Identification of an Additional Member of the Cytochrome c Oxidase Subunit VIIa Family of Proteins*

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We report the cloning, nucleotide sequence, evolutionary analysis, and intracellular localization of SIG81, a silica-induced cDNA from mouse macrophages. The cDNA encodes a 111-amino acid protein with extensive sequence identity with members of the mammalian cytochrome c oxidase subunit VIIa (COX7a) family. A human SIG81 sequence >80% identical with the mouse cDNA was deduced from homologous sequences in the human expressed tags database. The deduced amino-terminal region shows features common to mitochondrial targeting sequences. A phylogenetic analysis of the carboxyl-terminal domain homologous to COX7a identifies SIG81 as a divergent member of the family with an ancient origin. Southern blot analysis showed that the mouse genome contains two to three copies of the SIG81 gene. Northern blot analysis revealed that the SIG81 transcript is approximately 1 kb and expressed in every tissue tested, with higher levels of expression observed in kidney and liver. Antibodies raised against a glutathione S-transferase SIG81 fusion protein detected a 135-kDa protein that co-fractionates with mitochondrial localized enzymatic activity. Taken together, our data suggest that SIG81 is a novel member of the COX7a family that is constitutively expressed in mouse cells.

The activation of macrophage cells characterized by an increase in the secretion of cytokines and inflammatory mediators can be achieved by silica particles in an in vitro model that mimics the initial development of certain fibrosing lung diseases (1). Using mouse RAW 264.7 macrophages, we constructed a subtracted cDNA library enriched in sequences overexpressed in silica-treated cells. The differential screening of the library led to the isolation of nine cDNAs corresponding to genes induced by silica (2). SIG81 (silica-induced genes, clone 81) was expressed as a 1-kb transcript induced more than 3-fold by silica in RAW 264.7 cells (2). When we studied the expression pattern of the SIG81 gene, we found that SIG81 responded to inflammatory mediators such as interferon-γ and bacterial lipopolysaccharide with modest increases in mRNA levels and consistently showed a more than 2-fold induction in response to the activation of protein kinase C or an increase in intracellular calcium (2). The analysis of a partial nucleotide sequence from SIG81 revealed a significant similarity to the liver isoform of cytochrome c oxidase subunit VIIa (COX7a).

COX (cytochrome c oxidase, EC 1.9.3.1) is an essential enzyme in the respiratory chain of eukaryotic organisms that catalyzes the transfer of electrons from cytochrome c to molecular oxygen in the terminal reaction of the mitochondrial electron transport chain (for review, see Ref. 3). The COX enzyme is a metalloprotein complex that, in mammalian cells, consists of 13 subunits: the three largest subunits are encoded in the mitochondrial DNA, and the remaining 10 smaller subunits are coded by the nuclear genome (4). The mitochondria-encoded I, II, and III subunits are the catalytic core of the enzyme, whereas it has been advanced that the nuclear subunits are regulators of the enzymatic activity of COX (5), either as allosteric receptors (6) or regulating proton translocation (7). In mammals, three of the nuclear subunits, among them COX7a, are present as tissue-specific isoforms (3), namely the liver (COX7a-L) and heart/muscle (COX7a-H) isotypes, which differ both in amino acid sequence and in expression patterns. Thus, in humans and bovines, COX7a-H is the predominant isoform in heart and skeletal muscles (8–11), although low levels are detected in smooth muscle (12). The COX7a-L isoform is widely expressed in many tissues, including heart and muscle (7, 10, 11). In several species such as rat (9) and mouse (13), COX7a-L is the only isoform so far identified. As deduced from the corresponding cDNAs described from human (14), bovine (15), rat (16), and mouse (2), COX7a-L is translated as an 83-amino acid precursor protein. The amino-terminal domain containing the first 23 residues of the protein is a mitochondrial targeting sequence that is cleaved after mitochondrial import (10) to generate the mature form of the protein. Although the function of COX7a in the COX complex is still undefined, its presence is required for maintaining normal levels of COX activity in human liver cells (17) and, in yeast, its homolog COX7 is implicated in the assembly of the functional COX complex (18).

