12

CONFOCAL LASER SCANNING MICROSCOPY OF HUMAN SKIN FIBROBLASTS SHOWING TRANSIENT EXPRESSION OF A GREEN FLUORESCENT CARNITINE PALMITOYLTRANSFERASE 1 FUSION PROTEIN

F. R. van der Leij*, H. Roelofsen[1], K. E. Niezen-Koning, E. A. A. Nollen[2], and J. R. G. Kuipers

Department of Pediatrics
[1]Department of Internal Medicine
[2]Department of Radiobiology
Groningen Utrecht Institute for Drug Exploration
Groningen University, Groningen
The Netherlands

The mitochondrial outer membrane enzyme carnitine palmitoyltransferase 1 (CPT1) is a main site of regulation of intracellular long-chain fatty acid transport. At least two isoforms of CPT1 are expressed in the body: L-CPT1 (the "liver-type" isoform) and M-CPT1 (the "muscle-type" isoform).[1] Skin fibroblasts from healthy humans are known to contain only one isoform of CPT1: the liver-type, which is encoded by the gene *CPT1A.* Skin fibroblasts from patients with a liver-type CPT1 deficiency do not express either of the two known CPT1 isoforms (neither liver- nor muscle-type), and therefore could provide an excellent background to study CPT1 by means of molecular complementation.

In this chapter, we describe the first experiments we carried out with a gene fusion of a complementary DNA of the human gene for muscle-type carnitine palmitoyltransferase (*CPT1B*)[2] and a gene encoding an "enhanced" green fluorescent protein (*GFP*).[3]

We wished to express the human *CPT1B* gene in human skin fibroblasts, taking the following facts into consideration:

* Corresponding author at the Pediatrics Research Lab. CMCV 2nd floor, Groningen University Hospital, POBox 30001 NL-9700 RB Groningen, The Netherlands, Tel: (31) 50 363 2787, (31) 50 361 3251; FAX: (31) 50 361 1746; E-mail: F.R.VAN.DER.LEY@MED.RUG.NL

Current Views of Fatty Acid Oxidation and Ketogenesis: From Organelles to Point Mutations edited by Quant and Eaton, Kluwer Academic / Plenum Publishers, New York, 1999.

(1) *CPT1B* is normally not expressed in skin fibroblasts, therefore the *CPT1B* promoter itself would not be suitable for efficient expression of a *CPT1B* transgene in these cells. A constitutive or skin fibroblast-specific promoter is needed to express *CPT1B,* for that reason we use the human cytomegalovirus immediate early (CMV-IE) promoter, a broadly used strong promoter for transgene expression in mammalian cells.
(2) Human skin fibroblasts are generally regarded as recalcitrant to transfection. Various transformation procedures have been applied by different groups,[4,5,6,7] each claiming different levels of relative and absolute success. We wished to transiently express CPT1-GFP in order to get an impression of what would be possible with regard to transfection efficiencies, in our hands. If promising efficiencies could be obtained, an optimised protocol may allow functional studies.
(3) As human skin fibroblasts are recalcitrant to transfection, we wished to include an easily traceable marker, which can either be used as an in-frame fusion protein, or as a separately expressed protein. The widely applicable green fluorescent protein (GFP) reporter system, originally isolated from the jellyfish *Aequorea victoria,* and adapted for efficient expression in human cells and for, elevated levels of fluorescence,[3,8] provides these properties. The GFP system has been used for example, to study protein trafficking to the mitochondrial matrix[9] and the mitochondrial outer membrane.[10] Therefore, by using confocal laser scanning imaging we may get an impression of the intracellular localization of a CPT1-GFP fusion protein after transient or stable transfection.
(4) Since we aim to study CPT1 deficiencies in a conditional knockout mouse model (see elsewhere in this volume), the application of a fused marker protein in gene alteration approaches would serve a long-term goal. Applications like the monitoring of gene expression before (knock-in) and after a conditionally mediated knockout event would provide important tools in gene function analyses. Using the human gene for M-CPT1, experiments with fusions to GFP may provide important data to be applied in gene-targeting of the murine *CPT1* genes.
(5) Once the localisation of mitochondrial enzymes in a two- or three-dimensional perspective is studied, it is important to realise what may be expected. In textbooks, schematic representations of the three-dimensional shape of mitochondria are often globular structures which resemble flattened balls rather than flexible tubes (see e.g. reference,[11] page 487). It should be stressed however, that in real life mitochondria may "line up" or may be tubular shaped and have a thread-like, sometimes branched, appearance, as shown in reference,[11] on page 483 and 485.

A nearly full length coding region for human muscle-type CPT1, based on a complementary DNA sequence of the *CPT1B* gene[2] was fused to enhanced *GFP* in the mammalian expression vector pEGFP-N2 (Clontech, Palo Alto, CA, USA). Since the CPT1 C-terminal part is expected to be a much larger domain than its N-terminal counterpart relative to the transmembrane domains,[1,12] we chose to fuse GFP to the C-terminus of CPT1 as we aimed to achieve optimal freedom of the GFP domain to reach its fluorescent state. In the resulting construct the CMV-IE promoter drives the expression of the fused genes, and the transcript encodes CPT1 as the N-terminal domain, with GFP at the C-terminus. The plasmid provides a *neo* gene, driven by two promoters for selection in bacteria and mammalian cells.

