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Expression of monocarboxylate transporter 4 in human platelets, leukocytes, and tissues assessed by antibodies raised against terminal versus pre-terminal peptides

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Abstract

We compared antibodies (Abs) raised in rabbits against two non-overlapping peptides, terminal (T) and pre-terminal (PT) of the human monocarboxylate transporter (MCT4) lactate transporter in a variety of human tissues. Upon stringent SDS extraction, the PT Ab recognized a major 32 kDa band in many tissues, but not in leukocytes, while the T Ab recognized a 45 kDa band in leukocytes but only in a few other tissues. In two cell lines, human adult retinal pigment epithelial and Madin–Darby canine kidney, however, both Abs identified the same 45 kDa band only, whether extracted by stringent SDS or by a mild Triton X-100 procedure. Applying Triton X-100 and milder SDS methods to human tissues led us to conclude that: (1) MCT4 is more labile to proteolysis than MCT1 or 2; (2) the proteolysis involves an enzyme system which is absent from the cell lines, is of variable content in human tissues, and is accelerated by SDS and/or heat; (3) a major product is the 32 kDa band, which is missing the C-terminal peptide, since it is seen by the Ab to MCT4-PT, but not the Ab to MCT4-T; (4) this truncated 32 kDa form is prone to aggregate, producing oligomers also detected only by the MCT4-PT; (5) the 32 kDa form may have a physiological function, since (except in the cell lines and monocytes) it is the major form seen with the PT Ab even with our mildest extractions, and since MCT4-PT stained two compartments that were not stained by the T Ab in our immunohistochemistry survey: the capsule of the muscle spindle, and the cytoplasm of the lymphocyte; (6) platelets contained MCT4, stained by both Abs, and verified by the 45 kDa band on Western blotting, in addition to the presence of MCT2 that we had demonstrated previously [N. Merezhinskaya, S.A. Ogunwuyi, F.G. Mullick, W.N. Fishbein, Presence and localization of three lactic acid transporters (MCT1, -2, and -4) in separated human granulocytes, lymphocytes, and monocytes, J. Histochem. Cytochem. 52 (2004) 1483–1493.

Keywords: Lactate transporters; Monocarboxylate transporter; Human leukocytes; Antibodies; Immunohistochemistry; Western blotting; Granulocytes; Monocytes; Lymphocytes; Platelets

Introduction

The family of monocarboxylate transporters carries out the proton-linked translocation of lactate, pyruvate, and other monocarboxylates across plasma membranes (for review, see [1–4]). This transport does not utilize energy input, and is controlled by the concentration gradients of the substrates and protons. The first four members of the family (MCT1–4) have been proven experimentally to transport monocarboxylates [5–9], and two other members have been recently discovered to transport thyroid hormones (MCT8) [10,11] and aromatic amino acids (TAT1) [12]. A mutated MCT8 has been linked to a severe arrested development neurologic syndrome [10,11], and mutations in MCT1 have been found in some patients with exercise intolerance [13]. The rest of the MCTs have been identified on the basis of sequence homology, and are yet to be characterized kinetically and physiologically.

Monocarboxylate transporter 4 (MCT4) has initially been identified as human MCT3, since it had a fairly high

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Fig. 1. Location of Pre-C-Terminal (MCT4-PT) and C-terminal (MCT4-T) peptides in MCT4 sequence.

homology with chicken MCT3 [14]. However, it was renamed MCT4 in 1999 [1] after it was found to have a much broader tissue distribution than chicken, mouse, and rat MCT3, all of which were expressed exclusively in retinal pigment epithelium [15,16].

We have previously studied expression of MCT 4 in human tissues [17] using a polyclonal antibody raised against a Pre-C-Terminal peptide of human MCT4 (anti-MCT4-PT, Fig. 1). The Pre-C-Terminal peptide sequence, selected for antigenic properties using PC Gene software, was run against a protein database (National Center for Biotechnology Information, National Library of Medicine) to exclude any four amino acid sequence matches with other proteins. Anti-MCT4-PT antibody gave a single 32–33 kDa band in seven of the human tissues tested, and this band was competed out by the specific peptide. However, anti-MCT4-PT produced no bands on blots of separated human granulocytes, lymphocytes, and monocytes, although we demonstrated the MCT4 mRNA in all three cell types [18] and other researchers had reported a 45 kDa MCT4 band in a mixed population of human leukocytes using an antibody raised against the C-terminal peptide, distinct from our anti-MCT4-PT [7]. We, therefore, prepared a second rabbit antibody against the same C-terminal peptide, i.e., anti-MCT4-T (Fig. 1). This new antibody recognized a single strong 45 kDa band in lymphocytes and monocytes, and a very weak band in granulocytes (which have extreme proteolytic activity) [18], and these bands were eliminated by preincubation of the antibody with the C-terminal peptide. We therefore utilized the anti-MCT4-T in our study of the fractionated white cells, and deferred a definitive comparison of the -T and -PT antibodies for the present work.

Materials and methods

Antiserum preparation and purification

MCT4-T and MCT4-PT antibodies were prepared from rabbits, using KLH conjugation, Freund's adjuvant, IgG isolation on protein A column, and subsequent affinity chromatography, as described previously [17,18].

