Predominant expression of the mitochondrial dicarboxylate carrier in white adipose tissue

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We report the identification of a novel mouse protein closely related to the family of mitochondrial uncoupling proteins and the oxoglutarate carrier. The cDNA encodes a protein of 287 amino acids that shares all the hallmark features of the mitochondrial transporter superfamily, including six predicted transmembrane domains. It is nearly identical to the sequence recently reported for the rat mitochondrial dicarboxylate carrier (DIC). We find that murine DIC (mDIC) is expressed at very high levels in mitochondria of white adipocytes and is strongly induced in the course of 3T3-L1 adipogenesis. To determine the consequences of the presence of mDIC on the mitochondrial membrane potential, we transiently expressed mDIC in 293-T cells. Overexpression of mDIC leads to significant mitochondrial hyperpolarization. In addition, exposure to cold down-regulates mDIC levels *in vivo*. In contrast, free fatty acids lead to an up-regulation of mDIC protein in 3T3-L1 adipocytes. This is the first report demonstrating preferential expression in white adipose tissue of any mitochondrial transporter. However, it remains to be determined which metabolic pathways most critically depend on high level expression of mDIC in the adipocyte.

Key words: adipocyte, membrane potential, tissue-specific expression.

INTRODUCTION

There are three major classes of proteins that make up the bulk of the proteins found in the mitochondrial inner membrane. These include (*a*) the electron transport chain complexes, (*b*) the F_1F_0 ATPase complex and (*c*) mitochondrial anion carriers. This latter family includes proteins such as the ADP/ATP carrier and the phosphate carrier that are essential for oxidative phosphorylation. Most other carriers serve a dual role, participating in several different metabolic processes. All members of the superfamily have polypeptide chains of approx. 300 amino acids that consist of three tandem related sequences of about 100 amino acids [1]. The tandem repeats of the different carriers are interrelated and in all likelihood share similar secondary structures. It is also likely that the overall topology of these proteins is conserved. A total of six transmembrane domains are predicted, and many members in the family are found as dimeric complexes, giving rise to a functional transporter with a total of 12 transmembrane domains – highly reminiscent of the signature structure of many transporter proteins, such as the glucose transporters. Both the N- and the C-terminus are exposed to the cytoplasm [2,3].

We are interested in the identification of adipose tissue-specific factors that could be potential targets to interfere with tissue-relevant metabolic processes. Mitochondrial transporters represent such a target and have remained vastly unexploited in adipose tissue. We therefore screened EST sequences in the database that bear homology to the known mitochondrial transporters, in particular UCPs. Potential candidates were analysed for their relative enrichment in white adipose tissue. We report the identification of one such candidate molecule. A full-length version of the protein was isolated from a 3T3-L1 adipocyte library and specific antibodies were raised. In the course of this work, Fiermonte and colleagues [4] also reported the molecular cloning of a novel member of the mitochondrial transporter family and determined that the recombinant, reconstituted protein encoded by the newly isolated cDNA corresponds to the mitochondrial dicarboxylate carrier, a protein well characterized at the biochemical level in liver mitochondria. However, due to its very low abundance, the cDNA for this activity had not yet been reported. Our cDNA isolated from mouse adipocytes corresponds to the sequence reported by Fiermonte and colleagues. While these authors primarily subjected the newly identified protein to an in-depth biophysical characterization, we find that mDIC is expressed at very high levels in adipose tissue. Potential implications of these high expression levels in adipose tissue are discussed.

MATERIALS AND METHODS

Materials

DME (Dulbecco's modified Eagle's Medium) was purchased from Cellgro Inc.; Digoxigenin reagents for screening were purchased from Boehringer Inc.; [α-^{32}P]dCTP was purchased from New England Nuclear. 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$), valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma. All other chemicals, unless indicated, were purchased from Fisher.

Cell culture

3T3-L1 mouse fibroblasts (a generous gift of Dr. Charles Rubin, Department of Molecular Pharmacology, Albert Einstein College of Medicine) were propagated and differentiated according to the

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; EST, expressed sequence tag; HSP60, heat shock protein 60; mDIC, mouse mitochondrial dicarboxylate carrier; (P_iC), phosphate carrier; UCP, uncoupling protein.

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protocol described in [20]. In brief, the cells were propagated in 'FCS' [DME containing 10% fetal calf serum (JRH Biosciences) and penicillin/streptomycin (100 units/ml each)] and allowed to reach confluence (day −2). After 2 days (day 0), the medium was changed to 'DM1' (containing 'FCS' and 160 nM insulin, 250 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine). Two days later (day 2), the medium was switched to 'DM2' ('FCS' containing 160 nM insulin). After another 2 days, the cells were switched back to 'FCS'.

Antibodies

To raise a polyclonal antibody against mDIC, the carboxy-terminal 12 amino acids (-CLRKHFGIKVPTT) were coupled to BSA via a unique carboxy-terminal cysteine residue in the peptide and injected into rabbits according to standard protocols. Hsp60 antibodies were purchased from Stressgen. Anti-CD4 antibodies coupled to phycoerythrin were purchased from Becton–Dickinson.

Animal treatment

For cold exposure experiments, 5-month-old female F1 mice from a CBA × C57Bl/6 cross were kept for 5 h either at room temperature or at 4 °C without food. Each group contained five animals. At the end of the experiment, animals were killed by cervical dislocation and periovarian fat pads were dissected and immediately snap frozen in liquid nitrogen.