We used two protocols for skin fibroblast transfection: one commercially available preparation (Lipofectamine, Life Technologies, Paisley, Scotland) was applied according to the manufacturers instructions, and the other protocol was according to the calcium precipitation method,[13] with slight adaptations.[14] The former protocol is used in our laboratories for transient expression in hamster lung fibroblasts, and the latter for stable transfection of hamster lung fibroblasts and both transient- and stable transfection of the human cervix epithelial carcinoma cell line HeLa. The selective dose of G418 (Geneticin, Life Technologies) for human skin fibroblasts was determined in a pilot experiment. Geneticin selection was applied at 250 µg/ml after calcium phosphate transfection. In the Lipofectamine transfection experiments the cells were split into one half of the population at which G418 selection was applied, and one half which was allowed to attach to coverslips for transient expression assessment without G418 selection.

Images were taken with a confocal laser scanning microscope (True Confocal Scanner 4D, Leica, Heidelberg, Germany) equipped with an argon-krypton laser and coupled to a Leitz DM IRB inverted microscope (Leica).[15] Excitation was at 488 nm, and emission was band-pass filtered at 515nm.

An assessment of the transfection efficiency of human skin fibroblasts, compared to transfection of hamster lung fibroblasts and Human HeLa cells, indicated that the protocols we used are not suitable to obtain stable transfected skin fibroblasts. We concluded this from the fact that no stable transfected colonies of human skin fibroblasts were obtained, regardless of being CPT1-defective or being from a patient with a disorder unrelated to β-oxidation. Only transient expression with the Lipofectamine method was successful. We found an efficiency of 0.0-0.2 % of the cells which survived transfection without selection. However, compared to the efficiencies of transfection in hamster lung fibroblasts (5-10% in a parallel experiment), this still was low. Therefore, for long-term applications it is clear that different procedures should be used than the ones we applied here.

The low transfection efficiencies are, however, compensated for by the confocal laser scanning images we obtained of the few cells transiently expressing CPT1-GFP. As illustrated, these cells showed a promising result with respect to intracellular localisation of green fluorescent signal (Fig. 1). Punctuated and thread-like structures are clearly more fluorescent than the background level in the cytosol, and the nucleus remains clear of green fluorescent signal. These images are comparable to the microscopical data known from a variety of cells with mitochondrion specific stains like Rhodamine 123,[11] or GFP fusions targeted to the mitochondrial matrix.[9] Moreover, in human skin fibroblasts, the compound 2-(4-(dimethylamino)styryl)-l-methylpyridinium iodide has been successfully used to image mitochondria, showing comparable signal distribution patterns,[16] albeit that we sometimes could observe more distally localized fluorescent signals, putatively from mitochondria situated near the sites where the cells were attached to the coverslip glass. The low number of cells we have observed does not allow firm conclusions with respect to intracellular distribution of CPT1-GFP in relation to cytoskeletal compounds near these attachment sites.[17]

Control experiments with non-fused enhanced GFP resulted in homogeneously distributed green fluorescent signal, not only in the cytosol, but also in the nucleus. This uniform distribution of non-fused GFP was expected from previous reports.[3,8] Taken these facts together, our results with transient expression of CPT1-GFP in human skin fibroblasts are highly suggestive for targeting of the fusion protein to mitochondria. To investigate the targeting and topology of the CPT1-GFP fusion in more detail

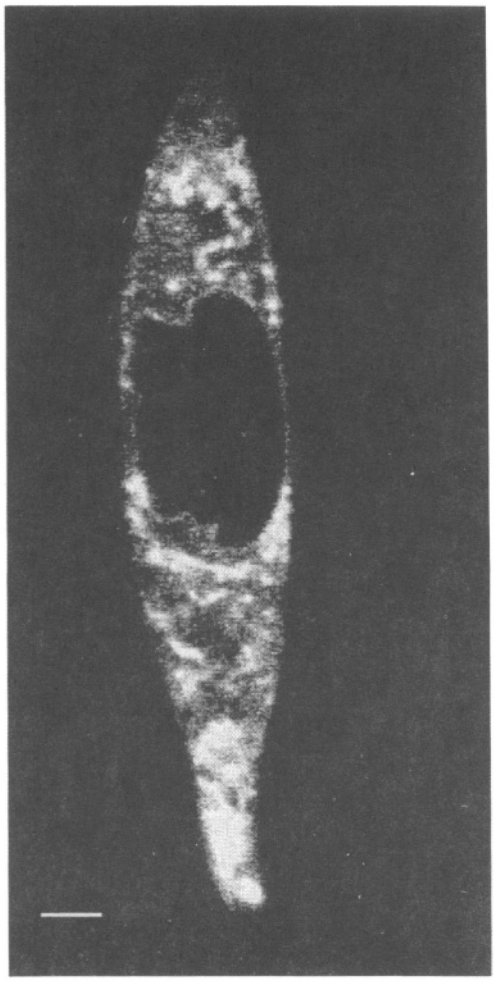

Figure 1. Transient expression of a green fluorescent carnitine palmitoyltransferase 1 fusion protein in a transfected human skin fibroblast. The intracellular distribution of the fusion protein is predominantly restricted to structures, putatively representing mitochondria. Bar represents 5 μm.

we recently have obtained stable transfected HeLa cells. These cells are currently subjected to more detailed microscopical and functional analyses (van der Leij *et al.,* in preparation).

