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A 24-residue presequence localizes human factor B to mitochondria ☆

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Abstract

We reported previously that the human factor B precursor is a 215-amino acid polypeptide, the first 40 amino acid residues of which function as a mitochondrial targeting presequence [G.I. Belogrudov, Y. Hatefi, J. Biol. Chem. 277 (2002) 6097–6103]. Confocal microscopy of live HEK293 cells, transiently transfected with factor B constructs tagged at the C-terminus with green fluorescent protein (GFP) revealed that either a 40- or 25-residue presequence localized factor B to mitochondria. Indirect immunofluorescent labeling of fixed, permeabilized HEK293 cells that were transiently transfected with a construct lacking a presequence, showed diffuse, intracellular staining that was consistent with targeting of ectopically expressed factor B to cellular compartments distinct from the mitochondria. Mutants in which either Met^{-25} or both Met^{-25}/Met^{-24} residues of the presequence were deleted exhibited decreased or undetectable levels, respectively, of the GFP-tagged factor B. The factor B presequence alone was shown to target a reporter polypeptide GFP to mitochondria. Our studies, therefore, demonstrate that a 24-residue presequence is sufficient to localize factor B to mitochondria, and suggest that the human factor B precursor is a 199-amino acid polypeptide.

Keywords: Factor B; Presequence; Precursor; Mitochondria; Confocal microscopy; HEK293 cells

The well-being of a eukaryotic cell depends critically upon a steady supply of sufficient amounts of ATP, the essential part of which is produced by mitochondria through oxidative phosphorylation. In mitochondria, the transfer of substrate-derived electrons by the respiratory chain complexes of the inner mitochondrial membrane culminates in the reduction of dioxygen to water, and is coupled to a vectorial, transmembrane proton translocation, whereby a proton-motive force Δp across the membrane is generated [1]. The Δp, in turn, drives ATP synthesis by a rotory motor ATP synthase complex, multiple transport processes including the transport of ions, metabolites and proteins into the mitochondria, and ultimately plays a crucial role in the cellular decision to live or die.

In the early biochemical experiments concerned with resolution and reconstitution of the oxidative phosphorylation system, a polypeptide component of the inner mitochondrial membrane, referred to as factor B, was identified [2]. Removal of factor B from the membrane by sonicating bovine heart mitochondria at a pH of ~8.8 in the presence of 0.6 mM EDTA rendered a preparation of inside-out vesicles, AE-SMP[1], deficient in their ability to synthesize ATP [2]. Surprisingly, antibiotic oligomycin, a specific inhibitor of proton translocation through the membrane sector F_O of the F_OF_1-ATPase, was found to restore the capability of such "non-phosphorylating" particles to catalyze the partial reactions of oxidative phosphorylation [3,4]. The finding that recoupling of AE-SMP could be achieved either with oligomycin or with partially purified

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[1] *Abbreviations used:* AE-SMP, ammonia-EDTA-treated bovine heart submitochondrial particles depleted of factor B; FB, a regulatory component of the ATP synthase complex factor B; GFP, green fluorescent protein.

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factor B preparations provided early evidence for the existence of a common target for both substances within the inner mitochondrial membrane and contributed to the view that coupling factor B is a component of the F_OF_1-ATPase that plays a role in proton translocation through the membrane sector F_O [5].

We recently cloned and expressed human [6] and bovine [7,8] factor B polypeptides in *Escherichia coli*, and demonstrated that reconstituting bovine AE-SMP, essentially devoid of endogenous factor B, with either recombinant polypeptide, restored the Δp-dependent reactions to levels observed in coupled SMP [6,7,9]. In agreement with the previously published N-terminal sequence of a ~22 kDa species of bovine heart mitochondria factor B [10], the N-termini of the recombinant polypeptides commenced with a sequence comprised of Phe-Trp-Gly-Trp-Leu-Asn-Ala amino acids.

To elucidate the role of factor B in mitochondrial bioenergetics at the cellular level, we are analyzing the effects of modulating the factor B gene product expression level in cultured mammalian cells. As an initial step, we identified the optimal length of the human factor B mitochondrial targeting presequence. We reported previously that human factor B is synthesized as a 215-amino acid precursor, the first 40 amino acid residues of which function as a mitochondrial targeting presequence [6]. The experiments reported herein demonstrate that a 24-residue presequence is sufficient for targeting factor B to the organelle, and suggest that the human factor B precursor is a 199-amino acid polypeptide.

Materials and methods

Reagents

MitoTracker Red CMXRos, goat anti-rabbit or anti-mouse Alexa 488 Fluor conjugated secondary antibodies, Image-iT FX signal enhancer, ProLong Gold antifade reagent, Optifect transfecton reagent, calcium-, magnesium-free PBS, 0.05% trypsin, restriction enzymes were purchased from Invitrogen (Carlsbad, CA). The human embryonic kidney HEK293 cell line was obtained from the American Type Culture Collection (Manassas, VA). The plasmid pCI-neo was purchased from Promega (Madison, WI). The plasmid pAcGFP1-N1 and the monoclonal anti-green fluorescent protein (GFP) antibody were purchased from BD Biosciences (Mountain View, CA). QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Monoclonal anti-hemagglutinin (HA) epitope antiserum and L-glutamine-penicillin-streptomycin solution were purchased from Sigma–Aldrich (St. Louis, MO). The Arrest-in transfection reagent was purchased from Open Biosystems (Huntsville, AL). Anti-glyceraldehyde phosphate dehydrogenase (GAPDH) monoclonal antibody was purchased from Ambion, Inc. (Austin, TX). Anti-rabbit and anti-mouse peroxidase conjugated secondary antibodies were purchased from EMD Biosciences-Calbiochem (La Jolla, CA). Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and sodium pyruvate with or without L-glutamine was purchased from Mediatech, Inc. (Herndon, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Inc. (Lawrenceville, GA). Poly-D-lysine coated 35 mm glass bottom culture dishes were purchased from MatTek Corp. (Ashland, MA). SuperSignal West Pico Chemiluminescent substrate was purchased from Pierce (Rockford, IL). All oligonucleotide primers were purchased from Operon Biotechnologies, Inc. (Huntsville, AL).