To gain some insight into the role of the SIG81 protein and its relationship to the bona fide COX nuclear subunits, we determined the complete sequence of the mouse SIG81 cDNA. In the present study, we demonstrate that the protein encoded in the SIG81 cDNA co-fractionates with mitochondria in subcellular fractionation experiments. We also report that SIG81 exhibits widespread expression and we present an evolutionary analysis showing that SIG81 is related to COX7a with a degree of divergence that points to an ancient origin. We propose that

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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) X80899.

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1The abbreviations used are: kb, kilobase(s); bp, base pair(s); COX, cytochrome c oxidase; COX7a, cytochrome c oxidase subunit VIIa; COX7a-H and -L, heart and liver isoform of cytochrome c oxidase subunit VIIa, respectively; PCR, polymerase chain reaction; EST, expressed sequence tag; GST, glutathione S-transferase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; UTR, untranslated region; PAGE, polyacrylamide gel electrophoresis; Tricine, N-tris(hydroxymethyl)methylglycine.

2M. Starborg and C. Höög, unpublished sequence.
SIG81 may be a hitherto unrecognized member of the family of COX7α proteins.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA modifying enzymes were from Boehringer Mannheim. [α-32P]dCTP (3000 Ci/mmol) and [α-35S]dATP (1000 Ci/mmol) were purchased from Amersham International. The GeneAmp PCR reagent kit was from Perkin-Elmer. All other chemicals, reagents or molecular biology grade and were obtained from Boehringer Mannheim, Merck, or Sigma.

SIG81 cDNA Cloning—Isolation of SIG81 cDNA by differential screening of a subtracted cDNA library from the murine macrophage cell line RAW 264.7 constructed in the λZAPII vector (Stratagene) has been described previously (2). Independent SIG81-containing clones were isolated after three rounds of screening of 40 × 106 clones of RAW 264.7 cDNA library in λZAPII (2) following standard procedures (19). The cDNA insert of SIG81 was labeled by random priming using the "RediPrime" (Amersham) labeling system in the presence of [α-32P]dCTP to be used as a probe. PBluescript SK− phagemids containing SIG81 cDNAs were excised in vivo from the λZAPII vector by confection of Escherichia coli XL1-Blue cells with VCS M13 helper phage (Stratagene), according to the supplier’s instructions.

DNA Sequencing and Analysis—Nucleotide sequences were determined by the deoxy chain termination method (20) on single- and double-stranded template DNA using Sequenase 2.0 (USB Amersham) with custom-designed oligonucleotide primers. Sequences were read manually from both strands and in some cases sequence ambiguities were resolved by using Sequenase 3.0 (USB Amersham) to obtain the radioactively-labeled probe at 42°C for 16 h. Blots were washed at 65°C in 0.2× SSC, 0.1% SDS. The phylogenetic tree was constructed by the Neighbor Joining Method (23), with branch length proportional to calculated genetic distance.

Characterization of Mouse SIG81

RNA Preparation and Northern Analysis—Total cellular RNA was extracted from Swiss mouse tissues by the guanidinium isothiocyanate phenol-acid method (24). For Northern analysis, 15 µg of total RNA were denatured with 2.2 × formamide, fractionated in 1.2% agarose, 2.2 × formaldehyde gels (19), and transferred by Hybond N (Amerham) membranes. RNA blots were hybridized with the appropriate radiolabeled DNA probe contained in 0.2 × SSC, 0.1% SDS at 65°C. In addition to the SIG81 insert, blots were hybridized with a mouse 28 S rRNA probe (2), and a 221-bp COX7a probe spanning positions 61 to 281 of the mouse COX7asequences (human, bovine, mouse, and rat) were downloaded from the GenBankTM/EMBL data base (see accession numbers in legend to Fig. 3). Amino acid sequences were aligned using the Pileup program, and refinements to the alignment were made manually. After sequences were optimally aligned, a matrix of pairwise distances expressed as the number of amino acid substitutions per 100 residues was generated with the Kimura method (22). The phylogenetic tree was constructed by the Neighbor Joining Method (23), with branch length proportional to calculated genetic distance.

