RESEARCH COMMUNICATION

A stress-regulated protein, GRP58, a member of thioredoxin superfamily, is a carnitine palmitoyltransferase isoenzyme

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We recently noted the association of carnitine palmitoyltransferase (CPT) activity with a 54 kDa microsomal protein [Murthy and Pande (1993) *Mol. Cell Biochem.* **122**, 133–138] that, based on amino-acid-sequence identity, seemed to be the protein previously described as a 'glucose-regulated protein-58' (GRP58), phosphoinositide-specific phospholipase C, hormone-induced protein-70, endoplasmic-reticulum protein-61 (ERp61), protein disulphide-isomerase, thiol protease, a protein affected in halothane anaesthesia and one that affects renal-tubular functions and the transcriptional activation of the interferon- α inducible genes. To ascertain the catalytic identity of this protein unambiguously, we have expressed the corresponding cDNA transiently and stably in human kidney 293 cells as well as in

HeLa cells. In each case we found that expression led to an increase in assayable and immunoreactive 54 kDa CPT activity, whereas the protein disulphide-isomerase activity was not increased. *In vitro* expression in a cell-free transcription and translation system led to the synthesis of a ~ 57 kDa (precursor) protein that was processed to a ~ 54 kDa (mature) protein when microsomes were present; in both these experiments again a large increase in CPT activity was seen. Thus the present data provide compelling evidence that the 54 kDa protein in question is a CPT isoenzyme. It remains to be seen now how the ability of this protein to interconvert acyl-CoA and acylcarnitine would relate to the diverse functions indicated for this protein *in vivo*.

INTRODUCTION

We recently reported that a ~ 900-fold-enriched preparation of a microsomal 54 kDa protein, showing carnitine palmitoyltransferase activity, seemed to be the protein, the cDNA of which had been cloned earlier but the catalytic activity of which was not established [1]. This clone was obtained by screening a cDNA expression library, using polyclonal antibodies against a preparation from guinea-pig uterus showing phosphoinositide-specific phospholipase C (PLC- α) activity [2]. The cDNA-derived amino acid sequence predicted a mature protein of ~ 54 kDa that was assumed to be for PLC- α ; however, the cDNA itself was not expressed to confirm this. Another previously studied protein, found induced in response to various stresses, including glucose deprivation, called 'glucose-regulated protein-58' (GRP58), was found subsequently [3,4], based on N-terminal amino-acid-sequence identity, to correspond to the protein product of the above cDNA. Lewis et al. [5], while studying the structure and assembly of endoplasmic-reticulum proteins, identified three major endoplasmic-reticulum-resident proteins from plasmacytoma 315 tumour cells; one of these was later [4] found to cross-react with antibodies to the PLC- α and showed the same N-terminal sequence as predicted for the PLC- α . Meanwhile, Srivastava et al. [6], while examining a protein disulphide-isomerase (PDI) activity from rat liver microsomes, found that the 110 amino acids sequenced in their protein showed 100% identity with the implied sequence of PLC- α . Urade et al. [7] reported that a ~ 60 kDa protein, purified from rat liver rough endoplasmic reticulum, had the same N-terminal amino acid sequence as expected for the PLC- α , but that it was devoid of PLC- α activity; they described, furthermore, that on two-

dimensional electrophoresis, their purified preparation resolved into eight spots all of which showed the same N-terminal sequence, and, when obtained by h.p.l.c. on a DEAE 5PW column, they all showed a feeble protease activity. Martin et al. [8], while studying trifluoroacetylation of rat liver microsomal proteins in regard to halothane anaesthesia, found that a major trifluoroacetylated protein of ~ 58 kDa, on amino acid sequence analysis, corresponded to the product of the above cDNA; they also reported that this protein, as isolated in the native, non-trifluoroacetylated, form, did not show any PLC- α activity. Wakui et al. [9], while purifying a 60 kDa heat-shock protein from porcine kidney, found that their enriched preparations were significantly contaminated with a ~ 54 kDa protein; the latter was subsequently assumed to be the PLC- α , as on sequence analysis, 186 out of the 194 amino acids sequenced were found to be identical with those assigned for rat liver PLC- α . And, on the basis of other related observations, a role for this protein in regional renal-tubular functions was suggested. Johnson et al. [10] found that an extranuclear protein, present in elevated levels in the myeloid cells of chronic-myelogenous-leukaemia patients, altered the electrophoretic mobility of DNA-protein complexes formed between nuclear proteins and the regulatory domains of interferon α -inducible genes, indicating that this protein plays an important role in the transcriptional activation of interferon-inducible genes. The purified protein was found to cross-react with the antibodies against the ~ 58 kDa trifluoroacetylated protein referred to above, and it was considered to be the same protein, the cDNA of which had been cloned by Bennet et al. [2], as 28 out of the 30 N-terminal amino acids were found to be identical with the cDNA-predicted amino acid sequence.

