Diss ETH No. 16215

Novel interaction partners of Creatine Kinase isoenzymes in the brain

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH

for the degree of

Doctor of Natural Science

Presented by

Tanja Simone Bürklen

Dipl.-Biologin, University of Konstanz, Germany

German citizen

accepted on recommendation of:

Prof. Dr. Theo Wallimann, examiner

Prof. Dr. Ulrike Kutay, co-examiner

Zürich, August 2005

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Acknowledgements

Here I take the opportunity to thank all the people who supported me during my PhD over the last four years. I appreciate that I would not be at this point in my life without the people that I will mention below.

First of all I would like to thank Prof. Theo Wallimann. From the first time we met he gave me the feeling that he respects the work that I am doing. He encouraged me in every situation with his positive and motivating attitude. With Theo as my PhD supervisor I always felt able to move as freely as a researcher ever wishes. He listened to me and helped me with private and scientific problems.

I would like to thank Prof. Ulrike Kutay for examining my thesis.

Thanks to the members of the Wallimann group.

With Nadine Straumann I had a great time in the lab. She was always there for me and helped in technical and private problems – thank you!

Many thanks go to Roland Türk who brought humour and music in our lab life. I thank Dr. Isabel Gerber for the nice and inspiring discussions about the fascination of the complexity of the human body. Many thanks to Dr. Marianne Suter, Dr. Malgorzarta Tokarska-Schlattner, Dr. Uwe Schlattner, Dr. Dietbert Neumann, Ramon Thali and Uwe Riek. I also would like to thank the former group members Dr. Kathryn Adcock and Dr. Oliver Speer for the inspiring scientific and private discussions and the culinary meetings after work. And Tina Leuenberger, Alexandra Wind and Dr. Thorsten Hornemann, thank you for the nice atmosphere in the lab.

Special thanks go to my friend Tina Thurnherr who was always there for me. She was always interested in the running of my projects and posed critical questions. She became one of my best friends during the last four years and I appreciate this friendship a lot.

Many thanks go to the IZB, where I enjoyed the nice and familiar atmosphere. Especially to Dr. Elisabeth Ehler for reading the manuscripts, Dr. Sonja Bonneick, Dr. Alain Hirschy, Dr. Stephan Lange and the Perriard group, Dr. Joao Relvas, Ursula Scheier, and the team of Rolf Huber.

Thanks to my former teachers in Konstanz: Prof. Werner Hofer, Prof. Dieter Brdiczka, Dr. Alexander Rück and Birgit Riede who taught me how to prepare rat heart mitochondria, performing coupled enzyme assays and pipetting accurately.

Special thanks go to Timo who is the most lovable man in the world. Especially during the hard times of the rehabilitation from my hip joint operation when he demonstrated unconditional love and support. He was always encouraging and full of respect for me and the work I was doing.

Thanks also to my friends that accompanied me during the last four years: Sabini Becker-Weimann, Holger Müller, Christa Henzler, Birgit Linkohr, Michi Kerschbaummaier.

And finally, I want to thank my family: thanks to my mother Regina who was always interested in the work and tried to understand the complexity of fundamental research. Thanks to Martin, my sister Steffi, my father Günther and Moni who always let me feel that they were proud of me. Particular thanks go to my grandpa Walter who showed me the most important thing in life: accepting love and fighting for it.

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1. Zusammenfassung

Gegenstand dieser Arbeit ist die Erforschung der zellulären Zusammenhänge zwischen der Kreatin Kinase (CK) und potentiellen Interaktionspartnern im Gehirn.

CK ist ein Schlüsselenzym, welches für die Bereitstellung und den Transport von Energie in eukaryotischen Geweben mit hohem und wechselndem Energiebedarf verantwortlich ist. CK ist in den Zellen isoenzym-spezifisch an Stellen der Energieproduktion und des Energieverbrauchs lokalisiert. Es gibt vier verschiedene Isoformen, welche alle die gleiche reversible Reaktion katalysieren, nämlich den Phosphotransfer von ATP auf das Substrat Kreatin, um die energiereiche Verbindung Phosphokreatin und ADP herzustellen. Die beiden zytosolischen Formen sind die „brain-type" CK (BCK) im Gehirn und anderen Geweben und die „muscle-type" CK (MCK) in Muskelgeweben. Zwei weitere Isoformen sind in den Mitochondrien lokalisiert, die ubiquitäre (uMtCK) und die sarkomerische CK (sMtCK).

Diese Arbeit ist in zwei große Themenbereiche unterteilt, wobei sich das erste mit der BCK und das zweite mit der uMtCK beschäftigt.

BCK spielt eine wesentliche Rolle beim Transport von Neurotransmittern in Neuronen und von Ionen in Astrozyten durch die Regenration von ATP. Es ist wenig bekannt über eine direkte Kopplung der BCK an Enzyme, die für die Gehirnfunktionen wichtig sind.

Ziel des Kapitels 1 ist daher die Identifizierung von Interaktionspartnern für die BCK im Gehirn. Dazu wurde mit Hilfe der „Yeast two-hybrid" (Y2H) Methode in einer humanen Gehirn cDNA Bibliothek nach potentiellen Interaktionspartnern von BCK gesucht.

Dabei wurde das Golgi Matrix Protein GM130 als Interaktionspartner identifiziert. Diese Interaktion wurde mit biochemischen, mikrobiologischen und zellbiologischen Experimenten bestätigt. Überdies konnte die Assoziation von BCK mit GM130 bei immunhistologischen Experimenten einer speziellen Phase des Zellzyklus zuordnen, der frühen Prophase der Mitose.

GM130 ist ein zytoplasmatisches Protein, welches fest an die Membranen des Golgi Apparates gebunden ist. Dort ist GM130 Teil eines größeren oligomeren Proteinkomplexes, der aus dem "Golgi reassembly stacking protein" GRASP65, p115 und

Giantin besteht. Die genaue Aufgabe dieses Komplexes ist noch unklar, wahrscheinlich ist jedoch eine stützende Wirkung auf die Struktur des Golgi Apparates. GM130 spielt eine Rolle bei der Golgifragmentierung während der Mitose. Dabei wird GM130 von der Cdc2 kinase phosphoryliert, was die Bindung zu p115 auflöst. Diese Phosphorylierung ist synchron mit der Umformung der Golgi Cristae in kleine Vesikel, was vermuten lässt, dass dies der erste Schritt zur Golgifragmentierung ist. Die Regulierung der Mitose und den damit zusammenhängenden Signalkaskaden benötigt Energie, welche durch die BCK Reaktion zur Verfügung gestellt werden könnte. Eine Beteiligung der BCK am Initialschritt zur Golgifragmentierung zu Beginn der Mitose scheint daher logisch. Die Identifizierung von GM130 als neuer Interaktionspartner von BCK ist daher höchst interessant und gibt einen neuen Aspekt der Energiebereitstellung durch das CK System für die Gewährleistung der lebenswichtigen Zellteilung.

Die Kapitel zwei und drei befassen sich mit der ubiquitären mitochondrialen CK (uMtCK). Mittels biochemischer Affinitätschromatographie konnte die Interaktion von uMtCK mit der Amyloid Precursor Proteinsuperfamilie (APSF) gezeigt werden.

Die APSF besteht aus drei hochkonservierten transmembranären Glykoproteinen, deren genaue Funktionen noch weitgehend unklar sind. Zur APSF gehören neben dem „Amyloid Precursor Protein" (APP) das „Amyloid Precursor-like Protein" 1 und 2 (APLP1, APLP2). Die APSF bestehen aus einem größeren extrazellulären und einem kleineren intrazellulären Bereich, welche durch eine Transmembrandomäne voneinander getrennt sind. Nur APP besitzt die Struktur für das A-beta Peptid (Aβ), welches die Hauptkomponente der senilen Plaques in der Alzheimer Demenz (AD) ist. Alle Proteine der APSF besitzen in ihrer intrazellulären C-terminalen Domäne eine kurze Sequenz, das NPXY Motiv. Für die biochemische Charakterisierung der Interaktion wurde für die APSF ein synthetisch hergestelltes Peptid verwendet, bestehend aus den 18 C-terminalen Aminosäuren von APLP1 inklusive des NPXY Motivs. Zusammen mit rekombinanter uMtCK konnten *in vitro* gezeigt werden, dass diese Interaktion direkt und von hoher Affinität ist (175 nM) ist. Obwohl sich die zytosolischen und die mitochondrialen CKs strukturell nur wenig unterscheiden, ist die Interaktion mit dem APLP1 Peptid isoform-spezifisch für uMtCK. Bei Co-Transfektion-Experimenten mit uMtCK und diversen Konstrukten der APSF, hauptsächlich jedoch mit dem C-Terminus des APP (C31), wurde das Erscheinen einer

hoch molekularen uMtCK Bande (HMW uMtCK, normal ca. 45 kDa) beobachtet. In der Literatur wird berichtet, dass CK sehr sensitiv mit reaktiven Substanzen, wie z.B. freien Radikalen, reagiert. Es ist daher möglich, dass CK durch eine oxidative Modifikation, ausgelöst durch die Co-Transfektion der APSF, ein erhöhtes molekulares Gewicht bekommt. Die Resultate der Oxyblot-Analyse konnten diese Annahme jedoch nicht bestätigen. Im Gegenteil scheint eine Überexpression von APLP1 eher eine schützende Wirkung auf oxidative Schädigung von zellulären Proteinen zu haben. Daher wurde die HMW uMtCK Bande durch Massenspektroskopie analysiert und als uMtCK inklusive mitochondrialer Importsequenz identifiziert. Außerdem war die Akkumulation der HMr uMtCK abhängig von der Menge des co-transfizierten C31. Um die Funktion der Interaktion von APSF und uMtCK zu untersuchen, wurde folgende Arbeitshypothese aufgestellt. Wenn man davon ausgeht, dass die Interaktion von APSF und uMtCK im Zytoplasma stattfindet, könnten die APSF Proteine entweder eine „abfangende" oder „begleitende" Funktion auf uMtCK haben. Dies wurde mit subzellulärer Fraktionierung von APLP1 überexprimierenden Zellkulturen untersucht. Im Falle der „abfangenden" Funktion sollte bei APLP1-Überexpression weniger uMtCK in die Mitochondrien gelangen, während bei der „begleitenden" Funktion eine Erhöhung der Proteinmenge in den Mitochondrien zu erwarten wäre. Die Überexpression von APLP1 hingegen zeigte keinen Einfluss auf die Gesamtproteinmenge in der ganzen Zelle und Lokalisierung der uMtCK in den Mitochondrien. Eine Co-Lokalisation von uMtCK und APLP1 wurde jedoch in der Mitochondrien Fraktion nachgewiesen. Interessanterweise wurde eine erhöhte enzymatische Aktivität der uMtCK in den Mitochondrien gemessen. Meine Hypothese konnte also nicht bestätigt werden und eine neue Interpretation der gefundenen Resultate wird in der folgenden Arbeit diskutiert.

2. Summary

The subjects of this dissertation are the cellular correlations between creatine kinase (CK) and its potential interaction partners in the brain.

CK is a key enzyme for providing and transporting energy in eukaryotic tissues that have a high and fluctuating energy turnover. CK is located, in an isoenzyme-specific manner, at subcellular sites of energy production and consumption. There are four different isoforms that all catalyze the same reversible reaction, namely the transfer of the γ-phosphoryl group of ATP to the substrate creatine (Cr) to build phosphocreatine (PCr) and ADP.

There are two cytosolic isoforms; the brain-type (BCK) in the brain and other tissues and the muscle-type CK (MCK) in muscle tissues. Two mitochondrial isoforms are located in the cristae and the intermembrane space of mitochondria; the ubiquitous mitochondrial (uMtCK) and the sarcomeric mitochondrial CK (sMtCK).

This work is divided into two main parts. The aim of chapter 1 in the first part was to identify and characterize novel interaction partners of cytosolic BCK in the brain.

By regenerating ATP, BCK plays an important role in the transport of neurotransmitters in neurons, and ions in astrocytes. Little is known about the direct coupling of BCK to enzymes that are important for brain functions. I therefore used the yeast two-hybrid method to screen a human brain cDNA library for potential interaction partners.

The Golgi Matrix Protein GM130 was identified as an interaction partner for BCK. This interaction was confirmed with biochemical, microbiological and cell biological experiments. Moreover, in our immunohistochemical experiments this interaction was associated to a certain phase of the cell cycle, namely the early prophase.

GM130 is a cytoplasmic protein tightly bound to the membranes of the Golgi apparatus. GM130 is part of a larger oligomeric complex consisting of the Golgi reassembly stacking protein 65 (GRASP65), p115 and giantin. The exact functions of this complex still remain unclear, however it is likely that it exerts a tethering function for the Golgi apparatus. GM130 plays a role in the fragmentation processes of the Golgi apparatus during mitosis. There, it is phosphorylated by the Cdc2 kinase and with that p115 can no longer bind to GM130. Phosphorylation of GM130 and dissociation of p115 are synchronized with the

conversion of Golgi cisternae into small vesicles which suggests that this is the initial step in Golgi fragmentation. It therefore seems logic that BCK participates at this initial step of Golgi fragmentation at the onset of mitosis. It is clear that there are many energy-requiring processes in signalling cascades that regulate mitosis where BCK could be involved in this energy provision. Thus, the results shown here are extremely important and reveal new aspects in the energy provision by the CK system involved in cell division.

The chapter 2 and 3 focus on the uMtCK. Using an affinity chromatography an interaction between uMtCK and the amyloid precursor super family (APSF) proteins was identified. The APSF is composed of three-highly conserved transmembrane glycoproteins; the amyloid precursor protein (APP) and the amyloid precursor-like proteins 1 and 2 (APLP1, APLP2) whose exact functions are still unclear. APSF proteins share a highly conserved large extracellular domain, a single membrane-spanning domain (TMR), and a smaller intracellular domain. APP is the precursor for the A beta peptide (Aβ), the main component of senile plaques found in brains of Alzheimer's Disease (AD) patients. The APSF proteins have the highly conserved NPXY motif sequence within their intracellular C-terminal domain in common.

For the biochemical characterization of the interaction, a synthetically processed peptide containing the NPXY motif surrounded of 18 amino acid residues corresponding to that of the APLP1 sequence was used. It was shown *in vitro* that the interaction between the peptide and a recombinant purified uMtCK is direct and of a high affinity (175 nM). Although the sequence homology between the cytosolic and the mitochondrial CK isoforms is high, the interaction between uMtCK and the APLP1 peptide is isoform specific and restricted to the uMtCK isoform. During the co-transfection experiments with uMtCK and diverse APSF constructs, mainly the truncated C-terminus of APP (C31), the appearance of a high molecular weight (HMW) band of uMtCK (normally about 45 kDa) was observed. It is reported in the literature that the CKs are highly sensitive to oxydative damage. The idea then was that co-transfection of uMtCK and the APSF leads to oxydative stress and modification of uMtCK in turn leads to a higher molecular weight. However, the results of oxyblot analysis revealed the opposite, namely decreased protein oxidation due to transfection. Then the HMW band of uMtCK was analyzed by mass spectrometry, which revealed the uMtCK sequence including the mitochondria import sequence. Further,

it was shown that the accumulation of the HMW band of uMtCK was dependent on the concentration of the amount of transfected C31. I proposed the hypothesis that if the interaction takes place in the cytoplasm the APSF proteins could act either as a scavenger or a chaperone for uMtCK. I analyzed this by subcellular fractionation experiments with APLP1 over-expressing HEK 293 cells. In the case of a scavenging function I expected less uMtCK in the mitochondria whereas in the case of a chaperone-like function more uMtCK in the mitochondria fraction. I did not observe any differences in the protein level in the whole cell lysates as well in the cytoplasm or the mitochondrial fraction. However, a co-localization of uMtCK and APLP1 in the mitochondria was observed. Interestingly, an increase of uMtCK activity in the mitochondria due to over-expression of APLP1 was detected. Thus, I could not verify my original hypothesis. However, the experimental results are discussed here within a different framework. The data suggest that the interaction between APLP1/C31 and uMtCK has beneficial effects on the cell by protecting them from oxidative stress and assuring uMtCK function in the mitochondria.

3. Introduction

3.1. Creatine Kinase

3.1.1. Cellular energy metabolism and creatine (Cr)

All the processes involved in growth and metabolism of cells require energy. In living cells, production, transport, conversion and utilization of energy are fundamental processes that are facilitated via metabolic pathways involving a large number of tightly regulated enzyme-catalyzed reactions. ATP is the universal energy currency for most of the energy-requiring processes in biological systems (Lehninger, 1982). However, even though cellular pools of ATP are rather small, no significant decrease in [ATP] is detected during cell activation (e.g., muscle contraction, brain stimulation, photo-transduction in retina or initiation of sperm motility, for review see (Mommaerts and Wallner, 1967). In all these tissues or cells with high and fluctuating energy requirements, ATP is continuously replenished from phospho-creatine (PCr) by the reaction of the creatine kinase (CK, EC 2.7.3.2) system. CK catalyzes the reversible transphosphorylation reaction between PCr and ADP, and belongs to the energy transmitting kinases because their reaction products are energy-rich phospho-compounds that can be used to regenerate ATP (Brdiczka and Wallimann, 1994).

Figure 1: CK reaction

Endogenous creatine (Cr) biosynthesis involves two sequential steps catalyzed by L-arginige:glycine amidinotransferase (AGAT) and S-adenosylmethionine:guanidinoacetate N-methyltransferase (GAMT) (Braissant et al., 2001).

Creatine is mainly synthesized in the liver and pancreas (Wyss and Kaddurah-Daouk, 2000), and continuously transported into the brain and muscles via Na+ - and Cl- - dependent creatine transporter (CRT). Cr transport runs against a creatine concentration gradient across the blood-brain barrier (Ohtsuki et al., 2002).

3.1.2. Creatine kinase (CK) isoenzymes

In vertebrates, at least four isoforms of CK are expressed in a tissue-specific manner: two cytosolic forms, the muscle-type (MCK) and brain-type (BCK) (Dawson et al., 1967), and two mitochondrial CKs (MtCK) (Wyss et al., 1990). The MtCK isoenzymes are further subdivided into an ubiquitous (uMtCK) and a sarcomeric (sMtCK) form, based on their tissue-specific expression and their relative isoelectric points (Haas and Strauss, 1990). In contrast to the cytosolic CK isoenzymes, which are always dimeric, uMtCK and sMtCK were found *in vivo* and *in vitro* to form predominantly octamers with an Mr of approximately 340 000 (Haas and Strauss, 1990; Wyss et al., 1990). Although MtCK is mostly isolated in its octameric state, a dynamic octamer/dimer equilibrium has been found *in vitro*, depending on various parameters like MtCK concentration, pH, and temperature (Schlattner et al., 1998). MtCK is localized in cristae and the intermembrane space of mitochondria (Wegmann et al., 1991).

In vivo, MCK and BCK subunits combine to give the three typical dimeric cytosolic MM, MB and BBCK isoenzymes with approximate Mr of 80 000 to 86 000 (Eppenberger et al., 1967). Whereas MCK is rather specific for differentiated sarcomeric muscle, BCK is found in brain and in a variety of other non-muscle tissues (Eppenberger et al., 1967).

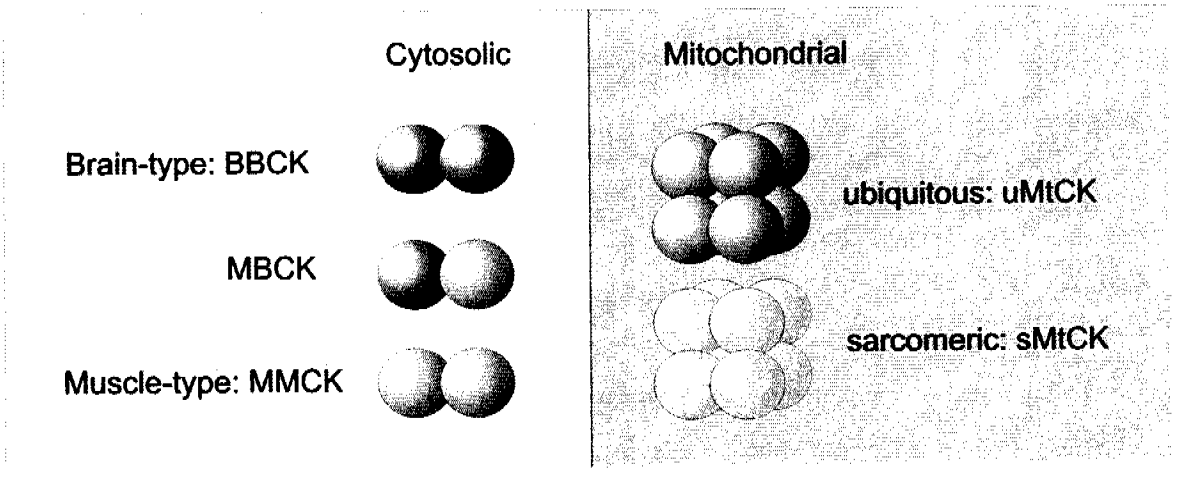

Figure 2: Creatine kinase isoenzyme structure

There are three cytosolic and two mitochondrial creatine kinase (CK) isoforms. Left panel shows the cytosolic homodimeric brain-type CK (BBCK), and muscle-type CK (MMCK) and the heterodimeric MBCK. This isoform is found only in the heart and is used as a sensitive diagnostical marker for heart

myocardial infarction (Hetland and Dickstein, 1996). The mitochondrial CK isoenzymes are active as homooctamers (Wallimann et al., 1989). Sarcomeric MtCK (sMtCK) is present in muscle whereas ubiquitous MtCK (uMtCK) is found in the mitochondria of non muscle tissues.

3.1.3. Subcellular compartmentation of the cytosolic CK isoenzymes

Most of the cytoplasmic CK isoenzymes that maintain the CK reaction at near equilibrium, are soluble in the cytoplasm (Matthews et al., 1982). However, significant amounts of CK are compartmentalized sub-cellularly with membranes or protein structures in a isoenzyme specific manner (Wallimann et al., 1983).

In skeletal muscle, cytosolic MCK is specifically bound to the myofibrillar M-line (Turner et al., 1973) where it is functionally coupled to the myofibrillar actin-activated Mg^{2+} - ATPase, acting as an efficient intramyofibrillar ATP regenerator (Ventura-Clapier et al., 1994; Wallimann et al., 1984). The ADP produced during muscle contraction is immediately rephosphorylated within this myofibrillar micro-compartment, resulting in a six-fold increase in the apparent affinity of the actomyosine ATPase for ATP (Wallimann et al., 1984). Additionally, some of the MCK was in skeletal muscle found to be associated with glycolytic enzymes within the myofibrillar I-band (Wallimann et al., 1989; Wegmann et al., 1992).

In the brain, BCK is the major cytosolic CK isoenzyme. In cerebellar cortex, higher levels of CK and PCr were measured in the molecular layer than in the white matter (Maker et al., 1973). However, Manos and co-workers found that oligodendrocytes contain nearly fourfold higher levels of CK activity than do neurons (Manos et al., 1991). BCK has been found in neurons associated with synaptic vesicles (Friedhoff and Lerner, 1977) and plasma membranes (Lim et al., 1983).

In adult retina, most of the CK (MtCK and BCK) is concentrated within the photoreceptor cell layer, as judged from immunofluorescence staining. In photoreceptors, BCK was found in the inner segments, as well as in the peripheral regions of the outer segments, partially in association with the plasma membrane of the outer segments (Wallimann et al., 1989; Wegmann et al., 1991).

High concentrations of BCK were also detected in smooth muscle and in epithelial cells of the human colon (Jockers-Wretou et al., 1977).

MtCK resides in the mitochondrial intermembrane space, where it is found along the entire inner membrane, including the cristae membranes, and at the peripheral 'contact sites' where inner and outer membranes are in close proximity (Kottke et al., 1991; Lim et al., 1983; Wegmann et al., 1991). Octameric MtCK is able to cross-link membranes at mitochondrial contact sites (Rojo et al., 1991). Human MtCK isoenzymes from heart and brain have been shown to differ in octamer stability and membrane binding (Schlattner and Wallimann, 2000). MtCK forms a functionally coupled micro-compartment with the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT), whereby an efficient 'energy transport channel' is formed (Brdiczka and Wallimann, 1994).

3.1.4. Functions of the CK isoenzymes and their coupling to signalling pathways

The main function of the CK/PCr system, is that of a 'temporal energy buffer' (Wallimann et al., 1992). Secondly, it serves as a 'spatial energy buffer' or 'transport system', and a third function of the CK/PCr system is to prevent a rise in intracellular [free ADP] (Wallimann et al., 1992). Further functions are proton buffering, providing appropriate local ATP:ADP ratios at sub-cellular sites where CK is functionally coupled to ATP consuming enzymes or processes (Wallimann et al., 1992). MtCK has 'privileged' access to ATP, produced in the mitochondrial matrix by oxidative phosphorylation, and is functionally coupled to proteins of the 'energy transport channel'. The ANT translocates ATP from oxidative phosphorylation through the inner membrane of mitochondria where it is directly transphosphorylated into PCr by MtCK (Saks et al., 1985), thereby producing ADP for an efficient stimulation of oxidative phosphorylation (Erickson-Viitanen et al., 1982; Gellerich et al., 1987; Saks et al., 1991). The stimulating effect of extra-mitochondrial Cr on the oxidative phosphorylation also suggests a functional coupling and a possible structural interaction with VDAC (Stachowiak et al., 1996). Such a tight functional coupling of CK to oxidative phosphorylation has been shown before (Kay et al., 2000). Furthermore, a direct interaction of MtCK and mitochondrial VDAC was demonstrated recently using surface plasmon resonance spectroscopy (Schlattner et al., 2001). In these latter studies the MtCK/VDAC interaction was shown to be Ca^{2+}-sensitive.

Figure 3 shows the CK/PCr energy transfer system, where the CK isoforms are

functionally and structurally linked to cellular sites of ATP production and consumption. MtCK metabolite channeling in mitochondrial micro-compartments (lower left): Octameric MtCK is found in two locations; (Gellerich et al., 1993) in the mitochondrial intermembrane space (IMS) where it associates with the adenine nucleotide translocator (ANT) and the voltage dependent anion channel (VDAC, or porin) in the so-called contact sites, and (Kottke et al., 1994) in the cristae associated with ANT only (not shown). In contact sites, octameric MtCK binds simultaneously to inner (IM) and outer mitochondrial membranes (OM), due to the identical top and bottom faces of the octamer. Binding partner in the IM is the twofold negatively charged cardiolipin, which allows a functional interaction with ANT that is situated in cardiolipin membrane patches. In the OM, MtCK interacts with other acidic phospholipids and, in a calcium-dependent manner, directly with the regulated pore-forming VDAC. This substrate channeling allows for a constant supply of substrates and removal of products at the active sites of MtCK. To prevent the dissipation of mitochondrially generated ATP into the cytosol, its energy content is directly transferred by MtCK to Cr, to give PCr, without loss of energy content. PCr, a metabolically inert compound, is then transported via VDAC into the cytosol, where it is available to CKs at the various locations for in situ regeneration of ATP. Vice versa, intramitochondrial regeneration of ADP stimulates oxidative phosphorylation (OX).

Micro-compartments of cytosolic MCK with the SR Ca^{2+}-ATPase pump (lower middle): A significant fraction of total cytosolic muscle MCK is tightly bound to the SR where it is functionally coupled to the Ca^{2+}-ATPase pump. ATP provided to the pump is preferentially delivered from PCr by bound CK and thereby lowering the apparent Km for ATP for the calcium pump. CK maintains a very high local ATP/ADP ratio in the vicinity of the energetically demanding Ca^{2+}-pump that operates close to thermodynamic equilibrium and thus, for efficient sequestration of Ca^{2+}, depends on a high local ATP/ADP ratio.

Micro-compartments of MCK in the sarcomeric M-band of the myofibrillar contractile apparatus (lower right): A further significant fraction of total cytosolic muscle MCK is tightly bound to the sarcomeric M-band of myofibrils, sufficient in activity to fully regenerate the ATP hydrolyzed by the acto-myosin ATPase at maximal contraction velocity. MCK is specifically anchored in an isoenzyme-specific manner at the M-band via

two 'lysine charge clamps' (Hornemann et al., 2003) that facilitate the binding of the enzyme to two structural proteins, M-protein, as well as to myomesin, at this sarcomeric location (from Saks et al. 2005, submitted).

