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Biochimica et Biophysica Acta 1279 (1996) 157–163

BBA Biochimica et Biophysica Acta

Cloning and sequencing of the monocarboxylate transporter from mouse Ehrlich Lettré tumour cell confirms its identity as MCT1 and demonstrates that glycosylation is not required for MCT1 function [1]

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Received 28 July 1995; revised 10 October 1995; accepted 18 October 1995

Abstract

Lactate transport is mediated in most tissues by H^+-monocarboxylate$^-$-cotransporters (MCTs). We have cloned and sequenced the lactate transporter from Ehrlich Lettré tumour cells by using the polymerase chain reaction (PCR) to amplify MCT1-related sequence from cDNA. The sequence is 93% and 87% identical to MCT1 from Chinese hamster and human respectively and so represents mouse MCT1. Most differences between MCT1 from Chinese hamster and mouse are conservative substitutions, located in hydrophilic parts of the molecule. Specific antipeptide antibodies confirm the presence of MCT1 protein in membranes from Ehrlich Lettré tumour cells. One difference between the mouse and Chinese hamster MCT1 is the absence of a predicted external consensus sequence for *N*-linked glycosylation in the mouse sequence. Using *N*-glycanase-F treatment and an in vitro translation system, we provide evidence that this glycosylation site is not actually utilised in Chinese hamster MCT1. These results are discussed in relation to current understanding of the roles of glycosylation of membrane proteins.

Keywords: Monocarboxylate transporter; cDNA sequence; Glycosylation; Lactate transport; Polymerase chain reaction; (Mouse Ehrlich cell)

1. Introduction

The majority of mammalian cells require lactic acid to be transported across the plasma membrane, either as an end product of glycolysis that must leave the cell, or as a substrate that must enter the cell for respiration or gluconeogenesis. This transport process is known to be mediated by a family of monocarboxylate transporters (MCT) all of which catalyse the H^+/lactate$^-$ cotransport, but with differing substrate and inhibitor specificities and tissue distribution [1]. Two of these transporter isoforms have now been cloned and sequenced; MCT1 from Chinese hamster ovary (CHO) cells [2] and MCT2 from Syrian hamster liver [3]. These proteins, which are 60% identical, are predicted to possess 12 transmembrane (TM) domains and form part of a new family of membrane transporters. The best characterised MCT is that found in the erythrocyte plasma membrane which we have partially purified [4] and whose N-terminal sequence suggests is identical to MCT1 [5].

Tumour cells often rely on glycolysis for their ATP production and thus the efflux of lactic acid from such cells is essential for their well-being. If it were possible to inhibit their lactate transport mechanism selectively, it might be a suitable strategy for chemotherapy. For this purpose we have performed extensive studies on the substrate and inhibitor specificity of the lactate transporter of Ehrlich Lettré tumour cells using the intracellular pH sensitive fluorescent indicator BCECF [6]. Our data strongly implied that these tumour cells possess a lactate carrier kinetically indistinguishable from MCT1. However, to confirm that MCT1 is responsible we have cloned and sequenced the carrier using the polymerase chain reaction (PCR) to amplify MCT1-related sequence from cDNA prepared from these tumour cells. We have obtained a sequence that is 93% identical to CHO MCT1 which we believe to correspond to mouse MCT1, and used specific

Abbreviations: MCT, monocarboxylate transporter; TM, transmembrane domain; CHO, Chinese hamster ovary; BzNLT, benzoyl-Asn-Leu-Thr-*N*-methylamide.

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[1] The sequence data reported in this paper have been submitted to the EMBL/GenBank Data Libraries under the accession number X82438.

SSDI 0005-2736(95)00254-5

antipeptide antibodies to confirm the presence of MCT1 in the plasma membranes of Ehrlich Lettré tumour cells. A notable difference between the mouse and Chinese hamster MCT1 is the absence of a putative external *N*-glycosylation site in the mouse sequence. We provide evidence that this glycosylation site is not actually used in Chinese hamster MCT1.

