RESEARCH COMMUNICATION

Cytosolic aspartate aminotransferase gene is a member of the glucose-regulated protein gene family in adipocytes

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Stress controls the expression of a cohort of genes. Among these, the glucose-regulated protein (GRP) genes are specifically activated by glucose deprivation, reducing agents, glycosylation block, intracellular calcium or *ex vivo* incubations of tissues or cells. We demonstrate that these stimuli induce the expression of

the cytosolic aspartate aminotransferase gene in adipocytes by a process involving the region of the promoter between –2405 and –26 bp. Therefore this transaminase is a new member of the GRP family.

INTRODUCTION

Cytosolic aspartate aminotransferase (cAspAT; EC 2.6.1.1) is a metabolic enzyme that participates in a ubiquitous function, the malate–aspartate shuttle, and is involved in tissue-specific functions, such as gluconeogenesis in the liver and glyceroneogenesis in adipocytes. During the course of our studies focussed on the hormonal and nutritional regulation of cAspAT gene expression, we have observed that glucose deprivation induces the expression of the mRNA encoding this protein in adipocytes. This finding has prompted us to study the regulation of cAspAT gene expression in response to other inducers of glucose-regulated proteins (GRPs).

The GRP family was first identified as a set of proteins induced specifically by glucose starvation. Subsequently, it has been found that a variety of effectors inducing aberrant protein folding, such as calcium ionophores, reducing agents or the protein glycosylation inhibitors, also enhance GRPs (reviewed in [1]). Most GRPs are ubiquitous endoplasmic reticulum (ER) proteins performing physiological functions (GRP94, GRP78/BiP, Erp72 and GRP58) [2–5]. More recently, it has been shown that two metabolic enzymes, the ubiquitous glucose transporter type-1 (GLUT-1) and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH), belong to the GRP family because of their similar pattern of regulation in response to treatment with calcium ionophores, protein glycosylation inhibitors, reducing agents or glucose deprivation [6,7].

We have analysed the effect of various GRP-inducing agents on cAspAT gene expression in adipocytes. We show that cAspAT mRNA is regulated by these agents in a manner similar to GRP78 [1]. Using stably transfected 3T3-F442A adipocytes, we have also demonstrated that the sequence from –2405 to –26 bp of the cAspAT gene promoter is the region responsive to induction by stress.

MATERIALS AND METHODS

Cell culture and treatment

3T3-F442A adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5g/l glucose at 37 °C in a humidified atmosphere of 10 % CO₂/90 % air. They were grown and differentiated in 10 % (v/v) fetal calf serum, penicillin (200 units/ml), streptomycin (50 mg/l), biotin (8 mg/l) and pantothenate (4 mg/l). At confluence, medium was supplemented with insulin (2 × 10^{–8} M) to favour triacylglycerol accumulation. Medium was changed every 2–3 days for a period of 8 days. Experiments were performed on mature adipose cells (8 days after confluence). Cells were shifted to serum-free, insulin-free medium during the treatment with the effectors. When glucose was omitted, medium was enriched with 1mM pyruvate and 0.1 mM lactate.

Culture of explants and of isolated adipocytes

Peri-epididymal fat-pads from 6-week-old male Wistar rats were cut with scissors into small pieces (2–3 mm³) and maintained at 37 °C in a humidified atmosphere of 10 % CO₂/90 % air for 18 h in DMEM containing 4.5 g/l glucose and 4 % BSA, before RNA extraction. Adipocytes were isolated as described by Ong et al. [8], then cultured as described for explants.

RNA extraction and analysis

RNA from tissue fragments and adipocytes was extracted by the method of Chirgwin et al. [9]. 3T3-F442A cells from two 60-mm dishes were pooled, and RNA was extracted according to the method of Chomczynski and Sacchi [10]. Total RNA was

Abbreviations used: cAspAT, cytosolic aspartate aminotransferase; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FAS, fatty acid synthase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GLUT-1, glucose transporter type-1; GRP, glucose-regulated protein; HSL, hormone-sensitive lipase; mAspAT, mitochondrial aspartate aminotransferase; PEPCCK, phosphoenolpyruvate carboxykinase.

