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Binding of amyloid β-peptide to mitochondrial hydroxyacyl-CoA dehydrogenase (ERAB): regulation of an SDR enzyme activity with implications for apoptosis in Alzheimer's disease

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Abstract The intracellular amyloid β-peptide (Aβ) binding protein, ERAB, a member of the short-chain dehydrogenase/reductase (SDR) family, is known to mediate apoptosis in different cell lines and to be a class II hydroxyacyl-CoA dehydrogenase. The Aβ peptide inhibits the enzymatic reaction in a mixed type fashion with a K_i of 1.2 μmol/l and a K_{iES} of 0.3 μmol/l, using 3-hydroxybutyryl-CoA. The peptide region necessary for inhibition comprises residues 12–24 of Aβ1–40, covering the 16–20 fragment, which is the minimum sequence for the blockade of Aβ polymerization, but that minimal fragment is not sufficient for more than marginal inhibition. The localization of ERAB to the endoplasmic reticulum and mitochondria suggests a complex interaction with components of the programmed cell death machinery. The interaction of Aβ with ERAB further links oxidoreductase activity with both apoptosis and amyloid toxicity.

Key words: ERAB; Short-chain dehydrogenases/reductase; Hydroxysteroid dehydrogenase; Alzheimer's disease; Amyloid β-peptide; Apoptosis

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting cholinergic pathways in the brain. The hallmarks of AD are extracellular senile plaques in the brain parenchyma, amyloid depositions in the blood vessels and intracellular neurofibrillary tangles [1]. The major constituent in plaques and amyloid depositions is the aggregated amyloid β-peptide (Aβ), a proteolytic product of the amyloid precursor protein (APP), whereas neurofibrillary tangles are mainly composed of hyperphosphorylated tau protein, derived from microtubules [2]. APP is found in all human cells and increasing evidence points to Aβ as a central effector in the pathogenesis of AD [3]. Underlying principles discussed in relation to Aβ-mediated neuronal toxicity include, besides direct cytotoxicity, activation of proinflammatory responses of the complement system [4], production of reactive oxygen species [5,6], increase of intracellular response to excitatory amino acids, and disruption of calcium homeostasis [7].

An intracellular protein, identified with a two-hybrid assay and termed ERAB, binds Aβ [8,9]. Upon binding Aβ, ERAB mediates neurotoxicity and apoptosis in neuronal cell lines [8]. This protein has been identified as an enzyme and member of the short-chain dehydrogenase/reductase (SDR) [10] superfamily, a protein family with currently over 1100 known structures [11]. Conserved sequence elements of this family include a nucleotide cofactor binding site and an active center with a Ser/Lys/Tyr triad [10]. ERAB has $>30\%$ sequence identity to its closest family members, prokaryotic 3-oxoacyl carrier protein reductases and hydroxysteroid dehydrogenases.

The aim of the present study was to investigate the interaction between Aβ and its intracellular target ERAB and to study the effect of the Aβ peptide on ERAB activity in the search for functional links between oxidoreductive actions and disease.

2. Materials and methods

2.1. ERAB cloning and expression of recombinant protein.

A human cDNA encoding ERAB was prepared with sequence-specific primers by RT-PCR from poly(A)$^+$ RNA (micro RNA prep, Pharmacia) of a human liver sample obtained from a transplantation donor. The resulting DNA was cloned into the expression plasmid pET15b (Novagen) resulting in a recombinant protein with an N-terminal poly-His tag and an integrated thrombin cleavage site, thus enabling rapid purification by metal-chelate chromatography. The construct was verified by DNA sequencing. After transformation into *Escherichia coli* strain BL21, recombinant protein was expressed by induction with 1 mM isopropylthiogalactoside. Cells were harvested 2 h after induction, lysed by sonication, and subjected to Ni^{2+}-chelate chromatography (His-bind resin, Novagen). After washing, pure recombinant ERAB protein was eluted with 1 M imidazole, 150 mM NaCl in 20 mM Tris-HCl, pH 7.5. Immediately after purification, the protein was subjected to a buffer change by gel filtration (PD 10, Pharmacia), and dithiothreitol was added to a final concentration of 10 mM to prevent precipitation. Protein concentrations during purifications were determined by the Lowry method, or in the case of the pure protein by hydrolysis with 6 M HCl and amino acid analysis with a ninhydrin-based instrument (Alpha Plus; Pharmacia-LKB).