2. Materials and methods

2.1. Materials

Chinese hamsters were obtained from a local breeder. Trizol Reagent was obtained from Gibco BRL, Life Technologies, Renfrewshire, UK. The reverse transcriptase cDNA synthesis kit, TNT T7/T3 Coupled Reticulocyte Lysate (rabbit) cell free translation system, and dog pancreatic microsomes were from Promega (Southampton, UK) and used according to the suppliers' instructions. Taq polymerase was from Boehringer-Mannheim UK (Lewes, East Sussex), and the restriction enzymes and general molecular biology reagents were either from Boehringer, Gibco or Pharmacia (Knowhill, Milton Keynes, UK). The QIAquick spin PCR purification kit was obtained from Qiagen (Dorking, Surrey, UK). [α-^{35}S]ATP (sequencing grade) and [^{35}S]methionine (translation grade) were obtained from DuPont (NEN Products, Stevenage, Herts., UK) and the Sequenase Version 2.0 DNA sequencing kit from USB (United States Biochemical, distributed by Amersham Life Science). Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC), SulfoLink coupling gel, and Immunopure gentle Ag/Ab elution buffer were obtained from Pierce, Chester, UK. The C-terminal peptide was synthesised by Zinsser Analytic, Maidenhead, UK, and Keyhole limpet haemocyanin (KLH) was supplied by Calbiochem, Nottingham, UK. Purified *N*-glycanase F and BzNLT (*N*-benzoyl-Asn-Leu-Thr-*N*-methylamide) were generous gifts of Prof. M.J.A. Tanner of this department. CHO MCT1 (77499) was obtained from the American Type Culture Collection (Maryland, USA). Reagents used for immunoblotting, such as the ECL (enhanced chemiluminescence) detection kits were obtained from the sources given previously [4].

2.2. Methods

2.2.1. Cloning and sequencing of MCT1 from Ehrlich Lettré tumour cells

Ehrlich Lettré tumour cells were cultured and harvested as described previously [6] and total cell RNA isolated using TRizol Reagent, according to the manufacturers' instructions. cDNA was prepared from the polyadenylated mRNA by reverse transcription and subjected to PCR using Taq polymerase. The primers used corresponded to the initiator methionine codon and last 27 bases of the 5′ noncoding region of CHO MCT1 (i) and the complementary 3′ terminal sequence corresponding to the stop codon and first 27 bases of the 3′ noncoding region (ii): (i) 5′-GCATTTTGGGATTCATCTACACTTAAAATG-3′ (ii) 5′-CACACAATGCTCTATTCATGCTTCAGTTCA-3′.

PCR amplification used a 7 min hot start at 94°C, with 35 cycles of 60 s at 55°C, 90 s at 72°C and 60 s at 94°C. The resulting PCR product, which was visible as a 1.5 kb fragment on agarose gel electrophoresis, was purified using a QIAquick spin column. The cDNA fragment was then ligated into *Sma*I-digested pUC18 vector treated with bacteria alkaline phosphatase. Both strands of one clone and the sense strand of two further clones (one with a truncated version of the full length cDNA), each derived from independent cDNA preparations and PCR, were sequenced manually or on a DuPont Genesis 2000 automated sequencer using the Sanger dideoxy method of chain termination and a custom primer walking strategy.

2.2.2. Anti-MCT-1 antibodies

A peptide corresponding to residues 478–494 of CHO MCT1, with the addition of an N-terminal Cys residue to enable coupling; i.e., CPQQNSSGDPAEEESPV, was used to raise antibodies in New Zealand White rabbits. The peptide was injected as a Keyhole Limpet Haemocyanin conjugate, which was prepared using the water-soluble heterobifunctional crosslinker sulfo-SMCC. IgG was obtained from the serum using caprylic acid [7]. The specific antipeptide antibodies were prepared from this IgG fraction by affinity purification using a peptide column. This affinity matrix was synthesised by linking the peptide (via the terminal Cys) to Sulfolink coupling gel (Pierce), according to the manufacturers' instructions. The coupling ratio for the reaction was 1 mg peptide/ml Sepharose. Antibody which bound to this column was eluted with Pierce 'gentle Ab/Ag elution buffer', the buffer exchanged to 20 mM Mops, 150 mM NaCl, pH 7.4 and purified antibody stored at −70°C.