Transfections and measurement of mitochondrial membrane potential *in vivo*

293-T cells were transfected by the calcium phosphate method with various cDNAs. To facilitate FACS analysis, a plasmid containing a truncated form of CD4 (pMACS4; Miltenyi Biotec, Auburn, CA, U.S.A.) was co-transfected at one fifth the stoichiometric amount of the target DNA. Cells were harvested 48 h post transfection, washed in PBS containing 5 mM EDTA but lacking K^+ (PBE), resuspended in PBE containing 1 mg/ml BSA (PBS–BSA) and exposed to phycoerythrin-labelled monoclonal anti-CD4 antibody. Cells were incubated for 15 min on ice, washed once in PBS–BSA and resuspended in PBS–BSA containing 1 μM $DiOC_6$ and where indicated with 75 μM CCCP or 10 μM valinomycin. Cells were immediately analysed by flow cytometry on a FACscan (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.). Dual fluorescence compensation was performed using prepared samples of CD4 only and $DiOC_6$ only samples. Live cells were selected for analysis by (forward scatter vs. side scatter) light scatter gating. CD4 positive cells and CD4 negative cells were derived by gating positive and negative cells on a forward scatter vs. CD4 fluorescence dot plot. Cells satisfying both the live light scatter and CD4 criteria gates were displayed on a green $DiOC_6$ fluorescence intensity distribution.

Total RNA isolation and Northern blot analysis

Isolation of mRNA from tissues and from 3T3-L1 cells at various stages of differentiation was performed as described in [21,22], as were ^{32}P labelling of DNA, agarose gel electrophoresis of mRNA and its transfer to nylon membranes.

Cloning of mDIC and DNA analysis

A full-length cDNA library generated with mRNA from 3T3-L1 adipocytes at day 8 of differentiation [22] was screened with a digoxygenin-labelled cDNA fragment obtained from EST clone W50987. Labelling, hybridization and detection were performed according to the manufacturer's instructions (Boehringer Inc.). Several of the positive clones obtained were subjected to automated sequencing. The entire 2.0 kb insert was sequenced at least two independent times on one and once on the complementary strand. The sequence has been submitted to Genbank and has accession number AF188712. Homology searches were performed at NCBI using the BLAST network service.

Immunoblotting

After SDS/PAGE, proteins were transferred to BA83 nitrocellulose (Schleicher & Schuell). Nitrocellulose membranes were blocked in PBS or TBS with 0.1% Tween-20 and 5% non-fat dry milk. Primary and secondary antibodies were diluted in the same buffers. Bound antibodies were detected by enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL, U.S.A.).

Subcellular fractionation of 293-T cells expressing mDIC

Subcellular fractionation of cells was performed as detailed in [23] with minor modifications. In brief, cells were scraped into 250 mM sucrose, 10 mM Tris, pH 7.4, 0.5 mM EDTA, homogenized and subjected to differential centrifugation at 500 ***g*** (5 min), 5000 ***g*** (15 min) and 100000 ***g*** (30 min). Nuclear, mitochondrial and cytosolic fractions were analysed by Western blot analysis for the presence of mDIC.

Preparation of cell lysates from adipocytes and 3T3-L1 cells during adipocyte differentiation

To determine protein levels at different stages of adipogenesis, 3T3-L1 cells at 2-day intervals of differentiation were washed twice with cold PBS, lysed in boiling 2 × SDS/PAGE sample buffer, and boiled again for another 3–5 min. Samples were then mildly sonicated to reduce viscosity. Sample loads from each time point were adjusted to give equal intensity on membranes by Ponceau S staining after SDS/PAGE and Western blotting onto nitrocellulose. Equal loading was confirmed by Western blotting for the constitutively expressed mitochondrial hsp60 marker. To isolate protein from adipose tissue, approx. 0.5 g of fat was incubated in 2 ml of PBS containing protease inhibitors, homogenized on ice in a loose-fitting Dounce homogenizer and centrifuged at 1000 ***g*** in a table top centrifuge. The fat cake was then removed by suction, and protein determination was performed with a BCA assay (Pierce) on the infranatant. Equal amounts of protein were used for Western blot analysis.

Two-dimensional gel analysis

Isoelectric focusing (IEF) followed by SDS/PAGE was performed as previously described in [24] on a Hoefer GT-1 tube gel apparatus.

Other methods

Separation of proteins by SDS/PAGE, fluorography and immunoblotting were performed as described previously [25]. Automated DNA sequencing was performed by the custom DNA sequencing facilities at Research Genetics and by the Einstein DNA Sequencing facility. Quantitation of Northern and Western blot data was performed on an Alpha Innotech ChemiImager4000 using the AlphaEase software package.

RESULTS

Identification and sequence of a protein with high homology to the family of uncoupling proteins

An Expressed Sequence Tag (EST) database search with the UCP1 coding sequence reveals a large number of clones that bear limited homology to members of the mitochondrial transporter family. We subsequently focused on a database entry (EST clone W50987) that was most closely related to the Uncoupling Protein family. We screened a 3T3-L1 adipocyte library using the 5′ end of clone W50987 and isolated several full-length clones. Figure 1 shows the cDNA sequence and the translation of the corresponding open reading frame. The size of the reported clone (2 kb) is in agreement with the size of the mRNA observed by Northern blot analysis (see Figure 2) within the resolution of our gels. The clone has an in-frame stop codon upstream of the starting ATG and polyA$^+$ tail at the 3′ end. We predict a protein of 287 amino acids that displays high homology to many mitochondrial transporter proteins. In particular, the sequence is nearly identical to the sequence of the rat dicarboxylate carrier that was recently reported [4]. We therefore refer to this clone as the mouse dicarboxylate carrier (mDIC). mDIC bears all the