2.2. Subcellular fractionation.

Subcellular fractionation was performed by density gradient centrifugation [12]. The liver homogenate (10%, w/v) was prepared with a Potter-Elvehjem homogenizer containing 0.25 M sucrose, 0.01 M HEPES (pH 7.5), 0.1% ethanol and 1 mM dithiothreitol. The homogenate (4°C) was centrifuged at $2000\times g$ for 15 min. The post-nuclear supernatant was then subjected either to centrifugation at $35\,000\times g$ for 45 min to obtain a mitochondrial fraction, or to sucrose gradient

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Abbreviations: Aβ, amyloid β-peptide; SDR, short-chain dehydrogenases/reductases

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centrifugation to obtain mitochondria, peroxisome- and ER-enriched fractions. In the latter procedure, the post-nuclear supernatant was layered on top of a pre-chilled 10–40% sucrose gradient and 65% sucrose as a cushion, and subsequently subjected to centrifugation at 100 000 × *g* for 16 h. Finally, fractions were collected from the bottom of the tube. Organelle-specific markers included catalase (peroxisomes), succinate dehydrogenase, cytochrome *c* (mitochondria), glucose 6-phosphatase and 11β-hydroxysteroid dehydrogenase type 1 (endoplasmic reticulum, ER), detected by enzymatic analysis and Western blotting.

2.3. Enzyme assays.

Analysis of enzyme activities was performed spectrophotometrically by recording the change of absorbance of NAD^+ at 340 nm, using a molar extinction coefficient of 6.22 M^{-1} cm^{-1} for NADH. One unit of enzyme activity was defined as 1 μmol product formed per min at 25°C. Dehydrogenase reactions were assayed in 50 mM Tris-HCl, pH 9.0, in the presence of 250 μM NAD^+, and reductase activities were assayed in 50 mM Tris-HCl, pH 7.0, with 250 μM NADH. Kinetic analysis was carried out with varying amounts of substrate (1–160 μM), and including 3-hydroxybutyryl-CoA, acetoacetyl-CoA (Sigma) and the steroids (Sigma) testosterone (3-oxo-reductase, 17β-HSD activities), isoursodeoxycholic acid (3β-HSD activity), cortisone (11-oxo-reductase activity), cortisol (11β- and 20β-HSD activities) and androsterone (3α-HSD activity). Recombinant 3β/17β-hydroxysteroid dehydrogenase from *Comamonas testosteroni* was produced and purified as described [13]. Type I hydroxyacyl-CoA dehydrogenase from pig heart was purchased from Sigma. Inhibition experiments with Aβ and its fragments were performed by preincubation of enzyme aliquots with different peptide concentrations for 30 min at 4°C. Kinetic data were collected and constants calculated using the Enzpack and FigP for Windows software packages (Biosoft). Synthetic human amyloid β-peptide 1–40 and fragments thereof were synthesized by Fmoc chemistry with reagents obtained from Sigma. Synthesized peptides were purified by RP-HPLC using an acetonitrile gradient in 0.1% aqueous trifluoroacetic acid, stored lyophilized at −20°C until use, and then redissolved in 20 mM Tris-HCl, pH 8.0. These peptide solutions were stable and did not aggregate for at least 24 h. For all inhibition experiments, only freshly prepared peptide solutions were utilized.

