Interaction of the Erythrocyte Lactate Transporter (Monocarboxylate Transporter 1) with an Integral 70-kDa Membrane Glycoprotein of the Immunoglobulin Superfamily

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Proton-monocarboxylate transporters (MCTs)1 are essential for the well being of almost all mammalian cells and play a central role in the transport of lactate between tissues (1). Recently two distinct MCTs (MCT1 and MCT2) have been cloned from mammalian cells (2–6). MCT1 is widely distributed in mammalian cells and has been well characterized at the functional (1, 7) and structural level (8), especially in erythrocyte membranes and an additional band of 130 kDa on Western blots of erythrocyte membranes and an additional band of 130 kDa after treatment of erythrocytes with 100 μM DIDS. The 70-kDa protein that is cross-linked to MCT1 was purified and shown to contain N-linked carbohydrate; the apparent core molecular mass is 40 kDa. Amino acid sequencing showed that the protein is the rat equivalent of the membrane-spanning mouse teratocarcinoma glycoprotein GP-70, a member of the immunoglobulin superfamily related to basigin (11). Cross-linking appears to occur via a site at or in communication with the substrate binding site of MCT1 and might play a role in regulating MCT1 activity. "In accordance with 18 U.S.C. Section 1734 solely to advertise", this article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. * This work was supported by the Wellcome Trust Grant 042249/Z/94/Z. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. † To whom correspondence should be addressed. Tel.: 44 117 928 8592; Fax: 44 117 926 8274; E-mail: A.Halestrap@Bristol.ac.uk.

1 The abbreviations used are: MCT, monocarboxylate transporter; CHC, a-cyano-4-hydroxycinnamate; CεNε, octaethenyl glycol monodecyl ether; DBDS, 4,4'-dibenzenamidostilbene-2,2'-disulfonate; DIDS, 4,4'-dipropionylamidostilbene-2,2'-disulfonate; DTSSP, 3,3'-dithiobis(sulfosuccinimidyl)-propionate; TM, transmembrane; PAGE, polyacrylamide gel electrophoresis; Triticine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MOPS, 4-morpholinopropanesulfonic acid.

*8* 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS) causes irreversible inhibition and chemical labeling of the lactate transporter, monocarboxylate transporter 1 (MCT1) (Poole, R. C., and Halestrap, A. P. (1992) Biochem. J. 283, 855–862). In rat erythrocytes DIDS also causes cross-linking of MCT1 to another protein in the membrane to give a product of 130 kDa on SDS-polyacrylamide gel electrophoresis. Cross-linking is markedly reduced by those compounds that protect against irreversible inhibition of lactate transport by DIDS and enhanced by imposition of a pH gradient across the plasma membrane to recruit the substrate binding site of MCT1 to an exofacial conformation. These data indicate that DIDS cross-linking is via the same site on MCT1 as is responsible for inhibition of transport. Antibodies raised against the cross-linked conjugate react with proteins of approximately 40 kDa (MCT1) and 70 kDa on Western blots of erythrocyte membranes and an additional band of 130 kDa after treatment of erythrocytes with 100 μM DIDS. The 70-kDa protein that is cross-linked to MCT1 was purified and shown to contain N-linked carbohydrate; the apparent core molecular mass is 40 kDa. Amino acid sequencing showed that the protein is the rat equivalent of the membrane-spanning mouse teratocarcinoma glycoprotein GP-70, a member of the immunoglobulin superfamily related to basigin (11). Cross-linking appears to occur via a site at or in communication with the substrate binding site of MCT1 and might play a role in regulating MCT1 activity.

EXPERIMENTAL PROCEDURES

Materials

Chemicals and biochemicals were obtained from the sources given previously (8, 9, 12). The protease Lys-C was from Sigma, Poole, Dorset, UK. Anti-peptide antibodies to various regions of MCT1 were raised and purified as described elsewhere (8).

