Trends Article:

The Oxygen-Dependent Modification of Triacylglycerols and Phospholipids, the Different Way of Initiating Lipid Body Mobilization

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For plant seedlings dependent on the breakdown of lipid reserves for gluconeogenesis the onset of lipid mobilization is a critically important process. Until recently, knowledge about the chemical mechanism of the initial breakdown steps has been limited, because the lipolytic activities, characterized by the intracellular localization and the type of substrate, have not always been found. As seedlings that depend on the reserves in lipid bodies may differ in morphology and biochemistry, it is likely that more than one pathway exists. Recent studies with cucumber (*Cucumis sativus*) cotyledons have shown that a lipid body-associated lipoxygenase, and an O_2-dependent reaction, initiate both the destabilization of the phospholipid monolayer and the breakdown of triglycerides. Both types of reactions rely on the particular properties of a lipoxygenase isoform exclusively present in lipid bodies.

Plants utilize different classes of chemical substances to store energy and reserves for carbon metabolism: carbohydrates in plastids, proteins in protein bodies, and lipids in lipid bodies. The processes used to mobilize starch in plastids (Beck and Ziegler, 1989) or proteins in protein bodies (Chrispeels, 1991) are understood at least conceptionally, whereas the onset of fat mobilization in lipid bodies is without a consistent concept. What is needed is not so much a hypothesis to explain the signals for activating genes governing hydrolysis than hypotheses to design how to study the first chemical and physical means by which an attack against the integrity of the storage organelle, i. e. lipid body, is elicited. At present, a new concept is arising which includes molecular oxygen as reagent for the activation of mobilization and a lipoxygenase as means for initiating the reaction sequence.

Occurrence and structural features of lipid bodies

The compartment for storing triacylglycerols is surrounded by a phospholipid monolayer and covered by a small set of characteristic proteins. One kind of surface proteins, called *oleosins*, stabilizes the organelle and thus prevents the oil structures from fusing (Murphy, 1990; Li *et al.,* 1993; Huang, 1994). The organelles are designated as lipid bodies, oleosomes, spherosomes or oil bodies. Oleosins characterize the lipid body surface from the beginning of the organelle formation, i.e. during seed maturation, whereas other proteins are synthesized and transported to the lipid body surface at a later developmental stage. Lipid bodies also acquire newly synthesized protein during lipid mobilization, i.e. during seed germination.

The most prominent protein synthesized de novo and transferred to lipid bodies is a particular isoform of *lipoxygenase*. Fig. 1 reveals that antibodies raised against lipoxygenase stain different structures when cells at an early and a late stage of germination are compared. The example shows a tissue at day 1 of germination when lipid bodies occupy a defined region and a not too small proportion of the intracellular space. At that stage, the immunoreactive zone seems to be a distinct layer of small organelles surrounding large organelles. In contrast, at day 4 of germination the expression of cytosolic lipoxygenase forms is overilluminating all other structures present.

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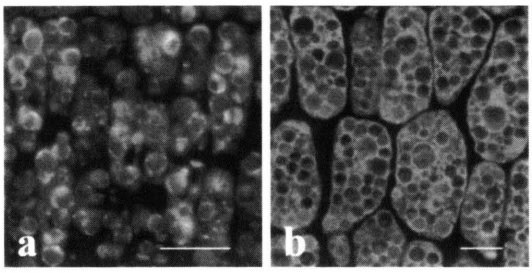

Fig. 1. Immunocytochemical staining of lipid bodies in cotyledons of cucumber seedlings. Applying anti-lipoxygenase antibodies and immunofluorescence it was revealed that lipid-bodies do not contain lipoxygenase prior to 4 h after imbibition but are decorated with this protein at day 1 of germination (a). A localized staining in a layer surrounding the large structures can be observed. Later, at day 4 (b), the lipid body-confined signal was outshone by the intensive immunoreaction with newly formed lipoxygenase localised in the cytosol. The bar indicates 20 μm. The picture was provided by Dr. B. Hause, Halle.