2.4. Western blots

Protein samples (about 20 μg per lane) were analyzed by SDS-PAGE (12% or 10–20% polyacrylamide). After transfer to nitrocellulose and blocking of unspecific binding sites with 5% dry milk in 1×TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), the blots were incubated with primary antibodies for 2 h, followed by washing in 1×TBST and incubation with secondary antibodies for 2 h. After washing, visualization was achieved by chemoluminescence (ECL, Amersham). Primary antibodies employed were polyclonal anti-ERAB, obtained from egg yolk by immunization of a chicken with 100 μg purified ERAB (secondary antibody: HRP-conjugated goat anti-chicken IgG; Sigma), polyclonal anti-11β-HSD-1 (mouse, secondary antibody goat-HRP-conjugated anti-rabbit IgG; Amersham) and monoclonal anti-cytochrome *c* antibodies (PharmIngen; secondary detection: HRP-conjugated protein A; Bio-Rad).

3. Results

Multiple sequence alignments revealed significant similarities between ERAB, bacterial hydroxysteroid dehydrogenases (about 40% identity) and vertebrate 3-hydroxyacyl-CoA dehydrogenases (about 80–90% identity) (Fig. 1), in agreement with other SDR assignments [8,9,14]. Sequence identity within

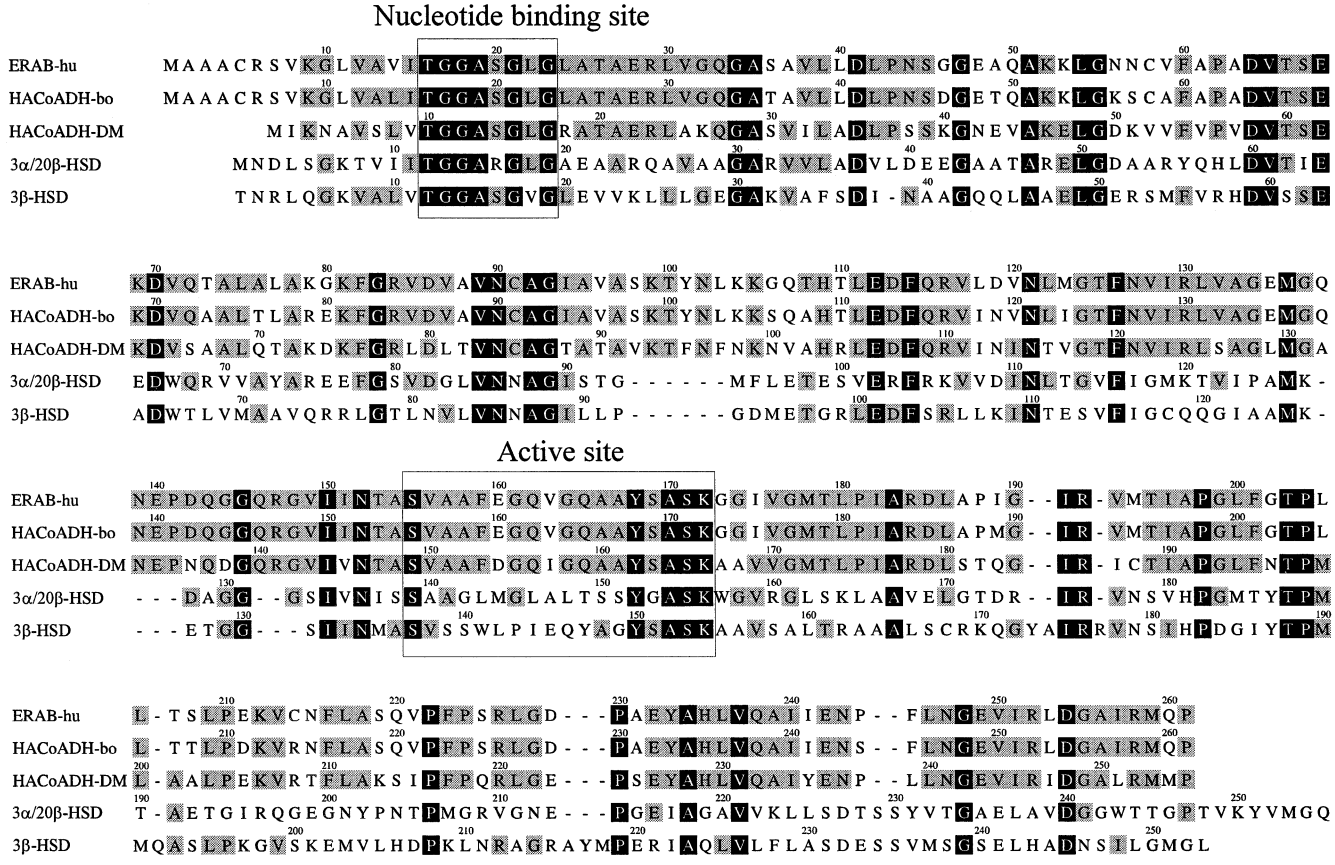

Fig. 1. Multiple sequence alignment of SDR enzymes, showing the similarity between type II hydroxyacyl CoA dehydrogenases (human, ERAB-hu; bovine, HACoADH-bo; and *Drosophila melanogaster*, HACoADH-DM), bacterial 3α/20β hydroxysteroid dehydrogenase (3α/20β-HSD from *Streptomyces hydrogenans*), and 3β/17β-hydroxysteroid dehydrogenase (3β-HSD from *Comamonas testosteroni*). Identities between all sequences are highlighted by black boxes, those between minimally three of the five sequences by gray boxes. Framed segments indicate the active site and nucleotide binding motifs common to SDR enzymes. The figure was created using the program PRALIN.