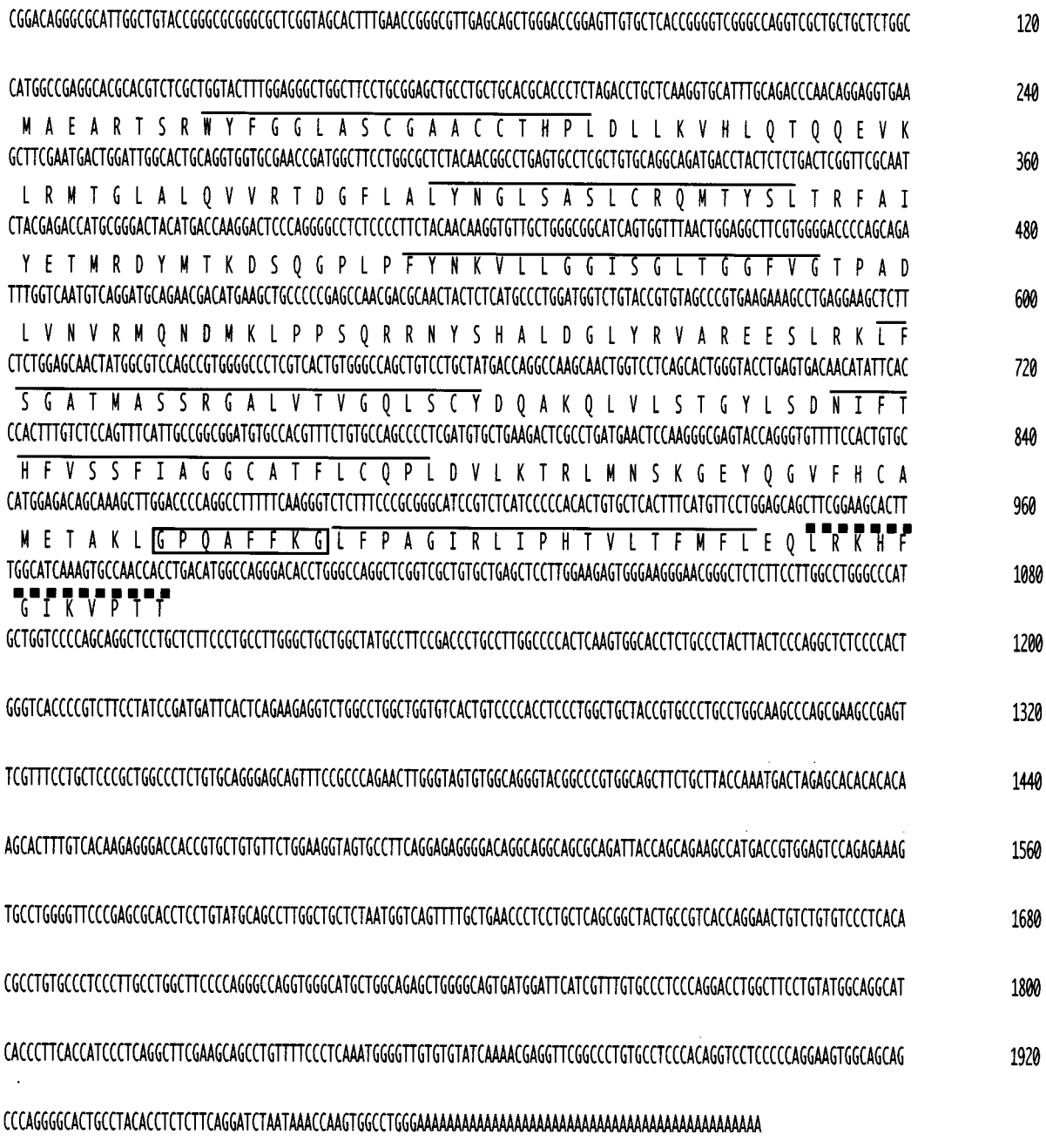

Figure 1 Nucleotide and predicted amino acid sequences of mDIC

The cDNA was cloned from a mouse 3T3-L1 adipocyte library. The carboxy-terminal epitope used to generate an anti-peptide antibody is marked with a dashed line, putative transmembrane domains are marked with a solid line and the predicted nucleotide binding domain is boxed.

