Muscle glycogen synthase translocates from the cell nucleus to the cytosol in response to glucose

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Abstract We have studied the intracellular localization of muscular glycogen synthase by fusing the green fluorescent protein (GFP) of the jelly-fish *Aequorea victoria* to the N-terminus of human muscle glycogen synthase (HMGS), and expressing the chimeric protein in C_2C_{12}, COS-1 cells, and primary cultured rat hepatocytes. In contrast to what we have recently found for the hepatic glycogen synthase (Fernández-Novell et al. (1997) Biochem. J. 321, 227–231), the GFP/HMGS fusion protein is localized to the nucleus of the cell in the absence of glucose, and in the presence of the sugar it is essentially found in the cytosol. Insulin is not required for the translocation of the enzyme.

Key words: Glycogen synthase; Translocation; Glucose; Glycogen metabolism; Green fluorescent protein

1. Introduction

Glycogen synthase (EC 2.4.1.11) catalyzes the synthesis of 1,4-α-linked glucose chains in glycogen. It is highly regulated by allosteric effectors and by covalent modification, and constitutes the control point of glycogen synthesis. To date, only two isoforms of glycogen synthase have been identified. The skeletal muscle type has been found in a number of organs, such as heart, brain, kidney, and fat, in addition to skeletal muscle, whereas the liver type isoform seems to be tissue specific [1].

In contrast to the high amino acid homology found for the enzymes characterized from the same tissue of different species (i.e. 92% between the rat and human liver synthase and 97% between the rabbit and human skeletal muscle enzymes), there is considerably less homology between the enzymes from two organs in the same species. The homology between human liver and human muscle glycogen synthase (HMGS) is only ~70%. An additional difference between the synthase from the two organs is a 34-amino acid truncation at the C-terminal end of the liver enzyme [2].

In a recent report we have shown that, in isolated rat hepatocytes, liver glycogen synthase (RLGS) translocates from the cytosol to the cell periphery in response to glucose [3]. Here, through the use of the *Aequorea victoria* green fluorescent protein (GFP) [4], we have analyzed the subcellular distribution of the muscle type isoenzyme. We have constructed the GFP/HMGS chimera and shown that in three different cell types, it concentrates in the nucleus in the absence of glucose, and that it translocates to the cytosol in response to this sugar.

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Abbreviations: GFP, green fluorescent protein; HMGS, human muscle glycogen synthase; RLGS, rat liver glycogen synthase; NLS, nuclear localization signal

2. Materials and methods

2.1. Plasmid construction

Standard molecular cloning techniques were used throughout [5]. The full coding sequence of HMGS, plus 410 bases of the 3′ untranslated region (GeneBank No. J04501) [6,7], was excised from the pET-3a/HMGS plasmid (a generous gift from R.J. Fletterick) with *Nde*I and *Sph*I, and ligated to pSL1180 (Pharmacia Biotech) previously cut with the same enzymes. The resulting plasmid was digested with *Nde*I and blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I and dNTPs, followed by digestion with *Sal*I. The fragment containing the coding sequence of HMGS was ligated into pEGFP-C1 (Clontech), which had been previously digested with *Bgl*II, Klenow-filled, and digested with *Sal*I. This ensured the in-frame fusion of HMGS at the C-terminus of the GFP coding sequence plus a linker of 5 amino acids, under the control of the constitutive immediate early promoter of the human cytomegalovirus. The final plasmid, pEGFP-C1/HMGS, was purified by ionic exchange chromatography (Qiagen) and finally dissolved in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0.

A *Sal*I restriction site between nucleotides 1817 and 1822 was introduced in the coding sequence of HMGS, using the QuickChange site-directed mutagenesis kit (Stratagene) and the two oligonucleotides CGGTATCTACATTGTCGACCGGCGGTTCC (sense) and GGAACCG CCGGTCGACAATGTAGATACCG (antisense). The pEGFP-C1/HMGSΔ553 plasmid, coding for a truncated form of HMGS lacking the C-terminal 183 amino acids, was constructed using the cloning strategy described for pEGFP-C1/HMGS. The DNA encoding the fusion proteins were sequenced using the ABI-PRISM DNA sequencing kit and the ABI-PRISM 377 automatic DNA sequencer (Perkin Elmer Applied Biosystems).