2.2.3. Studies of the glycosylation status of MCT1

Plasma membranes from mouse or Chinese hamster erythrocytes, and from CHO cells and mouse Ehrlich-Lettré tumour cells were prepared by hypotonic lysis of the cells in 5 mM sodium phosphate (pH 8.0) containing the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) at 2 mM [4]. CHO cells were initially removed from the culture dishes by scraping whilst the mouse Ehrlich-Lettré tumour cells were disrupted by passage through a narrow bore needle (0.5 × 16 mm, 25G) up to 10 times. Approx. 50 μg of membrane protein was then incubated in 25 μl of PBS buffer before addition of SDS (0.5% final) followed by NP40 (0.6% v/v final) and mercaptoethanol (8 mM final). A proteinase inhibitor cocktail (100 μM leupeptin, 2 μg/ml aprotinin, 1 mM benzamidine, 1 μM pepstatin and 0.5 mM PMSF; final concentrations) was also added to each assay, and the final volume brought to

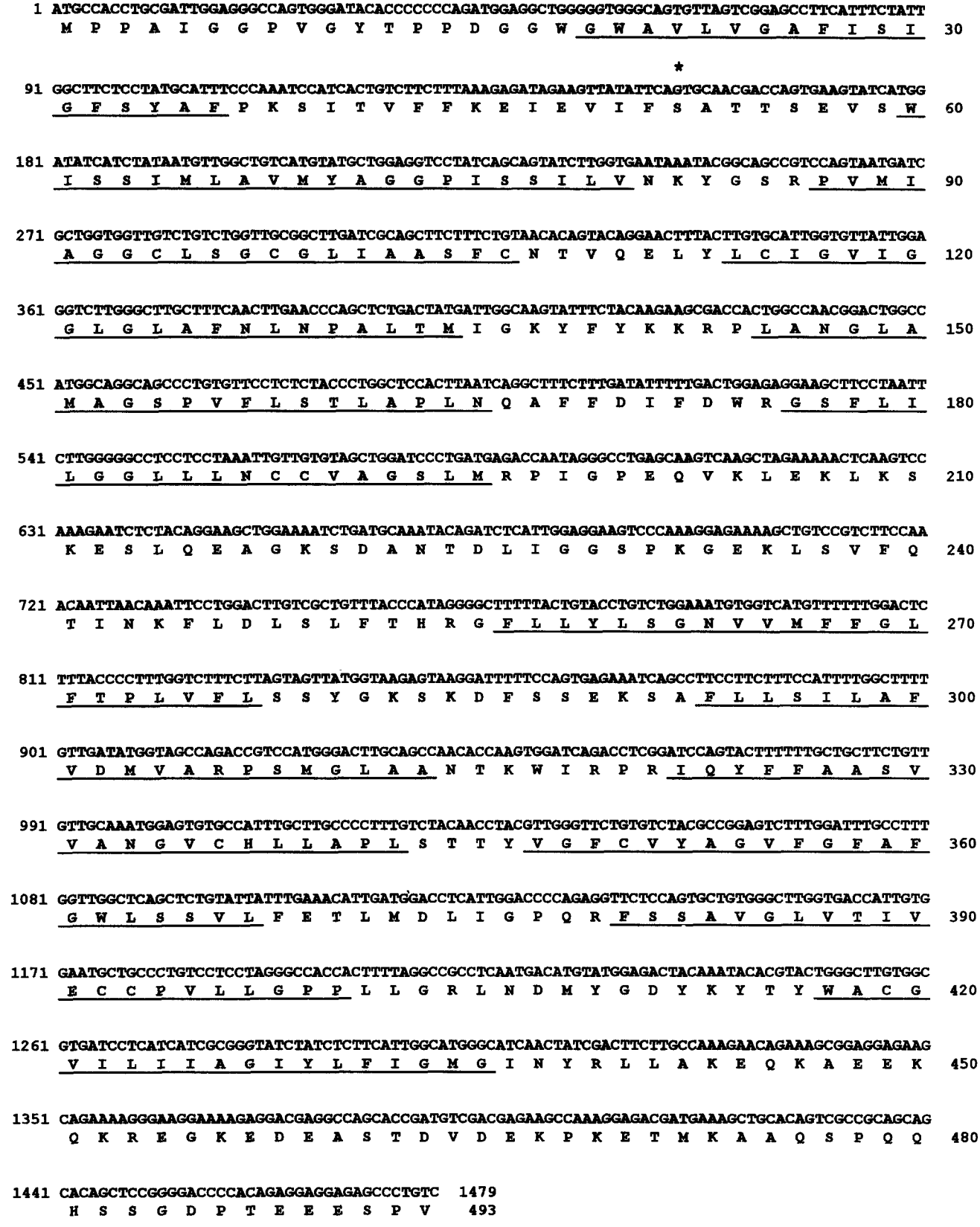

Fig. 1. Nucleotide and deduced amino acid sequences of mouse Ehrlich-Lettré tumour cell MCT1 cDNA. MCT from mouse Ehrlich-Lettré tumour cells was cloned after PCR amplification and sequenced by the Sanger method of dideoxy mediated chain termination, as described in Section 2.2. Numbers on the left refer to nucleotides, whilst those on the right refer to the amino acids. The underlined regions of the protein sequence denote the predicted transmembrane regions, assigned on the basis of hydrophobicity using the Kyte and Doolittle algorithm [26]. The asterisk denotes the amino acid substitution of Ser^{52} in mouse MCT1 in place of Asn^{52} in CHO MCT1. This leads to the loss of the putative consensus sequence for glycosylation.

50 μl. Where required, *N*-glycanase-F (20 Oxford Glycosystems units total). was added and samples incubated at 37°C for 4 h. Samples containing about 5 μg of protein were analysed by SDS-PAGE and Western blotting with the anti-MCT1 antibody using ECL for detection of immunoreactive bands.