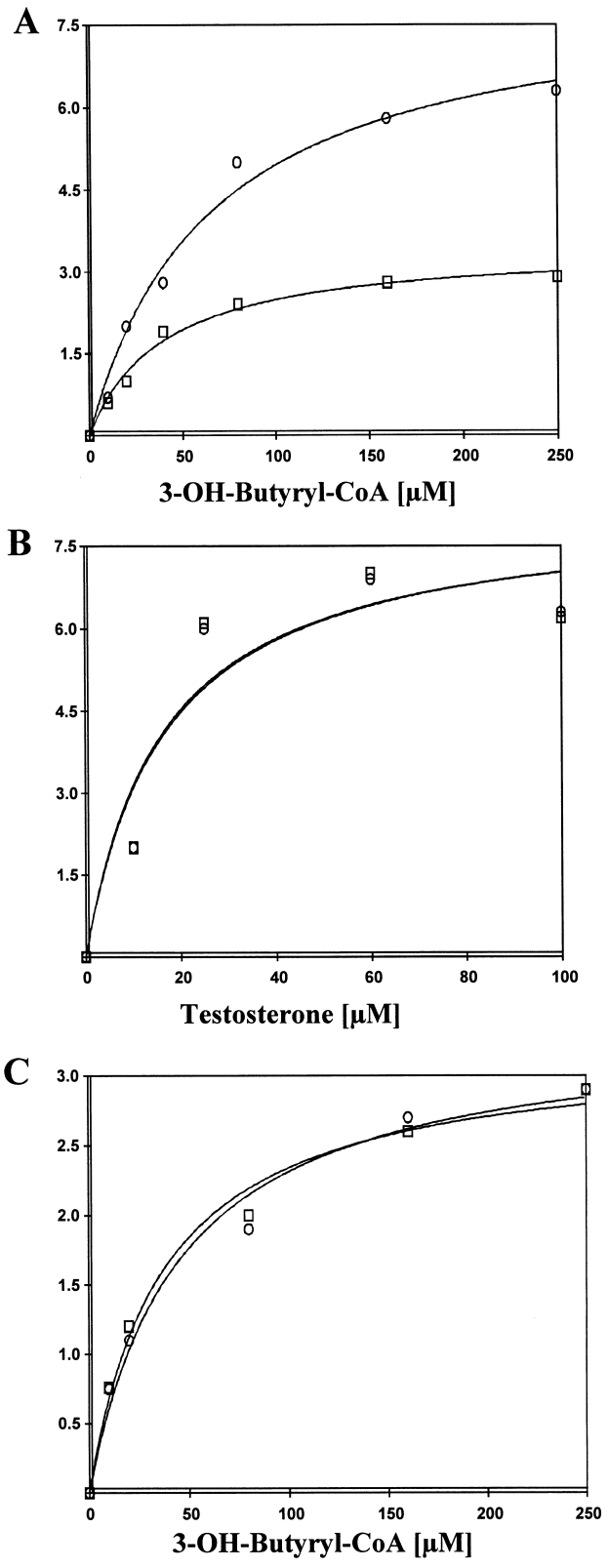

Fig. 2. Inhibition of enzyme activity by Aβ, showing the specificity for ERAB (3-OH-acyl-CoA dehydrogenase). The enzymes (ERAB, panel A, substrate 3-OH-butyryl-CoA; 3β/17β-HSD, panel B, 17β-dehydrogenase, substrate testosterone; and type I hydroxyacyl-CoA dehydrogenase from pig heart mitochondria, panel C, substrate 3-OH-butyryl-CoA) were preincubated in the absence (open circles) or presence (open squares) of 0.5 μM Aβ1–40 and then assayed for dehydrogenase activities. Strong inhibition of ERAB activity is observed but no inhibition of the other enzymes is detected.

the SDR family is otherwise 15–25%, and ERAB therefore displays a surprisingly large positional identity to the bacterial proteins, showing a clear evolutionary relationship. The similarities to bovine and *Drosophila* type II hydroxyacyl-CoA dehydrogenases [14] suggest that ERAB is the human orthologue of these enzymes. We therefore performed a substrate screening of the recombinant ERAB protein, including 3-OH-acyl-CoAs, 3-ketoacyl-CoAs and steroids with hydroxy and keto functions at positions C3, C11, C17 and C20, as given in Section 2. Whereas no steroid dehydrogenase activities could be detected with these substrates, recombinant human ERAB catalyzed the reduction and oxidation of acetoacetyl-CoA and 3-OH-butyryl-CoA (K_m 22.7 μmol/l, 1.1 U/mg; K_m 65.7 μmol/l, 9.8 U/mg, respectively). Thus, in agreement with other studies reported during the course of this work [9,15], it is fully established that ERAB is the human orthologue of the type II hydroxyacyl-CoA dehydrogenase, as predicted [14].

