Basigin (CD147) Is the Target for Organomercurial Inhibition of Monocarboxylate Transporter Isoforms 1 and 4

THE ANCILLARY PROTEIN FOR THE INSENSITIVE MCT2 IS EMBIGIN (gp70)*

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Translocation of monocarboxylate transporters MCT1 and MCT4 to the plasma membrane requires CD147 (basigin) with which they remain tightly associated. However, the importance of CD147 for MCT activity is unclear. MCT1 and MCT4 are both inhibited by the cell-impermeant organomercurial reagent p-chloromercuribenzenesulfonate (pCMBS). Here we demonstrate by site-directed mutagenesis that removal of all accessible cysteine residues on MCT4 does not prevent this inhibition. pCMBS treatment of cells abolished co-immunoprecipitation of MCT1 and MCT4 with CD147 and enhanced labeling of CD147 with a biotinylated-thiol reagent. This suggested that CD147 might be the target of pCMBS, and further evidence for this was obtained by treatment of cells with the bifunctional organomercurial reagent fluorescein dimercurate acid that caused oligomerization of CD147. Site-directed mutagenesis of CD147 implicated the disulfide bridge in the Ig-like C2 domain of CD147 as the target of pCMBS attack. MCT2, which is pCMBS-insensitive, was found to co-immunoprecipitate with gp70 rather than CD147. The interaction between gp70 and MCT2 was confirmed using fluorescence resonance energy transfer between the cyan fluorescent protein- and yellow fluorescent protein-tagged MCT2 and gp70. pCMBS strongly inhibited lactate transport into rabbit erythrocytes, where MCT1 interacts with CD147, but not into rat erythrocytes where it interacts with gp70. These data imply that inhibition of MCT1 and MCT4 activity by pCMBS is mediated through its binding to CD147, whereas MCT2, which associates with gp70, is insensitive to pCMBS. We conclude that ancillary proteins are required to maintain the catalytic activity of MCTs as well as for their translocation to the plasma membrane.

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1 The abbreviations used are: MCT, monocarboxylate transporter; BCECF, 2-7-bis(carboxyethyl)-5-6-carboxylfluorescein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GFP, green fluorescent protein; CD147-cYFP, CD147 tagged on the C terminus with cyan fluorescent protein; CD147-YFP, CD147 tagged on the N terminus with yellow fluorescent protein; CD147-VGF, CD147 tagged on the C terminus with yellow variant of green fluorescent protein; CD147nYFP, CD147 tagged on the N terminus with yellow variant of green fluorescent protein; CHC, α-cyan-4-hydroxycinnamate; DIDS, 4,4′-disothiocyanostilbene-2,2′-disulfonate; FDA, fluorescein dimercury acetate; MCT1/cFP, MCT1 tagged on the C terminus with cyan variant of green fluorescent protein; pCMBS, p-chloromercuribenzenesulfonate; TM, transmembrane domain; FRET, fluorescence resonance energy transfer; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PEO, polyethylene oxide-iodoacetamino biodot.
MCT Interactions with CD147, gp70, and Organomercurials

The continued interaction between these proteins is essential for transporter activity is not known, but disruption of this interaction by pCMBS is another potential target for organomercurial inhibition of transport. This is an attractive hypothesis, because MCT2 is pCMBS insensitive and does not interact with CD147. However, MCT2 does appear to require another ancillary protein, as yet unidentified, to be properly expressed at the cell surface (15, 16). Here we explore the effects of organomercurials on the interactions between CD147 and MCT1/4. We demonstrate that pCMBS inhibits MCT1 and MCT4 activity by attacking a reactive disulfide bond in the Ig-like C2 domain of CD147 and disrupting its interaction with MCT1 or MCT4. We also identify the ancillary protein with which MCT2 interacts as embigin (gp70), a CD147 homologue with an un-reactive Ig-like V domain in place of the C2 domain (17). This provides an explanation for the insensitivity of MCT2 toward inhibition by pCMBS. Our data imply that ancillary proteins are required to maintain the catalytic activity of MCTs as well as for their translocation to the plasma membrane.

MATERIALS AND METHODS

All reagents were obtained from Sigma (Poole, UK) unless otherwise stated. Fluorescein dimercury acetate (FDMA) was synthesized as described by (18). EZ-Link® PEO-Iodoacetate biotin was from Pierce (Pergo Science UK Ltd., Cramlington, Northumberland, UK).

Antibodies—Polyclonal antibodies were raised in rabbit against the C-terminal 16 amino acids of rat MCT1, MCT2, and CD147, and human MCT2 and MCT4 using keyhole limpet hemocyanin-conjugated peptides and affinity-purified using the immobilized peptide as described previously (10, 19, 20). Polyclonal antibodies were also raised against the cytoplasmic C-terminal region of rat embigin (gp70, amino acids 287–328) and rat MCT2 (amino acids 437–489), respectively, using the glutathione S-transferase (GST)-conjugated proteins as described previously for MCT9 antibodies (8). The mouse monoclonal antibody RBT2 against rat CD147 (21) was a generous gift of Dr. Neil Barclay (University of Oxford). Anti-rat MCT2 raised in chicken was obtained from Chemicon International. Secondary antibodies for Western blotting were from Amersham Biosciences (anti-rabbit) and Sigma (anti-chicken).