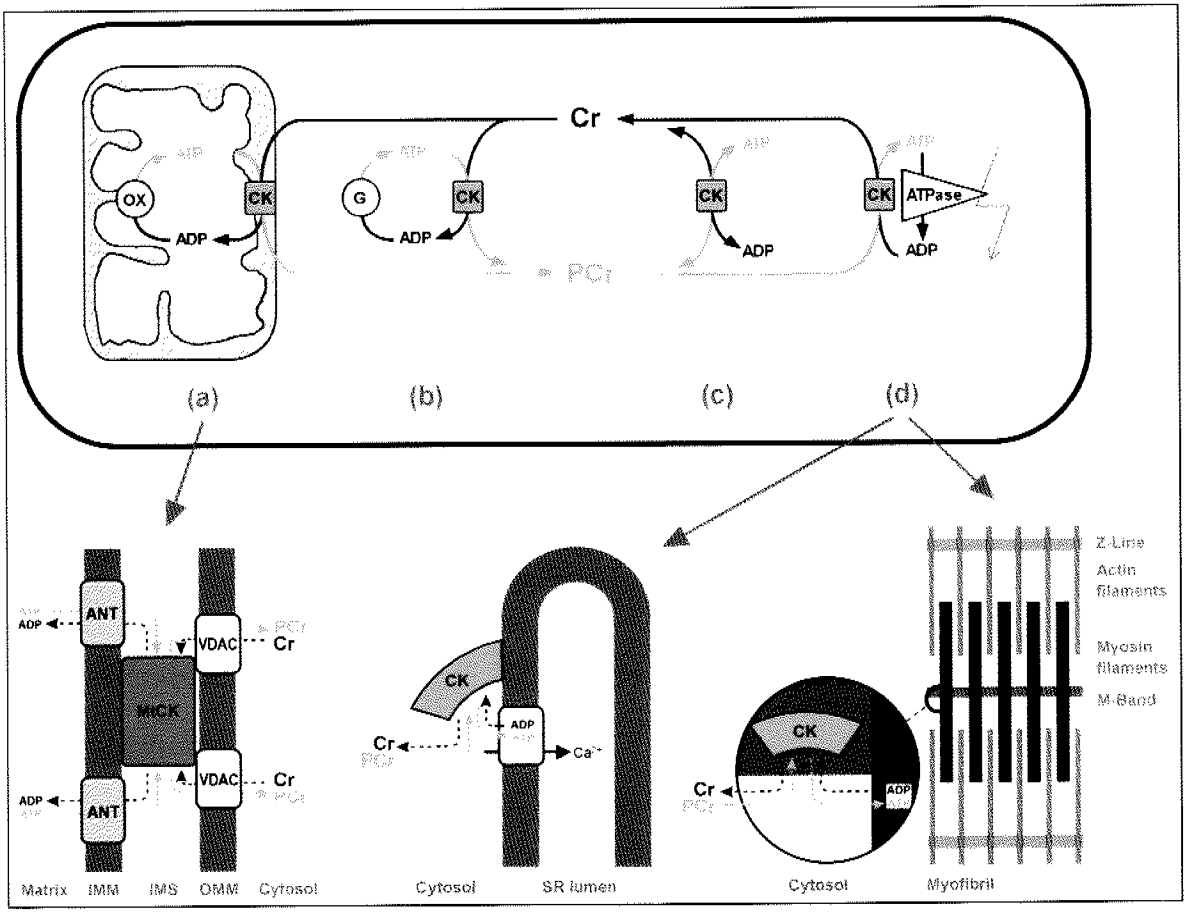

Figure 3: The creatine kinase (CK) / phosphocreatine (PCr) energy transfer system

Overview (top): Isoenzymes of CK are found in different compartments like mitochondria (a) and cytosol (b-d), in a soluble form (c) or associated to a different degree with ATP-delivering (a,b) or -consuming processes (d). A large cytosolic PCr pool up to 30 mM is built up by CK using ATP preferentially from oxidative phosphorylation (OX), e.g. in heart (a) or from glycolysis (G), e.g. in fast-twitch glycolytic muscle (b). PCr is then used to buffer global (c) and local (d) ATP/ADP ratios. In cells that are polarized and/or have very high or localized ATP consumption, these CK isoenzymes, together with easily diffusible PCr, also maintain an energy shuttle between ATP-providing or -consuming processes (a,d). Metabolite channeling occurs where CK is associated with ATP-providing or -consuming transporters, pumps or enzymes (a,b,d). Substrate and product fluxes between CK and the associated proteins are depicted as arrows.

In the doctoral thesis presented here, a full-fletched search for other CK micro-compartments and their binding partners involved in a direct interaction with CK in the brain is described and novel interaction partners for BCK and uMtCK are presented.

3.2. The Golgi matrix protein GM130 and the Golgi apparatus

3.2.1. The Golgi Matrix Protein GM130

So far, little is known about the Golgi Matrix protein GM130 and its function in the cell. In 1995, Nakamura and co-workers identified GM130 as a peripheral membrane protein with a Mr of 130 000 which is tightly bound to the Golgi membrane on the cytoplasmic side (Nakamura et al., 1995). GM130 belongs to the golgin protein family (Chan and Wong, 1998) and is predicted to adopt a coiled-coil structure similar to the myosin heavy chain and the intermediate filament proteins with short non-coil N- and C-terminal regions (Nakamura et al., 1995). The N-terminal region of GM130 binds to the vesicle tethering transcytosis-associated protein (TAP)/p115 and this binding is suggested to be required for the membrane fusion step of intercisternal transport in the Golgi stack (Barroso et al., 1995). The C-terminal region is required for Golgi localization and binds to the Golgi reassembly stacking protein 65 (GRASP65) (Barr et al., 1998). The N-terminal p115-binding region of GM130 is phosphorylated during mitosis and this is suggested to trigger the disassembly of the Golgi apparatus (Levine et al., 1996; Lowe et al., 1998; Nakamura et al., 1997a). GRASP65 and GM130 both localize to the cis face of the Golgi (Nakamura et al., 1995). GM130 is known to play an important role in mitotic fragmentation of the Golgi apparatus (Lowe et al., 1998), but its exact role is still unclear.

3.2.2. Structure and function of the Golgi apparatus

Structurally, the Golgi complex is localized in the perinuclear region of most mammalian cells and is characterized by stacks of membrane-bound cisternae as well as a functionally distinct trans- and cis-Golgi networks (TGN, CGN, Figure 3). The Golgi apparatus performs three major functions essential for growth, homeostasis and division of eukaryotic cells. First, it operates as a carbohydrate factory for the processing and modification of proteins and lipids moving through the secretory pathway (Farquhar and Palade, 1998). Second, it serves as a station for protein sorting and transport, receiving

membranes from vesicles containing membrane proteins from the endoplasmic reticulum (ER) and delivering them to the plasma membrane or other intracellular sites (Rothman, 1994). Finally, it acts as a membrane scaffold onto which diverse signalling, sorting and cytoskeleton proteins adhere (Altan-Bonnet et al., 2003; De Matteis and Morrow, 2000; Donaldson and Lippincott-Schwartz, 2000). With its composition as a stacked array of cisternae and connecting tubules/vesicles, its enormous diversity of protein components (Wu et al., 2000) and its unrivalled capacity to dynamically transform in response to specific stimuli, the structure of the Golgi apparatus is unique among sub-cellular organelles. Many proteins associated with the Golgi are part of large protein complexes (Whyte and Munro, 2002) and remain associated only transiently as they move through other pathways in the cell (Altan-Bonnet et al., 2004).

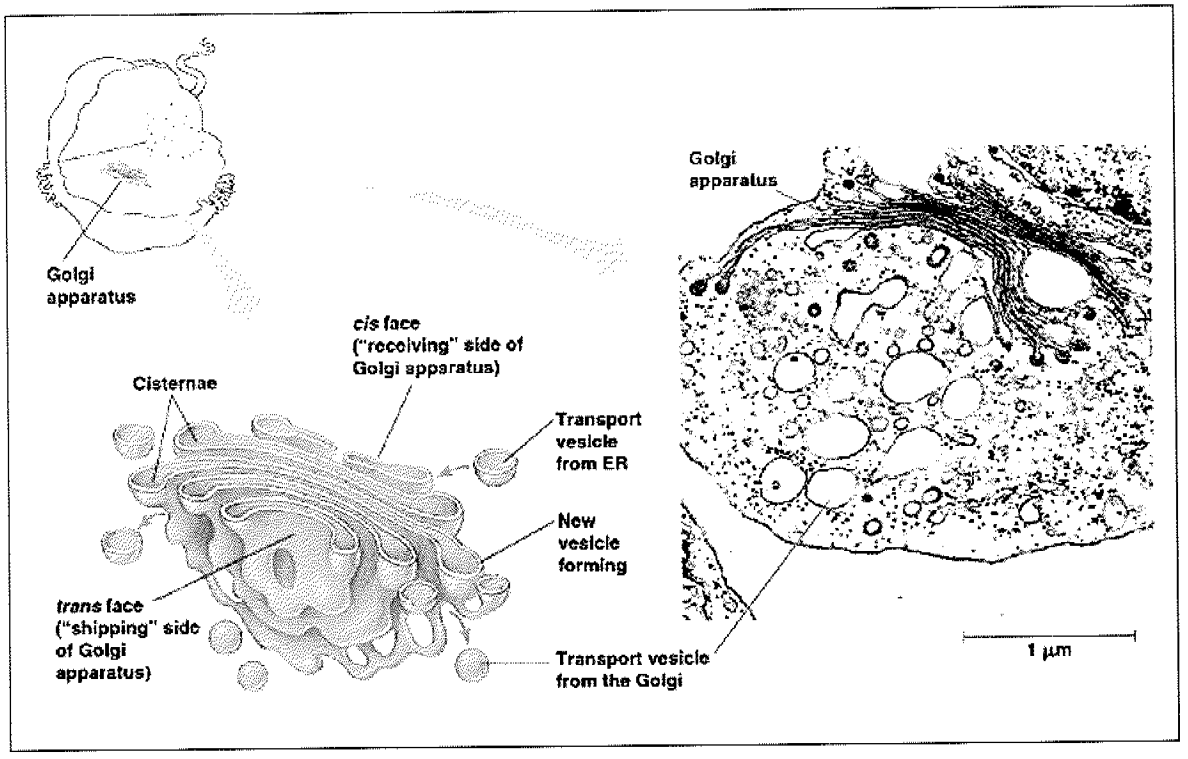

Figure 4: Structure of the Golgi apparatus and its functioning in vesicle-mediated transport.

The Golgi apparatus is located near the nucleus (red structure in the cell top left) and is orientated in a trans face, abandoned from the nucleus, and a cis face next to the ER facing the nucleus. Vesicles received from the ER go through the Golgi network leaving at the trans face. Right image shows an electron micrograph of the Golgi apparatus. (Images from Addison Wesley Longman Inc., Massachusetts, USA)

3.2.3. Cell cycle specific Golgi fragmentation

Observations based on immunofluorescence and electron microscopy suggest that at the onset of mitosis, the mammalian Golgi apparatus undergoes a dramatic breakdown of its interphase structure (Puthenveedu and Linstedt, 2001). There is a general agreement amongst the 'mitotic Golgi fragmentors' that Golgi membranes undergo sequential disassembly during mitosis (Fig. 4) in late prophase/pro-metaphase. There, the pericentriolar Golgi stacks break down into smaller pieces, so called 'mitotic blobs' (Nelson, 2000). Subsequently, between pro-metaphase and early anaphase, Golgi membranes undergo further fragmentation and are found diffusely dispersed in the cytosol (known as the mitotic Golgi 'haze'). Two components have been identified that mediate this conversion: the mitogen-activated protein kinase (MAPK) kinase 1 (MEK1) and polo-like kinase 1 (Plk1) (Acharya et al., 1998; Sutterlin et al., 2001). Mitotically activated MEK1 is found on Golgi membranes in late prophase, and it is likely that this association triggers Golgi fragmentation (Colanzi et al., 2003).

During anaphase, mitotic fragments comprising clusters of small and large vesicles (50-70 nm in diameter) and tubules disperse into the entire space of the cytoplasm and segregate stochastically into the dividing cell (Warren, 1993).

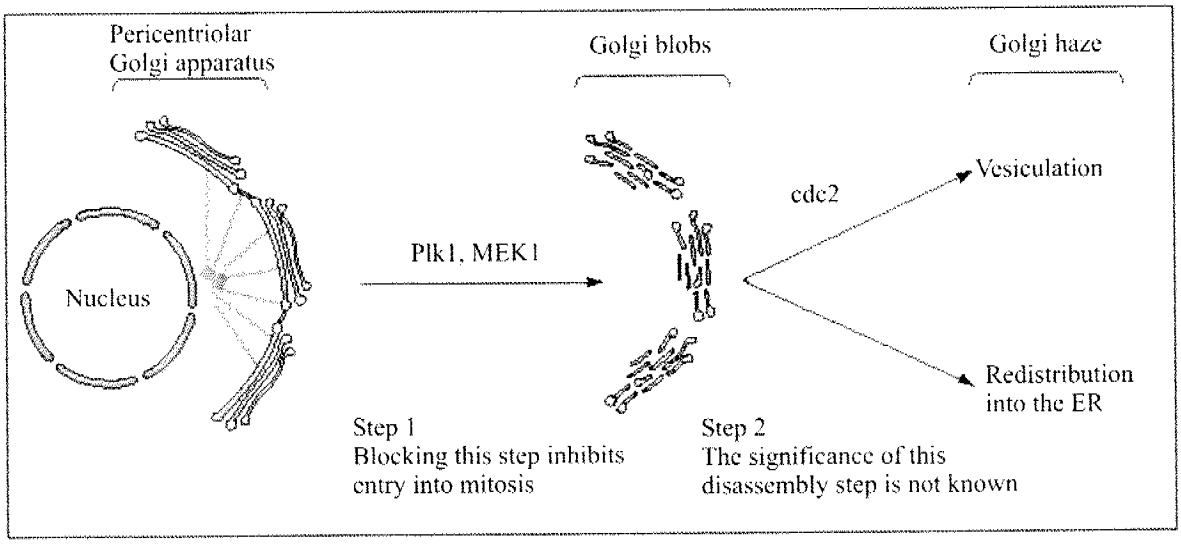

Figure 5: Golgi fragmentation occurs in two sequential steps

The pericentriolar stacks of Golgi cisternae undergo two sequential fragmentation reactions during mitosis in mammalian cells. Plk1 and MEK1 are required for converting these stacks into tubulo-reticular elements

(Golgi blobs). These Golgi blobs undergo further disassembly. The extent to which Golgi blobs are processed in this second disassembly reaction is controversial. The current extreme proposals are conversion into small vesicles by a process that requires cdc2 kinase, or fusion with the ER. It hast been reported recently that preventing the fragmentation of the pericentriolar Golgi stacks into Golgi blobs inhibits entry of mammalian cells into mitosis. It would be most valuable to ascertain the effect of blocking the second disassembly step on the fate of the Golgi membranes *in vivo* and on the progression of cells trough mitosis (modified after Nelson, 2000).

The vesicle-docking protein p115 is required for intra-Golgi transport (Waters et al., 1992) and has also been implicated in docking of transcytotic vesicles with the plasma membrane (Barroso et al., 1995). While p115 can bind to COP1 vesicles under both interphase and mitotic conditions (Sonnichsen et al., 1998), its binding to Golgi membranes is significantly inhibited in mitosis (Levine et al., 1996). This is because it can no longer bind its Golgi membrane receptor GM130 (Nakamura et al., 1997b; Nakamura et al., 1995). GM130 is mitotically phosphorylated and when this occurs, p115 can no longer bind to GM130 (Nakamura et al., 1997b).

3.3. The Amyloid precursor super family (APSF)

3.3.1. Domain characterization and alignment of the APSF

The amyloid precursor super family (APSF) is composed of three highly conserved transmembrane glycoproteins, the amyloid precursor protein (APP) and amyloid precursor-like proteins 1 and 2 (APLP1, APLP2) (Lyckman et al., 1998). Although the APP gene is the only APSF that encodes the amyloid beta peptide (Aβ the conservation of gene structure (Wasco et al., 1993) and domain homology (Slunt et al., 1994) among the APSF proteins suggests that they may share significant overlap in function and expression. Thus, amyloidogenic processing of APP may be influenced in parallel with processing of APLP1 and/or APLP2. For instance, APSF proteins from various phyla share highly conserved extracellular zinc and heparin binding domains (Bush et al., 1994), single membrane-spanning domains (TMR, Fig. 6) (Kang et al., 1987; Wasco et al., 1993), and intracellular phosphorylation sites (Suzuki et al., 1997). Additionally, APP and APLP2 genes both contain N-linked glycosylation sites (Dyrks et al., 1988), splice-specific chondroitin sulphate glycosaminoglycan (CSGAG) attachment sites (Shioi et al., 1995; Thinakaran and Sisodia, 1994; Thinakaran et al., 1995), and, alternatively, spliced exons that encode the

Kunitz protease inhibitor (KPI) domain (Tanzi et al., 1988; Wasco et al., 1993).

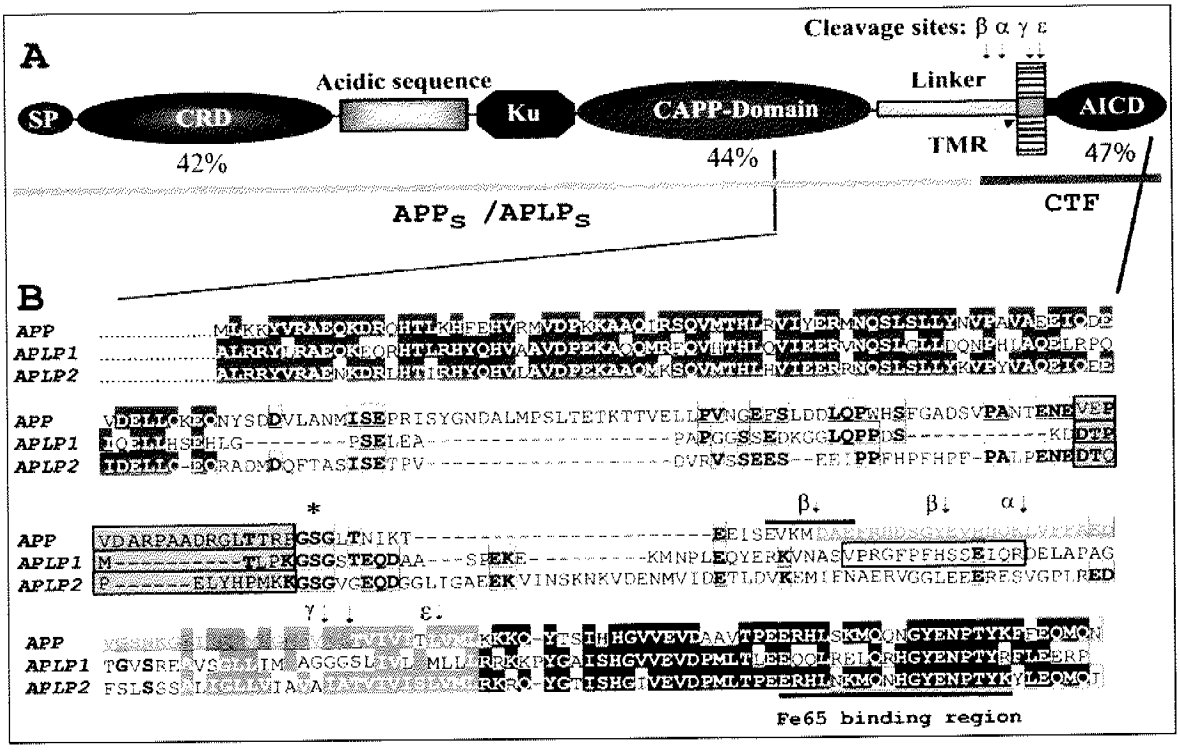

Figure 6: Structures and conservation of APP, APLP1, and APLP2

(A) Diagram of the domain structure of the three proteins of the APSF (APP, APLP1, and APLP2). The extracellular sequences of APP and APLPs are composed of six regions, an N-terminal signal peptide (SP), a cysteine-rich domain (CRD), an acidic sequence that chelates metal ions, a Kunitz domain (Ku) that is only present in APP and APLP2 and is alternatively spliced (Kitaguchi et al., 1988; Tanzi et al., 1988), a cysteine-poor bit conserved sequence (central APP domain, CAPP-domain), and a C-terminal linker that connects the extracellular domains to the transmembrane region (TMR). Positions of the α-, β-, γ-, and ε-cleavage sites in the linker and the TMR of APP are indicated. γ-Cleavage and ε-cleavage (which are presumably carried out by the same presenilin-dependent enzymes (Sastre et al., 2001; Yu et al., 2001) release the cytoplasmic tail to generate the APP intracellular domain (AICD). The percent identity (fully conserved residues) between human APP, APLP1 and APLP2 is shown only below those domains (the CRD, and AICD) that are significantly conserved among all three proteins. The size and cleavage location of the secreted extracellular APP/APLP fragment (APPs/APLPs) and the CTF generated by α-/β-cleavage is indicated.

(B) Alignment of the C-terminal sequences of APP, APLP1, and APLP2 starting with the C-terminal half of the CAD. Identical residues are highlighted with a domain-based colour code (blue, CAD; yellow, linker; green, TMR; black, AICD). The alternatively spliced sequences in the linker are boxed on a pink background, and excision of this sequence creates a recognition site for chondroitin sulphate glycosaminoglycans, which are attached to the serine residue marked with a sequence in APP and APLPs and the internal APLP1 sequence that were used to raise antipeptide antibodies are boxed. Note that the linker

between the extracellular domain and the TMR exhibits no sequence similarity between APP, APLP1, and APLP2 (image from Li and Sudhof, 2004).

3.3.2. APP processing and Alzheimer's Disease (AD)

The functions of the ubiquitous membrane protein APP (Kang et al., 1987; Kitaguchi et al., 1988; Tanzi et al., 1988) remain unknown, although numerous activities have been ascribed to it (De Strooper and Annaert, 2000). In contrast, the metabolism of APP is well characterized, mediated by a series of enzymes termed secretases (α, β, γ and ε) (Haass and De Strooper, 1999) (see Fig 7).

First, cleavage by α- or β-secretases release the large extracellular part of APP (sAPP) leaving the C-terminal fragment (CTF), which is composed of a small remaining extracellular stub, the TMR, and the cytoplasmic tail to remain. The CTF of APP is then cut by γ-secretase at multiple positions in the TMR (Sastre et al., 2001; Yu et al., 2001). γ-Cleavage liberates an intracellular cytoplasmic fragment referred to as AICD (Cupers et al., 2001; Kimberly et al., 2001) that may function as a transcriptional activator (Cao and Sudhof, 2001; Gao and Pimplikar, 2001) and has other signalling roles (Lu et al., 2000). In addition, γ-cleavage generates small peptides that are derived from the TMR and adjacent extracellular sequences. These peptides include β-amyloid$_{40\text{-}42}$ (Aβ), the major component of amyloid plaques in Alzheimer's Disease (AD) (Leissring et al., 2002; Lu et al., 2000).

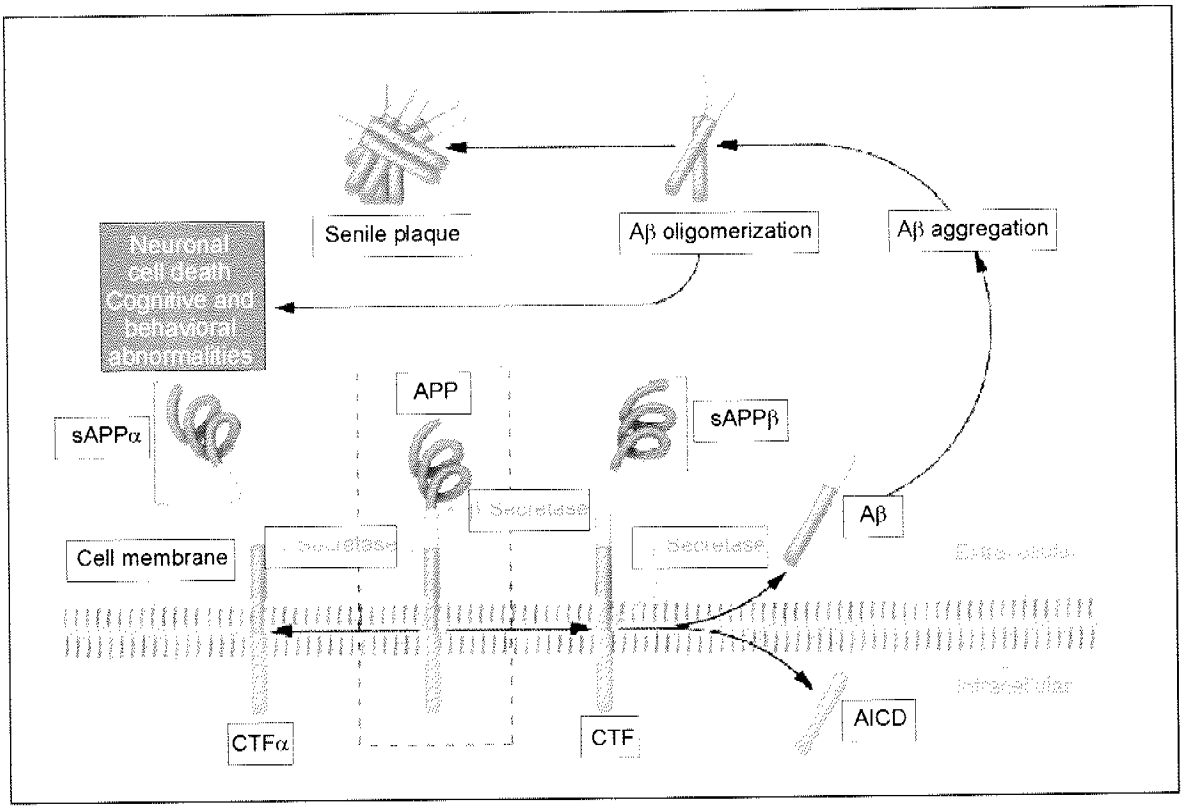

Figure 7: APP processing and Aβ accumulation

Mature APP (centre, inside dashed box) is metabolized by 2 competing pathways, the α-secretase pathway that generates $sAPP_{\alpha}$ and CTFα (left) and the β-secretase pathway that generates sAPPβ and CTF (right). All carboxyterminal fragments (CTFα and CTF) are substrates for γ-secretase, generating the APP intracellular domain (AICD), and Aβ (right). Aβ aggregates into small multimers (dimers, trimer, etc.) known as oligomers. Oligomers appear to be the most potent neurotoxins, while the end stage senile plaque is relatively inert (modified after Gandy, 2005).

From Alois Alzheimer's description of Auguste D.'s brain in 1907 to George Glenner's biochemical section of β-amyloid (Aβ) in 1984 (Glenner and Wong, 1984), the 'amyloid hypothesis' of Alzheimer's Disease has continued to gain support over the past two decades, particularly from genetic studies. The neuropathology of Alzheimer's Disease (AD) is characterized by two types of lesions, senile plaques and neurofibrillary tangles (NFT). Senile plaques are composed of Aβ, the cleavage product of the APP, and NFTs which consist of aberrantly phosphorylated tau, a microtubule-associated protein (Fig. 8) (Glenner and Wong, 1984; Lee et al., 1991). Figure 8 shows neurons in a normal and a modified situation, as it is the case in AD.