Next, we investigated the effect of Aβ binding on the enzymatic activity of ERAB. Preincubation of ERAB in the presence of Aβ1–40 and subsequent enzymatic assays revealed inhibition of the ERAB 3-hydroxyacyl-CoA dehydrogenase activity (Fig. 2A) in agreement with data from another study [9]. Kinetic analysis indicated a mixed-type inhibition, with a K_i of 1.2 μmol/l and a K_{iES} of 0.3 μmol/l using 3-hydroxybutyryl-CoA as substrate. The specificity for 3-hydroxyacyl-CoA dehydrogenase was evaluated by performing the same sets of experiments with the structurally related bacterial 3β/17β-HSD (Fig. 1) and the type I hydroxyacyl-CoA dehydrogenase from pig heart mitochondria. No inhibition of the reactions catalyzed by these enzymes could be observed with Aβ1–40 (Fig. 2B,C), indicating that Aβ interacts specifically with ERAB.

We studied the effects on ERAB activity by fragments of Aβ in order to define the Aβ region that interacts with ERAB (Fig. 3). Compared to full-length Aβ1–40 peptide, the region necessary for inhibition of ERAB activity can be restricted to the sequence 12–24, displaying 84% of the inhibition of the full-length Aβ1–40 peptide under the conditions employed. This segment is close to the minimal aggregate forming region 13–22 [16] of Aβ, and includes fragment 16–20 (KLVFF; Fig. 3), which is able to block polymerization [16], but that shorter segment itself gives only marginal inhibition.

