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Identification of a Unique Monocarboxylate Transporter (MCT3) in Retinal Pigment Epithelium

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The retinal pigment epithelium transports lactate between two tissue compartments, the interphotoreceptor matrix and the choriocapillaris. In this report we describe a 2.45-kb cDNA isolated from a chick cDNA RPE library that encodes a membrane protein found only in RPE cells. The deduced protein has 542 amino acids with twelve putative membrane spanning domains. The cDNA has been designated MCT3 based on its 45% identity in amino acid sequence and structural similarity with the monocarboxylate transporters MCT1 and MCT2. Stable transfectants (*p*Cl-neo/MCT3), made in a rat thyroid epithelial cell line (FRTL-5), express MCT3 RNA. Transfectants had enhanced pyruvate uptake (used as a measure of lactate uptake) which was proton-dependent and inhibited by α-cyano-4-hydroxycinnamate. In summary, MCT3's unique expression in RPE cells, multiple potential phosphorylation sites, and basolateral distribution suggest that MCT3 may regulate lactate levels in the interphotoreceptor space.

Retinal pigment epithelium (RPE) forms the outer blood-retinal barrier and regulates the vectorial movement of fluid, ions, and metabolites between the neural retina and the choroidal circulation (reviewed in 1). A close association between RPE and neural retina is established early in development and is essential for maintaining photoreceptor cell viability (2,3). The maintenance of visual cell function depends on glycolysis. The neural retina with its high rate of metabolism, uses glucose and produces substantial quantities of lactate, in both light and dark (4-6). Since the outer retina is avascular, the RPE moves glucose and lactate into and out of the subretinal space for use by the retina (7-10). By regulating the lactate concentration in the interphotoreceptor space (IPS), the RPE will influence photoreceptor metabolism and viability (1,4,7).

Recently, we identified an integral membrane protein in the basolateral membrane of differentiated chick RPE cells (11). The protein was identified using a monoclonal antibody (MAb 3C4) and called REMP (retinal epithelial membrane protein). REMP was found only in chick RPE cells and not in other chick tissues such as neural retina, intestine, kidney or liver (11). In this report, we describe the heterologous expression of a full length cDNA clone that encodes this protein. The deduced amino-acid sequence of the cDNA (isolated from a chick RPE expression library) is homologous with the recently cloned monocarboxylate transporters MCT1 and MCT2 (12,13). The heterologous expression of a cDNA in FRTL-5 cells demonstrated that REMP is a proton coupled monocarboxylate transporter and is designated MCT3.

MATERIALS AND METHODS

Isolation of MCT3 clones. Preparation of the chick RPE cDNA library used in this study is described elsewhere (11). The library was screened with a digoxigenin-labeled random primed DNA probe prepared from the 300 bp EcoRI-DraII fragment of REMP cDNA (11). Positive clones were plaque purified; phage DNA was digested with EcoRI; and cDNA inserts subcloned into pBluescript SK- (Stratagene). DNA was sequenced using ABI 373A Stretch sequencer at the Genetics Core Facility at the University of Pennsylvania, Philadelphia, PA. The sequence of one clone, with a 2.2 kb insert DNA, added 710 nucleotides to the 5′ proximal end of the previously reported REMP sequence (11). The combined cDNA sequence was named MCT3 based on its sequence homology to MCT1 and MCT2. Sequence comparisons were generated using the Genetics Computer Group (Madison, WI).

Stable transfection of FRTL-5 cells. Characteristics and propagation of FRTL-5 cells (ATCC CRL 8305, F1 clone), are described elsewhere (14,15). To generate stable transfectants, MCT3 cDNA was ligated into the EcoRI site of the mammalian expression vector, pCI-neo (Promega). FRTL-5 cells were trypsinized and suspended in 0.35 ml of cytomix (16) at a concentration of 4.5×10^6 cells per cuvette with 10 μg of plasmid DNA. The cells were electroporated at 300 Volts, 960 μF and plated in one 100 mm dish in medium containing 0.5% penicillin/streptomycin (Biofluids Inc.). Selection with 400 μg/

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ml active G418 (Life Technologies Inc.) was initiated at 48 hrs. After 3 weeks, colonies with MCT3 mRNA were expanded.