2.2. Cell culture and transfection

C_2C_{12} myoblasts (ATCC # CRL-1772) were grown on glass coverslips in Dulbecco's Modified Eagle's medium (DMEM; Whittaker), supplemented with 25 mM glucose, 10% fetal bovine serum (FBS; Biological Industries) and penicillin/streptomycin (Boehringer Mannheim). Differentiation into myotubes was induced by incubating the cells in DMEM containing 10% horse serum (HS; Life Technologies) instead of FBS. Fused myotubes were observed 3–4 days after the medium change [8]. COS-1 cells (ATCC # CRL-1650) were grown in DMEM supplemented with 25 mM glucose, 10% FBS and penicillin/streptomycin. Transfection of C_2C_{12} cells was performed at 50–60% of confluence for the studies in isolated myoblasts, and at 80–90% of confluence for the studies in fused differentiated cells. COS-1 cells were transfected at 70–80% of confluence. In both cases, liposome-mediated transfection was performed using 4 μg of Clonfectin (Clontech) and 4 μg of plasmid DNA per 35 mm dish, following the manufacturer's instructions. After transfection (4–5 h) at 37°C in humidified 5% CO_2:95% air, cells were washed in phosphate-buffered saline (PBS) and C_2C_{12} cells were incubated in DMEM supplemented with 25 mM glucose and 10% HS. COS-1 cells were incubated in DMEM containing 25 mM glucose and 10% FBS. Experiments were performed 48–72 h after transfection. Prior to the experiment,

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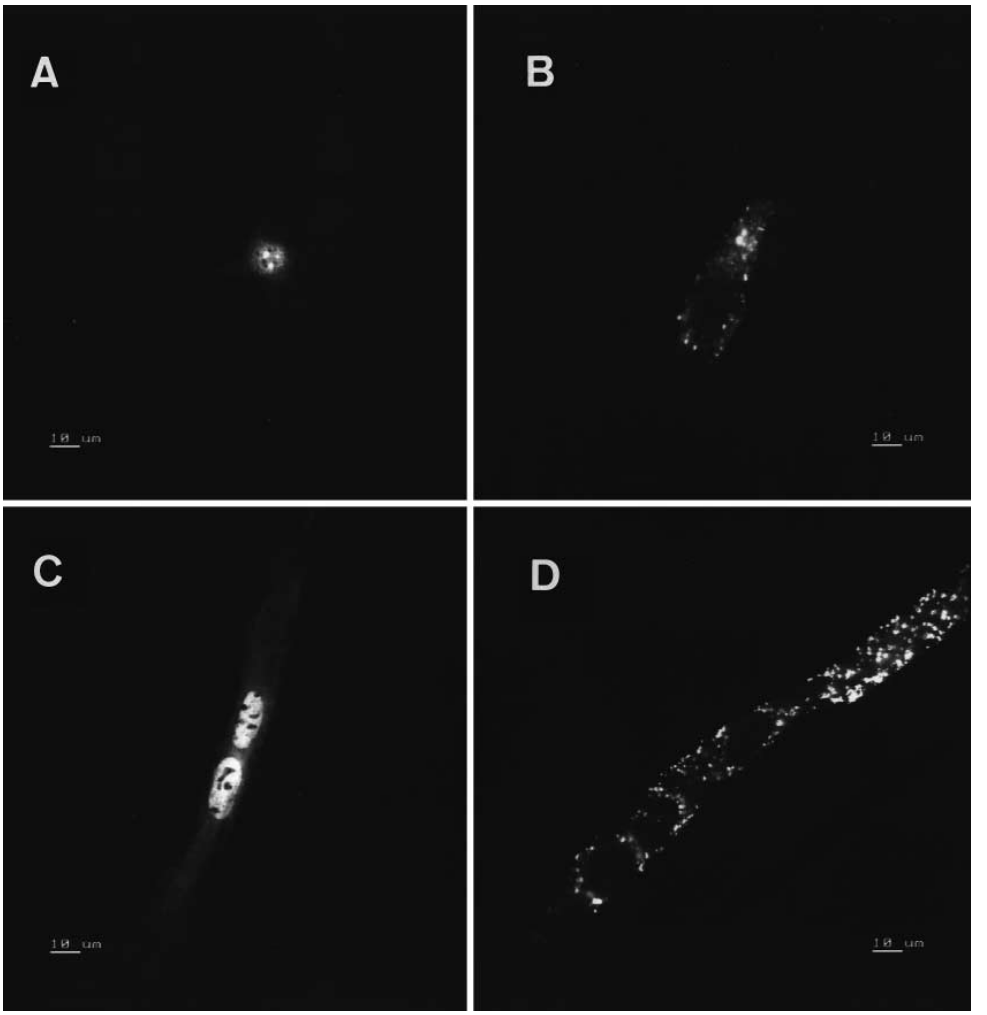