Methods

Labeling of Erythrocytes with DIDS and Analysis of Products—Erythrocytes were collected in a citrate buffer (84 mM sodium citrate, 1 mM EGTA, pH 7.4). Cells were then usually washed once in a bicarbonate-buffered saline buffer (121 mM NaCl, 25 mM NaHCO3, equilibrated with 95% O2, 5% CO2), and then at least twice more in citrate buffer before resuspending in the same buffer to 10% hematocrit and adjusting the pH to 7.4. Incubations with DIDS, removal of nonbound inhibitor, and preparation of ghost membranes were performed as described previously (10). Proteolytic digestion of red cell ghosts and separation of membrane proteins by SDS-PAGE were performed as described elsewhere (8). Anti-(MCT1-70-kDa Binding Protein Conjugate) Antibody—Rat erythrocyte membranes, prepared from cells treated with 100 μM DIDS, were subjected to chromatography on aminomethyl-Sepharose, essentially as described previously (12). Elution of the cross-linked product of 130 kDa was monitored both by Western blotting with anti-MCT1 antibodies, and by silver staining of protein. The peak fractions containing the cross-linked product (which co-eluted with free MCT1) were concentrated by centrifugal filtration (Amicon, Centriprep 10) and separated on a 6% (w/v) SDS-PAGE gel, and the protein was located with copper staining (13). The band of 130 kDa was excised, destained, and concentrated on a 6% (w/v) SDS-PAGE gel, and the protein was located by copper staining (13). The band of 130 kDa was excised, destained, and then electroeluted as described below. This preparation was used to immunize a New Zealand White rabbit, as described previously (8).

Purification of the 70-kDa Binding Protein and Sequencing of Lys-C

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4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), which is the rat equivalent of the mouse teratocarcinoma glycoprotein GP-70, which is a member of the immunoglobulin gene superfamily related to basigin (11). Cross-linking appears to occur via a site at or in communication with the substrate binding site of MCT1 and might play a role in regulating MCT1 activity.

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Purification of the 70-kDa Binding Protein and Sequencing of Lys-C
RESULTS AND DISCUSSION

DIDS Causes Cross-linking of MCT1 to a 70-kDa Protein—The data of Fig. 1 show that the 130-kDa band formed following incubation of rat erythrocytes with DIDS was shown to co-elute with MCT1 on ion-exchange fractionation (Q-Sepharose). Thus membranes (from either control or 5 μM DIDS-treated erythrocytes) were stripped of peripheral membrane proteins and solubilized with 1% (w/v) C$_{12}$E$_8$ at a protein concentration of 1 mg/ml, prior to batch purification using Q-Sepharose (10). Nonbound protein was removed by washing with buffer containing 0.5% C$_{12}$E$_8$ and a 0.2 M NaCl eluate was prepared by mixing the washed matrix with an equal volume of buffer containing 0.5% C$_{12}$E$_8$ and 0.4 M NaCl. The eluate was concentrated by centrifugal filtration to 2 ml, and proteins were separated by SDS-PAGE. A broad band at approximately 70 kDa (identified by staining with Coomassie Blue) was electroeluted overnight in buffer containing 20 mM Tris, 2 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, and 0.1% SDS (w/v), pH 8.0, before concentrating to 1.5 ml by centrifugal filtration. C$_{12}$E$_8$ was added to 1% (v/v), and the protein was incubated with N-glycanase F (400 Oxford Glycosystems units/ml) for at least 2 h, and usually overnight. The sample was then subjected to SDS-PAGE, where the deglycosylated binding protein migrated at approximately 40 kDa. Direct N-terminal microsequencing was performed after transfer onto a ProBlot membrane and staining with Serva Blue G (12). Alternatively, the polyaerylamide gel was stained with Coomassie Blue, and the 40-kDa band was excised, electroeluted overnight, and concentrated by centrifugal filtration. After addition of C$_{12}$E$_8$ (1% (v/v) in a final volume of 200 μl), a 50-μl aliquot was incubated over night at 37 °C with 0.2 μg of Lys-C, and the products were separated by SDS-PAGE using a Tricine buffer system able to separate small peptides (14). Peptides were transferred onto ProBlot was performed after transfer onto a ProBlot membrane and staining with Serva Blue G to reveal discrete bands of about 11 and 3.5 kDa. These were cut out for N-terminal microsequencing.