Pyruvate uptake in FRTL-5 cells. Cells (1×10^5 cells per well) were plated in 12 well dishes (Costar, Cambridge, MA) 2 days prior to assay, and fed fresh medium 6-8 hrs before uptake measurements. Cells were washed and incubated in glucose free Hanks' balanced salt solution (HBSS) [0.4 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 0.44 mM K_2HPO_4, 5.4 mM KCl, 1.3 mM $CaCl_2$, 138 mM NaCl, 4 mM $NaHCO_3$, 0.5 mM $MgCl_2$] with indicated additions. To assay for pyruvate uptake, cells were incubated in 0.5 ml of HBSS containing 0.2 mM Na-pyruvate and [^{14}C]-Na-pyruvate ($\sim 1.5 \times 10^3$ dpm/nmole). Pyruvic acid, sodium salt, [2-^{14}C] was from American Radiolabeled Chemicals Inc. Pyruvate uptake was terminated by two rapid washings with ice-cold HBSS/10 mM PIPES (piperazine-N,N′-bis[2-ethanesulfonic acid], United States Biochemical Corp.), pH 6.5, containing 300 μM phloretin (ICN) followed by the addition of 1 ml absolute alcohol for 30 mins. Radioactivity in the alcohol extract was counted along with 10 ml Hydrofluor (National Diagnostics) using a Beckman LS 5001 liquid scintillation counter. Uptake values presented are the mean of 3 independent experiments from which pyruvate uptake measured in the presence of 100 μM FCCP [carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (Sigma)] was subtracted. Protein was measured in each well on material not extracted by ethanol using BCA (Pierce) and was 25-66 μg/well.

RESULTS

Cloning of MCT3. In a previous study, an embryonic chick RPE expression library was screened with a monoclonal antibody that recognized a 50 kd membrane protein uniquely expressed in chick RPE cells (11). The cDNA was used to re-screen the chick RPE library to obtain additional cDNA clones and the full length coding sequence (GenBank accession No. U15685). The combined cDNA sequence was named MCT3 based on its sequence homology to MCT1 and MCT2 (12,13).

MCT3 has a 1626 bp open-reading frame, 446 bp 5′ untranslated region and a 234 bp 3′ untranslated region. There is an in-frame stop codon, six bases before the start codon. Ten nucleotides in MCT3 cDNA (encoding six different amino acids) differed from that reported previously (11). The genomic sequence confirmed the revised nucleotide assignments (unpublished data).

MCT3 like other transport proteins has twelve predicted membrane spanning domains. The 6th and 7th membrane spanning regions are separated by a long cytoplasmic loop while the rest of the transmembrane domains are separated by relatively short hydrophilic segments (Fig. 1). The amino- and carboxy termini are on the cytoplasmic side of the membrane (11, 17). Despite the structural similarity with many transport proteins, MCT3 has sequence homology only with the monocarboxylate transport proteins, MCT1 and MCT2 (Fig.1). From multiple sequence alignment, MCT3 has 43% and 45% sequence identity with MCT1 and MCT2, respectively.

The polypeptide sequence predicted from translation of MCT3 cDNA is 542 amino acids long. In the stretch

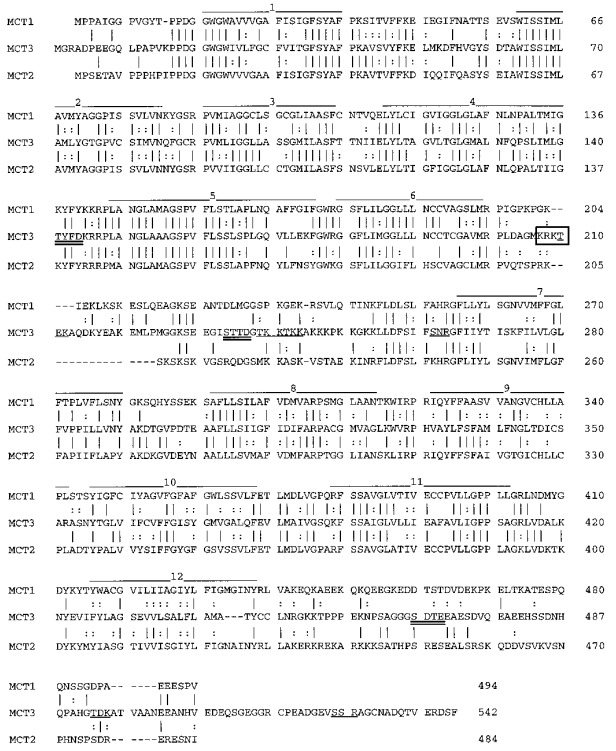

FIG. 1. Comparison of the deduced protein sequences of MCT1, MCT2 and MCT3. The predicted amino acid sequences for hamster MCT1 and MCT2 and chick MCT3 are numbered on the right. The numbered lines, above the sequences, show the proposed membrane spanning domains. Conserved amino acids are indicated by vertical lines and conservative replacements by double dots. Sequences were aligned using the Genetics Computer Group program and gaps, shown by dashes, were introduced for maximum alignment. Potential sites for protein kinase C phosphorylation are underlined, casein kinase II phosphorylation sites double underlined, and the cAMP/cGMP-dependent protein kinase phosphorylation site boxed.