Fig. 1. Expression of GFP/HMGS in cultured C_2C_{12} cells. Confocal images of transfected myoblasts (A, B) and myotubes (C, D), incubated in the absence (A, C) or the presence of 25 mM glucose for 4 h (B, D).

both cell types were preincubated for 4–5 h in plain DMEM without glucose.

Hepatocytes were isolated from 24 h starved male Wistar rats (Interfauna) by collagenase perfusion as described [9]. Cells were resuspended in DMEM supplemented with 10 mM glucose, 10% FBS, 100 nM insulin (Sigma), 100 nM dexamethasone (Sigma), and penicillin/streptomycin and seeded (6×10^4 cells/cm^2) onto gelatin-coated glass coverslips placed on tissue culture plates. After 4 h at 37°C in humidified 5% CO_2:95% air, unattached cells were removed by washing with PBS, and transfection was performed as described above. After transfection, cells were washed in PBS and incubated in plain DMEM without glucose for 24 h.

On the day of the experiment the three cell types were incubated in plain DMEM without glucose (control) or with 25 mM glucose and/or 10 nM insulin, for 1 or 4 h. At the end of the incubations, cells were fixed for 20 min at room temperature in PBS containing 4% paraformaldehyde, and washed several times with PBS. Coverslips were air-dried and finally mounted onto glass slides, using the Immuno Fluore mounting medium (ICN Biomedicals, Inc.).

2.3. Confocal microscopy

Fluorescence images were obtained with a Leica TCS 4D (Leica Lasertechnik, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope and 63× and 100× (NA 1.4 oil) Leitz Plan-Apo objectives. The light source was an argon/krypton laser (75 mW). Green fluorescence from GFP recombinants was excited at 488 nm with the laser and optical sections (0.1 μm) were obtained.

3. Results

Cultured C_2C_{12} cells, a line derived from mouse muscle, were transiently transfected with the pEGFP-C1 and the pEGFP-C1/HMGS plasmids. Two (for myoblasts) or three (for myotubes) days after transfection the cells were deprived of glucose for 4–5 h and subsequently incubated in a glucose-free medium (control) or in a medium containing 25 mM glucose and/or insulin.

After fixation, the cells expressing GFP showed a uniform diffuse labeling of the cytosol and the cell nucleus, with a slight concentration of the fluorescence in this last compartment, as has been observed in other cell types [10]. This distribution did not change upon incubation with 25 mM glucose and/or insulin (data not shown). In contrast, C_2C_{12} myoblasts transfected with pEGFP-C1/HMGS and incubated in a medium without glucose exhibited a clear localization of the fluorescent label to the cell nucleus (Fig. 1A). Conversely, in the presence of 25 mM glucose in the medium, the fusion protein was found exclusively in the cytosol, mainly as round-shaped aggregates, with a small fraction showing a diffuse pattern (Fig. 1B). The same kind of particles could be observed in the nucleus of the cells incubated without glucose

