Archives of Biochemistry and Biophysics xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

N-terminus of seed caleosins is essential for lipid droplet sorting but not for lipid accumulation

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ARTICLE INFO

Article history:
Received 24 February 2015
and in revised form 19 May 2015
Available online xxxx

Keywords:
Lipid droplets
Seed
Caleosin
Structure–function study
Saccharomyces cerevisiae
Lipid accumulation

ABSTRACT

Caleosin, a calcium-binding protein associated with plant lipid droplets, stimulates lipid accumulation when heterologously expressed in *Saccharomyces cerevisiae*. Accumulated lipids are stored in cytoplasmic lipid droplets that are stabilised by incorporated caleosin. We designed a set of mutants affecting putative crucial sites for caleosin function and association with lipid droplets, i.e. the N-terminus, the EF-hand motif and the proline-knot motif. We investigated the effect of introduced mutations on caleosin capacity to initiate lipid accumulation and on caleosin sorting within cell as well as on its association with lipid droplets. Our results strongly suggest that the N-terminal domain is essential for proper protein sorting and targeting to lipid droplets but not for enhancing lipid accumulation.

Introduction

Lipid droplets, also called lipid bodies, are organelles actively involved in lipid metabolism. They are widespread among species from all kingdoms, in which they have general function or are associated with special metabolism or living conditions. The composition of lipid droplets is simple: the highly hydrophobic lipid core is surrounded by a phospholipid monolayer and associated proteins. The number and type of stored lipids as well as the exact phospholipids and proteins involved depend on the specific origin of lipid droplets. In the last decade, proteomic and genetic studies on this cellular compartment have revealed that lipid droplets are not inert fat deposits, but complex, dynamic organelles with a crucial role in various metabolic activities, e.g. metabolism regulation, cell signalling, insulin response and response to abiotic and biotic stress [1–4].

In plants, lipid droplets can be found in several tissues, but the most typical site is in endosperm tissue in seeds, where they are a source of energy and carbon needed during seedling growth. The protein coat of plant lipid droplets contains proteins specific to lipid droplets (e.g. oleosins) as well as non-specific proteins (e.g. enzymes of lipid metabolism). The so-called structural proteins associated with plant lipid droplets, namely oleosin and caleosin, have an original "tri-block" amphiphilic structure consisting of a long hydrophobic inner domain, which ensures interaction with the lipid core, flanked by two hydrophilic domains (N- and C-termini), which allow interactions with the headgroups of phospholipids and cellular components [5]. These proteins stabilise lipid droplets and ensure their integrity as distinct particles inside the cells. Lipid droplets stabilised uniquely by caleosin are smaller and possess higher thermostability [6–9].

Oleosins and caleosins differ in the length of their hydrophobic domains: in oleosin, the hydrophobic domain reaches up to 76 amino acid residues, whereas caleosins contain a hydrophobic domain of approximately 30–40 amino acids. Interestingly, the hydrophobic domain in oleosins is proportionally as long as each flanking hydrophilic domain, whereas the hydrophobic domain in caleosins reaches only up to half of the length of each flanking hydrophilic domain. *In vitro* and *in vivo* experiments suggest that the central hydrophobic and the N-terminal domains [10–13] are both important for the incorporation of oleosin into the lipid droplet. In contrast, oleosins lacking the C-terminal domain are capable of stable membrane insertion [12,14]. The hydrophobic domain possesses a central, highly conserved "proline-knot motif" that consists of several prolines separated by a variable number of amino acid residues. This motif (P-6x-S-P-3x-P) and, more specifically, the prolines, form a loop that leads to a deeper and more stable incorporation of the protein inside the triacylglycerols (TAGs) of the lipid core [12]. Mutations in the proline positions

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http://dx.doi.org/10.1016/j.abb.2015.05.008
0003-9861/

