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Membrane-bound fatty acid desaturases are inserted co-translationally into the ER and contain different ER retrieval motifs at their carboxy termini

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Summary

Fatty acid desaturases (FADs) play a prominent role in plant lipid metabolism and are located in various subcellular compartments, including the endoplasmic reticulum (ER). To investigate the biogenesis of ER-localized membrane-bound FADs, we characterized the mechanisms responsible for insertion of *Arabidopsis* FAD2 and *Brassica* FAD3 into ER membranes and determined the molecular signals that maintain their ER residency. Using *in vitro* transcription/translation reactions with ER-derived microsomes, we show that both FAD2 and FAD3 are efficiently integrated into membranes by a co-translational, translocon-mediated pathway. We also demonstrate that while the C-terminus of FAD3 (-KSKIN) contains a functional prototypic dilysine ER retrieval motif, FAD2 contains a novel C-terminal aromatic amino acid-containing sequence (-YNNKL) that is both necessary and sufficient for maintaining localization in the ER. Co-expression of a membrane-bound reporter protein containing the FAD2 C-terminus with a dominant-negative mutant of ADP-ribosylation factor (Arf)1 abolished transient localization of the reporter protein in the Golgi, indicating that the FAD2 peptide signal acts as an ER retrieval motif. Mutational analysis of the FAD2 ER retrieval signal revealed a sequence-specific motif consisting of Φ-X-X-K/R/D/E-Φ-COOH, where -Φ- are large hydrophobic amino acid residues. Interestingly, this aromatic motif was present in a variety of other known and putative ER membrane proteins, including cytochrome P450 and the peroxisomal biogenesis factor Pex10p. Taken together, these data describe the insertion and retrieval mechanisms of FADs and define a new ER localization signal in plants that is responsible for the retrieval of escaped membrane proteins back to the ER.

Keywords: fatty acid desaturase, endoplasmic reticulum, co-translational translocation, dilysine motif, aromatic motif, ER retrieval.

Introduction

The secretory pathway of eukaryotic cells consists of a series of discrete, membrane-bound compartments, including the endoplasmic reticulum (ER), Golgi, and vacuoles (reviewed by Hawes *et al.*, 1999; Vitale and Denecke, 1999). Transport of proteins and lipids between these various subcellular locations is continuously in flux in both anterograde and retrograde directions and is mediated by a vesicle carrier system that allows each organelle to preserve a unique set of resident proteins (and lipids), while at the same time selectively packaging other proteins destined for transport to different sites in the pathway. The final location of proteins in the pathway depends, therefore, on their ability to be incorporated into, or excluded from, transport vesicles, a process that is influenced by the presence of different molecular targeting signals contained in the protein's primary structure.

It is now widely accepted that the localization of ER resident proteins is dependent upon at least two types of molecular targeting signals: one signal that is responsible for initial sorting to the ER and a second signal that is

responsible for either static retention in the ER or retrieval from other compartments in the secretory pathway. For instance, the sorting of soluble proteins (reticuloplasmins) to the ER lumen is mediated by a cleavable, N-terminal hydrophobic signal sequence that is recognized by the signal recognition particle (SRP) and directs co-translational translocation (reviewed by Kermode, 1996). Maintenance of these proteins in the ER is then mediated by a C-terminal tetrapeptide motif –H/K/RDEL, an ER retrieval signal that is recognized by its cognate receptor, Erd2p, in a Golgi compartment and leads to retrograde transport of the escaped proteins back to the ER (reviewed by Denecke, 2003). Extensive mutational analysis of the –H/K/RDEL signal in evolutionarily diverse organisms revealed that any non-conserved amino acid changes to the motif result in a complete loss of ER retrieval and subsequent secretion of the modified protein from the cell (Denecke *et al.*, 1992; Haugejorden *et al.*, 1991).

