Biol. Chem., Vol. 381, pp. 619 – 622, July 2000

Short Communication

Association of Betaine-Homocysteine S-Methyltransferase with Microtubules

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In mammals, betaine of the mitochondrial matrix is used in the cytosol by betaine-homocysteine S-methyltransferase for methionine synthesis. The resulting dimethylglycine is shuttled back into the mitochondrial matrix for further degradation. Nanospray tandem mass spectrometry and N-terminal amino acid sequencing of microtubule-associated proteins from rat liver tubulin revealed that betaine-homocysteine S-methyltransferase is microtubule associated. This was confirmed by confocal laser scanning microscopy of HepG2 cells labeled with betaine-homocysteine S-methyltransferase- and α-tubulin-specific monoclonal antibodies. The association of betaine-homocysteine S-methyltransferase with the cytoskeleton may functionally integrate the mitochondrial and cytoplasmic compartments of choline degradation.
Key words: Choline / Cytoskeleton / Mitochondria / Tubulin.

The metabolic fate of choline in mammals is threefold. Choline constitutes a part of some phospholipids, including phosphatidylcholine, the most abundant phospholipid; it is required for acetylcholine biosynthesis, and it plays an important role as a C1 donor for S-adenosylmethionine- and folate-dependent C1 transfer reactions. The C1 units are produced during the oxidative degradation of choline, which takes place predominantly in liver and kidney mitochondria (Sunden *et al.*, 1997), although enzymes of this metabolic pathway are also expressed in other tissues (Lang *et al.*, 1994; Bergeron *et al.*, 1998). Choline oxidation starts with choline oxidase, an enzyme of the inner mitochondrial membrane. The betaine aldehyde formed is transformed into betaine by a NAD^+-dependent dehydrogenase of the mitochondrial matrix. One methyl group of betaine is transferred to homocysteine by betaine-homocysteine S-methyltransferase (EC 2.1.1.5; BHMT), yielding methionine and dimethylglycine. One of the two remaining methyl groups is removed from dimethylglycine by dimethylglycine dehydrogenase and transferred to tetrahydrofolate with the formation of methylene-tetrahydrofolate and sarcosine. Sarcosine dehydrogenase then transfers the last methyl group to tetrahydrofolate, yielding a second molecule of methylene-tetrahydrofolate and glycine. Finally, glycine may be further metabolized by the glycine cleavage system into methylene-tetrahydrofolate, NH_4^+ and CO_2.

One pecularity of this pathway is the fact that BHMT resides in the cytoplasm. Thus, betaine has to leave the mitochondrion and dimethylglycine must enter this organelle. Accordingly BHMT, which in mammals is the only alternative to the vitamin B12-dependent methionine synthase, plays an important role at the intersection of the cytosolic and mitochondrial C1 pool. BHMT is an abundant protein in hepatocytes, where it can account for up to 0.5% – 2% of the total soluble protein (Garrow, 1996; Millian and Garrow, 1998). Expression of the enzyme is induced by a methionine-deficient diet (Park and Garrow, 1999), reflecting its physiological role to conserve homocysteine, and thus, methionine. BHMT is also detectable in the human liver hepatoblastoma cell line, HepG2, but its expression is down-regulated (Park and Garrow, 1999) as compared to normal liver cells.

During the last years it has become increasingly apparent that the cytosolic enzymes of various metabolic pathways are organized to be at specific cellular locations in contact with particular cellular structures like the plasma membrane, the endoplasmic reticulum (ER) membrane or the mitochondrion. The microtubular cytoskeleton is assumed to play an important role in the intracellular organization and connection of these metabolic subcompartments.

This work was started with the aim of identifying tubulin-associated proteins (MAPs) from rat liver. Here we show that a fraction of BHMT is found tightly associated with the tubulin fraction of hepatocytes, and a significant amount of BHMT in HepG2 cells is associated with the microtubules. Such an association of BHMT with the cytoskeleton may help to functionally link the mitochondrial betaine-dimethylglycine shuttle with the enzyme involved in demethylation of betaine and methionine synthesis.