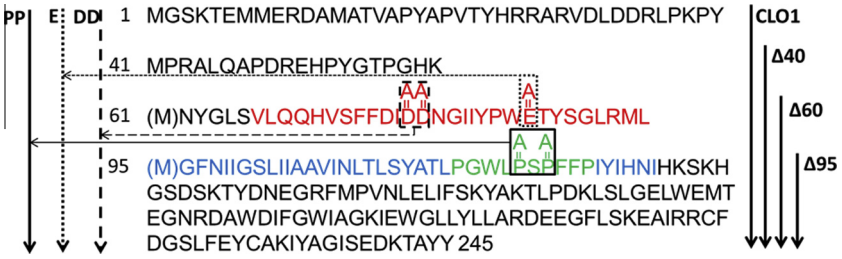

Fig. 1. Caleosin amino acid sequence. Caleosin is a 245 amino acid protein with an identified domain, the calcium-binding EF-hand domain, between amino acids 66 and 94 (in red), and a hydrophobic region between amino acids 95 and 133 (in blue). The caleosin variants used in this study are indicated. The truncated proteins started at position 41 (Δ40 mutant), 61 (Δ60 mutant), and 95 (Δ95 mutant) with additional methionine for the convenient translation of constructs when necessary. Point mutations were also generated to induce amino acid replacement in the EF-hand or proline-knot motif (in green). Proteins with the modification of aspartic acids 77 and 78 (DD mutant), glutamic acid 86 (E mutant), or proline 122 and 124 (PP mutant) into alanine were constructed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the proline-knot motif prevent oleosins from incorporating lipid droplets and result in intrinsic instability in the oleosin polypeptide during trafficking [12]. Caleosin and oleosin may be spontaneously targeted to artificial oil emulsions *in vitro* in a co-translational mode [12,15]. Although there may be a signal recognition particle for targeting oleosins to lipid droplets and the endoplasmic reticulum (ER) [11,12,16,17], no specific signal sequence has been detected in the N-terminal domain of these proteins. In contrast, sequences essential for oleosin and caleosin targeting to the ER are present in the hydrophobic domain and the N- and C-terminal domains play a minor or insignificant role in this process [16,17]. *In vitro*, the hydrophobic domain is required for caleosin incorporation into lipid droplets and thus their stabilisation, suggesting that the subdomain comprising the proline-knot motif for caleosins (P-3x-P-x-P-2x-P) is crucial for targeting them to lipid droplets [15]. For caleosin, a truncated hydrophobic domain negatively affects the stability of artificial lipid droplets, suggesting that the whole length of the hydrophobic domain (ca. 40 residues) is required for stable lipid droplets [18].

Caleosin, initially described only as a structural analog of oleosin, is a protein that is instrumental in stabilising lipid droplets. Furthermore, in contrast to oleosin, caleosin is clearly a multifunctional protein that also participates in the signalling cascade during the stress response, biogenesis and mobilisation of lipid droplets [9,19–28]. Calcium, a common cellular signalling and messenger molecule, is an important partner for caleosin function in these dynamic processes [23,29,30]. Calcium interacts with caleosins through a binding domain, the EF-hand motif situated in the N-terminus just before the central hydrophobic domain (see Fig. 1) [6,19,20]. Highly conserved aspartates or glutamates are crucial residues for calcium binding by providing oxygen ligands that can chelate the calcium ion [31–33].

Plant lipid droplets have recently emerged as a new target for plant biotechnology to improve lipid storage, i.e. oil production, in various oleaginous seeds or as a tool for heterologous plant expression systems for pharmaceutical proteins [34,35]. Identification of the molecular and cellular factors involved in the biogenesis and mobilisation of storage lipid droplets is therefore vital for determining the key factors involved in the stability of these organelles. Stable lipid droplets are central to developing economical and efficient production methods for the agricultural or biotechnology sectors to provide alternative and renewable sources of lipid-rich biomass for biofuels and other biomaterials from plants to microorganisms.

Saccharomyces cerevisiae is a powerful tool for the production and functional study of eukaryotic recombinant proteins. It is a good model for studying the incorporation of caleosin and other lipid droplets integral proteins in lipid droplets [17,24,36–38]. Here, *S. cerevisiae* was used as an *in vivo* model expressing a mutated form of caleosin to determine the structural motif necessary for lipid droplet targeting and stabilisation.

Results

Mutations in the N-terminal and central domains do not influence the ability of caleosin to promote lipid accumulation in yeast

In a previous study, we expressed *Arabidopsis thaliana* caleosin CLO1 (AtClo1, At4g26740)[1] in *S. cerevisiae* [39]. This protein is targeted to lipid droplets and constitutes their major protein. To investigate the role of the domains or residues involved in lipid droplet targeting, full-length caleosin and its variants were fused with the green fluorescent protein (GFP) for co-localisation experiments in yeast. A set of N-terminal-truncated mutants (Δ40, Δ60 and Δ95) was prepared together with point mutants (in the EF-hand motif: D77A + D78A (DD), E86A (E) and in the proline-knot motif: P122A + P124A (PP), see Fig. 1) and cloned under the GAL1-inducible promoter. These constructs were first introduced in the BY4741 strain (Table 2). After 4 h of induction in galactose-containing medium, the size and the expression level of mutated proteins were checked by Western blot on total protein extracts using GFP antibodies and GAS1p antibodies as loading controls (Fig. 2A). We observed the presence of bands with the expected sizes for each construct. Moreover, the expression of the caleosin isoforms was similar to the GFP fusion protein: GAS1p signal ratio: between 3.2 and 5.2. For the Δ40- and Δ60-expressing cells, we observed an additional band with an apparent molecular weight identical that observed in the GFP-expressing cells. We concluded that these bands correspond to free GFP, a possible consequence of partial degradation of the fusion protein. This additional band was not observed for the other caleosin isoforms.

[1] *Abbreviations*: CLO1, caleosin, protein AtClo1, At4g26740; DD, caleosin isoform mutated at positions D77A and D78A; DPM1, dolichol-phosphate mannosyltransferase; E, caleosin isoform mutated at position E86A; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; ERG6p, sterol 24-C-methyltransferase; FAME, fatty acid methyl esters; FID, flame ionization detection; GAL1, galactokinase; GAS1p,glycosylphosphatidylinositol-anchored plasma membrane glycoprotein; GC, gaschromatography; GFP, green fluorescent protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPTLC, high performance thin layer chromatography; Δ40, N-terminal-truncated caleosin mutant lacking the first 40 amino acids; Δ60, N-terminal-truncated caleosin mutant lacking the first 60 amino acids; Δ95, N-terminal-truncated caleosin mutant lacking the first 95 amino acids; MOPS, 3-(N-morpholino)propanesulfonic acid; NB, neutralisation buffer; PP, caleosin isoform mutated at positions P122A and P124A; PVDF, polyvinylidene difluoride; RFP, red fluorescent protein; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE, sterol esters; TAG, triacylglycerols; TCA, trichloroacetic acid; UOD, unit of optical density; wt, wild-type protein.