GRIM-19, a Cell Death Regulatory Gene Product, Is a Subunit of Bovine Mitochondrial NADH:Ubiquinone Oxidoreductase (Complex I)*

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The sequences of 42 subunits of NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria have been described previously. Seven are encoded by mitochondrial DNA, whereas the remaining 35 are nuclear gene products imported into the organelle from the cytoplasm. An additional protein, which does not correspond to any previously known subunit of the complex I assembly, has now been detected. Denaturing gels of subcomplex Iα, the hydrophilic arm of complex I, clearly show a hitherto unidentified band, which was digested with trypsin and subjected to mass-spectrometric analysis to provide several peptide sequences, used in cDNA cloning and sequencing. Measurement of the intact protein mass indicated that the N terminus is acetylated. The new complex I subunit (B16.6) is the bovine homolog of GRIM-19, the product of a cell death regulatory gene induced by interferon-β and retinoic acid, thus providing a new link between the mitochondrion and its electron-transport chain and apoptotic cell death.

Mitochondrial NADH:ubiquinone oxidoreductase (complex I) catalyzes the first step in the electron transport chain, the oxidation of NADH to NAD+ coupled to proton translocation across the inner mitochondrial membrane (1–4). Complex I from bovine heart mitochondria is a highly complex, multisubunit, membrane-bound assembly, with a molecular mass of over 900 kDa. The sequences of 42 of the subunits of complex I have been reported previously (3, 5–7); seven are mitochondrial gene products, whereas the rest are encoded in the nucleus and imported into the organelle from the cytoplasm. Prokaryotic complex I systems are simpler assemblies of 13–14 subunits, all of them conserved in eukaryotes, and contain an equivalent set of redox cofactors (8, 9). Mitochondrial complex I is a roughly L-shaped assembly (10–12), with one arm in the plane of the lipid bilayer. The other, containing the more hydrophilic subunits, protrudes from the membrane and can be dissociated from it, producing subcomplex IA (13, 14). The oxidation of NADH is catalyzed at an active site containing non-covalently-bound flavin mononucleotide, and electrons are transported through the assembly to the ubiquinone acceptor via iron-sulfur clusters. Subcomplex IA contains 15 subunits of complex I and all of its known redox cofactors but has no bound ubiquinone; it is thus active in NADH oxidation but only when coupled to artificial electron acceptors such as ferricyanide or hexaammineruthenium.

With the aim of unambiguously defining the subunit composition of subcomplex IA (13, 14), each of the bands resolved by SDS-PAGE analysis was examined by peptide-mass mapping. In this way, 14 known subunits of mitochondrial complex I were confirmed as being present in the subcomplex. In addition, a 15th band, not corresponding to any known subunit, was observed, running close to subunit B17.2. This additional protein, which is tightly associated with subcomplex IA, was investigated by mass mapping and tandem MS of its tryptic peptides, and the corresponding cDNA was sequenced. The mature protein, of 143 amino acids, has an acetylated α-amino group. The presence of the new protein has also been confirmed in intact complex I, and following the nomenclature for mitochondrial complex I it has been named B16.6 (3). The amino acid sequence clearly identifies subunit B16.6 of mitochondrial complex I to be the bovine homolog of the GRIM-19 protein, the product of a cell death regulatory gene induced by interferon-β and retinoic acid. GRIM-19 has previously been detected in HeLa cells, predominantly in the nucleus, though punctate staining of the cytoplasm was also observed (15, 16).

EXPERIMENTAL PROCEDURES

Isolation of Bovine Mitochondrial Complex I and the IA Subcomplex—Mitochondria were isolated from bovine hearts (17), and mitochondrial membranes were prepared by disruption with a glass tissue grinder or a Waring blender in the presence of potassium chloride (18). Complex I, from the first ion-exchange column above, was purified according to Sazanov et al. (14), by solubilization with dodecylmaltoside (Anatrace, Maumee, OH) and ion-exchange chromatography on Q-Sepharose HP media (Amersham Pharmacia Biotech). Pooled complex I fractions were either used to prepare subcomplex IA (see below) or subjected to further purification using a second ion-exchange separation, ammonium sulfate precipitation, and gel filtration (14). All purification steps were carried out at 0–4 °C.

