Alternative translation initiation generates a novel isoform of insulin-degrading enzyme targeted to mitochondria

Malcolm A. LEISSRING*, Wesley FARRIS*, Xining WU*, Danos C. CHRISTODOULOU†, Marcia C. HAIGIS†, Leonard GUARENTE† and Dennis J. SELKOE*¹

*Center for Neurologic Diseases, Department of Neurology, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02115, U.S.A., and †Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

IDE (insulin-degrading enzyme) is a widely expressed zinc-metallopeptidase that has been shown to regulate both cerebral amyloid β -peptide and plasma insulin levels *in vivo*. Genetic linkage and allelic association have been reported between the IDE gene locus and both late-onset Alzheimer's disease and Type II diabetes mellitus, suggesting that altered IDE function may contribute to some cases of these highly prevalent disorders. Despite the potentially great importance of this peptidase to health and disease, many fundamental aspects of IDE biology remain unresolved. Here we identify a previously undescribed mitochondrial isoform of IDE generated by translation at an in-frame initiation codon 123 nucleotides upstream of the canonical translation start site, which results in the addition of a 41-amino-acid N-terminal mitochondrial targeting sequence. Fusion of this sequence to the N-terminus of green fluorescent protein directed

this normally cytosolic protein to mitochondria, and full-length IDE constructs containing this sequence were also directed to mitochondria, as revealed by immuno-electron microscopy. Endogenous IDE protein was detected in purified mitochondria, where it was protected from digestion by trypsin and migrated at a size consistent with the predicted removal of the N-terminal targeting sequence upon transport into the mitochondrion. Functionally, we provide evidence that IDE can degrade cleaved mitochondrial targeting sequences. Our results identify new mechanisms regulating the subcellular localization of IDE and suggest previously unrecognized roles for IDE within mitochondria.

Key words: Alzheimer's disease, diabetes mellitus, insulin-degrading enzyme, mitochondrion, metallopeptidase.

INTRODUCTION

IDE (insulin-degrading enzyme; also known as insulysin or insulinasin; EC 3.4.24.56) is a member of the M16 family of zinc-metalloendopeptidases that contain at their catalytic centre a tetrad of conserved residues [His-Xaa-Xaa-Glu-Xaa₇₆-Glu] that are involved in zinc binding and peptide hydrolysis [1]. Although the insulin-degrading abilities of IDE have been studied for more than 50 years, IDE is expressed in both insulin-sensitive and insulin-insensitive tissues, suggesting that there may be unrecognized functions for this highly conserved endopeptidase. Within the cell, IDE is localized predominantly to cytoplasm [1], but in different cell types it can be secreted into the extracellular space [2] or associated with the cell surface [3,4]. In addition, IDE is present in several subcellular compartments, including peroxisomes, endosomes and the nucleus (see [1]). The localization of IDE to peroxisomes is explained by the presence of a conserved type-I peroxisomal-targeting sequence (Ala-Lys-Leu) at the C-terminus [5]. However, the mechanisms responsible for the targeting of IDE to other subcellular compartments are unknown.

Renewed interest in IDE has emerged following the observation that it can degrade A β (amyloid β -protein), which accumulates in the brains of AD (Alzheimer's disease) patients [2,6–8]. Building on this *in vitro* observation, we [9] and others [10] have recently shown that genetic deletion of IDE in mice leads to significant elevations in both brain A β and plasma insulin levels, confirming an important role for IDE in the regulation of these

peptides *in vivo*. Our laboratory has also shown that up-regulation of IDE in neurons prevents AD-type pathology in transgenic mice overexpressing the A β precursor protein [11], supporting a potential therapeutic role for IDE in the treatment of AD.

The finding that IDE can degrade A β prompted a search for possible genetic linkage and/or allelic association of late-onset AD to the IDE region on chromosome 10q, evidence for which was first reported by Tanzi and colleagues [12]. Since this initial report, this finding has been corroborated by several independent groups studying various patient cohorts around the world [13–15]. Genetic association has also been reported between the IDE gene and DMII (Type II diabetes mellitus) [16,17]. These reports are tantalizing in light of epidemiological studies that suggest a significant degree of co-morbidity between AD and DMII [18–20] or between AD and hyperinsulinaemia [21,22]. However, despite considerable effort, no pathogenic mutations have been uncovered within the coding region of IDE, raising the likelihood that the underlying pathogenic alterations are instead manifested at the transcriptional and/or translational levels.

Additional support for the possible involvement of IDE dysfunction in DMII and AD comes from a well known model of DMII, known as the GK rat [23]. This compelling animal model was generated by inbreeding Wistar rats and selecting for animals with glucose intolerance, resulting in the production of a genetically homogeneous strain that spontaneously develops a diabetic phenotype. The GK rat has been subjected to extensive genetic analysis to uncover the elements underlying its susceptibility to

Abbreviations used: A β , amyloid β -protein; AD, Alzheimer's disease; CHO, Chinese hamster ovary; DMII, Type II diabetes mellitus; EGFP, enhanced green fluorescent protein; HA, haemagglutinin; IDE, insulin-degrading enzyme; Met¹-IDE and Met⁴²-IDE, IDE isoforms beginning at Met¹ and Met⁴² respectively; MPP, mitochondrial processing peptidase; NRD_C, N-arginine dibasic convertase.

¹ To whom correspondence should be addressed: Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA 02115, U.S.A. (email DSelkoe@rics.bwh.harvard.edu).

DMII, leading to the discovery of two missense mutations in IDE ($\text{His}^{18} \rightarrow \text{Arg}$ and $\text{Ala}^{890} \rightarrow \text{Val}$) that were reported to decrease the ability of IDE to degrade insulin [24]. Our group has recently confirmed these findings and shown further that these mutations also impair the degradation of $\text{A}\beta$ [25]. Moreover, primary neuronal cultures from these animals display defects in $\text{A}\beta$ degradation that lead to substantial accumulation of $\text{A}\beta$ in their conditioned medium. Although the missense mutations in IDE may only partially explain the diabetic phenotype of the GK rat, IDE knockout mice show a remarkably similar phenotype; in addition to showing defects in insulin and $\text{A}\beta$ degradation, these mice also manifest impaired glucose tolerance [9]. Together, these findings provide compelling support for the hypothesis that co-morbidity between AD and DMII may in some cases be attributable to impairments in IDE function.