of hydrophilic amino acids in the long cytoplasmic loop, there are four potential sites for protein kinase C phosphorylation (underlined) and a potential site for cAMP/cGMP dependent phosphorylation (boxed). There are two additional protein kinase C phosphorylation sites in the carboxy terminus (underlined). There are three possible sites for casein kinase II phosphorylation (double underlined).

One potential ASN-linked glycosylation site is in the loop between the 9th and 10th membrane spanning domains, but no carbohydrate was detected in purified MCT3 (Grollman and Philp, unpublished observation). The loop does not have 10 residues on either side of the asparagine residue, required for the oligosaccharide transferase (18).

The cytoplasmic loop of MCT3, which separates the 6th and 7th membrane spanning domain, is 66 amino acids in length, 6 amino acids longer than in MCT1, and 17 longer than in MCT2 (Fig. 1). This loop in MCT3 is rich in lysine residues, and contains phosphorylation

TABLE 1

Pyruvate Uptake in MCT3 Transfected FRTL-5 Cells

Clone	Pyruvate uptake (nmoles per mg protein ± SE)
pCl-neo/MCT3	
1-1	33.2 ± 3.0
1-3	37.0 ± 6.6
1-4	37.0 ± 3.5
1-5	44.2 ± 4.7
pCl-neo/MCT3 antisense	
14-1	22.1 ± 1.1
14-2	14.7 ± 5.4
pCl-neo	
5-1	26.5 ± 6.9
5-2	28.0 ± 3.7
5-3	29.6 ± 2.7

Note. [^{14}C] pyruvate uptake was measured in transfected FRTL-5 cells with the indicated constructs. Cells were preincubated 15 mins at room temperature in glucose-free HBSS containing 5 mM pyruvate and 10 mM PIPES, pH 6.5. After washing, the cells were incubated in HBSS with 10mM PIPES, pH 6.5, containing 0.2 mM Na-pyruvate and [^{14}C] pyruvate. After incubation for 5 mins at room temperature, the uptake assay was terminated as detailed in *Materials and Methods.* Pyruvate uptake in the presence of 100 μM FCCP was 21.4 nmol/mg protein (n=17) and this value was substracted from the values shown.

sites not found in MCT1 or MCT2. The carboxy terminus of MCT3 has the least identity with those of MCT1 and MCT2, and is 35 amino acids longer.

Pyruvate transport in pCl-neo/MCT3 transfectants. A mammalian cell line was transfected with an expression plasmid containing MCT3 cDNA. C-14 pyruvate was used as a probe of lactate transport (19). A thyroid epithelial cell line, FRTL-5 cells, was selected for heterologous transfection since this cell line is suitable for ion transport studies (15). Cells were transfected using a mammalian expression plasmid pCI-neo/MCT3 containing a G418 resistance marker and the MCT3 cDNA insert under the control of a CMV promoter. Stable cell lines were selected with G418 as were control transfectants containing the vector alone, or MCT3 cDNA in the antisense orientation. [^{14}C]-pyruvate uptake was measured in these transfected cells (Table 1). Pyruvate uptake in FRTL-5 transfectants was measured after 5 mins at room temperature. Under these conditions, [^{14}C]-pyruvate uptake is linear and proportional to the amount of cell protein (not shown).

Pyruvate uptake was measured in clones transfected with MCT3 plasmid, clones with vector alone or clones with the anti-sense cDNA (Table 1). Of 8 MCT3 clones analyzed in this screening assay, 4 had a higher uptake of pyruvate than cells transfected with control plasmids. All clones with enhanced pyruvate uptake had MCT3 mRNA on Northern blot analysis as shown for clone 1-5 in Fig. 2. Immunofluorescence staining of MCT3 with MAb 3C4 is shown for this same clone in Fig. 3. All cells stained, but only ~10-20% of the cell population stained intensely. Clones transfected with vector alone, or antisense constructs, did not immunostain with MAb 3C4 nor had MCT3 mRNA on Northern blot analysis (not shown).