Membrane-spanning resident ER proteins can also contain a cleavable N-terminal signal sequence for initial targeting from the cytosol to the ER. However, more often, the first transmembrane domain (TMD) acts as a non-cleavable signal sequence that interacts with SRP, directs co-translational protein synthesis and then acts as a stop transfer sequence for anchoring the protein in the membrane (Johnson and van Waes, 1999). In yeast and mammalian cells, the residency of membrane proteins in the ER is maintained by a number of well-defined signals, including cytoplasmically exposed C-terminal dilysines (-KKXX or -KXKXX), N-terminal or internally positioned di-arginines (-XXRR- or -RXR-; Zerangue *et al.*, 1999), or various C-terminal aromatic amino acid-enriched motifs (e.g. -YXXL- and -W/YX$_{2-3}$W/Y/F-), as well as determinants present in the TMD or the oligomeric status of the protein (reviewed by Gomord *et al.*, 1999; Pelham, 2000; Teasdale and Jackson, 1996). Resident ER membrane proteins may contain more than one of these signals, and a disruption of these signal(s) normally results in the modified protein being mislocalized to a post-ER compartment such as the vacuolar membrane (in yeast: Gaynor *et al.*, 1994; Roberts *et al.*, 1992) or the plasma membrane (in mammalian cells; Jackson *et al.*, 1990). In plants, the molecular signals responsible for maintaining membrane-bound proteins in the ER are far less understood. For example, only three studies have provided a role of either C-terminal (di)lysine residues (Barrieu and Chrispeels, 1999; Benghezal *et al.*, 2000) or the length of the TMD (Brandizzi *et al.*, 2002a) as ER retrieval signals. A great deal of attention has been focused, however, on the localization of proteins such as Erd2p, Sar1p and Arf GTPases, and Rer1p that serve as part of the machinery responsible for mediating the sorting of membrane and/or soluble protein cargo through the plant secretory pathway (reviewed by Denecke, 2003). Overall, the results of these studies have led to a working model whereby most plant resident ER membrane proteins, like their yeast and mammalian counterparts, are transported back from Golgi compartments to the ER by interacting directly or indirectly with specific protein factors, including the coat protein I (COPI) complex, a membrane coat composed of seven subunits that assembles on transport vesicles and directs Golgi-derived retrograde transport (reviewed by Aniento *et al.*, 2003). Any disruption of an ER retrieval signal that would normally mediate the transport back to the ER results in the accumulation of the membrane protein in the plasma membrane (Brandizzi *et al.*, 2002a).

Many resident ER membrane proteins play a central role in lipid metabolism. For example, ER membrane-bound fatty acid desaturases (FADs) synthesize linoleic (18 : $2\Delta^{9cis,12cis}$) and linolenic acids (18 : $3\Delta^{9cis,12cis,15cis}$), both of which are common components of cellular membranes and commercial vegetable oils (reviewed by Ohlrogge and Browse, 1995). The synthesis of these two fatty acids proceeds by sequential desaturation of oleic acid (18 : $1\Delta^{9cis}$) to linoleic acid by Δ^{12} oleate desaturase (FAD2), and then linoleic acid to linolenic acid by ω-3-fatty acid desaturase (FAD3). In addition to FAD2 and FAD3, many diverged FAD2 enzymes have been identified in non-traditional crop plant species that synthesize a variety of structurally unrelated fatty acids (e.g. hydroxy, epoxy, acetylenic, and conjugated fatty acids) that accumulate primarily in the seed oils (reviewed by Shanklin and Cahoon, 1998). Despite the availability of cDNAs encoding both normal and so-called diverged FADs, however, efforts to produce high amounts of these unusual fatty acids in transgenic oilseed crops have been largely unsuccessful. Based on these results, future success in producing genetically engineered oilseed crops with desired traits will likely require a better understanding of the cellular aspects of lipid biosynthesis, including the biogenesis of enzymes such as FADs and the interrelationships between the various subcellular compartments (e.g. plastids, ER, and oil bodies) that participate in these biochemical events (see Dyer and Mullen, 2003 for review).