Sequence Analysis of the SIG81 cDNA—Nucleotide sequences were determined by the deoxy chain termination method (20) on single- and double-stranded template DNA using Sequenase 2.0 (USB Amersham) with custom-designed oligonucleotide primers. Sequences were read manually from both strands and in some cases sequence ambiguities were resolved by using Sequenase 3.0 (USB Amersham) to obtain the radioactively-labeled probe at 42°C for 16 h. Blots were washed at 65°C in 0.2× SSC, 0.1% SDS.

DNA Preparations from Cell and Tissues—Cellular RNA was extracted from Swiss mouse tissues by the guanidinium isothiocyanate/phenol-acid method (24). For Northern analysis, 15 µg of total RNA were denatured with 2.2 × formaldehyde, fractionated in 1.2% agarose, and transferred by Hybond N (Amerham) membranes. RNA blots were hybridized with the appropriate radiolabeled DNA probe contained in 0.2 × SSC, 0.1% SDS at 65°C. In addition to the SIG81 insert, blots were hybridized with a mouse 28 S rRNA probe (2), and a 221-bp COX7a probe spanning positions 61 to 281 of the mouse COX7a cDNA (GenBankTM accession number X58486) obtained by reverse transcription-PCR from RAW 264.7 poly(A) RNA (3).

Bacterial Expression of GST-SIG81 Fusion Protein—On the basis of the size of SIG81 cDNA and the two oligonucleotide primers, Oligo F 5′-GAATTCATGACTGATACCAAG-3′ (positions 26–39) and Oligo S′ 5′-CGGAATTCTTCATTGTTT-3′ (positions 364–350), were designed for a PCR amplification of the entire SIG81 coding region. Recognition sites for BamHI and EcoRI were included at the 5′ ends of OF26 and OF27, respectively, to ensure a correct in-frame ligation to the expression vector. With SIG81 cDNA as a template, a 354-bp fragment was generated and cloned into pBluescript SK−. The fragment was inserted into the multiple cloning site of pGEX-4X (Pharmacia Biotech Inc.) to construct pGEX-SIG81. Large scale (500 ml of culture) production of GST-SIG81 from a 500-ml culture was induced with 0.5 mM IPTG for 6 h. Bacterial lysis was according to the manufacturer’s instructions. The GST-SIG81 fusion protein was affinity-purified from the bacterial lysate by using the SulfoLink Coupling Gel (Pierce). Coupling of GST-SIG81 fusion protein to the gel and affinity purification of the antibodies followed the manufacturer’s instructions.

RESULTS

Sequence Analysis of the SIG81 cDNA—SIG81 is one of the nine silica-induced cDNAs isolated by differential screening of a subtracted RAW 264.7 macrophage cDNA library (2). The comparison of a partial SIG81 sequence against the GenBankTM/EMBL nucleotide data bases using the FASTA sequence alignment algorithm (31) revealed that SIG81 cDNA