Leukocyte fractionation

The fractionation was performed as described earlier [18] with modifications to further improve the fraction purity and decrease red cell contamination. Blood samples from donors (with written consent) were collected in heparin tubes and then poured into EDTA tubes to ensure minimal clotting. Blood was diluted with PBS + 2 mM EDTA (1:3 v/v) and layered on an equal volume of Histopaque 1077. The cells were spun down for 400*g* for 35 min at 20 °C. Granulocyte pellets were treated with a 10-fold volume of buffer A (155 mM NH_4Cl, 10 mM $KHCO_3$, and 10 mM EDTA, pH 7.3) at 20 °C for 10 min to lyse red cells. Upon clearing, the solution was centrifuged at 300*g* for 10 min at 20 °C and the treatment was repeated once more. The pellet was washed to a barely pink color in 0.5% BSA, 2 mM EDTA in PBS, pH 7.3. After resuspension in PBS, and preparation of droplet and smear microscope slides, the remaining granulocyte fraction was subdivided and stored at −80 °C.

The mononuclear cells at the Histopaque 1077 interface were harvested, washed, and spun for 10 min at 200*g* and 24 °C. Monocytes were separated from lymphocytes by selective adsorption on anti-CD14-coupled magnetic microbeads (Miltenyi Biotech, Auburn, CA) as detailed [18] with the following modification. The flow-through lymphocyte–platelet fraction was left standing at room temperature for 30–60 min to allow a pellet consisting primarily of lymphocytes to form. This pellet was resuspended in PBS, used to prepare droplet and smear slides, then aliquoted, and stored at −80 °C. The supernatant containing the smaller platelets and rare lymphocytes was spun down at 100*g* for 5 min, and the platelet pellet was resuspended in PBS. Droplet and smear slides were prepared immediately for evaluating platelet to lymphocyte ratios, and the remainder was frozen at −80 °C for Western blotting.

Tissue and cell membrane preparation

Stringent SDS extraction of tissues was performed as previously described [17]. For Triton X-100 extraction, tissues were thawed and minced on ice. One milliliter of imidazole buffer (10 mM imidazole, 100 mM KCl, 1 mM EDTA, and 5 mm $MgCl_2$, pH 7.4), with Complete Protease Inhibitor Cocktail (Boehringer, Indianapolis, Indiana) was added, and the tissues were homogenized on ice with a Pro-Tissue homogenizer for 10–30 s. The mixture was sonicated on ice at 40% cycle and 1.25 output for 10 s in pulse mode with the microprobe (Heat Systems/Ultrasonics, Plainview, NY). The suspension was spun in an Eppendorf centrifuge at 16,500*g* for 30 min at 4 °C (low speed spin) or in the Beckman Airfuge at 134,000*g* for 60 min at 24 °C (high speed spin). The membrane pellet was resuspended in Triton X-100 buffer (the imidazole buffer with added 1% Triton X-100) with freshly added protease inhibitors, and incubated either on ice or at 24 °C for 30 min. The mixture was spun down at 16,500 rpm for 30 min at 4 °C, and the supernatant (Triton X-100 solubilized membrane fraction) was either used immediately or frozen on dry ice and stored at −130 °C.

Fractionated white blood cells, and human ARPE19 and canine MDCK cell lines kindly sent to us by Dr. Nancy Philp (Department of Pathology, Anatomy, Cell Biology, Thomas Jefferson University, Philadelphia) were treated the same except for omitting the homogenization step. In initial trials with anti-MCT4-PT, we used a 10 min freeze (dry ice) and 10 min thaw (37 °C) sequence repeated twice more for cell disruption; but later on we discarded this procedure as unnecessary, and moreover a likely candidate for aggravating protein degradation, which had then come under suspicion. For the same reason, upon revisiting the SDS procedure, we decreased the concentration for extraction to 2.5%, and limited the temperature to 24 °C, to provide milder extraction conditions.

Western blot analysis

SDS-extracted membranes were initially heated at 100 °C for 5 min as in the past [17,18], whereas Triton X-100 extracted membranes were incubated at 37 °C for 5 min with sample buffer (62.5 mM Tris–HCl, pH 6.8, 50 mM dithiothreitol, 10% glycerol, 0.1% bromphenol blue, and 2.5% SDS) prior to loading on a gel for SDS–PAGE. The rest of the procedure was as previously described [17]. The milder SDS method employed a 24 °C incubation for 5 min with the same sample buffer.

Immunohistochemical analysis

Both 6 μm sequential cryostat section of frozen human skeletal muscle, and freshly separated white blood cells or platelets that had been drop-dried or smeared on glass slides and fixed with 100% methanol for 30 min

at 4 °C were used for antibody staining by the method described earlier [18]. Photomicrography was performed on the Olympus Provis AX-70 microscope equipped with the DP-70 12-megapixel camera, using an oil-immersion lens.

Other procedures

Protein concentrations were measured with the Lowry procedure [19], modified to 0.8 ml final volume to triple the sensitivity, or DC Protein Assay (Bio-Rad Lab, Hercules, CA) for Triton X-100 samples; BSA provided the reference standard. For rabbit antisera purification, UV absorption was measured; and the protein concentration was calculated using the known specific absorption of rabbit IgG, $A^{1\%} = 13.5$ at 280 nm, 1 cm. The reverse ATPase reaction was used to evaluate the three muscle fiber types in frozen sections as described by Gregory and Griffin [20].

Results

Western blot analysis of MCT4-T and -PT on human cell culture lines

The first piece of evidence that the anti-MCT4-PT also recognizes the full-length MCT4 came from Dr. Nancy Philp, who informed us that our donated sample of anti-MCT4-PT reacted with the same 45 kDa protein as her own anti-MCT4-T in a 1% Triton X-100 extract of the human ARPE-19 cell line, and kindly sent us samples of that cell line and also of the canine MDCK cell line. Since we had used 3.75% SDS to prepare extracts from human tissues and cells, we reasoned that this harsher procedure may cause proteolysis of the 45 kDa band, and extracted both cell lines using our standard SDS procedure. However, both anti-MCT4-PT and -T still reacted only with a single 45 kDa band in both cell lines (Fig. 2). Supernatant fractions from the 16,500*g* centrifugation were also positive for this band (Figs. 2 and 3, lanes S) but after 127,000*g* for 30 min, the upper half of the supernatant was devoid of detectable antigen with either antibody, indicating that all of the antigen was particulate (Fig. 3, lanes S1). Since biochemical and physiological composition of immortalized cell lines can differ significantly from cells and tissues, we extended the evaluation of extraction procedures to human tissues.