Cloning of cDNA Constructs—The coding regions of gp70 and rat MCT2 were subcloned into the EcoRI site of the pcI-neo mammalian expression vector (Promega). Human MCT4 was subcloned into the NotI site of the pcI-neo vector. The pcI-neo constructs required to express rat MCT1 and rat CD147 tagged on the N and C termini were obtained from Chemicon International. Secondary antibodies for Western blotting were from Amersham Biosciences (anti-rabbit) and Sigma (anti-chicken).

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange kit (Stratagene, UK), and the presence of the desired mutation was confirmed by sequencing.

CD147-antisense Experiments in Oocytes—The antisense nucleotide TTCTCATAAAAGAATTTGTTG was chemically synthesized; this sequence was derived from the consensus sequence of several partial Xenopus CD147 sequences (e.g. CK741473) identified by a BLAST search of the expressed sequence tag data base. The amino acid sequence encoded by this nucleotide sequence is identical to amino acids 228–235 of rat CD147, but the corresponding nucleotide sequence is different in six places. This is important to allow prevention of endogenous CD14 expression without an effect on the expression of exogenous rat CD147.

Measurement of Transport—For MCT activity expressed in Xenopus oocytes, the procedure employed was as described previously (7). CRNA was prepared by in vitro transcription from the appropriate linearized pGHL plasmid (mMessage mMachin, Ambion). Oocytes were injected with 25 ng of the relevant cDNA (MCT and CD147) and, where required, 200 ng of CD147 antisense cDNA. Transport was measured 3 days after injection using the ratemetric pH-sensitive fluorescent dye BCECF. Stock solution of pCMBS (Toronto Research Chemicals Inc, Toronto, Canada) were freshly prepared (30 mM in water) and applied at the required concentration 1 min before addition of 30 mM l-lactate to initiate transport. Lactate transport into rat and rabbit erythrocytes was studied by measuring the change in extracellular pH with a pH-sensitive electrode as described previously (22). The cells were used at 5% hematocrit in lightly buffered saline (150 mM NaCl, 2 mM Hapes, pH 7.0) supplemented with 5 µM DIDS and 100 µM acetazolamide to prevent bicarbonate/CO2-mediated proton movements. Transport was initiated by addition of monosaccharide at the required concentration, and data were analyzed by first order regression analysis of the time course of pH change.

Preparation of Plasma Membrane Fractions—Plasma membrane fractions from rat tissues were prepared as described previously (10). A crude plasma membrane fraction from cultured cells was prepared as follows. Cells were harvested and washed in PBS. The resulting pellet was resuspended in Tris-saline buffer (2.5% (v/v) Tris 40, 0.14 M NaCl, 10 mM Tris-HCl, pH 7.4, containing proteinase inhibitors phenylmethylsulfonyl fluoride (0.5 mM), benzamidine (0.5 mM), leupeptin (4 µg/ml), and pepstatin (4 µg/ml) and incubated with rolling for 1 h at room temperature. The cells were homogenized by passage through a syringe and fine needle and subjected to centrifugation at 1,500 × g for 10 min at 4 °C to remove cell debris. The resulting supernatant was centrifuged at 200,000 × g for 30 min at 4 °C to sediment the plasma membrane fraction that was resuspended in Tris-saline buffer (without Tween 40 and assayed for protein concentration with the Bio-Rad protein assay). Plasma membranes from rat and rabbit red blood cells were obtained using a standard erythrocyte ghost preparation (19).

Co-immunoprecipitation Studies—These were performed essentially as described previously (15). RBL-2H3 cells were harvested by scraping, collected by centrifugation, and washed twice in ice-cold PBS. Following RBT2 (5 µl of GST-rMCT2 or GST-rgp70) in PBS (1 ml) were incubated for 2 h at room temperature with the following inhibitors as required: 5 mM CHC, 500 µM pCMBS, and 100 µM DIDS. Cells were collected by centrifugation and washed three times with PBS. The pellet was re-suspended in cold lysis buffer (1% (w/v) Brij97, 10 mM Tris, 140 mM NaCl, 1 mM EDTA, pH 7.4, containing the proteinase inhibitors phenylmethylsulfonyl fluoride (0.5 mM), benzamidine (0.5 mM), leupeptin (4 µg/ml), antipain (4 µg/ml), and pepstatin (4 µg/ml) and incubated for 30 min at 4 °C, and then the insoluble fraction removed by centrifugation for 10 min at 10,000 × g. The lysate was incubated with the appropriate anti-rat CD147 antibody or avidin beads for 2 h (or overnight) at 4 °C. For antibody immunoprecipitations samples were then incubated for 2 h at 4 °C with Protein A immobilized on Sepharose beads that had been pre-washed in lysis buffer and then washed three times (100 µl samples) with lysis buffer before re-suspending in sample buffer containing 5% β-mercaptoethanol. Following SDS-PAGE, Western blotting was performed as described previously (15). For co-immunoprecipitation of MCT2 and MCT1, kidney membranes (100 µg of protein) were solubilized in lysis buffer as above and incubated with 5 µl of antibodies GST-SMCT2 or GST-rgp70 and 50 µl of monoclonal RET.PE-2 (anti-rat CD147) for 2 h at 4 °C. The immunoprecipitate was retrieved with immobilized Protein A, washed, and subjected to SDS-PAGE and Western blotting as described below.