In order to investigate the possibility of putative glycosylation sites being used in MCT1, an in vitro translation system was used in the presence and absence of dog pancreatic microsomes and the competitive inhibitor of core glycosylation, BzNLT (a tripeptide glycosylation acceptor) as described by Groves and Tanner [8]. cDNA encoding either mouse Ehrlich-Lettré tumour cell or CHO MCT1 was subcloned into KS Bluescript, and expressed in a cell free rabbit reticulocyte lysate by coupling transcription to translation with T7 RNA polymerase. The assay was performed with 0.25 μg cDNA in a total of 12.5 μl lysate buffer containing [^{35}S]methionine (0.4 mM, 5 μCi per assay) in the presence or absence of dog pancreatic microsomes (1.25 μl per reaction) and 30 μM BzNLT [8]. The translation reaction was incubated for 90 min at 30°C and subsequently, in some cases, for 4 h at 37°C in the presence or absence of *N*-glycanase F (10 Oxford Glycosystems units total), with the methods as above. BSXG1, a plasmid vector with cDNA encoding for Band 3, a known membrane glycoprotein, was employed as a positive control in these experiments [8]. The in vitro translation products were separated by SDS-PAGE and detected by either autoradiograph, or fluorography essentially as described by Bonner and Laskey [9].

3. Results

3.1. Cloning and sequencing of MCT1 from mouse Ehrlich Lettré tumour cells

In Fig. 1 we show the nucleotide sequence and derived amino acid sequence of the PCR product amplified using the primers derived from the 5′ and 3′ non-coding regions of the published sequence of CHO MCT1. Three separate clones derived from separate cDNA preparations and PCR were used to confirm the final sequence. An alignment of the mouse tumour cell MCT1 protein sequence with those of Chinese hamster and human MCT1 is shown in Fig. 2. The mouse MCT1 sequence is 93% identical with that from CHO cells, the differences being mainly conservative substitutions occurring primarily in putative loops and the C-terminal domain as was noted for human MCT1 [10], with which the mouse MCT1 shows 87% identity. Comparing mouse with Chinese hamster MCT1, the mouse protein has a single deletion in the C-terminal domain. Of greater possible significance is the loss of a consensus site for *N*-glycosylation site on the first putative extracellular loop (Asn^{52}Ser). This consensus site for glycosylation is also absent in human MCT1 [10].

