underlined nucleotide sequences indicate the PCR primers used to amplify the 1,359-bp genomic DNA fragment linked to the PCR primers used to amplify the 1,359-bp genomic DNA fragment linked.
alternative splicing patterns of the IDH subunits for IDH activity, we isolated and characterized nearly full-length cDNA clones for human IDHβ and IDHγ subunits in this study. Using the co-expression system that we developed (Fig. 1), we have successfully produced IDH proteins of various combinations together with the IDHα subunit (17). Based on the relative activities of IDH proteins of various subunit combinations, our data established that all three IDH subunits are required for the maximal IDH activity and that IDHα is the catalytic subunit, while IDHβ or -γ subunit has an essential supporting role in constituting IDH activity. This conclusion is also in agreement with our previous data (17) of structural similarity between H-IDHα and yeast IDH2 subunit, the catalytic subunit in yeast IDH (11). Furthermore, the catalytically active recombinant H-IDHαβγ protein (316 kDa) most likely exists as an octamer of 2(2αβγ) (32, 33), although we cannot rule out other possible oligomeric structures, such as 2(αβ2γ) or 2(αβ,2γ). However, our data clearly disprove the possibility of a heteropentamer of 2α,2β,γ (25) or the mixture of other oligomeric structures (34).

Based on the significant difference in its C-terminal sequence from monkey IDHβ (29) and pig IDH (16), careful analyses of the nucleotide and deduced protein sequences of H-IDHβα cDNA revealed the presence of an intron-like cassette located near the 3'-end of the cDNA sequence (Fig. 2). Surprisingly, the C-terminal 26 amino acids deduced from the alternatively spliced IDHβα variant (Fig. 3) were highly homologous to those of monkey IDHβ (29) and the partial tryptic peptide of pig IDH (16). The actual presence of the two IDHβ cDNA isoforms was verified using DNA amplification by PCR and subsequent sequencing of the amplified DNA fragments near the 317 bp in question (Figs. 2 and 3). This was further supported by Northern analyses for human and mouse tissues (Fig. 5).

It is well established that multiple mRNA transcripts and their corresponding protein isoforms can be produced by a variety of different mechanisms (18). For example, a different set of mRNA transcripts can be generated by using alternative promoters (35) or from spatially separated exons in the genome (30, 35) by either mutually exclusive splicing as in the cases of

**DISCUSSION**

To investigate the functional role of each of the three IDHα, IDHβ, and IDHγ subunits for IDH activity, we isolated and expressed the recombinant H-IDH proteins produced in E. coli using the plasmid pHIDHαβγ and pHIDHαβγγ. Optimal pH profiles for the mitochondrial IDH activity in mouse tissues. IDH activity was measured at 25 °C as NADH production at 340 nm in the reaction mixture containing 50 mM MOPS buffer containing 5 mM threo-DS-1,3bisphosphoglycerate, 35.5 mM triethanolamine, 2 mM NAD+, 1 mM ADP, 2 mM MgCl2, and 1 μg/ml rotenone, as described by Rutter and Denton (25).

Vmax values caused by the substitution of IDHβ isoforms further support the role of IDHβ in the regulation of IDH activity in a tissue-specific manner.

**pH-dependent Expression of H-IDHβα in Cultured Cells**—To determine whether changes in pH levels can cause a shift in the alternative splicing patterns of the IDHβ mRNA transcript, the plasmid pIDHβGFP, in which GFP was allowed to be expressed only under the same manner of alternative splicing for IDHβα mRNA was constructed (Fig. 8) and then stably transfected into HT 1080 cells. At about 70% confluence, HT1080 cells were subjected to growth for another 16 h under fresh culture media at different pH values (pH 6.2, 6.6, 7.0, 7.4, and 7.8). As shown in Fig. 9, A and B, the levels of GFP expression from pIDHβGFP in HT1080 cells decreased as the pH of the culture media increased. Relative intensity assessed by GFP fluorescence at pH 6.2 was 1.7-fold higher than that at pH 7.8 (Fig. 9B). These results indicate that pH levels can regulate the levels of IDHβ isoforms by favoring one splicing pattern over another. A similar pH-dependent splicing was observed in human ATP synthase γ subunit (28).