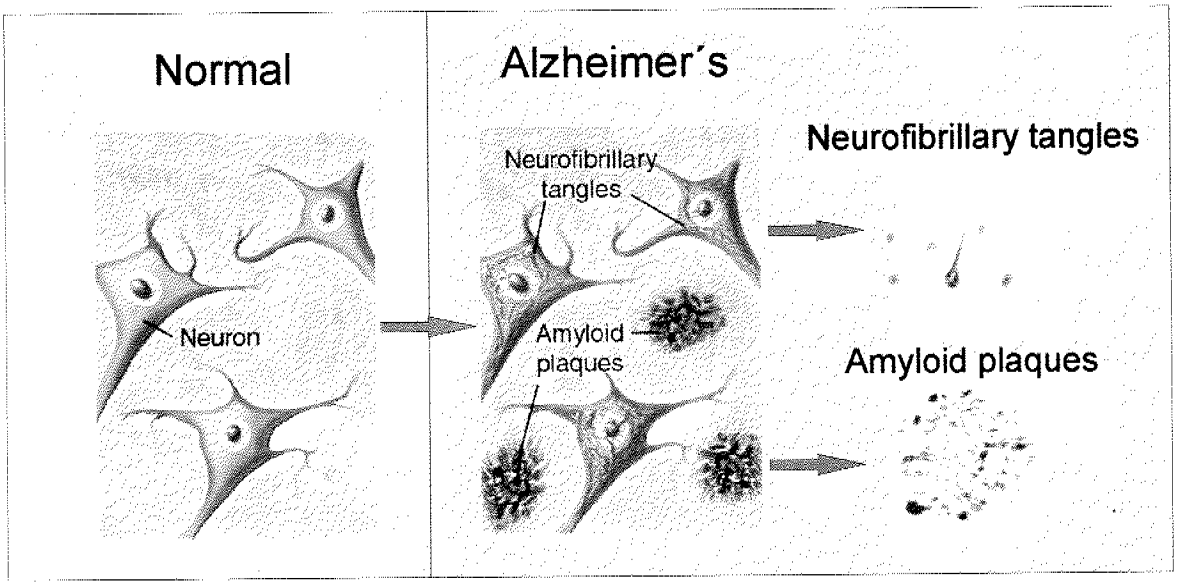

Figure 8: Amyloid plaque and neurofibrillary tangle formation in neurons of Alzheimer's Disease patients

The formation of amyloid plaques and neurofibrillary tangles are thought to contribute to the degradation of the neurons in the brain and the subsequent symptoms of Alzheimer's Disease. (Modified image from the American health assistance foundation homepage, National Institute of Aging NIA senior health homepage).

Many neurological disorders are characterized by an abnormal accumulation of macromolecules inside cells as, for example, the senile plaques in AD. These 'inclusion bodies' are also found, for example, in the brain of patients suffering from prion disease, amyotrophic lateral sclerosis (ALS), Parkinson's Disease (PD) and a group of nine so-called poly-glutamine diseases, of which Huntington´s Disease (HD) is the most widely known (Orr, 2004). HD is caused by abnormal polyglutamine expansion within the protein huntingtin, which then is aggregated into microscopic intracellular deposits called 'inclusion bodies' (DiFiglia et al., 1997). The common factor in polyglutamine diseases is a genetic mutation that produces abnormal repeats of the amino-acid glutamine in the encoded protein, with more repeats being more pathogenic (van Dellen and Hannan, 2004). With the advent of studies by molecular geneticists and cell biologists, it became clear that, in the inherited forms of each of the 'inclusion body diseases', inclusion bodies contain the protein encoded by the gene containing the disease-causing mutation. These observations reignited long-standing questions of whether and if the inclusions contribute directly to the disease process. However, in HD, inclusion-body formation actually prolonged survival

and protected neurons (Arrasate et al., 2004).

Thus, determining whether a particular change is pathogenic, incidental or beneficial has important implications for understanding mechanisms of disease and for identifying therapeutic targets. In this context, the results that are showing a direct interaction of APLP1 and a C-terminal region of APP with uMtCK are presented as a second important finding in this doctor thesis.

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4. Chapter 1

Brain-type Creatine Kinase BCK interacts with the Golgi Matrix Protein GM130 in early prophase

Tanja Bürklen*, Alain Hirschy, Theo Wallimann

Institute of Cell Biology, Swiss Federal Institute of Technology (ETH) Zürich, Hönggerberg HPM, CH-8093 Zürich, Switzerland

*Corresponding author:

Tanja Bürklen

Institute of Cell Biology

HPM D22

Schafmattstr. 18

8093 Zürich, Switzerland

Phone: (0041) 44-6333339

Fax: (0041) 44-6331069

E-mail: tanja.buerklen@cell.biol.ethz.ch

Submitted to the Journal of Cell Science

4.1. Abstract

Creatine kinase (CK) isoenzymes are essential for storing, buffering and intracellular transport of high phosphate-bound energy compounds in tissues with high energy demand such as muscle and brain. In neurons, cytosolic brain-type CK (BCK) and mitochondrial CK (MtCK) act as components of a shuttle system to distribute and maintain cellular energy. BCK plays an essential role in the regeneration of ATP to support the rapid reactions of signal transduction in astrocytes, as well as for the formation of neuronal synapses. To date, little is known about direct coupling of BCK to enzymes important for brain functions. Here, we identify the cis-Golgi matrix protein (GM130) as an interacting partner of BCK using a yeast two-hybrid (Y2H) screen followed by characterization of the GM130-BCK complex by GST-pulldown experiments and co-localization studies with confocal microscopy. GM130 plays an important role in Golgi fragmentation during mitosis. We report here that GM130 and BCK co-localize specifically in a transient fashion during early prophase and propose that BCK may provide the energy required for initiating the signalling cascades during early phases of mitosis that eventually lead to the process of Golgi fragmentation.

Keywords: creatine kinase, energy metabolism, Golgi Matrix protein GM130, mitosis, yeast two-hybrid, energy shuttle

4.2. Introduction

The creatine kinase (CK) isoenzymes catalyze the reversible transfer of a high-energy phosphate group between phosphocreatine (PCr) and ADP to maintain intracellular ATP levels in cells of high and fluctuating energy demands. In vertebrates, at least five distinct CK isoenzymes are expressed in a tissue specific manner and localize to different intracellular compartments (Eppenberger et al., 1967; Wallimann et al., 1992).

The maintenance of a high energy state in the brain is still an ill understood and much debated aspect of metabolism. In the brain, ATP can be formed by mitochondrial oxidative phosphorylation and (an)aerobic glycolysis and is consumed by electrogenic activity of Ca^{2+}, Na^{+}/K^{+} ATPases, glutamine-glutamate neurotransmitter cycling, and molecular synthesis and transport of cell constituents (Streijger et al., 2004). Two different CK isoforms constitute the components for a functional intracellular energy shuttle in the

brain. Di- or octameric ubiquitous mitochondrial CK (uMtCK) generates PCr in the peripheral intermembrane space and in the cristae of mitochondria (Brdiczka, 1994; Speer et al., 2004), which is then used by the dimeric cytosolic brain-type CK (BCK) to generate ATP at sub-cellular sites of high ATP turnover (Friedman and Roberts, 1994; Wallimann and Hemmer, 1994; Wallimann et al., 1992).

BCK plays an essential role in the transport of neurotransmitters in neurons (Lim et al., 1983) and ions in astrocytes (Magistretti et al., 1999; Trachtenberg and Pollen, 1970), as well as in the regeneration of ATP during thrombin receptor signalling (Mahajan et al., 2000). Although it has been shown that the brain of BCK knockout mice can metabolically compensate to some for the loss of BCK activity, these mice revealed diminished habituation and spatial learning acquisition (Jost et al., 2002). Moreover, BCK deficient mice display a larger intra-infra-pyramidal mossy fibre field size and enlarged ventricular space (Jost et al., 2002). BCK together with its substrate PCr and Cr in the brain might not only be important for energy homeostasis but can also exert a general neuroprotective role, as shown by the supplemental feeding of Cr in animal models for seizures, hypoxia, or neurodegenerative disorders (Holtzman et al., 1998; Tsuji et al., 1995).

To ascertain the role of BCK in the brain as a protein structurally and functionally linked to energy requiring enzymes, we performed a yeast two-hybrid (Y2H) to identify BCK-interacting proteins out of a brain cDNA library. Here, we report the human Golgi Matrix protein 130 (GM130) as a novel interaction partner of human BCK. In a LexA based Y2H screen using a N-terminally truncated form of human BCK as a bait (amino acids 103-381) we show that the amino acid residues 433–679 of GM130 are responsible for this interaction (Fig. 11).

GM130 belongs to the golgin protein family (Fig. 9) (Barr, 1999) and is involved in the assembly and maintenance of the Golgi apparatus (Rabouille and Jokitalo, 2003). Golgins are coiled-coil proteins associated with the Golgi apparatus, that are believed to be involved in the tethering of vesicles and the stacking of cisternae, as well as other functions such as association with the cytoskeleton. Many golgin proteins are peripheral membrane proteins recruited by GTPases. Several have been described in animal cells, and some in yeast, but the relationships between golgins of different species is hard to define because their protein sequences are quite divergent although they share common structural features

(Fridmann-Sirkis et al., 2004). Structurally, the Golgi-localized proteins are characterized by an extended coiled-coil. Figure 1 shows the structural framework of all golgin protein family members. A subset of the golgins contains a carboxy-terminal region, called the GRIP domain that mediates binding to the Golgi membranes. The GRIP domain was named after the four proteins in which it was originally found (golgin-97, RanBP2-α, Imh1p and p230/golgin-245) (Kjer-Nielsen et al., 1999; Munro and Nichols, 1999). The N-terminal 73 amino acid residues of GM130 interact with the vesicle docking protein p115 (Nakamura et al., 1997), which suggests a role for GM130 in the vesicular traffic between endoplasmic reticulum (ER) and Golgi stacks. GM130 also interacts with GRASP65, a Golgi reassembly and stacking protein of 65 kDa that is involved in the reformation of the Golgi complex after mitotic cell division (Barr et al., 1997). The interaction between p115 and GM130 is inhibited upon mitotic phosphorylation of GM130 by cyclin-dependent kinase I (Lowe et al., 1998).

Finally, we show for the first time that GM130 and BCK interact *in vivo* and that co-localization of endogenously expressed proteins occurs specifically in the early prophase of mitosis. Altogether, these data reveal new aspects on the role of creatine kinase for energy provision in the regulation of the cell cycle.

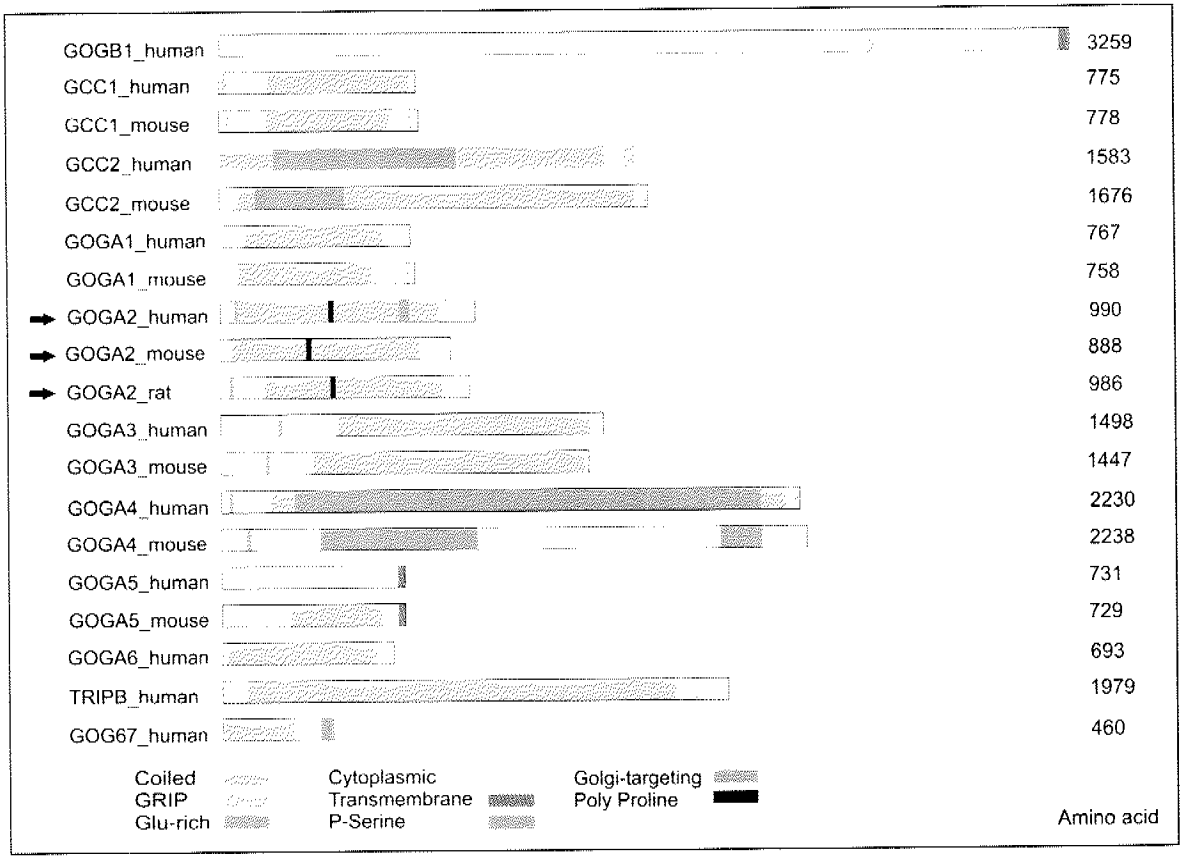

Figure 9: Domain structure of the golgin protein family members

Among the golgin protein family members many have similarities, like the coiled-coil domains (yellow), the GRIP domains that mediate binding to Golgi membranes (green), a glutamate-rich domain (light blue), a cytoplasmic region (lavender), a transmembrane domain (turquoise) and a Golgi-targeting domain (grey). The GM130 proteins (GOGA2) from human, mouse and rat are indicated with an arrow. All three share a high homology within their domain structure compared to the other golgin proteins (raw structural data from the ExPasy Proteomics Server).

4.3. Materials and Methods

4.3.1. Plasmid constructs

For the yeast two hybrid screen, human BCK cDNA was amplified by PCR from the cDNA template (kindly provided by Dr. Jean-Paul Steghens, Lyon, France) and inserted into the pLexA-dir bait vector (Dualsystems Biotech, Zürich, Switzerland) resulting in a 480 amino acid fusion protein. The human brain cDNA library in the pACT2 vector was obtained from Clontech Laboratories, Inc Palo alto, USA). The full-length rat GM130 construct, as well as its deletion constructs with amino acids 433-679, 679-986, 75-271 and

272-986 in the pACT2 vector were kindly provided by Dr. Francis A. Barr, Munich, Germany (Preisinger et al., 2004). For the GST construct, full-length human BCK cDNA was amplified by PCR and subcloned into the Glutathione S-transferase (GST) vector pET42(+) (Novagen Inc, Madison, USA). All clones derived by PCR were verified by DNA sequencing. Protein expression was verified by Western blotting using specific antibodies.

4.3.2. Bacterial recombinant fusion proteins

Glutathione S-transferase (GST) fusion protein of BCK was prepared according to the manufacturer's instructions. Briefly, expression of fusion protein in *E.coli* BL21 cells (Invitrogen, Carlsbad, USA) was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3-5 hours. Cells were pelleted at 4°C, 4000g for 20 minutes. The pellet was lysed in lysis buffer (0.5 M sucrose, 20 mM Tris pH 8.0, 15 mM β-Mercaptoethanol, with addition of protease inhibitor cocktail (Roche, according to the manufacturer's instruction). After incubation with lysozyme and DNase I (400U/ml) for 30 minutes on ice, cells were sonicated and debris were removed by centrifugation for 15 minutes at 4°C 10000 g. The supernatant was affinity purified on glutathione-agarose (Sigma, Buchs, Switzerland). Expression and purification was controlled by SDS polyacrylamide gel electrophoresis.

4.3.3. Yeast two-hybrid assays

Yeast two-hybrid screening assays were performed as described previously (Cuomo et al., 1994). Briefly, after excluding self-activation of the BCK bait construct, the yeast reporter strain DSY-1 (Dualsystems Biotech, Zürich, Switzerland) was co-transformed with the bait and the cDNA library and transformants were grown on selection plates lacking histidine, leucine and tryptophan. Positive transformants were tested for β-Galactosidase activity using a filter assay (Serebriiskii and Golemis, 2000). Library plasmids were isolated from positive clones and assayed in a bait dependency test and with a bait encoding a LexA-laminC fusion as control, using a mating strategy (Kolonin et al., 2000). The identity of positive interactors was determined by DNA sequencing.

4.3.4. Preparation of rat liver Golgi stacks

Rat liver Golgi stacks were prepared as described previously (Slusarewicz et al., 1994). Briefly, rat liver was homogenized with a glass-teflon Potter in ice-cold 0.5 M sucrose-PM buffer (0.1 M sodium phosphate, pH 6, 7.5 mM $MgCl_2$) to a final concentration of 0.6g liver/ml. The homogenate was applied to a two-step gradient comprising 1.3 and 0.86 M sucrose-PM buffer. After centrifugation at 25000 rpm using a SW28.Ti rotor (Beckmann) for 2 hours at 4°C, the Golgi fraction was collected from the 0.5/0.86 M sucrose interface. After dilution to 0.25 M sucrose using PM buffer, the membranes were centrifuged at 6500 g for 30 minutes at 4°C, washed in 0.25 M PM, re-centrifuged and resuspended in the same buffer. Golgi fraction was verified by Western blotting (WB) using GM130 as Golgi marker protein.

4.3.5. GST-pull down assay

Pulldown of GM130 from rat liver Golgi stacks was achieved by mixing 20 µl of 50% glutathione-agarose equilibrated in binding buffer (20 mM Tris pH 7.4, 65 mM NaCl, 2.5 mM MgCl, 0.05% NP-40, 1 mM DTT) with 20 µg GST-BCK or GST alone. After incubation at 4°C for 1 hour, 10 µg of the rat liver Golgi stacks preparation was added and incubated for 1 hour at 4°C. Glutathione beads were then recovered by centrifugation and washed three times vigorously with binding buffer. Proteins bound to the beads were eluted by SDS sample buffer and analyzed by Western blotting using mouse-anti GM130 (BD Bioscience Pharmingen, San Diego, USA) and rabbit-anti GST antibody (Sigma, Buchs, Switzerland).

4.3.6. Western blotting

SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gels. For Western blot analysis, proteins were transferred to nitrocellulose membranes (Schleicher&Schuell, Bottmingen, Switzerland). Primary antibodies were overlaid with goat-anti rabbit (Amersham Pharmacia Biotech, Sweden) or anti-mouse (Calbiochem-Novabiochem International, Inc., USA) horseradish peroxidase-coupled secondary antibodies, and chemiluminescence was detected using the Pico detection kit (Pierce Biotechnology, Rockford, USA).

4.3.7. Cell culture, immunofluorescence staining and confocal imaging

Monolayer cultures of HeLa cells were grown at 37°C and 5% CO_2 in DMEM high glucose medium, containing 10% FCS (Invitrogen). For immunhistological analysis, HeLa cells were grown on gelatine coated glass coverslips, washed with pre-warmed MP buffer (65 mM PIPES pH 6.9, 25 mM HEPES, 10 mM EGTA, 3 mM $MgCl_2$), pre-fixed for 2 minutes with 3% PFA in MP buffer, permeabilized for 4 minutes with 0.2% Triton X100 in MP. After extensive washing with MP, cells were fixed for another 6 minutes then blocked with blocking buffer (5% BSA in MP). Primary and secondary antibodies were diluted in blocking buffer and incubated for 60 and 40 minutes, respectively. Dilutions were: chicken anti-human BCK (Schlattner et al., 2002b) 1:50, rat anti-alpha tubulin (Abcam) 1:100, mouse anti-GM130 (BD Pharmingen) 1:100, anti-chicken Cy3 (Molecular Probes, Leiden, the Netherlands), 1:500, anti-mouse Cy5 (rat Ig absorbed, Jackson, Bar Harbor, USA) 1:100, anti-rat Cy2 (mouse Ig absorbed; Jackson) 1:100.

The imaging system consisted of a Leica inverted microscope DM IRB/E, a Leica true confocal scanner TCS SP1 (Leica Microsystems AG, Glattbrugg, Switzerland) and a Fujitsu-Siemens workstation (Fujitsu Siemens computer Bv., Regensdorf, Switzerland). The images were recorded using a Leica PL APOx63/1.4 oil immersion objective. The system was equipped with Ar and He/Ne lasers. Image processing was done using Imaris 4.0 (Bitplane AG, Zurich, Switzerland). This software was used to visualize single sections from the confocal data set. Final assembly of figures was made with Adobe Photoshop CS (Adobe systems, San José, USA). For each phase of mitosis, three independent cells showing the same staining pattern were recorded and pictures representing one single layer of the sections were shown.

4.4. Results

4.4.1. Identification of GM130 as an interaction partner of BCK using a Y2H screen

To identify putative novel interaction partners of BCK, we screened a human brain cDNA library using a LexA based yeast two-hybrid system (Cuomo et al., 1994). As preliminary Y2H screens with full-length BCK did not reveal any positive interaction partners (data not shown), we fused the C-terminal 278 amino acid domain of human BCK to lexA (Fig. 10A). This fusion protein includes the isoform specific box B260 and the highly conserved Cys 283 near the catalytic site (Stolz and Wallimann, 1998). Expression of the fusion protein in yeast was verified by Western blot (data not shown).

Our screen with 8.6 x 10^6 transformants revealed 64 positive clones (Fig. 10B). Strong binders are indicated as dark blue colour in the β-Galactosidase assay at 30 minutes and with higher intensity at 3 hours (Fig. 10B and C, orange squares). DNA sequencing identified the human Golgi Matrix protein GM130 as interaction partner for BCK in 8 of the 64 positive clones. This result suggests that BCK interacts with a Golgi structural protein and might be involved in the assembly or turnover of the Golgi apparatus.

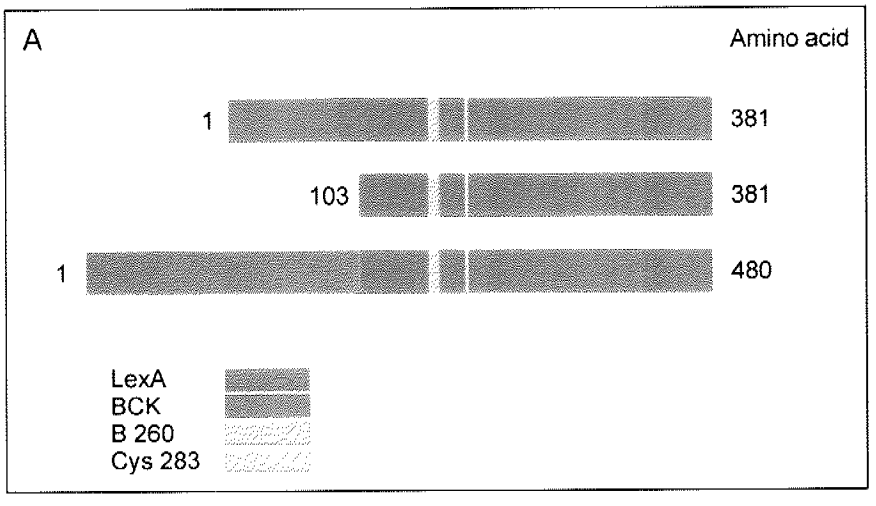

Figure 10 A

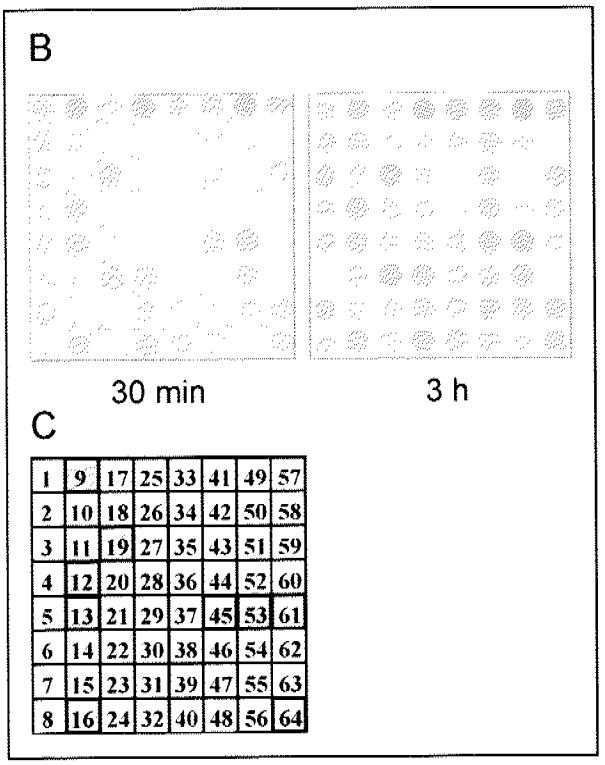

Figure 10 B: Domain structure of the BCK bait construct and identification of Golgi Matrix protein GM130 as interaction partner in the Y2H

(A) The full-length BCK (upper panel) is composed of 381 amino acids. A truncated form of 278 amino acids (103 – 381) was N-terminally fused to the LexA DNA binding domain and used as bait for the Y2H screen (lower panel). The isoform specific box B 260 as well as the highly conserved Cys 283 near the catalytic site is also included within the bait construct. (B, C) 64 clones rescued from the library screen were isolated and bait dependency was verified by measuring β-Galactosidase activity for 30 minutes up to 3 hours. Blue colour indicates protein-protein interaction via X-Gal cleavage by the reporter β-Galactosidase. (B) The differences in the blue colour intensities indicate the strength of the interaction (C). DNA sequencing of strongest binders (orange squares) identified human Golgi Matrix protein 130 (GM130) in 8 of 64 clones.

4.4.2. GM130 interacts via its 433-679 amino acid domain with BCK in the Y2H

GM130 binds via its N-terminus to the vesicle-tethering protein p115 (Nakamura et al., 1997) and via its C-terminus to the N-myristoylated Golgi reassembly stacking protein (GRASP) 65 (Barr et al., 1997). To identify the precise region in GM130 that is required for the interaction with BCK we performed a Y2H interaction assay with various deletion constructs of rat GM130 (Fig. 11). Interaction as indicated by β-Galactosidase activity

could be shown for constructs 433-679 (amino acid residues), 272-986 and the full-length construct. No interaction could be detected for the constructs 679-896 and 75-271, indicating that the amino acids 433-679, which include the predicted poly-proline domain (grey, dark framed) (Preisinger et al., 2004) play a role in this interaction. The helical coiled-coil structures and non-helical termini of the golgin proteins are proposed to be a clue to their function (Kjer-Nielsen et al., 1999). These results suggest that the central part of GM130 is sufficient for the interaction with BCK, however the increased signal in case of the full length constructs might indicate a structural stabilisation that aids this interaction.

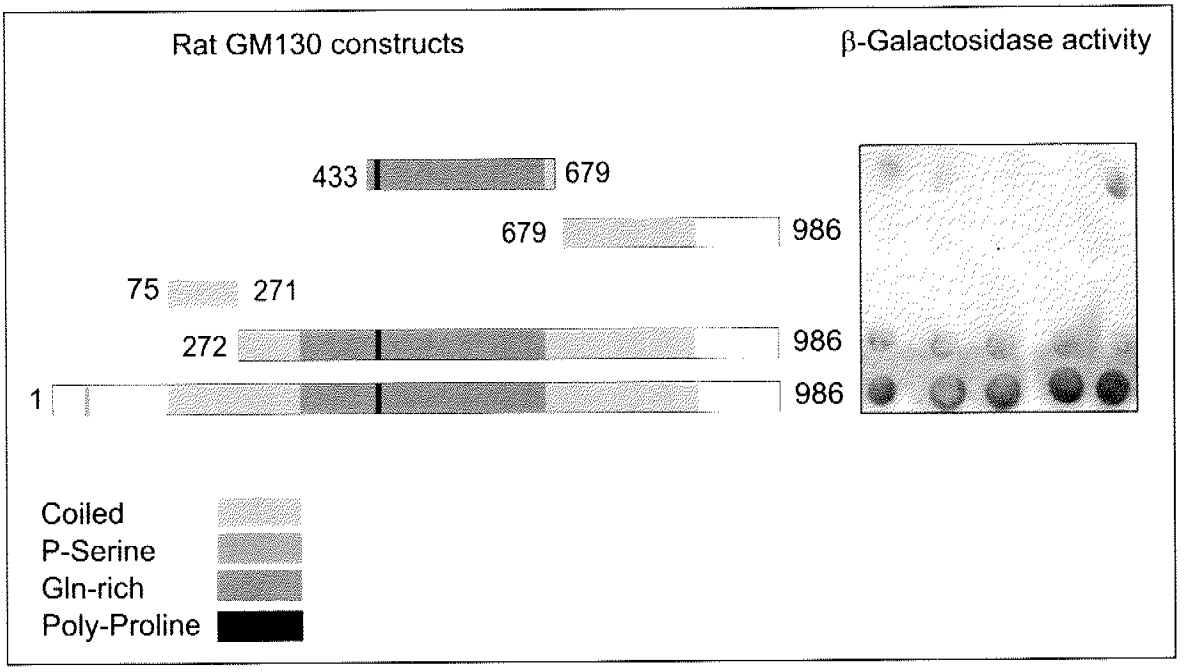

Figure 11: Characterization of the Golgi Matrix protein GM130 binding domain that is responsible for the binding to the BCK bait construct

Full-length GM130 and indicated deletion constructs of rat GM130 with different sizes (length in amino acids) were tested against the human BCK bait construct for β-Galactosidase activity. Blue colony colour indicates protein-protein interaction via X-Gal cleavage by the reporter β-Galactosidase. All constructs containing the poly-proline domain interact with BCK and amino acid residues 433-679 of GM130 are responsible for this interaction.