Subcellular fractionation of human liver samples using density gradient centrifugation was carried out to investigate the intracellular distribution of ERAB (Fig. 4). Using different markers for organelle specificity, ERAB could be detected by Western blot analysis and enzymatic activity measurements to reside in enriched mitochondrial and ER fractions, a finding which is in accordance with immunofluorescence data [8], which describe ERAB as localized mainly to the ER. The absence of ERAB-positive immunological signals in peroxisomal fractions but a simultaneous presence of 3-hydroxyacyl-CoA dehydrogenase activity in those fractions gives evidence for the existence of enzymes structurally unrelated to ERAB in peroxisomes.

4. Discussion

The fact that intracellular binding of Aβ to the ERAB protein leads to apoptotic cell death has added a new dimension of β-amyloid-mediated effects in Alzheimer's disease [8]. In the present study, we aimed at defining the subcellular

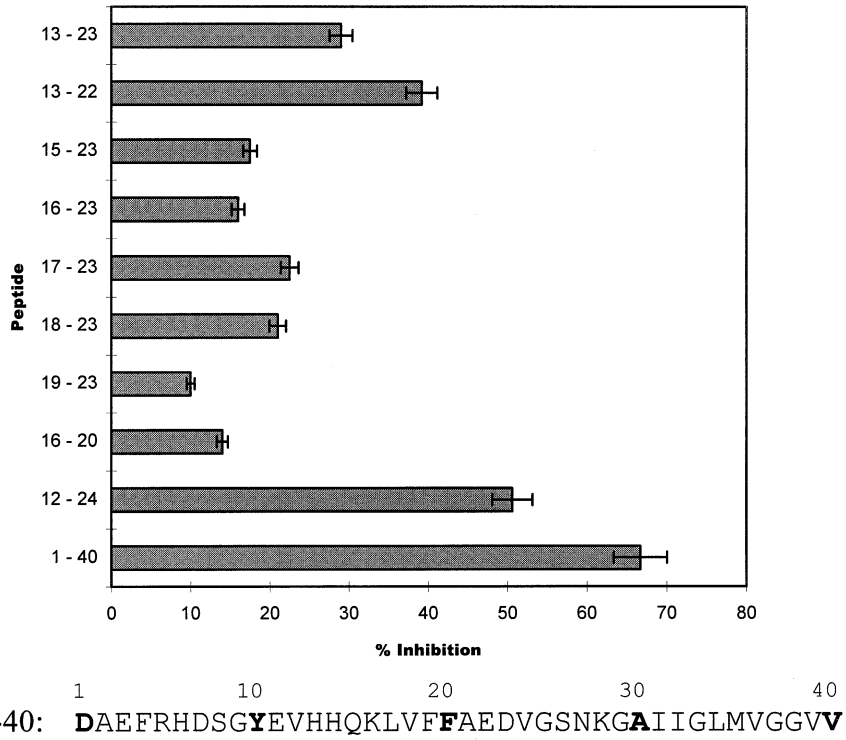

Fig. 3. Inhibition of ERAB hydroxyacyl-CoA dehydrogenase activity by different Aβ fragments. Inhibition of enzymatic activity was measured in the presence of 0.5 μM peptide at substrate concentrations of 160 μM 3-OH-butyryl-CoA and 250 μM NAD^+. Values are given as percent inhibition (number of experiments 3–7) compared to the uninhibited control reaction (no peptide).

distribution of ERAB, and the nature of the Aβ/ERAB interaction. From initial alignments (Fig. 1) and previous data [8,14] we realized that ERAB was a SDR enzyme homologue to vertebrate type II hydroxyacyl-CoA dehydrogenases. During the course of our substrate screening study, this was confirmed, and independently two reports recently suggested ERAB to be identical to hydroxyacyl-CoA dehydrogenase [9,15]. We evaluated possible effects of Aβ binding on the hydroxyacyl-CoA dehydrogenase activity, and demonstrated an ER and mitochondrial distribution of ERAB, inhibition of ERAB enzyme activity by Aβ, and a suggestion how these events may be connected to the pathophysiology of neurodegenerative disorders and apoptotic processes.