3 F. Segade, unpublished data.
Corresponded to a novel transcript with a significant similarity to mammalian COX-7a at its 5' end. For full-length sequencing of the SIG81 cDNA and to circumvent the possible errors being introduced during the enzymatic manipulations performed on the cDNAs for the construction of the subtracted cDNA library (2), 12 independent cDNA clones were isolated from a RAW 264.7 macrophage cDNA library by screening with the radio-labeled 1.0-kb SIG81 insert. In addition to the original SIG81 cDNA, the complete sequences of four of the additional clones and an average of 250 bp from the 5' end of the remaining eight cDNAs were determined. The 5' end of every cDNA sequence was located within the first 11 bp of the sequence depicted in Fig. 1A. Therefore, it is likely that this cDNA represents a near full-length copy of the SIG81 transcript. The mouse SIG81 cDNA spans 1035 bp followed by a poly(A) tail. The ATG at base 29 is probably the genuine initiator since it is located within a favorable environment for translation initiation with similarity to processed COX7a proteins. The putative polyadenylation signal is shown in boldface type B, Northern analysis of total RNA extracted from RAW 264.7 (lane M) and human T47D (lane H) cells. Blots were hybridized with random-primed labeled mouse SIG81 (upper panel) or 28 S rRNA (lower panel) probes. C, schematic representation of the pairing of human ESTs with homology to mouse SIG81. EST identification number (data not shown). The sizes of mouse and human SIG81 mRNAs were estimated by Northern blot analysis of total RNA prepared from mouse RAW 264.7 macrophages and human T47D cells, respectively. Using mouse SIG81 as a probe, we found a unique 1-kb hybridizing band in both RNA preparations, thus confirming the size estimated from the cDNA sequences (Fig. 1B). The conceptual translation of the open reading frame in the mouse SIG81 cDNA resulted in a 111-amino acid protein with a calculated molecular mass of 12,385 Da. Although the inferred human homolog translated from the assembled ESTs encompassing the putative SIG81 coding region (H09013, H60257, R10896, R10947, R56795, R91485, and T39299) showed a higher degree of sequence conservation (77–83% identity) than the ESTs pairing with the 3' UTR (D62561, H09580, H06563, and R98944), Thus, the 3' UTRs, although they exhibit 66–72% identity are more divergent that would be expected for sequences with few evolutionary constraints. A putative human SIG81 cDNA sequence was assembled from EST and, by analogy to mouse SIG81, results in a cDNA with an estimated size of 900 pb comparable to that of mouse cDNA (data not shown). The sizes of mouse and human SIG81 mRNAs were estimated by Northern blot analysis of total RNA prepared from mouse RAW 264.7 macrophages and human T47D cells, respectively. Using mouse SIG81 as a probe, we found a unique 1-kb hybridizing band in both RNA preparations, thus confirming the size estimated from the cDNA sequences (Fig. 1B).
Characterization of Mouse SIG81

A

Mouse SIG81  
Human SIG81  
Mouse COX7a-L  
Human COX7a-L  
Pat COX7a-L  
Bovine COX7a-L  
Human COX7a-H  
Bovine COX7a-H

Phylogenetic Analysis of SIG81—As depicted in Fig. 3A, a multiple sequence alignment of the COX7a-homology domains in the deduced mouse and human SIG81 and the mature mammalian COX7a proteins available in GenBank reveals conserved clusters of short amino acid sequences. Within a species, SIG81 and COX7a proteins showed a degree of homology comparable with that between L- and H-isofoms. Thus, there is 55% identity between mouse SIG81 and COX7a-L, and 59% identity for human SIG81 and COX7a-L, while human COX7a-L and -H are just 51% identical. Hence, a phylogenetic distance analysis was performed to elucidate the evolutionary relationship between SIG81 and COX7a proteins. After aligning the carboxyl-terminal homologous regions of COX7a and SIG81 proteins, evolutionary distances between each pair of sequences were calculated by the Kimura protein distance method (22). The topology of the resulting dendrogram indicates that SIG81 proteins clearly constitute a monophyletic group most closely related to the COX7a-H cluster (Fig. 3B). However, the rate of sequence divergence is significantly different not only among the amino acid positions present in a majority of the sequences. Dots indicate gaps introduced during the alignment to maximize amino acid identity. COX7a sequences were retrieved by GenBank accession numbers: X15822 (human COX7a-L), M38136 (human COX7a-M), X15235 (bovine COX7a-L), M83299 (bovine COX7a-M), X58486 (mouse COX7a-L), and X54080 (rat COX7a-L). B, phylogenetic distance tree based on the SIG81 and COX7a protein sequences. Distances between sequences are represented by the horizontal length of the branches. The scale bar indicates the distance corresponding to 10 differences in 100 positions.

Hydrophobicity plot of mouse SIG81 protein. The hydrophathy plot was obtained with the method of Kyte and Doolittle (33) using a moving window of 7 amino acids. The amino acid positions are indicated on the x-axis; the hydrophobic (+) or hydrophilic (−) indices are shown on the y-axis.