DIDS Causes Cross-linking of MCT1 to a 70-kDa Protein—The data of Fig. 1 show that the 130-kDa band formed following incubation of rat erythrocytes with 100 μM DIDS was recognized by specific anti-MCT1 antibodies. It is of note that this cross-linking is highly specific; normally no other additional bands were detected upon treatment with DIDS. These observations indicate that the cross-linking is probably a reflection of a close association of the two proteins in the erythrocyte membrane, which may in turn be of functional relevance. Since DIDS is membrane impermeant, the binding protein must be either a membrane-spanning protein or an exofacial peripheral protein.

To investigate more fully the cross-linking reaction, we performed the labeling reaction in the absence and presence of various inhibitors of MCT1 activity. DBDS and CHC, two potent inhibitors of lactate transport that also inhibit labeling of MCT1 by DIDS (9, 10), reduced markedly the DIDS-induced cross-linking of MCT1, whereas the poor inhibitor 4,4’-dinitrostilbene-2,2’-disulfonate (DNSD) (9) had little effect (Fig. 2). These results demonstrate that the cross-linking of MCT1 is via a site on the transporter that is either at the substrate binding site, consistent with competitive inhibition of lactate transport by DIDS, or affected by conformational changes induced by substrate or inhibitor binding. In Fig. 3 we show that the cross-linking reaction is dependent upon the nature of the buffer used for the reaction. The reaction was performed in either a citrate buffer, pH 7.4, which induces a large pH gradient (0.6–0.8 pH unit, alkaline inside) or a saline buffer, also at pH 7.4, in which there is very little gradient (approximately 0.2 pH unit, acid inside) (17). It is clear that in citrate buffer the rate of cross-linking of MCT1 is more rapid and the extent is greater. This result might be expected since an alkaline-inside pH gradient would be predicted to cause recruitment of an outward-facing binding site of the transporter, according to the accepted kinetic model for the carrier in which proton binding precedes monocarboxylate binding (1). Thus the empty carrier will preferentially take up the conformation in which the sub-
MCT1 Interaction with GP-70

The Binding Protein Is the Rat Homologue of the Mouse Teratocarcinoma Glycoprotein, GP-70—Membranes from DIDS-treated erythrocytes were treated with N-glycanase F, which resulted in an apparent reduction in molecular mass of the binding protein on SDS-PAGE, to approximately 100 kDa (Fig. 5). Since MCT1 is not glycosylated (6), this must reflect N-linked glycosylation of the binding protein. Antibodies raised against the MCT1-binding protein conjugate, as described under “Experimental Procedures,” recognized two proteins on Western blots of control erythrocyte membranes. These were a weak band at 40 kDa that reacted with antibodies against MCT1 (not shown) and a stronger band at 70 kDa, which is likely to represent the unconjugated binding protein (Fig. 6). In membranes prepared from cells pretreated with 100 μM DIDS, these bands were still detected, but an additional band at 130 kDa was detected that presumably represents the DIDS cross-linked MCT1 conjugate to which the antibody was raised.

We attempted to purify the putative 70-kDa binding protein by replacing DIDS as cross-linker with the cleavable membrane impermeant cross-linking reagent 3,3'-dithiobis(sulfosuccinimidyl)propionate (DTSSP). Preliminary experiments showed that erythrocytes incubated for 1 h at 37 °C with 1 mM DTSSP gave a 130-kDa cross-linked product that reacted with MCT1 antibody on Western blots. Partial purification of this protein was performed on aminoethyl-Sepharose and SDS-PAGE, followed by electroelution of the 130-kDa band as described for the DIDS-labeled conjugate under “Methods.” Cleavage of the eluted protein was achieved by incubation with β-mercaptoethanol (5% v/v) for 45 min, and the cleaved products were separated by SDS-PAGE. A broad band of about 70 kDa was obtained, but N-terminal sequencing showed it to contain more than one polypeptide. Thus we attempted to purify the native binding protein.
Western blotting with the antibody raised against the 130-kDa MCT1-binding protein conjugate showed that the 70-kDa putative binding protein eluted from Q-Sepharose at a similar salt concentration to MCT1 (0.2 M NaCl in batch elution experiments). The eluted protein fraction was further purified by SDS-PAGE, and a broad band around 70 kDa, presumably composed of a number of polypeptides, was electroeluted and then subjected to treatment with N-glycanase F. The 70-kDa band, now free of MCT1, was clearly detected with the antibody, and treatment with N-glycanase F reduced its apparent molecular mass to 42 kDa. Silver staining of these fractions revealed the 42-kDa band to be a major component of the deglycosylated samples as shown in Fig. 7. The core (deglycosylated) protein prepared in this manner was subjected to N-terminal microsequencing, but no sequence was obtained. Thus it was digested with the protease Lys-C, and the resulting peptides were separated by SDS-PAGE before transferring to ProBlot membrane for N-terminal microsequencing. Two peptides were visible of approximately 12 and 3.5 kDa, but only the 3.5-kDa peptide gave a good sequence of 15 amino acids (MG-DTLYNQYRTVFN). This sequence was used in a BLAST search of the protein sequence data bases via the National Center for Biotechnology Information (NCBI) and was found to have a strong identity to an internal sequence of mouse teratocarcinoma glycoprotein GP-70 (SWISS-PROT accession no. P21995) as shown in Fig. 8. This 70-kDa glycoprotein is a cell surface member of the immunoglobulin superfamily that has a developmentally regulated carbohydrate moiety (11). It deglycosylates to yield a core protein with a mobility on SDS-PAGE corresponding to about 40 kDa; the true molecular weight being 37,102. Thus it is probable that our MCT1-binding protein is the rat equivalent of mouse GP-70, or a closely related protein. The identity is further strengthened by the presence of a lysine on the N-terminal side of the methionine of the GP-70 peptide, since our peptide was derived by Lys-C cleavage.