More recently, the two groups working with endoplasmic-

Abbreviations used: CPT, the carnitine medium/long-chain acyltransferase; PLC- α , phosphoinositide-specific phospholipase C- α ; PDI, protein disulphide-isomerase; ERp61, endoplasmic-reticulum protein-61; GRP58, glucose-regulated protein-58.

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reticulum protein-61 (ERp61) and GRP58 have shown in a joint publication that ERp61 and GRP58 are in fact one and the same protein, based on sequence identities and immuno-cross-reactivities [11]. However, when the corresponding cDNA was expressed, the protein product as found to be devoid of any PLC- α or PDI activity, and consequently the catalytic activity of this protein was considered as unknown. In view of the foregoing, and our evidence suggesting association of a carnitine palmitoyltransferase (CPT) activity with this protein [1], we have examined whether the expression product of the cDNA in question would show CPT activity; as described below, our results have shown that this indeed is the case. These findings have not only established the elusive catalytic identity of this protein, but, importantly, have raised questions of how the ability of this protein to catalyse the conversion of acyl-CoA plus carnitine to acylcarnitine plus CoA would relate to the various functions ascribed to this protein *in vivo*.

EXPERIMENTAL

Materials

[1- 14 C]Decanoyl-CoA was synthesized and purified by h.p.l.c. as described previously [12]. Porcine 125 I-insulin (116 μ Ci/ μ g) was from New England Nuclear. [35 S]Methionine (~ 1000 Ci/mmol) was purchased from Amersham International. The TNT transcription/translation kit was obtained from Promega. Rat liver microsomes were prepared by the procedure of Walter and Blobel [13] with the modification described in [14].

Plasmids and transfections

The mammalian expression plasmids were constructed as follows: pcD-61 with simian-virus-40 early promoter containing the full-length cDNA for GRP58/ERp61 was a gift from Michael Green, Saint Louis University, St. Louis, MO, U.S.A.; Mazzarella et al. [11] had obtained this clone previously by screening the murine cDNA library in pcD vector using the putative PLC- α rat cDNA described by Bennet et al. [2] as the probe. The N-terminal sequencing of ERp61 had shown that 19 amino acids analysed were identical with those ascribed to the PLC- α [11]. pcDNAI(neo)-61 was constructed by ligating the *Bam*HI fragment of pcD-61, containing the full-length cDNA of the 54 kDa protein, in to the *Bam*HI site next to the cytomegalovirus promoter of the pcDNAI(neo) from Invitrogen. After transfection in 293 cells, the clones were screened for the expression of immunoreactive 54 kDa protein using antibody to the microsomal 54 kDa CPT. pGRE5-61 was constructed by ligating the *Hind*III-*Eco*RV fragment of the pcDNAI(neo)-61 with *Sma*I-*Hind*III-cut pGRE5 (a gift from John White, McGill University, Montreal, Canada); the pGRE5 contains a synthetic promoter with five high-affinity glucocorticoid response elements [15]. Both the transient and stable transfections in human kidney 293 cells and HeLa cells were done by the calcium phosphate precipitation procedure [16]. The cells were maintained in Opti-MEM from Gibco-BRL. In the stable transfection experiments, selection was done with 800 μ g/ml G418 (geneticin; Gibco-BRL) for 3 weeks. In transient transfection experiments the cells were harvested 72 h post transfection. In the controls, the cells were transfected with the respective vector alone (i.e., lacking the cDNA insert). The cells were collected by scraping and washed twice in medium A [220 mM mannitol/80 mM sucrose/10 mM Hepes (pH 7.4)/0.1 mM EGTA/5 mM carnitine (helps stabilize the CPT activity [17])], 0.5 mM phenylmethanesulphonyl fluoride, and 50 μ g/ml each of leupeptin, bestatin and pepstatin).