3.2. Studies on the glycosylation state of MCT1

In order to assess the significance of the loss of the consensus sequence for *N*-linked glycosylation it was necessary to establish whether this site was used in CHO

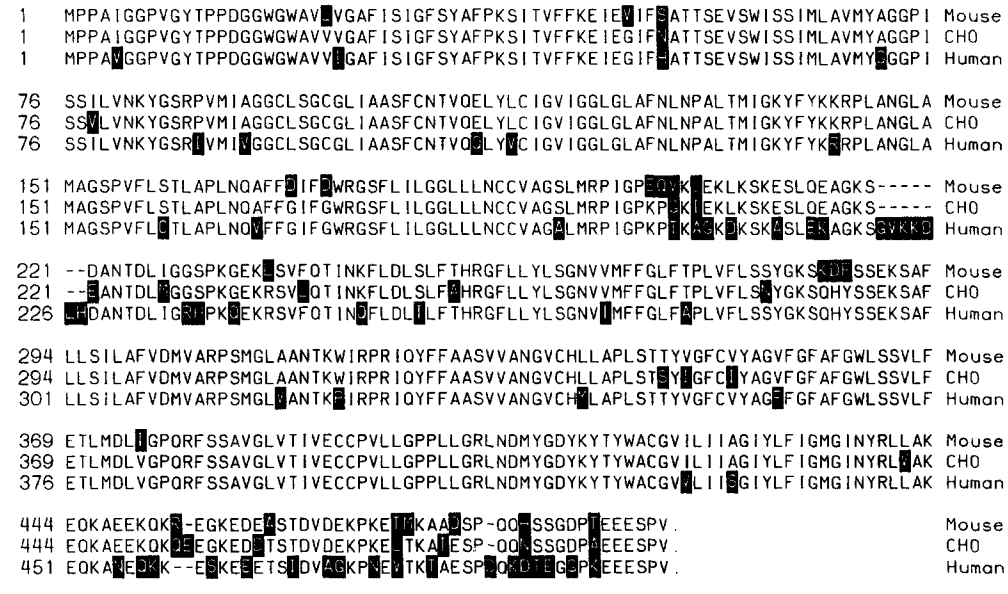

Fig. 2. Alignment of the protein sequences of MCT1 from mouse Ehrlich-Lettré tumour cells with Chinese hamster and human MCT1. The deduced protein sequence of mouse Ehrlich-Lettré tumour cell MCT1 is aligned against that for Chinese hamster and human MCT1 taken from published data [2,10]. Alignments were performed using Megalign software (DNAStar) with the Clustal algorithm (gap penalty = 10, gap length penalty = 10). Residues which are not identical in all three sequences are highlighted. The deduced amino acid sequence of mouse MCT1 has 93% identity to CHO MCT1 and 87% identity to human MCT1.

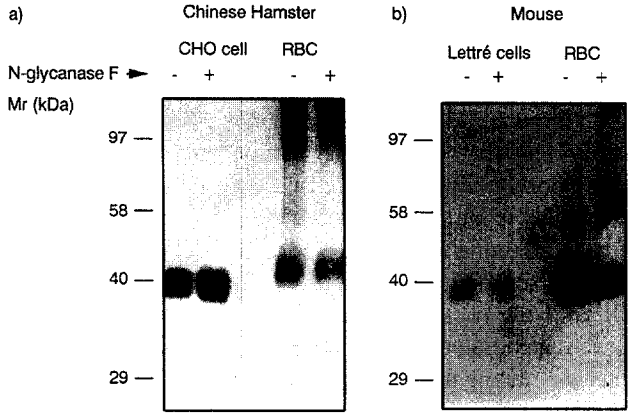

Fig. 3. *N*-Glycanase-F treatment of MCT1 in membrane preparations from Chinese hamster and mouse cells. Membranes were prepared from CHO cells, mouse Ehrlich-Lettré tumour cells (Lettré cells) and red blood cells (RBC) from Chinese hamster or mouse, and treated with *N*-glycanase-F as described in Section 2.2. 5 μg of each membrane preparation, with (+) and without (−) treatment with *N*-glycanase F were separated by SDS-PAGE and immunoblots probed with an anti-MCT1 antibody. The ECL developed blots were exposed for 2 min (a) and 10 min (b).

MCT1. We have previously noted a difference in the mobility of MCT1 from rabbit, rat and guinea pig which would be consistent with different glycosylation states [4]. However rabbit MCT1, which runs on SDS-PAGE at a higher apparent molecular weight than MCT1 from other species, showed no change in mobility following treatment with *N*-glycanase-F [5]. In Fig. 3 we show that CHO MCT1 was also unaffected by *N*-glycanase F treatment under conditions which caused a sharpening and increased mobility of human GLUT 1 (data not shown). MCT1 from Chinese hamster red blood cell membranes also showed no change in mobility under these conditions, although surprisingly the protein ran at a higher apparent molecular weight than CHO MCT1. MCT1 from mouse red blood cell or Ehrlich Lettré tumour cells exhibited identical mobility on SDS-PAGE and this was also unaffected by *N*-glycanase F treatment. Thus, our results suggest that MCT1 is not glycosylated in CHO cells despite the presence of a potential glycosylation site. However, it is possible that the *N*-glycanase F is sterically prevented from removing the carbohydrate moiety, which might be present in greater quantity in Chinese hamster red blood cells, thus accounting for its decreased mobility.