We are characterizing *Arabidopsis thaliana* FAD2 and *Brassica napus* FAD3 proteins to gain insight into the mechanisms involved in the assembly and regulation of plant FADs in general. Previously, we showed that both of these enzymes were located in the ER of tobacco bright yellow (BY)-2 suspension cells and that their N- and C-termini were oriented on the cytosolic side of the ER membrane (Dyer and Mullen, 2001). Here, we report on the mechanism by which nascent FAD2 and FAD3 are inserted into ER membranes, and we characterize the different C-terminal ER retrieval signals in these two proteins that are responsible for maintaining their steady-state localization in the ER. We also present data that a novel ER retrieval motif present at the C-terminus of *Arabidopsis* FAD2, which resembles aromatic amino acid-enriched ER retrieval signals in a few yeast and mammalian ER membrane proteins,

is also present at the C-terminus of other FAD2 and diverged FAD2 enzymes from different plant species, as well as other putative or known ER membrane proteins in *Arabidopsis*, including several isoforms of cytochrome P450. The implications of these results and the nature of the FAD2 ER retrieval signal in general are discussed.

Results

FADs are inserted co-translationally into ER membranes in vitro

To begin to examine the biogenesis of plant FADs, we investigated whether *Arabidopsis* FAD2 and *Brassica* FAD3 were inserted co- or post-translationally into ER membranes *in vitro*. Figure 1(a,b, lanes 1–3; Co) shows that when FAD2 and FAD3 mRNAs were translated in the presence of ER membranes in a reticulocyte lysate, the majority (>50% for each protein) of the total radiolabeled protein product from each reaction pelleted with membranes in the bottom fraction following centrifugation. In contrast, relatively low amounts of FAD2 (23%) and FAD3 (13%) protein were recovered in bottom fractions when these FADs were incubated with ER microsomes post-translationally (Figure 1a,b, lanes 4–6). The amount of FADs recovered in these bottom fractions was similar to the amounts obtained when no membranes (−mbs) were included in the reactions (lanes 7–9), indicating that a portion of the FAD proteins pellet because of either protein misfolding and/or aggregation rather than membrane binding. Control experiments with rat liver cytochrome b_5 (Cb_5), which inserts post-translationally into the ER (Janiak *et al.*, 1994; Kim *et al.*, 1997), revealed that most of the Cb_5 protein (91%) pelleted with membranes that were added after translation was terminated (Figure 1c, lane 6). Additional control experiments (Figure 1d) using bovine pre-prolactin (PPL), a soluble protein co-translocated into the ER lumen (Gilmore *et al.*, 1982), revealed that the protein was efficiently incorporated into the ER during co-translational reactions (68%), as evidenced by its signal sequence being processed and the formation of lower molecular mass lumenal prolactin (compare lanes 1–3). These control experiments with Cb_5 and PPL indicate that the microsomes used in these assays were competent for both co- and post-translational insertion of proteins, and that FAD2 and FAD3 were inserted co-translationally, and not post-translationally, into ER membranes.