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electrophoresed on a 1% (w/v) agarose gel containing 2.2 M formaldehyde, and blotted on to a nylon membrane. The integrity and relative amounts of RNA were assessed by Methylene Blue staining. Prehybridization and hybridization of the blots were performed according to Yang et al. [11]. Membranes were hybridized overnight at 65 °C in 0.27 M NaCl/1.5 mM EDTA/15 mM sodium phosphate (pH 7.7)/7% (w/v) SDS/10% (v/v) poly(ethylene glycol)-6000/250 g/ml sonicated salmon sperm DNA/250 g/ml heparin/10⁶ c.p.m./ml of cDNA labelled with [α -³²P]dCTP (ICN Pharmaceuticals) by random priming, according to the manufacturer's recommendation. Membranes were washed twice for 15 min at room temperature in 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7), 0.1% (w/v) SDS, and then for 30 min at 60 °C with 0.1 × SSC and 0.1% (w/v) SDS.

Probes

Specific cDNA probes used were cAspAT [12], mitochondrial AspAT (mAspAT) [12], fatty acid synthase (FAS) [13], GRP-78 [14], hormone-sensitive lipase (HSL) [15], phosphoenolpyruvate carboxykinase (PEPCK) [16] and β -actin [17].

Stable transfections

Exponentially growing 3T3-F442A preadipocytes (10⁵ cells per 100-mm plates) were co-transfected by calcium phosphate/DNA co-precipitation [18] with 2 g of pSV2-NEO [19], the expression of which confers resistance to the antibiotic geneticin, together with 20 g of the plasmid construct containing nts -2405 to -26 of the cAspAT gene promoter linked to the chloramphenicol acetyltransferase (CAT) gene [20]. Cells were treated as described previously [18]. Transfection efficiency was about 5 × 10⁻⁵. After 2 weeks exposure to geneticin (0.4 mg/ml), ten colonies of geneticin-resistant cells were isolated and analysed. Cells from 90% of these colonies were still able to differentiate at a frequency similar to that of the parental line. About 70% of these expressed CAT. Fully differentiated adipocytes were shifted to serum-free medium, and either deprived of or incubated with glucose for 24 h before CAT assay.

Preparation of cell homogenates for CAT assays was performed as described previously [21]. The method of Seed and Sheen [22] was used for CAT activity determination (one unit of CAT converts 1 mol of chloramphenicol into butyryl chloramphenicol per min at pH 7.8 and 37 °C).

RESULTS AND DISCUSSION

During the course of our studies focussed on the regulation of gene expression by nutrients, we observed that the cAspAT mRNA was strongly up-regulated by 24–48 h glucose deprivation in 3T3-F442A adipocytes (Figure 1). The increase in cAspAT activity occurred in tandem with the induction of mRNA (results not shown). mAspAT was also affected by this treatment, although the magnitude of the glucose effect was much lower than that observed for cAspAT (Figure 1). Conversely, expression of the FAS gene was down-regulated by glucose deprivation (Figure 1) as previously described [23,24]. β -Actin mRNA remained unchanged, and was used as a control. An increase in GRP-78 mRNA was observed with a magnitude equivalent to that of cAspAT mRNA (Figure 1).

GRPs, such as GRP-78, are known as stress-inducible proteins. They respond to reducing stress, inhibition of protein glycosylation or depletion of the ER calcium stores [1]. We tested

Figure 1 Effect of glucose deprivation on gene expression in 3T3-F442A adipocytes

Cells were deprived of glucose for 24 h or 48 h before RNA extraction and analysis. The same Northern blot was hybridized sequentially with the cDNA probes, as indicated. Results of typical autoradiograms are shown.