The term lipid body connotes that, irrespective of the occurrence in dormant organs like endosperm or cotyledons, the lipid-protein structures may consist also of lipids other than triacylglycerols. There is a number of reports on lipid-protein particles which can be isolated by flotation centrifugation of the cytosol, e.g. from senescing tissues (McKegney *et al.*, 1995). The large low-density particles are distinguishable from lipid bodies in that they contain a lower percentage of triacylglycerols, but more sterols. If it turns out that they also encompass oleosins, then they should also be discussed like lipid bodies.

Chemical reactions involved in triacylglycerol/phospholipid mobilization

Triacylglycerols containing a high proportion of linoleoyl moieties are energy and building block currencies frequently used by plants in storage tissues. Previous hypotheses of lipid body mobilization mostly implied a hydrolytic reaction as a first step taking place at the lipid body surface (Huang, 1992). However, lipases with the appropriate properties were not found always at the right intracellular site. Furthermore, one may anticipate that several different mechanisms exist for the initiation of lipid bodies. In maize scutellum (Wang and Huang, 1987), and possibly in cotton, does a true lipase appear at the right developmental stage. In castor bean seedlings, a Ca^{2+}-stimulated neutral lipase has been described besides an acidic lipase (Hills and Beevers, 1987). The neutral lipase is absent from dry seeds, but appears at day 3 of germination. Other seed-specific lipases do not fully fit into the scheme of lipase as a constituent of lipid body during the onset of mobilization. Instead, glyoxysomes seem to possess a hydrolytic activity, though in the form of a monoacylglycerol or diacylglycerol esterase.

An initiation of lipid body mobilization mediated by an O_2-dependent reaction rather than by a hydrolytic reaction was recently found. For cotyledons of cucumber and soybean (Feussner and Kindl, 1992; Feussner *et al.*, 1995), it has been shown that a lipoxygenase is being transferred to lipid bodies during germination (Sturm *et al.*, 1985; Radetzky *et al.*, 1993; Feussner *et al.*, 1996). In addition, it was demonstrated by in vivo biosynthesis (Höhne *et al.*, 1996), mRNA levels, and immunocytochemical staining (Feussner *et al.*, 1996) that the decoration of the lipid body surface with a newly synthesized lipoxygenase is a very early process during germination.

The lipoxygenase form located at the lipid body surface can be differentiated from other plant lipoxygenases (Hildebrand, 1989; Rosahl, 1996). It was characterized by its product specificity: by converting linoleic acid or the linoleoyl moiety of triacylglycerols or phospholipids exclusively to S-13-hydroperoxydes of 9-*cis*,11-*trans*-octadecadienoyl derivatives (Fig. 2). This characterization of lipoxygenase *in vitro* was paralleled by the analysis of the primary products formed in lipid bodies during the onset of lipid body mobilization: S-13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoyl moieties increase in lipid bodies substantially in the fraction of triacylglycerols and also amount to a very high percentage in the phospholipid fraction. Almost at the same time, free S-13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid appears in the cytosol; here, the ratio of S-13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid to linoleic acid is higher than 10:1, suggesting a preferential hydrolytic cleavage of oxidized triacylglycerols. The product analyses show the same extremely high regio- and stereoselectivity for both the activity of the isolated enzyme and the unimpaired process *in vivo* (Feussner *et al.*, 1995). Also other lipoxygenases

