# Mitochondrial β-Barrel Proteins, an Exclusive Club?

Mitochondria, chloroplasts, and Gram-negative bacteria have transporter proteins in their outer membranes called β-barrel proteins. The mechanism of integration of these proteins into the outer membrane of bacteria has been partly elucidated, including the identification of a C-terminal motif consisting of the terminal amino acid phenylalanine and additional hydrophobic residues close to the C terminus (Robert et al., 2006). However, little is known about the sequence characteristics that allow β-barrel proteins to integrate into the outer membrane of mitochondria. In their *Cell* paper published earlier this year, Kutik et al. (2008) identified a signal sequence, the β-signal, involved in the sorting of mitochondrial β-barrel proteins and also the receptor for the β-signal, the protein Sam35. The β-signal is the first sorting signal reported to be specific for mitochondrial β-barrel outer membrane proteins (MBOMPs).

The β-signal consists of the motif Po.G..Hy.Hy (Po, polar residue; G, glycine; Hy, large hydrophobic residue) and occurs near the end of the most C-terminal β-strand of MBOMPs. A potential β-signal appears in every known MBOMP, but there may not be as many undiscovered MBOMPs as once thought. Upon publication of the β-signal in the Kutik et al. (2008) paper, we attempted to investigate through statistical analysis of candidate MBOMPs if it could be refined. We were able to confirm that all known MBOMPs have potential β-signals and to propose a minor refinement of the motif pattern. Despite some effort, we were able to find only one potential new MBOMP candidate in a bioinformatics analysis using this new motif.

After grouping clear MBOMP homologs together, there are only five known families of MBOMPs (Neupert and Herrmann, 2007). Kutik et al. (2008) used mutational analysis (Tom40, Porin, Mdm10) and evolutionary conservation revealed by multiple sequence alignment (Tom40, Porin, Mdm10, Sam50) to demonstrate the β-signal in four of the five MBOMP families. The remaining known MBOMP family, Mmm2 and its orthologs, was not mentioned by Kutik et al. presumably because, unlike the other four MBOMP family members, the occurrence of the β-signal is far from the C terminus. However, secondary structure prediction by PSIPRED (Bryson et al., 2005) suggests that the position of the motif coincides with the most C-terminal β-strand (see Figure S1 available online). Not all secondary structure prediction programs agree, so our conclusions must remain tentative, but it does seem possible that Mmm2 contains a functional β-signal.

Although only five families of MBOMPs are known, multiple sequence alignment of their homologs reveals a set of 54 distinct octomers, which presumably can act as β-signals. Analysis of these octomers reveals that in 53 of 54 cases the residue following glycine is hydrophobic (leucine, 21; isoleucine, 12; valine, 8; phenylalanine, 4; alanine, 4; cysteine, 2; tryptophan, 1; methionine, 1), with the single exception being threonine found in a fungal porin (VDAC_NEUCR). On the other hand, the C-terminal sequence of the Mdm10 protein, an MBOMP in the fission yeast *Schizosaccharomyces pombe* (O13814_SCHPO; not included in the 54 octomers analyzed above), suggests that the polar residue in the β-signal may not be strictly necessary. The Mdm10 protein family has a conserved match to the β-signal near the C terminus, which Kutik et al. showed to be essential in *Saccharomyces cerevisiae*; but in the *S. pombe* homolog, the polar position is occupied by the nonpolar residue phenylalanine.

These observations prompt us to propose a slightly altered motif—Po.GHy.Hy.Hy—whose alternating hydrophobic residues probably reflect the dyad repeat structure of β-strands. This interpretation is supported by the recent NMR structure of the human porin VDAC-1 (Hiller et al., 2008), an MBOMP in which the β-signal occurs in the last of 19 β-strands. The residues constituting the β-signal form hydrogen bonds with the penultimate strand in antiparallel orientation and the first strand in parallel orientation, thus completing the barrel. This is in contrast to known bacterial outer membrane protein structures, which have an even number of strands connected exclusively in an antiparallel orientation (White, 2008).

The integral outer membrane proteins of Gram-negative bacteria, which represent 2%–3% of the bacterial proteome (Wimley, 2003), are thought to be only β-barrel proteins. From preliminary bioinformatic analyses, it was expected that the yeast proteome would contain more than 100 MBOMPs (Wimley, 2003). Yet, 5 years and many complete genome sequences later, we still only know of five MBOMP families. The new β-signal may represent an opportunity to search for new candidate MBOMPs, so we devised a protocol (see Table S1; http://seq.cbrc.jp/mitoBetaSignal/) using the β-signal motif, UniProt and Gene Ontology annotation, evolutionary conservation, and predicted secondary structures to identify promising MBOMP candidates. This protocol was necessary because we found that MBOMP predictors trained on bacterial sequences did not effectively separate known MBOMPs from non-MBOMP control proteins.