In order to assess more directly whether CHO MCT1 can be glycosylated we used the coupled rabbit reticulocyte in vitro transcription and translation system, in the presence and absence of dog pancreatic microsomes to enable glycosylation to occur. Human erythrocyte band 3 (AE1) cDNA was used as a control since this is known to be glycosylated under such conditions in a manner that is sensitive to BzNLT, a tripeptide glycosylation acceptor that acts as a competitive inhibitor of core glycosylation, and that this glycosylation can be reversed by *N*-glycanase F treatment [8]. In Fig. 4a we show that human band 3 behaved as expected under our conditions. Thus, in the

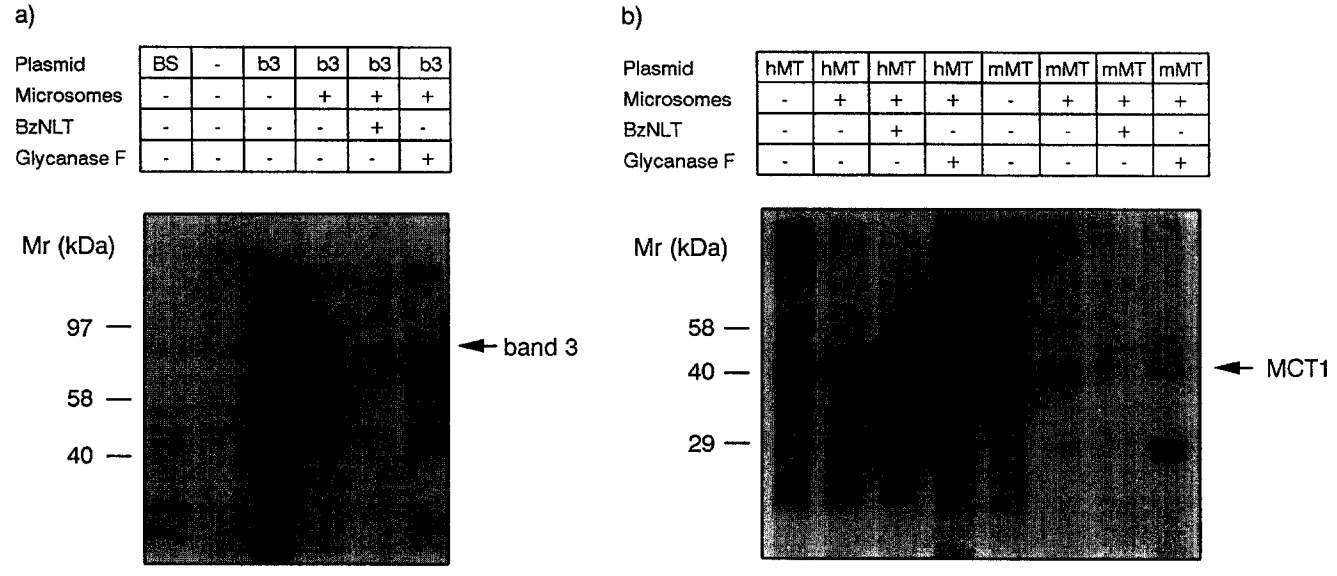

Fig. 4. Cell free translation of MCT1 from Chinese hamster and mouse in the presence and absence of microsomes, BzNLT and *N*-glycanase-F. A coupled rabbit reticulocyte lysate was used for cell free transcription and translation to express human Band 3 (b3) cDNA, or MCT1 cDNA (MT) from Chinese hamster (hMT) and mouse (mMT) cells, as described in Section 2.2. In all cases, assays contained 0.25 μg of the relevant cDNA, and where specified, dog pancreatic microsomes (1.25 μl) and BzNLT (30 μM). Once expression was complete, where indicated, the lysate was also treated with 10 U of *N*-glycanase-F as described in Section 2.2. 1 μl of each lysate mix (2 μl for the *N*-glycanase-F digest) were separated using SDS-PAGE on gels containing 8% (band 3) or 10% (MCT1) (w/v) polyacrylamide. The gel in Fig. 4a was subjected to autoradiography to visualize the ^{35}S-labelled proteins whilst the gel in Fig. 4b was subjected to fluorography. Negative control assays in Fig. 4a contained either KS Bluescript vector (BS) or water (−).