The cell pellets were then homogenized in medium A and the homogenates centrifuged at 500 *g* for 10 min. The supernatant was centrifuged at 110000 *g* for 1 h. The resulting pellets were suspended in medium A and assayed for various activities.

Enzyme assays

CPT was assayed using 25 μ M [1- 14 C]decanoyl-CoA plus 5 mM carnitine as described previously [1]. Protein disulphide-isomerase (PDI) was assayed with 125 I-insulin as described by Srivastava et al. [6]. Glucose-6-phosphatase activity was monitored by the procedure of Arion [18].

Western-blot analyses

Human kidney 293 cells were transfected with pcD-61 (for transient expression) or with pcDNAI(neo)-61 (for stable expression) and the membrane fractions (110000 *g*) were prepared from the cells as described above. From each preparation 50 μ g of protein was loaded on to an SDS/10%-PAGE gel. After electrophoresis, the separated proteins were blotted on to a poly(vinylidene difluoride) membrane. The blotted proteins were probed with anti-(54 kDa CPT) antibody (raised in rabbits) and the ECL kit (Amersham). The time of exposure of the blot to the X-ray film was 30 s.

In vitro cell-free transcription and translation

The pcDNAI(neo)-61 plasmid with the cDNA insert for the microsomal 54 kDa protein with the 24 amino acid N-terminal signal sequence, under the control of the T_7 -promoter, was used. This plasmid, supercoiled, without linearization, was used in the TNT (transcription/translation) system (Promega), the manufacturer's instructions being followed. The 25 μ l incubation mixture contained 12.5 μ l of TNT rabbit reticulocyte lysate, TNT reaction buffer, T_7 -RNA polymerase, amino acid mix, 20 units of RNasin and 10 mM carnitine. For [35 S]methionine labelling experiments, the amino acid mix minus methionine, supplemented with [35 S]methionine (~ 1000 Ci/mmol), was used. Where indicated, rat liver microsomes (22 μ g of protein) were also present. After 2 h incubation at 30 $^{\circ}$ C, the tubes received 175 μ l of 10 mM Hepes (pH 7.4)/5 mM carnitine and 5% glycerol. This was followed by centrifugation at 150000 *g* for 2 h and removal of the supernatant; the resulting pellets were suspended, by sonication, in 100 μ l of 25 mM Tris/HCl (pH 8.0)/20 mM dithiothreitol/5 mM carnitine/5% glycerol. These pellet suspensions were processed either for CPT assays or for SDS/PAGE/fluorography. In experiments with unlabelled methionine, where CPT activity was to be measured, a positive control with pcDNAI plasmid (Invitrogen) containing the cDNA for the peroxisomal soluble CPT (pcDANI-px) with preceding T_7 -promoter, and a negative control with pPOLY(A)-*luc* plasmid (from Promega) expressing luciferase enzyme were included. Monitoring of the 35 S-labelled products showed that most of it was recovered with the 150000 *g* pellet fraction; it is known that at times this protein associates with membranes [2] and that the cleavable 24-amino-acid N-terminal of the precursor protein resembles a membrane anchoring domain [2].

RESULTS AND DISCUSSION

We reported recently [1] that the microsomal malonyl-CoA-insensitive CPT seemed to be the protein that had been described previously as GRP58 [3], ERp61 [4] and HIP-70 (hormone-induced protein-70) [19]. That ERp61 and GRP58 are the same protein has now been established [11]. To ascertain unequivocally whether this protein indeed shows CPT activity, we have

Table 1 Enzyme activities in control and the 54 kDa-protein-expressing cells

Plasmid construction, transfections, preparation of membrane fractions from the control and experimental (Exptl.) cells, and assays of various enzyme activities were carried out as described in the Experimental section. Results are means $\pm$ S.E.M. for three to eight observations. Values for glucose-6-phosphatase are averages for two separate experiments.