To study a more homogenous population of cells, clone 1-5 was subcloned. Pyruvate uptake was measured in cells that were grown for one week without TSH, to reduce basal uptake of pyruvate. Pyruvate uptake in clone 1-5 A3 was linear over the first 2 mins and two-fold higher than in non-transfected cells (Fig. 4). Moreover, pyruvate accumulation in transfected cells was decreased 50% with 200 μM α-cyano-4-hydroxycinnamate, a compound that inhibits monocarboxylate transporters (12,13,19).

DISCUSSION

Lactate is used to fuel mitochondrial oxidative metabolism in photoreceptor cells with Müller glial cells being an important source of this lactate (20). Müller cells have few mitochondria and use glucose trans-

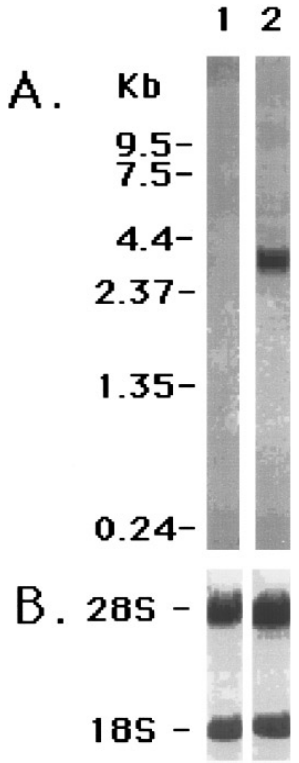

FIG. 2. RNA expression of MCT3 in transfected FRTL-5 cells. Total RNA (5 μg) from cells transfected with the vector alone (lane 1) or the plasmid containing MCT3 cDNA (lane 2) was separated on a 1% agarose gel and hybridized with a digoxingenin labeled MCT3 riboprobe as detailed in ref. 11. Detection was with alkaline phosphatase conjugated anti-digoxigenin antibody and NBT/BCIP (A). Methylene blue acid staining of 28S and 18S ribosomal RNA is shown in (B).

ported by the RPE from the choroidal blood. Lactate is released by Müller cells into the interphotoreceptor space (IPS) where it can be used by photoreceptor cells (4,20). The IPS is circumscribed by photoreceptor and Müller cells on one side, and the RPE on the other side (1). The RPE can influence lactate levels in the IPS by delivering lactate from the outer retina to the blood (7-10). Regulation of lactate levels in the IPS is essential for maintaining the pH and osmolarity in the fluid surrounding photoreceptor cells (8).

Transport studies of FRTL-5 cells, expressing MCT3, show that MCT3 is a monocarboxylate transporter. Pyruvate uptake in the transfected cells was proton-dependent and blocked by an inhibitor of MCTs, α-cyano-4-hydroxycinnamate. The increase in pyruvate uptake in transfected cells was related to MCT3 mRNA expression, and cell surface MCT3 immunoreactivity.

In summary, the present report characterizes a new monocarboxylate transporter, MCT3 located on the basal membrane of the RPE. MCT3 is a unique and abundant monocarboxylate transporter and is detected only in RPE (11). Features, distinctive to MCT3, include: multiple potential phosphorylation sites, and expression confined to one tissue. These differences suggest that MCT3 activity may respond to as well as regulate lactate levels in the subretinal space.

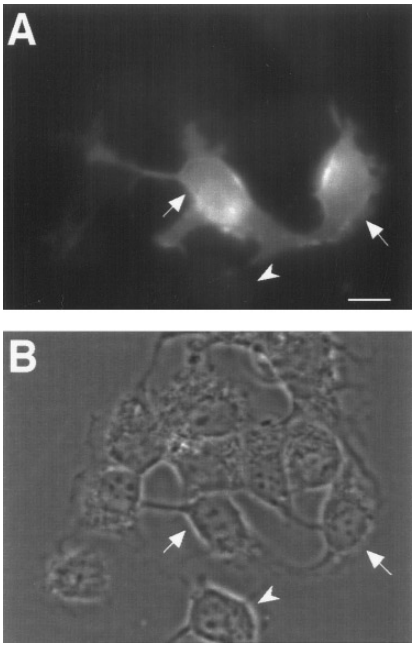

FIG. 3. Immunofluorescence staining of MCT3 in transfected FRTL-5. Cells transfected with MCT3 cDNA plasmid were grown on 2-well Permanox chamber slides (Lab-Tek) and stained with MAb 3C4 as described in ref. 11. The secondary antibody was Texas Red conjugated donkey anti-mouse IgG (Jackson Immuno-Research Labs. Inc.). In (A), arrows indicate brightly staining cells, while arrowheads show cells that are more faintly labeled. The phase contrast of the same field is shown in (B). Bar equals 10 μm.