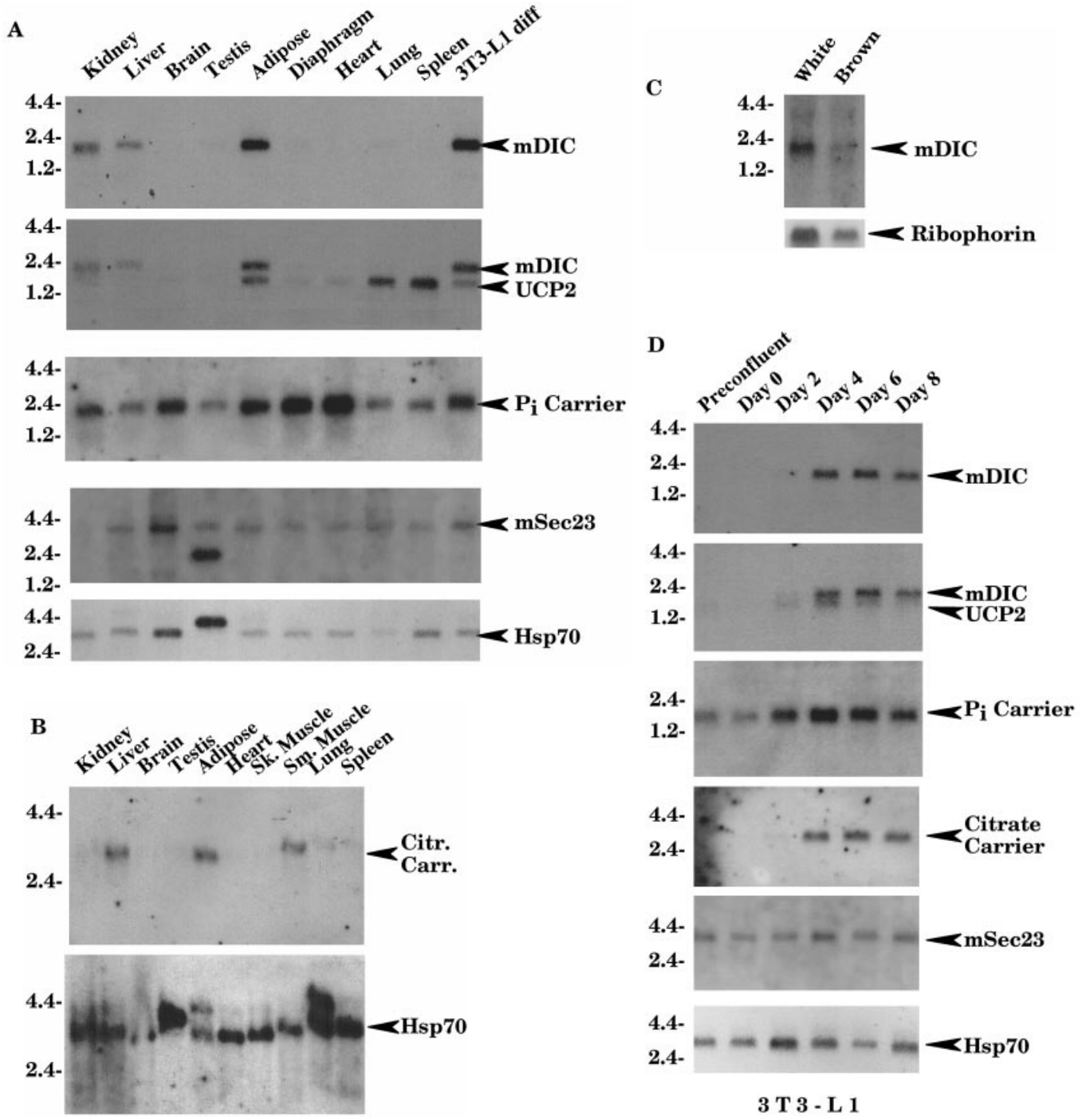

Figure 2 Northern blot analysis

(**A**, **B**) Tissue Northern blot analysis was performed sequentially with fragments encoding the full-length open reading frames of mDIC, UCP2, P_i carrier, mSec23 and hsp70. For the citrate carrier, a fragment comprising position 59–584 of the open reading frame was used. All samples contain 0.5 μg of mRNA and equal loading was confirmed by blotting with the cDNA for the constitutive markers mSec23 and hsp70. The last lane contains an equivalent amount of mRNA isolated from 3T3-L1 adipocytes. Importantly, the Northern blot was not stripped between the mDIC and UCP2 hybridizations, such that the signal for mDIC in the second panel is still present from the first hybridization. (**C**) mRNA levels in white and brown adipose tissue were compared. A blot containing 0.5 μg of the respective mRNAs was probed with a fragment comprising the open reading frame of mDIC. As a control, a probe for ribophorin was used. (**D**) Northern blot analysis on differentiating 3T3-L1 cells was performed sequentially with fragments in A and B. All samples contain 0.5 μg of mRNA and equal loading was confirmed by blotting with the cDNA for the constitutive markers mSec23 and hsp70. Importantly, the Northern blot was not stripped between the hybridization with the mDIC and UCP2 probes, such that the signal for mDIC in the second panel is still present from the first hybridization.

hallmarks of a mitochondrial carrier protein. It has six predicted transmembrane domains (overlined) and has an apparently intact nucleotide binding domain (boxed). The carboxy-terminal epitope used to generate the polyclonal antibody used in this study is indicated with a dashed line. Within the mitochondrial transporter family, it is most closely related to the uncoupling proteins and the oxoglutarate carriers.

Northern blot analysis of mDIC mRNA

We have used EST clone W50987 for Northern blot analysis to determine its tissue distribution. Figure 2A shows that the mRNA of approx. 2 kb is predominantly expressed in white adipose tissue. Low levels of mRNA are detected in kidney and liver (top panel). In contrast to the dicarboxylate carrier, UCP2 (the more ubiquitously expressed uncoupling protein homologue) shows an overlapping but rather distinct distribution. While UCP2 is also present in white adipose tissue, it can be found in many other tissues with predominant expression in spleen and lung. A trivial explanation for the strong induction of mDIC in the course of differentiation and the enrichment in adipose tissue could be that all mitochondrial proteins are induced during adipogenesis. To address this issue, we re-probed the same Northern blots with another member of the mitochondrial carrier family [the phosphate carrier (P_iC)] that should be a more accurate indicator of the overall level of mitochondrial proteins. This protein translocates phosphate across the inner mitochondrial membrane to meet the requirement of oxidative phosphorylation for inorganic phosphate. As expected, the phosphate carrier displays a relatively ubiquitous distribution with enrichment in tissues known to contain high levels of mitochondria, such as muscle tissue. White adipose tissue does not express disproportionate amounts of phosphate carrier, suggesting that the relative enrichment we observe for the dicarboxylate carrier truly reflects tissue-specific enrichment. The two bottom panels show two hybridizations with probes encoding the constitutively expressed proteins mSec23 and Hsp70 to assess differences in loading of the mRNA.

To determine the expression levels of a mitochondrial transporter that is predicted to be involved in providing metabolites for fatty acid biosynthesis, we probed a Northern blot with a fragment encoding the mitochondrial citrate carrier (Figure 2B). In contrast to the pattern observed for mDIC, we find equally high level expression in liver and adipose tissue. In addition, smooth muscle cells express significant levels of the citrate carrier.