Transfection expt.		Activity (nmol/min per mg of protein)					
		CPT		PDI		Glucose-6-phosphatase	
		Control	Exptl.	Control	Exptl.	Control	Exptl.
A.	Transient; 293 cells with pcD-61	1.3 $\pm$ 0.1	3.6 $\pm$ 0.2	0.05 $\pm$ 0.003	0.053 $\pm$ 0.002	0.19	0.20
B.	Stable; 293 cells with pcDNA1(neo)-61	1.7 $\pm$ 0.2	5.1 $\pm$ 0.5	—	—	—	—
C.	Transient; 293 cells with pGRE5-61	1.1 $\pm$ 0.1	4.9 $\pm$ 0.2	0.06 $\pm$ 0.010	0.059 $\pm$ 0.005	0.21	0.22
D.	Transient; HeLa cells with pGRE5-61	2.0 $\pm$ 0.3	4.3 $\pm$ 0.5	—	—	—	—

Figure 1 Western-blot analysis of preparations from human kidney 293 cells, transfected with vector alone (control) or vector carrying the cDNA for the microsomal 54 kDa protein

Transfection of 293 cells was with pcD-61 (transient) and pcDNA1(neo)-61 constructs. Control cells were transfected with vector alone. Membrane preparations from these cells were processed for Western blotting using an antibody against the purified microsomal 54 kDa CPT. Additional details are described in the Experimental section. Lane 1, purified rat liver microsomal 54 kDa CPT; lane 2, pcD vector-transfected control; lane 3, pcD-61-transfected; lane 4, pcDNA1(neo)-vector-transfected control; and lane 5, pcDNA1(neo)-61-transfected.

expressed the full-length mouse cDNA of the ERp61, both transiently and stably, in two different cell lines. As Table 1 shows, in each case, a 2–4-fold increase in CPT activity was seen. As the contribution of this microsomal malonyl-CoA-insensitive CPT to total cellular CPT activity is modest ($\leq 15\%$ in rat liver), the observed increase in cellular CPT activity represents a relatively large increase in this microsomal CPT activity. And in line with this, the results of Western blots, probed with antibody to the 54 kDa CPT, showed a large increase in the 54 kDa peptide content (Figure 1) (expression of the corresponding rat liver cDNA [2] also led to an increase in the CPT activity and the content of the corresponding 54 kDa peptide monitored in Western blots [19a]). Experiments A and C of Table 1 showed that, concurrently, the activity of PDI did not increase; this is in agreement with the results of Mazzarella et al. [11], but not with those of Srivastava et al. [20], and is consistent with our observations that the purified microsomal CPT preparations are devoid of the PDI activity [1]. Table 1 shows also that the expression did not increase the activity of glucose-6-phosphatase, a microsomal marker; this eliminated the remote possibility that the expression protocols were causing an unspecific microsomal proliferation. Western blots, with antibodies to the mitochondrial outer CPT (a gift from J. D. McGarry, University of Texas, Dallas, TX, U.S.A.) and to the inner CPT, showed that there was no noticeable increase in the signals for these major cellular CPT proteins. When, as a positive control, a cDNA of peroxisomal ~ 63 kDa CPT (a gift from B. Chatterjee, University of Texas, San Antonio, TX, U.S.A.) in pGRE5 plasmid was expressed in

Figure 2 [35 S]Methionine labelling of the expression product of pcDNA1(neo)-61 synthesized in a cell-free system in the absence and in the presence of microsomes

Transcription and translation reactions using TnT reticulocyte lysate system were carried out, and the reaction products were processed as described in the Experimental section. SDS/PAGE was on 10% gels with 20 μ l of the reaction pellet preparations. After electrophoresis, the gels were treated with Amplify (Amersham), dried, and exposed to X-ray film for 2 h at -80°C . Lane 1, TnT control incubation without added DNA; lane 2, incubation with 1 μ g of pcDNA1(neo)-61; lane 3, incubation with microsomes (22 μ g of protein) only; lane 4, incubation with 1 μ g of pcDNA1(neo)-61 and microsomes (22 μ g of protein).

the 293 cells and in HeLa cells, in both the experiments a marked increase was seen in the assayable CPT activity (~ 3 -fold) as well as in the immunoreactive ~ 63 kDa peptide content seen in Western blots probed with antibody to the peroxisomal soluble CPT (results not shown).