absence of microsomes, the synthesised protein ran as a fairly sharp single band which in the presence of microsomes became a doublet with the higher glycosylated band having a slightly lower mobility. When BzNLT was added in the presence of the microsomes, the major product was the non-glycosylated lower band and this was also seen if the product from the microsomal incubation was incubated with *N*-glycanase F. In direct contrast to this, the data of Fig. 4b show that when CHO MCT1 cDNA was added to the assay, a protein with an apparent molecular mass identical to MCT1 (40 kDa) was synthesised, but neither the presence of microsomes nor the addition of BzNLT or *N*-glycanase F had any effect on its mobility. Taken together, our data strongly suggest that the potential *N*-glycosylation site of CHO MCT1 is not utilised.

4. Conclusions

The data we present in this paper demonstrate that mouse Ehrlich Lettré tumour cells express MCT1, and provide strong support for our previous kinetic data [6] which indicated that the properties of the monocarboxylate carrier in these cells corresponded almost exactly to those of MCT1. We cannot completely rule out that these cells contain another MCT isoform in addition to MCT1, but this is unlikely in view of the similar kinetic characteristics of lactate transport into tumour cells and erythrocytes where MCT1 is known to be responsible. Since MCT1 appears to be the major isoform present in tumour cells, erythrocytes and CHO cells, it would seem likely that it is the standard 'housekeeping' monocarboxylate carrier for those cells whose requirement for lactate transport is purely to allow efflux of glycolytically derived lactic acid. In this regard it may be the MCT counterpart of GLUT1 which is the glucose transporter found in similar cells [11,12]. In contrast, liver parenchymal cells that use lactic acid as a substrate for gluconeogenesis and lipogenesis have a distinct isoform that has recently been cloned and sequenced and termed MCT2 [3]. In heart cells, which can both utilise lactic acid as a respiratory substrate and produce it glycolytically during hypoxia, both MCT1 and MCT2 can be detected by immunofluorescent microscopy, but only at low levels and restricted to the intercalated disk region ([2,3] and X. Wang and R.C. Poole unpublished data). However, our own studies suggest the presence of substantial activities of two other isoforms of MCT which exhibit kinetics and substrate and inhibitor specificities distinct from MCT1 and MCT2 [13–16]. These isoforms have yet to be cloned and sequenced. It is significant however that basal levels of MCT1 do appear to be present in both liver and heart cells despite the majority of transport being mediated by different isoforms [1]. This is also the case with GLUT 1 which is often present at low levels in cells where another isoform dominates [11].

The mouse and Chinese hamster MCT1 sequences are 93% identical with the mouse and human MCT1 sequences being 87% identical. The majority of changes are conservative substitutions and in putative loop regions or the C-terminal domain. It is a characteristic of membrane transporters that hydrophilic portions of the protein are more poorly conserved between species and isoforms than membrane spanning segments [17]. The species variation in the C-terminal sequence also explains why in Western blots the sensitivity of detection of MCT1 by our antipeptide antibody (raised to residues 478–494 of CHO MCT1) varies depending on the source of the erythrocyte ghosts or other membrane preparation used, when transport activities are similar.