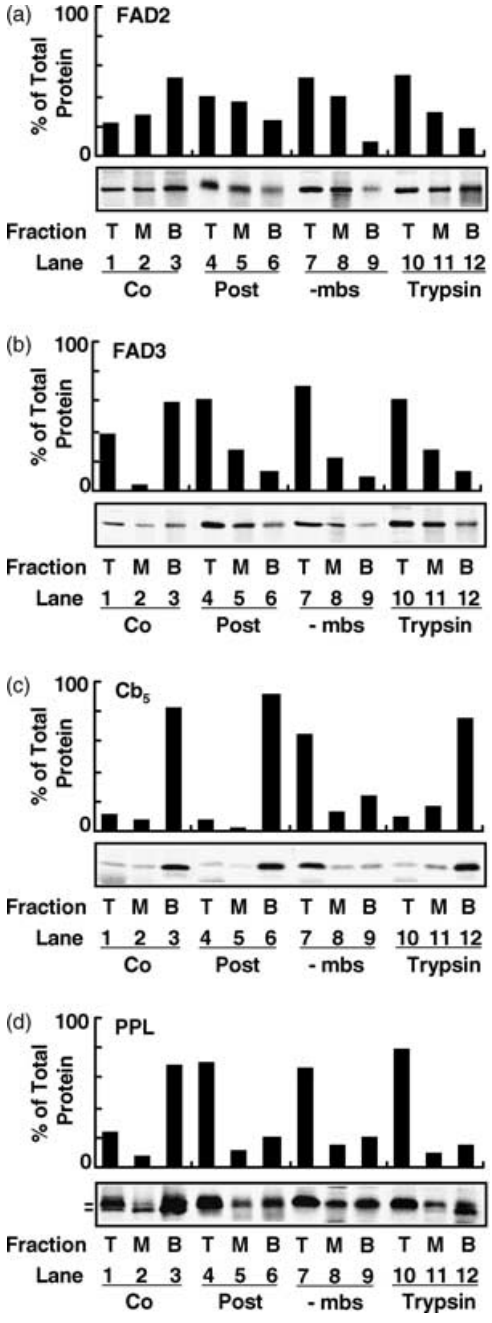

Figure 1. Co- or post-translational insertion of various proteins into ER membranes *in vitro*.

Plasmids containing sequences coding for either (a) FAD2, (b) FAD3, (c) Cb_5, or (d) PPL were transcribed *in vitro* using SP6 polymerase, and transcription products were then treated as follows: Co, transcription products were translated in reticulocyte lysate containing canine pancreatic microsomes; Post, microsomes were added after the reticulocyte lysate translation reactions were terminated; −mbs, no microsomes were added to the reticulyte lysate translation reaction; and Trypsin, transcription products were translated in reticulocyte lysate with microsomes that were pre-treated with trypsin. All reactions were layered on top of a 0.5 M sucrose cushion, and the microsomes were pelleted by centrifugation as described in the Experimental procedures. The resulting gradients were then divided into top (T), middle (M), and bottom (B) fractions. The top and middle fractions contain soluble proteins, and the bottom fraction contains microsomes and microsome-bound proteins. Equivalent amounts of each fraction were analyzed by SDS–PAGE, and the percentage of the total protein recovered from each fraction was determined using a phosphorimager. The migration of higher molecular weight PPL and the lower molecular weight (mature) translocated prolactin are indicated (bars) to the left side of lane 1 in the autoradiogram shown in (d). The data shown are representative of three independent experiments.

Additional evidence for the co-translational binding of FADs to ER membranes came from experiments in which the classical SRP-dependent targeting pathway was disrupted. The SRP receptor α subunit is exposed on the cytosplasmic surface of ER membranes and is susceptible to a mild trypsin treatment. Trypsinization does not however affect other required components of the translocation machinery (Gilmore *et al.*, 1982). Figure 1 (lanes 10–12) shows that membranes incubated with trypsin prior to co-translation reactions were unable to support binding of either FAD2 (only 8% in bottom fraction), FAD3 (8%), or the control protein PPL (13%). However, trypsin digestion had only a slight negative effect on membrane binding of Cb_5 in the reactions (76% versus 82%; compare Figure 1c, lanes 3 and 12) because it is targeted to ER membranes by an SRP-independent pathway (Janiak *et al.*, 1994).