In the present study, we identify a novel mitochondrial isoform of IDE generated from translation beginning at an in-frame initiation codon located upstream of the canonical start site. We show that this longer isoform contains an N-terminal mitochondrial targeting sequence and that this indeed targets IDE to mitochondria. In addition, we provide evidence that IDE functions to degrade cleaved mitochondrial leader peptides. Our results identify new mechanisms regulating IDE subcellular localization and suggest novel functions for IDE that may play a role in both health and disease.

MATERIALS AND METHODS

Generation of cDNA constructs

The cDNA for full-length IDE containing its authentic termination codon was amplified from human brain RNA by reverse transcription-PCR and cloned into the pEF6/V5-His-TOPO vector (Invitrogen), using the primers IDE-Fwd ($5'\text{-GGCTAATGCGGT-ACC GGCTAGC-3'}$) and IDE-TA-Rev ($5'\text{-ATCTTCAGAGTTT-GCAGCC-3'}$), to generate pEF6-IDE-STOP. A second cDNA clone was generated using a primer lacking the termination codon and thus permitting the in-frame addition of the C-terminal V5-6 $\times$ His fusion tag encoded by the vector, to generate pEF6-IDE-V5-His. Constructs encoding IDE beginning obligatorily at Met¹ or Met⁴² (Met¹-IDE or Met⁴²-IDE) were generated from pEF6-IDE-STOP by mutagenesis of the codons encoding Met⁴² or Met¹ respectively to Ala using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). EGFP (enhanced green fluorescent protein) fusion constructs containing the N-terminal portion of Met¹-IDE or Met⁴²-IDE were generated by subcloning BamHI–BamHI fragments of the IDE cDNA (encoding peptides comprising residues Met¹ or Met⁴² to Pro¹⁰⁰) into pEGFP-N1 (Clontech). An internal HA (haemagglutinin) tag was added to the wild-type, Met¹-IDE or Met⁴²-IDE constructs immediately after Arg⁴³ using a PCR-based method. A Kozak consensus sequence was also incorporated into the Met¹-HA-IDE construct by PCR using the primers ATG1/Koz-IDE-Fwd ($5'\text{-CGCGGATCCGCC-ACCATGCGGTACCGGCTAGCGTGGCTTCTG-3'}$) and IDE-TA-Rev.

Generation of stable cell lines

Because we wished to compare the relative expression levels of the different HA-tagged IDE cDNA constructs, we elected to use Invitrogen's Flp-In system, which uses homologous recombination to stably integrate a single copy of each construct into the same genomic locus of a single cell line. To this end, the HA-tagged cDNAs were subcloned into pcDNA5/FRT and transfected into a CHO (Chinese hamster ovary) cell line stably expressing

the Flp-In receiving vector, pFRT/*lacZeo*. Stable isogenic clones were selected and characterized according to the manufacturer's recommendations.

Subcellular fractionation by differential centrifugation

Groups of cells were washed two times in PBS, collected in ice-cold buffer A (5 mM Tris/HCl, 1 mM EDTA, 0.25 M sucrose, pH 7.4) and gently disrupted using a Dounce homogenizer. Cell nuclei and unbroken cells were pelleted (P_1) by centrifugation at 500 *g* for 10 min. Pelleted fractions enriched for various subcellular organelles were obtained from the post-nuclear supernatant by successive centrifugation at 3000 *g* for 15 min (P_2 ; enriched for heavy mitochondria and plasma membrane fragments), 10 000 *g* for 15 min (P_3 ; enriched for light mitochondria, lysosomes, peroxisomes and Golgi) and 100 000 *g* for 60 min (P_4 ; containing endoplasmic reticulum vesicles, plasma membrane, endosomes, etc.), and the high-speed supernatant was retained as the soluble fraction (S_1) (see [26]). After each centrifugation step, the resulting pellets were washed two times by resuspension in an excess of buffer A, followed by centrifugation at the appropriate relative centrifugal force (*g*).

Purification of mitochondria from HEK-293 cells

Confluent monolayers of cultured cells (2×10^8) were washed with PBS, collected by scraping, and pelleted at 1000 *g* for 20 min. Cells were resuspended in 6 vol. of ice-cold cell homogenization medium (150 mM NaCl, 10 mM KCl, 10 mM Tris/HCl, pH 6.7) and disrupted with a Dounce homogenizer. The cell homogenate was supplemented with $\frac{1}{3}$ vol. of cell homogenization medium containing 1 M sucrose, and nuclei and unbroken cells were removed by centrifugation at 1000 *g* for 20 min. The supernatant was centrifuged at 5000 *g* for 20 min, and the resulting pellet was resuspended in ice-cold sucrose/ Mg^{2+} medium (0.25 M sucrose, 150 mM MgCl_2 , 10 mM Tris/HCl, pH 6.7). Following repeated washing and centrifugation at 5000 *g* for 20 min to remove trapped peroxisomes and other organelles, the purified mitochondria were resuspended in mitochondrial suspension medium (0.25 M sucrose, 10 mM Tris/HCl, pH 7.0).

Confocal microscopy

CHO cells plated on to sterile glass-bottomed cell chambers (Nalge Nunc International) were transfected with the IDE_{NTF}::EGFP cDNA constructs either alone or together with cDNAs encoding DsRed directed either to the mitochondrion or to the endoplasmic reticulum (DsRed2-mito and DsRed2-ER respectively; Clontech). After 32 days, fluorescence in the green and red channels was visualized in the living cells using a Zeiss LSM 510 confocal microscope fitted with a 63 $\times$ Plan NeoFluar oil-immersion objective using the 488 nm and 568 nm laser lines of argon and krypton lasers respectively.

Western blotting

Western blots were performed as described in [9]. The IDE-5 antibody was generated by vaccination of rabbits with a peptide corresponding to amino acids 28–41 of the human IDE sequence and was affinity purified using this same peptide (Research Genetics). Other antibodies used in the present study include α IDE-1 [4], 3F10 (reactive with the HA tag; Roche), α -hsp60 (Stressgen Biotechnologies), α -catalase (Rockland, Inc.) and anti- α -syntaxin 13 (Stressgen Biotechnologies).