Western Blotting—Following SDS-PAGE, Western blotting was performed using polyclonal antibodies against rat MCT1, human MCT4, GST-rat gp70, and rat CD147 at 1 in 1000 (15). MCT2 was detected with chicken anti-rat MCT2, 1:5000. Blots were developed with enhanced chemiluminescence (ECL detection kit, Amersham Biosciences) using anti-rabbit or -chicken Ig secondary antibody conjugated to horseradish peroxidase.

Transfection of COS-7 Cells—COS cells were maintained in Dulbecco’s modified Eagle’s medium (from Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen), penicillin 100 units/ml, and streptomycin 100 µg/ml. For transfection (below), coverslips were placed in 6-well tissue culture plates and seeded with 1–3 × 105 cells per well in 2 ml of complete medium. Cells were incubated at 37 °C in a CO2 incubator until they were 60–70% confluent, and then transfection was carried out essentially as described previously (15). DNA (2 µg) was mixed with 6 µl of FuGENE 6 (Roche Applied Science) in 100 µl of serum-free medium and incubated for 20 min before adding to the cells and incubating for 24 h. At this point the
medium was replaced, and live imaging performed after a further 24 h. Live Imaging of Cells and FRET—

FIG. 1. Predicted structure of MCT4 modeled on the crystal structures of the E. coli glycerol-3-phosphate transporter and the lactose permease. The Cn3D program (www.ncbi.nlm.nih.gov/Structure/CN3D/cn3dtut.shtml) was used to align the two known structures from published co-ordinates (glycerol-3-phosphate transporter IPW4_A and lactose permease IPV6_A) and then the sequences for human MCT1 (gi 13432183), MCT2 (gi 6225703), and MCT4 (gi 6225705) threaded on to this structure to give the best fit. All three isoforms showed the same basic transmembrane helix arrangement and that for MCT4 is shown. The key residues in rat MCT1 identified by Broëer et al. (41, 42) from site-directed mutagenesis as being critical for activity are shown in their equivalent positions on MCT4 and labeled (Arg-143), 2 (Arg-153), 3 (Asp-302), 4 (Arg-306), 5 (Phe-360), and 6 (Glu-389). The cysteine residues modified by site-directed mutagenesis in the current experiments are shown with arrows and labels, whereas other cysteine residues that are not found in equivalent positions in either MCT1 or MCT2 are labeled a (Cys-28), b (Cys-81), c (Cys-81), d (Cys-87), e (Cys-161), and f (Cys-409). The color coding is shown to represent the progression of sequence from N to C termini (red to mauve).

RESULTS

MCT4 Cysteine Mutants Remain Sensitive to pCMBS Inhibition—We initially sought to identify an exofacial cysteine residue, present in both MCT1 and MCT4 but not MCT2, that might be the target of pCMBS inhibition. The MCT family is a member of the major facilitator superfamily, and the crystal structures of two members of this family, the Escherichia coli glycerol-3-phosphate transporter and lactose permease, have now been solved (23, 24). We have used the Cn3D program (www.ncbi.nlm.nih.gov/Structure/CN3D/cn3dtut.shtml) to model the MCT1, MCT2, and MCT4 structures and so predict more accurately the probable location of all cysteines within the MCTs. The predicted structure of MCT4 is shown in Fig. 1 and suggests that Cys-108, located in the short extracellular loop between TMs 3 and 4, is the only exofacial cysteine residue in MCT4. Furthermore, the equivalent residue in MCT1 is also a cysteine in all species for which sequences are available, whereas MCT2 does not have a cysteine in this position. We mutated this residue to serine (to match MCT2) and expressed the protein in Xenopus oocytes, which contain endogenous CD147 (25), to measure lactate transport. The mutant was found to remain sensitive to pCMBS when expressed as shown in Fig. 2. Further examination of the sequences revealed another candidate cysteine (Cys-190), located in TM6, that was also present in MCT1 and MCT4 but absent in MCT2. Therefore, although an unlikely target, we generated the double mutant of MCT4, C108S/C190A. For completion we also mutated Cys-191 (present in both MCT1 and MCT2 of all species), Cys-193 (present in neither MCT1 or MCT2 of any species), and Cys-325 (present in neither MCT1 or MCT2 of any species) to alanine. All mutants gave similar rates of transport in the absence of pCMBS (data not presented), but as shown in Fig. 2, their sensitivity toward pCMBS was increased, rather than decreased. This was most notable when Cys-325 was mutated to alanine as in C108S/C190A/C191A/C325A and C108S/C190A/C193A/C325A. Whatever the reason for this increase in sensitivity (see “Discussion”) it is clear that none of these cysteines is responsible for inhibition of MCT4 transport activity by pCMBS.

