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INVITED EDITORIAL
Mitochondrial Protein Transport—A System in Search of Mutations

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From the time nearly 20 years ago when it was first proposed that nuclear-encoded proteins were specifically transported into the mitochondrion through a combination of recognition elements on the protein precursors and translocation complexes in the mitochondrial membranes, it was anticipated by human geneticists that a variety of new targets for mutation in human disease would become apparent within this apparatus. Mutations affecting the recognition elements (now, leader sequences or leader peptides) might lead either to lack of targeting or aberrant targeting or might allow targeting but not translocation or proteolytic cleavage of the precursors. Mutations altering the translocation apparatus might affect the rate or extent of this process and might involve single precursors or many precursors pleiotropically, depending on the specificity of the transporters involved. It was hoped, in accord with the classic paradigm of human genetics, that uncovering such mutations in patients with inherited disease and studying their physiologic and biochemical sequelae would provide insights into the mechanisms of recognition, protein-protein interaction, translocation, and proteolysis involved in mitochondrial protein transport.

We have, on the whole, been sorely disappointed in these expectations. After years of searching in the cDNA of patients with defects in specific mitochondrial proteins and, with somewhat less diligence, in patients with undefined mitochondrial dysfunction, a legion of investigators have uncovered only a handful of mutations that lead to aberrant, inefficient, or absent mitochondrial localization. This issue of the *Journal* contains a report of such a mutation in ornithine aminotransferase (OAT) (Kobayashi et al. 1995), bringing the total to a mere *five* or so, depending on how one chooses to count. How can one rationalize this dearth of mutational effects, in terms of the present understanding of the mechanisms of recognition, translocation, and proteolysis? What insights, if any, can these observations provide into the means by which this complex system achieves its high level of specificity and efficiency?

There are two fundamentally distinct targets for mutation in the mitochondrial protein transport pathway: the translocation system itself and the leader peptides that interact with it. Although it was speculated initially that there might be a number of translocators with specificities for individual proteins or classes of proteins, subsequent data support the current hypothesis that a single apparatus is responsible for the recognition and transport of almost all cytoplasmically synthesized mitochondrial proteins, with a few exceptions. Our current understanding (for reviews, see Hartl et al. 1989; Pfanner and Neupert 1990; Kiebler et al. 1993; Pfanner et al. 1994; and Stuart et al. 1994) suggests that the process begins as newly synthesized precursor emerges from cytosolic polysomes and binds, as an unfolded protein, to cytosolic Hsp70 class chaperones, keeping it in an "import-competent" conformation. Simultaneously, a "presequence binding factor" may recognize and interact specifically with the leader peptide. These proteins deliver precursor through the cytosol to the mitochondrial translocation complex by an unknown mechanism. In the mitochondria, two apparently independent subcomplexes, one in the outer membrane and one in the inner, take part. The outer membrane has two proteins that recognize a leader peptide (or other targeting signals, in those cases in which they are not amino-terminal), accept the precursor from its delivery complex, and transfer it to the outer-membrane translocation complex, which consists of at least six proteins. The leader peptide is inserted through the outer membrane via a proteinaceous pore and interacts with the inner-membrane complex, so far known to contain four proteins, and is inserted through the inner membrane in an energy-dependent step. Once exposed to the mitochondrial matrix, the emerging peptide contacts mitochondrial Hsp70 (mtHsp70), and, by an ATP-hydrolysis–driven

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cycle of interactions between the peptide, mtHsp70, and one of the components of the inner-membrane complex, most or all of the precursor is translocated into the matrix. Concomitant with or subsequent to this step, the leader peptide is removed by the general matrix peptidase, known as "MPP," and the protein is delivered to the chaperonin Hsp60 for folding and to its submitochondrial location for assembly into active enzyme or complex.

A number of these components, including some of the proteins of the outer- and inner-membrane complexes, mtHsp70, Hsp60, and MPP, have been shown to be essential in yeast under all growth conditions. If we assume that higher eukaryotes, including man, have similar requirements for intact mitochondrial metabolic function for normal growth and development, it is perhaps not surprising that no human disease attributable to mutations in these genes has been uncovered. Unlike mutations in mtDNA, which lead to deficient ATP synthesis and energy metabolism and which largely affect the function of individual tissues with large or specialized energy requirements (e.g., muscle or certain nerves), mutations in the translocation-apparatus genes would likely produce effects in all cells and organ systems, from the earliest stages of development, leading to early embryonic lethality. It remains possible that partial deficiencies in this pathway might be compatible with development to term and survival, but it is difficult to predict the phenotype of such individuals. Surely it would include mitochondrial myopathy and encephalopathy, along with a wide range of metabolic disturbances in fatty-acid metabolism, amino acid catabolism, and ammonia detoxification, to name only a few of the pathways with obligate mitochondrial steps. The developmental sequelae of such pleiotropic metabolic defects are unknown. So far, no patients fitting this profile have been described.