A	M ₁		M ₄₂
	human	MR ^Y RLAWLLHPALPSTFRSVLGARLPPPERLCGFGOKKTYSKMNNPAI...	
	rat	MR ^N GLVWLLHPALPSTL ^I SILGARPPPVKRLCGFPKQIYSTMNNPAI...	
	mouse	MR ^N GLVWLLHPALPGTLRSILGARPPPAKRLCGFPKQIYSTMNNPAI...	
B	AUG1		AUG2
	human	TGGCTAA <u>U</u> GC	AGCAAA <u>U</u> GA
	rat	CGGGT <u>A</u> GC	AGCACA <u>U</u> GA
	mouse	CGGGT <u>A</u> GC	AGCACA <u>U</u> GA
	Kozak	GCCPCCA <u>U</u> GP	GCCPCCA <u>U</u> GP

Figure 1 The amino acid and nucleic acid sequences corresponding to the N-terminus of IDE are conserved among mammalian species

(A) The amino acid sequences of human, rat and mouse IDE all feature two alternative in-frame initiating methionines, Met¹ and Met⁴² (bold). The high degree of sequence similarity among the different species implies a functional role for this region. The amino acid mutated in the GK rat (His¹⁸ → Arg) is highlighted in black. The epitope for the antibody IDE-5 is underlined. The arrow indicates the predicted cleavage site of the Met¹-IDE isoform by MPP. (B) The mRNA sequence flanking the second initiation codon (encoding Met⁴²) is closer to the Kozak consensus sequence than that flanking the first initiation codon (encoding Met¹). The initiation codons are underlined, and nucleic acids matching the Kozak consensus sequence are shown in bold. Purines (A or G) are denoted by P.

Immunogold-electron microscopy

Cryosections were prepared from frozen blocks of formaldehyde-fixed cultured cells infiltrated with 2.3 M sucrose in PBS and transferred to formvar-carbon-coated copper grids for gold labelling. All antibodies and Protein A-gold were diluted in 1% (w/v) BSA. Grids were floated on drops of 1% BSA for 10 min to block unspecific labelling, transferred to 5 µl drops of primary antibody and incubated for 30 min. The grids were then washed in four drops of PBS for a total of 15 min and transferred to 5 µl drops of Protein A-gold (Amersham). Contrasting/embedding of the labelled grids was carried out on ice in 0.3% (w/v) uranyl acetate in 2% (w/v) methyl cellulose for 10 min. The grids were examined in a JEOL 1200EX transmission electron microscope, and images were recorded at a primary magnification of ×20 000–30 000.

Peptide degradation assays

A peptide corresponding to the cleaved mitochondrial targeting sequence of the E1α subunit of human pyruvate dehydrogenase [27] was synthesized by Fmoc (fluorenylmethoxycarbonyl) chemistry at the Tufts University Core Facility (Boston, MA, U.S.A.). Degradation assays were performed at 37 °C using the HPLC-purified peptide dissolved in PBS to a final concentration of 100 µM and reacted with 50 nM recombinant IDE [11]. Aliquots of the reaction mixture were removed at various time points and terminated by boiling or by the addition of 2 mM 1,10-phenanthroline. Degradation products were separated by reverse-phase HPLC using a Vydac C-18 column (250 mm × 4.6 mm) with a 50-min linear gradient of 7–70% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid at 1.5 ml/min and detected by absorbance at 226 nm.

RESULTS

Alternative initiation codons are a conserved feature of mammalian IDE homologues

The open reading frames of all known mammalian IDE cDNAs contain two in-frame translation initiation codons, and therefore potentially encode proteins beginning either at the first or the 42nd amino acid (designated Met¹-IDE and Met⁴²-IDE; respectively; Figure 1A). Current experimental evidence indicates that Met⁴²-

IDE is the predominant, if not exclusive, isoform expressed in tissues and cultured cells, and previous attempts to detect Met¹-IDE have proved unsuccessful [28,29]. Factors influencing translation efficiency have been postulated to account for this preference, as the nucleotide sequence surrounding the second initiation codon contains a better Kozak consensus sequence for initiation of translation (Figure 1B) [30]. Although predicted to be less efficiently translated, the Met¹-IDE isoform nevertheless could account for a significant fraction of total cellular IDE. Moreover, the novel N-terminal sequence contained within the Met¹-IDE protein might determine an alternative subcellular localization for this translational isoform.

Translation beginning at Met¹ encodes a mitochondrial targeting sequence

To address this issue, we performed bioinformatic analyses on the Met¹-IDE protein sequence using several N-terminal sorting sequence/subcellular localization prediction programs available on the ExPASy on-line proteomics server (www.expasy.ch). Two such programs predicted the presence of a signal peptide within the N-terminus of Met¹-IDE (SignalP [31] and PSortII [32]), while two others predicted a mitochondrial targeting sequence in this region (MitoProt [33] and TargetP [34]). To resolve this issue experimentally, we generated DNA constructs that encoded fusion proteins consisting of N-terminal fragments (ending at Pro¹⁰⁰) of either Met¹-IDE (with Met⁴² mutated to Ala) or Met⁴²-IDE (with Met¹ mutated to Ala) fused in-frame to the N-terminus of EGFP, which is normally cytosolic (Figure 2A). These fusion constructs, designated Met¹-IDE_{NTF}::EGFP and Met⁴²-IDE_{NTF}::EGFP respectively, or the unmodified EGFP construct were transfected separately into CHO cells and visualized by confocal microscopy. Like EGFP itself, the Met⁴²-IDE_{NTF}::EGFP fusion protein was expressed overwhelmingly in the cytoplasm (Figure 2B). This result is consistent with the principally cytosolic localization reported for wild-type IDE and suggests that the Met⁴²-IDE isoform lacks an organelle-specific targeting sequence at its N-terminus. In marked contrast, the Met¹-IDE_{NTF}::EGFP fusion protein was expressed within subcellular organelles possessing a vermiform morphology (Figure 2B). To determine the identity of these organelles, the Met¹-IDE_{NTF}::EGFP construct was co-transfected with cDNAs encoding the DsRed fluorescent protein directed to different subcellular compartments. Met¹-IDE_{NTF}::EGFP co-localized perfectly with mitochondrion-directed DsRed (Figure 2B, lower panels), but did not overlap with endoplasmic reticulum-directed DsRed (results not shown). These results demonstrate that the N-terminal sequence present in Met¹-IDE includes a mitochondrial targeting sequence that is necessary and sufficient for transport into mitochondria.

To investigate the subcellular localization of the full-length IDE translational isoforms, we generated stable cell lines expressing the following HA-tagged expression constructs (Figure 3A): wtHA-IDE, encoding full-length wild-type IDE (i.e. containing both initiation codons); Met⁴²-HA-IDE, beginning obligatorily at Met⁴² and lacking the first initiation codon; Met¹-HA-IDE, beginning obligatorily at Met¹ and with Met⁴² mutated to an alanine; and Koz-Met¹-HA-IDE, comprising the latter construct with a Kozak consensus sequence engineered to precede Met¹. We generated these cell lines using a homologous recombination-based strategy (Invitrogen Flp-In System) in order to integrate each expression construct into the same genomic locus of a single parental CHO cell line. Because the expression constructs in the different cell lines are integrated at the same site(s), the relative expression levels of the different isoforms can be validly compared.