In parallel, a Northern blot containing mRNA from white and brown adipose tissue was probed (Figure 2C). mDIC is preferentially expressed in white adipocytes and to a much smaller extent in brown adipose tissue.

Usually, such enrichment in white adipose tissue reflects a dramatic increase in expression levels in the course of differentiation of a tissue culture model for adipogenesis, such as 3T3-L1 cells. This cell line can be propagated indefinitely in the form of fibroblasts. To induce differentiation, cells are allowed to reach confluence. Cells are subsequently treated with a hormonal cocktail that triggers the differentiation process. Most adipocyte-specific markers are induced between day 2 and day 4 of differentiation. In the case of mDIC, we can indeed observe a very strong induction on day 4 (Figure 2D; top panel). Surprisingly, UCP2 expression peaks on day 4 and then decreases in the course of maturation (second panel). In analogy to Figure 2A, we have blotted sequentially with mDIC and UCP2 probes, such that both signals are seen in the second panel. Equal loading was confirmed with two constitutive markers (mSec23 and Hsp70).

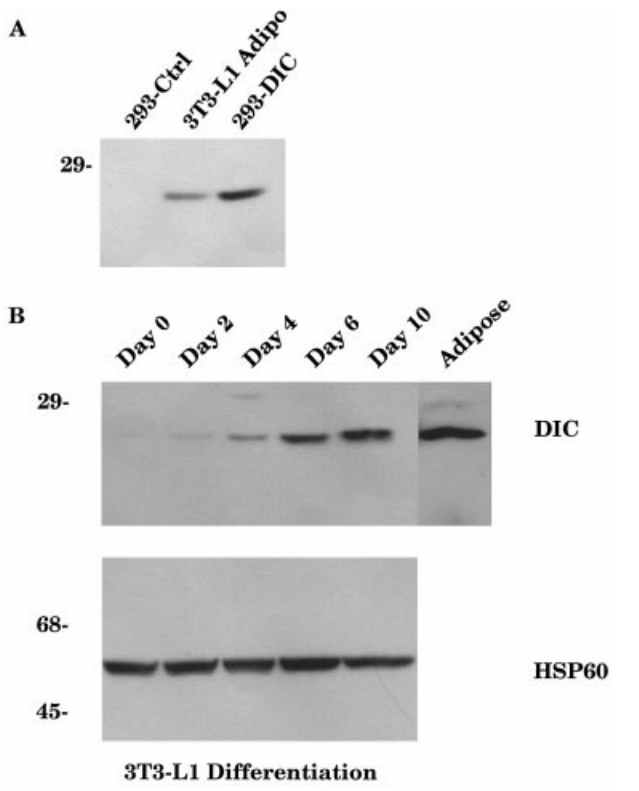

Figure 3 Western blot analysis

(**A**) The cDNA clone isolated from the 3T3-L1 library was transiently transfected into 293-T cells. 48 h post transfection, cells were lysed in SDS–PAGE sample buffer and briefly sonicated. A day 8 3T3-L1 adipocyte lysate was prepared in parallel. 50 μg of all cell lysates were analysed by Western blotting for the presence of mDIC. Note that the translation product from the cDNA construct isolated gives rise to a protein that co-migrates with endogenously expressed mDIC in adipocytes. No other crossreacting bands were observed. (**B**) 50 μg of total protein lysate from 3T3-L1 cells at different stages of differentiation and from mouse adipocytes were analysed by SDS–PAGE and Western blot analysis with anti-mDIC and anti-Hsp60 antibodies. mDIC is strongly induced in the course of adipogenesis. No other crossreacting bands were observed.

We also probed for the levels of P_iC during differentiation. While the signal peaks around day 4, it levels off towards day 8 to the point where we find only two- to three-fold higher levels of P_iC mRNA at day 8 compared with day 0. Interestingly, we also observed a peak for UCP2 around day 4, suggesting that UCP2 levels follow the generalized expression pattern of all mitochondrial proteins under the standard conditions employed for 3T3-L1 differentiation. Finally, we have also tested the expression pattern of the citrate carrier during differentiation and found that this carrier displays an induction pattern similar to that observed for mDIC during differentiation.

mDIC expression at the protein level

In the light of our finding that the mDIC mRNA levels are very high in adipose tissue, we generated an antibody that specifically recognizes mDIC. The carboxy-terminal 12 amino acids served as an epitope. The antiserum produced recognizes a band of approx. 28 kDa in 3T3-L1 adipocytes. Importantly, upon transient transfection of the mDIC cDNA into 293-T cells, a protein of identical electrophoretic mobility is produced as in 3T3-L1 adipocytes, indicating that the cDNA encodes the full-length version of the protein (Figure 3A).

In agreement with the induction observed at the mRNA level,

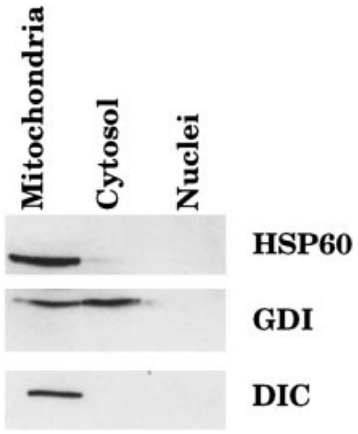

Figure 4 Subcellular distribution of mDIC

50 μg of total protein lysate of the indicated subcellular fractions isolated from 293-T cells transfected with the mDIC cDNA were analysed by Western blotting for the presence of mDIC and Hsp60.