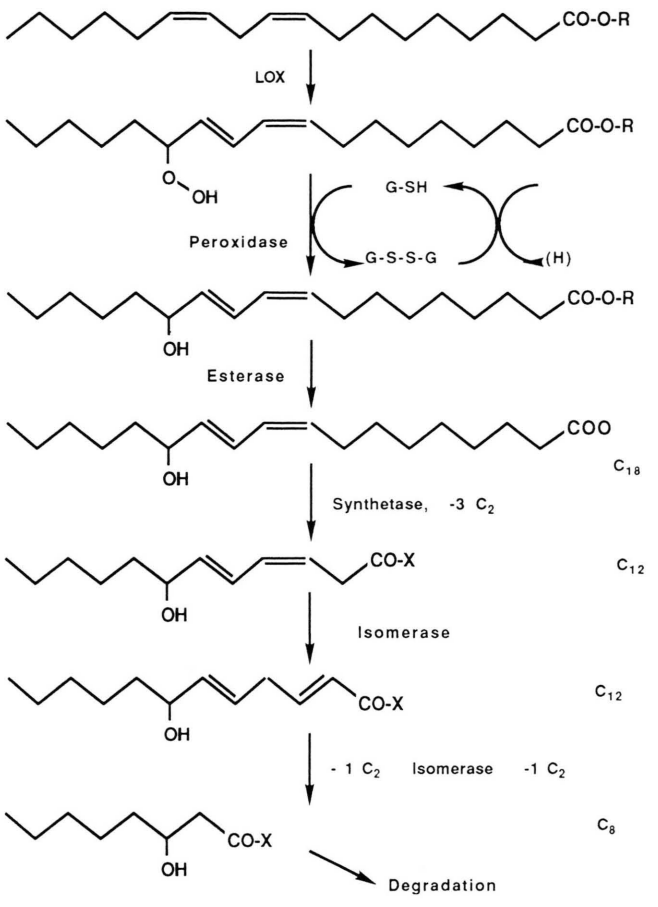

Fig. 2. Formation and degradation of a 13-hydroxy acid moiety deriving from linoleoyl groups. The pathway involves an O_2-dependent step, reduction, hydrolysis and a synthetase reaction at the level of C_{18}. Chain shortening (symbolized as $-C_2$) and isomerization eventually lead to L-3-hydroxyoctanoyl-CoA.

have been found which are able to act on trilinolein (Matsui and Kajiwara, 1995).

Four days after germination, half the amount of linoleoyl groups in the phospholipid fraction were converted into S-13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoyl moieties. Thus, it is conceivable that membranes lose structural and functional integrity under these conditions. Furthermore, it may be that the introduction of a hydroperoxy- or hydroxy-group into the phospholipid side chain forces the molecule to flip out the membrane layer, thus possibly causing holes. It has been shown that the incorporation of more than 3% oxidized phospholipid into a bilayer alters the structural and functional characteristics of model membranes (Isaacson *et al.*, 1990). Membrane dysfunction therefore plays a decisive role in initiation of attack of triacylglycerols. Lipid peroxidation and membrane destabilization are also fundamental features in plant senescence processes and the hypersensitive response (Keppler and Novacky, 1987). The onset of mobilization on the lipid body surface also seems to be comparable to the dose-dependent increase of lipid peroxidation produced by UV light in the liposomal membranes (Bose *et al.*, 1990).

It is hypothesized (Fig. 2) that the formation of S-13-hydroxyoctadecadienoic acid which is subsequently released into the cytosol proceeds by the sequential action of three enzymes: the lipoxygenase, the glutathione-dependent peroxidase, and an esterase distinguished by a preferrence for S-13-hydroxyoctadecadienoyl esters compared to linoleoyl esters. A phospholipid hydroperoxide glutathione peroxidase has recently been described in salt-stressed citrus cells (Beeor-Tzahar *et al.*, 1996). A similar enzyme in *Avena fatua* is expressed early during imbibition of seeds (Johnson

et al., 1995). For mammalian cells, it has been shown that the phospholipid hydroperoxide glutathione peroxidase is suited to reduce hydroperoxy ester lipids originating by the function of 15-lipoxygenase (Schnurr *et al.*, 1996). This indicates that cooperation between lipoxygenase and glutathione peroxidase may be a general property in the alteration of membrane properties. For lipid bodies, destabilization of the phospholipid monolayer maybe the prerequisite for the action of both the hydroperoxide peroxidase and the lipolytic enzyme already present at the surface of the lipid bodies.

Less clear is the question as to whether the action of the as yet studied classical lipase always is responsible for the triacylglycerol degradation. In cases when modified rather than unmodified triglycerides are the actual substrates, also the enzyme acting on them may be a particular hydrolase. As the available data do not provide a general and consistent view, is it necessary to look for further candidates: co-lipases, endoplasmic reticulum-bound hydrolases or constituents of lytic vesicles?