the effects of a potent reducing agent (2-mercaptoethanol) of the calcium ionophore A23187 and an inhibitor of protein glycosylation (tunicamycin), at concentrations known to be efficient for induction of GRPs [25], on the expression of the cAspAT and GRP-78 genes (Table 1). A 24 h treatment of 3T3-F442A adipocytes with these drugs, in the presence of 25 mM glucose, induced cAspAT mRNA 3–5 fold. These increases were similar to those observed for GRP-78 mRNA in the same cells (Table 1). We then analysed the effect produced by a shift in temperature from 37 °C to 42 °C on cAspAT mRNA content. Such a treatment is known to favour the induction of another class of stress proteins, the heat-shock proteins [26]. To achieve this aim, 3T3-F442A adipocytes were cultured at 42 °C for 24 h before RNA analysis. This treatment did not appreciably alter cAspAT gene expression and, as expected [27], had no effect on GRP-78 mRNA (Table 1). Other treatments, such as incubation for 2–14 h at 42 °C followed by 10 h at 37 °C, were also ineffective (results not shown). Thus alteration of cAspAT gene expression is produced specifically by GRP-inducing treatments.

Another property shared by members of the GRP family is their response to stress induced by the *ex vivo* incubation of tissues or cells [6]. To determine whether cAspAT gene expression followed such a pattern, we cultured explants of epididymal adipose tissue or isolated adipocytes for 18 h in DMEM before RNA analysis. For both systems, a 2.5–3-fold increase in cAspAT mRNA occurred (Figure 2). mAspAT mRNA content was also slightly increased by the *ex vivo* incubation of adipocytes (Figure 2). In contrast, mRNAs encoding PEPCK or HSL were drastically reduced, as has been previously observed [28]. This showed that the increase in cAspAT mRNA was not a general phenomenon linked to the culture systems. As expected, GRP-78 mRNA was induced (Figure 2). Therefore cAspAT and GRP-78 mRNAs were co-ordinately regulated in response to the *ex vivo* incubation of adipose tissue. Thus the cAspAT gene belongs to the GRP gene family. GRPs are usually defined as ubiquitous

Table 1 Effect of various GRP-inducing agents on cAspAT and GRP-78 mRNAs in 3T3-F442A adipocytes

Cells were treated for 24 h in glucose-supplemented medium containing 2-mercaptoethanol (2-ME), A23187 or tunicamycin (Tunica) at the indicated concentrations, heat-shocked at 42 °C (HS) or deprived of glucose for 24 h (No glucose) before RNA extraction and analysis. Northern blots were hybridized sequentially with cAspAT, GRP-78 and actin cDNA probes. Data were normalized to the β -actin mRNA signal and expressed as the percentage of the cAspAT or GRP-78 RNA signal from cells cultured in the presence of 25 mM glucose. None of the treatments affected β -actin mRNA. Each value represents the mean $\pm$ S.E.M. of data obtained from three independent experiments with duplicate dishes.

	Glucose	No Glucose	2-ME		A23187		Tunica	HS
mRNA/ β -actin (% of control)	25 mM		0.1%	0.25%	1 μ M	7 μ M	2 g/ml	
cAspAT	100	376 $\pm$ 26.9	387 $\pm$ 29	432 $\pm$ 54	465 $\pm$ 104	455 $\pm$ 79	536 $\pm$ 165	157 $\pm$ 13
GRP-78	100	371 $\pm$ 2	236 $\pm$ 31	373 $\pm$ 37	570 $\pm$ 61	444 $\pm$ 46	312 $\pm$ 144	87.5 $\pm$ 12

Figure 2 Evolution of the expression of various genes in adipose-tissue explants or in isolated adipocytes incubated *ex vivo*

Adipose-tissue explants or isolated adipocytes were incubated for 0h (t0) or 18 h, as indicated in the Materials and methods section, before RNA extraction and analysis. The same Northern blot was hybridized sequentially with the cDNA probes, as indicated. Results of typical autoradiograms are shown. Abbreviation: ND, not determined.