Figure 3B demonstrates a similar induction at the protein level. Cell extracts were prepared at different stages of 3T3-L1 differentiation and from adipose tissue and analysed by immunoblot analysis for the presence of mDIC (top panel) and the mitochondrial matrix protein Hsp60 (bottom panel). In agreement with our observations at the mRNA level, mDIC is strongly induced at the protein level as well. In contrast, the levels of the mitochondrial matrix marker Hsp60 do not significantly increase.

We have subjected extracts from 3T3-L1 cells to two-dimensional gel analysis. Western blotting with anti-mDIC antibodies reveals a single spot, suggesting a uniform population of mDIC transporters within adipocytes (data not shown). We are unaware of any reports demonstrating post-translational modifications of any members of the mitochondrial transporter family. The uniformity of the signal observed for mDIC is consistent with the absence of such post-translational modifications.

Subcellular distribution of mDIC

To confirm mitochondrial localization of mDIC, a crude subcellular fractionation was performed on 293-T cells stably transfected with mDIC cDNA (Figure 4). As expected, mDIC indeed co-fractionates with the mitochondrial marker Hsp60 and is not found to any significant extent in the cytosol and nuclear fractions.

mDIC overexpression leads to hyperpolarization of mitochondria

To address the functional relevance of mDIC expression in cells, we transiently transfected the cDNA for mDIC into 293-T cells. A substoichiometric amount of a truncated CD4 cDNA was also co-transfected. 48 h post transfection, the cells were stained with a fluorescently labelled anti-CD4 antibody to identify transfected cells. $DiOC_6$ (a potential-sensitive dye that allows qualitative assessment of the mitochondrial membrane potential *in vivo*) was added to the cells and the relative intensity of the $DiOC_6$ signal within the CD4-positive population of cells was determined by FACS analysis. As shown in Figure 5, expression of mDIC leads to a marked hyperpolarization of mitochondria in comparison with control cells transfected with the expression vector lacking the mDIC cDNA insert. To establish the amplitude of the $DiOC_6$ signal, control cells were either treated with valinomycin (leading to a marked hyperpolarization of mitochondria in intact cells resuspended in PBS) or treated with CCCP, a chemical uncoupler of the mitochondrial membrane potential. As predicted, valinomycin leads to increased intensity of the $DiOC_6$ signal as indicated by a shift to the right, whereas a complete collapse of the mitochondrial membrane potential by CCCP treatment leads to a shift of the $DiOC_6$ signal to the left. The marked mitochondrial hyperpolarization observed with valinomycin is caused by loss of intracellular K^+ to the extracellular medium (lacking K^+) due to the action of valinomycin at the level of the plasma membrane. In parallel, valinomycin incorporated into the inner mitochondrial membrane will cause a net efflux of K^+ from the matrix into the cytoplasm, leading to a further net increase of the membrane potential [5].

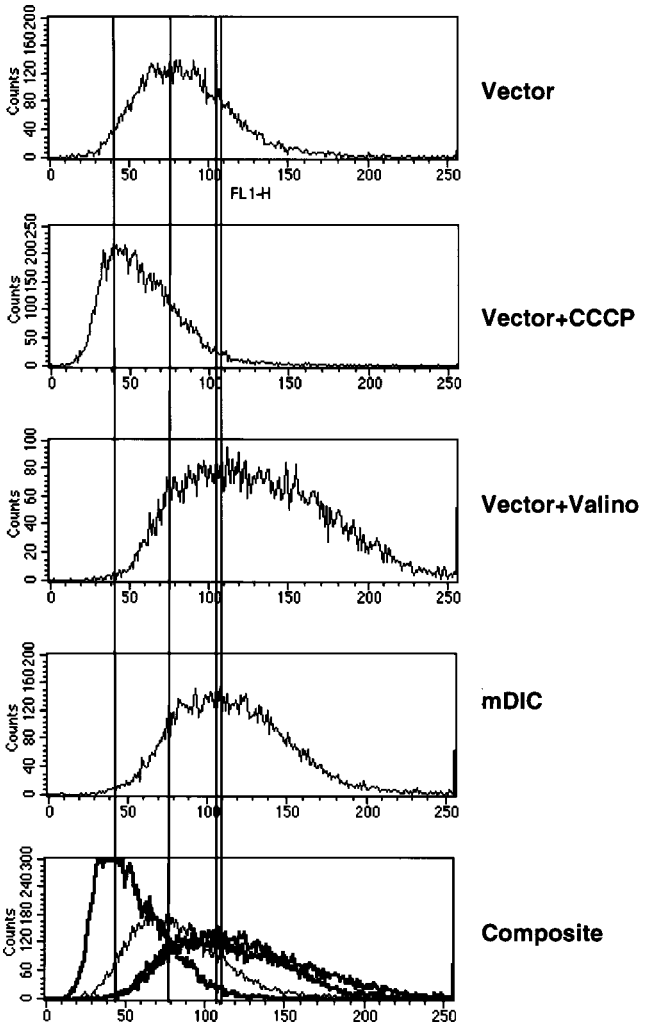

Figure 5 mDIC overexpression leads to mitochondrial hyperpolarization

The cDNA for mDIC in vector pcDNAI was transiently transfected into 293 cells. A substoichiometric amount of a truncated CD4 cDNA was also co-transfected as described in the Materials and methods section. 48 h post transfection, the cells were stained with a fluorescently labelled anti-CD4 antibody to identify transfected cells. $DiOC_6$ was added to the cells and the relative intensity of the $DiOC_6$ signal within the CD4-positive population of cells was then determined by FACS analysis. Expression of mDIC leads to a marked hyperpolarization of the mitochondria in comparison with control cells transfected with the same expression vector lacking the mDIC cDNA insert. When indicated, cells were either treated with valinomycin or treated with CCCP. A superposition of the top four panels is shown in the bottom panel.