4.4.3. Interaction of BCK with GM130 in rat liver Golgi stacks

To confirm the Y2H *in vivo* data biochemically, GST-pulldown assays were performed. For this, full-length human BCK was N-terminally fused to GST and expressed in E.*coli*. Purification of fusion protein (80 kDa) and GST (37 kDa) alone was achieved by affinity binding to glutathione agarose-beads (Fig. 12A).

To enrich GM130, rat liver Golgi stacks were prepared as described previously (Slusarewicz et al., 1994). GM130 is a cytoplasmic protein that is tightly bound to Golgi membranes (Nakamura et al., 1995) and remains attached to the Golgi membrane stacks during the preparation procedure (input Fig. 12B). The proteins eluted from the pulldown complex were analyzed by Western blotting. Antibodies used were anti-GST and anti-GM130 (Fig. 4B). We demonstrate here, that GM130 from the Golgi membrane fraction

(Fig.12B input) binds to GST-BCK *in vitro* but not to the GST control. Thus the interaction between BCK and GM130 could also be confirmed by a biochemical assay.

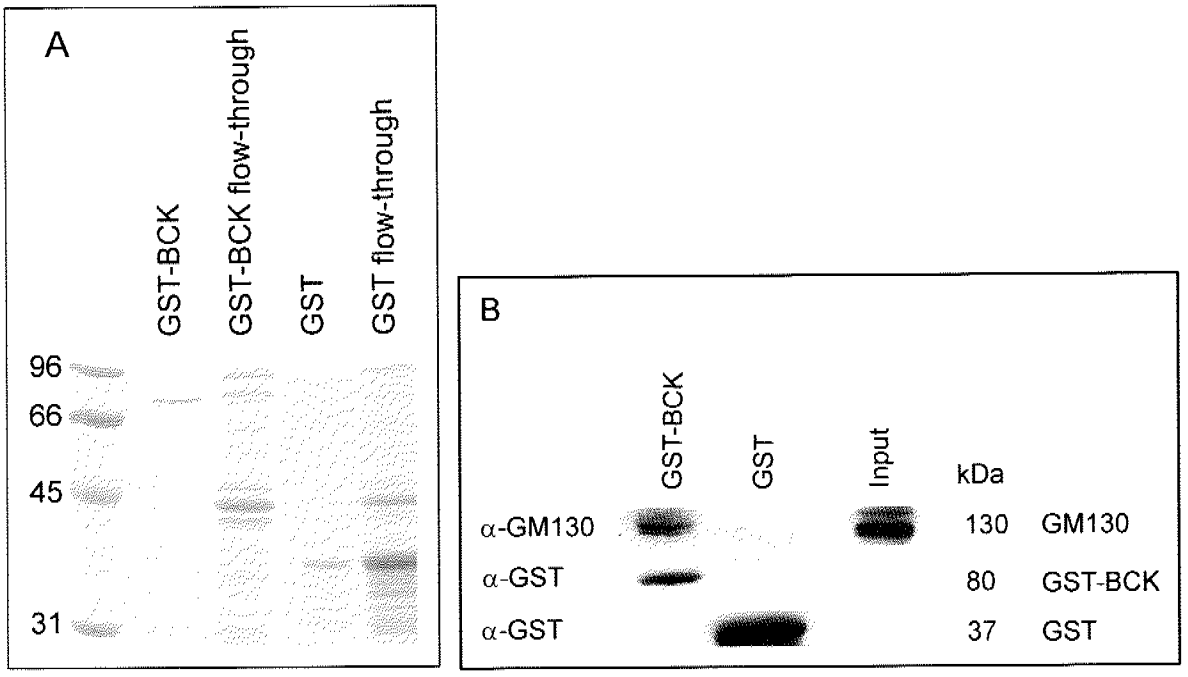

Figure 12: GST-pulldown experiment with GST-BCK fusion protein and rat liver Golgi stacks

(A) N-terminally GST-tagged BCK and GST alone were expressed in E.*coli* and purified on glutathione-agarose. GST-BCK has a calculated molecular weight of 80 kDa and GST alone 37 kDa. (B) rat liver Golgi stacks were prepared as described in the text and incubated with GST-BCK and GST, respectively bound to glutathione-agarose. Recovered complexes were analyzed by Western blotting. GM130 in the Golgi preparation bound specifically to GST-BCK but not to the GST control.

4.4.4. Co-localization of GM130 and BCK at early prophase in mitotic HeLa cells

GM130 plays an important role in the Golgi fragmentation during mitosis. Fragmentation is induced by phosphorylation of GM130 on Serine 25 by the Cdc2 kinase (Fig. 9) (Lowe et al., 1998; Nakamura et al., 1997). The dephosphorylation of GM130 at the end of mitosis is performed by the trimeric form of PP2A with the Bα regulatory subunit(Lowe et al., 2000). Since BCK catalyzes the reaction, which leads to the transfer of high energy phosphates between γ-ATP and phospho-creatine (PCr) it can be the provider of energy for ATP-dependent processes including cell division. To find out whether BCK is associated with GM130 during certain mitotic phases, we immunostained endogenously expressed

BCK and GM130 (Fig. 13 and 14) in cultured HeLa cells. Mitotically active cells were identified by staining of the microtubuli (green, alpha-tubulin), allowing the observation of all mitotic stages (Fig. 13 and 14). In interphase cells, BCK shows a diffuse distribution in the cytoplasm, as reported by previous studies (Friedman and Roberts, 1994; Schlattner et al., 2002a) and is almost excluded from the nucleus. However, in early prophase, a clear condensation of BCK to one perinuclear site can be observed. This correlates with the condensation of the GM130 and the increased tubulin staining at the same site, as indicated in the inset with a white arrow in Fig. 13 (early prophase). The co-localization of BCK and tubulin was reported earlier (Cande, 1983; Suginta et al., 2001). However during late prophase, where the separation of the centrosomes begins, the accumulation of BCK disappears, indicating that the interaction between BCK and GM130 is only transient. Therefore, the specific co-localization of BCK and GM130 is clearly restricted to the onset of mitosis (Fig. 13, compare the column labelled "early prophase" with the other stages of mitosis in 13 and 14). The transient interaction of BCK and GM130 at that early mitotic stage may be necessary to provide the energy that is required to start the signalling cascade that leads to fragmentation of the Golgi apparatus during cell division.

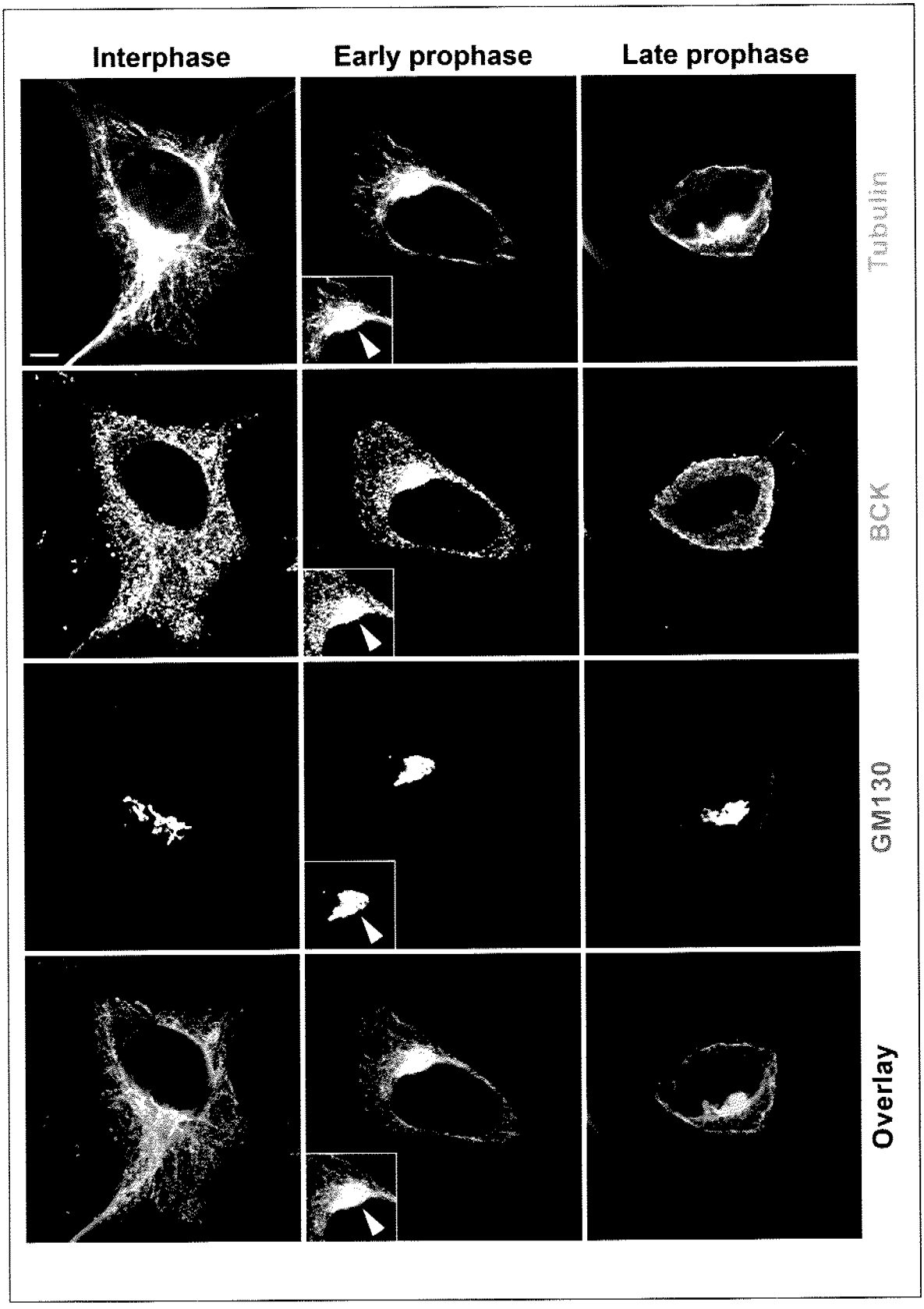

Figure 13: Co-localization of BCK and GM130 is found during early prophase of mitotic HeLa cells

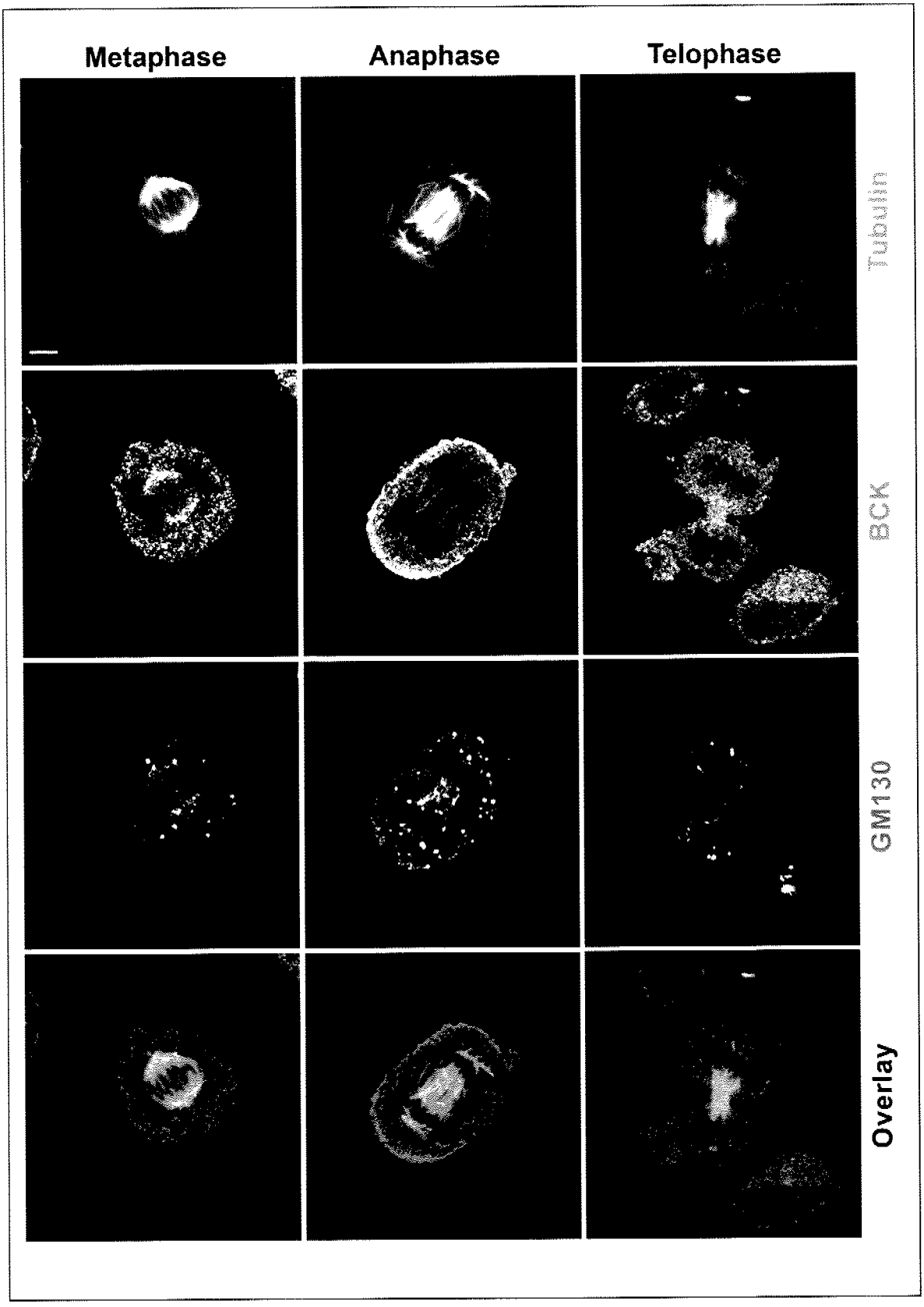

Figure 14: Co-localization of BCK and GM130 is found during early prophase of mitotic HeLa cells

(A, B) Cells were stained with antibodies against endogenously expressed alpha-tubulin (green) to verify the mitotic status of the cell, BCK (red) and GM130 (blue). Pictures shown here represent single confocal sections of the overlays and the individual channels. The BCK staining shows an overall cytoplasmic distribution. In the prophase, the early and the late anaphase, BCK condenses to certain cellular structures but this condensation overlaps with the GM130 staining specifically only in the early prophase (A), indicated by the white signal in the overlay (arrow in inset). Pictures were taken as sequential laser scans to prevent any possible overlapping of fluorescence signals emitted by the secondary antibody-coupled fluorochromes of neighbouring excitation wave lengths (scale bar 5 μm).

4.5. Discussion

In this study, we report that human BCK binds specifically to human GM130 *in vitro* and *in vivo*. Screening a human brain cDNA library with a LexA-BCK bait construct with the C-terminus (103-381) of BCK, including the isoform specific box B260 and the highly conserved Cys 283 near the catalytic site, resulted in the isolation of 64 clones of which 8 were identified as GM130 (Fig. 10B). This interaction was confirmed by a GST pulldown assay (Fig. 12B) and co-localization in confocal microscopy (Fig. 13).

GM130 is a cytoplasmic protein that is tightly bound to Golgi membranes and represents a part of a larger oligomeric complex. This complex consists of Golgi reassembly stacking protein 65 (GRASP65), p115 and giantin which are referred to as tethering proteins (Sonnichsen et al., 1998). GM130 belongs to the golgin protein family and predictions from the protein sequence suggest that GM130 is an extended rod-like protein with coiled-coil domains (Fig. 9). The so called "string theory" (Orci et al., 1998) proposes a predominant role for the GM130 protein in holding transport vesicles in place (Diaz et al., 1997; Warren and Malhotra, 1998). Our deletion analysis showed that a region around the predicted poly-proline domain of GM130 in Fig. 11 (amino acids 433-679) is involved in the interaction with BCK. To determine whether this domain causes the interaction, deletions of different residues, e.g. the prolines within this region may give further insights.

The Golgi apparatus occupies a central position in the classical secretory pathway, where it receives de novo synthesized proteins from the ER, and functions to distill posttranslationally processes and to sort cargo to their final destinations (Mellman and Simons, 1992). Its mode of inheritance after cell division is still under debate and several models have been proposed: de novo formation (Barr, 2002), fission (Pelletier et al., 2002), and disassembly-reassembly (Uchiyama et al., 2003). The latter process is regulated by Cdc2-cyclin B kinase (Misteli and Warren, 1994). GM130 is a substrate of Cdc2 (Nakamura et al., 1997; Sohda et al., 1998) and upon phosphorylation on serine 25, the vesicle tethering factor p115 can no longer bind to GM130 (Nakamura et al., 1997). Mitotic Golgi fragmentation *in vitro* depends on Cdc2-cyclin B activity. Phosphorylation of GM130 and dissociation of p115 are synchroniszed with the conversion of Golgi

cisternae into small vesicles. Since cyclin B2 has been localized to the Golgi complex in interphase cells (Jackman et al., 1995) it is possible that Cdc2 phosphorylates GM130 during prophase to initiate the disassembly of the Golgi complex (Lowe et al., 2000). Interestingly, many different ATP requiring processes are taking place in late prophase at the Golgi: protein phosphorylation reaction by mitogen-activated protein kinase 1 (MEK1) and Polo-like kinase 1 are required for Golgi complex fragmentation (Sutterlin et al., 2001), while RAF1 is necessary to activate MEK1 in the first place. Moreover MEK1 is found on the Golgi membranes in late prophase (Colanzi et al., 2000).

As high energy phosphates are needed in all these phosphorylation processes, it is likely that the interaction between GM130 and BCK would facilitate GM130 phosphorylation by ATP-requiring protein kinases. However, the interaction between BCK and GM130 seems to be transient and restricted to the prophase of the cell cycle, as demonstrated with immunostaining of HeLa cells at different phases of mitosis for endogenously expressed GM130 and BCK. As BCK is distributed diffusely throughout the entire cytoplasm exact co-localization was difficult to show, however in early prophase a clear condensation of BCK that clusters together with GM130 (Fig. 13 and 14) can be demonstrated very clearly and reproducibly. Thus, we suggest that BCK may facilitate the provision of energy to protein kinases that are recruited to signalling steps upon initiation of prophase. The action of BCK would be to maintain locally high ATP levels and a high ATP/ADP ratio that would favour protein kinase reaction.

Additionally, BCK was recently shown to be in a complex together with the chloride intracellular channel (CLIC), dynamin I, α-tubulin, β-actin and 14-3-3 (Suginta et al., 2001). Interestingly, GM130 targets the protein kinase YSK1 to the Golgi, which has 14-3-3 as a substrate (Preisinger et al., 2004). 14-3-3 is a protein enriched in brain that modulates ion channel functions. It regulates diverse cell signalling pathways, including activation of protein kinases, cell cycle control, neural development, cell migration and polarization and pathogenesis of bacteria and viruses (Santoro et al., 2003). Taken together, these data confirm that BCK is structurally and functionally coupled in a dynamic and cell cycle dependent manner to the Golgi apparatus. There, BCK is most likely a member of a large protein complex that also comprises several protein kinases.

This raises a new aspect to the creatine kinase family and is a first indication that creatine kinases are linked to signalling cascades regulating the integrity of the Golgi apparatus and thus may be involved in the control of the cell cycle.

Acknowledgements

We kindly thank Dr. Francis Barr for generously providing the rat GM130 constructs, Dr. Uwe Schlattner for the anti BCK antibody and Dr. Jean-Paul Steghens for providing the human BCK cDNA; Thanks to Tina Thurnherr and Dr. Elisabeth Ehler for critical reading of the manuscript. This work was supported by a Swiss National Foundation Grant No.3100A0-102075.

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5. Chapter 2

Stabilization of ubiquitous mitochondrial creatine kinase preprotein by APP family proteins

Xiaofan Li[‡], Tanja Bürklen[+], Xianglin Yuan[§#], Uwe Schlattner[+], Dominic M. Desiderio[§‖#], Theo Wallimann[+] and Ramin Homayouni[§‡¶]

From the Departments of [§]Neurology, [‡]Anatomy and Neurobiology, [‖]Molecular Sciences, and [#]Charles B. Stout Neuroscience Mass Spectrometry Laboratory, University of Tennessee Health Science Center, Memphis, TN; [+]Institute of Cell Biology, Swiss Federal Institute of Technology (ETH Zurich), Honggerberg HPM, Zurich, Switzerland

Corresponding author:

Ramin Homayouni

Department of Neurology

855 Monroe Ave, Rm 416 Link

Memphis, TN 38163

Phone: (901) 448-6180

Fax: (901) 448-7440

E-mail: rhomayouni@utmem.edu

Submitted to Molecular & Cellular Neuroscience

5.1. Abstract

Amyloid precursor protein (APP) is involved in the pathogenesis of Alzheimer's Disease (AD). However the physiological role of APP and its family members are still unclear. To gain insights into APP function, we used a proteomic approach to identify APP interacting proteins. We report here for the first time a direct interaction between the C-terminal region of APP family proteins and ubiquitous mitochondrial creatine kinase (uMtCK). This interaction was confirmed *in vitro* as well as in cultured cells and in brain. Interestingly, expression of full length and C-terminal domain of APP family proteins stabilized uMtCK preprotein in cultured cells. Our data suggest that APP may regulate cellular energy levels and mitochondrial function via a direct interaction and stabilization of uMtCK.

5.2. Introduction

Amyloid precursor protein (APP), a type I transmembrane protein, is intimately involved in AD pathogenesis (Selkoe, 2000). In addition to a role in AD pathogenesis, several lines of evidence indicate that APP has important biological functions (De Strooper and Annaert, 2000; Turner et al., 2003). APP belongs to a gene family that is highly conserved throughout evolution. In mammals, there are three APP family members, including amyloid precursor like protein-1 and -2 (APLP1 and APLP2). Whereas mice with targeted disruption of the APP gene display subtle abnormalities (Zheng et al., 1995; Steinbach et al., 1998), disruption of a combination of APLP1 and APLP2 or APP and APLP2 results in perinatal lethality (von Koch et al., 1997; Heber et al., 2000). These results suggest that the APP family members have essential and redundant roles during development. Importantly, whereas the large extracellular domain of APP family proteins is quite variable in sequence, their intracellular domains are highly conserved, suggesting that APP family proteins may participate in similar intracellular signaling pathways.

Some clues into the cellular function of APP have come from identification of APP interacting proteins (King and Scott Turner, 2004). Different regions in the cytoplasmic tail of APP have been shown to bind to heterotrimeric Gα protein (an intracellular signal transducer) (Nishimoto et al., 1993), kinesin light chain (axonal transport protein) (Kamal et al., 2000; Kamal et al., 2001) or PAT1 (a microtubule associated protein) (Zheng et al., 1998). In addition, the C-terminal region of APP family proteins, containing the amino

acid sequence motif NPxY, interacts with the phosphotyrosine-binding (PTB) domain of a number of intracellular adapter proteins such as Dab1, X11, and Fe65, which are involved in distinct cellular processes (Homayouni et al., 1999; Kimberly et al., 2001; King et al., 2003). Notably, most of these interactions were identified by yeast-two hybrid approaches that only detect one-to-one protein interactions in an artificial yeast nuclear environment. Thus alternative approaches are necessary to identify multi-protein complexes or APP interactions that require specific cellular environments or post-translational modifications. For instance, using a biochemical approach, Zhou et al (2004) recently identified a novel interaction between tyrosine-phosphorylated APP cytoplasmic domain and growth factor receptor-bound protein 2 (Grb2) (Zhou et al., 2004). In addition, Ho and Sudhof used a functional proteomic approach to identify F-spondin as a novel extracellular ligand of APP (Ho and Sudhof, 2004).

We hypothesize that the C-terminal regions of APP family proteins engage in similar intracellular functions through interactions with multi-protein complexes. Therefore, we used a functional proteomic strategy, involving biochemical affinity chromatography and peptide mass fingerprinting, to identify new APP interacting proteins. Here we report identification of ubiquitous mitochondrial creatine kinase (uMtCK, EC 2.7.3.2) with the C-terminal region of APP family proteins. This association was confirmed by surface plasmon resonance spectroscopy *in vitro* and by co-immunoprecipitation (co-IP) from cell culture and brain extracts. The interaction of APP with uMtCK stabilized the immature form of uMtCK in cultured cells. Creatine kinase (CK) catalyzes the reversible transfer of high energy phosphate from ATP to creatine, yielding phosphocreatine for energy storage and transport (Wallimann et al., 1992). There are several isoenzymes of CK with distinct tissue and cellular distributions. In the brain, the cytosolic brain-type CK (BCK) and uMtCK comprise an energy shuttling system, which transfers energy efficiently from the site of ATP production (mitochondria) to distal parts of the cell where energy is consumed (Wallimann et al., 1998; Joubert et al., 2002). In addition, uMtCK may affect mitochondrial transport and ionic homeostasis by forming a high energy channeling complex consisting of porin and adenine nucleotide translocator (ANT) (Dolder et al., 2001; Schlattner et al., 2001). Taken together our studies suggest a new role for APP in modulation of uMtCK levels and possibly cellular energy metabolism.

5.3. Experimental Methods

5.3.1. Materials

A1 (CELQRHGYENPTYRFLEE) peptide and control randomized peptide (CFEYRNRHQETPELLGET) were described previously (Homayouni et al., 1999). The antibodies used in this study were anti-myc (Invitrogen Life Technologies), anti-eGFP (Invitrogen Life Technologies), anti-flag (clone M2, Sigma-Aldrich), anti-APP-Cterm (Sigma-Aldrich), anti-APLP1 (CT11, Calbiochem), anti-APLP2 (CT12, Calbiochem) and anti-huMtCK described in Schlattner et al. (2002) (Schlattner et al., 2002). CK isoenzymes were expressed in E.Coli and purified as described in Schlattner et al. (2000) (Schlattner et al., 2000).

5.3.2. Plasmid construction

Several expression constructs used in this study were described previously: PcDNA3-VLDLR-HA by D'Arcangelo et al (1999) (D'Arcangelo et al., 1999), PcDNA3-mAPP695 and PcDNA3-mAPLP1 by Homayouni et al (1999) (Homayouni et al., 1999). PcDNA3-APLP1-flag was generated by inserting the full-length mouse APLP1 cDNA (Homayouni et al., 1999) into PcDNA3-flag construct described in Homayouni et al (2001) (Homayouni et al., 2001). pCS2+-eGFP fusion constructs containing APP-C31 was generated by PCR amplification using nested EcoRI and XbaI primers and the full-length PcDNA3-APP as template. PcDNA3-uMtCK-myc and PcDNA3-BCK-myc constructs were made by reverse transcription PCR using wild type mouse brain RNA library. All PCR products were confirmed by sequencing. Primers used for these constructs are listed in supplemental Table 1.

5.3.3. Affinity binding and 2-D electrophoresis

Synthetic A1 and control peptides were cross-linked to agarose resin using SulfoLink kit (Pierce, USA) and packed into 1ml columns. Wild type mice brains were homogenized in brain homogenization buffer (50 mM Tris-HCl, 1% Triton X-100, 5% glycerol, 150 mM NaCl, 2 μM leupeptin and Aprotinin) and pre-cleared by centrifugation at 20,000 g for 30 minutes. The supernatants were passed through the columns and washed extensively with brain homogenization buffer. After a final wash with 50 mM Tris-HCl, the bound proteins

were eluted with rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% IPG buffer and trace of bromophenol blue) supplemented with DTT and loaded onto pH3-10L strip (Amersham Pharmacia Biotech). Isoelectric focusing electrophoresis (IEF) was performed in IPGphor (Amersham Pharmacia Biotech), followed by the second dimension SDS-PAGE on the protean II cell following manufacturer's protocols (BioRad laboratories). Proteins were visualized by silver staining as described by Yuan et al (2002) (Yuan et al., 2002).