ER proteins, which are induced when the ER is subjected to stress [1]. cAspAT does not belong to the ER compartment; however, two other proteins which are not located in the ER are considered to be GRPs, namely GLUT-1 [6] and GAPDH [7]. For the latter, opposite effects of glucose have been reported, depending on the cell type [7,29,30], showing that cell-specific regulation occurs. This may also apply to the regulation of cAspAT gene expression, which is induced by glucose deprivation in adipocytes (Figure 1), but not in hepatoma or myocyte cell lines (results not shown). What could be the physiological meaning of the effect of stress on cAspAT and, to a lesser extent, mAspAT? One reasonable hypothesis, implicating the role of cAspAT in NADH transport, would be that cellular ATP synthesis is required when adipocytes are subjected to stress. Experiments aimed at confirming this hypothesis have yet to be carried out. To determine whether the effect of stress was mediated by the promoter region of the cAspAT gene, we used the p(−2405/−26)–CAT construct, previously shown to be responsive to hormones when transfected into Fao hepatoma

Table 2 Effect of glucose deprivation and A23187 treatment on CAT expression in the (−2405/−26 bp cAspAT)–CAT fusion gene-3T3-F442A stable transfectants

Differentiated FEP103.3 and FEP103.4 stable transfectants were incubated for 24 h in glucose-deprived medium (No glucose) or in glucose-supplemented medium ([glucose] = 25 mM) in either the absence or presence of 1M A23187, before assaying for CAT activity. Results are expressed in m-units of CAT/mg protein. Each value represents the mean $\pm$ S.E.M. of data obtained from three independent experiments with duplicate dishes.

m-Units of CAT/mg protein	Glucose (25 mM)	No glucose	A23187 (1 μ M)
FEP103.3	2.1 $\pm$ 0.2	4.3 $\pm$ 0.3	6.6 $\pm$ 1.3
FEP103.4	4.6 $\pm$ 0.7	27.6 $\pm$ 2.6	195 $\pm$ 7.3

cells [20]. This construct contains −2405 to −26 bp of the cAspAT gene fused to the CAT reporter gene. We co-transfected 3T3-F442A adipoblasts with p(−2405/−26)–CAT and pSV2–NEO, which conferred resistance of the transfected cells to the neomycin analogue G418. Among twelve individual stable transfectants, five were selected for their combined characteristics of adequate differentiation and expression of CAT at a substantial level, with specific activities ranging from 2.4 to 35 m-units/mg of protein after adipocyte differentiation. All these transfectants responded to 24 h glucose deprivation by an increase in CAT activity, although to a variable extent (results not shown). A detailed analysis of the effect of stress on two of these transfectants (FEP103.3 and FEP103.4) was carried out. CAT activity in FEP103.3 and FEP103.4 adipocytes increased by 2–6-fold following 24 h glucose deprivation, and by 3–42-fold with A23187 treatment (Table 2). Such a difference in the magnitude of response of the transgene to effectors in various stable transfectants is a commonly observed phenomenon [21,31]. Nevertheless, these results clearly show that the region of the cAspAT gene between nts −2405 and −26 is responsive to inducers of GRPs. Among the other stress-responsive gene promoters, that of GRP-78 has been analysed in great detail by Wooden et al. [32]. Using a series of 5′-deletions of the GRP-78 gene promoter, they have shown that a proximal region containing a CCAAT-box-like motif mediates the response to GRP inducers. Several elements such as these are present in the cAspAT gene promoter [33]. The potential involvement of these elements constitutes the basis for our future investigations.

We thank Dr. Howard Green (Boston, U.S.A.) for providing us with the 3T3-F442A cell line, Dr. Erol Cerasi (Jerusalem, Israel), Daryl Granner (Nashville, TE, U.S.A.), Alan Goodridge (Iowa, U.S.A.), Dominique Langin (Toulouse, France) and Bruce Spiegelman (Boston, U.S.A.) for the GRP-78, PEPCK, FAS, HSL and actin cDNA

respectively. E.P.-G was a recipient of a fellowship from the French Ministère de l'Education Nationale, de la Recherche et de la Technologie (1993–1996) and the 1996 Laureate of the award of the Société de Nutrition de Langue Française. We are grateful to Dr. Armelle Leturque (Meudon, France) and Erol Cerasi for helpful discussions. This work was supported by CNRS, INSERM and the Université Paris-Val de Marne.

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