Subcomplex IA was prepared by a procedure developed from that of Finel et al. (13). Complex I, from the first ion-exchange column above, was precipitated by addition of dodecylmaltoside to 1%, sodium cholate (Dojindo Laboratories, Kumamoto, Japan) to 1.8%, and ammonium sulfate to 50% saturation. After centrifugation, the resulting pellet was dissolved into 1% LDAO (Calbiochem), 100 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol (Melford Laboratories, Ipswich, UK), to a final concentration of ~10 mg ml−1. Portions were layered onto linear sucrose gradients (12 ml, 5–15% sucrose, 0.5% LDAO, 500 mM potassium phosphate, pH 7.5), and this step was repeated three times. The final pellet was resuspended in 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 1% LDAO, and 0.5% ammonium sulfate. Complex I was purified as described previously (14, 15). The final pellet was resuspended in 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 1% LDAO, and 0.5% ammonium sulfate. Complex I was purified as described previously (14, 15).
sium phosphate, pH 7.5, 1 mM diithiothreitol) and centrifuged for 18 h at 200,000 × g. The sharp yellow-brown band in the center of the gradient was concentrated to ~6 mg ml⁻¹ by ultracentrifugation (YM-100; Millipore, Bedford, MA), and portions (0.5 ml) were injected into a Superose 6 HR 10/30 gel filtration column (Amersham Pharmacia Biotech) and eluted (0.3 ml/min) in a buffer containing 0.1% LDAO, 100 mM potassium phosphate, pH 7.5, and 1 mM diithiothreitol. The colored fractions spanning the apex of the symmetrical absorbance peak (280 nm) were pooled and stored in liquid nitrogen (concentration ~1 mg ml⁻¹).

Protein concentrations were determined by the BCA protein assay (Pierce) using bovine serum albumin as standard. Unless otherwise stated, all chemicals were purchased from Merck and were of analytical grade.

**SDS-PAGE Gel Systems, Western Blotting, and N-terminal Sequence Analysis—**SDS-PAGE was carried out by the method of Laemmli (19) using a 12–22% acrylamide gradient, and gels were stained with either 0.2% Coomassie Blue R250 (7% acetic acid, 50% methanol) or 0.1% colloidal Coomassie G250 (3% phosphoric acid, 6% ammonium sulfate) (20). For N-terminal sequence analysis, proteins separated by SDS-PAGE were transferred to an Immobilon P membrane (Millipore, Bedford, MA) in a solution of 25 mM Tris, 192 mM glycine, and 10% methanol. Excised Coomassie Blue-stained bands were analyzed by automated Edman degradation using a model 494 Procise protein sequencer (Applied Biosystems, Warrington, UK).

**Mass Spectrometry—**Proteins separated by SDS-PAGE were identified by peptide mass fingerprinting. Bands were excised from the gel, cleaved "in-gel" with trypsin (21), and the mixture of tryptic peptides was analyzed with MALDI-TOF MS using a ToF Spec 2E mass spectrometer (Micromass, Altrincham, UK) and α-cyano-4-hydroxy-trans-cinnamic acid as the matrix. Spectra were calibrated using peptides from trypsin autopepsin at 2163.057 and 2273.160, and a matrix-related ion at 635.015. MS and tandem MS data were acquired automatically.

**CID fragmentation** of a synthetic peptide, [Glu1] fibrinopeptide B (Sigma-Aldrich). Peptide mixtures from in-gel tryptic digest were desalted on-line a Poros R2 50-Å column (Perseptive Biosystems, Bedford, MA) in a solution of 25 mM Tris, 192 mM glycine, and 10% methanol. The new protein was also identified in intact complex I and the inaccessibility of the N terminus provide likely explanation for this. The new subunit of the assembly. The new subunit is a component of the complex I heavy chain, as shown by co-purification with the 30-kDa subunit. Porcine complex I is also present in subcomplex I, or precipitation by dialysis against a low ionic strength buffer. The new protein was also identified in complex I of this Figure 1, which also revealed a further band that did not correspond to any known complex I subunit. The new protein is not adventitiously bound to complex I, and because it is present in the complex at approximately the same molar ratio as other subunits (as estimated by gel band intensities and the relative yields in HPLC experiments) it is reasonable to conclude that it is a bona fide subunit of the assembly. The new protein gave no N-terminal sequence by Edman degradation, suggesting that the N terminus is modified (see below) and in accordance with the nomenclature for bovine mitochondrial complex I has been named B16.6 (3). Subunit B16.6 is also present in subcomplex I (Fig. 1) and also revealed a further band that did not correspond to any known complex I subunit. The new protein was also identified in complex I of this Figure 1. It could not be removed from the complex by any of the following procedures: extended washing of complex I bound to an ion-exchange column, gel filtration, dialysis, treatment with 2 M NaCl followed by dialysis, ammonium sulfate precipitation, or precipitation by dialysis against a low ionic strength buffer. In addition, the new protein is also present in subcomplex I, prepared by disrupting complex I with LDAO, and purified using a sucrose gradient and gel filtration. Therefore, the new protein is not adventitiously bound to complex I, and because it is present in the complex at approximately the same molar ratio as other subunits (as estimated by gel band intensities and the relative yields in HPLC experiments) it is reasonable to conclude that it is a bona fide subunit of the assembly. The new protein gave no N-terminal sequence by Edman degradation, suggesting that the N terminus is modified (see below) and in accordance with the nomenclature for bovine mitochondrial complex I has been named B16.6 (3). Subunit B16.6 is observed reproducibly in all our preparations of bovine complex I and subcomplex I. The complicated SDS-PAGE patterns of complex I and the inaccessibility of the N terminus provide likely explanation for why B16.6 has been overlooked previously.
Tandem Mass Spectrometry—The tryptic peptide mixture from the unidentified protein band was examined by tandem MS, and amino acid sequences from several tryptic peptides were determined (Table I). These were assembled into Protein Sequence Tags (23), and comparison with protein sequence data bases revealed a precise match of one tag ((879.5) L/I E D (522.3)), to the human protein GRIM-19 (accession number AF286697). A number of other peptide sequences (Table I) also aligned readily with the human sequence, suggesting B16.6 to be a homolog of human GRIM-19; they later confirmed the amino acid sequence deduced from cDNA sequencing (see below).
cDNA Sequence and Molecular Mass of the New Subunit—The nucleotide sequence encoding B16.6 and the deduced amino acid sequence are displayed in Fig. 2A. The 5’-sequence of the cDNA extends 31 bases upstream of the proposed initiator methionine codon and does not contain an in-phase stop codon. The mass of the intact protein measured by ESI MS (16585 Da) was found to be consistent with the mass calculated from the sequence (16584.3 Da), provided that the initiator methionine is cleaved from the mature protein and that the N-terminal alanine residue is acetylated.