The central position of S-13-hydroxyoctadecadienoic acid as transport metabolite between lipid bodies and glyoxysomes is well documented (Feussner and Kuhn, 1995; Feussner *et al.*, 1995). For fatty acids β-oxidation taking place in the glyoxysomes (Kindl, 1987) the fact that S-13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid and not 9-*cis*,12-*cis*-octadecadienoic acid (linoleic acid) is the substrate has some implications. If linoleic acid acts as substrate for the β-oxidation the enzyme machinery not only requires acyl-CoA synthetase, the multifunctional protein and thiolase (Kindl, 1993) but also auxiliary enzymes that resolve the *cis*-double bond at bond 9 and 12. Any *cis*-double bond extending form an uneven numbered C-atom needs an additional isomerase eventually converting a 3-*cis* compound into a 2-*trans*-derivative (Engeland and Kindl, 1991) whereas a *cis*-double bond extending from an even-numbered C-atom relies on the function of an epimerase (Engeland and Kindl, 1991; Preisig-Müller *et al.*, 1994). Since S-13-hydroxyoctadecadienoic acid rather than linoleic acid is the product released from lipid bodies the metabolic sequence of the fatty acid degradation does not have to handle a 2-*cis*-enoyl-CoA and does not need an epimerase. The 9-*cis* bond originally found in linoleoyl moiety, and the 15-*cis*-bond in linolenoyl moiety, are still to be circumvented by an isomerase acting on 3-*cis*-enoyl-CoA. The 12-*cis*-bond in the structure of linoleoyl moieties, however, is already changed to an 11-*trans*-bond by the lipoxygenase reaction. In addition, the hydroxy-function originally found as S-13-hydroxyoctadecadienoic acid affords an L-3-hydroxyacyl-CoA at the level of C_8 thus leading directly into the sequence of standard β-oxidation (Fig. 2).

This concept and this pathway imply that the linoleoyl moiety is the primary target at the triacylglycerols. Subsequently, an as yet known lipase or an esterase acting preferentially on hydroxyoctodecadienoyl residues is required at the lipid body surface whereas diacylglycerols are split by glyoxysomes. But the question arises: is there a generally applicable sequence of steps in mobilizing the lipid body? Or is the onset of fat mobilization much more dependent on the special equipment of the individual seed species? Do we have to imply several pathways proceeding in parallel? Do alternative pathways occur in one and the same tissue, or do they have to be considered when different plants with greatly differing linoleic acid content are compared? Clearly, the oxygen-dependent pathway will not be effective when seeds have undetectable levels of polyunsaturated lipids.

The interfaces of an endoplasmic reticulum-lipid body-glyoxysome network

Electron micrographs frequently show lipid bodies and glyoxysomes pressed against each other. This may be explained just by considering the tight arrangement of organelles in the fat-rich tissue. However, specific interactions and extensive exchange of metabolites have been implicated to account for the phenomenon. Allowing for the reaction sequence outlined in Fig. 2, it may be that 13-hydroxyoctadecadienoic acid is a metabolite transferred from the lipid body surface to the glyoxysomal membrane. However, it is presently unknown whether diacylglycerols represent the other class of metabolites being passed over to the glyoxysomal membrane. Corresponding to the equipment of a particular plant tissue with triacylglycerol- or diacylglycerol-hydrolyzing enzymes, one,

two or all of the following steps may occur: (1) hydroxyoctadecadienoic acid is cleaved off by a lipid body-bound lipase, and a diglycerol rich in oleoyl groups passes over to the glyoxysomal membrane where it is further hydrolized; (2) all acyl groups are cleaved off by a lipid body-associated lipase and only salts of fatty acid reach the glyoxysomal surface; (3) a glyoxysomal lipase comes into close contact with the destabilized lipid body surface and starts attacking the 13-hydroxyoctadecadienoyl esters.

All data presently available indicate that in soybean and cucumber seedlings step (3) operates or a variation of it applies. The glyoxysomal lipase hydrolyses tri-, di-, and monoacylglycerols of linoleic acid (Lin *et al.,* 1982; K. Schwennesen and H. Kindl, unpublished). As substrates for the glyoxysomal lipase, lipid bodies modified during the onset of germination but not lipid bodies from dry seeds were accepted. Both lipid body lipoxygenase and glyoxysomal lipase exhibit a rather alkaline pH optimum. In maize and cotton, however, the lipid mobilization may take place according to step (2): that is visualized by the equipment of lipid bodies with a true lipase acting only at an oil-water interphase. Interestingly, the lipase activity was found slightly behind the schedule of lipid degradation. The lipase activity was not present in dry seeds, started to appear on lipid bodies at day 3 of germination and reached maximal values ot day 6 (Lin *et al.,* 1983). In rape seed, a germination-specific lipase was detected in a microsomal fraction (Theimer and Rosnitschek, 1978).

