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A functional test of Neandertal and modern human mitochondrial targeting sequences

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ABSTRACT

Targeting of nuclear-encoded proteins to different organelles, such as mitochondria, is a process that can result in the redeployment of proteins to new intracellular destinations during evolution. With the sequencing of the Neandertal genome, it has become possible to identify amino acid substitutions that occurred on the modern human lineage since its separation from the Neandertal lineage. Here we analyze the function of two substitutions in mitochondrial targeting sequences that occurred and rose to high frequency recently during recent human evolution. The ancestral and modern versions of the two targeting sequences do not differ in the efficiency with which they direct a protein to the mitochondria, an observation compatible with the neutral theory of molecular evolution.

1. Introduction

Most mitochondrial proteins in humans are encoded in the nuclear genome [1,2]. The immature forms of these proteins are synthesized on cytosolic ribosomes and contain N-terminal mitochondrial targeting sequences that initiate binding to the outer mitochondrial membrane and translocation of the unfolded polypeptide through mitochondrial membranes. After translocation, the targeting sequence is cleaved off and degraded [2]. Targeting sequences for mitochondrial matrix proteins differ in the efficiency with which they direct mitochondrial import, and some proteins are localized in both mitochondria and cytosol, a situation that is mediated in part by their targeting sequence: the cleavage rate of the targeting sequence can determine the equilibrium between forward translocation into the mitochondrial matrix and backward translocation into the cytosol [3,4].

Changes in the subcellular localization of proteins may be a mechanism by which proteins obtain new functions during evolution [5]. One such case is glutamate dehydrogenase, where one isoform acquired mitochondrial localization in the common ancestor of humans and apes 18–25 million years ago, and subsequently diverged functionally from the cytosolic form [4]. However, it is not clear how frequently such evolutionary relocalizations occur.

With the determination of a draft Neandertal genome sequence [6,7], it has become possible to identify amino acid substitutions that occurred on the human lineage either after or shortly before its separation from the Neandertal lineage. Analysis of a data set where nucleotide positions encoding amino acids that changed on the human evolutionary lineage after its separation from the chimpanzee lineage were isolated and sequenced in a ~43,000-year-old Neandertal individual [7] revealed two mitochondrial targeting sequences where the Neandertal carried an ancestral amino acid residue shared with chimpanzees, while the modern human reference genome encodes another, derived variant. We decided to test if one or both of these substitutions had a functional effect.

2. Materials and methods

Coding differences between the Neandertal genome and the modern human reference sequence (from NCBI HomoloGene, build 58) [7] were considered if the Neandertal carried the version shared with both chimpanzee and orangutan, and if it was located within a region tagged by UniProt as "mitochondrial transit peptide". Positions that were tagged as polymorphic in dbSNP (April 2009) were excluded. The modern and ancestral versions of the two mitochondrial targeting sequences (TK2 and CCDC90B) and a control (aconitase 2) were ordered as oligonucleotides from MWG Biosciences (Ebersberg, Germany) with overhanging ends for insertion into pEGFP-N1 (Mountain View, CA), using *Nhe*I and *Age*I sites. As the sequences were too long for one-step synthesis, they were ordered as two halves with complementary