5.3.4. Peptide fingerprinting by MALDI-TOF mass spectrometry

Gel pieces were minced, de-stained and digested with trypsin (Promega, WI) overnight at 370C. The tryptic peptides were extracted with 60% acetonitrile/5% TFA by sonication. The peptide mixture was purified by Ziptip C18 (Millipore, MA) and eluted directly onto the MALDI plate with α-cyano-4-hydroxycinnamic acid (CHCA) solution in 50% acetonenitrile/0.1% TFA. The MALDI spectra were collected using a Voyager DE-RP mass spectrometer (Framingham, MA) in the delayed extraction and reflector mode with internal calibration of trypsin autolysis products. The unique peaks in the spectra from one gel spot were compared to the tryptic peptide patterns of proteins in the non-redundant protein database at NCBI using Mass-Fit program available on the website of http://prospector.ucsf.edu.

5.3.5. Liquid chromatography Mass spectrometry (LC-MS)

These methods were described in detail previously by Yuan and Desiderio (2003) (Yuan and Desiderio, 2003). Samples were prepared as described above. Tryptic peptides extracted in 60% acetonitrile /5% TFA were concentrated by vacuum centrifugation and dissolved in 0.1% formic acid followed by separation with home-made on-line HPLC column (10 cm in length; i.d. 75 μm; packing material: C18, 200Å) and then were sequenced with nano-ESI method using an LCQdecaLC-ESI-Q-IT MS instrument (ThermoFinnigan, CA). LCQ sequence data were used to query the SWISS-PROT database using the SEQUEST program.

5.3.6. Surface plasmon resonance (SPR) spectroscopy

The interaction of A1 and random peptides with CK isoforms was analyzed by surface plasmon resonance (SPR) with a Biacore 2000™ instrument (Biacore, Uppsala). Peptides were dissolved in 10 mM Na-citrate pH 4.5 to a concentration of 0.05 mg/ml. About 300RU peptide per lane (400 fmole/mm2) were immobilized by thiol disulphide exchange between their C-terminal cysteines and PDEA that was previously coupled to the carboxydextran surface of CM5 sensorchips according to the supplier's instructions (PDEA thiol coupling kit, Biacore, Uppsala). Excess reactive groups were deactivated by 50 mM cysteine in 1 M NaCl. CK protein stocks were exchanged against running buffer (50 mM TES pH 7.4, 100 mM NaCl, 0.1% Triton X-100) via gel filtration (PD10 columns, Amersham). CK-peptide association and dissociation kinetics were recorded at 25°C in running buffer at a flow rate of 1.2 ml h-1 with CK concentrations ranging from 25 to 1600 nM. The peptide surface was recovered by a final injection of 1M NaCl. Kinetic data were corrected for background binding to the empty PDEA surface. The affinity was derived from the slope of a plot Req/c versus c, analogous to a Scatchard plot. Data are given as mean ± standard deviation (SD).

5.3.7. Cell culture and co-immunoprecipitation

COS-7 cells were maintained in DMEM containing 10% FCS at 370C in 5% CO2. Cells were transiently transfected with plasmids using FuGene 6.0 following the manufacture's protocol (Roche, Germany). About 48 hours post-transfection, the cells were harvested in lysis buffer (PBS containing 1% Triton X-100, 5% glycerol, 150 mM NaCl and 2 μM leupeptin and Aprotinin). The lysates were cleared at 20,000g for 20 minutes in a microcentrifuge before incubation with primary antibody at 4°C overnight and followed by incubation with protein-G sepharose beads (Pierce Chemical Co., Rockford, IL) at 4°C for 2 hours. The Protein complexes were washed three times with lysis buffer and the bound proteins were eluted with 2× Laemmli buffer and analyzed by SDS-PAGE and Western blotting. For brain co-IP, the protein complexes were washed 3 times in RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.2, 1% Triton X-100, 0.1% SDS, 1% Deoxycholate, 5 mM EDTA).

5.3.8. SDS-PAGE and immunoblot analysis

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane for immunoblot analysis. The membrane was blocked in 5% non-fat milk in TBST (263 mM NaCl, 150 mM Tris-HCl, 0.5% Tween-20, pH 7.4) at room temperature for one hour and incubated with anti-myc antibody (1:5,000), anti-huMtCK (1:2,000), anti-flag M2 (1:5,000), anti-APP (1:8000), anti-APLP1 (1:5,000), anti-APLP2 (1:2,000); or anti-EGFP (1:350). The membrane was subsequently incubated with either anti-rabbit or anti-mouse IgG-HRP conjugated secondary antibody for another hour. Proteins were visualized using chemiluminescence Supersignal West Pico or West Femto kit (Pierce Chemical Co., Rockford, IL).

5.3.9. Differential centrifugation

The crude mitochondria preparations from transfected COS-7 cells were collected using Mitochondria Isolation Kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's protocol. Briefly, transfected cells were pelleted at 1000×g for 5 minutes, washed with 1× DPBS and resuspended in reagent A. After addition of reagent B and C, the supernatant was centrifuged at 700×g and again at 3000×g to obtain the mitochondria-enriched fraction.

5.3.10. Metabolic labeling and IP

Transfected COS7 cells were starved in labeling medium (DMEM without L-Met, L-Gln and L-Cys, 10% dialysed FBS, 1% P/S, 2 mM Glu) for 1hr, and then incubated in labeling medium containing 0.2 mCi/ml [35S]-protein labeling mix (NEG-772, PerkinElmer Life Sciences, Inc.) for 8 minutes at 37°C. Immediately after labeling, the cells were washed in normal DMEM medium. At specific chase time points, the cells were lysed in 1% Triton lysis buffer as described above. uMtCK was immunoprecipitated using anti-myc antibody and protein G sepharose beads at 4°C for 2 hours. The immunoprecipitates were washed three times with RIPA buffer and subjected to SDS-PAGE and autoradiography.

5.3.11.Primary neuronal culture and immunocytochemistry

Embryonic day 18 mouse telecephalon was dissected and the cells were dissociated by trituration in Hank's balanced salt solution (HBSS) w/o Ca^{2+} and Mg^{2+}, supplemented with 1.0 mM sodium pyruvate and 10 mM HEPES. Approxiamately 3×106 cells were plated on poly-D-lysine-coated coverslips in a 60 mm dish. Cells were maintained in B27 supplemented neurobasal medium for 4 days and then fixed and permeabilized in 4%PFA/0.1%Triton in 1×PBS. After blocking in 3% horse serum at room temperature for 15 minutes, the cells were incubated with anti-uMtCK (1: 500) and anti-APP (1:500) in 1×PBST for 1 hr at RT, washed 3×5 min with PBST, then incubated with FITC conjugated anti-rabbit (1:1,000) and TR conjugated anti-mouse (1:1,000) for 30 min at room temperature. Fluorescence was examined by confocal laser microscopy (BIO-RAD MRC-1024, BioRad laboratories).

5.4. Results

5.4.1. Identification of ubiquitous mitochondrial creatine kinase as an APLP1 interacting protein.

To identify protein complexes associated with cytoplasmic domain of APP proteins, we used a functional proteomic approach. Mouse brain homogenates were passed through columns containing Sepharose beads cross-linked with synthetic peptide (A1 peptide) corresponding to the last 18 amino acids of APLP1 cytoplasmic domain, which is highly conserved among the APP family proteins (Homayouni et al., 1999). After extensive washing, the bound proteins were eluted and subjected to 1-D or 2-D electrophoresis and silver staining. We identified a number of proteins that preferentially bound to A1 peptide, and not to the randomized control peptide, by tryptic peptide fingerprinting using MALDI-TOF spectrometry (data not shown). Among these proteins were several subunits of adaptor-related protein complex AP-2, which are known to bind to NPxY-containing sequences and are necessary for clathrin-mediated endocytosis (Bonifacino and Traub, 2003). Another protein that preferentially bound to the A1 peptide was identified as uMtCK (NP_034027) (Figure 15A and B).

To confirm the interaction between uMtCK and A1 peptide, we performed biochemical

pulldown experiments using cell lysates transiently transfected with either mitochondrial uMtCK-myc or cytosolic BCK-myc constructs. Figure 15C shows that A1 peptide specifically interacted with uMtCK-myc but not with BCK-myc. Interestingly, a higher molecular weight band of uMtCK-myc seemed to preferentially bind to the A1 peptide.

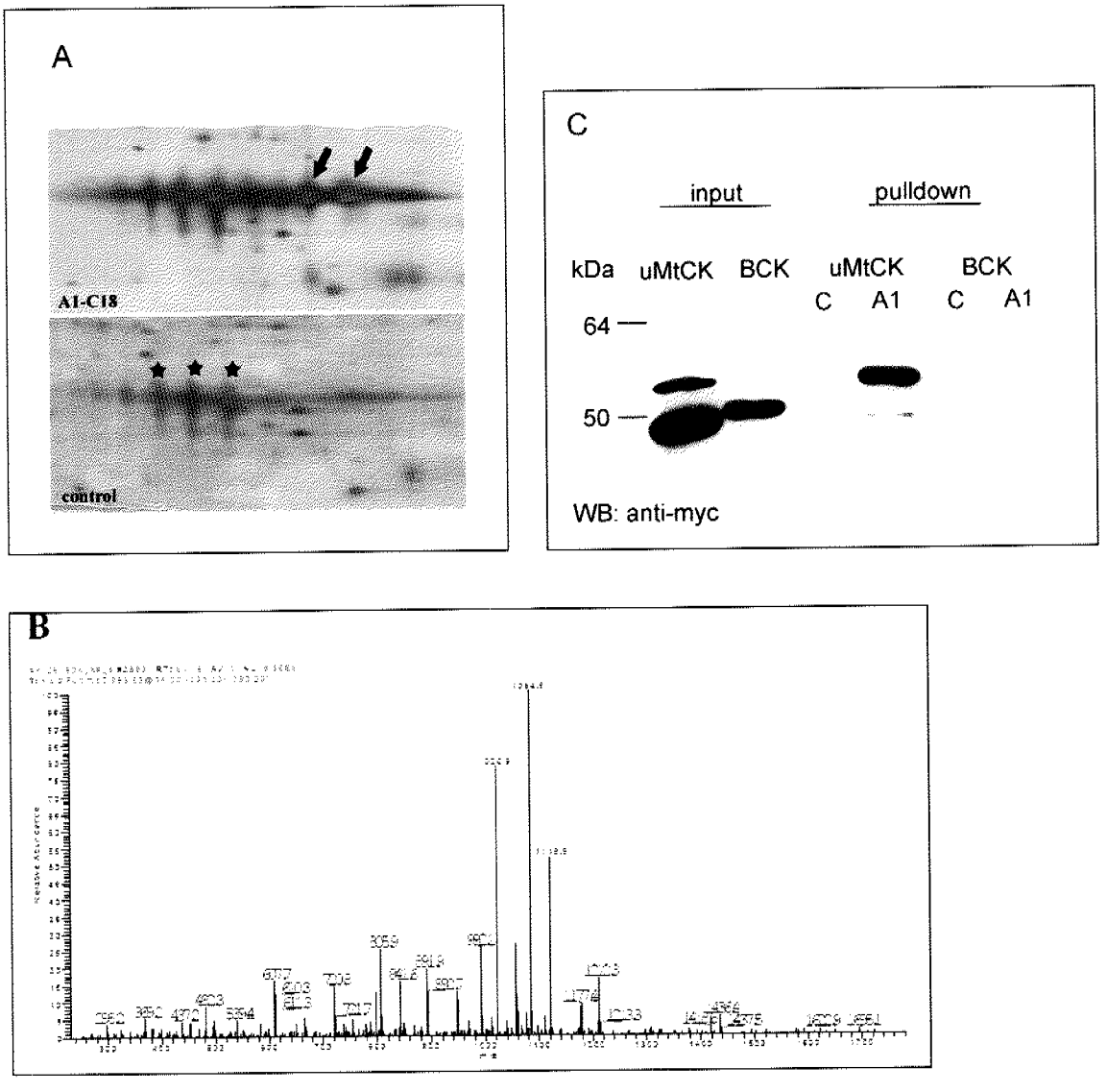


Figure 15: Identification of ubiquitous mitochondrial creatine kinase as a novel APLP1 interacting protein.

(A) 2-D gel images of proteins that bound to A1 peptide (top panel) compared with control peptide (bottom panel). Two proteins that preferentially bound to the A1-peptide (arrows) were excised from the gel and processed for tryptic peptide fingerprinting. Stars indicate proteins that non-specifically bound to the control peptide. (B) Mass spectra of peptides obtained from the proteins denoted by arrows in A. Stars indicate the peptides that corresponded to uMtCK. (C) Confirmation of uMtCK-A1 interaction by biochemical pulldown

experiments. Lysates from COS7 cells transiently transfected with either uMtCK-myc or BCK-myc were incubated with either A1-peptide (A1, lanes 4 and 6) or control randomized peptide (C, lanes 3 and 5) immobilized to sepharose beads. The protein complexes were separated by SDS-PAGE and examined by Western Blotting using anti-myc antibodies. As a control, 10% of input lysates were also loaded on the gels (lanes 1-2).

Since biochemical affinity experiments can identify multi-protein complexes, we tested whether the interaction of A1 peptide with uMtCK is direct or indirect by using surface plasmon resonance (SPR) spectroscopy. The association and dissociation kinetics of A1 peptide binding to purified recombinant BCK or uMtCK was determined in 'physiological' buffer conditions (0.1% Triton, 100 mM NaCl). Consistent with the pull-down experiments above, we found that uMtCK bound preferentially to A1 peptide as compared to the random peptide (Figure 16A and B), while cytosolic BCK bound neither peptide (Figure 16C and D). Because the uMtCK-A1 interaction showed a very fast association rate (Figure 16B), a direct fit of the kinetics was impossible. However, Scatchard plot analysis revealed an affinity of 175 ± 30 nM for the A1 peptide, as compared to 430 ± 115 nM for the low level binding to random peptide. These data demonstrate that interaction of A1 peptide with uMtCK is direct and of rather high affinity. Given the fact that A1 and the control peptide have identical amino acid compositions, the differences in their binding properties obtained under these conditions are considerable.

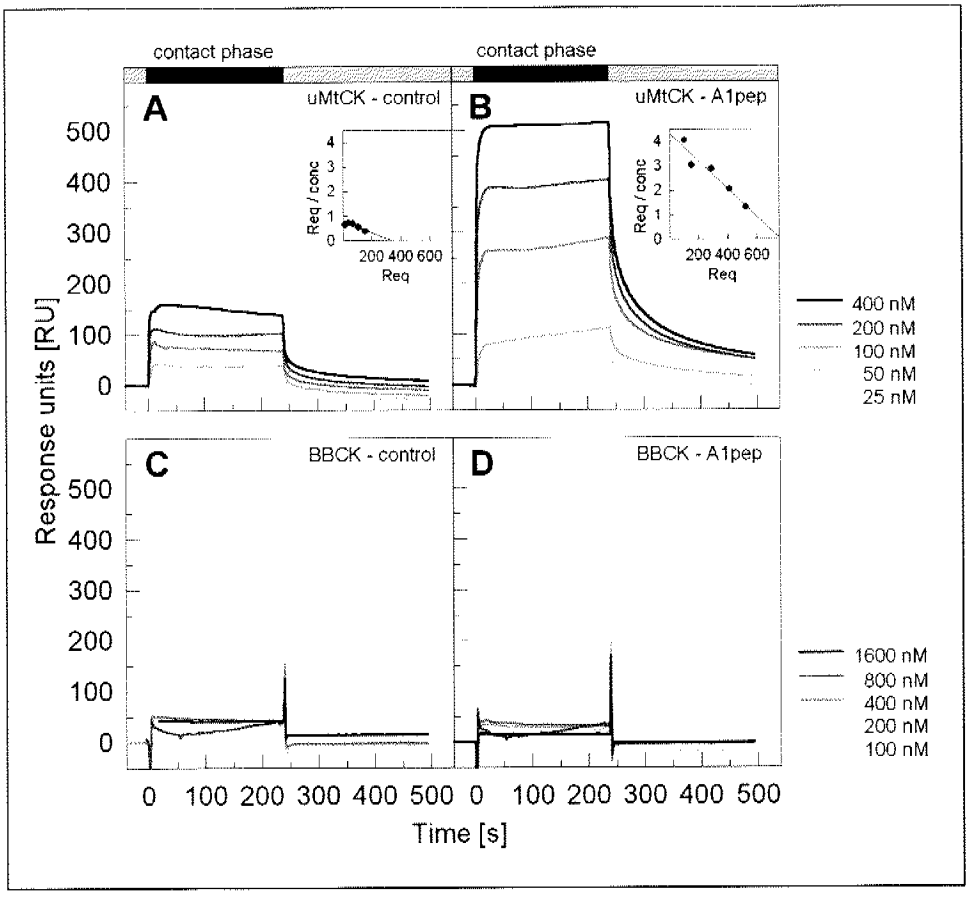


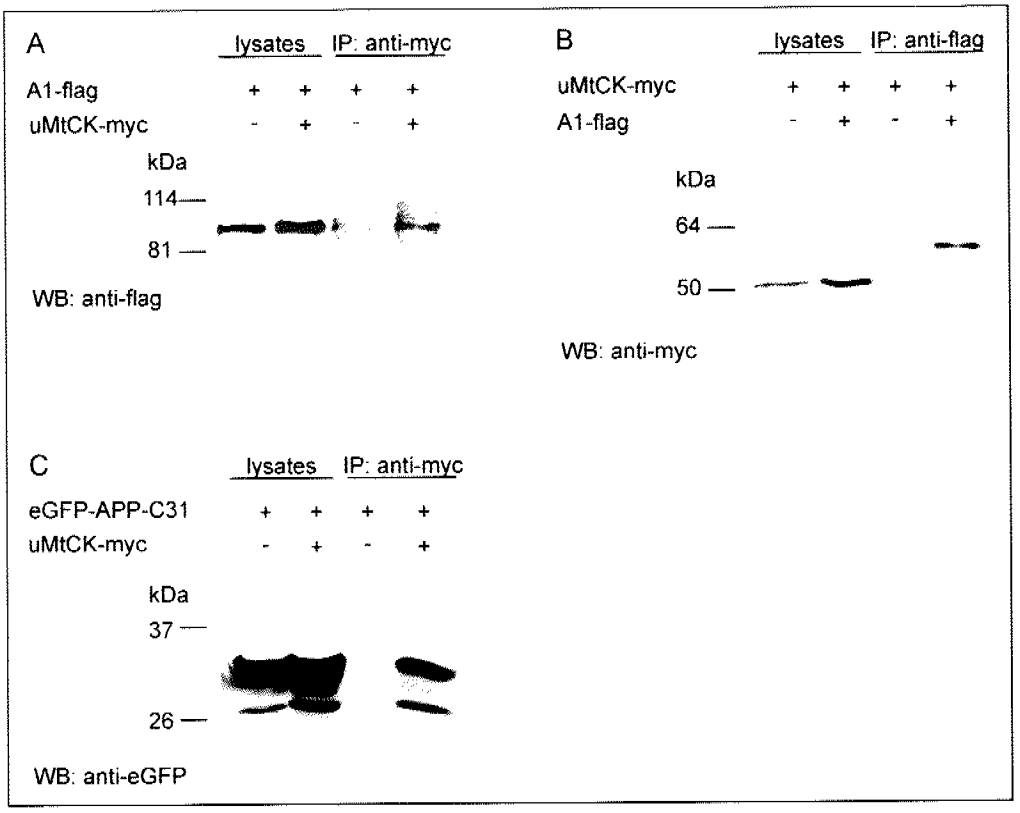
Figure 16: Interaction of uMtCK and APLP1 peptide is direct, specific, and of high affinity.

Representative SPR traces of contact and dissociation phase of human uMtCK (A, B) or human BCK (C, D) with randomized control peptide (A, C) or specific APLP1 peptide (B, D). Representative traces for 25, 50, 100, 200, and 400 nM octameric uMtCK and 100, 200, 400, 800, 1600 nM dimeric BCK are shown. Inserts in A and B, Scatchard plots derived from the relationship between octamer concentration (conc, in nM) and SPR signal at equilibrium (Req, in RU) to estimate affinity KD. SPR data were recorded at 25°C and a flow rate of 1.2 ml h-1 with 50 mM TES pH 7.4, 100 mM NaCl and 0.1% Triton X-100 as running buffer. Response units (RU) are proportional to the amount of bound CK.

uMtCK associates with full-length APLP1 and C-terminal fragment of APP in cultured cells. To examine whether full-length APLP1 and uMtCK interact in cells, we carried out co-immunoprecipitation experiments using transiently transfected COS7 cells. We found an association between uMtCK-myc and APLP1-flag in both uMtCK and APLP1 immunoprecipitates (Figure 17A and B). Consistent with our earlier results, we found that only the large molecular weight form of uMtCK-myc associated with full-length APLP1 in COS7 cells.

APP family proteins can be proteolytically cleaved by secretases and caspases, which lead to intracellular release of their cytoplasmic domains (Schienfeld, et al., 2002; Eggert et al., 2004; Galvan et al., 2002). Therefore, we tested if the soluble intracellular domain of APP also interacts with uMtCK. Co-immunoprecipitation experiments were performed using lysates from COS7 cells which were transiently transfected with uMtCK-myc and an eGFP fusion construct containing the C-terminal 31 amino acids in APP (eGFP-APP-C31). We found that eGFP-APP-C31 strongly associated with uMtCK (Figure 17C).

To investigate the subcellular site of interaction between APP and uMtCK, we performed laser confocal microscopy on COS7 cells transiently transfected with uMtCK-myc along with either full-length APLP1-flag or eGFP-APP-C31 (Figure 17D and E). As expected, uMtCK staining was observed in irregular structures resembling mitochondria around the nucleus and throughout the cytoplasm. Similar structures were observed using the mitochondrial marker MitoTracker (Molecular Probes) in COS7 cells (data not shown). On the other hand, APLP1 staining was observed primarily in the ER/Golgi and in smaller vesicular structures throughout the cytoplasm. Very little overlap in staining was observed between uMtCK and APLP1 staining. This result suggests that APLP1 is not targeted to mitochondria, the predominant site of uMtCK protein. However, it is possible that the interaction of the cytosolic uMtCK preprotein with APLP1 occurs in the ER/Golgi at concentrations that are below detection in this assay. In contrast to APLP1-myc, eGFP-APP-C31 fluorescence was intense in the nucleus and diffuse throughout the cytoplasm (Figure 17D and E). Interestingly, the eGFP-APP-C31 staining was absent in the mitochondria and more pronounced around the mitochondria in the perinuclear region, resulting in a honeycomb like staining pattern.



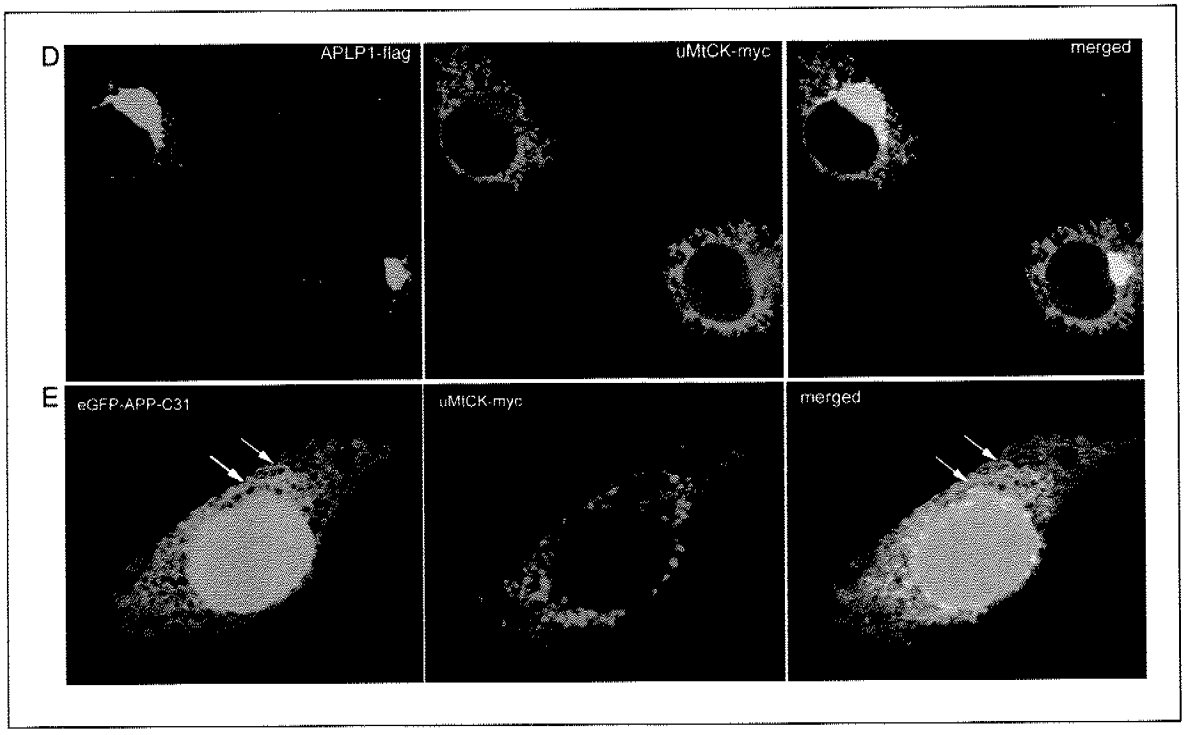


Figure 17: uMtCK interacts with full length APLP1 in cultured cells.

(A) Protein lysates from COS7 cells transfected with APLP1-flag alone (lane 3) or APLP1-flag plus uMtCK-myc (lane 4) constructs were immunoprecipitated with anti-myc and immunoblotted with anti-flag

antibodies. (B) Protein lysates from COS7 cells transfected with uMtCK-myc alone (lane 3) or uMtCK-myc plus APLP1-flag (lane 4) constructs were immunoprecipitated with anti-flag and immunoblotted with anti-myc antibodies. (C) Protein lysates from COS7 cells transfected with eGFP-APP-C31 alone (laned 3) or eGFP-APP-C31 plus uMtCK-myc (lane 4) constructs were immunoprecipitated with anti-myc antibody and immunoblotted with anti-eGFP antibodies. As a control, 3% of input lysates were loaded on the gels (lanes 1 and 2, panels A-C). D, COS7 cells were transfected with APLP1-flag and uMtCK-myc. Subcellular localization of APLP1-flag and uMtCK-myc was examined by immunofluoresence using laser confocal microscopy. APLP-Flag was visualized using anti-APLP1 polyclonal antiserum (CT11) and Alexsa480-red conjugated anti-rabbit antibody (Green fluorescence, left panel) and uMtCK-myc was visualized using monoclonal anti-myc antibody and Alexsa594-conjugated anti-mouse antibody (Red Fluorescence, middle panel). A merged image is shown in the right panel. (E) COS7 cells were transfected with eGFP-APP-C31 and uMtCK-myc. Subcellular localization of eGFP-APP-C31 and uMtCK-myc was examined by immunofluoresence using laser confocal microscopy. eGFP-APP-C31 is shown in green fluorescence (left panel) and uMtCK-myc was visualized using monoclonal anti-myc antibody and Alexsa594-conjugated anti-mouse antibody (red fluorescence, middle panel). A merged image is shown in the right panel. Arrows mark the honeycomb staining pattern observed for eGFP-APP-C31 protein due to its absence in the mitochondria, marked uMtCK staining.

5.4.2. uMtCK associates with all three APP family members in brain.

To determine whether the interaction between uMtCK and APP family proteins occur in physiological conditions, we performed co-IP experiments using brain homogenates. uMtCK was immunoprecipitated from adult mouse brain homogenates using anti-human uMtCK (anti-huMtCK) antiserum. The protein complexes were stringently washed with RIPA buffer and then examined by SDS-PAGE and Western blotting using anti-APP, anti-APLP1 or anti-APLP2 antibodies. We found that anti-huMtCK antiserum, but not the pre-immune serum precipitated all three APP family members (Figure 18A). Interestingly, only the lower molecular weight forms, presumably the immature un-glycosylated forms (Lyckman et al., 1998), of APP and APLP2 associated with uMtCK. In addition, using confocal laser microscopy on cultured cortical neurons, we found that APP co-localizes with uMtCK predominantly in the perinuclear region, although some co-localization was also observed along the processes (Figure 18B). Co-localization of APP and uMtCK in neurons is different from our observations in COS7 cells (above) and may account for the high efficiency of the co-IP in brain (Figure 18A) as compared to COS7 cells (Figure 17A and B). Taken together, these data suggest that interaction of uMtCK and APP family

proteins can occur under physiological conditions in the brain.