To eliminate the remote possibility that the above expression experiments in intact cells somehow led to a fortuitous increase in assayable CPT activity and 54 kDa peptide content, we also expressed this protein using the cell-free TnT (transcription–translation) system (Promega) with T_7 -RNA polymerase. These incubations were carried out in the absence and presence of microsomes and the product was monitored for [35 S]methionine labelling and CPT activity. Figure 2 shows that a single labelled ~ 57 kDa peptide was synthesized in the absence of microsomes, but, when microsomes were present, labelled bands were seen at ~ 57 kDa and ~ 54 kDa zones. These data showed, consistent with the expectation, that the full-length expression product, with the N-terminal 24-amino-acid cleavable signal peptide [2], was produced initially, and that it became partially processed to the mature form when microsomes were present. Importantly, a marked increase in the assayable CPT activity was found in both

Table 2 The protein product of the pcDNA1(neo)-61 in a cell-free system supplemented without and with microsomes showed CPT activity

The plasmid pcDNA1(neo)-61 was used in the cell-free TnT expression system (Promega) as described in the Experimental section with a non-radioactive-methionine-supplemented amino acid mixture. The additions of the plasmid and microsomes were as indicated below. CPT was assayed at 30 °C using 25 μ M [$1\text{-}^{14}\text{C}$]decanoyl-CoA plus 5 mM carnitine in a total volume of 250 μ l. The data shown are means $\pm$ S.E.M. for four separate TnT incubations; CPT assays were carried out in duplicate.

Additions to the TnT incubations		CPT activity (pmol/5 min per 5 μ l)
A.	pPOLY(A)-luc (a negative control)	34 $\pm$ 5.7
B.	pcDNA1(neo)-61, 1 μ g	99 $\pm$ 7.4*
C.	pcDNA1(neo)-61, 2 μ g	181 $\pm$ 16.1*
D.	pcDNA1(neo)-61, 1 μ g plus microsomes	147 $\pm$ 13.1**
E.	Microsomes	84 $\pm$ 8.7*
F.	pcDNA1-px, 1 μ g (a positive control)	94 $\pm$ 5.1*

* $P < 0.01$ as compared with the negative control, A.

** $P < 0.01$ as compared with the microsomes-alone group, E.

the absence and presence of microsomes (Table 2); this confirmed that the expressed protein has CPT activity, and showed, moreover, that the unprocessed CPT precursor also displays CPT activity. It is noteworthy that the expression of the rat liver mitochondrial inner CPT in *Escherichia coli* showed that the unprocessed precursor of this protein also shows CPT activity [21].

Collectively, the present data provide compelling evidence that this microsomal 54 kDa protein, assigned different functions, has CPT activity. Whether this catalytic activity itself would account for the stress-related and other functions [22] indicated for this protein as described in the Introduction, and if so, how, remains to be investigated. Meanwhile, we do know that this protein has nuclear localization signal [23] and that it has been localized in the nucleus [24]. Perhaps in the nuclear microenvironment, by acting as a CPT, this protein modulates the levels of acyl-CoA and carnitine, both of which have been implicated in the regulation of gene transcription [25–27]. It is also known that dynamic protein acylation–deacylation is crucial for the proper functioning of certain proteins and that this requires acyl-CoA [28]. Reportedly, oestrogen and luteinizing-hormone-releasing hormone induce this 54 kDa protein $\sim$ 100-fold in the hippocampus region of the brain [29]. The presence in this protein of an apparent oestrogen-binding domain [30] and of two [2] thioredoxin active-site sequences, CGHC, were recognized previously. It should be noted that proteins with one or more CGHC sequence are now classified as members of the thioredoxin superfamily [31]. It remains to be seen now how the possible

interaction of the oestrogen-binding and CGHC domains, together with the CPT activity, would link to the diverse apparent functions ascribed to this protein *in vivo*.

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