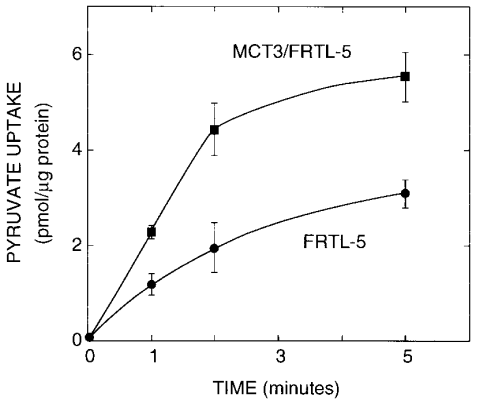

FIG. 4. Proton-coupled pyruvate uptake in a subclone of FRTL-5-MCT3 cells. Control and MCT3 transfected FRTL-5 cells were grown without TSH for 1 week. Cells were rinsed and pre-incubated in HBSS with 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid], ICN), pH 7.4, for 30 mins and assayed for [^{14}C]-pyruvate uptake in choline-HBSS with 10 mM PIPES, pH 6.5. For choline HBSS, NaCl was replaced with equi-molar concentrations of choline Cl. Uptake for the times indicated is shown for FRTL-5-MCT3 subclone (●) and parental FRTL-5 cells (■). The mean of 3 separate determinations ± SD is shown. The value in the presence of 100 μM FCCP was subtracted.

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REFERENCES

1. Adler, R., and Hewitt, A. T. (1994) *in* Retina (Ryan, S. J., Ed.), 2nd ed., pp. 1857–1867, Mosby-Year Book, Inc., St. Louis, MO.
2. Bok, D. (1993) *J. Cell Sci. Suppl.* **17,** 189–195.
3. Coulombre, A. J. (1995) *Am. J. Anat.* **96,** 153–189.
4. Ames, A., Li, Y., Heher, E. C., and Kimble, C. R. (1992) *J. Neurosci.* **12,** 840–853.
5. Li, J. -D., Gallemore, R., Dmitriev, A., and Steinberg, R. (1994) *Invest. Ophthalmol. Vis. Sci.* **35,** 2700–2711.
6. Winkler, B. J. (1981) *Gen. Physiol.* **77,** 667–692.
7. Adler, A. J., and Southwick, R. E. (1992) *Ophthalmic Res.* **24,** 243–252.
8. Kenyon, E., Yu, K., la Cour, M., and Miller, S. S. (1994) *Am. J. Physiol.* **267,** C1561–C1573.
9. la Cour, M., Lin, H., Kenyon, E., and Miller, S. S. (1994) *Invest. Ophthalmol. Vis. Sci.* **35,** 434–442.
10. Lin, H., la Cour, M., Andersen, M. V., and Miller, S. S. (1994) *Exp. Eye Res.* **59,** 679–688.
11. Philp, N. J., Chu, P., Pan, T. C., Chu, M. L., Stark, K., Boettiger, D., Yoon, H., and Kieber-Emmons, T. (1995) *Exp. Cell Res.* **219,** 64–73.
12. Garcia, C. K., Goldstein, J. L., Pathak, R. K., Anderson, R. G. W., and Brown, M. S. (1994) *Cell* **76,** 865–873.

13. Garcia, C. K., Brown, M. S., Pathak, R. K., and Goldstein, J. L. (1995) *J. Biol. Chem.* **270,** 1843–1849.
14. Grollman, E. F., Saji, M., Shimura, Y., Lau, J. T. Y., and Ashwell, G. (1993) *J. Biol. Chem.* (1993) **268,** 3604–3609.
15. Fanelli, A., Berlin, W. K., and Grollman, E. F. (1995) *Thyroid* **5,** 223–230.
16. van den Hoff, M. J. B., Moorman, A. F. M., and Lamers, W. H. (1992) *Nucleic Acids Res.* **20,** 2902.
17. Poole, R. C., Sansom, C. E., and Halestrap, A. P. (1996) *Biochem. J.* **320,** 817–824.
18. Landolt-Marticorena, C., and Reithmeier, R. A. F. (1994) *Biochem. J.* **302,** 253–260.
19. Poole, R. C., and Halestrap, A. P. (1993) *Am. J. Physiol.* **264,** C761–C782.
20. Poitry-Yamate, C. L., Poitry, S., and Tsacopoulos, M. (1995) *J. Neurosci.* **15,** 5179–5191.