Figure 2 The N-terminus of the Met¹-IDE isoform contains a mitochondrial targeting sequence

(A) Design of EGFP fusion constructs used to test for potential sorting sequences within the N-terminus of Met¹-IDE or Met⁴²-IDE. EGFP alone served as a control for cytosolic localization. (B) Confocal microscopy showing the subcellular localization of EGFP alone (upper left panel), Met⁴²-IDENTF::EGFP (upper middle panel) and Met¹-IDENTF::EGFP (upper right panel). The lower panels shows a cell co-transfected with Met¹-IDENTF::EGFP and the mitochondrion-directed DsRed fluorescent protein.

Figure 3 The full-length Met¹-IDE isoform is expressed in a cell fraction enriched in mitochondria

(A) Design of full-length, HA-tagged IDE expression constructs designed to exclusively express the Met¹-IDE and Met⁴²-IDE isoforms. Note that the first initiation codon is deleted in the Met⁴²-HA-IDE construct, while Met⁴² is mutated to Ala within the Met¹-HA-IDE and Koz-Met¹-HA-IDE constructs. The wt-HA-IDE construct contains both initiation codons. (B) Western blots showing expression of the different HA-tagged constructs in fractions (10 μ g/well) generated by differential centrifugation. Labels to the left of (A) apply also to (B). P₂, post-nuclear 3000 g pellet; P₃, 10 000 g pellet; P₄, 100 000 g pellet; S₁, 100 000 g supernatant fraction. Note that the Met⁴²-HA-IDE construct is expressed predominantly in the S₁ fraction, which contains exclusively soluble (cytosolic) proteins, while the Koz-Met¹-HA-IDE construct is expressed almost exclusively in the P₂ pellet, which is highly enriched in mitochondria (see D). (C) The Met¹-HA-IDE construct (lacking a Kozak consensus sequence) is expressed at low levels in the P₂ fraction. The panel shows an overexposed anti-HA Western blot (30 μ g/well) of fractionated cells expressing Met¹-HA-IDE compared with empty vector. The Met¹-HA-IDE isoform is increased exclusively in the P₂ fraction (arrow). Note the presence of non-specific bands (*) of equal intensity in the two P₂ fractions. (D) The relative distribution of different subcellular compartments in our fractionation method is exemplified by fractions from the Koz-Met¹-HA-IDE cell fractions subjected to Western analysis with several subcellular markers: hsp60 (mitochondrial matrix protein; enriched in P₂), catalase (peroxisomal marker; enriched in P₃) and α -syntaxin 13 (α -synt 13; endosomal marker; enriched in P₄).

Cells expressing the different constructs were homogenized in an isotonic buffer, fractionated by differential centrifugation (see the Materials and methods section), and analysed by Western blot for the presence of the HA-tagged IDE proteins. Consistent

with the known subcellular localization of endogenous IDE, the wtHA-IDE construct was expressed predominantly in the high-speed supernatant fraction (cytosol), but was also found at lower levels in each of the pelleted fractions (Figure 3B). Met⁴²-IDE was

Figure 4 Endogenous IDE is present in purified mitochondria

(A) Detection of IDE in purified mitochondria. Note that mitochondrial IDE migrates at a similar size as cytosolic/peroxisomal IDE. (B) Mitochondrial (m) but not cytosolic (c) IDE is protected from digestion with trypsin. The arrowhead indicates an IDE species of a size consistent with that predicted for uncleaved Met¹-IDE (i.e. still containing the mitochondrial targeting sequence). Note the absence of the latter species in the cytosolic IDE and in the mitochondrial IDE treated with trypsin.

expressed in a closely similar pattern (Figure 3B), confirming previous studies showing that the majority of endogenous IDE protein represents this isoform. In striking contrast, the Koz-Met¹-HA-IDE construct was present most abundantly in the post-nuclear 3000 g pellet (P₂), which is enriched in mitochondria (Figure 3B), as confirmed by Western analysis of marker proteins (Figure 3D). It initially appeared that the Kozak consensus signal within Koz-Met¹-HA-IDE was essential for translation beginning at Met¹, since the Met¹-HA-IDE protein (lacking the Kozak consensus sequence) did not appear to be present on Western blots that detected the other isoforms (Figure 3B). However, upon longer exposure, expression of Met¹-HA-IDE could, in fact, be detected solely in the mitochondrion-enriched P₂ fraction (Figure 3C). Taken together, the results in Figure 3 indicate that translation of Met¹-IDE can occur, albeit at lower levels than that of Met⁴²-IDE, and they show that the Met¹-IDE isoform is localized to a mitochondrion-enriched subcellular fraction. Similar results (not shown) were obtained with transiently transfected COS cells.

Endogenous IDE is present within mitochondria

To attempt to confirm that an endogenous mitochondrial IDE isoform exists, we performed Western blots on extensively washed mitochondria purified from untransfected HEK-293 cells (see the Materials and methods section) and detected using our well characterized anti-IDE antibody (IDE-1). This antibody is highly specific for IDE (both isoforms), recognizing a ~110 kDa band on Western blots of whole-cell lysates or tissue homogenates of numerous mammalian species that is absent from samples prepared from IDE knockout mice [9]. Endogenous IDE was readily detected in purified mitochondrial preparations (far left lanes in Figures 4A and 4B), which were shown by Western analysis to be free of the peroxisomal marker catalase, but highly enriched in the mitochondrial marker hsp60 (Figure 4A). The IDE present in these fresh intact mitochondria was protected from digestion by trypsin (Figure 4B), excluding the possibility of contamination with the cytosolic Met⁴²-IDE isoform and consistent with the predicted intramitochondrial localization of Met¹-IDE based on its N-terminal targeting sequence.