The majority of integral membrane-bound FAD polypeptides are exposed to the cytosol

To determine whether the FAD2 and FAD3 proteins that were observed to bind microsomal membranes in the *in vitro* assay were actually integrated into the membrane bilayer, pellet fractions (Figure 1, lane 3) were re-suspended in 0.1 M sodium carbonate (pH 11.5) to release lumenal and peripheral membrane proteins and then integral membrane proteins were pelleted by centrifugation. As shown in Figure 2(a), the majority of FAD2 (80%) and FAD3 (90%), as well as almost all of the control integral membrane protein Cb_5 (98%), was recovered in the membrane pellet fraction (P) after sodium carbonate extraction, whereas most of the lumenal prolactin protein (86%) was extracted into the supernatant (S). In contrast, FAD2, FAD3 and Cb_5 were extracted in the supernatant when carbonate-extracted membranes were solubilized with Triton X-100 (data not shown), indicating that the FAD proteins were not sedimenting with membranes simply because of aggregation. These results demonstrate that FAD2 and FAD3 were fully integrated into the lipid bilayer during the *in vitro* co-translational reactions.

Next, the topological orientation of ER membrane-integrated FAD2 and FAD3 was assayed by proteolysis with proteinase K. As shown in Figure 2(b), treatment of pellet fractions (lane 3 in Figure 1) with either 0.4, 0.1, or 0.01 μg ml^{-1} proteinase K resulted in almost complete degradation of both FAD2 (compare lane 1 with lanes 2–4) and FAD3 (compare lane 5 with lanes 6–8) proteins, consistent with a previous model that predicts the proteins contain four transmembrane domains and the majority of each polypeptide, including their N- and C-termini, exposed on the cytosolic side of ER membranes (Dyer and Mullen, 2001; Shanklin *et al.*, 1994). Lower molecular mass polypeptides (<14.5 kDa) observed in protease treatments were likely the products of partial digestion as they were more prominent with decreasing amounts of proteinase K. Figure 2(b) also shows the

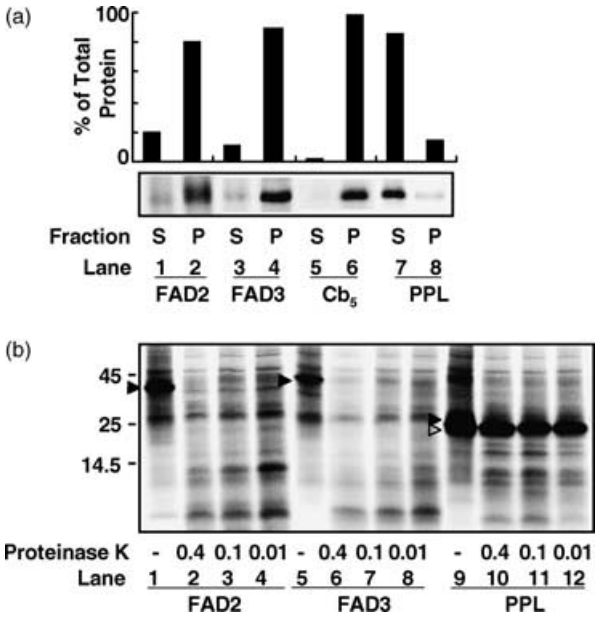

Figure 2. Membrane integration and topological orientation of FAD enzymes.
(a) Sodium carbonate extractions of ER membrane-bound FADs and control proteins. Microsome-associated proteins (bottom fraction from *in vitro* translation assay) were incubated in translation buffer and adjusted to pH 11.5 with Na_2CO_3 for 30 min at 0°C, and then lumenal and perhiperal proteins (S) were separated from integral membrane proteins (P) by centrifugaton. Equivalent amounts of each fraction were then analyzed by SDS–PAGE, and the percentage of the total protein synthesized in the translation reaction that was recovered from each fraction was determined using a phosphorimager.
(b) Protease susceptibility of microsome-associated proteins. Microsome-bound proteins obtained from *in vitro* cotranslation reactions were either left untreated (–) or incubated with different amounts of proteinase K (0.4, 0.1, or 0.01 μg ml^{-1}) for 15 min at 0°C. Proteolysis was terminated by the addition of PMSF, and equivalent amounts of each reaction were then analyzed by SDS–PAGE. The migration positions of molecular mass markers (in kDa) are shown to the left of the panel. The position of full-length translation products of FAD2, FAD3, and PPL are each marked with a solid arrowhead to the left of lane 1, 5, and 9, respectively. The open arrowhead left of lane 9 indicates the position of mature translocated prolactin (PL).The data shown in (a) and (b) are a representative of three independent experiments.