The B16.6 subunit has a predicted isopotential point of 9.7. Values above pH 9.5 are predicted for approximately half the subunits of mitochondrial complex I and for almost all of the 12 nuclear-encoded subunits with modified N termini. The hydrophathy profile (not shown) demonstrates the amphiphilic nature of subunit B16.6 (24), with one potential transmembrane a-helical domain between residues 29 and 47 (25). These properties are consistent with B16.6 comprising part of subcomplex Iα; whereas it is expected to be predominantly hydrophilic, isolated subcomplex Iα aggregates in the absence of detergent, suggesting the presence of hydrophobic surface patches. Because of its strong association with subcomplex Iα, subunit B16.6 is likely to be located predominantly on the matrix side of the inner mitochondrial membrane, but its topology in the inner membrane is not known, and either residues 1–28 or 48–143 could lie in the intermembrane space.

Identification of the New Complex I Subunit, B16.6, as GRIM-19—The sequences of human GRIM-19 (16) and B16.6 from bovine complex I are 83% identical (Fig. 2B). Data base entries (EMBL and EST) for other vertebrate homologs also show high identities with the bovine protein: 94, 83, 81, 73, 73, and 70% for pig, mouse, rat, zebrafish, oryzias, and chicken, respectively. In all species (see also Fig. 2B), the N-terminal sequences are highly conserved, though deletion of the N-terminal 50 amino acids of GRIM-19 did not inhibit cell death (16). The 25 C-terminal amino acids are markedly less conserved. In addition, there are a number of plant homologs, for example from soybean, sorghum, cotton fiber, wheat, barley, and barley medic, which show high identity with each other but a maximum of 46% identity with bovine B16.6. However, the sequence of residues 97–109 remains highly conserved. Deletion of the C-terminal 43 amino acids of GRIM-19 showed their importance in promoting cell death (16), consistent with an important role for residues 97–109. However, this motif does not appear to have the characteristics of an ATP binding domain (26) as was suggested by Angell et al. (16).

Post-translational Modification and Targeting of B16.6 to Mitochondria—The acetylation of the alanine N terminus and the presence of a methionine at position –1 indicate that, in common with 18 other subunits of complex I, no cleavable presequence is used to target B16.6 to mitochondria (3, 6). Of these 18 subunits, 12 are modified at the N terminus, 11 by acetylation and 1 by myristylation; for such proteins, the localization signal must lie within the mature protein (27, 28). The cellular distribution of GRIM-19 (B16.6) is complex. It has been detected, with antibodies, primarily in the nucleus of HeLa cells, though “punctate” staining in the cytoplasm, possibly corresponding to mitochondria, was also observed (16). Furthermore, transcripts of GRIM-19 are particularly elevated in human heart and skeletal muscle and to a lesser extent in liver, kidney, and placenta, correlating approximately with the relative abundance of mitochondria in these tissues (16). Although only one human gene for GRIM-19 has been detected (29), it remains possible that differential splicing provides the means to target the protein to more than one site. A second possibility is that targeting is controlled by a post-translational modification such as acetylation or phosphorylation. Angell et al. (16) have suggested that human GRIM-19 is phosphorylated constitutively, but there is no indication from our mass spectrometric analysis for a phosphoryl group in mitochondrial B16.6. Another mitochondrial protein, endonuclease G, has been shown to be released from mitochondria during apoptosis and subsequently translocated to the nucleus (30). Clearly the targeting of GRIM-19 (B16.6) to either nucleus or mitochondria requires further study.

Concluding Remarks—B16.6 is the 43rd subunit of bovine mitochondrial complex I to be identified. It is possible that it represents the completion of the primary structure of this complex enzyme, although a protein with a molecular mass of 10566 (±2) Da, observed in some preparations, has not yet been explained (5).

The GRIM-19 protein has now been found to fulfill two roles within the cell. The first is as part of the interferon-β- and retinoic acid-induced pathway of cell death (15, 16). The second is as part of the mitochondrial complex I assembly. These two seemingly disparate functions may be linked by the involvement of mitochondria in apoptotic cell death.

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