The mitochondrial presequence of IDE is probably removed upon import into mitochondria

The Met¹-IDE and Met⁴²-IDE isoforms are predicted to differ in size and could conceivably be discriminated on this basis. In

Figure 5 Detection of the mitochondrial isoform of IDE by immuno-electron microscopy

Electron micrographs of CHO cells expressing wt-HA-IDE (A) or Koz-Met¹-HA-IDE (B) and labelled with gold-coupled anti-HA antibodies. (A) Wild-type IDE is present in cytosol (c), as expected, but also in mitochondria (m), with a much weaker signal in the nucleus (n). (B) The Met¹-HA-IDE isoform is expressed exclusively in mitochondria. Scale bars = 200 nm.

practice, however, we could not detect a reliable size difference between the Met¹-IDE and Met⁴²-IDE HA-tagged isoforms even following separation on 4% (w/v) polyacrylamide gels, although we were readily able to detect a sizable mobility shift in constructs containing a 45-residue tag at the C-terminus, even on 8% (w/v) polyacrylamide gels (results not shown). This result can be explained if the mitochondrial targeting sequence is cleaved upon import to the mitochondrion by MPP (mitochondrial processing peptidase), as is true for most mitochondrial presequences. Consistent with this idea, the signal prediction program MitoProt predicts cleavage of Met¹-IDE at the Leu³¹-Cys³² peptide bond ('probability' = 0.9770 [33]) (arrow in Figure 1A). Cleavage at this site would leave the proteolytically processed Met¹-IDE isoform with only 10 additional amino acids beyond the 978 amino acids present in Met⁴²-IDE, explaining the difficulty in discriminating between Met⁴²-IDE and cleaved Met¹-IDE. We were also unable to directly detect the non-cleaved Met¹-IDE isoform using an affinity-purified polyclonal antibody (IDE-5) that was raised to a peptide corresponding to amino acids 28–40 within the predicted mitochondrial targeting sequence (Figure 1A, underlined). However, cleavage at the predicted site (Leu³¹-Cys³²) would be expected to remove significant portions of the epitope recognized by IDE-5, and this could explain its inability to recognize the proteolytically processed variant of Met¹-IDE. These results are therefore consistent with the idea that the Met¹-IDE isoform represents a mitochondrial precursor whose targeting sequence is removed proteolytically by MPP upon import into the mitochondrion. In this regard, we noticed that, in purified mitochondria, the IDE-1 antibody does recognize a faint band of the predicted size of the unprocessed Met¹-IDE isoform (Figure 4B, arrowhead) that is susceptible to trypsin digestion. We speculate that this band represents a small pool of Met¹-IDE that has been translocated to mitochondria (e.g. by cytosolic hsc70 or other mitochondrial presequence chaperones) but that has not yet been proteolytically cleaved by MPP.

Ultrastructural evidence for the presence of IDE within mitochondria

To confirm the mitochondrial localization of IDE at the ultrastructural level, we performed immuno-electron microscopy on CHO cells expressing HA-tagged IDE constructs. In CHO cells (Figure 5A) expressing the wild-type isoform, IDE was clearly detected within mitochondria, in addition to its expected cytoplasmic

Figure 6 IDE efficiently degrades a prototypical mitochondrial targeting sequence

(A) Amino acid sequence of the cleaved leader peptide of the E1 α subunit of human pyruvate dehydrogenase. (B) HPLC elution profiles illustrating that the peptide in (A) is hydrolysed by IDE at multiple sites in a time-dependent fashion.

localization, and similar results were obtained with COS cells (results not shown). In CHO cells expressing the Koz-Met¹-HA-IDE construct, IDE was detected exclusively in mitochondria (Figure 5B).

Potential function of IDE within mitochondria

IDE within peroxisomes has been shown to degrade the cleaved leader sequences removed from peroxisomal precursor proteins [35]. Moreover, likely IDE orthologues in plants have been shown to degrade N-terminal sorting sequences cleaved from both mitochondrial and chloroplast proteins [36,37]. To determine whether IDE may perform a similar function in mitochondria, we tested the ability of IDE to degrade a synthetic peptide corresponding to the cleaved mitochondrial targeting sequence for the E1 α subunit of human pyruvate dehydrogenase [27] (Figure 6A). This peptide was indeed hydrolysed at several sites by purified recombinant IDE (Figure 6B), and the degradation was completely inhibited by 1,10-phenanthroline, a broad-spectrum zinc-metalloprotease inhibitor (results not shown). Analysis of the rate of hydrolysis of this substrate suggested that degradation of this peptide was highly efficient: based on disappearance of the intact peptide as monitored by HPLC, we estimated a k_{cat} value of $\sim 200 \text{ min}^{-1}$. Although this value is likely to reflect a lower boundary of the true value, it is comparable with the k_{cat} values determined for the degradation of A β ($\sim 100\text{--}200 \text{ min}^{-1}$) [38,39], another avid IDE substrate.

DISCUSSION

Here we provide evidence for the existence of a previously unidentified isoform of IDE that is localized to mitochondria. This isoform is generated by translation beginning at an alternative initiation codon upstream of the canonical start site, producing a novel protein with 41 amino acids added to the N-terminus. We show that this N-terminal sequence, which we find is predicted to contain a mitochondrial targeting signal, is both necessary and sufficient to transport normally cytosolic proteins (IDE and EGFP) into mitochondria, where, as for most nuclear-encoded mitochondrial proteins, the signal appears to be removed proteolytically. Within mitochondria, the functional role of IDE may include the degradation of cleaved mitochondrial targeting sequences.

IDE appears to be unique among mammalian zinc-metalloproteases in using alternative translation initiation to regulate the expression of multiple protein isoforms with different subcellular

localizations. By way of comparison, a mitochondrial variant of another metalloendopeptidase, neurolysin (EC 3.4.24.16), is regulated via an alternative promoter, the use of which generates a splice isoform that encodes a protein containing a cleavable mitochondrial targeting sequence [40]. However, the translational regulation apparently used by IDE is not unprecedented. For example, such a mechanism has been found to regulate the expression of mitochondrial and cytosolic variants of fumarate [41]. As compared with transcriptional regulation, which can be influenced by numerous *cis*- and *trans*-acting factors, regulation via translation would appear to be relatively inflexible, being influenced only by the primary nucleotide sequence of the mRNA. In the case of fumarate, however, RNA species were found that could suppress translation of the cytosolic variant and enhance expression of the mitochondrial one [41]. Such *trans*-acting factors might also regulate the translation of Met¹-IDE and Met⁴²-IDE. In this regard, we found a larger relative proportion of endogenous mitochondrial IDE (Figure 4A) than was expected on the basis of our studies in stable cell lines expressing the HA-tagged IDE constructs (Figure 3B). It is possible that the latter constructs, which lack the complete 5' and 3' untranslated regions present in endogenous IDE mRNAs, may have escaped the influence of *cis*- or *trans*-acting factors regulating the endogenous transcripts. In view of the potentially important role of IDE in health and disease, factors that regulate the translation of the two IDE isoforms should be viewed as attractive targets for future genetic studies and drug development.