Plasmid construction

An earlier described [6] plasmid, pDrive-hFB, which contains the human factor B precursor cDNA with a 5′ UTR including 340 nucleotides, was used throughout the present study as a template for the PCR amplification of human factor B cDNA. The cDNA encoding the human factor B precursor with a 40-amino acid presequence was PCR-amplified with the forward primer GB112, ccg*CTCGAG***ATG**TGCTGTGCGGTC TCTGAGCAGCG (the restriction site is in italics), and the reverse primer GB115, ccc*AAGCTT*CTTCAATTGTAATTTTAGTTCCAGAGAAG GC, lacking the stop codon. The PCR product was digested with *Xho*I and *Hin*dIII restriction enzymes and then ligated into the *Xho*I-, *Hin*dIII-cut pAcGFP1-N1 plasmid encoding the monomeric GFP. The cDNA encoding the human factor B precursor with a 25-amino acid presequence was PCR-amplified using the forward primer GB113, ccg*CTCGAG***AT GATG**CTATTTGGAAAAATTTCCCAGC, and the reverse primer GB115. To express the human factor B with a 25-amino acid presequence from the pAcGFP1-N1 plasmid without the GFP tag, a stop codon was introduced in the reverse primer GB114, ccc*AAGCTT***TTA**CTTCAATT GTAATTTTAGTTCCAGAGAAGGC. A cDNA encoding a putative splice variant of the human factor B comprising 96 amino acids (GenBank™ Accession No. U79253) was amplified with the forward primer GB113 and the reverse primer GB117, ccc*AAGCTT*CTGCAAATCAT CTGCATTTTCTATTAGC, using plasmid DNA prepared from I.M.A.G.E. clone 24431 as a template. The PCR product was digested with *Xho*I and *Hin*dIII restriction enzymes and then ligated into *Xho*I-, *Hin*dIII-cut pAcGFP1-N1 plasmid. To express the splice variant from the pACGFP1-N1 plasmid backbone without GFP, a stop codon was introduced in the reverse primer GB116, ccc*AAGCTT***TCA**CTGCAAATCAT CTGCATTTTCTATTAGC. The resulting plasmids were designated pAcGFP1-N1-FB, pAcGFP1-N1-Δ1-15FB, pAcGFP1-N1-Δ1-15FBstop, pAcGFP1-N1-Δ1-15FB_S and pAcGFP1-N1-Δ1-15FB_Sstop.

To subclone the cDNA encoding the human factor B precursor with a 25-amino acid presequence into the pCI-neo mammalian expression vector, a PCR was performed using the forward primer GB113 (described above), the reverse primer GB126, tgc*TCTAGA***TTA**CTTCAATTGTAA TTTTAGTTCCAGAGAAGGC, and the pDrive-hFB plasmid as a template. The PCR product was digested with *Xho*I and *Xba*I restriction enzymes and then ligated into *Xho*I-, *Xba*I-cut pCI-neo plasmid. A cDNA lacking the mitochondrial targeting sequence was amplified using the forward primer GB136, ccg*CTCGAG***ATGATG**TTCTGGGGCTGGTTGA ATGC, in which two consecutive codons encoding Met residues were introduced upstream of the codon encoding a Phe residue from which the mature human factor B commences, and the reverse primer GB126. A cDNA encoding the human factor B precursor with a 25-amino acid presequence and an HA epitope at the C-terminus was amplified using the forward primer GB113 and the reverse primer GB137, tgc*TCT AGA***TTA**AGCGTAATCTGGAACATCGTATGGGTACTTCAATTG TAATTTTAGTTC, in which the HA epitope Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala was placed immediately downstream of the C-terminal Lys^{175} of the human factor B, followed by a stop codon. The prepared plasmids were designated pCI-FB, pCI-Δ1-40FB, and pCI-FB-HA.

To prepare a plasmid harboring a 24-residue human factor B precursor fused alone to GFP, an *Apa*I GGGCCC restriction site was created within the nucleotide sequence encoding the amino acids Trp^2 and Gly^3 of the mature factor B, using a plasmid pAcGFP1-N1-Δ1-15FB. We inserted a CC nucleotide pair in the preexisting nucleotide sequence via the QuikChange method (Stratagene, La Jolla, CA), using the primer GB171, TGGTCATGTGACTCCAGATACTTCTGGG**CC**CTGGTTGAATGC AGTGTTTAATAAGG, and its reverse counterpart (the position of the inserted nucleotides is shown in bold). The resulting plasmid, which, in addition to a newly created restriction site, also contained an *Apa*I site in its MCS region, was digested with *Apa*I restriction enzyme, the ~4.7 kb plasmid fragment was gel-purified and subsequently re-ligated, yielding a plasmid designated pGB-GFP. The latter plasmid encodes a fusion polypeptide in which a 24-residue human factor B presequence, followed by an 8-amino acid linker, is fused in-frame to GFP cDNA. Among the 8 amino acids of the linker intervening between the factor B presequence and GFP,