Because there are no other cysteine residues in MCT4 that are present in the equivalent location in MCT1, or are likely to be accessible to the extracellular medium (Fig. 1), two alternative conclusions are possible. First, the cysteine attacked by pCMBS is on a protein other than MCT1 and MCT4, the likely candidate being CD147. Second, although pCMBS is generally
used as a cysteine-modifying reagent (because of its mercuric nature), its effects might be mediated through the sulfonate groups. This is unlikely because p-chloromercuribenzoic acid and mersalyl are also potent inhibitors of MCT1 (11, 26, 27). However, to confirm this, the non-mercuric compound p-chlorobenzoic sulfonate was substituted for pCMBS but no inhibition of MCT1 or MCT4 expressed in Xenopus oocytes was observed (data not shown).

**CD147 Is Reactive toward Organomercurials**—For pCMBS to bind to CD147, it must react with a cysteine residue on the extracellular (N-terminal) domain of the protein. Inspection of the amino acid sequence of CD147 reveals that there are only four cysteine residues conserved across species. These are thought to be involved in the disulfide bridges of immunoglobulin-like folds. However, some cysteine residues can remain reactive to thiol reagents as has been shown for the Ig-like C2 domain (IPR003598) of CD4 (28). The Prosite data base (us.expasy.org/prosite/) also identifies an Ig-like C2 domain between residues 22 and 103 of CD147 that might be reactive toward thiol reagents. Our first evidence that pCMBS might attack CD147 was obtained by cross-linking CD147 with a monoclonal antibody and a fluorescein isothiocyanate-labeled secondary antibody against mouse IgA. Such antibody treatment causes CD147 to coalesce together with MCT1 or MCT4 into caps in Y3 cells (15). We were able to demonstrate that this capping of CD147, MCT1, and MCT4 was abolished by exposure of Y3 cells to 0.5 mM pCMBS for 5 min (see Supplementary Fig. S1). In contrast, two other inhibitors of MCT1, 4-4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) and α-cyano-4-hydroxycinnaminate (CHC), were without effect. These data imply that following pCMBS treatment the conformation of CD147 changes such that the antibodies cannot bind. This is strongly suggestive of a direct interaction of pCMBS with CD147. This conclusion was confirmed directly by the series of experiments described below.

We anticipated that fluorescein dimercury acetate (FDMA) would fluorescently label CD147 and allow its detection in an immunoprecipitate. Initially, rat basophilic leukemia cells (RBL.2H3) were used for these studies, because we had previously demonstrated substantial co-immunoprecipitation of MCT1 with CD147 in such cells (15). However, following FDM treatment of the RBL cells, our monoclonal antibody no longer immunoprecipitated CD147 (data not shown). This suggested that the conformation of CD147 was perturbed following FDM treatment such that the monoclonal antibody could no longer bind. Furthermore, when probing a Western blot of RBL cells lysates with our polyclonal CD147 antibody we found that FDM significantly decreased the intensity of the CD147 band visible at 50 kDa as shown in Fig. 3A. This was sometimes accompanied by the appearance of a weak higher band at about 100 kDa. In rabbit erythrocytes FDMA also reduced the CD147 signal at 50 kDa, but in this case no band became visible at 100 kDa. Inspection of the Coomassie Blue-stained blots (not shown) revealed similar levels of protein loading in the presence and absence of FDM. Parallel blotting with the MCT1 antibody showed that FDM also decreased the intensity of the MCT1 band at 45 kDa, and this was associated with the appearance of a band at ~100 kDa that probably represents dimeric MCT1 (Fig. 3A). The stability of such dimers in SDS-PAGE is not uncommon in membrane proteins and has been observed previously with MCT1 (14).