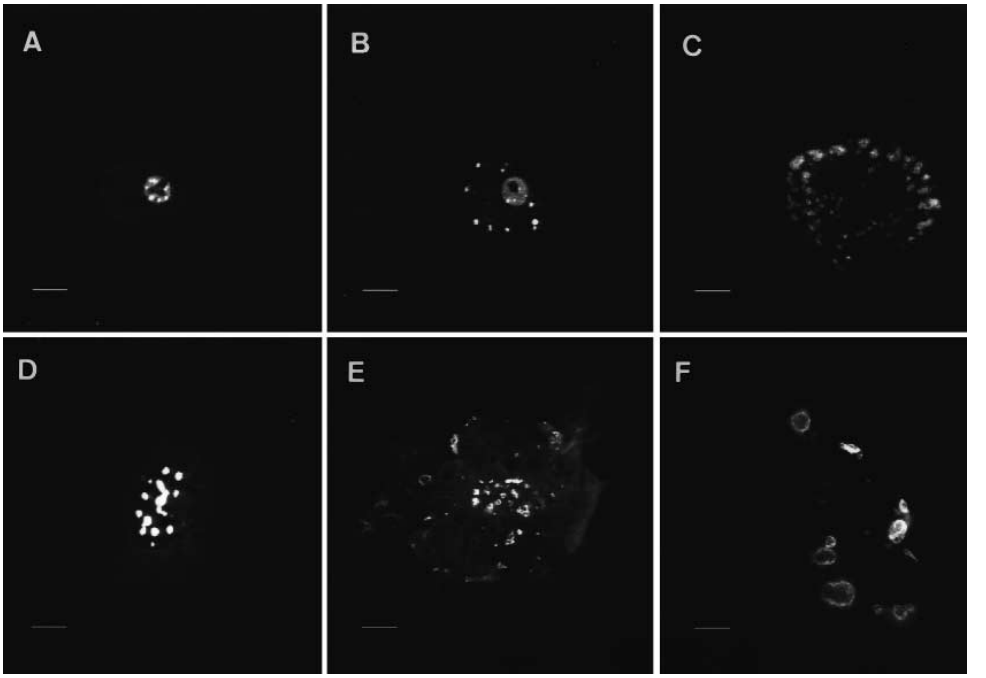

Fig. 2. Expression of GFP/HMGS in cultured rat hepatocytes (A–C) and COS-1 cells (D–F). Confocal images of cells incubated in the absence (A, D) or the presence of 25 mM glucose for 1 h (B, E) or for 4 h (C, F). Scale bar = 10 μm.

(Fig. 1A). Similar results were obtained when the C_2C_{12} cells were induced to differentiate into myotubes after transfection (Fig. 1C, D).

The expression of GFP/HMGS in other cell types, such as primary cultured rat hepatocytes (Fig. 2A–C) or COS-1 cells (Fig. 2D–F), showed a similar distribution of the protein. In both cell types, the fluorescent label was essentially found in the nucleus in the absence of glucose (Fig. 2A, D). A short incubation (1 h) with the sugar led to the distribution of the chimeric protein between the nucleus and the cytosol (Fig. 2B, E), and, after a longer incubation period (4 h), all the fluorescence was found exclusively in the cytosol (Fig. 2C, F). As in the case of the muscle cells, the fusion GFP/HMGS protein exhibited a particulate pattern. The size and the apparent complexity of the particles in the cytosol increased with time in the cells incubated with glucose.

In all the cell types studied, the incubation with insulin alone had no effect on the cellular localization of the GFP/HMGS chimera, while the cells incubated with glucose and insulin showed the same pattern of fluorescence as those incubated with glucose alone (data not shown).

To rule out the possibility that the fusion of the HMGS cDNA to the GFP adventitiously generated a nuclear localization signal in the chimeric protein, a truncated form of the HMGS, lacking the C-terminal 183 amino acids, was fused to GFP. The resulting fusion protein was found exclusively in the cytoplasm and was excluded from the nucleus, both in the absence and in the presence of glucose (data not shown).

4. Discussion

We have used the intrinsic fluorescence of the GFP to study the subcellular distribution of the human muscle isoform of glycogen synthase. The expression of the chimeric GFP/HMGS fusion protein in all cell types studied led to its accumulation in the cell nucleus in the absence of glucose, and its translocation to the cytosol in the presence of the sugar. In all instances, the GFP/HMGS chimera showed a particulate pattern reminiscent of the structural organization of the glycogen molecule [11]. This suggests that the fusion protein remains bound to its substrate and product. It has been proposed that the enzymes involved in glycogen metabolism are associated to the polysaccharide and that the glycogen-protein complex forms morphologically distinct cell organelles, named glycosomes [12].