We conclude that for long-term expression studies in human skin fibroblasts, the transfection methods we applied in this study are not appropriate. This may be improved when other transfection methods are applied. In that respect, a combination of a receptor-targeted transfection method and an immortalization strategy[7] seems attractive, since this would overcome the limited number of cell cycles of skin fibroblasts. High resolution imaging of the few cells we obtained made this study a valuable exercise, however. Much could be learned already from the few transfected cells we observed. The intracellular heterogenous distribution of CPT1-GFP points to mitochondrial targeting of this fusion protein, and these results are an encouraging basis for ongoing and future experiments.

ACKNOWLEDGEMENTS

We thank Mrs. W. Lemstra for help with various cell culture techniques, and Mrs. T. Bos for the supply of human skin fibroblasts. We are grateful to Prof. B. Poll-The at Utrecht University for providing a CPT1-deficient skin fibroblast culture. Part of our research is funded by the Netherlands Heart Foundation (NHS), and the Netherlands Scientific Organisation (NWO).

REFERENCES

1. McGarry, J.D. & Brown, N.F. (1997). *Eur J Biochem* **244**:1–14. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis.
2. van der Leij, F.R., Takens, J., van der Veen, A.Y., Terpstra, P. & Kuipers, J.R.G. (1997). *Biochim Biophys Acta* **1352**:123–128. Localization and intron usage analysis of the human CPT1B gene for muscle-type carnitine palmitoyltransferase I.
3. Zhang, G., Gurtu, V. & Kain, S.R. (1996). *Biochem Biophys Res Commun* **227**:707–711. An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells.
4. Fountain, J.W., Lockwood, W.K. & Collins, F.S. (1988). *Gene* **68**:167–172. Transfection of primary human skin fibroblasts by electroporation.
5. Ray, J. & Gage, F.H. (1992). *Biotechniques* **13**:598–603. Gene transfer into established and primary flbroblast cell lines: comparison of transfection methods and promoters.
6. Mayne, L.V., Priestley, A., James, M.R. & Burke, J.F. (1986). *Exp Cell Res* **162**:530–538. Efficient immortalization and morphological transformation of human fibroblasts by transfection with SV40 DNA linked to a dominant marker.
7. Veelken, H., Jesuiter, H., Mackensen, A., Kulmburg, P., Schultze, J., Rosenthal, F., Mertelsmann, R. & Lindemann, A. (1994). *Hum Gene Ther* **5**:1203–1210. Primary fibroblasts from human adults as target cells for ex vivo transfection and gene therapy.
8. Zolotukhin, S., Potter, M., Hauswirth, W.W., Guy, J. & Muzyczka, N. (1996). *J Virol* **70**:4646–4654. A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells.
9. Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R.Y. & Pozzan, T. (1996). *Curr Biol* 6:183–188. Double labelling of subcellular structures with organelle-targeted GFP mutants in vivo.
10. Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. & Youle, R.J. (1997). *J Cell Biol* 139:1281–1292. Movement of Bax from the cytosol to mitochondria during apoptosis.
11. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J.D. (1983). *Molecular biology of the cell.* Garland Publishing, Inc., New York and London.
12. Fraser, F., Corstorphine, C.G. & Zammit, V.A. (1997). *Biochem J* **323**:711–718. Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane.
13. Graham, F.L. & Eb, A.J.v.d. (1973). *Virology* **52**:456–467. A new technique for the assay of infectivity of human adenovirus 5 DNA.
14. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. & Chasin, L. (1979). *Proc Natl Acad Sci USA* **76**:1373–1376. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells.
15. Roelofsen, H., Vos, T.A., Schippers, I.J., Kuipers, F., Koning, H., Moshage, H., Jansen, PL. & Muller, M. (1997). *Gastroenterology* **112**:511–521. Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells.
16. Ruiters, M.H., van Spronsen, E.A., Skjeldal, O.H., Stromme, P., Scholte, H.R., Pzyrembel, H., Smit, G.P., Ruitenbeek, W. & Agsteribbe, E. (1991). *J Inherit Metab Dis* **14**:45–48. Confocal scanning laser microscopy of mitochondria: a possible tool in the diagnosis of mitochondrial disorders.
17. Bereiter-Hahn, J., Luck, M., Miebach, T., Stelzer, H.K. & Voth, M. (1990). *J Cell Sci* **96**:171–188. Spreading of trypsinized cells: cytoskeletal dynamics and energy requirements.