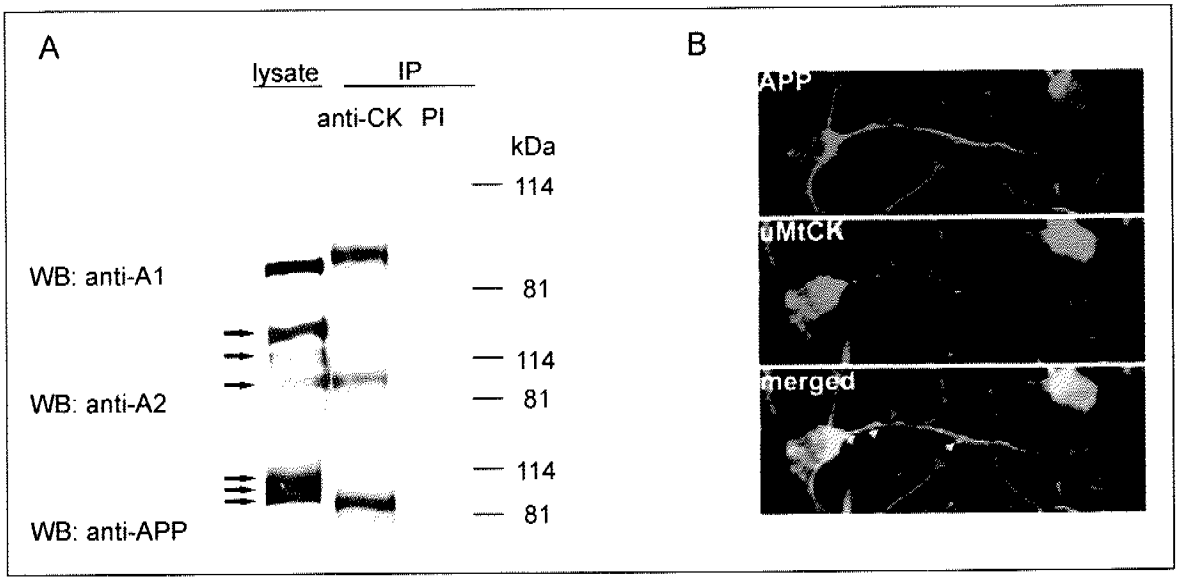


Figure 18: uMtCK interacts with APP family proteins in brain.

(A) Approximately 200 µg of total protein from mouse brain homogenates were immunoprecipitated with either anti-huMtCK antiserum (anti-CK) or the pre-immune serum (PI). Protein complexes were washed in RIPA buffer and examined by immunoblotting using anti-APLP1 (top panel), anti-APLP2 (middle panel) and anti-APP (bottom panel) antibodies. Approximately 20 µg of total protein was loaded on the gel as control (lysate). Arrows indicate multiple forms of APLP2 and APP, presumably due to glycosylation. (B) Subcellular localization of uMtCK and APP in primary neuronal cultures (4 days *in vitro*) was examined by immunofluoresence using laser confocal microscopy. APP was visualized using anti-APP monoclonal antiserum (22C11) and Texas-red conjugated anti-mouse antibodies (top panel) and uMtCK was visualized using rabbit anti-human uMtCK and FITC-conjugated anti-rabbit antiserum (middle panel). A merged image is shown in the bottom panel to better illustrate the subcellular co-localization (yellow). Arrows point to punctate staining along the neuronal processes.

5.4.3. Stabilization of uMtCK preprotein by APP family proteins.

During the co-IP experiments, we observed that the steady state level of the higher molecular weight form of uMtCK (HMr-uMtCK) was increased by co-transfection of all three family members of APP using cultured COS7 cells (data not shown). We investigated this phenomenon further in a new series of experiments. We found that expression of full-length APLP1-flag or eGFP-APP-C31 protein led to an accumulation of HMr-uMtCK (Figure 19A). These effects were specific since co-transfection of very low-density lipoprotein receptor (VLDLR), another transmembrane protein containing a cytoplasmic NPxY sequence, did not increase the HMr-uMtCK. In addition, the effect of eGFP-APP-C31 was concentration dependent, as increasing amounts of eGFP-APP-C31 produced more HMr-uMtCK (Figure 19B). Notably, the lower Mr form of uMtCK was also increased, especially under the lower amount of eGFP-APP-C31 when the accumulation of HMr-uMtCK was not so dramatic.

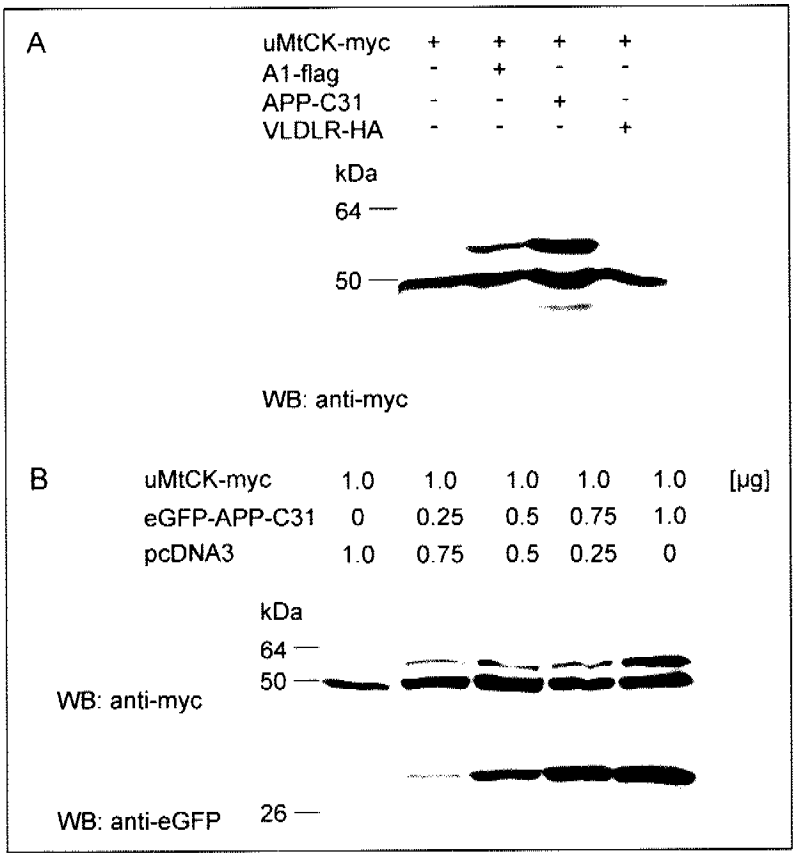


Figure 19: Stabilization of high molecular weight (HMr) uMtCK by APP family proteins.

(A) Immunoblotting was performed using anti-myc antibody on 30μg of total protein prepared from COS7 cells transfected with uMtCK-myc alone (lane 1) or co-transfected with APLP1-flag (lane 2), eGFP-APP-C31 (lane 3) or VLDLR-HA (lane 4). (B) Immunoblotting was performed using anti-myc (top panel) or anti-eGFP (bottom panel) on COS7 cells transfected with uMtCK-myc alone (lane 1) or along with increasing amounts of eGFP-APP-C31 construct (lanes 2-5).

uMtCK is highly susceptible to oxidative damage (Wendt et al., 2003). Thus one possibility is that transfection of APP proteins increases oxidative stress, resulting in a modification of uMtCK that retards its migration in SDS gels. On the other hand, APP proteins may stabilize the immature unprocessed form of uMtCK, which contains a N-terminal mitochondrial targeting sequence. Therefore, we set out to identify the HMr-uMtCK that accumulated after APP co-expression. First, the higher molecular weight form of uMtCK appeared to be approximately 5 kDa larger than expected. This is consistent with the calculated Mr of the 39 amino acid residues leader sequence, which is cleaved after its mitochondrial targeting. In crude fractionation experiments, we found that the HMr-uMtCK was not associated with the mitochondria whereas the lower uMtCK band was enriched in the mitochondrial fraction (Figure 20A). We next examined the turnover rate and degradation of the two uMtCK bands by performing pulse chase analysis (Figure 20B). We found that both bands were labeled with [35S] -Met/Cys immediately after the 8 min labeling period. Within 2 hrs after the labeling period, the upper band was decreased substantially, while the lower band persisted. This is consistent with the notion that the upper band is the pre-processed form of uMtCK that is rapidly converted into the more stable mature mitochondrial form after cleavage of the signal sequence. When cells were co-transfected with eGFP-APP-C31, both upper and lower bands of uMtCK were increased. If indeed the HMr-uMtCK were derived from oxidative or post-translational modification of the mature uMtCK form, we would expect to see a decrease in the amount of the lower band in proportion to an increase in the amount of the upper band. Thus our results suggest that APP stabilizes the uMtCK preprotein. Lastly, we examined whether the HMr-uMtCK contained the signal sequence using electrospray tandem mass spectrometry. COS7 cells were transiently transfected with uMtCK-myc and eGFP-APP-C31 to enrich the HMr-uMtCK band and then processed for A1 affinity binding as described above. Two bands (~Mr 47 and 52 kDa) were excised from gels and digested with trypsin. The resulting peptides were separated by LC/MS and sequenced by electrospray mass

spectrometry. Both bands were identified as uMtCK, however, only the upper band contained a peptide containing the signal sequence for uMtCK (Figure 20C). Taken together these results indicate that the high molecular weight form of uMtCK, which is stabilized by APP, is indeed uMtCK pre-protein and not an oxidative derivative.

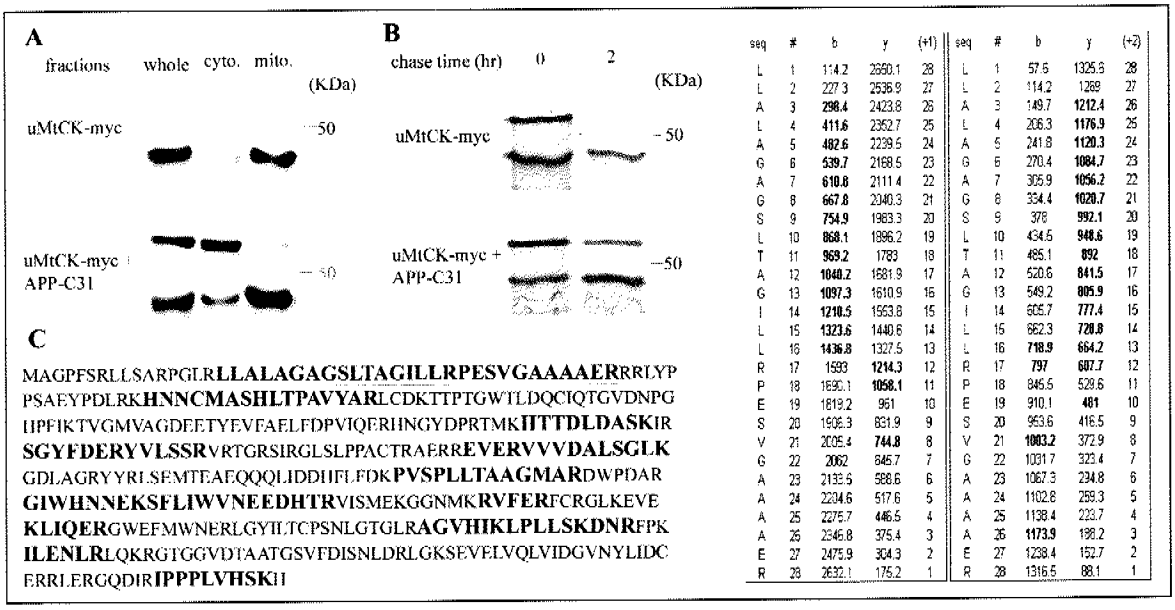


Figure 20: Identification of the high Mr band of uMtCK as uMtCK preprotein.

(A) Differential centrifugation was performed on COS7 cells transfected with uMtCK-myc alone (upper panel) or along with eGFP-APP-C31 (lower panel). uMtCK was detected by immunoblotting using anti-myc antibodies on whole lysate (whole), cytosolic (cyto.) and mitochondria-enriched fractions (mito.). (B) COS7 cells transfected with uMtCK-myc alone (upper panel) or along with eGFP-APP-C31 (lower panel) were metabolically labeled with 35S-Met/Cyst for 8 minutes and then chased with cold medium for 2 h. uMtCK was immunoprecipitated using anti-myc antibodies, separated by SDS-PAGE and visualized by autoradiography. (C) The high Mr band observed in COS7 lysates transfected with uMtCK-myc and eGFP-APP-C31 was excised from a SDS gel, trypsinized and then subjected to electrospray tandem mass spectrometry. We identified and sequenced 18 peptides covering 36.8% of uMtCK protein sequence (bolded, left panel). The sequence of one peptide (right panel) corresponded to the N-terminal signal sequence in uMtCK (underlined, left panel). Arrow in left panel indicates the predicted cleavage site of uMtCK signal sequence. The right panel shows the theoretical masses of b- and y-fragment ions of this peptide. The detection of continuous y-ion series and b-ion series (shaded) confirms the amino acid sequence of this peptide.

5.5. Discussion

In this study, we show that uMtCK interacts with APP family proteins in cultured cells as well as in the brain using co-immunoprecipitation assays. This interaction is direct and of high affinity as shown by surface plasmon resonance experiments. Importantly, we found that overexpression of APP cytoplasmic domain in cultured cells stabilized a higher molecular weight form of uMtCK, which we identified as the uMtCK preprotein containing the mitochondrial targeting sequence.

uMtCK is encoded by the nuclear genome, synthesized by ribosomes, and then translocated to mitochondria via its N-terminal signal sequence. In the mitochondria, uMtCK dimmers or octamers are localized in the cristae and the intermembrane space (contact sites) of mitochondria (Schlattner et al., 1998). It is likely that mitochondrial targeting of uMtCK involves cytosolic chaperones such as mitochondria-import stimulating factor (MSF) or arylhydrocarbon receptor-interacting protein (AIP) (Mihara and Omura, 1996; Yano et al., 2003) as well as the translocase of outer mitochondrial membrane (TOM) protein family (Pfanner and Wiedemann, 2002). Mitochondrial chaperones assist in the import of mitochondrial proteins by directly binding to the preprotein and preventing their aggregation, misfolding and proteolysis. Overexpression of mitochondrial chaperone proteins in cultured cells has been shown to stabilize mitochondrial preproteins (Yano et al., 1998). Consistent with these observations, we found that APP C31 expression decreased the turnover and increased steady state levels of uMtCK preprotein. Indeed, we have found that over-expression of full-length APLP1 in HEK293T cells results in an increase in dimeric and octameric CK activity (Bürklen and Wallimann, unpublished observations, 6. Chapter 3). These results suggest that APP may function as a cytosolic chaperone for uMtCK preprotein and consequently regulate CK activity and cellular energy metabolism.

The hypothesis that APP may function as a chaperone protein was proposed over ten years ago by Liautard (1994) (Liautard, 1994). APP is known to be an acute phase response gene induced by cellular stress and neuronal injury. For example, APP expression is induced together with several stress response proteins such as C-FOS and heat shock protein 70 after cerebral ischemia (Hall et al., 1995; Lin et al., 1999). Since ischemia is an energy

deficiency insult, it is reasonable to assume that neurons would respond by stabilizing enzymes that are critical for production and maintenance of energy. Indeed, APP has been shown to play a neuroprotective role in neurons and in cultured cells. For example, Koegel et al (2003) recently demonstrated that APP, but not APPswe, protects PC12 cells against the unfolded protein response induced by prolonged treatments with inhibitors of glycosylation (tunicamycin) or ER-to-Golgi transport (brefeldin A) (Kogel et al., 2003).

Accumulating evidence suggest that mitochondria play a central role in neurodegeneration as well as AD through modulation of cellular energy, calcium levels and reactive oxygen species (Swerdlow and Khan, 2004). An alternative hypothesis has been proposed for late-onset AD, which states that neurodegeneration in AD results from mitochondria genome deficits which lead to accumulation of damage caused by reactive oxygen species (Mattson, 1997; Swerdlow and Khan, 2004). Clearly, there is mitochondrial dysfunction and increased oxidative stress in AD brains (Smith et al., 1991; Gibson et al., 1998; Eckert et al., 2003). Positron Emission Tomography studies showed reduced brain glucose utilization in early stages of AD brain (Rapoport et al., 1996). In addition, a group of genes involved in mitochondrial metabolism were up-regulated in the early stage of pathological changes in the APP transgenic mouse model (Reddy et al., 2004). Together, these evidence point to the close link between mitochondria and early pathogenesis of AD. However the exact role APP plays in this model is not clear. An N-terminal sequence in APP was shown to target APP to mitochondria in a membrane arrested form and cause disruption of mitochondrial metabolism (Anandatheerthavarada et al., 2003). However, in our studies, we did no observe a significant targeting of full-length APLP1 or APP C-terminal fragment to mitochondria (Figure 3D & E).

uMtCK has been shown to be important to mitochondrial function in several ways. It has a protective role against oxidative or toxic insults to the mitochondria (O'Gorman et al., 1997; Kanazawa et al., 1998; Dolder et al., 2003; Hatano et al., 2004). uMtCK is also important for mitochondrial energy channeling (Wallimann et al., 1992; Wallimann et al., 1998) and permeability transition (O'Gorman et al., 1997; Dolder et al., 2003). Interestingly, the fact that transgenic ablation of uMtCK leads to impaired learning and memory in mice (Streijger et al., 2004) suggests that uMtCK may play a role in cognitive deficits such as in AD. Therefore, the identification of a direct interaction between APP

and uMtCK may provide important insights not only into the physiological roles of APP family proteins but also on the pathological roles of APP and the mitochondria in the neurodegeneration associated with AD.

Acknowledgements

This work was supported by University of Tennessee, Center for Neurobiology of Brain Diseases and University of Tennessee, Center for Genomics and Bioinformatics (to R.H.); The National Institutes of Health RR 10522, RR 14593 and the National Science Foundation DBI 9604633 (to D.M.D.); and The Swiss Society for Research on Muscle Diseases and the Swiss Natl. Science Foundation grant Nr. 3100/-0-/02075/1 (to T.W). We thank members in Homayouni lab for helpful discussions.

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6. Chapter 3

Additional results: Amyloid precursor protein (APP) and ubiquitous mitochondrial creatine kinase (uMtCK) interaction: a mitochondrial study

6.1. Abstract

We recently identified an interaction between the amyloid precursor protein family members (APP) and the ubiquitous mitochondrial creatine kinase (uMtCK). The APP family consists of the amyloid-like proteins 1, 2 (A1, A2) and the amyloid precursor protein (APP). They all share a high homology within their C-terminus and have the consensus motif NPxY in common (Borg et al., 1996; Homayouni et al., 1999). The interaction between APP and uMtCK was shown by affinity chromatography, using a peptide corresponding to an 18 amino acid sequence of the A1 protein including the NPTY motif coupled to a matrix and a mouse brain homogenate (X.Li et al. 2004). uMtCK was then identified by 2D gel-electrophoresis and mass spectrometry.

Creatine kinase (CK) isoenzymes catalyze the reversible transfer of the phosphoryl group from phospho-creatine (PCr) to ADP, thus regenerating ATP. CK is found in tissues with high and fluctuating energy turnover, e.g. skeletal and cardiac muscle, brain and photoreceptor cells, and spermatozoa (Wallimann et al., 1989). In vertebrates, four isoforms are expressed in a tissue specific manner: two cytosolic forms, MCK and BCK, and two mitochondrial forms, ubiquitous and sarcomeric MtCK (Eppenberger et al., 1971).

Surface Plasmon resonance (SPR) analysis showed that *in vitro* the binding between uMtCK and APP is direct and of high affinity (175 nM). We confirmed this interaction *in vivo* by co-immunoprecipitation experiments with mouse brain homogenates, as well as with transfected cell lysates. Interestingly, co-transfection of uMtCK and APP or the C-terminal construct of APP (C31) in COS7 cells revealed the appearance of a high molecular weight band probably due to the accumulation of APP or C31. We identified this band by mass spectrometry as uMtCK preprotein with its mitochondrial import sequence still present.

From these data we hypothesize that APP may act as a scavenger or chaperon for uMtCK and its over-expression may lead to an altered mitochondrial CK localization and activity. We therefore analyzed function, localization of mitochondria and enzymatic activity of uMtCK in A1 over-expressing HEK 293 cells. We were also looking at the oxidation status of proteins in these cells to exclude an inhibitory effect on CK activity, as CK enzymes are known to be highly sensitive to oxydative damage (Mihm et al., 2001; Wendt et al., 2002). Oxydative damage and mitochondrial dysfunction are discussed in the context of neurodegenerative disorders such as Alzheimer's Disease (AD). Recent studies in a transgenic mouse model of Alzheimer disease revealed mitochondrial dysfunction, ATP depletion, reduced cytochrome c odixase activity and decreased basal levels of mitochondrial membrane potential (Baloyannis et al., 2004; David et al., 2005; Gotz et al., 2004; Schlattner et al., 2002a). It is therefore important to elucidate the role in energy metabolism of the creatine/phospho-creatine/CK (Cr/PCr/CK) system in the context of an altered APPs expression.

Our data suggest that the interaction between A1 and uMtCK has beneficial effects on the cell by protecting them from oxidative stress and assuring MtCK function in the mitochondria. Further, A1 expression leads to an up-regulation of CK activity.

6.2. Experimental Procedures

6.2.1. Materials

The antibodies used in this study were anti-APP-Cterm (Sigma, Buchs, Switzerland), anti-huMtCK described in Schlattner et al. 2002 (Schlattner et al., 2002b), anti-HA (Roche), anti-Calnexin (C-term, Chemicon international), anti-VDACI and anti-DNP (Chemicon international).

6.2.2. Plasmid Constructs

pcDNA3-mAPLP1 was described previously by Homayouni et al (1999) (Wallimann et al., 1977), pcDNA3-APLP1-HA (A1), pcDNA-APLP2-HA (A2), pcDNA-APP-HA (APP) were kindly provided by Xiaofan Li (University of Tennessee).

6.2.3. Cell Culture and transfection

HEK 293 cells were grown in DMEM high glucose and stable glutamine medium (Invitrogen) supplemented with 10% FCS, 1% penicillin/streptomycin in 5% CO2. Cells were transiently transfected with A1, A2 or APP plasmids using Fugene 6 transfection reagent following the manufacturer's instructions. After 24 hours, cells were lysed in lysis buffer (20mM Tris, pH 7,4, 75mM NaCl, 2,5mM MgCl2, 0,1% Nonidet P40, 1mM DTT, protease inhibitor Complete tablets (Roche)). DNA was degraded by adding Benzonase (Roche) and incubating for 20 on ice. Cell debris was removed by centrifugation for 30 min, 4°C at 13000 rpm on a tabletop centrifuge. The pellet was resuspended in lysis buffer and protein concentration was determined using the DC Protein Assay (BioRad).

6.2.4. Cell fractionation

HEK 293 cells were grown to 90% confluency and harvesting was carried out with the sub cellular proteome extraction kit (S-PEK, ProteoExtract, and Calbiochem). By following the manufacturer's instructions for the protocol of adherent cells different fractions were obtained. Preparation resulted in a cytosolic fraction, an organelle/membrane fraction including mitochondria, a nucleic fraction and cytoskeleton proteins. Protein concentration was determined using the DC Protein Assay (BioRad). For functional studies (CPE, CK activity measurement) mitochondria were used immediately. For further biochemical analysis (WB, Oxyblot) sample was stored at -20°C.

6.2.5. Immunoblotting

Protein separation was performed using the NuPAGE® Bis-Tris Gels 4-12% (Invitrogen) loading 20 µg of total protein. Proteins were trans-blotted onto a nitrocellulose membrane (Schleicher&Schuell, Bottmingen, Germany) and were then stained by Red Ponceau S (Sigma, Buchs, Switzerland). The membrane was blocked in blocking solution (5% fatty acid-free milk powder in TBS-T (150 mM NaCl, 25 mM Tris-HCl, pH 7,4, 0,05% Tween 20) for 1 hr at room temperature. After washing for 10 min, membranes were incubated with the primary antibodies (anti-uMtCK 1:1000, anti-APP (C-term) 1:4000, anti-Calnexin 1:1000, anti-VDAC 1: 2000, anti-HA 1:20000 and anti-DNP 1:250 in blocking solution) and the appropriate secondary antibodies.

6.2.6. CK activity measurement with a coupled enzyme assay

CK activity for the forward reaction was determined using coupled enzyme assay modified after Wallimann et al [13]. Briefly, 50 µg of total lysate were added to the reaction mixture (5,13 U/µl lactate dehydrogenase, 1,6 U/µl pyruvat kinase, 1 mM phopshoenolpyruvate, 0.5 mM NADH, 0.2 mM MgCl2+ ATP, 5 mM MgCl2, 10 mM DTT, 10 mM creatine in 10 mM HEPES pH 7.1). NADH consumption was measured at 340 nm for 20 min after a short equilibration time of 5 min at 30°C. For each sample, three measurements with and without creatine were made and the CK activity was calculated as the difference of the respective means.

6.2.7. Cellulose Polyacetat electrophoresis (CPE)

Cellulose Polyacetat electrophoresis was performed as described earlier (Friedman and Roberts, 1994). Briefly, 15 µg of total protein was loaded onto a cellulose strip and a constant voltage of 150 V was applied for 50 min at RT. CK and adenylate kinase (AK) isoenzyme bands were visualized at 37°C with an overlay gel technique in a reaction protocol similar to the one described above for the measurement of enzymatic activity. NADPH was reacted with nitrobluetetrazolium in the presence of phenazine methosulfate to yield formazan. For visualization of CK bands only, Ap5A was added to the overlay gel to inhibit AK activity.

6.2.8. Oxyblot procedure

DNPH derivatization was carried out in a solution containing 6% SDS and 1 mM DNPH as described in the manufacturer's instruction for the OxyBlot oxidized protein detection kit (Chemicon international). The reaction was stopped after 15 min incubation by neutralization with the neutralization solution. As a control, an aliquot of the samples was prepared in the absence of DNPH. The samples were then loaded on 10% SDS gel and trans-blotted as described above.

6.3. Results

6.3.1. Effect of APLP1, APLP2 and APP expression on CK activity

To see whether the over-expression of A1, A2 or APP in HEK 293 cells has an effect on the specific CK activity, we transiently transfected the cells and analyzed them by Western blotting (Fig. 21A). Over-expression of APP family proteins was detected with the anti-APP (C-term) antibody, whereas in control mock cells only a faint band at the size of APP appeared indicating endogenously expressed APP (~ 120 kDa). A strong APP signal was obtained in APP-transfected cells. These results differ clearly from the findings with co-transfected COS7 cells where the high molecular weight (HMW) band of uMtCK appears due to APP accumulation (X. Li et al., 2005, unpublished data). Obviously, transfection of uMtCK is necessary to see the HMW band.

The cell lysates were further analyzed with a coupled enzyme assay, where CK activity was determined indirectly by measuring NADH consumption of pyruvat kinase, subject to the addition of the specific CK substrate creatine (10 mM) (Fig. 21B). To prevent oxidation of NADPH by adenylate kinase (AK), we added the specific inhibitor P1,P5-di (adenosine-5') pentaphosphate (Ap5A) to a final concentration of 40 nM. No significant difference in the specific CK activities was observed between A1, A2 or APP over-expressing cells and non-transfected cells. But we observed a slight increase in total CK activity compared to the control. From these data we cannot say anything about effects of A1 on specific CK isoforms except that A1 does not seem to significally inhibit CK. To interpret the slight increase in CK activity in transfected cells, we have to optimize the assay conditions to reduce the variability of the measurement.