In contrast to the RBL cells, the majority of MCT1 of the rabbit erythrocytes appeared to run on SDS-PAGE as a dimer even in the absence of FDMA, with a small fraction remaining as a monomer. However, FDMA caused both monomeric and dimeric MCT1 signals to decrease in parallel with the decrease in CD147 signal. Thus in both cell types FDMA treatment decreased the total amount of CD147 (monomer and dimer) detected on the blot. We have previously shown that CD147 is present in the plasma membrane as a dimer together with a dimer of MCT1 (14, 16). Thus a likely explanation of our data is that the two mercuric groups of FDMA produce a cross-link between two CD147 molecules. This chemical modification may then reduce the interaction between CD147 and MCT1 leading to aggregation of both proteins, first to dimers and then, for CD147, higher SDS-insoluble oligomeric forms that do not enter the polyacrylamide gel. In this context it is of interest that thiol reagents such as HgCl2 and 5,5′-dithiobis(2-nitrobenzoic acid) facilitate dimerization and oligomerization of CD4 (29, 30), which also has reactive extracellular cysteine residues (28).

For final confirmation that organomercurials such as pCMBS attack a thiol group on CD147 we used the biotin containing thiol reactive agent EZ-Link® PE0-Iodoacetyl biotin (Pierce) to label exofacial reactive cysteines on RBL cells as has been performed to demonstrate the reactivity of disulfide

![Image](http://www.jbc.org/)

**Fig. 3.** FDMA and PEO-Iodoacetyl biotin modify cysteine residues on CD147. In A, rabbit erythrocytes or rat basophilic leukemia cells (RBL.2H3) were incubated for 10 min at room temperature with 0.2 mM FDMA prior to preparation of ghosts or plasma membranes and protein separation by SDS-PAGE. Western blots were then performed using polyclonal antibodies against CD147 and MCT1 (1:1000). In B, RBL cells were incubated in the absence (lane 1) or presence of 0.5 mM pCMBS (lanes 2 and 4) and or 0.12 mM PEO-Iodoacetyl biotin (lanes 3 and 4) for 90 min prior to washing, lysis in 1 ml and binding of biotinylated proteins to avidin beads or immunoprecipitation with the polyclonal C-terminal CD147 antibody as described under “Materials and Methods.” The bound or immunoprecipitated proteins and a 10-ng sample of lysed cells were separated by SDS-PAGE, blotted, and probed with either avidin peroxidase or polyclonal anti-CD147 antibody as indicated. The inset contains data of avidin-bound protein from two separate experiments to confirm that the higher of the two CD147 bands (marked with an arrowhead) was only detected when cells were treated with both pCMBS and PEO and not with PEO alone. Note that the lysate blot shows that the labeling of several bands with PEO is greatly increased with pCMBS treatment, but this is not observed in the avidin pull-down, which selectively enriches the PEO-labeled protein.
bridges in CD4 (28). Following preparation of a plasma membrane fraction, labeled proteins were revealed using SDS-PAGE and Western blotting with avidin-peroxidase. As shown in Fig. 3B, several labeled proteins were detected, and these could be pulled down with agarose-avidin beads. Labeling of several minor bands was reduced by the pCMBS reflecting the competition of the two reagents for the thiol groups. However, the labeling of three bands at 45, 55, and 65 kDa in the total cell lysate was greatly increased following pCMBS treatment of the cells, suggesting that the reagent had unmasked latent thiol groups. It should be noted that this increased labeling was not seen in the avidin-bound fraction, because the avidin-beads bind only the labeled protein causing their enrichment. The band at 55 kDa ran slightly higher than CD147 (running at 50–52 kDa) as might be predicted for PEO-labeled CD147. Confirmation that CD147 is present in this band was obtained using SDS-PAGE of the avidin-bound proteins and Western blotting with anti-CD147 antibodies. A little CD147 (50–52 kDa) was present in control (untreated) samples and is taken to represent nonspecific binding. However, an additional band, running slightly higher (55 kDa) and marked with an arrowhead, was always detected only in samples that had been treated with both PEO and pCMBS. Three representative blots are shown in Fig. 3B to demonstrate the consistency of this result. As noted above, the change in mobility is likely to reflect the covalent attachment of the PEO-biotin. For completion, we confirm that MCT1 is targeted to the plasma membrane as shown in Fig. 5A, allowing us to conclude that MCT1 and CD147 remain closely associated even 1 h after treatment with pCMBS. Thus inhibition of transport by this agent is unlikely to be caused by the rapid dissociation of the two proteins within the membrane. Two other transport inhibitors, DIDS (100 μM) and CHC (1 mM), or the presence of l-lactate (1 mM) gave a slight decrease in 480 nm/530 nm fluorescence ratio (increased FRET) that was statistically significant (p < 0.05) only for l-lactate perhaps indicating conformational changes occurring upon substrate and inhibitor binding.