First, the tubulin fraction of rat liver extract was isolated by taxol precipitation and sucrose gradient centrifugation.

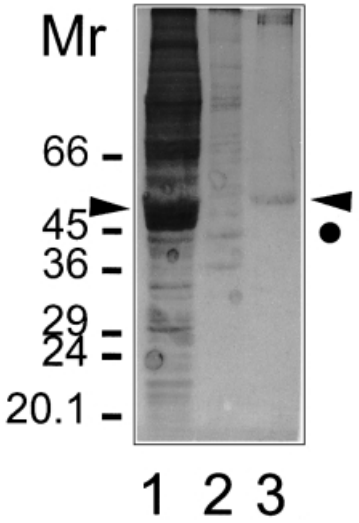

Fig. 1 Analysis of the Tubulin-MAP Fraction from Soluble Rat Liver Extract following Taxol Cosedimentation and Sucrose Gradient Centrifugation.
Rat liver extract was prepared according to Stoltz *et al.* (1997) and tubulin and MAPs were isolated following the protocol of Nick *et al.* (1995). Proteins were analyzed by SDS-PAGE on a 12% polyacrylamide gel and stained by Coomassie Brilliant Blue. Lane 1, tubulin fraction after elution of MAPs with 3 M KCl; lane 2, MAP fraction; lane 3, 2 μg of bovine neurotubulin. The molecular mass of protein molecular markers is given in kDa. The left arrowhead indicates rat liver tubulin, the right arrowhead bovine neural tubulin, the black dot a protein band identified as BHMT by N-terminal amino acid sequencing (Hewick *et al.*, 1981) and nanospray tandem mass spectrometry (Wilm and Mann, 1996).

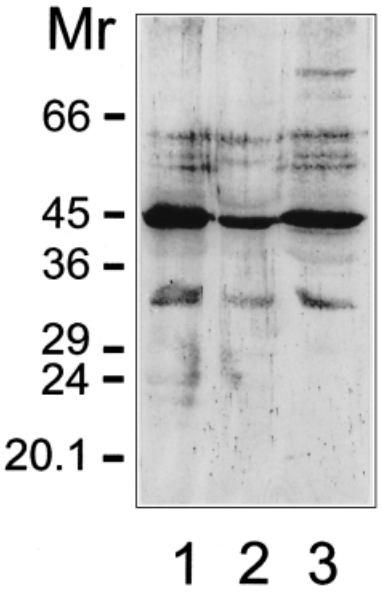

Fig. 2 Western Blot Analysis of Rat Liver Extract, Rat Liver Tubulin and the Rat Liver MAP Fraction.
Western blots were prepared with an electroblotting device from Bio-Rad (Munich, Germany) as recommended by the supplier and decorated with BHMT antiserum. Lane 1, supernatant of liver extract after taxol precipitation of tubulin; lane 2, tubulin fraction following centrifugation through a 20% sucrose cushion and release of MAPs with 3 M KCl; lane 3, MAP fraction.

MAPs were released from tubulin with a small volume of 3 M KCl and analyzed by SDS-PAGE (Figure 1). One main protein band in the MAP fraction was identified by nanospray tandem mass spectrometry (Nano ESI MSMS) as BHMT. N-terminal amino acid sequencing of this protein band revealed the sequence NH_2-MAPIAGKKAKCOOH (one letter code of amino acids), which corresponds exactly to the N-terminal amino acid sequence of BHMT.

To gain additional evidence for the association of BHMT with tubulin, the distribution of the enzyme in rat liver fractions was analyzed following SDS-PAGE and Western blotting with the aid of a rabbit antiserum raised against human BHMT. This antiserum was also specific for the rat protein (Park and Garrow, 1999; Figure 2). The supernatant after taxol precipitation of tubulin (Figure 2, lane 1) still contained BHMT, probably due to the high amount of this protein in hepatocytes, indicating that the amount of tubulin might be limiting. The tubulin pellet that had been treated with salt (Figure 2, lane 2) still gave a signal with the BHMT-antiserum. Apparently a fraction of BHMT was tightly associated with tubulin. However, a large part of the bound BHMT was detached by treating the taxol-precipitated tubulin fraction with 3 M KCl (Figure 2, lane 3).