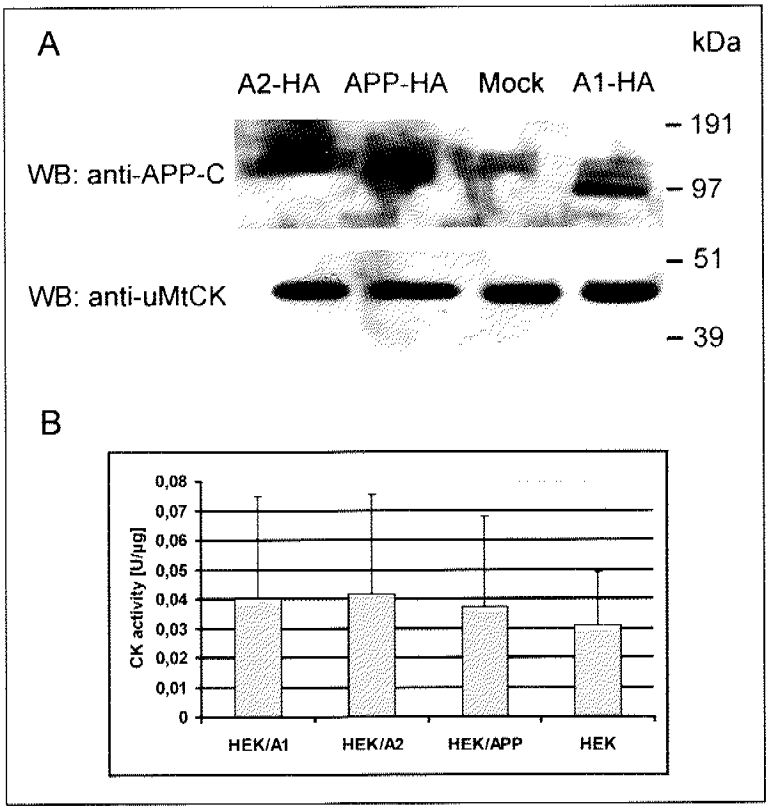

Figure 21: Influence of APPs expression on CK activity in HEK 293 cells

(A) HEK 293 cells were transiently transfected with either A1, A2, APP or pcDNA3 using Fugene 6 transfection reagent. Expression of A1, A2, APP and endogenously expressed uMtCK was confirmed by loading 20 µg protein and Western blotting using anti-APP (C-term) and anti uMtCK antibodies. The APP-HA constructs have the following calculated sizes: A1 95kDa, A2 140 kDa and APP 120 kDa. (B) Specific CK (total) activity of HEK lysates (n=3) transfected with A1, A2, APP and a mock control was analyzed using a coupled enzyme assay of lactate dehydrogenase (LDH) and pyruvat kinase (PK) consuming NADPH. CK activity was not affected significantly in A1, A2 or APP transfected cells.

6.3.2. Co-localization of APLP1 and uMtCK in subcellular fractions of A1 over-expressing cells

If the interaction between A1 and uMtCK had a scavenging effect, we would expect less uMtCK within the mitochondria after over-expression of A1. To check this hypothesis, we isolated mitochondria from over-expressing HA tagged A1 HEK 293 cells. The resulting organelle fraction contains endoplasmic reticulum (ER), as well as mitochondria. Calnexin is an ER marker and for mitochondria the voltage-dependent anion channel I (VDACI) and uMtCK were used as mitochondrial markers. With the HA-antibody we detected the A1

protein mainly in the cytoskeletal and to a lower extend in the organelle fraction (Fig. 22). Thus, the co-localization of uMtCK and A1 appears mainly in the organelle fraction indicating that the interaction appears within the mitochondria. As judged by the expression level of uMtCK, we could not observe any significant differences between the A1 over-expressing or the control cells. This suggests that A1 over-expression does not affect protein level of uMtCK in the mitochondria or other fractions and that A1 does not seem to function as scavenger for uMtCK.

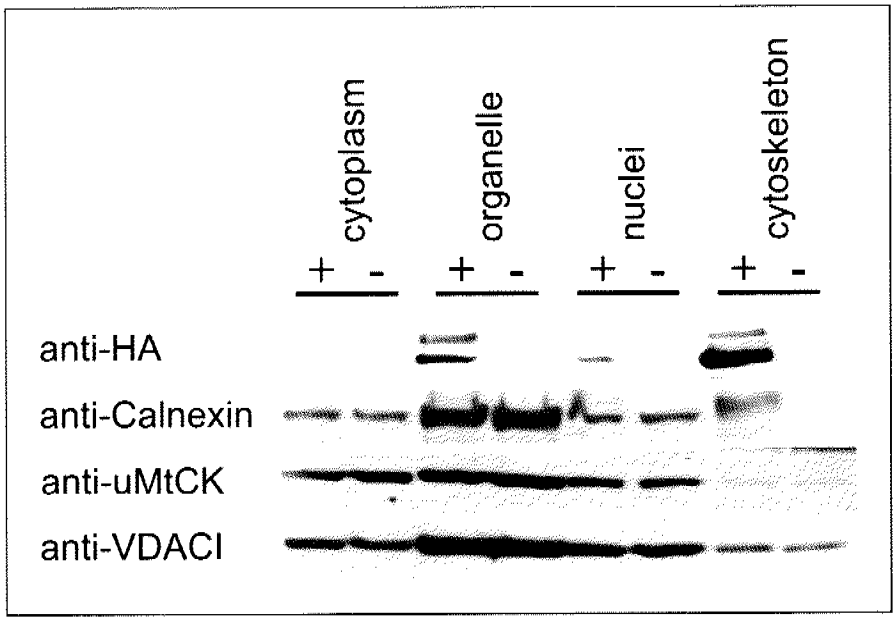

Figure 22: Co-localization of uMtCK and APLP1 in subcellular fractions

HEK 293 cells were transiently transfected with A1-HA and different sub-cellular fractions were obtained by using the sub-cellular proteome extraction kit (S-PEK, Calbiochem). uMtCK and VDACI were enriched in the organelle fraction from APLP1-HA over-expressing (+) and control (-) cells (lane 3 and 4). Calnexin was used as ER marker showing enrichment in the organelle fraction. A1 and uMtCK mainly co-localize in the mitochondria and no differences in the protein level of uMtCK in mitochondria due to A1-HA over-expression were observed.

6.3.3. Effect of APLP1 over-expression on CK isoform activity

Next we measured specific activity of uMtCK in the different fractions obtained by the sub-cellular fractionation experiment. If A1 would act as a chaperone, we would expect more CK activity within the mitochondria, whereas a scavenging function of A1 would decrease uMtCK activity. We separated the dimeric (MtCKd) from the octameric MtCK

(MtCKo) isoforms as described earlier (Wendt et al., 2003). We show an activity of MtCK within the organelle fraction using the CPE method and interestingly, we observe an increase of MtCK activity in the mitochondria from A1 over-expressing cells (Fig. 23). Thus, A1 over-expression leads to a higher uMtCK activity within the mitochondria.

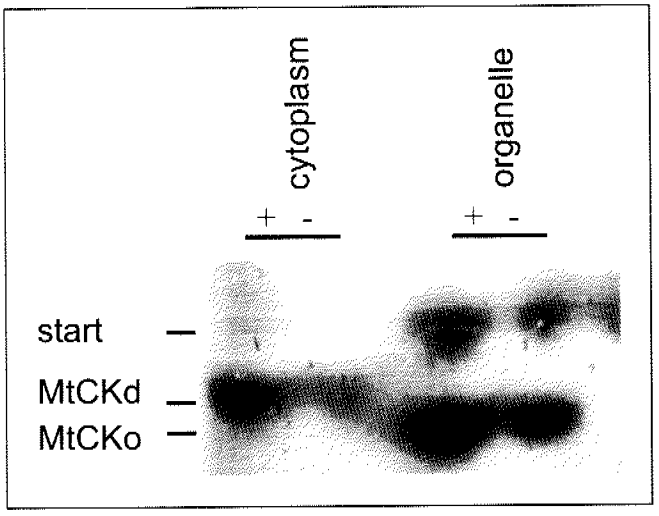

Figure 23: APLP1 (A1) increases uMtCK activity in mitochondria

15 µl of the organelle and cytoplasmic fraction + A1 and – A1 were loaded onto a cellulose strip and CK isoforms were separated by cellulose polyacetate electrophoresis (CPE) at 150 V for 50 min (start = place of sample application). CK activity was displayed by formazan building in a coupled enzyme assay at 37°C. uMtCK isoform could be separated in the dimeric and octameric form (MtCKd, MtCKo). MtCK activity is increased in mitochondria and cytoplasm of + A1 compared to – A1 cells.

6.3.4. Effects of APLP1-overexpression on the protein oxidation level

To exclude any oxidative side effects of A1 over-expression to the cells, we analyzed the oxidation level of these cells with the OxyBlot detection kit. The carbonyl content of total proteins in the cell lysates was measured immunochemically with an anti-DNP antibody. As shown in Fig. 24, over-expression resulted in significantly lower levels of protein carbonylation compared to the control lysate. Thus, one role of A1 may be the protection of cells from endogenously generated oxidative damage.

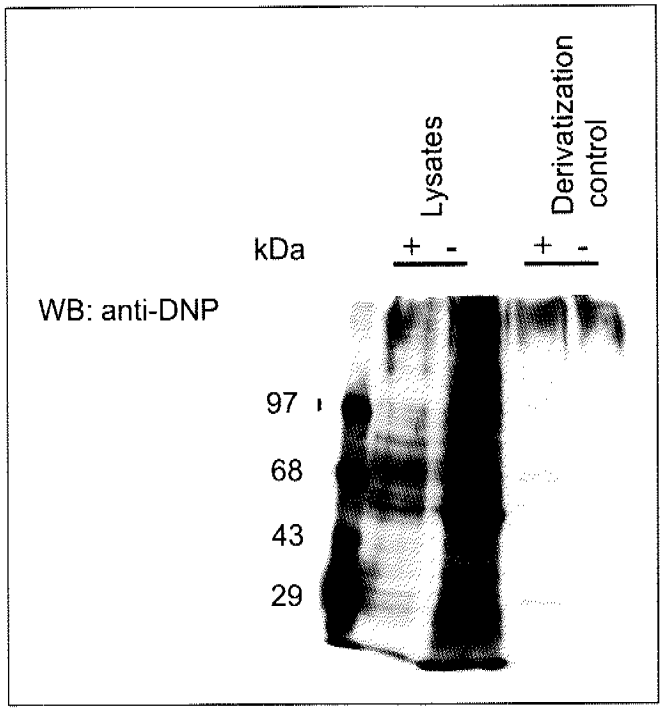

Figure 24: Protein oxidation levels in APLP1 (A1) over-expressing cells

HEK 293 cells were transiently transfected with A1 and protein carbonyls in the total cell lysates were drivatized with or without DNPH, and analyzes by western blotting with anti-DNP. We observed a lower oxidation in the lysate + A1 compared to the control – A1.

6.4. Discussion

In this study, we wanted to characterize the interaction between A1 and uMtCK. From recent results together with our co-workers (X.Li et al. 2005), we hypothesized that the APPs may act either as a scavenger or as chaperones for the immature form of uMtCK. In this work, most of the experiments were done with the APP isoform or only the truncated C-terminal APP peptide. But as the initial experiment was done with a C-terminal sequence from the A1 gene where uMtCK was identified as interaction partner, we used the A1-HA tagged construct for the following experiments. If A1 would act as a scavenger or chaperone for uMtCK, we would expect a difference in the content of uMtCK in the mitochondria or changes in CK enzyme activity of A1 over-expressing cells.

We analyzed the distribution of uMtCK in subcellular fractions, specific CK activity and protein carbonylation of A1 over-expressing HEK293 cells. Table 1 shows a summary of the results found in this study.

	+ A1 transfected	**- A1 non-transfected**
total uMtCK protein level (Fig. 21A)	unchanged	unchanged
Mitochondrial uMtCK protein level (Fig. 21A)	unchanged	unchanged
uMtCK localization (Fig. 22)	mitochondria, cytoplasm, nucleus	mitochondria, cytoplasm, nucleus
A1 localization (Fig. 22)	mitochondria, nucleus, cytoskeleton	no A1 detectable
Total CK activity in total lysate (Fig. 21B)	high	low
Specific MtCK activity in mitochondria (Fig. 23)	high	low
Protein oxidation (Fig. 24)	low	high

Figure 25: Table: Overview of the results found by analyzing the A1-uMtCK interaction

We first analyzed the effect of the over-expression of all APP family members on total CK activity in cell lysates. There, we could not detect any significant differences in total CK activity between cells over-expressing either A1, A2 or APP and mock transfected control cells (Fig 21B).

In sub-cellular fractions we looked at the A1 and uMtCK co-localization (Fig. 22). uMtCK was enriched in the organelle and nuclear fraction whereas A1 occurred mainly in the cytoskeleton fraction but was also associated to the nuclear and organelle fraction (Fig. 22). Localization of A1 to the nucleus was reported earlier but its function there is still unclear (Friedman and Roberts, 1994). Thus co-localization of the two proteins occurs mainly in the organelle fraction, suggesting that A1 and uMtCK meet in mitochondria.

We have not found any differences in the protein levels of uMtCK between A1 over-expressing and the control cells (Fig. 22), but when we analyzed the isoform specific enzyme activity (Fig. 23), we observed an increase of uMtCK activity in the mitochondrial fractions due to A1 over-expression.

A1 over-expression does not lead to any differences on MtCK protein levels in the mitochondria (Fig. 22) and specific uMtCK activity is even increased. This suggests that there might be a regulatory mechanism induced by A1 which leads to increased CK activity.

Mitochondrial dysfunction is a hallmark in neurodegenerative disorders like Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's Disease (AD) and accumulation of full-length APP in the mitochondrial compartment causes mitochondrial dysfunction and impaired energy metabolism (David et al., 2005; Gotz et al., 2004). Transgenic mice over-expressing the P301L mutant human Tau protein exhibit an accumulation of hyperphosphorylated Tau and develop neurofibrillary tangles. Metabolically related proteins including mitochondrial respiratory chain complex components, antioxidant enzymes and synaptic proteins were identified as modified in the proteome pattern of P301L Tau mice. Functional analysis demonstrated a mitochondrial dysfunction in P301L Tau mice, together with reduced NADH-ubiquinone oxidoreductase activity and, with age, impaired mitochondrial respiration and ATP synthesis. Mitochondrial dysfunction was associated with higher levels of reactive oxygen species in aged transgenic mice (Takuma et al., 2005). Over-expression of A1 in our study may have a compensatory beneficial effect on CK activity and other enzymes that are susceptible to ROS damage in the mitochondria which then in turn can provide sufficient energy for cellular processes.

Interestingly, our oxidation analysis showed that A1 expression leads to a decrease of protein carbonylation (Fig. 24). Whereas neurons cultured from transgenic mice with

targeted over-expression of a mutant form of amyloid precursor protein and Amyloid-beta peptide (A-beta) binding alcohol dehydrogenase (ABAD) (Tg mAPP/ABAD) displayed spontaneous generation of hydrogen peroxide and superoxide anion, and decreased ATP, as well as subsequent release of cytochrome c from mitochondria (Keil et al., 2004). Generation of reactive oxygen species (ROS) was associated with dysfunction at the level of mitochondrial complex IV (cytochrome c oxidase, or COX). In neurons cultured from Tg mAPP/ABAD mice, COX activity was selectively decreased. *In vivo*, Tg mAPP/ABAD mice displayed reduced levels of brain ATP and COX activity, diminished glucose utilization, as well as electrophysiological abnormalities in hippocampal slices compared with Tg mAPP mice (Keil et al., 2004).

In a future set of experiments we could analyze mitochondrial respiration of A1 over-expressing cells. If we would analyze specific activities of the respiratory chain proteins, we further could examine which effects Cr supplementation would have on this *in vitro* system. As uMtCK activity is increased in mitochondria of A1 over-expressing cells, we would expect a positive stimulation of CK activity by Cr supplementation.

We conclude that A1 exerts an effect on CK activity in the mitochondria, as well as in the entire cell, but not on the expression level of MtCK. While over-expression of APP leads to protein oxidation, A1 seems to protect the proteins from ROS or other oxidants. It is still unclear which role the interaction between uMtCK and A1 may play. But our data suggest that the creatine/phospho-creatine/CK (Cr/PCr/CK) system is up-regulated by A1 which is shown by increased CK activity. It could be possible that A1 over-expression possibly caused by a determinate cellular energy state, could lead to a compensatory up-regulation of the CK system, such that cells may be protected from energy failure.

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7. Chapter 4

The creatine kinase/phosphocreatine system in the neurodegeneration of Alzheimer's Disease: potential beneficial effects of creatine supplementation

Tanja Bürklen*, Theo Wallimann

Institute of Cell Biology, Swiss Federal Institute of Technology (ETH) Zürich, Hönggerberg HPM, CH-8093 Zürich, Switzerland

*Corresponding author:

Tanja Bürklen

Institute of Cell Biology

HPM D22

Schafmattstr. 18

8093 Zürich, Switzerland

Phone: (0041) 44-6333339

Fax: (0041) 44-6331069

E-Mail: tanja.buerklen@cell.biol.ethz.ch

Submitted to Medical Hypothesis

7.1. Abstract

Alzheimer's disease (AD) is the fourth most frequent cause of death for people over 65 years of age. Studies of postmortem brain tissue from AD patients have provided evidence of increased levels of oxidative stress, mitochondrial dysfunction, impaired glucose uptake and energy loss in neuronal populations. As the creatine kinase (CK) isoenzyme family plays a fundamental role in eukaryotic energy metabolism, they could help to diminish the energetic deficits occurring in these patients. In this review, preference will be given to more recent developments in the field of mitochondria involvement and energy depletion in the pathogenesis of AD. In the brain, two isoforms of CK enzymes constitute as an energy shuttle; the mitochondrial CK (MtCK) and the cytosolic brain-type CK (BCK). The creatine kinase isoenzyme family catalyzes the reversible transfer of the γ-phosphate group of ATP to creatine (Cr) to yield ADP and phosphocreatine (PCr). PCr can be easily transported, stored and used as a high energy buffer and transport molecule. The CK enzymes are highly sensitive to oxidation and BCK is oxidatively modified in AD brain. Recent studies in cell culture have shown enhanced mitochondrial activity and increased density of synapses after the addition of Cr. Cr supplementation is already successfully employed in a broad range of applications, e.g. in patients with muscular and neuromuscular diseases. The goal of this article is to provide a comprehensive overview of the potential roles of the creatine kinase/phosphocreatine (CK/PCr) system in Alzheimer's Disease (AD). Stimulation of this system by Cr supplementation could potentially provide a new therapeutic approach to treat, delay or influence the course of neurodegenerations like AD.

Keywords: Neurodegeneration, Alzheimer's Disease, energy metabolism, creatine kinase, creatine supplementation, mitochondrial dysfunction, protein oxidation.

7.2. Introduction

7.2.1. Brain energy metabolism and creatine

Glucose is the mandatory energy substrate in the brain and it is almost entirely oxidized to CO_2 and H_2O to produce ATP. The main energy-consuming process in neural cells is the transport of ions by the Na^+/K^+-ATPase (Magistretti and Pellerin, 1999). Even though the cellular pools of ATP are rather small, no significant change in overall ATP levels can be detected during activation of excitable tissues (Mommaerts et al., 1977). This is because ATP is continuously and efficiently replenished from the large pools of PCr through the reaction controlled by the CK (Focant and Watts, 1973). The CK isoenzymes catalyze the reversible transfer of the γ-phosphate group of ATP to the guanidino group of Cr to yield ADP and PCr.

$$\mathbf{PCr^{2-} + MgADP^{-} + (x)H^{+} \longleftrightarrow MgATP^{2-} + Cr}$$

In vertebrates, Cr is synthesized mostly in the liver and kidney, but not in muscle, and is then transported through the blood and taken up by tissues with high energy demands (Fitch et al., 1979; Wyss and Kaddurah-Daouk, 2000). Cr biosynthesis involves two sequential steps catalyzed by L-arginine:glycine amidinotransferase (AGAT) and S-adenosylmethionine:guanidinoacetate N-methyltransferase (GAMT) (Walker and Hannan, 1976). Small amounts of Cr is synthesized in the brain itself (Defalco and Davies, 1961). However, the majority of Cr is continuously taken up through the blood/brain barrier by a Na^+/Cl^- dependent creatine transporter (CRT) against a Cr gradient (Ohtsuki et al., 2002). Considering that PCr generates ATP much faster from the pool of PCr than it is obtained from glycolysis or oxydative phosphorylation and that the expression of Cr biosynthetic and metabolic enzymes is tightly regulated, it is very likely that the CK/PCr shuttle system plays a fundamental role in the brain energy homeostasis. (Tachikawa et al., 2004).

7.2.2. The creatine kinase/phosphocreatine shuttle system the in the brain

The concept of the creatine kinase/phosphocreatine (CK/PCr) shuttle system describes the functional association of CK isoenzymes with discrete intracellular compartments of ATP production and hydrolysis, and the suitability of PCr and Cr to serve as cytosolic energy

transducers (Bessman and Carpenter, 1985; Wallimann et al., 1992). Thus, the function of Cr and PCr is to form an essential links between sites of ATP synthesis and consumption.

The CK/PCr system mainly functions as a temporal energy buffer, a spatial energy buffer and as an energy transport system (Bessman and Geiger, 1981; Wallimann and Eppenberger, 1985). Another role of this system is to prevent a rise in intracellular free ADP, thus avoiding an inactivation of cellular ATPases and a net loss of adenine nucleotides (Iyengar et al., 1982). The CK/PCr system also provides appropriate local ATP/ADP ratios at subcellular sites where CK is functionally coupled to ATP-consuming enzymes or processes (Levin et al., 1990) (Fig. 26). Thus, the CK/PCr system is a rapidly available source for ATP synthesis in brain. The high activity of CK in the brain justifies the assumption that CK is a key enzyme in the energy metabolism of this tissue (Norwood et al., 1983).

Figure 26 shows the subcellular compartmentation of CK enzymes and the co-localization of the CK/PCr system with ATP-producing and -consuming sites within the cell.

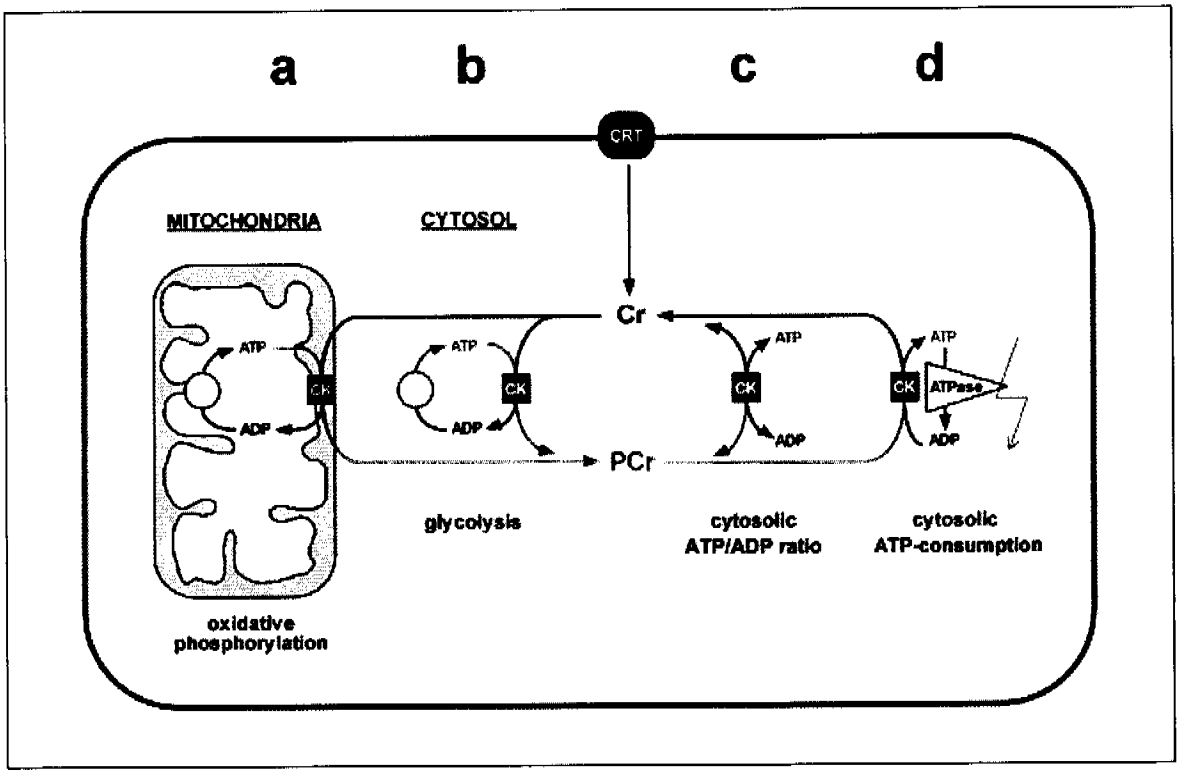

Figure 26: The CK/PCr system.

Isoenzymes of CK are found in different compartments such as mitochondria (a) and the cytosol (b-d) in a

soluble form (c) or associated to a different degree to ATP-delivering (a,b) or -consuming processes (d). A large cytosolic PCr pool up to 30 mM is built up by CK using ATP generated by oxidative phosphorylation e.g. in the heart (a) or glycolysis e.g. in fast-twitch glycolytic muscle (b). PCr is then used to buffer global (c) and local (d) ATP/ADP ratios. In cells that are polarized and/or have a very high or localized ATP consumption, these CK isoenzymes, together with easily diffusible PCr, also maintain an energy shuttle between ATP-providing or -consuming processes (a, d). Metabolite channeling occurs where CK is associated with ATP-providing or -consuming transporters, pumps or enzymes (a, b). Cr is synthesized in only few cell types (e.g. liver and kidney) and has to be taken up from the blood stream by a specific CRT. (From: Schlattner U, and Wallimann T. (2004) Metabolite channeling: creatine kinase microcompartments. In: *Encyclopedia of Biological Chemistry* (W.J. Lennarz, and M.D. Lane, eds.), Academic Press, New York, USA)

7.2.3. Function and localization of the CK isoenzyme family

In vertebrates at least four isoforms of CK are expressed in a tissue-specific manner. In sarcomeric muscle, dimeric cytosolic muscle-type CK (MCK) is localized to the M-band (Wallimann et al., 1977), the sarcoplasmic reticulum (SR) (Korge et al., 1993; Rossi et al., 1990) and the plasma membrane. At these sites, MCK is functionally coupled to the myofibrillar acto-myosin ATPase (Krause and Jacobus, 1992; Ventura-Clapier et al., 1987; Wallimann et al., 1984), the SR Ca^{2+}-ATPase (Korge et al., 1993; Rossi et al., 1990) and at the plasma membrane Na^{+}/K^{+} ATPase (Grosse et al., 1980), respectively, and utilizes PCr for in situ regeneration of ATP.

Mitochondrial CK (MtCK), is located in the mitochondrial intermembrane space (Kottke et al., 1991), where it is found along the entire inner membrane, and also at peripheral sites where inner and outer membranes are in close proximity (Biermans et al., 1990; Brdiczka, 1994). There, MtCK can directly transphosphorylate intra-mitochondrially produced ATP into PCr (Jacobus, 1985), which is then exported into the cytosol. In brain tissue, it was reported that CK activity is associated with mitochondria, which was later identified and characterized as the brain ubiquitously MtCK (uMtCK) (Booth and Clark, 1978). The uMtCK is localized preferentially as octamers to mitochondrial membrane contact site of brain mitochondria (Kottke et al., 1988). In muscle tissues sarcomeric MtCK (sMtCK) is expressed and localized as dimers and octamers to the mitochondria.

The dimeric cytosolic brain-type CK (BCK) is the main isoform in the brain (Eppenberger et al., 1967). BCK has been found in association with synaptic vesicles (Lerner and Friedhoff, 1980) and synaptic plasma membranes (Lim et al., 1983). In the rat hypothalamus, BCK plays an essential role in regenerating ATP for glutamate clearance during excitatory synaptic transmission (Oliet et al., 2001). In glial cells, the generation of ATP functions to induce and stabilize synapses in the central nervous system (CNS). Thereby, number of synapses can be profoundly regulated by non-neuronal signals, which raises the possibility that glia may actively participate in synaptic plasticity (Ullian et al., 2001). Based on the finding that BCK and uMtCK levels are increased during postnatal cerebellar development the existence of a functional CK/PCr energy shuttle system has been proposed (Friedman and Roberts, 1994).

Furthermore, there is a coincidence in the timing of maximal expression of BCK and myelin basic protein (MBP) in the cerebellum which may be a further indication for a role of CKB in myelination (Shen et al., 2002). The CK/PCr circuit also plays a role in the formation and maintenance of hippocampal mossy fibre connections, processes that involve habituation, spatial learning and seizure susceptibility in mouse brain (Jost et al., 2002). Thus, the most prevalent CK enzymes in the brain and neural tissues are BCK and uMtCK which together constitute an efficient energy shuttle system (Wallimann and Hemmer, 1994).

7.2.4. Neurodegenerative diseases: Alzheimer's Disease

Degenerative diseases of the central nervous system (CNS) are a heterogeneous group of slowly progressive disorders. A common feature of this group, which includes AD, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), is the gradual loss of specific populations of neurons (Waggie et al., 1999). Dementia of Alzheimer's type is the commonest form of dementia, the other two forms being vascular dementia and mixed dementia. This report will focus on AD and therefore describe some pathological features of the disease.