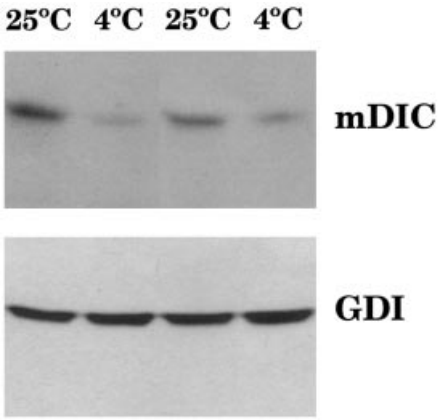

Figure 6 Cold exposure reduces mDIC protein levels

A group of five mice and the equivalent number of control mice were exposed for 5 h either to 4 °C or to 25 °C. Animals were then killed, periovarian adipose tissue was collected and snap frozen. Total protein from adipocytes was isolated, and equal amounts of cell extracts were analysed by Western blot analysis with anti-mDIC antibodies and with anti-GDI antibodies (as a control for equal protein loading). Two representative extracts are shown for each temperature. Quantitation of cold-induced down-regulation of mDIC by densitometric scanning of the signals obtained indicates a reduction by $48 \pm 5\%$ difference vs. control value ($P < 0.05$).

Cold exposure down-regulates mDIC levels *in vivo*

Previous studies have demonstrated that chronic cold exposure activates the sympathetic nervous system, increases energy expenditure, improves glucose uptake in peripheral tissues [brown (BAT) and white adipose tissues (WAT) and muscles]. It does so by stimulating facultative thermogenesis, mitochondriogenesis and glucose utilization in BAT and WAT and lowering systemic insulin levels [6]. Under these conditions of increased energy demand, it is physiologically not desirable either to esterify free fatty acids into triglycerides or to sustain high levels of fatty acid biosynthesis. One of the potential roles of mDIC could be an involvement in glyceroneogenesis, generating the backbone required for the esterification of free fatty acids. Alternatively, mDIC could directly participate in lipogenesis providing substrates for fatty acid biosynthesis. We therefore tested the expression levels of mDIC at the protein level upon cold exposure for 5 h. Figure 6 shows that there is a significant reduction of approx. 50 % of mDIC protein expression upon cold exposure in a group of five mice when compared with mice from a control group. The specific repression of mDIC in white adipose tissue under these conditions correlates with the decreased need for glyceroneogenesis/biosynthesis of free fatty acids. Cold adaptation generally results in an upregulation of mitochondrial function and protein content in brown adipose tissue [7]. UCP1, the protein primarily responsible for increased thermogenesis, shows a 10-fold increase in expression levels in brown adipose tissue under conditions similar to the ones employed in our experiment [8].

Insulin down-regulates mDIC, whereas free fatty acids induce the expression of mDIC in adipocytes

To test whether expression levels of mDIC are subject to regulation by insulin, we exposed 3T3-L1 adipocytes for various times to insulin. Figure 7A determines the effects of an overnight treatment with insulin on mDIC mRNA levels. Insulin causes a rather dramatic suppression of mDIC mRNA levels (approx. 80 % reduction) that is even more pronounced than the well-

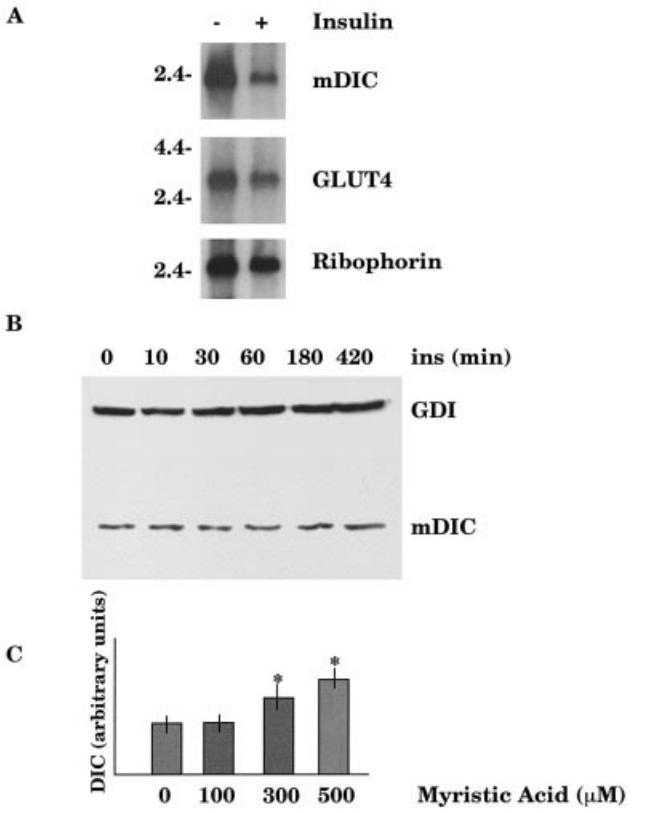

Figure 7 Effects of treatment of 3T3-L1 adipocytes (A) with insulin on mDIC mRNA levels, (B) with insulin on mDIC protein levels, (C) with myristic acid on mDIC protein levels

(**A**) 3T3-L1 adipocytes were treated o/n in serum free medium containing 0.75% BSA (w/v) either in presence or absence of 160 nM insulin. Poly(A^+) RNA was subsequently isolated and analysed by Northern blot analysis with probes for mouse ribophorin, mouse GLUT4 and mouse DIC. (**B**) 3T3-L1 adipocytes were serum starved o/n in DME containing 0.75% BSA (w/v) and were then treated for the indicated amount of time with 160 nM insulin. Cells were washed with PBS and lysed in 3 × Laemmli Buffer (6% SDS, 0.5 M Tris), boiled for 5 min and sonicated. Samples were analysed by SDS/PAGE and Western blot analysis with anti-mDIC and anti-GDI. (**C**) 3T3-L1 adipocytes were treated o/n in serum free medium lacking glucose containing 1% BSA (essentially fatty acid free). The next morning, the indicated amount of myristic acid was added. After 6 h, cells were lysed and analysed as indicated in (**B**). The results show an increase of $58 \pm 5\%$ difference vs. control value ($P < 0.05$).