To directly visualize an association of BHMT with microtubules, the rabbit human-BHMT antiserum and a monoclonal mouse α-tubulin antibody, directed angainst a highly conserved N-terminal epitope (Amersham, Little Chalfont, UK; Petràšek *et al.*, 1998), were used for double-immunofluorescent staining of HepG2 cells and analyzed by confocal laser scanning microscopy. Figure 3 shows the colocalization of BHMT (Figure 3A) with microtubules (Figure 3B) in the region of the perinuclear ring. The superposition of the two signals is shown in Figure 3C; the yellow color indicates regions where both signals overlap. At high magnification it can also be detected along cytoplasmic microtubules (Figure 3C) and in pericellular protrusions of the cell (Figure 3D – E). To check for the specificity of staining and for potential filter leakage contaminating the BHMT signal by the microtubule signal, control experiments were performed where the BHMT antiserum was replaced by a rabbit normal serum. Although microtubules could be clearly visualized in these controls (Figure 3G), there was virtually no BHMT signal detectable (Figure 3F), demonstrating the specificity of the BHMT signal.

The results presented here show that BHMT can be found associated with microtubules. The cosedimentation of BHMT with tubulin in the taxol assay demonstrates that BHMT can coassemble with tubulin dimers into microtubules. In rat liver, where the amount of BHMT is high, not all of the protein was tubulin-associated. However, since a 3 M KCl wash did not dissociate all BHMT from the tubulin fraction, the association of BHMT with tubulin appears not to be an experimental artefact due to its high expression in liver. Whether the free and the tubulin-associated forms of BHMT in rat liver represent two functionally different forms of the enzyme is not clear.

The argument for a genuine association of BHMT with the microtubular cytoskeleton is strenghtened by the

