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Ectopic expression of the human adenine nucleotide translocase, isoform 3 (ANT-3). Characterization of ligand binding properties

A.K. Carroll*, W.R. Clevenger, T. Szabo, L.E. Ackermann, Y. Pei, S.S. Ghosh, S. Glasco, R. Nazarbaghi, R.E. Davis, C.M. Anderson

MitoKor, Inc., San Diego, CA 92121, USA

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Abstract

The adenine nucleotide translocase (ANT) is a key component in maintaining cellular energy homeostasis, and has also been implicated in formation of the mitochondrial permeability transition pore. Human ANT-3 was cloned from a human heart cDNA library and expressed as a histidine-tagged fusion protein in the mitochondria of the *Trichoplusia ni*. cell line. Over-expression resulted in a concomitant decrease in the endogenous ANT content, allowing for the characterization of binding of known ANT ligands to the human protein. Binding affinities for bongkrekic acid (BKA), ADP, and atractyloside (ATR) were measured in mitochondria from the human ANT-3 expressing cell line, and compared to similar preparations from bovine heart mitochondria by use of a novel radioiodinated derivative of ATR. Binding to ANT-3 by the high affinity inhibitors BKA and ATR, as well as the lower affinity natural ligand ADP, was similar to that measured in bovine heart mitochondria, and to that previously reported for mammalian heart mitochondria. Characterizations such as these of human ANT isoforms may lead to drug development for enhanced mitochondrial function and cellular viability.

Keywords: Adenine nucleotide translocase; Ligand; Bongkrekic acid; Atractyloside

1. Introduction

The adenine nucleotide translocase (ANT) is an integral membrane protein of the inner mitochondrial membrane where it plays a key role in maintaining cellular energy metabolism by acting as the sole means of exchanging cytosolic ADP for ATP synthesized in the mitochondrial matrix. The 1:1 molar exchange ratio of ADP for ATP across the inner mitochondrial membrane drives the rate of oxidative phosphorylation and is the most active transport system in aerobic cells (Lunardi and Attardi, 1991).

It is clear that disrupting ANT through the use of selective inhibitors (Obatomi and Bach, 1996; Isenberg and Klaunig, 2000), oxidative damage (Costantini et al., 2000), or by knocking out the protein entirely (Graham et al., 1997) leads to loss of mitochondrial function and damage at the cellular

* Corresponding author. Address: Department of Cell and Molecular Biology, Amylin Pharmaceuticals, Inc., 9360 Towne Center Drive, San Diego, CA, USA. Tel.: +1-858-458-8472; fax: +1-858-824-7583.

E-mail address: acarroll@amylin.com (A.K. Carroll).

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level. Such observations suggest that damage or dysfunction of the ANT in vivo may have pathological consequences. In fact, there is a significant body of evidence implicating the role of mitochondria and possibly the ANT in the apoptotic death process (Zoratti and Szabo, 1995; Zamzami et al., 1996; Susin et al., 1998). This death induction associated with the release of mitochondrial contents is reported to be due to the formation of a mitochondrial permeability transition (MPT) pore, wherein the ANT is implicated as a key component (Halestrap et al., 1997; Marzo et al., 1998; Jacotot et al., 2000; Belzacq et al., 2001). By understanding all the ANT functions in different cell types, we may be able to protect the ANT from insults that alter its activity and improve cell viability.

Regardless of its potential role in permeability transition pore formation, the primary function of the ANT is to transport adenine nucleotides to and from the mitochondria. The critical nature of maintaining ADP/ATP transport is reflected in the highly conserved nature of the protein across species, with $>80\%$ sequence homology between humans and *Drosophilia sp.* The protein is approximately 300 amino acids long, and spans the inner mitochondrial membrane six times with three repeat loops. Adenine nucleotides bind to ANT at two distinct high-affinity (50 nM) and low-affinity (5 μM) binding sites (Dupont et al., 1982). Transport occurs when the protein binds adenine nucleotides at the M2 site and undergoes a change in conformational state (Klingenberg, 1989). There are two described states of the ANT related to this conformational change, the so-called c- (cytosolic) and m- (matrix) conformations (Weidemann et al., 1970). If the ANT is locked into either conformation by an inhibitor, nucleotide translocation ceases. Two inhibitors of the ANT, atractyloside (ATR) and bongkrekic acid (BKA), bind to the ANT in a dimeric state and lock it in the c- or m-conformation, respectively. There is strong evidence that the high-affinity site for nucleotide binding is influenced by the binding of ATR or BKA (for review see Fiore et al., 1998), and may in fact be the site of inhibitor binding (Klingenberg, 1989; Muller et al., 1996; Majima et al., 1998). Because of their inhibitory effects by either direct or indirect interaction with the M2 loop, ATR and BKA have been useful tools to examine nucleotide binding and transport (Block and Vignais, 1984; Klingenberg et al., 1984; Huber et al., 1999).

One approach to elucidate the function of the ANT is to produce a functional, recombinant ANT protein, and to study its characteristics. To this end, we have initiated studies on the human ANT protein by defining the ligand binding characteristics of one of the three known human ANT isoforms. Although many attempts have been made to express mammalian ANT isoforms in bacterial (Miroux and Walker, 1996) and yeast (Giraud et al., 1998; Hashimoto et al., 1999) expression systems, it has proven difficult to produce a functional protein by classical techniques; thus characterization of the human protein has been limited.

Here we describe the ectopic expression of the human ANT-3 isoform. By expressing the protein in an insect cell line, we observed proper localization of ANT-3 to the mitochondrial inner membrane and have measured ligand binding using a novel ATR-derivative. The system described herein has lead to the first in-depth characterization of binding of known ligands to an intact human ANT protein.

2. Material and methods

2.1. Production of human ANT-3 construct

The coding region for human ANT-3 was amplified by PCR from human brain cDNA (BD Biosciences Clontech, Palo Alto, CA) using forward and reverse primers to the published sequence for human ANT isoform 3 (Genbank Accession: BC014775). Unique restriction sites were introduced to forward and reverse primers. The single amplicon PCR product of the appropriate size was ligated into the pBlueBacHis2 transfer vector 'B' version (Invitrogen, Carlsbad, CA). Exact DNA sequence of the insert was confirmed to be human ANT-3, as compared to the published sequence for the human ANT-3 isoform.

2.2. Expression and detection of ectopic ANT-3

Purified pBlueBacHis2/ANT-3 plasmid DNA was supplied to the Invitrogen Corporation (Carlsbad, CA), for production of high titer viral stocks using