**FIG. 7.** Optimal pH values for IDH activity for the recombinant H-IDH proteins and in mouse tissues. A, optimal pH profiles for IDH activity of the recombinant H-IDH proteins produced in E. coli using the plasmid pHIDHαβγ and pHIDHαβγγ. B, optimal pH profiles for the mitochondrial IDH activity in mouse tissues. IDH activity was measured at 25 °C as NADH production at 340 nm in the reaction mixture containing 50 mM MOPS buffer containing 5 mM threo-δ-ketoisocotate, 35.5 mM triethanolamine, 2 mM NAD+, 1 mM ADP, 2 mM MgCl2, and 1 μg/ml rotenone, as described by Rutter and Denton (25).
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Fig. 9. Effect of pH on the expression of IDHβ isoform by alternative splicing. Human HT1080 fibrosarcoma cells with permanently transfected pHIDHβGFP gene were grown in a culture media at pH 7.4 pre-adjusted by sodium bicarbonate (15 mM). Upon 70% confluence, cells were grown in new media at various pH values pre-adjusted by different concentrations of sodium bicarbonate, as described by Endo et al. (28). (A) The photomicroscopy of GFP fluorescence in the cells (x 200) were taken by an Inverted microscope equipped with a GFP filter. (B) The relative intensities of GFP fluorescence from the cells cultured at various pH values were compared with GFP intensity from the cells grown at pH 7.4. The intensity of GFP fluorescence for each cell lysate (0.1 mg protein/ml) was determined using a fluorescence spectrometer with excitation and emission at 488 and 510 nm, respectively.

β-tropomyosin (36) and MEF2D (37) or single exon-excluding splicing for ATP synthase F_{1,γ} subunit (28), myocyte-specific enhancer factor 2A (MEF2A) (38), and neural cell adhesion molecule (N-CAM) genes (39). In the case of the two IDHβ mRNA transcripts, it is most likely that the two isoforms are produced from a single gene via an alternative splicing by using a common 5′-splicing site (GT dinucleotide at nucleotides 819 and 820 in Fig. 6A) and different 3′-splicing sites (AG dinucleotides) in a tissue-specific manner: AG at nucleotides 1503 and 1504 for H-IDHβ1 and at 1820 and 1821 for H-IDHβ2. Therefore, the H-IDHβ2 transcript is likely to be produced by the exclusion of a part of one exon (exon E1 in Fig. 6B) like an intron cassette. A similar pattern of the alternative splicing has been reported in several genes and has been previously categorized as an alternative splicing mechanism mediated by the internal intron acceptor site (18).

Recently, the tissue-specific alternative splicing of ATP synthase F_{1,γ} mRNA isoforms in the heart and liver has been reported to be dependent on the extracellular pH and other physiological factors such as a transactivating factor produced via the protein kinase C-mediated pathway in HT1080 fibrosarcoma cells (28). In acidic pH, the heart-specific ATP synthase F_{1,γ} transcript is mainly produced through the exclusion of exon 9, whereas the liver-specific transcript containing the exon 9 is predominantly expressed in alkaline pH (28). In addition, the possible roles of MymD in this pH-dependent alternative splicing in cultured mouse myoblasts have been suggested (40), although the significance of the alternatively spliced F_{1,γ} transcript isoforms relative to the regulation of ATPase activity was not clearly demonstrated (41). Our current data indicate that the alternative splicing of H-IDHβ transcripts, as measured by the level of GFP expression in HT1080 cells, is also inversely affected by increasing the extracellular pH. The GFP expression at pH 6.2 was about 1.7-fold higher than that expressed at pH 7.8 (Fig. 9, A and B). In contrast to the ambiguous biological implication for the alternatively spliced heart- and liver-specific ATPase F_{1,γ} transcript isoforms, the substitution of H-IDHβ (liver-, brain-, and kidney-specific) with H-IDHβ2 (heart- and muscle-specific) in the recombinant IDHβγ protein lowered the optimal pH value for IDH activity from pH 8.0 to 7.6 (Fig. 7A). This result is consistent with the values observed in mouse tissues: pH 7.6 for the heart and pH 8.0 for the brain and kidney (Fig. 7B). The substitution of H-IDHβ1 with H-IDHβ2 in the recombinant IDHβγ protein also increased the $K_m$ and $V_{max}$ values by 2.4- and 2.7-fold, respectively. These data together with the tissuespecific expression of IDHβ transcripts (Fig. 5) indicate a potential regulatory role for the IDHβ subunit in constituting IDH activity. Because of the differences in amino acid sequences and secondary structures of the C termini (Fig. 3) between H-IDHβ1 and H-IDHβ2, it can be concluded that the C-terminal region of IDHβ expressed in a tissue-specific manner, probably plays an important role in constituting the total IDH activity.

In conclusion, the data presented provided evidence for at least two different IDHβ isoforms existing in a tissue-specific manner via an alternative splicing mechanism using a part of an exon as an intron-like cassette. Functional analyses of the recombinant IDH proteins suggested that the IDHβ or γ subunit performs a supporting role for constituting IDH activity with the catalytic subunit IDHa. Furthermore, the replacement of one β isoform with the other in the recombinant IDHβγ protein suggested that IDHβ isoforms appear to have a regulatory role in determining optimal pH values, as previously observed for the mitochondrial IDH in various mouse tissues. The pH-dependent regulation of the alternative splicing of IDHβ isoforms also indicated a complex regulatory mechanism for the production of IDHβ isoforms. To our knowledge, this is the first report of a pH-regulated alternative splicing pattern with the resultant isoforms causing a different pH optimum of the enzyme.

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