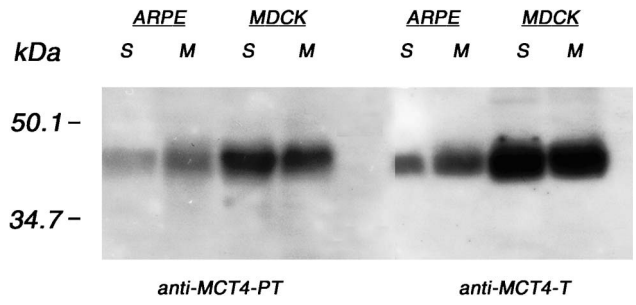

Fig. 2. Western blot of MCT4 in low speed supernatant (S) and membrane (M) fractions of ARPE and MDCK cell lines probed with either pre-terminal (MCT4-PT) or terminal (MCT4-T) antibodies. The blot is from a single gel with extraneous lanes removed. The nearest molecular weight standards are shown on the left.

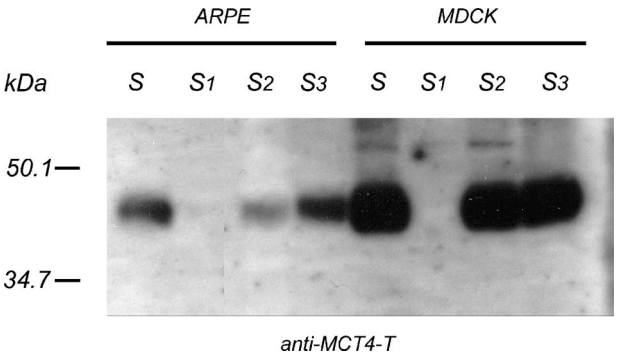

Fig. 3. Western blot of MCT4 in low and high speed supernatant fractions of ARPE and MDCK cell lines. Supernatants after 16,500*g* (S) were further spun down at 127,000*g* for 30 min and aliquots from the high speed supernatant upper half (S1), lower half (S2), and from the pellet resuspended in the initial volume (S3) were run on SDS–PAGE, transblotted, and probed with anti-MCT4-T antibody. Molecular weight markers are shown on the left.

Methodologic effects on Western blots of tissues with anti-MCT4-T and -PT

We compared our standard conditions (3.75% SDS extraction at 37 °C, and boiling the sample with loading dye prior to SDS–PAGE) with milder extraction (1% Triton X-100 incubation either on ice or at 37 °C, and 37 °C incubation with loading dye) on two human tissues, muscle and esophagus. As shown in Fig. 4, these modifications had dramatic effects. The milder Triton procedure now revealed a major 45 kDa band by anti-MCT4-PT as well as -T; and it was stronger with the 4 °C than with the 37 °C incubation. MCT4-PT also reacted with the 32 kDa band we had seen in our earlier experiments. The stringent SDS-boiling procedure completely eliminated (in muscle) or greatly reduced (in esophagus) the 45 kDa band for both antibodies (Fig. 4, SDS 37/100 °C). Under these conditions, anti-MCT4-T was negative on the muscle blot and reacted very weakly only with some high MW bands in esophagus. Anti-MCT4-PT reacted with the 32 kDa band and also with high MW forms which are likely to be aggregates of this band, since they were also competed out by preincubation with the specific peptide.

The SDS/heat treatment cannot be solely responsible for the protein degradation, as can be seen in Fig. 4 (last lane), where the MDCK membranes treated with SDS still maintain the 45 kDa band. Rather, SDS/heat treatment in tissues must activate proteolysis which involves the C-terminus, thus removing the MCT4-T antigenic site. This must also render the truncated protein susceptible to aggregation, and the aggregates will still carry the antigenic site for MCT4-PT, but not for MCT4-T.

A number of factors promoted the formation of the 32 kDa band with simultaneous decrease of the 45 kDa band. Storage of membrane extracts and tissues at temperatures higher than −80 °C, or employing high sonication intensities, led to such conversion. Fig. 5 compares Western