Senile plaques, neurofibrillary tangles and inflammation are the three major pathologies that characterize AD (Goedert, 1993; Su et al., 1996). Extracellular senile plaques are found particularly in the hippocampus, neocortex and amygdala. They consist of

aggregates of a 39-43 amino acid containing peptide, known as Aβ. Aβ is derived from a larger precursor protein the amyloid precursor protein (APP), upon proteolytic cleavage (Jarrett et al., 1993; Kang et al., 1987). Neurofibrillary tangles are intracellular structures of abnormal fibres, called tau, which forms a paired helical filament. Hyperphosphorylated tau in neurofibrillary tangles is also known to be ubiquitinated (Lee et al., 1991; Morishima-Kawashima et al., 1993). In some families AD is inherited as an autosomal dominant disorder, although in most cases inheritance appears to be multifaktoriell (Nee et al., 1987). To date, three genes have been identified in which mutations result in an early onset of familial AD. These are the APP, presenilin 1 (PS-1), and PS-2 genes (Goate, 1997; Levy-Lahad et al., 1995; Van Broeckhoven et al., 1992). Additionally, genetic risk factors as for example the apolipoprotein E (apoE) ε4 allele are discussed, which are not sufficient to cause the disease, but may greatly increase the risk for AD (Corder et al., 1993).

7.2.5. The role of Aβ in the pathogenesis of Alzheimer's Disease

The amyloid precursor super family (APSF) is composed of three highly conserved transmembrane glycoproteins, APP and amyloid precursor-like proteins 1 and 2 (APLP1, APLP2) (Lyckman et al., 1998). Although the APP gene is the only APSF that encodes Aβ, the conserved gene structure (Wasco et al., 1993) and domain homology (Slunt et al., 1994) among the APSF proteins suggests that they may share significant overlap in function and expression. The APP gene contains 18 exons, spanning over 170 kb. The region encoding the Aβ sequence comprises part of exons 16 and 17 and contains between 39 and 43 amino acid residues that extend from the ectodomain into the transmembrane domain of the protein (Fig. 27). It is now known that Aβ is an abnormal product of APP cleavage (da Cruz e Silva and da Cruz e Silva, 2003). The major route of APP processing is by α-secretase, an enzyme that cleaves within the Aβ sequence (Selkoe et al., 1996). Cleavage by β- and γ-secretases at the N- and C-terminal ends of the Aβ sequence liberates the Aβ polypeptide, which is subsequently secreted from cells (Sinha and Lieberburg, 1999). There are several hypotheses about the onset of AD. The 'amyloid hypothesis' posits a central role for Aβ in initiating the AD pathogenic cascade and argues that the neurodegenerative disease process, including the development of neurofibrillary tangles, is

a consequence of an imbalance between the generation and clearance of Aβ (Hardy and Selkoe, 2002).

Recent studies of a cellular model of Huntington's Disease (HD) showed that inclusion body formation improved survival and lead to decreased levels of mutant huntingtin. Inclusion body formation can function as a coping response to toxic mutant huntingtin (Arrasate et al., 2004). As HD and AD are both neurodegenerative diseases, these findings provoke the debate as to whether Aβ is a cause or an effect of the pathogenic process of AD.

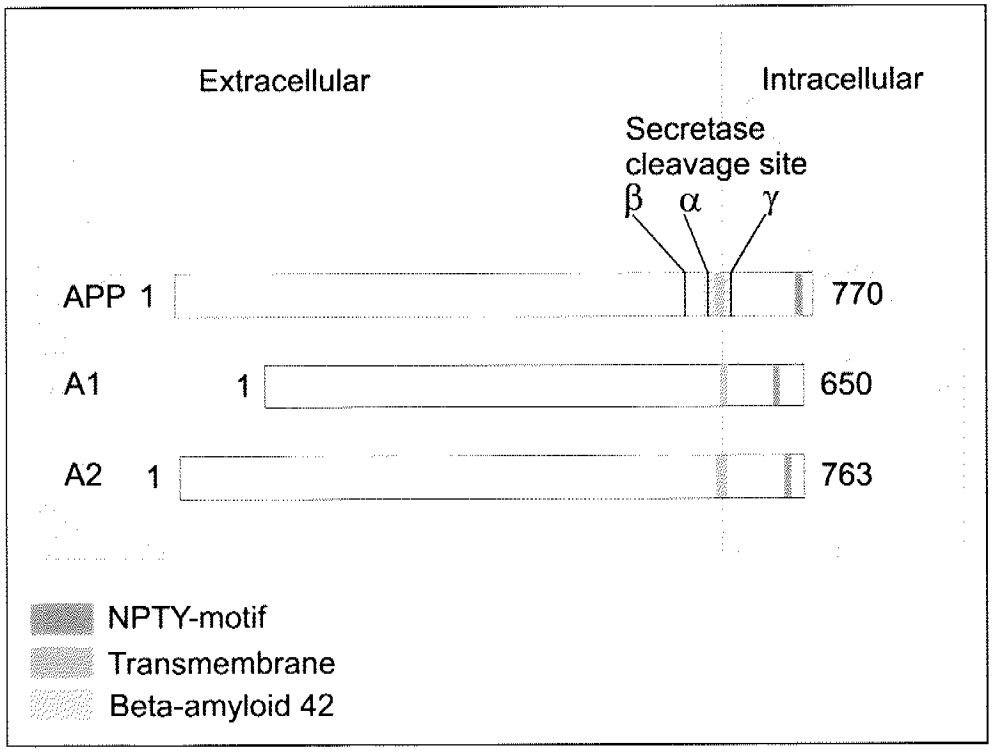

Figure 27: Protein alignment of the human amyloide precursor protein family

The amyloid precursor proteins (APP) consist of a large extracellular and a short intracellular domain. The APP and amyloid precursor-like protein 1 (APLP1) have the NPXY motif in common, whereas in the amyloid precursor-like protein 2 (APLP2) this motif is missing. Three secretases (α, β, γ) cleave the APP at specific sites resulting in different cleavage.

7.2.6. Disturbed energy metabolism in neurodegenerative diseases

The actual energy state of the living cell and the whole body is a measurement for the functionality of the organism. Disturbed energy metabolism leads to severe degenerative

disorders such as HD, ALS, PD and AD (Beal, 2000). A common factor in all diseases is mutated mitochondrial DNA or nuclear DNA mutations that lead to secondary mitochondrial dysfunction. Cellular energy reserves appear to have an important role in apoptosis and necrosis and mitochondria are essential in controlling specific apoptotic pathways (Green and Reed, 1998).

In patients with ALS, a chronically deficient intake of energy (Kasarskis et al., 1996) increased mitochondrial volume and elevated Ca^{2+}-levels (Siklos et al., 1996), decreased complex I activity, increased oxidative damage, loss of mitochondrial membrane potential and elevation in cytosolic Ca^{2+} concentrations due to mutations in copper-zinc superoxide dismutase (SOD1) (Carri et al., 1997).

Decreases in cerebral metabolic rate (CMR) characteristically occur in AD and in other dementias. Inducing decreases in CMR leads to clinical disabilities which are similar to the ones observed in AD patients or animal models of (Blass, 2001). The AD brain is characterized by a variable, but often marked, loss of neurons, the deposition of extracellular plaques and intracellular neurofibrillary tangles (Selkoe, 1999; Small and McLean, 1999). A decrease in energy metabolism and altered cytochrome c oxidase activity are among the earliest detectable defects in AD (de la Monte et al., 2000; Maurer et al., 2000; Parker, 1991; Valla et al., 2001).

7.2.7. Mitochondrial aspects of neurodegeneration

Nowadays, the involvement of mitochondria in neurological disorders is discussed with increasing frequency. It is known that mitochondrial dysfunction leads to the generation of free radicals and subsequent oxidative damage (Boveris and Cadenas, 2000).

Studies of AD patients have identified decreased complex IV activity, and mitochondrial DNA mutations (Aksenov et al., 1999; Cardoso et al., 2004). Recently, a role for mitochondria has been indicated in Aβ-induced apoptosis. The Aβ-binding alcohol dehydrogenase (ABAD) has been reported to interact with Aβ in the mitochondria of AD patients and transgenic mice (Lustbader et al., 2004) and to potentiate Aβ-induced apoptosis and free-radical generation in neurons. Furthermore, in brains from patients with autopsy-confirmed AD and clinical dementia ratings (CDRs) before death, the activity of tricarboxylic acid (TCA) cycle enzymes of mitochondria were decreased. These include

the pyruvate dehydrogenase complex, isocitrate dehydrogenase and the alpha-ketoglutarate dehydrogenase complexes were significantly decreased. Changes in TCA cycle activities correlated with the clinical state of the disease, suggesting a coordinated mitochondrial alteration (Lustbader et al., 2004). Zheng Li et al uncovered a structural and functional interplay between dendritic mitochondria and spines/synapses. A small fraction of mitochondria is present within dendritic protrusions of cultured neurons (Li et al., 2004). Interestingly, Cr enhances mitochondrial activity and causes a higher density of spines and synapses. Remarkably, the ability of neurons to form new excitatory synapses in response to stimulation is also correlated with increased activity of dendritic mitochondria. Neuronal activity itself affects the motility, fusion/fission balance, and subcellular distribution of mitochondria in dendrites, depending on calcium influx. This seems to be physiologically relevant, because repetitive depolarization that stimulates synapse formation causes the redistribution of mitochondria into dendritic protrusions. These results suggests a local involvement of mitochondria in synapse formation and development (Li et al., 2004). Taken together, these findings raise the possibility that the characteristic loss of synapse in such disorders arises in part from mitochondrial dysfunction.

7.2.8. Altered CK functions and PCr/Cr ratios are linked to neurodegenerative diseases

Oxidative alterations of proteins have been implicated in the progression of neurodegenerative disorders (Butterfield and Lauderback, 2002; Markesbery, 1997). Protein carbonyls, considered as detectable marker of protein oxidation, are increased in AD (Smith et al., 1991). In a proteomic approach BCK, glutamine synthase (GS) and ubiquitin carboxy-terminal hydrolase l-1 (UCH L-1) were identified as specifically oxidized proteins in the brain of AD patients (Castegna et al., 2002).

Other reports show that CK in AD brains is modified such that the nucleotide binding site of CK is blocked and that abnormal partitioning of CK between the soluble and pellet fractions occurs. In addition, CK activity in AD brain homogenates is decreased to 86 %, shown with the [alpha 32P]8N3ATP incorporation and the amount of CK is decreased by less than 14 % (David et al., 1998).

Loss of BCK activity (Aksenov et al., 2000; Hensley et al., 1995; Smith et al., 1991),

resulting from its oxidation (Aksenov et al., 2000), implies that less energy is available to neurons and synaptic elements in AD brain. Mildly demented Alzheimer's patients have reduced levels of PCr and probably ADP and an increased oxidative metabolic rate compared to healthy persons. As the dementia worsens, the levels of PCr and ADP increase and the oxidative metabolic rate decreases (Pettegrew et al., 1994).

Adult mice lacking both CK isoforms, BCK and uMtCK, (CK--/-- double knockout mice display a reduced body weight, a severely impaired spatial learning in both a dry and a wet maze, lower nest building activity and diminished acoustic startle reflex responses when compared to control wild type mice. In contrast, their visual and motor functions, exploration behaviour, prepulse inhibition and anxiety-related responses were not changed, suggesting no global deficit in sensory motor function, hearing or motivation. Morphological analysis of CK--/-- double knockout brains revealed a reduction of brain weight and hippocampal size, a smaller regio-inferior area and relatively larger supra-pyramidal and intra-infra-pyramidal mossy fiber area. These results suggest that the lack of both brain-specific CK isoforms renders the synaptic circuitry less efficient in coping with sensory or cognitive activity related challenges in the adult brain (Streijger et al., 2005).

In vitro studies of the AMP activated protein kinase (AMPK) showed that this enzyme inhibits CK activity by phosphorylation (Ponticos et al., 1998) AMPK, a heterotrimeric complex, consists of the catalytic α-subunit and β- and γ-subunits (Salt et al., 1998), is highly sensitive to the ATP:AMP ratio and is activated by AMP. AMPK is reversibly phosphorylated and once activated, AMPK phosphorylates a number of enzymes involved in biosynthetic pathways, causing their inactivation and preventing further ATP utilization (Ponticos et al., 1998). Because the CK/PCr system is involved in maintaining the delicately balanced ATP:ADP ratios within the cell, a functional link to the AMPK seems logical. AMPK also co-localizes with MCK *in vivo* and a fall in the PCr:Cr ratio leads to gradual activation of AMPK, which is analogous to the activation in response to a fall in the ATP:AMP ratio in liver (Corton et al., 1994; Hardie and Carling, 1997). An increase in AMPK activity will result in the phosphorylation and inactivation of MCK. Thus, if the ATP:ADP ratio decreases AMPK inhibits CK activity to prevent further ATP depletion by PCr synthesis.

In a Drosophila model for neurodegeneration, a mutation in the AMPK γ-subunit causes progressive loss of neurons (Tschape et al., 2002). AMPK has a central role in cholesterol metabolism by regulating hydroxymethylglutaryl-CoA (HMG-CoA) reductase (Kemp et al., 1999). HMG-CoA reductase regulates cholesterol synthesis in neurons (Frears et al., 1999), which influences the production of the pathogenic Aβ peptide (De Strooper and Annaert, 2000; Refolo et al., 2000). It is shown that the Aβ processing occurs within detergent-insoluble sphingolipid-cholesterol rafts, whereas the non-amyloidogenic α-processing occurs outside (Kojro et al., 2001; Lee et al., 1998; Simons et al., 1998). Thus, AMPK could be linked by two different mechanisms link to neurodegenerative processes. On the one hand this link could be by the cholesterol metabolism and Aβ processing and on the other hand by altered PCr:Cr ratios in AD brains. It is still unknown whether ATP decrease is the initial step or a consequence of dysregulation and dysfunction of enzymes involved in signalling and metabolic pathways.

7.3. Hypothesis

7.3.1. Potential beneficial effects of Cr supplementation in neurodegenerative diseases

One of the most important hallmarks in the pathogenesis of senile dementia of the Alzheimer type is the marked decrease of cerebral glucose metabolism, cholinergic deficit caused by a disturbed acetyl-CoA synthesis and critically lowered oxidative phosphorylation. A decrease of the oxidative energy metabolism in senile dementia and the resulting ATP deficit may change protein degradation, synaptic transmission and ion homeostasis (Meier-Ruge et al., 1994). Further, disturbed function and abnormal morphology of mitochondria are associated with neurodegenerative diseases such as AD and PD (Castellani et al., 2002).

Recent data from our laboratory show an interaction between the uMtCK and the APSF proteins (unpublished data) and this interaction stabilizes the immature precursor form of uMtCK. These data link the CK/PCr system to the APSF proteins that are involved in the pathogenesis of AD.

Over the past decade, the ergogenic benefits of synthetic Cr monohydrate have made it a popular dietary supplement, particularly among athletes (Bemben and Lamont, 2005). The anabolic properties of Cr also offer hope for the treatment of diseases characterized by weakness and muscle atrophy. Recent data from cell culture experiments identified Cr as a rather potent natural survival- and neuroprotective factor in a model for PD (Andres et al., 2005). ALS is characterized by a progressive degeneration of motoneurons, resulting in weakening and atrophy of skeletal muscles. In patients with this condition, Cr offers potential benefits by facilitating residual muscle contractility as well as improving neuronal function (Ellis and Rosenfeld, 2004; Mazzini et al., 2001). To date, two clinical trials of Cr monohydrate in ALS have been completed without demonstration of significant improvements in overall survival or a composite measure of muscle strength (Shefner et al., 2004). These trials brought up new questions about the optimal dosage of Cr and its beneficial effects on muscle fatigue, a measure distinct from muscle strength. A large multicenter clinical trial is currently underway to further investigate the efficacy of Cr monohydrate in ALS and to address these unresolved issues. To date, evidence shows that Cr supplementation has a good safety profile and is well tolerated by patients with ALS. The pharmacokinetics and rationale for the use of Cr are described along with available evidence from animal models and clinical trials for ALS and related neurodegenerative or neuromuscular diseases described in (Ellis and Rosenfeld, 2004). In an additional trial of patients with HD, Cr supplementation lowered brain glutamate level (Bender et al., 2005) and in patients with a novel cytochrome b mutation Cr supplementation attenuated the production of free radical and paracrystalline inclusions (Tarnopolsky et al., 2004).

Furthermore, Cr has direct antioxidant properties *in vitro* (Lawler et al., 2002) and could therefore prevent oxidative damage that occurs in AD (Aliyev et al., 2005; Wang et al., 2005).

Thus, we hypothesize, that Cr supplementation may compensate for the disturbed energy metabolism in subjects with AD by replenishing the energy pools, activating mitochondrial respiration (Kay et al., 2000; O'Gorman et al., 1997) and protecting cells from apoptosis (Dolder et al., 2003; O'Gorman et al., 1997) and oxidative damage.

Cr supplementation preferentially given to patient at an early stage of AD at a dosage of 5-10g/day may then prolong life quality, enhance or maintain memory and learning functions

(Rae et al., 2003) and thus delay the entrance of the patients into disability. Together with other clinical interventions, Cr supplementation may by a very valuable adjunct therapy for AD patients and in addition extend a huge socio-economic benefit on our health-cost ridden society. This is especially true since long-term Cr supplementation is safe (Kreider et al., 2003) and cheap at the same time.

Acknowledgements

We kindly thank Tina Thurnherr for critical reading of the manuscript and Dr. Uwe Schlattner for the discussions. This work was supported by a Swiss National Foundation Grant No.3100A0-102075.

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8. General Conclusions

This work contributes substantially to the expansion of our knowledge about the involvements of the CK isoenzymes in the regulation of cellular processes.

Because the CK enzymes play an important part in stabilizing the cellular energy state that is needed for many cellular processes, the results in this work link the CK/PCr system to cell cycle events at the onset of mitosis as well as to a potential involvement in the functions and processing of APSF. Interestingly both findings associate CK with cellular processes in the brain.

With the identification of GM130 from a human brain cDNA library as interaction partner of BCK, and the fact that GM130 plays a role in Golgi fragmentation during mitosis where it is phosphorylated, and that BCK associates with GM130 especially in early prophase, I strongly suggest a regulatory role of BCK for cell cycle events. In the future, two main questions will have to be resolved. Is BCK involved in correct Golgi fragmentation during mitosis and whether it can thereby act as a regulator oft the entry into mitosis?

As BCK associates with GM130 specifically at the early prophase it is very likely that BCK is involved in Golgi events at the onset of mitosis. Recent data show that the fragmentation and dispersal of the pericentriolar Golgi complex is required for entry into mitosis in mammalian cells (Sutterlin et al., 2002). There, a reagent was developed that blocked mitotic Golgi fragmentation but which by itself was not essential for regulating entry into mitosis. The authors suggested that the Golgi apparatus as a sensor in regulating entry into mitosis. If BCK is indeed involved in these regulatory mechanisms, the entry into mitosis should be carried out in a modified version of their experimental setup by stimulating or inhibiting BCK activity, or in a BCK knock-out animal model.

In a further set of experiments, direct energy provision of the BCK reaction could be analyzed in the sequential Golgi fragmentation actions by MEK1 and Plk (Acharya et al., 1998). This would be a challenging project because of the complexity of the *in vivo* situation. One would have to consider a number of controls that assure some important points: CK activity and ATP provision would have to be related specifically to BCK, and other ATP producing reactions should have to be excluded. But if it could be shown that BCK is essentially related to initial regulatory processes at the entry to mitosis this would

be an absolute novel aspect in the research field of CK and in the involvement of CK in energy metabolism of cell division.

In another aspect of this work uMtCK is associated with proteins that play a role in the pathogenesis of AD. Here, it was shown that uMtCK interacts with APLP1 and a C-terminal region of APP (C31). They both belong to the APSF and APP possesses the sequence for the Aβ peptide present in senile plaques of AD. These interactions were confirmed by *in vitro* analysis, but their function for the cellular processes is still unclear. Modification and dysfunction of mitochondria is thought to be involved in many neurological disorders such as AD and impaired energy availabilty in this context leads to neuronal loss. It was shown that APLP1, and to a higher extent C31, stabilizes the immature precursor of uMtCK and co-localization of these two is found in the mitochondria. This finding seems to be confusing, particularly if we consider that APLP1 and C31 have neither scavenging- nor chaperone-like function to uMtCK as first hypothesized.

Additionally, as the APLP1 is a transmembrane protein with a large extracellular domain, there must be some mechanisms that process and release it into the cytoplasm. This is consistent with the fact that C31 stabilizes also the preform of uMtCK as well. Interestingly, the activity of uMtCK in the mitochondria is increased by the overexpression of APLP1 in the cell culture which draws the conclusion on a potential side effect.

Thus, two main points will have to be cleared. In the future, the question of what is the exact role for the interaction between uMtCK and APLP1 or C31 in the living cell has to be answered second whether this a physiological type of interaction? To elaborate this, I suggest performing *in vitro* interaction analysis of truncated APLP1 and APP constructs, obtained by secretase cleavage. The determination of the sequence from the APSF that interacts with the preform of uMtCK could give information about the function of this interaction. The second question is whether the CK/PCr system can be linked to the pathogenesis of AD? There are many possible links discussed in this work. There are some animal models available showing attributes of AD (Sykova et al., 2005; Sadowski et al., 2004). Answers to that question could be given by feeding these animals with Cr and analyzing parameters like habituation and survival of the animals, oxidation level of the proteins, plaque formation and mitochondrial function.

At all events, the results presented in this work allow new insight in the manifold general energy providing functions of the CK isoenzyme family and motivate to elucidate their exact roles in the contexts of cell cycle events and the pathogenesis of AD.

8.1. References

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9. List of Figures

10. Abbreviations

ABAD	Aβ-binding alcohol dehydrogenase
AD	Alzheimer`s Disease
ADP	Adeonosine diphosphpate
AGAT	L-arginige:glycine amidinotransferaase
AK	Adenylate kinase
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPK	AMP activated kinase
ANT	Adenylate translocator
Ap5A	P^1,P^5-di(adenosine 5′)-pentaphosphate
APLP1,2	Amyloid precursor-like protein 1,2
APP	Amyloid precursor protein
APSF	Amyloid precursor super family
ATP	Adenosine triphosphate
BCK	Brain-type creatine kinase
bp	basepair
CDR	Clinical dementia ratings
CK	Creatine kinase
CLIC	Chloride intracellular channel
CMR	Cerebral metabolic rate
CNS	Central nervous system
COX	Cytochrome c oxidase
CPE	Cellulose polyacetate electrophoresis
Cr	Creatine
CRT	Creatine transporter
DNA	Deoxyribonucleic acid
E.*coli*	Escherichia coli
e.g.	Exempli gratia
ER	Endoplasmic reticulum
Fig.	Figure
GAMT	S-adenosylmethionine:guanidinoacetate N-methyltransferase
GM130	Golgi matrix protein 130
GRASP65	Golgi reassembly and stacking protein 65
GRIP	Golgin-97, RanBP2-a, Imh1p, p230
GS	Gluthamine synthase
GST	Glutathione S-transferase
HD	Huntington`s Disease
HK	Hexokinase
HMG-CoA	Hydroxymethylglutaryl-CoA
IPTG	Isopropyl-b-D-thiogalactopyranoside
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase

MCK	Muscle-type creatine kinase
MEK1	MAPK extracellular signal-regulated kinase 1
Mr	Molecular mass
NBT	Nitroblue tetrazolium
PBS	Phosphate-buffered saline
PCr	Phosphocreatine
PD	Parkinson`s Disease
PDH	Pyruvate dehydrogenase
Plk1	Polo-like kinase1
PCR	Polymerase chain reaction
PTP	Permeability transition pore
ROS	Reactive oxygen species
Rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
sMtCK	Sarcomeric mitochondrial creatine kinases
SOD1	Superoxide dismutase1
SPR	Surface plasmon resonance
SR	Sarcoplasmic reticulum
TCA	Tricarboxylic acid
UCH L-1	Ubiquitin carboxy-terminal hydrolase l-1
uMtCK	Ubiquitous mitochondrial creatine kinases
VDAC1	Voltage-dependent anion channel1
Y2H	Yeast two-hybrid

Curriculum Vitae

Tanja Simone Bürklen

Dr. sc. nat.

Apfelbaumstr. 39
CH-8050 Zürich
Phone private +41-44-3125736
Mobile +49-179-6835689
Phone work +41-44-6333339
Email: tanja@buerklen.de

Curriculum Vitae

Tanja Simone Bürklen

Dr. sc. nat.

Personal Data

Born in August 15th 1974, in Ostfildern, Citizenship: german
Unmarried

Actual Position

10/2005 – today	Post-Doc, Prof. T. Wallimann, Institute of Cellbiology, ETH Zürich

Education

06/2001 – 09/2005	PhD Study: Novel interaction partners of Creatine Kinase isoenzymes in the brain, Prof. T. Wallimann, Institute of Cellbiology, ETH Zürich
11/2004	Further education: dementia: Differencial diagnostic, rehabilitation and trainings
03/2003	Didactics-Seminar: presenting, publishing, communicating, Didaktikzentrum ETH Zürich
10/1995 – 04/2001	Course of studies in Biology, University of Konstanz, Diploma: Interactions between T-cell protein tyrosin phosphatase and potential interaction partners, Prof. W. Hofer, degree grade: 2
1985 – 1994	Matriculation standard, Wirtemberg–Gymnasium, Stuttgart, degree grade: 2,4

Practical experience

2001 – 2005	Supervision of collegiate practical courses, ETH Zürich
2003 – 2004	PhD representative in the institute council, Institute of Cellbiology, ETH Zürich
06/1999	Scientific assistant, Prof. V. Ullrich, Medical Chemistry, University of Konstanz, Regulation of vascular tone in bovine coronary arteries
02/1999	Scientific assistant, Prof. W. Welte, Biophysics and Sturctural Biology: University of Konstanz: X-Ray structure analysis of proteins: Molecule modelling
07/1998	Scientific assistant, Prof. Dr. D. Brdyczka, Biochemistry, University of Konstanz: Mitochondrial physiology
06/1997	Practical course, Prof. W. Welte, Biophysics und Sturctural Biology, University of Konstanz: Proteinpurification for X-Ray structure analysis of proteins
03/1995 – 09/1995	Assistant, private nursing service Stuttgart
10/1994 – 02/1995	Voluntary social year at the Paul–Lechler Krankenhaus, Tübingen
1998 – 2000	Groupleader children´s physical education TV Obertürkheim

Congresses

2005	Joint Meeting, Molecules to mind, ETH Zürich, posterpresentation
2004	D-Biol Symposium ETH Zürich, Davos, posterpresentation
2003	ELSO 2003 - European Life Science Organisation, Dresden
2002	D-Biol Symposium ETH Zürich, Davos
2001	ELSO 2001 - European Life Science Organisation, Nice

Stay abroad

10/1991	Language stay abroad Fernet-Voltaire, France

Additional skills

Communication	Moderator in group discussions, empathy, supervising and motivating, good interpersonal skills, international co-laboration
Research	Project planning and coordinating, creating ideas, identifying problems, understanding information, good data presentation
Computer applications	PC: MS-Windows 95-XP, MS-Office, div. image processing software Internet: Internet/email application, scientific database research
Techniques	Biochemical standard techniques, cellculture (primary, cell lines), molecular and microbiological techniques, confocal microscopy, surface plasmon resonance
Languages	English (fluent), French (good)

Hobbies

Walking, hiking, swimming, cooking and society, reading contemporary and classical literature

Publications

In process

- **Bürklen T**, Wallimann T. Potential benefit of the creatine kinase/phosphocreatine system in the neuro-degeneration of Alzheimer´s Disease
- **Bürklen T**, Hirschy A, Wallimann T. Brain-type Creatine Kinase interacts with the Golgi Matrix protein GM130 in early prophase

published

- Li X, **Bürklen T**, Yuan X, Schlattner U, Desiderio DM, Wallimann T and Homayouni R. (2005) Stabilization of ubiquitous mitochondrial creatine kinase preprotein by APP family proteins. Mol. Cell. Neurosci., in press
- Speer O, Bäck N, **Bürklen T**, Brdiczka D, Koretsky A, Wallimann T and Eriksson O. (2005) Octameric mitochondrial creatine kinase induces and stabilizes contact sites between the inner and outer membrane. Biochem. J. 385 (445–450)