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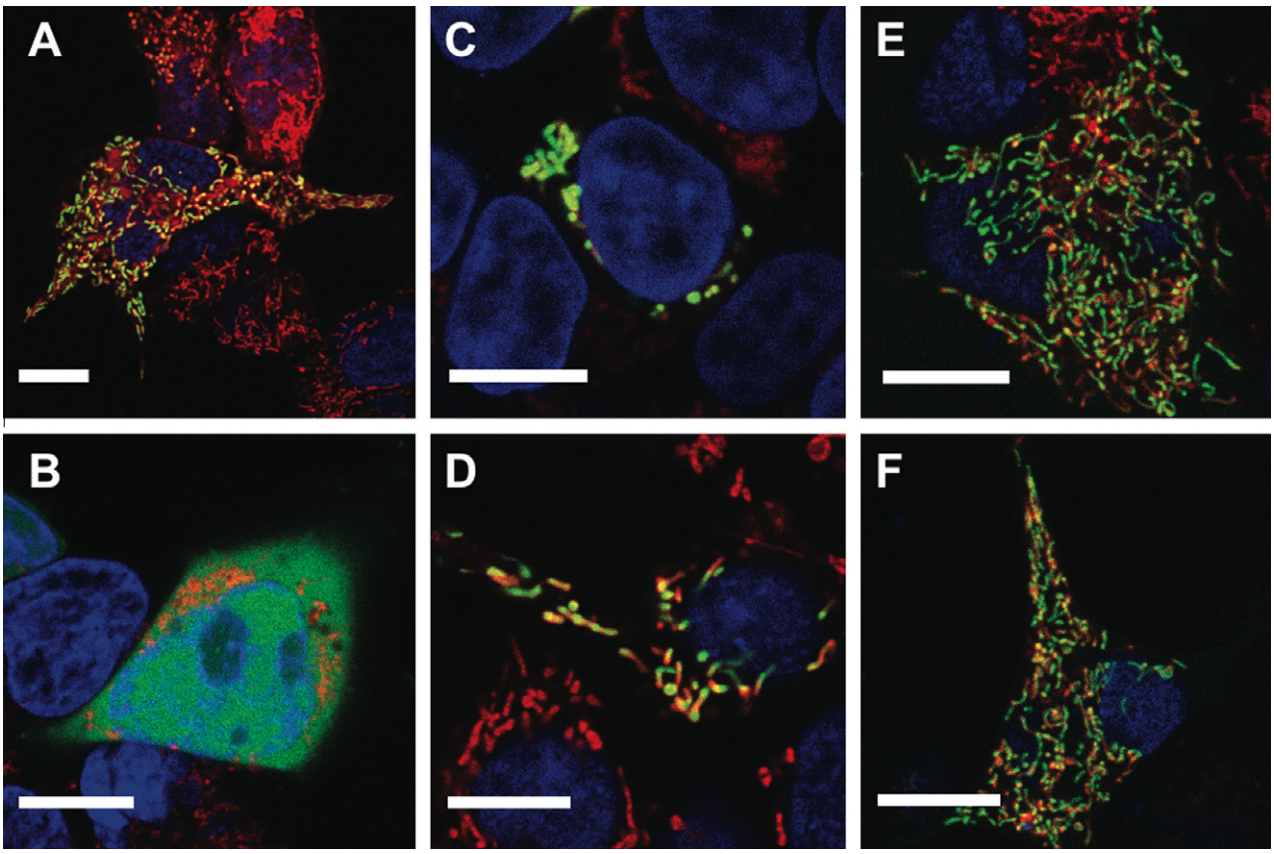

Fig. 1. Localization of constructs containing ancestral and modern human mitochondrial targeting sequences (MTS). (A) Fusing the strong MTS from aconitase 2 to GFP leads to an exclusively mitochondrial localization of GFP (green) in live HEK cells. All images were taken on a confocal microscope. Red: MitoTracker Deep Red 633. Blue: Hoechst 33342. (B) GFP without any MTS is cytosolic. (C and D) The MTS from modern (C) and ancestral (D) TK2 leads to strong mitochondrial localization of GFP, though cytosolic background is higher than in positive control (A). There is no difference between the ancestral and the modern versions of the MTS. (E and F) The MTS from modern (E) and ancestral (F) CCDC90B also leads to strong mitochondrial localization, though cytosolic background is higher than in positive control (A). There is no difference between the ancestral and the modern versions of the MTS. Scale bars: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

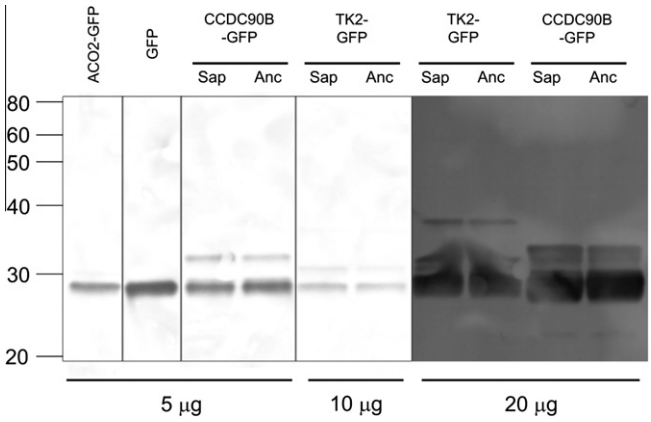

Fig. 2. Efficiency of mitochondrial maturation of GFP fused to ancestral and modern human mitochondrial targeting sequences. Lysates from HEK cells transfected with the indicated amounts of each construct were immunoblotted with anti-GFP antibody. *GFP* indicates no MTS at all. The mature versions of all other constructs are very similar to GFP, while immature versions have a higher molecular weight. *ACO2-GFP*: almost only mature protein. *CCDC90B-GFP*: considerable amounts of immature protein are present; the absolute amounts and ratio of immature to mature protein are the same for the modern *Homo sapiens* (Sap) and ancestral human (Anc) MTS using either 5 μg or 20 μg of DNA. *TK2-GFP*: two different immature proteins are present, due to two different start codons. Again, absolute amounts and ratios of immature to mature protein are the same for modern (Sap) and ancestral (Anc) human MTS using either 10 μg or 20 μg of DNA. Vertical lines indicate immunoblots performed on different days and from different lysates.

overlaps. After annealing the appropriate forward and backward sequences, the digested plasmid was ligated with the two halves in a three-point ligation reaction. All constructs were verified by sequencing of the entire open reading frames.