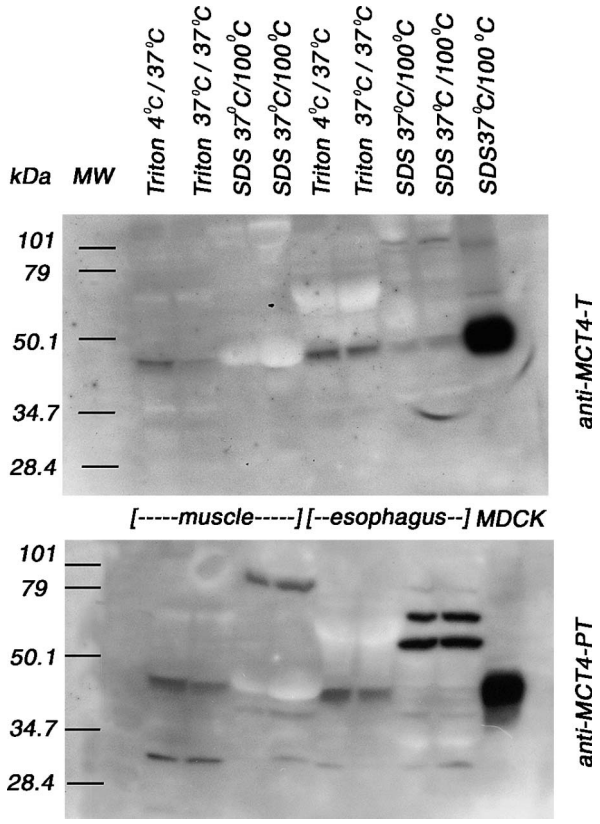

Fig. 4. Western blot of muscle and esophagus membranes extracted either with 1% Triton or SDS and probed with either anti-MCT4-T (upper panel) or anti-MCT4-PT (lower panel). Detergent extraction temperatures are indicated above, to the left, and loading dye incubation temperatures are indicated above, to the right of the forward slash. Control MDCK membranes are shown on the right and molecular weight markers are on the left. White bands occur where high level of immunologically unreactive protein impede background staining.

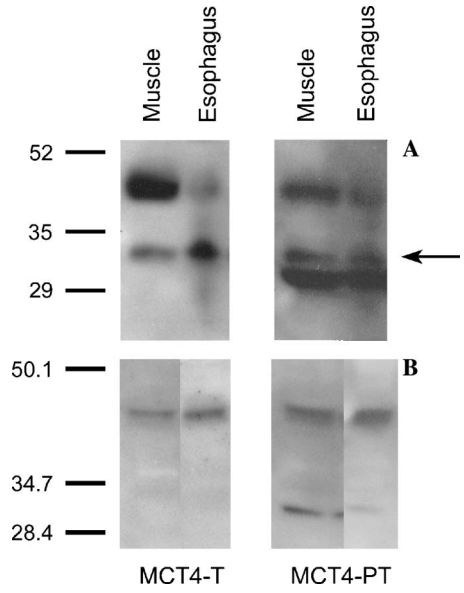

Fig. 5. Western blot of muscle and esophagus Triton X-100 membrane extracts stored at −20 °C (A) and −130 °C (B). Lanes in (B) are duplicates of the 4 °C Triton X-100 extracts from Fig. 4. Note the appearance of a new 34 kDa band recognized by both antibodies (arrow). The 32 kDa band still reacts with anti-MCT4-PT only, and is now greater in quantity than the 45 kDa band. Molecular weight markers are shown on the left.

blot analysis of Triton X-100 membrane extracts from muscle and esophagus after storage for a week at −20 °C (panel A), with the storage at −130 °C (panel B). At either storage temperature, anti-MCT4-PT recognized 45 and 32 kDa bands in muscle and esophagus (two right lanes), but the amount of the 32 kDa band was significantly greater than that of the 45 kDa band at −20 °C (panel A). In contrast, the same tissues stored at −130 °C exhibited levels of the 45 kDa form higher than (esophagus) or equal to (muscle) the 32 kDa band (panel B). After storage at −20 °C, an additional 34 kDa band appeared and it was recognized by anti-MCT4-T as well as by anti-MCT4-PT (Fig. 5, arrow). This shows that anti-MCT4-T antibody can recognize bands other than 45 kDa; so the presence of 45 kDa band on a Western blot cannot be postulated as the sole criterion for the specificity for any given anti-MCT4 antibody. Instead, the presence of smaller molecular weight bands suggests the MCT4 C-terminus may be cleaved at several sites, and some of resulting products might still have an epitope detectable by anti-MCT4-T (as well as by anti-MCT4-PT). Further degradation leads to the complete elimination of the MCT4-T epitope and consequently results in negative Western blots in several tissues that remain positive to the anti-MCT4-PT antibody by virtue of the 32 kDa band.

We were able to demonstrate the 45 kDa band in a number of other human tissues by using the milder extraction, low sonication intensity, moderate temperature for loading dye incubation, and running SDS–PAGE and blot transfer immediately after membrane extraction. Fig. 6 shows a multiple tissues Western blot with MCT4-T and MCT4-PT. Both antibodies reacted with the 45 kDa band in stomach, kidney, colon, esophagus, muscle, and lung. We were able to demonstrate the 45 kDa band in heart and brain only with MCT4-PT, because of the limited availability of fresh tissue. In addition to the 45 kDa band, MCT4-PT reacted with the 32 kDa band in all tissues mentioned. Except for colon (Fig. 6), the 32 kDa band was stronger than the 45 kDa; but both bands were eliminated after preincubation of either antibody with its specific peptide.

Anti-MCT4-PT as well as anti-MCT4-T identified the 45 kDa band in Triton X-100 extracted membranes of monocytes and lymphocytes (Fig. 7). We had demonstrated earlier [18] that MCT4-T sometimes showed a faint 45 kDa band in granulocytes as well, but its recovery was hindered by proteolytic activity of these highly degradative cells. MCT4-PT, but not MCT4-T, also recognized a 32 kDa band in lymphocytes (Fig. 7, lane L).