Identifying the Cysteine on CD147 That Is Responsible for pCMBS Inhibition of Lactate Transport—To identify which of the four N-terminal extracellular cysteines of CD147 might be the target of pCMBS, we have used site-directed mutagenesis to replace them with valine or alanine. CD147 mutants, tagged with the GFP variants cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) and demonstrating FRET when both fluorescent tags were intracellular (referred to as MCT1cCFP and CD147cYFP) (16). In Fig. 4C we confirm the occurrence of FRET in cells transfected with MCT1cCFP and CD147cYFP. Data are expressed as the 480 nm/530 nm fluorescence ratio, with FRET being detected as a decrease in this ratio. Thus the emission ratio for the FRET pair MCT1cCFP/CD147cYFP was about 0.85, whereas that for the non-FRET pair MCT1cCFP/CD147nYFP was about 1.1. We have previously confirmed by fluorescence bleaching that these differences in fluorescence ratios reflect FRET rather than some fluorescence artifact (16). Only very small decreases in FRET were observed following treatment with pCMBS for 5 min and 1 h, allowing us to conclude that MCT1 and CD147 remain...
larly to wild-type proteins (data not shown). Using Xenopus oocytes expressing MCT1 plus the C188V/C126V mutant of CD147 we were able to confirm that transport remained sensitive to inhibition by pCMBS as shown in Fig. 5B. These experiments required removal of endogenous CD147 from Xenopus oocytes by coinjection of antisense against endogenous CD147 (25). This greatly reduced the rate of lactate transport detected following co-injection of MCT1 cRNA, but rates were restored by co-injection of cRNA encoding the double mutant of rat CD147 (C188V/C126V). These do not reach the membrane and do not show FRET. In B, Xenopus oocytes were injected with cRNA (25 ng) for MCT1 and CD147 (wild type or C126V/C188V) in the presence of antisense cDNA to Xenopus CD147 (200 ng). The uptake of 10 mM L-lactate followed using BCECF fluorescence. Where indicated 0.2 mM pCMBS was added 5 min prior to the L-lactate.

![FIG. 5](image)

**FIG. 5.** The effects of replacing extracellular cysteines of CD147 on the plasma membrane expression of MCT1 and CD147 and the pCMBS-sensitivity of lactate transport. Cysteine site-directed mutants of CD147cYFP were made and co-expressed in COS cells with MCT1cCFP. The confocal images (A) show plasma membrane expression of the double mutant C126VC188VC147cYFP with MCT1cCFP. Although the data are not shown, FRET occurred between these proteins and between MCT1cCFP and the two single CD147 mutants C126VC147cYFP and C188VC147cYFP. A also shows confocal images of cells co-expressing MCT1cCFP with either the single mutants C87VC147cYFP or C41VC147cYFP, and the triple mutant C87VC126VC188VC147cYFP. The uptake of 10 mM L-lactate followed using BCECF fluorescence. Where indicated 0.2 mM pCMBS was added 5 min prior to the L-lactate.

Similarly, the Golgi apparatus (Fig. 5A). Similar results were obtained if the cysteines were mutated to alanine rather than valine (data not shown). Because we were also unable to express these CD147 mutants at the surface of Xenopus oocytes, it was not possible to confirm directly that either Cys-41 or Cys-87 is the target of pCMBS inhibition of lactate transport. However, our data do suggest that these residues play a critical role in the correct folding of CD147 that is essential for its interaction with MCT1 and subsequent translocation to the plasma membrane. Interestingly, others have shown that mutation to alanine of the two cysteines (Cys-130 and Cys-159) in the Ig-like C2 domain of CD4 prevents correct plasma membrane expression of this protein at the plasma membrane (28).

**FIG. 6.** The ancillary protein for MCT2 is gp70. A, kidney membranes were solubilized and immunoprecipitation performed as described under “Materials and Methods.” For MCT2, the positive control (left lane) was the detection of MCT2 in an immunoprecipitate obtained with a rabbit polyclonal MCT2 antibody, whereas for MCT1, solubilized kidney membranes were employed. Detection of MCT2 was with chicken C-terminal antipeptide antibody (1:5000) and of MCT1 with rabbit C-terminal antipeptide antibody (1:1000). In B, COS cells were co-transfected with gp70 and rat (r) or human (h) MCT2, and the plasma membrane localization was confirmed with Western blotting. In C similar experiments were performed with gp70cCFP and rat MCT2cYFP and plasma membrane localization demonstrated with confocal microscopy. In D data are shown for the FRET occurring between MCT2cYFP or MCT2nYFP and gp70cCFP but not between MCT2nCFP and gp70nYFP. Data are presented as mean ± S.E. of the number of observations shown.