It is instructive to compare the present findings with a study by Hospital and colleagues [42], published after initiation of our work, which examined translation initiation and subcellular localization of a close homologue of IDE, NRD_C (N-arginine dibasic convertase; nardilysin). Like IDE, NRD_C also contains two in-frame initiation codons, the second of which is believed to be used preferentially if not exclusively. When translated beginning at the first initiation codon, the N-terminal amino acid sequence is predicted by both MitoProt and PSORT II to encode a mitochondrial targeting sequence, as we found for IDE. When Hospital and co-workers added a Kozak consensus sequence in front of the first initiation codon (but did not mutate the second), they observed a longer isoform of NRD_C in addition to the usual isoform in whole-cell lysates. Intriguingly, they could detect both isoforms in the cytosol as well as on the cell surface, where biotinylation could take place. Although these investigators did not report on the possible localization of the longer isoform within mitochondria, their results nonetheless show that this N-terminal sorting signal is not absolutely required for the transport of NRD_C to the cell surface (nor were C-terminal sorting signals involved, since Hospital and colleagues analysed constructs containing C-terminal tags). Given the very high degree of sequence similarity between NRD_C and IDE, it seems likely that the N-terminal extension of IDE that we describe here is also not required for the targeting of wild-type IDE to the cell surface, where IDE can be biotinylated [3,4].

In the present study, we show that IDE efficiently degrades a prototypical cleaved mitochondrial targeting sequence. Such sequences are characterized by the presence of numerous positively charged residues, particularly Arg. In this regard, it is notable that several IDE homologues show a preference for substrates containing Arg residues. For example, IDE's closest homologue, NRD_C, shows a preference for cleavage within the motif -Xaa↓Arg-Lys-, or less commonly at -Arg↓Arg-Xaa-, where Xaa represents any amino acid except Arg or Lys [43]. Moreover, the cleavage of leader peptides by MPP frequently fits the motif Arg-Xaa↓Xaa, where Xaa represents any amino acid [44]. Furthermore, we have recently found that IDE itself shows a strong preference for

cleavage of peptides containing Arg residues at the +1 and +2 positions, based on active-site mapping experiments using combinatorial peptide mixtures (M. A. Leissring, B. E. Turk, L. C. Cantley and D. J. Selkoe, unpublished work). Taken together, these observations suggest that cleaved mitochondrial targeting sequences are innately good substrates for IDE, lending support to the view that their degradation represents a physiological function of mitochondrial IDE. We emphasize, however, that we cannot rule out other, more vital functions for IDE within mitochondria. Indeed, other members of the M16 metalloprotease family have evolved to play essential roles in mitochondrial functions, as exemplified by the core subunits of the mitochondrial cytochrome *bc₁* complex [45], which play an indispensable structural role in the integrity of this respiratory complex. Moreover, outside of mitochondria, IDE and its homologues have been reported to participate in diverse cellular functions, such as regulation of the proteasome [46], interaction with androgen and glucocorticoid receptors [47], and regulation of bud site selection in haploid *Saccharomyces cerevisiae* [48], the latter process being independent of its proteolytic activity.

As mentioned in the Introduction, an inbred rat model of DMII has been found to harbour two missense mutations in IDE, namely His¹⁸ → Arg and Ala⁸⁹⁰ → Val [24]. It is notable that one of these mutations, His¹⁸ → Arg, lies within the mitochondrial targeting sequence that we have identified in Met¹-IDE. Interestingly, an Arg residue is normally present at this position in both the human and murine IDE homologues (Figure 1A). This Arg residue appears to contribute to the positive charge that is essential for the function of mitochondrial signal sequences [49], suggesting that this mutation may alter the subcellular localization of rat IDE. Interestingly, when Fakhrai-Rad and colleagues [24], who originally identified the missense mutations in IDE in the GK rat, transfected cells with cDNAs that harboured either both mutations together or the individual mutations alone, they observed defects in insulin degradation only in cells transfected with constructs containing both mutations. We recently revisited this question [25] and found significant differences in the catalytic efficiency of IDE within the cytosolic fraction of GK rat brains relative to wild-type controls. Because most IDE within this fraction lacks the mitochondrial targeting sequence and therefore the His¹⁸ → Arg mutation, our results suggest that the Ala⁸⁹⁰ → Val mutation alone is sufficient to confer significant impairments in the degradation of insulin and A β . Nonetheless, it remains possible that the His¹⁸ → Arg mutation could contribute independently to the diabetic phenotype in the GK rat, perhaps by altering the subcellular localization of the Met¹-IDE isoform. Intriguingly, mitochondria isolated from the brains of GK rats were recently reported to exhibit increased vulnerability to exposure to A β peptides [50]. Our discovery that IDE is present within mitochondria should help to elucidate the mechanism(s) underlying this intriguing finding.

It is presently uncertain whether the mitochondrial IDE precursor identified in the present study plays any role in human disease. However, emerging evidence implicates mitochondrial dysfunction in the pathogenesis of DMII [51,52], and mitochondria also play important roles in neurodegeneration [53]. Because both variants can be translated from the same mRNA, mutations or other factors affecting the relative abundance of the Met¹-IDE isoform would necessarily affect the abundance of the Met⁴²-IDE isoform, which is presumably the principal form involved in the degradation of A β and insulin *in vivo*. However, one cannot rule out the possibility of a more direct role for the Met¹-IDE isoform in these diseases. While significantly more research will be required to fully uncover the physiological and pathophysiological roles of IDE within mitochondria, such efforts should be facilitated by the finding that mitochondrial IDE is derived from

a unique translational isoform that can be manipulated experimentally.

We thank Dominic Walsh for providing CHO cell lines stably expressing the pFRT/*lacZeo* vector of the Flp-In system, Maria Ericsson for performing electron microscopy, Chittaranjan Das for assistance with HPLC, and Kostas Vekrellis and Stefan Mansourian for assistance with the generation and purification of the α IDE-5 antibody. This work is supported by National Institutes of Health grants AG12749 (to M. A. L., W. F. and D. J. S.) and K08 NS046324-01 (to W. F.).