We had reported in our previous paper [18] that platelets showed plasmalemmal staining with anti-MCT2, but did not show convincing staining with MCT1 or 4, and our platelet preparations were then not pure enough to confirm the immunohistochemistry (IHC) by Western

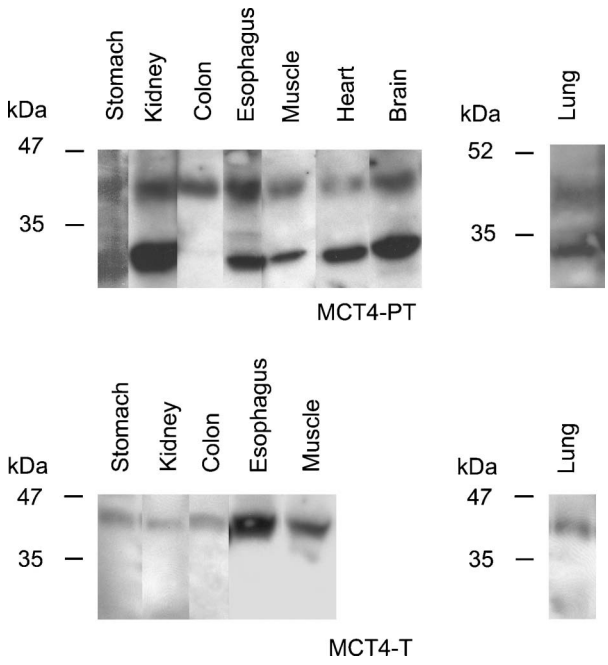

Fig. 6. Western blot of tissue membranes probed with either anti-MCT4-PT (upper panel) or anti-MCT4-T (lower panel). Results from several experiments were combined with extraneous lanes removed. Heart and brain tissues were not available for the MCT4-T Western blot. The amount of protein loaded on the gel was optimized to give the strongest signal without elevating the background staining. Molecular weight markers are indicated on the left.

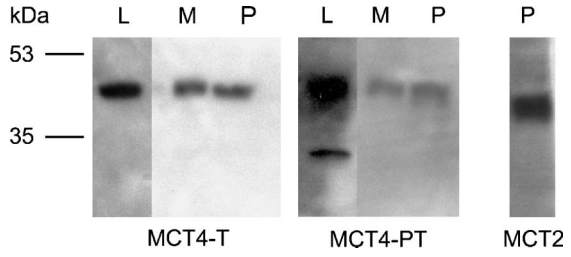

Fig. 7. Western blot of MCT4 in lymphocytes (L), monocytes (M), and platelets (P) probed with anti-MCT4-T, anti-MCT4-PT, and anti-MCT2. The results are from two different blots with extraneous lanes removed. Note that only the lymphocytes show a 32 kDa band. Molecular weight markers are indicated on the left.

blotting. In this study, we therefore prepared highly purified platelets (see Materials and methods) having <1% cellular contamination by volume (estimating a 30-fold greater lymphocyte volume). These platelet membranes were Triton X-100 extracted and analyzed by Western blotting (Fig. 7). The membranes were negative for MCT1 in accordance with our earlier IHC data (not shown). Anti-MCT2 antibody produced a 43 kDa band corresponding to that in human tissues which express MCT2, also as expected. Both anti-MCT4-T and anti-MCT4-PT, however, were also positive, each reacting with the same 45 kDa band in the purified platelet membranes. All of these bands were blocked upon preincubation of each antibody with its specific peptide prior to membrane application.

Immunohistochemical analysis of MCT4 expression

Methanol-fixed serial frozen sections of human skeletal muscle were used for the IHC experiments. Methanol fixation does not introduce artifacts when compared with unfixed frozen muscle [18] and early fixation should prevent the proteolysis encountered during membrane extraction, SDS–PAGE, and Western blotting. The use of muscle cross-sections also permits us to track the same cell in many

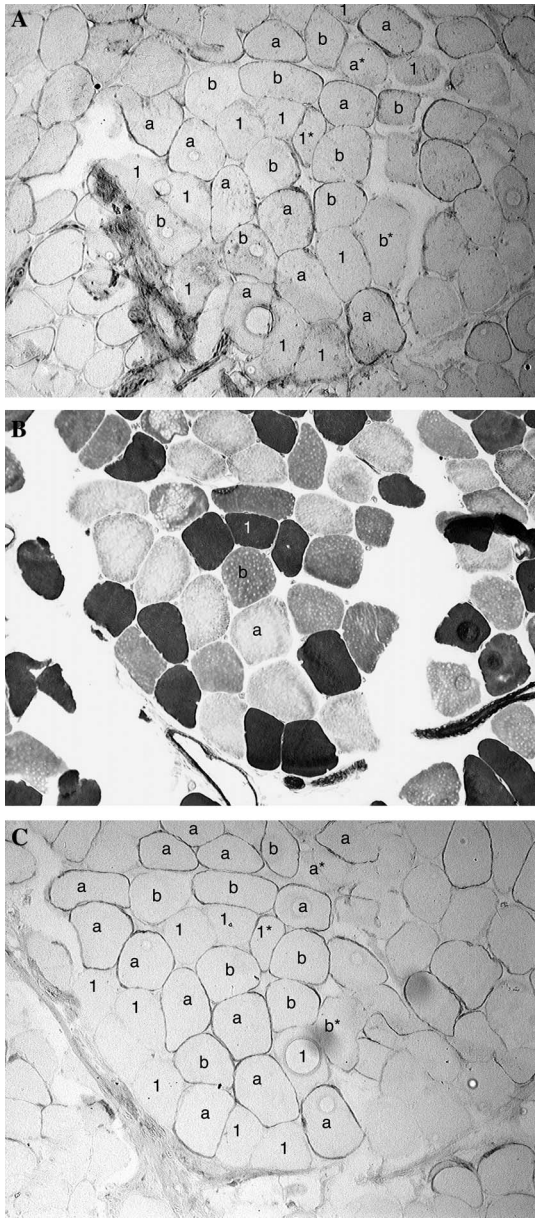

Fig. 8. IHC staining of MCT4 in serial sections of methanol-fixed frozen normal human skeletal muscle with anti-MCT4-T (A) and anti-MCT4-PT (C) antibodies, fiber types accorded by the reverse ATPase stain (B). Dark = type 1; light = type 2a (coded here as "a"); intermediate = type 2b (coded here as "b"). Magnification ~60×. Both antibodies reacted with the same subset of predominantly type 2a and 2b fibers. Three anomalous fibers were also identified by both antibodies, and are marked by asterisks: staining of a single type 1 fiber, and absence of staining of a single type 2a fiber and a single type 2b fiber.