MCT2 Interacts with gp70 Rather Than CD147—The observation that MCT2 does not associate with CD147 (15) and is not inhibited by pCMBS (4, 13) might be explained if MCT2 interacts with another ancillary protein that is insensitive to pCMBS. An obvious candidate for this would be gp70 (embigin), a close homologue of CD147 (17). gp70 is less widely expressed than CD147, and thus is not the normal binding partner for MCT1 and MCT4 (15), although it does associate with MCT1 in rat erythrocytes where the two proteins can be cross-linked by DIIDS (14). In Fig. 6A we used immunoprecipitating antibodies...
against CD147 and gp70 to investigate their interaction with MCT1 and MCT2 in solubilized kidney plasma membranes that contain all four proteins. MCT2 was found to co-immunoprecipitate with gp70, but not with CD147, whereas for MCT1 the opposite was the case. To confirm this interaction we tagged MCT2 and gp70 with CFP or YFP on their C and N termini with CFP and YFP and used live cell imaging to demonstrate their co-expression at the plasma membrane as shown in Fig. 6B. These data contrast markedly with the inability of CD147 to support MCT2 expression at the plasma membrane (15). Expression of MCT2 was confirmed by SDS-PAGE of plasma membrane preparations followed by Western blotting with MCT2 antibodies (Fig. 6C). Furthermore, the use of CFP and YFP constructs allowed us to investigate FRET between the tagged proteins in the plasma membrane much as we have done previously for MCT1 and CD147 (16). Expression of MCT2 was confirmed by SDS-PAGE of plasma membrane preparations followed by Western blotting with MCT2 antibodies (Fig. 6C). Furthermore, the use of CFP and YFP constructs allowed us to investigate FRET between the tagged proteins in the plasma membrane much as we have done previously for MCT1 and CD147 (16). Data are shown in Fig. 6D. For gp70 tagged with CFP on its cytosolic C terminus, FRET was observed with MCT2 tagged with YFP on either the N or C terminus, both of which are on the cytosolic face of MCT2. In agreement with earlier studies using MCT1 and CD147 (16), no FRET was observed when the two fluorophores were on opposite sides of the plasma membrane (MCT2 with CFP on the C terminus and gp70 with the YFP on the N terminus). These data act as a negative control and confirm the validity of the FRET measurements.

**MCT1 Bound to gp70 Is Insensitive to pCMBS Inhibition—**

Our data imply that MCT2 is insensitive to pCMBS because it interacts with gp70 rather than CD147. If this were the case then it would be anticipated that lactate transport into cells such as rat erythrocytes, in which MCT1 binds to gp70 rather than CD147, should be insensitive to inhibition by pCMBS. It should be noted that early studies demonstrating the sensitivity of lactate transport to low concentrations of pCMBS (26, 31) were performed in human erythrocytes where CD147 rather than gp70 is known to be expressed (32, 33). Thus we decided to compare directly the pCMBS sensitivity of MCT1-mediated lactate transport in erythrocytes that contain primarily gp70 with those containing mainly CD147. In Fig. 7D we demonstrate that erythrocytes from rodents (rats, guinea pigs, and hamsters) contain mainly gp70, whereas in rabbit erythrocytes, as found by others (34), there is a large amount of CD147, but little gp70. Bovine erythrocytes express neither, but it is also known that they do not express any monocarboxylate transporter (35, 36). Rates of lactate transport were found to be faster in rabbit than rat erythrocytes and were much more sensitive to inhibition by 0.2 mM pCMBS treatment (Fig. 7, A and B). This difference in sensitivity was not shared by the stilbene disulfonate DIDS (Fig. 7C), which is not surprising because DIDS can cross-link MCT1 to both gp70 and CD147 (14, 15). Furthermore, when rat erythrocytes were treated with PEO-Iodoacetyl biotin with or without pCMBS treatment and avidin pull-down used to detect biotin-labeled proteins, no gp70 bound could be detected by SDS-PAGE and Western blotting of the bound fraction (data not shown). This is in contrast to the data obtained with CD147 (Fig. 3B) and confirms that gp70 is unreactive toward thiol reagents as expected from the insensitivity of lactate transport to pCMBS inhibition when MCT1 is associated with gp70 (Fig. 7B). We also determined the $K_m$ values (millimolar ± S.E.) of MCT1 in both rat and rabbit erythrocytes at 6 °C for L-lactate (2.8 ± 0.3 and 2.8 ± 0.5), D-lactate (10.3 ± 3.1 and 16.1 ± 3.5), and pyruvate (0.94 ± 0.12 and 1.45 ± 0.19). These data suggest that the presence of gp70 versus CD147 does not have any major effect on the transport properties of MCT1 other than its pCMBS sensitivity.