REFERENCES

- Duckworth, W. C., Bennett, R. G. and Hamel, F. G. (1998) Insulin degradation: progress and potential. *Endocr. Rev.* **19**, 608–624
- Qiu, W. Q., Ye, Z., Kholodenko, D., Seubert, P. and Selkoe, D. J. (1997) Degradation of amyloid β -protein by a metalloprotease secreted by microglia and other neural and non-neural cells. *J. Biol. Chem.* **272**, 6641–6646
- Seta, K. A. and Roth, R. A. (1997) Overexpression of insulin degrading enzyme: cellular localization and effects on insulin signaling. *Biochem. Biophys. Res. Commun.* **231**, 167–171
- Vekrellis, K., Ye, Z., Qiu, W. Q., Walsh, D., Hartley, D., Chesneau, V., Rosner, M. R. and Selkoe, D. J. (2000) Neurons regulate extracellular levels of amyloid β -protein via proteolysis by insulin-degrading enzyme. *J. Neurosci.* **20**, 1657–1665
- Chesneau, V., Perlman, R. K., Li, W., Keller, G. A. and Rosner, M. R. (1997) Insulin-degrading enzyme does not require peroxisomal localization for insulin degradation. *Endocrinology* **138**, 3444–3451
- Kurochkin, I. V. and Goto, S. (1994) Alzheimer's β -amyloid peptide specifically interacts with and is degraded by insulin degrading enzyme. *FEBS Lett.* **345**, 33–37
- McDermott, J. R. and Gibson, A. M. (1997) Degradation of Alzheimer's β -amyloid protein by human and rat brain peptidases: involvement of insulin-degrading enzyme. *Neurochem. Res.* **22**, 49–56
- Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M. B., Rosner, M. R., Safavi, A., Hersh, L. B. and Selkoe, D. J. (1998) Insulin-degrading enzyme regulates extracellular levels of amyloid β -protein by degradation. *J. Biol. Chem.* **273**, 32730–32738
- Farris, W., Mansourian, S., Chang, Y., Lindsley, L., Eckman, E. A., Frosch, M. P., Eckman, C. B., Tanzi, R. E., Selkoe, D. J. and Guenette, S. (2003) Insulin-degrading enzyme regulates the levels of insulin, amyloid β -protein, and the β -amyloid precursor protein intracellular domain *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 4162–4167
- Miller, B. C., Eckman, E. A., Sambamurti, K., Dobbs, N., Chow, K. M., Eckman, C. B., Hersh, L. B. and Thiele, D. L. (2003) Amyloid- β peptide levels in brain are inversely correlated with insulin activity levels *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6221–6226
- Leissring, M. A., Farris, W., Chang, A. Y., Walsh, D. M., Wu, X., Sun, X., Frosch, M. P. and Selkoe, D. J. (2003) Enhanced proteolysis of β -amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. *Neuron* **40**, 1087–1093
- Bertram, L., Blacker, D., Mullin, K., Keeney, D., Jones, J., Basu, S., Yhu, S., McInnis, M. G., Go, R. C., Vekrellis, K. et al. (2000) Evidence for genetic linkage of Alzheimer's disease to chromosome 10q. *Science* **290**, 2302–2303
- Ait-Ghezala, G., Abdullah, L., Crescentini, R., Crawford, F., Town, T., Singh, S., Richards, D., Duara, R. and Mullan, M. (2002) Confirmation of association between D10S583 and Alzheimer's disease in a case-control sample. *Neurosci. Lett.* **325**, 87–90
- Edland, S. D., Wavrant-De Vriese, F., Compton, D., Smith, G. E., Ivnik, R., Boeve, B. F., Tangalos, E. G. and Petersen, R. C. (2003) Insulin degrading enzyme (IDE) genetic variants and risk of Alzheimer's disease: evidence of effect modification by apolipoprotein E (APOE). *Neurosci. Lett.* **345**, 21–24
- Prince, J. A., Feuk, L., Gu, H. F., Johansson, B., Gatz, M., Blennow, K. and Brookes, A. J. (2003) Genetic variation in a haplotype block spanning IDE influences Alzheimer disease. *Hum. Mutat.* **22**, 363–371
- Ghosh, S., Watanabe, R. M., Valle, T. T., Hauser, E. R., Magnuson, V. L., Langefeld, C. D., Ally, D. S., Mohlke, K. L., Silander, K., Kohtamaki, K. et al. (2000) The Finland-United States investigation of non-insulin-dependent diabetes mellitus genetics (FUSION) study. I. An autosomal genome scan for genes that predispose to type 2 diabetes. *Am. J. Hum. Genet.* **67**, 1174–1185
- Karamohamed, S., Demissie, S., Volcjak, J., Liu, C., Heard-Costa, N., Liu, J., Shoemaker, C. M., Panhuysen, C. I., Meigs, J. B., Wilson, P. et al. (2003) Polymorphisms in the insulin-degrading enzyme gene are associated with type 2 diabetes in men from the NHLBI Framingham Heart Study. *Diabetes* **52**, 1562–1567
- Ott, A., Stolk, R. P., Hofman, A., van Harskamp, F., Grobbee, D. E. and Breteler, M. M. (1996) Association of diabetes mellitus and dementia: the Rotterdam Study. *Diabetologia* **39**, 1392–1397