The chimeric GFP/HMGS protein (111 kDa) is too large to diffuse freely between the cytoplasm and the nucleus. The accepted exclusion limit for the passive diffusion between these two compartments is approximately 45 kDa [13]. The fact that, in the absence of glucose, the GFP/HMGS chimera concentrates in the nucleus is an indication that the protein is actively transported into this compartment and that it must possess a nuclear localization signal (NLS) in its primary sequence. A truncated form of the GFP/HMGS fusion protein, in which the C-terminal 183 amino acids have been eliminated, is found exclusively in the cytosol. This fact rules out the possibility of an artifactually generated NLS in the GFP/HMGS chimera and indicates that the C-terminal end of the HMGS is necessary for the nuclear localization of the protein. However, HMGS does not have any sequence analogous to the NLS of the SV40 large T antigen (PKKKRKV) or to the bipartite motif found in other nuclear proteins, comprising two basic amino acids, a spacer of any ten amino acids and a basic cluster in which three out of the next five amino acids are basic [14]. Furthermore, in the presence of glucose, GFP/HMGS exits the nucleus and is found almost exclusively in the cytoplasm, indicating that the NLS present in HMGS cannot be constitutive, but must be regulated.

The GFP/HMGS chimera behaves very similarly in C_2C_{12}, COS-1 and hepatic cells, suggesting that its intracellular local-

ization is an intrinsic characteristic of the protein, dependent on the metabolic conditions, but independent of the cell type. Interestingly, this change in cellular localization is induced by glucose alone with no need of any hormonal signal, such as insulin.

We have recently shown, in isolated rat hepatocytes, that RLGS translocates from a uniform cytosolic distribution in the absence of glucose to the cell cortex in the presence of the sugar [3]. Therefore, both known isoforms of glycogen synthase undergo a change in their cellular localization in response to glucose, but their exact position in the cell in either condition is clearly different. This observation suggests that glycogen synthesis and its regulation are different in hepatic and muscular tissues. Other authors have suggested the existence of such differences, based on the different relative abundance of glycogenin, the protein that mediates the de novo synthesis of glycogen, in hepatic and muscular cells [15].

An additional outcome of this study is that it may help settle the long-standing controversy about whether or not glycogen synthase is present in the cell nucleus. Larner and coworkers, using rat hepatocytes, reported that RLGS was found exclusively in the cytoplasm [16], while Granzow and co-authors claimed that intact nuclei isolated from HD33 Ehrlich-Lettré ascites tumor cells possess high autochthonous glycogen synthase activity [17,18]. Although the glycogen synthase present in HD33 cells has not been fully characterized, the second observation could be explained assuming that these cells express an isoform of glycogen synthase similar to the muscle type enzyme, which, in contrast to what occurs for the hepatic isoform, can be found in the nucleus under certain metabolic conditions.

A comparison of the primary sequences of the RLGS and the HMGS reveals that these two enzymes have 70% overall identity. However, the degree of homology varies throughout the sequences. The central region of the proteins is the most conserved, while the amino and the carboxyl termini, which contain the phosphorylation sites that regulate the activity of the enzyme, show a much lower degree of homology [19]. Since the differences between the two isoforms accumulate in the N- and C-terminal ends, and these regions are responsible for the regulation of the enzyme activity, we speculate that they also contain the sequences that control the cellular localization of the liver and muscle enzymes. There are known examples of proteins that shuttle between different cellular compartments, whose location inside the cell is regulated by their phosphorylation state. These include regulated migration from the cytosol to the plasma membrane [20] and translocation from and into the nucleus [21]. It is tempting to speculate that, as well as the activity, the subcellular localization of glycogen synthase is also controlled by a phosphorylation-dephosphorylation mechanism. Based on the results obtained with the truncated form of the GFP/HMGS protein, we have initiated a systematic study to identify the exact sequences in the amino and carboxyl termini of HMGS and RLGS that contain the information for the translocation of the enzymes from the nucleus to the cytosol, in the first case, and from the cytosol to the cell cortex in the second, and to determine how these processes are regulated at the molecular level.

It has been shown that certain proteins, like glyceraldehyde-3-phosphate dehydrogenase and others [22], which can be found in the nucleus and in the cytoplasm of the cell, are involved in other cellular activities unrelated to their primary function. More specifically, there are examples of proteins whose combined functions are believed to link the energy metabolism to certain activities related to the regulation of gene expression [22]. The accumulation of muscular glycogen synthase in the cell nucleus and its translocation to the cytosol in response to glucose may only constitute an additional regulatory mechanism of its activity. Conversely, the muscular isoform of the glycogen synthase may possess some additional functions not yet described, which involve its shuttling between these two cellular compartments.

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