These concepts are rather hypothetical in detail and may thus need some further refinements. E.g. if the ER does play a direct role in the interplay of lipid bodies and glyoxysomes. Rather than a lipase formed on free polysomes and targetted by an as yet unknown mechanism to the lipid body surface a germination-specific ER-bound hydrolase may take over the task of ester hydrolysis.

How can we visualize a direct contact between lipid body and ER? This is a question persistent for the biosynthesis of oleosins, the function of lipoxygenase and, probably, for the operation of a putative hydrolase synthesized and first transported into the ER. In all three cases, we have to envisage a subsequent lateral transfer along a membrane phase.

While the interface between lipid bodies and glyoxysomes may still turn out to be a thin layer of cytosol, the interface between ER and lipid bodies may be a more direct one. It seems to be not so surprising if it were necessary to re-consider the term appendix (Wanner and Theimer, 1978), an intermediary structure characterized by properties of both the continuum to lipid body surface layer and to the double membrane of the ER.

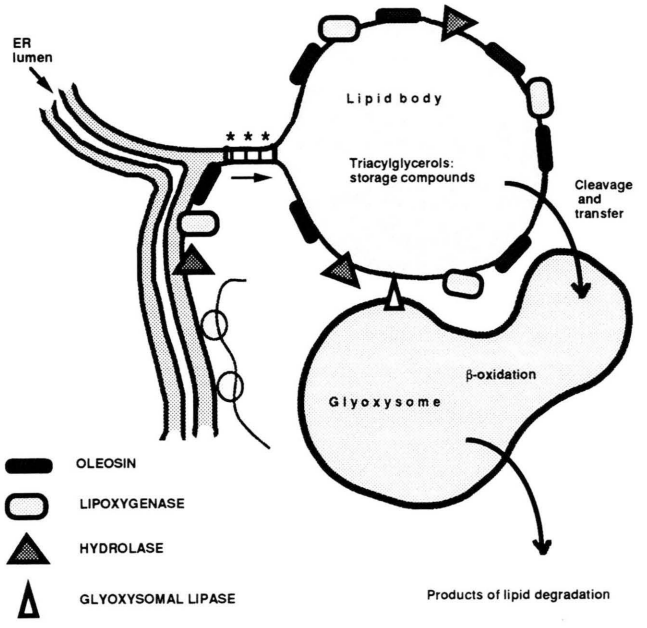

Fig. 3. A schematic drawing of interphases emphasizes the salient point of a working hypothesis. For the transfer of lipoxygenase from the ER to the lipid body, a lateral diffusion along different membrane phases is proposed. Most critically, a bridge between the double layer membrane of the ER and the monolayer of the lipid body surface is delineated (indicated by stars) in analogy to the ER-like annex observed on lipid bodies.

Fig. 3 is based on the existence of such an appendix, and illustrates the main features of a hypothesis that is basically in agreement with morphological and biochemical data. However, whether there is an opening between the ER lumen and the lipid phase of the lipid body is still controversial among electron microscopists. If there is continuity between ER and lipid bodies, it excludes a role of Golgi vesicles as intermediary structures of the intracellular transport. This is in contrast to previous results which have Golgi vesicles not totally eliminated from functioning in the biosynthesis of lipid bodies (Sturm *et al.*, 1985; Radetzky *et al.*, 1993).

How does the appearance and localization of lipase fit into the concept of an ER-lipid body-glyoxysome network? Lipase in the scutellum of maize seedlings is synthesized on free polysomes (Wang and Huang, 1987) and transferred to lipid bodies by an as yet unknown mechanism. As suggested, oleosins might be utilized as tools for targeting proteins such as lipase to the lipid body surface. In contrast, it may be hypothesized that the intracellular targetting processes are the same for all protein constituents of lipid bodies, i.e. identical to the targeting process effective for oleosins. This would not apply to the kind of lipase investigated in the last decade, but would be true for an ER-associated hydrolase.

It remains to be established whether other metabolic sequences starting with linoleoyl moieties, such as the pathway to eicosanoids (Weller and Dvorak, 1994), jasmonate (Sembdner and Parthier, 1993) or pheromones (Wendel and Juttner, 1996), originate from lipid bodies. This would require the targeting of additional enzymes to the lipid body surface.