- 19 Leibson, C. L., Rocca, W. A., Hanson, V. A., Cha, R., Kokmen, E., O'Brien, P. C. and Palumbo, P. J. (1997) The risk of dementia among persons with diabetes mellitus: a population-based cohort study. *Ann. N.Y. Acad. Sci.* **826**, 422–427
- 20 Ott, A., Stolk, R. P., van Harskamp, F., Pols, H. A., Hofman, A. and Breteler, M. M. (1999) Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology* **53**, 1937–1942
- 21 Razay, G. and Wilcock, G. K. (1994) Hyperinsulinaemia and Alzheimer's disease. *Age Ageing* **23**, 396–399
- 22 Stolk, R. P., Breteler, M. M., Ott, A., Pols, H. A., Lamberts, S. W., Grobbee, D. E. and Hofman, A. (1997) Insulin and cognitive function in an elderly population. The Rotterdam Study. *Diabetes Care* **20**, 792–795
- 23 Goto, Y., Kakizaki, M. and Masaki, N. (1976) Production of spontaneous diabetic rats by repetition of selective breeding. *Tohoku J. Exp. Med.* **119**, 85–90
- 24 Fakhrai-Rad, H., Nikoshkov, A., Kamel, A., Fernstrom, M., Zierath, J. R., Norgren, S., Luthman, H. and Galli, J. (2000) Insulin-degrading enzyme identified as a candidate diabetes susceptibility gene in GK rats. *Hum. Mol. Genet.* **9**, 2149–2158
- 25 Farris, W., Mansourian, S., Leissring, M. A., Eckman, E. A., Bertram, L., Eckman, C., Tanzi, R. E. and Selkoe, D. J. (2004) Partial loss-of-function mutations in insulin-degrading enzyme that induce diabetes also impair degradation of amyloid β -protein. *Am. J. Pathol.* **164**, 1424–1434
- 26 Dealtry, G. B. and Rickwood, D. (1992) *Cell Biology Labfax*, BIOS Scientific Publishers, Oxford
- 27 Dahl, H. H., Hunt, S. M., Hutchison, W. M. and Brown, G. K. (1987) The human pyruvate dehydrogenase complex. Isolation of cDNA clones for the E1 α subunit, sequence analysis, and characterization of the mRNA. *J. Biol. Chem.* **262**, 7398–7403
- 28 Perlman, R. K. and Rosner, M. R. (1994) Identification of zinc ligands of the insulin-degrading enzyme. *J. Biol. Chem.* **269**, 33140–33145
- 29 Chesneau, V. and Rosner, M. R. (2000) Functional human insulin-degrading enzyme can be expressed in bacteria. *Protein Expr. Purif.* **19**, 91–98
- 30 Kozak, M. (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283–292
- 31 Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**, 1–6
- 32 Nakai, K. and Horton, P. (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* **24**, 34–36
- 33 Claros, M. G. and Vincens, P. (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur. J. Biochem.* **241**, 779–786
- 34 Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **300**, 1005–1016
- 35 Authier, F., Bergeron, J. J., Ou, W. J., Rachubinski, R. A., Posner, B. I. and Walton, P. A. (1995) Degradation of the cleaved leader peptide of thiolase by a peroxisomal proteinase. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3859–3863
- 36 Stahl, A., Moberg, P., Ytterberg, J., Panfilov, O., Brockenhuus Von Lowenhielm, H., Nilsson, F. and Glaser, E. (2002) Isolation and identification of a novel mitochondrial metalloprotease (PreP) that degrades targeting presequences in plants. *J. Biol. Chem.* **277**, 41931–41939
- 37 Bhushan, S., Lefebvre, B., Stahl, A., Wright, S. J., Bruce, B. D., Boutry, M. and Glaser, E. (2003) Dual targeting and function of a protease in mitochondria and chloroplasts. *EMBO Rep.* **4**, 1073–1078
- 38 Mukherjee, A., Song, E., Kihiko-Ehmann, M., Goodman, Jr, J. P., Pyrek, J. S., Estus, S. and Hersch, L. B. (2000) Insulysin hydrolyzes amyloid beta peptides to products that are neither neurotoxic nor deposit on amyloid plaques. *J. Neurosci.* **20**, 8745–8749
- 39 Leissring, M. A., Lu, A., Condrón, M. M., Teplow, D. B., Stein, R. L., Farris, W. and Selkoe, D. J. (2003) Kinetics of amyloid β -protein degradation determined by novel fluorescence- and fluorescence polarization-based assays. *J. Biol. Chem.* **278**, 37314–37320
- 40 Kato, A., Sugiura, N., Saruta, Y., Hosoiri, T., Yasue, H. and Hirose, S. (1997) Targeting of endopeptidase 24.16 to different subcellular compartments by alternative promoter usage. *J. Biol. Chem.* **272**, 15313–15322
- 41 Sass, E., Blachinsky, E., Karniely, S. and Pines, O. (2001) Mitochondrial and cytosolic isoforms of yeast fumarate are derivatives of a single translation product and have identical amino termini. *J. Biol. Chem.* **276**, 46111–46117
- 42 Hospital, V., Nishi, E., Klagsbrun, M., Cohen, P., Seidah, N. G. and Prat, A. (2002) The metalloendopeptidase nardilysin (NRDc) is potently inhibited by heparin-binding epidermal growth factor-like growth factor (HB-EGF). *Biochem. J.* **367**, 229–238
- 43 Chesneau, V., Pierotti, A. R., Prat, A., Gaudoux, F., Foulon, T. and Cohen, P. (1994) N-arginine dibasic convertase (NRD convertase): a newcomer to the family of processing endopeptidases. An overview. *Biochimie* **76**, 234–240
- 44 Miura, S., Amaya, Y. and Mori, M. (1986) A metalloprotease involved in the processing of mitochondrial precursor proteins. *Biochem. Biophys. Res. Commun.* **134**, 1151–1159
- 45 Xia, D., Yu, C. A., Kim, H., Xia, J. Z., Kachurin, A. M., Zhang, L., Yu, L. and Deisenhofer, J. (1997) Crystal structure of the cytochrome *bc*₁ complex from bovine heart mitochondria. *Science* **277**, 60–66
- 46 Hamel, F. G., Bennett, R. G. and Duckworth, W. C. (1998) Regulation of multicatalytic enzyme activity by insulin and the insulin-degrading enzyme. *Endocrinology* **139**, 4061–4066
- 47 Kupfer, S. R., Wilson, E. M. and French, F. S. (1994) Androgen and glucocorticoid receptors interact with insulin degrading enzyme. *J. Biol. Chem.* **269**, 20622–20628
- 48 Adames, N., Blundell, K., Ashby, M. N. and Boone, C. (1995) Role of yeast insulin-degrading enzyme homologs in pheromone processing and bud site selection. *Science* **270**, 464–467
- 49 Horwich, A. L., Kalousek, F. and Rosenberg, L. E. (1985) Arginine in the leader peptide is required for both import and proteolytic cleavage of a mitochondrial precursor. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4930–4933
- 50 Moreira, P. I., Santos, M. S., Moreno, A. M., Seica, R. and Oliveira, C. R. (2003) Increased vulnerability of brain mitochondria in diabetic (Goto-Kakizaki) rats with aging and amyloid- β exposure. *Diabetes* **52**, 1449–1456
- 51 Maechler, P. (2002) Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic β -cells. *Cell Mol. Life Sci.* **59**, 1803–1818
- 52 Petersen, K. F., Dufour, S., Befroy, D., Garcia, R. and Shulman, G. I. (2004) Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N. Engl. J. Med.* **350**, 664–671
- 53 Jordan, J., Cena, V. and Prehn, J. H. (2003) Mitochondrial control of neuron death and its role in neurodegenerative disorders. *J. Physiol. Biochem.* **59**, 129–141

Received 24 June 2004/16 July 2004; accepted 30 July 2004

Published as BJ Immediate Publication 30 July 2004, DOI 10.1042/BJ20041081