**DISCUSSION**

**CD147 Rather Than MCT1 and MCT4 Is the Target for pCMBS Inhibition of Monocarboxylate Transport—**

pCMBS is widely used as a membrane-impermeable thiol-modifying reagent that targets cysteine residues. The inability of pCBS (which lacks the active mercurial group of pCMBS) to inhibit lactate transport by MCT4 confirms that covalent modification of a cysteine by the mercuric group of pCMBS is likely to be important for the inhibition of transport. Furthermore, other organomercurial thiol reagents are also inhibitors of lactate transport, including mersalyl (11, 27), pCMB (13), and FDMA (Fig. 7), whereas maleimides and alkylating reagents such as iodoacetate (and PEO-Iodoacetyl biotin, data not shown) are not (31, 36, 37). However, we have shown that site-directed mutagenesis of Cys-108, the only cysteine of MCT4 that
is likely to be accessible to pCMBS from the extracellular medium, to Ser did not prevent inhibition by pCMBS, whereas mutation of four others (Cys-190, Cys-191, Cys-193, and Cys-325) to alanine actually increased the sensitivity of transport to inhibition by the reagent. The predicted location of these cysteine residues is shown on Fig. 1. Cys-325 is on TM helix 10 at the interface with helix 9 both of which are predicted to undergo major conformational changes during the translocation cycle (23). Cys-190, Cys-191, and Cys-193 all lie on the same external surface of MCT4 in TM helices 3 and 6 that might interact with the TM helix of CD147. Although the exact cause of the increased sensitivity to pCMBS when these cysteines are mutated to alanine is not known, it is likely that they perturb the interaction between MCT4 and CD147 such that the accessibility or reactivity to pCMBS of an exofacial cysteine residue on CD147 is increased (see below).

We have provided several lines of evidence that CD147 is the site of action of pCMBS. First, the agent prevents cross-linking antibodies from causing CD147 to aggregate into caps on the cell surface (Supplementary Fig. 1). Second, it enhances labeling of CD147 with the biotinylated thiol reagent PEO-Iodoacetyl biotin (Fig. 3B) an effect not seen with gp70 (not shown). Third, FDMA, with two reactive mercury groups, causes CD147 to dimerize and then aggregate (Fig. 3A). Fourth, when MCT1 is associated with gp70, as it is in rat erythrocytes, rather than CD147 as in rabbit erythrocytes, it becomes insensitive to pCMBS inhibition (Fig. 7, A and B). The Disulfide Bridge of the Ig-like C2 Domain of CD147 Is the Probable Locus of pCMBS Binding—Although CD147 and gp70 are closely related proteins, and both are predicted to have two extracellular immunoglobulin-like domains with disulfide bridges, there are significant structural differences. In gp70, there are two Ig-like V domains (IPR003599) between residues 38–161 and 162–256, whereas CD147 has one Ig-like C2 domain (IPR003598) between residues 22 and 103 and one Ig-like V domain (residues 105–203). Our data suggest that it is the C2 domain of CD147 that contains the reactive cysteines. This is not without precedent, because in CD4 one of the Ig-like C2 domains is known to remain partially reduced and to be highly reactive toward thiol reagents (28). Furthermore, mutation of the two cysteines (Cys-130 and Cys-159) of the C2 domain of CD4 to alanine prevented correct plasma membrane expression, exactly as we observe for the C8V and C41V mutants of the domain of CD147 that contains the reactive cysteines. This is V domain (residues 105–203). Our data suggest that it is the C2 

Mechanism by Which pCMBS Binding to CD147 Inhibits Transport by MCT1 and MCT4—We have shown previously that CD147 acts as a chaperone for expression of active MCT1 and MCT4 at the plasma membrane where the proteins remain closely associated as revealed by co-immunoprecipitation and FRET studies (15, 16). The ability of pCMBS to abolish or greatly reduce co-immunoprecipitation of MCT1 and MCT4 with CD147 (Fig. 4, A and B) implies that this reagent reduces the affinity of interaction between the two proteins such that they dissociate following detergent solubilization. However, the strength of the association is still sufficient to prevent their complete dissociation within the membrane, because FRET is maintained (Fig. 4C). Our data do not allow us to conclude whether or not the decreased interaction induced by organomercurials is the cause of transporter inhibition, but they do imply that a change in the conformation in CD147 is transmitted to the transporter to inhibit its activity. What is clear is that CD147 is more than just a chaperone to take MCT1 and MCT4 to the membrane; its continued presence and correct conformation are critical for transporter function. This is consistent with our previous observation that, when purifying lactate transporter activity from red blood cell membranes, it was not possible to separate MCT1 from its ancillary protein (gp70 rather than CD147 in rat red blood cells) without losing the ability to reconstitute transport activity (38).

gp70 Is the Ancillary Protein for MCT2 and Explains Its Insensitivity to Inhibition by pCMBS—MCT2 is insensitive to inhibition by pCMBS (4, 13) and also requires an ancillary protein other than CD147 to translocate to the plasma membrane (15). Here we show that it is gp70 that fulfills this role. This is an important observation in view of the critical role that MCT2 is thought to play in lactate uptake as a fuel for neurons, especially in the synapses (39, 40). Our data also provide a satisfying explanation as to why transport by MCT2 is insensitive to organomercurials while remaining sensitive to CHC and stilbene disulfonates that do not disrupt the MCT/ancillary protein interaction (Fig. 4A).

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Basigin (CD147) Is the Target for Organomercurial Inhibition of Monocarboxylate Transporter Isoforms 1 and 4: THE ANCILLARY PROTEIN FOR THE INSENSITIVE MCT2 IS EMBIGIN (gp70)
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