B

Mouse SIG81  
Human SIG81  
Mouse COX7a-L  
Pat COX7a-L  
Bovine COX7a-L  
Bovine COX7a-H

Protein Expression and Subcellular Localization of SIG81—

Phosphorylation site (amino acid 84), and two N-myristoylation sites (amino acids 14 and 94). Interestingly, their similarity is nevertheless restricted to a 57-residue carboxyl-terminal stretch (hereafter termed the COX7a-homology domain), whereas the amino-terminal domains differ both in size (55 residues in SIG81, 23 residues in COX7a-L, and 21 residues in COX7a-H) and sequence (see below).

Phylogenetic Analysis of SIG81—As depicted in Fig. 3A, a multiple sequence alignment of the COX7a-homology domains in the deduced mouse and human SIG81 and the mature mammalian COX7a proteins available in GenBank reveals conserved clusters of short amino acid sequences. Within a species, SIG81 and COX7a proteins showed a degree of homology comparable with that between L- and H-isofoms. Thus, there is 55% identity between mouse SIG81 and COX7a-L, and 59% identity for human SIG81 and COX7a-L, while human COX7a-L and -H are just 51% identical. Hence, a phylogenetic distance analysis was performed to elucidate the evolutionary relationship between SIG81 and COX7a proteins. After aligning the carboxyl-terminal homologous regions of COX7a and SIG81 proteins, evolutionary distances between each pair of sequences were calculated by the Kimura protein distance method (22). The topology of the resulting dendrogram indicates that SIG81 proteins clearly constitute a monophyletic group most closely related to the COX7a-H cluster (Fig. 3B). However, the rate of sequence divergence is significantly different not only among the amino acid positions present in a majority of the sequences. Dots indicate gaps introduced during the alignment to maximize amino acid identity. COX7a sequences were retrieved by GenBank accession numbers: X15822 (human COX7a-L), M38136 (human COX7a-M), X15235 (bovine COX7a-L), M83299 (bovine COX7a-M), X58486 (mouse COX7a-L), and X54080 (rat COX7a-L). B, phylogenetic distance tree based on the SIG81 and COX7a protein sequences. Distances between sequences are represented by the horizontal length of the branches. The scale bar indicates the distance corresponding to 10 differences in 100 positions.

A single transcript that hybridized with the SIG81 cDNA probe. Although the highest steady state levels of SIG81 mRNA were found in kidney and liver (Fig. 4A), the transcript was also very abundant in skeletal muscle and heart (Fig. 4C). There seems to be no direct relation between SIG81 mRNA abundance and the specific metabolism of a tissue. Rehybridization of the same blot with a probe for COX7a-L revealed a strong hybridization signal in kidney whereas substantially lower levels of COX7a-L mRNA were detected in the remaining tissues (Fig. 4B). COX7a-L is the only COX7a isoform known so far not to be present in the mouse genome. Although at least two SIG81 gene copies may be present in the mouse genome that could correspond to a small family of genes and/or pseudogenes.

Genomic Analysis of Mouse DNA—As a preliminary study on the SIG81 gene(s) present in the genome, we performed a Southern blot analysis of total mouse genomic DNA cleaved with four restriction enzymes (BamHI, HindIII, PstI, and XbaI). When probing the radiolabeled full-length SIG81 cDNA, 2–3 strongly and 1–2 more weakly hybridizing bands were revealed in each lane (Fig. 5). The result suggests that at least two SIG81 gene copies may be present in the mouse genome that could correspond to a small family of genes and/or pseudogenes.

Protein Expression and Subcellular Localization of SIG81—
Characterization of Mouse SIG81

Fig. 4. Expression of SIG81 mRNA in mouse tissues. Northern blot analyses were performed with 15 μg of total cellular RNA extracted from six adult Swiss mouse tissues: heart, liver, skeletal muscle, kidney, brain, and testes. Blots were subsequently hybridized to radiolabeled mouse SIG81 (A), COX7a-L (B), and 28 S rRNA (C) probes.

Fig. 5. Southern analysis of mouse genomic DNA. Twenty μg of mouse thymus DNA were digested with the indicated restriction enzymes, fractionated by electrophoresis, blotted, and probed with the entire radiolabeled SIG81 cDNA probe. The scale at the left side shows the sizes in kilobases of HindIII-digested λ-DNA.