For confocal microscopy, HEK cells growing on 35 mm glass bottom culture dishes (MatTek, Ashland, MA) were transfected with the appropriate plasmid at 2.0 μg DNA and 6.0 μl TransIT-LT1 (Mirus, Madison, WI) per well. 18–24 h after transfection, 2 μM Hoechst 33342 and 100 nM MitoTracker Deep Red 633 (Invitrogen, Carlsbad, CA) were added directly to the medium and incubated at 37 °C for another 30 min. Cells were then washed in PBS and analyzed on an inverted confocal laser scanning microscope Leica TCS SP5 equipped with an HCX PL APO 63 1.4 oil immersion objective (Leica Microsystems, Wetzlar, Germany). All images were acquired in the sequential scan mode using laser lines at 405 nm, 488 nm or 633 nm wavelength for excitation.

For immunoblotting, HEK cells grown to near confluency in 60 mm dishes were transfected using 8.0 μg DNA and 20 μl Lipofectamine 2000 per dish. Medium was changed 4 h after transfection. 24–30 h after transfection, cells were detached and collected in 5 mM EDTA and lysed using ProteoJet (Fermentas, St. Leon, Germany). The indicated amounts of total protein were applied on Tris polacrylamide gels containing a gradient of 10.5–14% acrylamide (BioRad, München, Germany). After transfer to nitrocellulose membranes, GFP was detected using an anti-GFP antibody (mab3580, Chemicon, Nürnberg, Germany).

3. Results and discussion

We investigate two changes in mitochondrial targeting sequences that occurred recently during modern human evolution: First, an Ala → Pro substitution at position 33 of thymidine kinase 2 (*TK2*). The derived allele occurs at a frequency of 91% among 50 individuals from various populations of the world [7] and 84–99% in several East Asian populations (information for rs3743715 on dbSNP); second, a Leu → Phe substitution at position 10 in the protein "coiled-coil domain containing 90B" (*CCDC90B*). The derived allele occurs at a frequency of 52% among 50 individuals from various populations of the world [7] and at 29% in the populations represented on dbSNP (rs494791). These two modern/ancestral signal peptide pairs, together with an efficient control sequence derived from aconitase 2, were expressed in human HEK293 cells as N-terminal fusions to green fluorescent protein (GFP). Mitochondrial localization of the positive control was scored as colocalization with the organic dye MitoTracker Deep Red (Fig. 1A), while GFP alone showed an exclusively cytosolic localization (Fig. 1B).

All four constructs showed largely mitochondrial localization, though cytosolic background in the cytosol was higher than for the positive control (Fig. 1C–F). The localizations of the ancestral and modern versions of each protein construct were very similar. Overexpression of the very long targeting peptide of TK2 may have caused cellular stress leading to mitochondrial swelling (Fig. 1C–D). These observations were consistent across three different transfections.

In order to quantify the efficiency of the ancestral and modern targeting sequences, lysates of transfected cells were analyzed by electrophoresis and transferred to membranes that were then analyzed with an antibody to GFP (Fig. 2). While the positive control targeting sequence was almost completely cleaved, so that it was difficult to detect the immature protein band, the other four were less efficient, so that both the mature and immature forms (residing either in the mitochondria or the cytosol) could be detected. However, the ratio between mature and immature forms was, for both the *TK2* and the *CCD90B* constructs, conserved between ancestral and modern human forms. This was true at different transfection levels and in three repeated experiments.

The absence of detectable differences between the modern and ancestral reporter proteins in this cell line shows that these two mitochondrial targeting sequence mutations that occurred recently on the modern human lineage had no general functional effects.

Obviously, we cannot exclude that functional effects might exist in some cell type different from the cell line we have used. However, the high-coverage Neandertal mitochondrial genomes [8,9] and the draft version of the Neandertal nuclear genome [6,7] do not suggest any general changes in the protein translocation or folding environment on the modern human lineage that might affect mitochondrial import function.

4. Conclusion

Our results are compatible with the neutral theory of molecular evolution [10]. Although extrapolation from only two substitutions may be dangerous, our present results, together with a recent study on signal peptides directing translocation into the endoplasmatic reticulum [11], suggests that many or most amino acid substitutions that have recently become fixed or reached high frequency during human evolution may be without functional consequence. Thus, comprehensive testing of all such substitutions in the future, as functional tests become available, will allow the presumably small fraction that does influence function to be identified.

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