We started with over 9000 eukaryotic proteins annotated as mitochondrial proteins (including by similarity, etc.) in either the UniProt or Gene Ontology databases. Then, we applied an automated procedure that reduced the number to 60 by requiring the refined β-signal motif to match within 40 residues of the C terminus of each homolog cluster and finally to 12 by consideration of predicted secondary structure and available annotation. Of these 12 clusters, 11 were members of the known MBOMP families: porin, Tom40, Sam50, and Mdm10. The remaining cluster contained Sun (*S*im1, *U*th1, *N*ca3) family proteins with a dual localization in both the cell wall and the mitochondria (Velours et al., 2002). One of the proteins in the cluster, yeast Uth1p, is promising because it is a mitochondrial integral outer membrane protein (Velours et al., 2002). However several (but not all) similar Sun family proteins also match the β-signal in the same (after alignment) position as Uth1p, including yeast Sun4p, which is localized to the mitochondrial matrix (Velours et

al., 2002). This discrepancy may be due to differences in targeting signals in their N-terminal regions, which are completely different.

This analysis is not comprehensive. Some unknown proteins that were annotated as not having a mitochondrial localization may have been missed, and there may be classes of MBOMPs without β-signals or that, like Mmm2, have β-signals distal from the C terminus. Nevertheless the process we describe finds 80% of known MBOMPs but only identifies one new candidate out of 2133 homolog clusters. We propose that the β-signal is found in all known MBOMPs and that there may be few MBOMPs remaining to be discovered.

**Supplemental Data**

Supplemental Data include one figure and one table and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01578-X.

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## Response

# The Mitochondrial β-Signal and Protein Sorting

The outer membranes of mitochondria, chloroplasts, and Gram-negative bacteria contain abundant β-barrel proteins that are essential for the transport of proteins and metabolites. Identification of the mitochondrial sorting and assembly machinery (SAM complex) revealed a new protein import pathway and sparked interest in mitochondrial β-barrel biogenesis. A central SAM component, Sam50, is conserved from bacteria to humans and a related protein is also found in chloroplasts, implying a conserved mechanism of β-barrel sorting in eukaryotes and prokaryotes (Pfanner et al., 2004; Paschen et al., 2005; Dolezal et al., 2006; Bos et al., 2007). However, the other three SAM subunits have no homologs in bacteria or chloroplasts, and analysis of the bacterial β-signature sequence did not lead to the identification of a similar mitochondrial signal. In our *Cell* paper (Kutik et al., 2008), we identified the sorting signal of mitochondrial β-barrel proteins in yeast and unexpectedly found that Sam35 functions as a receptor for the β-signal (Kutik et al., 2008).

In their Correspondence, Imai et al. report a detailed bioinformatics analysis of mitochondrial β-barrel proteins using the information derived from the β-signal. They propose a refinement of the β-signal by inclusion of a further hydrophobic residue in the motif. We experimentally demonstrated that four conserved residues of the β-signal are critical for its function, yielding the motif Po.G..Hy.Hy (Po, large polar residue; G, glycine; Hy, large hydrophobic residue) (Kutik et al., 2008). The glycine residue is present in all known β-signals, whereas the other three residues are conserved in the vast majority of species. The amino acid following glycine is usually hydrophobic in most species, and so Imai et al. propose inclusion of this residue in a motif Po.GHy.Hy.Hy. However, at this position small hydrophobic residues like alanine are also found, and we experimentally demonstrated that replacement of the large hydrophobic residues in the C-terminal portion with alanine inhibits the function of the β-signal (Kutik et al., 2008). Thus the motif Po.GHy.Hy.Hy proposed by Imai et al. (Hy, all hydrophobic residues) is inaccurate. It will be critical to discriminate between large and small hydrophobic residues, that is, the motif could be Po.Ghy.Hy.Hy (hy, hydrophobic residue; Hy, large hydrophobic residue). A detailed experimental analysis as performed for the other residues will be needed to validate this refined motif.

Imai et al. note that the polar residue is not present in all β-signals as there is one example (Mdm10 in *Schizosaccharomyces pombe*) out of more than 50 β-signals with a hydrophobic residue at this position. This variation was already pointed out in our Kutik et al. (2008) paper. Given that the polar residue is highly conserved (>98% conserved in all known β-signals) and that we experimentally demonstrated its importance in *Saccharomyces cerevisiae*, we think this residue should remain in the β-motif. Imai et al. also propose that two more proteins, Mmm2 and Uth1, contain β-signals and should be included in our list of β-barrel proteins imported into mitochondria. These suggestions are valuable yet require experimental validation. So far we have not been able to obtain experimental evidence for the functional relevance of these putative β-signals and for import of Mmm2 or Uth1 via the SAM pathway.