The most interesting difference between the mouse and Chinese hamster sequence is the lack of an externally disposed consensus sequence for *N*-linked glycosylation in mouse MCT1. This site is also lacking in the human MCT1 sequence [10]. As expected from the sequence, we found no *N*-linked glycosylation of mouse MCT1. Hence, it is clear that MCT1 can be expressed and function correctly without glycosylation. This is perhaps not surprising since *N*-linked carbohydrate has been removed from many transporters either enzymatically or by site-directed mutagenesis, with little if any effect on function (see for example, [18–21]). Whilst the non-glycosylated nature of mouse MCT1 was fully expected, we were also unable to provide any evidence for *N*-linked glycosylation of CHO MCT1, despite the presence of a consensus site. There are a number of possible explanations for this observation. Firstly, the consensus acceptor sequence (N-X-S/T where X is not P) at N^{52} in CHO MCT1 may not be externally disposed and hence not available for glycosylation. Secondly, and perhaps more likely, the size of the loop between putative TM1 and TM2 may not be sufficiently large to be an acceptor for *N*-linked carbohydrate. Studies of the in vitro expression of a membrane protein in which the glycosylation site was engineered to be at increasing distances from the membrane, implied that glycosylation only occurs when there are at least 10 residues on either side of the asparagine acceptor in the exposed loop [22,23]. Furthermore, a recent comprehensive survey of the occurrence and utilisation of consensus sites for *N*-linked glycosylation in multiple-spanning membrane proteins also concluded that the asparagine acceptor must be at least 10 residues away from a transmembrane domain, and that a loop of at least 30 residues in length is normally required [24]. The only exception to these rules was the CHIP28 water channel (loop approx. 20 residues), but only approx. 25% of the protein is actually glycosylated in this case [25]. The estimated size of the loop between transmembrane segments 1 and 2 of MCT1 is approx. 23 residues, with only 8 residues on the C-terminal side of N^{52} (see Fig. 1), and hence this asparagine is unlikely to be glycosylated.

It is interesting to note that the vast majority of multi-spanning membrane proteins are glycosylated. Indeed, a

large proportion of the few which are not glycosylated (e.g., α-subunit of Na/K-ATPase) are known to be tightly associated with other, glycosylated, subunits/proteins [24]. It is therefore tempting to speculate that MCT1 might be associated with another protein in the membrane.

The one observation that is difficult to explain is the higher apparent molecular weight of MCT1 in erythrocytes from Chinese hamster as compared with CHO cells. This is unlikely to be due to *N*-glycosylation since in Chinese hamster erythrocyte ghosts there is no increase in mobility or sharpening of the MCT1 band on SDS-PAGE after treatment of ghosts with *N*-glycanase F sufficient to remove carbohydrate from human GLUT 1. There can be little doubt that MCT-1 is the isoform expressed in these cells since a variety of anti-(MCT1 peptide) antibodies react with the protein in Chinese hamster erythrocytes (R.C. Poole, unpublished work). We have also observed that the MCT1 present in rat heart cell plasma membranes shows slightly greater mobility on SDS-PAGE than does MCT1 in red cell ghosts (X. Wang, unpublished data). It is of note that MCT1 expressed in erythrocytes from a number of different mammalian species does have different mobility on SDS-PAGE [4]. These differences between MCT1 of membranes from different cells and different species might be related to some other post-translational modification of the protein, as yet unidentified. Although consensus sequences for a variety of protein kinase phosphorylation sites can be identified in the rat, mouse and Chinese hamster MCT1 sequences, we have been unable to demonstrate any changes in mobility of MCT1 following alkaline phosphatase treatment (unpublished data of R.C. Poole).

In conclusion, our data indicate that MCT1 is the lactate/monocarboxylate transporter expressed in mouse ascite tumour cells, and presumably other tumour cells. Whilst this may appear to make MCT1 a less promising target for cancer chemotherapy, this may not be the case. It is now clear that the major tissues of lactate production and utilisation (white skeletal muscle, liver, heart) utilise isoforms other than MCT1 for lactate transport [2,3,15]. Those cells/tissues which express MCT1 as the major or sole isoform, e.g., erythrocytes, may survive even if this process is inhibited, whereas tumour cells have a clear requirement for high rates of lactate efflux.

5. Addendum

We have recently cloned and sequenced MCT1 from a rat skeletal muscle cDNA library and shown that it is 96% identical to mouse MCT1 at the protein level and also lacks a consensus glycosylation site [27].

Acknowledgements

This work was funded by a grant from the Wellcome Trust. L.C. is supported by a Studentship from the BBSRC. We thank Dr. J.D. Groves and Professor M.J.A. Tanner for their help with the in vitro glycosylation studies and for fruitful discussions. We thank Drs. L. Hall and N.T. Price for their advice on cloning and sequencing and the Molecular Recognition Centre, University of Bristol for providing sequencing facilities.

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