DISCUSSION

We report herein the isolation and characterization of the cDNA encoding mouse SIG81, a novel gene whose product appears to be closely related to the nuclear subunit COX7a of the mitochondrial COX complex. Remarkably, the average 91% identity between the inferred mouse and human SIG81 protein sequences is significantly higher than the homologies between mouse and human COX7a-L (69% identity), or human and bovine COX7a-H (61% identity). For the carboxyl-terminal region of SIG81, which we have termed the COX7a-homology domain, the similarity between SIG81 and the COX7a isoforms is comparable to the values found between the isoforms themselves. Close examination of the SIG81 sequences confirmed that the COX7a-homology domain includes 11 amino acids that are present in the mature COX7a peptides from yeast to mammals (36) located in human SIG81 in identical positions, and one highly conserved proline is missing in mouse SIG81 due to a two-codon deletion. SIG81 proteins also contain the motif ELKFFFQKAD (amino acids 61–70) homologous to the sequence EKQLFFQED proposed as a functional core domain in mammalian COX7a (10). However, in nonmammalian vertebrates such as rainbow trout, COX7a-L contains the slightly modified sequence EKQLLFQAX (37) whereas in yeast COX7a, homologies in this region are reduced just to the amino acid pairs QK and FQ (10, 36), thus suggesting that a great deal of
for a retarded migration of a processed peptide, we think it is unlikely in the case of SIG81 given that only one tyrosine phosphorylation site and one N-myristoylation motif are present in the carboxyl-terminal region of SIG81. In fact, the hydrophobic stretch (amino acids 35–44 in mouse SIG81) found within the putative mitochondrial targeting sequence is reminiscent of the so-called “stop-transfer” domain present in proteins to be localized to the mitochondrial membranes (35). In many cases, targeting sequences containing stop-transfer domains are not cleaved (35). It is tempting to speculate that the divergence in the amino-terminal domains of SIG81 and COX7a reflect distinct intramitochondrial localization and thus justify the occurrence of two highly homologous proteins being coincidentally expressed in the same tissue. It is peculiar that, if SIG81 arose from the duplication of an ancestral gene, it has maintained a widespread expression in various tissues instead of exhibiting the more specialized pattern such as that for COX7a-H (10, 36) and by many other genes that arise by duplication. The tight regulation of SIG81 gene expression is exemplified by the fact that no measurable differences were found in mRNA levels in RAW 264.7 and J774A.1 macrophages and NIH 3T3 fibroblasts that were serum-starved and then growth-stimulated by addition of serum to increase their metabolic rates. Likewise, SIG81 expression was unaltered in RAW 264.7 cells cultured in the presence of insulin or thyroid hormone, subjected to stress provoked by heat shock, or altered in their redox state. It is noteworthy that a similar lack of response has also been reported for several nuclear-encoded COX subunits, including COX7a (38–40). Alternatively, SIG81 expression may be regulated through a post-transcriptional mechanism. SIG81 mRNA contains a GC-rich 5′-UTR indicative of a highly structured transcript that could function in translational regulation (32). In fact, preliminary data on SIG81 protein levels estimated by Western blotting with anti-GST-SIG81 antibodies suggest that the levels of SIG81 protein may be lower than expected from such an abundant transcript. Why has SIG81 not been identified previously as a member of the COX7a family of proteins? Since nucleotide sequence homology between SIG81 and COX7a cDNAs from the same species is low (61% identity in a 177-bp stretch between mouse SIG81 and COX7a-L), it is unlikely that a standard screening of a cDNA library with a COX7a probe would identify SIG81 sequences, even at low stringency hybridization conditions. At the protein level, an identification failure may be even more complicated. One possibility is that SIG81 is associated with the COX complex as a weakly bound subunit which could be removed easily by the detergent treatments usually employed in COX isolation, as reported for other COX subunits such as III, VIa, VIb, and VIIa, that sometimes are missing totally or in part from COX preparations (3, 41). Alternatively, SIG81 is simply very homologous to one COX subunit but it is not a bona fide COX subunit and, as suggested above, might not necessarily be located in the same mitochondrial compartment. Further studies are in progress to elucidate the role of SIG81 in the mitochondria and its putative functional relation to the COX7a family of proteins.

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