serial sections and to correlate the immunostaining with the fiber type in a neighboring section stained by the reverse ATPase reaction, as shown in Fig. 8B. The dark fibers are type 1, the light fibers are type 2a, and the intermediate fibers are type 2b. Serial sections of the same muscle, with fiber types annotated, were immunostained with anti-MCT4-T (Fig. 8A) or anti-MCT4-PT (Fig. 8C). Both antibodies stained exactly the same subset of muscle fibers, predominantly type 2a and b. Three anomalous fiber-type-stains were also expressed by both antibodies and are marked by asterisks: staining of a single type 1 fiber, and absence of staining of a single type 2a fiber and a single type 2b fiber.

Methanol-fixed serial sections of a muscle spindle were separately stained with anti-MCT4-T and -PT (Fig. 9B and C). The spindle intrafusal fibers could not be traced across sections, like the longer and larger extrafusal muscle fibers, so we could only evaluate the general staining pattern, rather than that of specific individual fibers. The minimal degree of non-specific staining obtained in the absence of primary antibody is shown in panel A. Both anti-MCT4-T and anti-MCT4-PT stained some of the small chain fibers, but, in addition, anti-MCT4-PT stained the spindle capsule, a unique structure heavily invested with nerves and vessels.

The results of IHC staining of fractionated white blood cells with anti-MCT4-T and anti-MCT4-PT are shown in Fig. 10. Both antibodies stained plasmalemma and cytosol of monocytes and granulocytes. Anti-MCT4-PT staining was somewhat stronger and sharper than anti-MCT4-T, but did not exhibit convincing staining of a distinctive compartment. Preincubation of the antibodies with specific peptides prior to membrane application significantly decreased the intensity of the staining (compare panel A with C, and B with D). In lymphocytes, anti-MCT4-T stained only the plasmalemma, whereas anti-MCT4-PT strongly stained the cytoplasm in addition (Figs. 10E and F).

Purified platelets exhibited membrane staining with both anti-MCT4-T and -PT with cobalt enhancement of the DAB stain (Figs. 10I and J), and this membrane staining was also detected in preparations stained with conventional DAB arrows (Figs. 10F and G). Many platelets also exhibit somewhat weaker but definite cytoplasmic staining with both antibodies, suggesting that subcellular organelles may well contain the antigen, just as we have suggested for fractionated leukocytes.

Discussion

We have demonstrated that the identification of MCT4 in Western blots is critically dependent upon experimental conditions. The "full length" MCT4 band (the quotes signify the phrase as figurative) with apparent SDS–PAGE MW (MW_{app}) of 45 kDa was identified by both anti-MCT4-T and anti-MCT4-PT in human cells and tissues extracted with warm or cool 1% Triton and incubated with loading dye at 37 °C. An additional prominent 32 kDa band, identified only with the MCT4-PT antibody and blocked by preincubation of anti-MCT4-PT with its specific peptide antigen, was also present. We note that experimentally observed MW_{app} for both "full length" 45 and 32 kDa MCT4 are lower than their theoretical MW calculated by the amino acid composition. This phenomenon is common for highly hydrophobic membrane proteins, and it reflects different metastable conformations often displayed by these proteins during electrophoresis [21]. Lower MW_{app} values have been reported for MCTs by other investigators. Halestrap et al. reported a value for human MCT4 as 42 kDa compared with MW_{calc} of 49.5 kDa [7]. Rat MCT2 has been reported as 50 kDa [22], 46 kDa [23], and 37 kDa [24], with MW_{calc} being 53 kDa [14]. We have reported "full length" MW_{app} for MCT1 as 46 kDa ($MW_{calc} = 54$ kDa) and for MCT2 as 43 ($MW_{calc} = 52$ kDa) [17]. In view of these discrepancies, it is clear that one can hardly presume that the "truncated 32 kDa form" will differ from the "full length 45 kDa form" by the MW_{calc} of the peptide removed.

An observed increase in the amount of 32 kDa band with the concomitant decrease in the 45 kDa band under permissive conditions (heat, ionic detergents), as evident from Fig. 5, indicated to us that the 32 kDa band was derived from the "full length" protein as a result of proteolysis. Although alternative splicing is frequently observed in transmembrane transporters [25,26], it can not explain the appearance of the 32 kDa band in our case. First, both MCT4-T and MCT4-PT sequences reside in the middle of

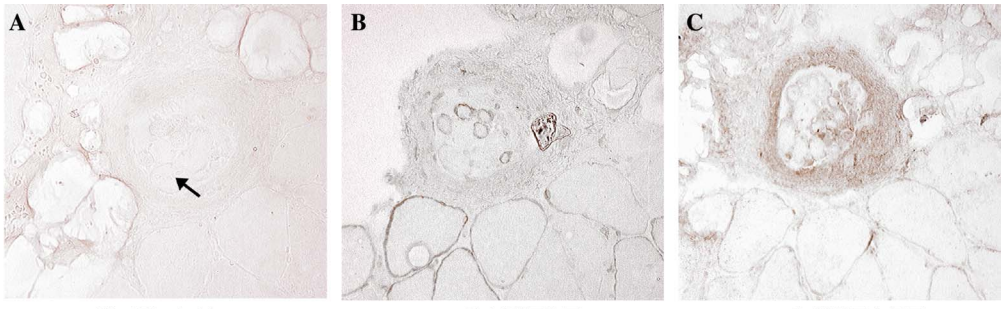

Fig. 9. IHC staining of muscle spindle with anti-MCT4-T (B) and anti-MCT4-PT (C) antibodies. The near absence of background staining upon omission of primary antibody is shown in (A). Both antibodies stain small chain fibers, but only anti-MCT4-PT strongly stains the capsule. A crystalline artifact is present in (B) (magnification ~80×).