results of control assays with PPL (lanes 9–12), whereby nearly all of the non-translocated PPL (solid arrow left of lane 9) was digested with each of the proteinase K concentrations employed, whereas lumenal (membrane protected) prolactin (open arrow left of lane 9) was undigested. These data for PPL illustrate that the microsomal membranes used for insertion and proteinase K reactions were of sufficient integrity to map topological orientation of proteins.

FAD2 and FAD3 enzymes contain different C-terminal sequences that each resemble ER retrieval motifs

Table 1 shows an alignment of the deduced C-terminal amino acid sequences of FAD2, including diverged FAD2, and FAD3 enzymes from a variety of different plant species. Overall, there is a higher degree of uniquely conserved amino acid residues in the C-termini of FAD2 and FAD3

Table 1 Alignment of C-terminal amino acid sequences from various plant FAD2, diverged FAD2 and FAD3 enzymes

Species	C-terminal sequence
Aleurites fordii FAD2	–YVEADDGDESKGVYWYNKKF
Arachis hypogaea FAD2	–YVEPDDGASQKGVYWYKNKF
Arabidopsis thaliana FAD2	–YVEPDREGDKKGVYWYNNKL
Calendula officinalis FAD2	–YVDKD–EEVKDGVYWYRNKI
Glycine max FAD2	–YVEPDEGTSEKGVYWYRNKY
	** * ***** * *
Aleurites fordii dFAD2	–FVEPDEGDNNNGVFWYSNKF
Crepis alpina dFAD2	–IFIEPEKGRGSKGVYWYNKF
Crepis palaestina dFAD2	–ECMYIEPDSKLKGVYWYHKL
Impatiens balsamina dFAD2	–YVESDVPGKNKGVYWYNNDI
Momordica charantia dFAD2	–DDGATSGSSSKGVFWYHNKL
Ricinus communis dFAD2	–FVEPDEGAPTQGVFWYRNKY
Aleurites fordii FAD3	–VFYQTDPD––IYKVDKSKLN
Arabidopsis thaliana FAD3	–VFYETDPDLYVYASDKSKIN
Brassica napus FAD3-1	–VFYETDPDLYVYASDKSKIN
Brassica napus FAD3-2	–VFYETDPDLYVYASDKSKIN
	*** **** * **** *

The CLUSTALW algorithm was used to create a sequence alignment among the C-terminal portions of either FAD2, diverged FAD2 (dFAD2) or FAD3 enzymes. Sources of all sequences (indicated in single-letter code) shown are from GenBank: *Af* FAD2, AF525534; *Ah* FAD2, AF272950; *At* FAD2, L26296; *Co* FAD2, AF343065; *Gm* FAD2, L43920; *Af* dFAD2, AF525535; *Ca* dFAD2, Y16285; *Cp* dFAD2, Y16283; *Ib* dFAD2, AF182520; *Mc* dFAD2, AF182521; *Rc* dFAD2, U22378; *Af* FAD3, AF047172; *At* FAD3, D265081; *Bn* FAD3-1, L01418; and *Bn* FAD3-2, L22962. Identical amino acid positions for each group of FADs are marked with an asterisk (*) and putative aromatic amino acid-enriched (in FAD2 and dFAD2) and dilysine (in FAD3) ER retrieval signals are underlined.