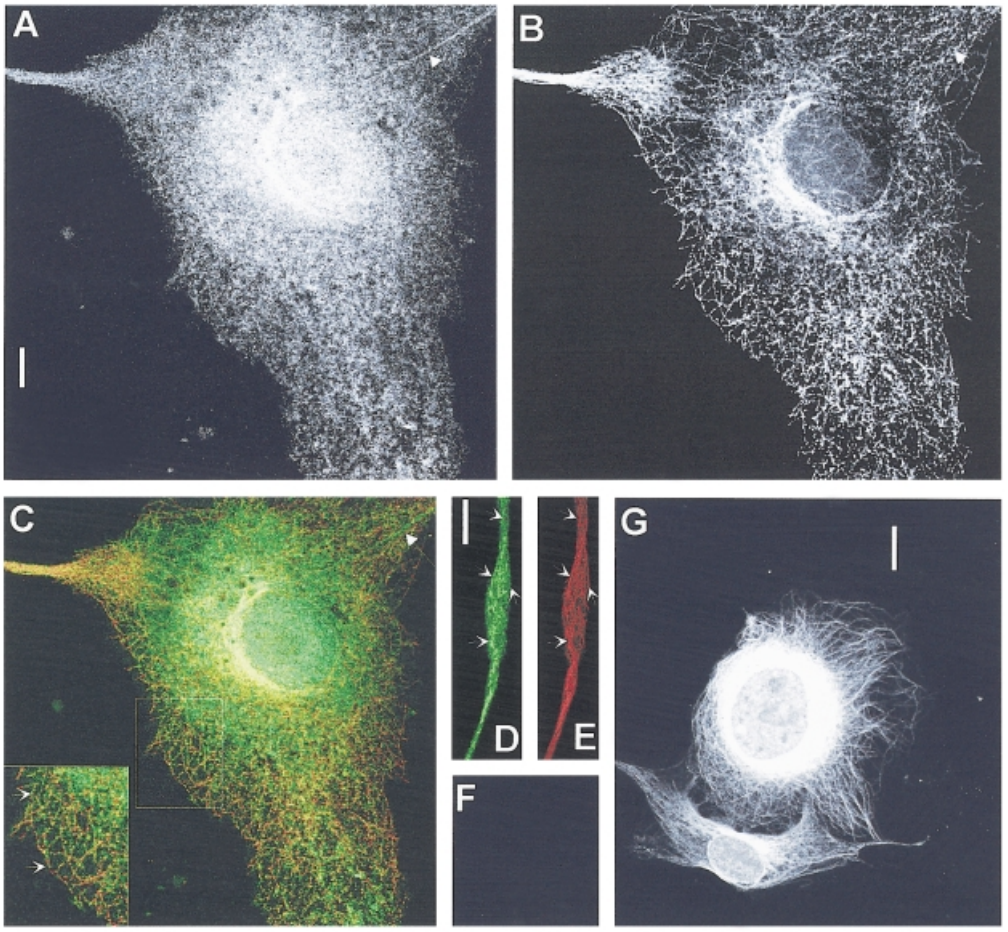

Fig. 3 Double Immunofluorescence Analysis of HepG2 Cells.
Cells of the human hepatoblastoma cell line HepG2 were processed for double immunofluorescence staining as described in detail in Petràšek *et al.* (1998). As first antibodies monoclonal mouse α-tubulin antibodies (1:100 in TTBS) and rabbit BHMT-antiserum (1: 250 in TTBS) were used, followed by the secondary antibodies (1: 25 in TTBS), which were TRITC-labeled goat anti-mouse IgG and FITC-labeled goat anti-rabbit IgG. As controls, HepG2 cells were treated with normal rabbit serum (BHMT-antibody free; 1:100 in TTBS) and monoclonal mouse α-tubulin antibodies, followed by FITC-fused goat anti-rabbit IgG and TRITC-fused goat anti-mouse IgG. The cells were visualized under a confocal laser microscope (DM RBE; Leica, Bensheim, Germany), using a two-channel scan with an argon-krypton laser at 488 nm and 568 nm excitation, a beam splitter at 575 nm, and 580 nm and 590 nm emission filters using a 36-image line averaging algorithm. The images were processed and superimposed using the Scanware software (Leica, Bensheim, Germany).
(A – C), colocalization of BHMT (A) with microtubules (B) in the region of the perinuclear ring. The superposition of the two signals is shown in (C); the yellow color indicates regions where both signals overlap. The insert in (C) shows a blow-up of the peripheral region to show the decoration (white arrows) of microtubules (red) with BHMT (green). The white arrowheads in (A), (B) and (C) indicate a microtubule that is densely covered by BHMT. The white bar represents 5 μm. (D – E), colocalization of BHMT (D) and microtubules (E) in pericellular protrusions. The white arrows indicate microtubules that are continuously decorated with BHMT. The white bar represents 2 μm. (F – G), negative controls checking for filter leakage of the tubulin signal into the BHMT signal and for specificity of the BHMT antibody. In these controls the BHMT-antiserum was replaced by a rabbit normal serum; the microtubular signal is shown in (G), the (absent) BHMT signal is shown for the same cell in (F).

immunofluorescent images of HepG2 cells. The BHMT-antiserum specifically identified reactive material associated with microtubules. Microtubule-associated proteins (MAPs) control the dynamics and the organization of the microtubular cytoskeleton, supporting its cellular motor and transport functions. Among the molecules transported along the microtubular network are proteins like growth factors, calmodulin, lysosomal enzymes and the glycolytic enzyme enolase (Brady and Lasek, 1982). An association with, and a movement along, microtubules was also demonstrated for mitochondria (Tyler, 1992). The association and movement of BHMT and mitochondria along microtubules can facilitate the biochemical and physiological function of BHMT, which is to transfer a methyl group from the betaine being synthesized in the mitochondrial matrix, to the cytosolically located homocysteine. Following methionine biosynthesis the demethylated product, dimethylglycine, would then be in a position to be readily transported into the mitochondrial matrix for further degradation. In this way a tight link in space between the different compartments of the metabolic pathways for choline demethylation and methionine synthesis

and the transport of metabolites between the cellular compartments could be achieved by their association with the cytoskeleton.

Acknowledgements

We wish to thank W. Fritz for help in preparing the manuscript and the figures. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to R. B.

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Received March 31, 2000; accepted May 18, 2000