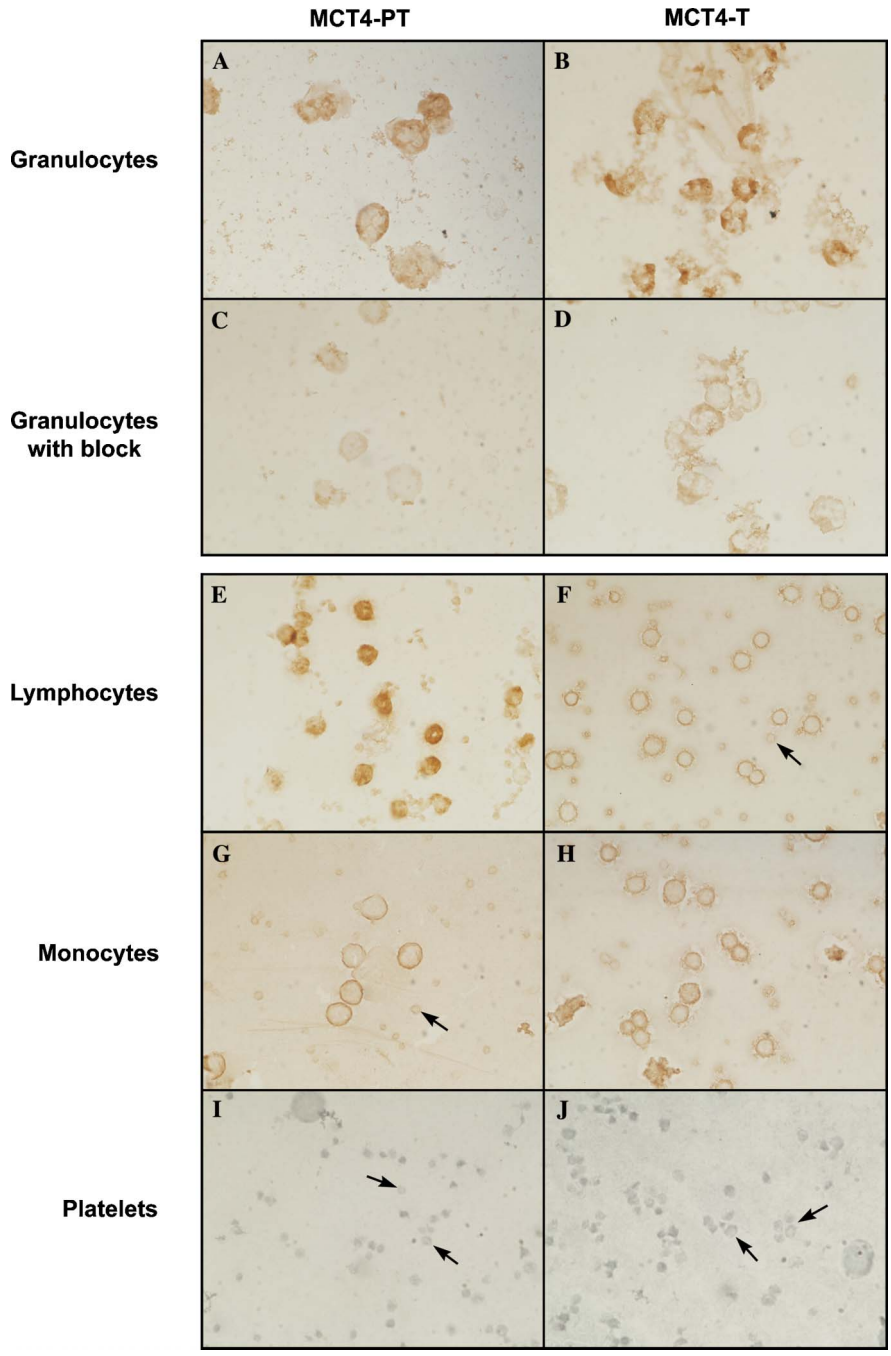

Fig. 10. IHC staining of MCT4 in the three human leukocyte fractions with anti-MCT4-T and anti-MCT4-PT antibodies. The arrows show plasmalemmal staining of platelets in lymphocyte (F) or monocyte fraction (G) by standard IHC, and also with cobalt enhancement (I and J). A single lymphocyte is present in (I and J) to provide a size reference (magnification ~400×). Note that many platelets show cytoplasmic staining.

exon 5, and no additional splicing sites in the C-terminal were identified by the GENSCANW program (MIT). Second, conversion of the 45 kDa band into a 32 kDa band was observed in a detergent solubilized membrane fraction, which eliminates the involvement of transcriptional and translational pathways. The proteolytic process must involve removal of the C-terminal segment, since anti-MCT4-PT, but not anti-MCT4-T, reacts with the truncated 32 kDa protein. The removal of the hydrophilic C-terminal would render the truncated form more hydrophobic [27] which, in turn, can explain the appearance of higher molecular weight aggregates, identified principally by MCT4-PT (Fig. 4).