described effects of insulin on the mRNA levels of the insulin-responsive glucose transporter GLUT4 (approx. 50 % under our conditions) [9]. Under the same conditions, mRNA levels of a control protein (ribophorin) are not significantly affected. These changes, however, are not reflected at the protein level (Figure 7B). Under similar conditions, we did not observe a significant decrease of mDIC protein, suggesting that mDIC has a rather long half-life at the protein level. Since mDIC levels are quite efficiently down-regulated *in vivo* within 5 h (Figure 6), the existence of additional factors that are playing a regulatory role *in vivo* leading to higher turnover rates has to be postulated.

In contrast, incubation of 3T3-L1 adipocytes with low levels of free fatty acid (myristate) leads to an induction of mDIC at the protein level (Figure 7C), consistent with a potential role of mDIC in triacylglycerol biosynthesis. When glucose is available to adipocytes, the endogenous synthesis of free fatty acids is high. Under these conditions, the effect that exogenous free fatty acids exert on gene expression is blunted. Therefore, this experiment was performed in absence of glucose. Cells were kept

for 12 h in glucose-free DME. They were subsequently exposed to increasing levels of myristic acid supplied in DME supplemented with 1% BSA (essentially fatty acid free) for 6 h. We observed an increase of mDIC protein of $\approx 60\%$ above basal levels, suggesting that long chain amino acids lead to an induction of mDIC production.

DISCUSSION

mDIC is a novel protein that belongs to the superfamily of mitochondrial transporters and is closely related to the uncoupling proteins and the oxoglutarate carrier. We show here that this protein is highly abundant in adipose tissue. Its transient expression in 293 cells leads to a dramatic hyperpolarization of mitochondria, and its expression levels are regulated by cold exposure, insulin and free fatty acids.

Biochemically, dicarboxylate transport activity has been well characterized for many years and inhibitors of it have been described in the past [10–13]. It preferentially translocates malonate, malate and succinate in exchange for phosphate, sulphate, sulphite or thiosulphate [10,14]. Fiermonte and colleagues [4] have identified a cDNA that, upon recombinant expression in bacteria and reconstitution in liposomes, mirrors the substrate specificity of the purified mitochondrial dicarboxylate transport activity. Given the nearly identical sequences of the cDNA reported here and that reported by Fiermonte and colleagues, it seems very likely that the cDNA described in this paper indeed encodes the dicarboxylate carrier. Conventionally, DIC is thought to be involved in gluconeogenesis and ureogenesis. In agreement with that, DIC is expressed in the liver and in kidney (this paper and [4]). However, neither gluconeogenesis nor the biosynthesis of urea are particularly prominent pathways in adipocytes. Another likely explanation for its strong presence in adipose tissue could be the involvement in fatty acid biosynthesis. This could be rationalized by the co-operative action of the citrate, dicarboxylate and phosphate carriers. The citrate carrier mediates efflux of citrate from the mitochondrial matrix into the cytoplasm in exchange for malate, an essential step for fatty acid biosynthesis. mDIC could then mediate the exchange of malate with phosphate, and the phosphate carrier would then mediate the exchange of phosphate with a hydroxyl ion, resulting in a net transport of citrate to the cytoplasm. In agreement with the increased potential for fatty acid biosynthesis during differentiation, other key lipogenic enzymes, such as pyruvate carboxylase (providing acetyl-CoA and NADPH for the *de novo* biosynthesis of fatty acids) are dramatically upregulated during 3T3-L1 differentiation [15].

Alternatively, mDIC could also be involved in glyceroneogenesis providing the necessary backbone for the esterification reaction of the free fatty acids to triglycerides. Phosphoenolpyruvate carboxykinase (PEPCK) is abundantly expressed in adipocytes [16], where its major role is its involvement in glyceroneogenesis, catalysing the conversion of oxaloacetate to phosphoenolpyruvate. This allows fatty acid reesterification in order to restrain fatty acid output during lipolysis [17]. Similarly to our observations for mDIC, PEPCK expression is stimulated by long chain fatty acids in adipose tissue [18].

It will be of great interest to characterize the factors that lead to increased turnover rates of mDIC protein *in vivo* upon cold exposure. Generally, little is known about post-translational regulation of mitochondrial transporter activity. While some metabolites (e.g. fatty acids and purine nucleotides in the case of uncoupling proteins) can directly affect transporter activity, increased turnover rates in response to metabolic changes offer another attractive possibility for cells to modulate transporter activity across the inner mitochondrial membrane. The up- and down-regulation of these mitochondrial transporters directly determines the availability of metabolites in the cytoplasm and in the mitochondrial matrix and, as a consequence, has a profound impact on the pathways that depend on the presence of these metabolites. A recent report by Sivitz and colleagues [19] looked at mRNA and protein levels of UCP-1, -2 and -3 in a number of different tissues in response to leptin injections and fasting. They came to the conclusion that the mRNA and protein levels for the three UCP isoforms are regulated in tissue- and subtype-specific fashion by leptin and food restriction. Importantly, under some of the experimental conditions, the effects of these perturbations on UCP mRNA and protein levels differ. This corroborates our observation that changes in mRNA levels of mitochondrial transporters, in particular mDIC, do not necessarily have to reflect changes in protein level, at least at the time scale of a few hours.

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