Individual leader-peptide sequences might have been expected to provide a more fertile source of disease-causing mutations, resulting in reduced or absent activity of single enzymes and avoiding the potentially lethal pleiotropy of translocation-apparatus mutations. Leader peptides range from <15 to >80 amino acids in length, show little homology to each other, and contain no consensus linear amino acid sequence that forms the targeting signal. Careful examination has revealed certain general features: a positive overall charge (i.e., no aspartates or glutamates), a high content of other hydrophilic amino acids, and a predicted tendency to form amphiphilic α-helices, particularly at the amino-terminus (Gavel and von Heijne 1990). Likewise, a consensus proteolytic cleavage sequence is not readily apparent. Surveys (Hendrick et al. 1989; von Heijne et al. 1989; Gavel and von Heijne 1990) indicate that many (~70%) but not all sites cleaved by MPP have an arginine residue two or three amino acids amino-terminal to the cleaved bond. This relaxed specificity is further complicated by the finding that a number of precursors are cleaved a second time by an independent protease (MIP) (Hartl et al. 1986; Sztul et al. 1987; Kalousek et al. 1992).

Moreover, in vitro mutagenesis of a few leader peptides has revealed a substantial plasticity in these sequences. Extensive studies of the precursor of ornithine transcarbamylase (OTC) (Horwich et al. 1985, 1986, 1987; Kraus et al. 1988) have indicated that most single amino acid substitutions have little or no effect on either transport into mitochondria or cleavage by the leader peptidases; even partial deletions have been tolerated to some degree. Multiple substitutions reducing the overall positive charge of the leader have been shown to be effective in disrupting these processes, as has one specific amino acid substitution, R23G, that changed the consensus arginine two residues before the MPP cleavage site. Other, less radical changes at this site were only partially disruptive, however. Transfection of some of these constructs into HeLa cells (Isaya et al. 1988) revealed that the multiple-substitution mutants were not transported, whereas R23G was transported but not processed, consistent with an effect on its recognition or cleavage by MPP. Mutational analyses in a number of other precursors have yielded similar results, namely that individual substitutions have little effect on the rate or efficiency of transport and processing. Furthermore, it has been shown (Isaya et al. 1991, 1992) that both MPP and MIP are sensitive to the structural context on both sides of their cleavage sites, extending into the mature protein sequence. These data, along with the structural predictions and lack of consensus sequences, suggest that secondary structure, along with positive charge, largely determines both mitochondrial targeting and leader-peptide removal. A corollary of this hypothesis is that single amino acid substitutions in leader peptides should largely be silent in their effect on the mitochondrial localization—and, hence, activity—of the mature passenger proteins, because most secondary-structural elements are probably sufficiently robust to withstand the majority of changes in their individual amino acid residues.

How do the mutations in leader peptides described so far fit into this picture? One, in the leader sequence of methylmalonyl-CoA mutase, is a nonsense mutation (Ledley et al. 1990); translation reinitiates at an in-frame Met codon within the mature protein sequence, leading to a truncated protein with no leader peptide, which, as would be predicted, remains in the cytosol and is degraded (Fenton et al. 1987). Another, R26Q in the OTC leader (Grompe et al. 1989), recapitulates a site-directed mutation studied in vitro by Horwich et al. (1986),

where it had little effect. In this patient, however, the mutation is in the last base of the first exon and results in the reduction of OTC mRNA levels to <5%, so it is impossible to judge the effect of the amino acid substitution on translocation and processing. A splicing error in the isovaleryl-CoA dehydrogenase gene results in deletion of the second exon, removing 30 amino acids beginning at mature residue 20 (Vockley et al. 1992). Even though the leader peptide and cleavage site are intact, the smaller precursor accumulates in the cytosol of fibroblasts in culture. Experiments in vitro suggest that mitochondrial recognition and binding are reduced, whereas proteolysis by purified MPP is normal. Finally, another mutation in the mature sequence of OAT, H319Y, has been reported to produce a protein that is transported into mitochondria but that is not cleaved (Inana et al. 1989).

Q90E, the mutation reported in this issue of the *Journal* (Kobayashi et al. 1995), is also in the mature sequence of OAT and apparently results in the complete absence of translocation, at least in the patient's cells. Whether the partial processing observed in insect cells overexpressing the mutant protein reflects partial mitochondrial transport and processing or is an artifact of the heterologous system remains to be established. The nature of this mutation is most similar to that of both the other OAT defect above and the isovaleryl-CoA dehydrogenase deletion—each is in its respective mature protein sequence but still leads to inefficient or absent translocation or processing. The only way to rationalize this observation is to invoke the apparent dependence of both targeting and proteolysis on structural elements that may extend beyond the immediate region of the leader peptide. How a single amino acid change disrupts the formation or recognition of such an element(s) remains unclear. The authors have proposed two possibilities: (1) a specific aberrant interaction between the introduced negative side-chain and a positive side-chain in the leader peptide and (2), as a result of the increased negative charge of the mature sequence, a disruption of the recognition of newly synthesized precursor by cytosolic import factors such as Hsp70 or presequence binding factor. Another possibility might be disturbance in the interaction between the translocating protein and mtHsp70. It has been proposed that ATP-driven cycles of binding and release among the translocating polypeptide, mtHsp70, and one of the components (MIM44) of the inner-membrane–translocation apparatus are responsible for moving a protein into the mitochondrion (Schneider et al. 1994). Disrupting this process could lead to partial translocation and even to partial processing, followed by release of the precursor back into the cytosol (Kang et al. 1990; Ungermann et al. 1994). Determining which of these mechanisms (or any others) is operating here may provide additional insights into the details of both the specificity and flexibility of the mitochondrial protein-transport system.

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