A search for the presence of possible protease sites between the two antigen sequences was performed using PeptideCutter (ExPASy). The analysis revealed several candidates, including Proteinase K and enterokinase, the latter having a single cut site within the whole MCT4 sequence. Both of these enzymes are known to retain their activity at wide pH and temperature values and in the presence of various detergents and denaturants [28]. Another possible proteolytic mechanism might involve the ubiquitinase pathway

[29]. There is a single TPETS sequence in human MCT4 (but not MCT1 or 2) just before the C-terminal V. Although this sequence does not fulfill a consensus sequence for ubiquitinase targeting, the latter has such redundancy that perhaps this possibility should not be dismissed. Indeed, the pronounced susceptibility of MCT4 to proteolysis may explain why Bonen's group failed to detect MCT4 protein in rat heart [30] whereas McClelland and Brooks [31], who were also using an anti-MCT4-T antibody, found it present.

Since the 32 kDa form is present even under our mildest extraction procedures and in freshly drawn blood, it may be physiologically relevant and present some new and distinct functional features. Elimination of the highly variable cytosolic C-terminal is not likely to render the transporter inactive, since mutational analysis points instead to highly conserved transmembrane domains as especially important for maintaining lactate transporter activity [32,5]. Indications of a potential role for this truncated form arose in our IHC survey comparing the two antibodies in methanol-fixed muscle biopsies. Although most of these comparisons showed equivalent staining with either antibody, there were two instances wherein a compartment stained only with anti-MCT4-PT (and none with only anti-MCT4-T): the capsule of the muscle spindle and the cytoplasm of the lymphocyte. Truncated MCT4 may be responsible for these reactions, especially since the lymphocytes, but not the monocytes, also showed the 32 kDa band on the Western blot (Fig. 6). It is possible that the absence of the C-terminal sequence targets the truncated MCT4 form to intracellular membranes rather than plasmalemma, and to specific membrane locations, such as the muscle spindle capsule. Such differential targeting has been shown for different isoforms of the Na^+-acid bile transporter [25]. Future elucidation of the kinetic properties and substrate specificity of the 32 kDa form may give us additional clues to its physiological role.

We have now demonstrated the expression of MCT4 and MCT2 in human platelets by IHC, and by Western blots of highly purified preparations; as before, MCT1 was not detected. The two transporters appear to be present in cytoplasm (organelles) as well as the plasma membranes by IHC. In this respect, platelets resemble granulocytes, monocytes, and lymphocytes which have been shown to have intracellular compartments stained with all three MCTs [18]. Since it is known that all but about 2% of platelet pyruvate is converted to lactate which then leaves the cell [33,34], it is reasonable to postulate that MCT2, which has high affinity for pyruvate, transports it, as it is begins to accumulate, across the mitochondrial membrane into the cytosol, and after reduction by LDH, MCT2, and MCT4 collaborate at low and high lactate levels, respectively, in transporting the lactate across the plasmalemma into the blood stream.

Platelets are similar to muscle in having active glycolysis and ample glycogen, and an actomyosin-like ATPase that utilizes energy to provide activated platelets with the reactions of aggregation, granule release, and clot retraction. These reactions, producing plugs in vascular leaks and subsequent thrombus formation, necessarily also block oxygenation of the platelet mass, requiring the takeover of energy generation by glycolysis, which can fully compensate [35].

Following our earlier report that MCT1, MCT2, and MCT4 are all expressed in human skeletal muscle [17], these three transporters have been identified in rat [36] and porcine muscles [37]. With regard to localization to subcellular organelles, MCT1 was initially identified in the membranes of cardiac and skeletal muscle mitochondria in Brooks' laboratory [38] and confirmed by Butz et al. [39]. Most recently, MCT1, MCT2, and MCT4 have all been reported to be present in skeletal muscle mitochondria [36]. Our finding that all three transporters are present in monocytes, lymphocytes, and granulocytes, and in the cytoplasm (presumably in organelles) as well as the plasmalemma [18] led us to suggest that, since the transporters have progressively higher K_m levels by about fivefold from MCT2 to 1–4, they would operate across the entire sweep of physiologic lactate levels to facilitate transport of this substrate out of the cell to prevent toxic acidity under the frequently hypoxic conditions encountered by these cells.

Another rationale arises from the evidence provided by the Brooks laboratory that cycling of NADH/NAD with pyruvate/lactate may underlie the fatty acid oxidation cycle in peroxisomes [31]. In this, and possibly other organelles, such cycling might help modulate the respective ratios in cytoplasm and in the organelles, by the presence of appropriate expression levels of two transporters, such as MCT2 for the low levels of pyruvate present, versus MCT1 or 4 for the moderate or high levels of lactate present in alternate compartments. Several examples of such partial combinations are known. We found that a subset of the highly oxidative, but low force, type 1 fibers in human muscle expressed MCT2 in the plasmalemma along with the characteristic MCT1, and the frequency of this subset varied among different muscles [17]. Philp et al. [40,41] has shown that MCT1 is present on the apical membrane, whereas MCT3 is present on the basal membrane of the same retinal pigment epithelial cells both in rats and humans. Similarly, MCT2 has been shown to be expressed specifically on the basolateral domain of rat medullary thick ascending limb of Henle, whereas the luminal domain possesses yet another uncharacterized organic anion transporter [24]. More recently, Bonen's group has found that rat skeletal muscle intermyofibrillar mitochondria (which can interchange only with the cytoplasm of the same fiber) express only MCT2, whereas the subsarcolemmal mitochondria (which face neighboring capillaries and muscle fibers, often of different type) express MCT1, 2, and 4 [36]. In this study, we report that human platelets express MCT2 and MCT4 (but not MCT1), and evidence indicating a potential physiological role for a truncated version of MCT4, proteolytically divested of its C-terminal segment. In the future it may be possible to fashion cogent rationales, were we to delineate

the substrate spectrum, specificity, turnover number, and expression level of each and every one of these transporters.

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