Confirmation that the MCT1-binding protein is the rat equivalent of the mouse GP-70 was obtained by searching the EST data base with the GP-70 nucleotide sequence. Two closely related EST sequences were identified (EMBL accession nos. H32620 and H39274) of which only the latter corresponded to the coding region of mouse GP-70. Fortunately this 276-base pair EST fragment encoded the region of the protein corresponding to the peptide we had sequenced. The translated sequence (in the third reading frame) is shown in Fig. 8 and agrees with our experimentally determined sequence in all but one residue. The incorrect residue was a Ser which we deter-

FIG. 7. The effect of N-glycanase F on the purified MCT1 binding protein. The protein to which MCT1 is cross-linked by DIDS was purified and, when required, incubated with N-glycanase F as described under "Methods." SDS-PAGE was performed on 10% gels that were either stained directly with silver (right tracks) or transferred to an Immobilon P membrane for Western blotting using the anti-MCT1-conjugate protein antibody as a probe.

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FIG. 8. Comparison of some partial amino acid sequence of the MCT1-binding protein with the mouse and rat GP-70 sequences. The top sequence represents amino acids 129–136 of the mouse membrane teratocarcinoma glycoprotein GP-70 (SWISS-PROT accession no. P21995). The middle sequence corresponds to that obtained from N-terminal microsequencing a 3.5-kDa peptide derived from Lys-C digestion of the purified and deglycosylated MCT1-binding protein as described in the text. The K at the N terminus is assumed in view of the specificity of the protease. The lower sequence is derived from translation of a rat EST sequence (EMBL accession no. H32724) that was identified by searching the EST data base with the GP-70 sequence as described in the text.
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could be separated from the 4D4 antigen (1). Thus it is possible that in rabbit erythrocytes it is basigin that fulfills the same function that GP-70 does in rat erythrocytes. However, we have not been able to detect a DIDS cross-linked MCT1-conjugate protein in rabbit erythrocytes (10), which suggests that, if basigin does interact with MCT1, lysines on the two molecules are not sufficiently close to allow cross-linking to occur.

Whether the interaction of GP-70 with MCT1 can regulate its activity remains to be established. An increased expression of OX-47 is observed in several cell types under conditions of metabolic activation that require enhanced glycolysis and are accompanied by an increase in the expression of glucose transporters (22). It is an attractive possibility that OX-47 and GP-70 may be involved in a parallel stimulation of monocarboxylate transport. There is evidence that a similar regulatory mechanism may be involved in the stimulation of neutral amino acid transport by system A in response to hypertonic shock (25). Another possibility might be that GP-70 is involved in the translocation of MCT1 from the endoplasmic reticulum to the Golgi apparatus, a role played by glycophorin in the expression of band 3 at the cell surface (26, 27). These possibilities await further investigation, by co-expression of the two proteins in cells lacking endogenous lactate transporter activity.

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