Temporal development of the mobilization and fat degradation machinery

The expression of lipid body lipoxygenase during germination precedes the expression of other lipoxygenase forms. Lipid body lipoxygenase is not present during the stage of dry seed. Comparing its biosynthesis and attachement to lipid bodies with the processes taking place at glyoxysomes during germination, the appearance of lipoxygenase at the lipid bodies coincides with the first steps prior to the enlargement of glyoxysomal precursor structures. Some initial steps in glyoxysomal differentiation precede the endowment of glyoxysomes with enzymes (Kindl, 1982). The enlargement of small glyoxysome-like vesicles is coupled with the establishment of chaperones on their surface (Preisig-Müller *et al.*, 1994). At the same time when by synthesis and transport of the lipoxygenase the mobilization of the lipid bodies is initiated the small glyoxysomal structures are prepared for the import of enzyme precursors (Fig. 4).

In contrast to the re-designing of lipid bodies during germination, lipid bodies have been established during seed maturation by integration of oleosins synthesized at the ER (Hills *et al.*, 1993; Loer and Herman, 1993). The information con-

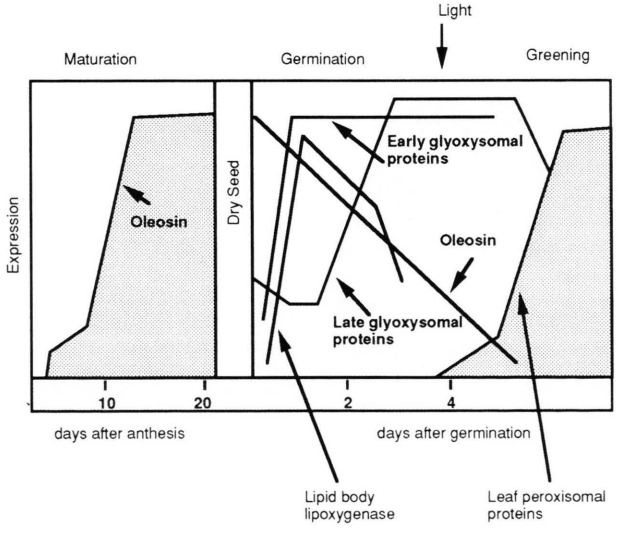

Fig. 4. Time course of levels of organellar proteins characterizing consecutive stages of lipid body and microbody development. During seed formation and maturation, the appearance of oleosins parallels the formation of lipid bodies whereas glyoxysomal forms are present at late stages only. At the onset of germination, de novo synthesis of glyoxysomal chaperones and lipid body lipoxygenase seem to be the earliest events. Later, decrease of triglycerides and increase in glyoxysomal β-oxidation enzymes are observed. With cucurbitaceae, leaf peroxisomal properties can be seen upon greening of the cotyledons.

tained within the oleosin peptide is sufficient that the peptide translated in an unrelated plant is correctly targeted to lipid bodies (Lee *et al.*, 1991; Batchelder *et al.*, 1994). Similar specificity in transport and sorting precision are to be expected for the intracellular transport of the lipid body form of lipoxygenase. Both oleosins and lipid body lipoxygenase are cotranslationally transported into the ER without proteolytic processing. In contrast to the early appearance of lipoxygenase on the lipid body surface (Fig. 4), the activity of lipase reaches maximal values 5 days after onset of germination. Thus, lipase activity persists beyond the time when oleosins are present. The odd correlation of the presence of lipase and the beginning of lipid mobilization strengthens the hypothesis that many plants use an oxygen-dependent step for the initial attack, rather than a hydrolysis.

Collectively, the recent studies support the notion that lipoxygenase is the enzyme that is synthesized, transported and becomes functionally active exactly at the time when the products of lipid body mobilization are required for the seedling's growth. This does not seem to apply fully for the other enzyme candidates implicated in lipid body mobilization. What do oleosins, lipoxygenase, and the postulated esterase have in common? Further biochemical studies will undoubtedly unravel the structure of the putative ER-lipid body interface. The knowledge of the sorting mechanisms between the ER and the lipid body surface will be the key for understanding of the fate of lipid bodies during formation and degradation.

Acknowledgements

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