Previous work has defined the segment at positions 16–20 of the Aβ-peptide as the area essential for aggregation and hence for fibril formation [16]. We now find that inhibition of ERAB activity is optimal with the sequence 12–24 of Aβ, i.e. an area covering the fibril-forming region 13–22, and overlapping the 16–20 segment, which iarea alone is insufficient for full inhibition but blocks fibril formation. It is intriguing that Aβ toxicity (apoptosis or fibril formation) is critically dependent on largely the same structural segment as the one inhibiting the enzyme activity of ERAB. This interaction appears to be specific for ERAB and Aβ, as demonstrated by the lack of enzyme inhibition with the structurally or functionally related enzymes 3β/17β-HSD and type I hydroxyacyl-CoA dehydrogenase. From inspection of 3D structures homologous to ERAB, the ER binding region cannot be deduced, whereas the mitochondrial ERAB localization can be attributed to the N-terminal sequence, possibly representing a non-cleavable import signal [14]. Recent data reveal a wider substrate specificity for ERAB than just fatty acid CoA derivatives, including aliphatic alcohols and estrogens [9]. In contrast, our data and those of He et al. [15], did not reveal 17β-hydroxysteroid dehydrogenase activity with other 17β-hydroxysteroids and at present no clear mechanism linking Aβ-ERAB interactions and apoptotic processes has been defined. Although the ERAB alcohol dehydrogenase activities could contribute di-

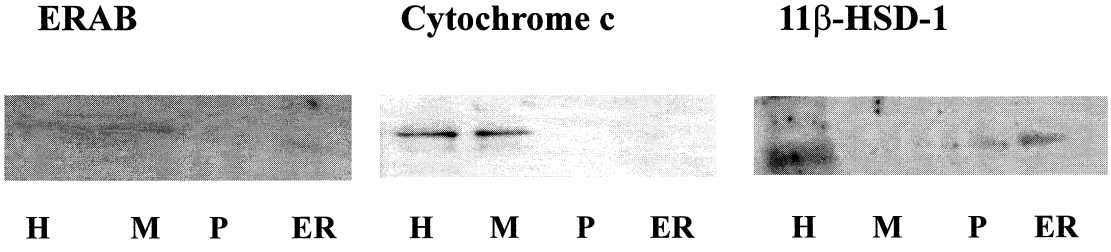

Fig. 4. Subcellular localization of ERAB to mitochondria and ER in human liver. Human liver homogenate was separated by gradient centrifugation, and fractions were analyzed for ERAB and organelle-specific markers by immunological assays. Western blots using anti-ERAB, anti-human cytochrome *c* and anti-11β-hydroxysteroid dehydrogenase antibodies, with total homogenate (H), enriched mitochondrial (M), peroxisomal (P), and ER fractions.

rectly to the lipid peroxidation products [9] this appears unlikely since there are other, more efficient cellular alcohol dehydrogenases. However, binding of Aβ to ERAB, occurring at levels reflecting intracellular Aβ concentrations [9], is not sufficient to promote cytotoxicity and cell death. Rather, functional ERAB appears to be required for these effects [9] and in vitro inhibition relationships might not reflect the in vivo situation.

Nevertheless, mediation of apoptosis by ERAB and Aβ adds a new possible route to cell death, emphasizing the role of mitochondria and oxidoreductases in this process [17,18]. Another possible, hypothetical scenario, supported by the localization of ERAB to ER and mitochondria, includes interruption of mitochondrial homeostasis or of energy supply subsequently leading to the formation of reactive oxygen species and release of apoptosis inducing factor and cytochrome *c*. The cytotoxic activity of these molecules highlights the importance of the mitochondrion in programmed cell death [19–24]. Since a similar segment of Aβ seems relevant for two different postulated mechanisms of Aβ toxicity, i.e. for fibril formation and for ERAB-mediated apoptosis, pharmacological inhibition of one process may also affect the other.

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