proteins than that in the C-termini of diverged FAD2 proteins. For instance, all FAD3s shown possess two conserved lysines at the −3 and −5 positions relative to the C-terminus. This conspicuous dilysine motif was previously recognized in *Arabidopsis* FAD3 (Arondel *et al.*, 1992; Gomord *et al.*, 1999), but whether it actually functions as an ER retrieval signal for this enzyme (or any FAD3 enzyme) has not been tested experimentally. In contrast, FAD2 and diverged FAD2 proteins do not possess a recognizable C-terminal dilysine motif at the −3 and −4/−5 positions, but do contain C-terminal sequences with similarity to aromatic amino acid-enriched motifs (-YXXL- and -W/YX$_{2-3}$W/F/Y-), shown previously to function in the localization of a few yeast and mammalian ER-resident membrane proteins, including *Saccharomyces cerevisiae* Sec71p (Cosson *et al.*, 1998) and human ε chain of the T-cell receptor complex (Mallabiabarrena *et al.*, 1992, 1995).

Deletion of the FAD2 and FAD3 C-termini results in loss of ER localization and reduced enzyme activity in yeast cells

To explore the functional role of FAD2 and FAD3 C-terminal sequences in maintaining the localization of these proteins in the ER, we expressed wild-type and C-terminal truncated myc-epitope tagged versions of FAD2 and FAD3 in *S. cerevisiae* and characterized both their subcellular localization and enzymatic activity. We chose yeast rather than plant cells to examine the necessity of FAD2 and FAD3 ER retrieval signals because computer-based secondary structure predictions revealed that both proteins contain a putative dimer interaction domain (data not shown), and therefore it was possible that in plant cells truncated versions of ectopically expressed FADs would have been retained in the ER by oligomerizing with their endogenous counterparts. This possibility was minimized in yeast cells, as *S. cerevisiae* contains a single ER-localized desaturase (Stukey *et al.*, 1990) that has low homology to plant FAD2 and FAD3 (13 and 11% identical, respectively). Figure 3 shows that yeast cells expressing either myc-FAD2 (panel a) or myc-FAD3 (panel g) proteins displayed similar immunofluorescence patterns that appeared as a ring around the nucleus and a reticula at the cell periphery. This fluorescence pattern was attributable to the localization of these two proteins to ER, as evidenced by the identical fluorescence pattern of the ER marker protein signal sequence-green fluorescent protein-HDEL (ss-GFP-HDEL) (Prinz *et al.*, 2000) co-expressed in the same cells (compare Figure 3a,b,g,h). In contrast, the immunofluorescence patterns of the C-terminal truncation mutants of myc-FAD2 and myc-FAD3 exhibited a more punctate staining pattern that co-localized with the fluorescence pattern of endogenous Tlg1p, a marker protein for yeast Golgi (Holthuis *et al.*, 1998; compare Figure 3d,e,j,k). These data indicate that the C-termini of FAD2 and FAD3 are necessary for the localization of these proteins in the ER of yeast cells, and that truncation of these sequences results in their mislocalization to the Golgi.

To determine whether FAD2 and FAD3 proteins that were mislocalized to the Golgi retained enzymatic activity, the fatty acid composition of yeast cells expressing either wild-type or C-terminally truncated versions of these proteins was examined by gas chromatography. Wild-type yeast cells have a simple fatty acid composition dominated by palmitic (16 : 0), palmitoleic (16 : 1), stearic (18 : 0), and oleic (18 : 1) acids, and thus, the enzymatic activity of FAD2 and FAD3 can be readily detected by appearance of polyunsaturated fatty acids not normally present in the yeast cells. Control experiments with yeast cells containing empty pYES2 plasmid revealed no detectable levels of linoleic (18 : 2) and linolenic (18 : 3) acids, the products of the FAD2 and FAD3 enzymes, respectively (data not shown). In contrast, yeast cells expressing myc-FAD2 contained approximately 30% linoleic acid (w/w total fatty acids; Figure 3m), indicating that the enzyme was functional in metabolizing endogenous oleic acid (18 : 1) into linoleic acid. Cells expressing the C-terminal truncated version of myc-FAD2, however, contained no detectable

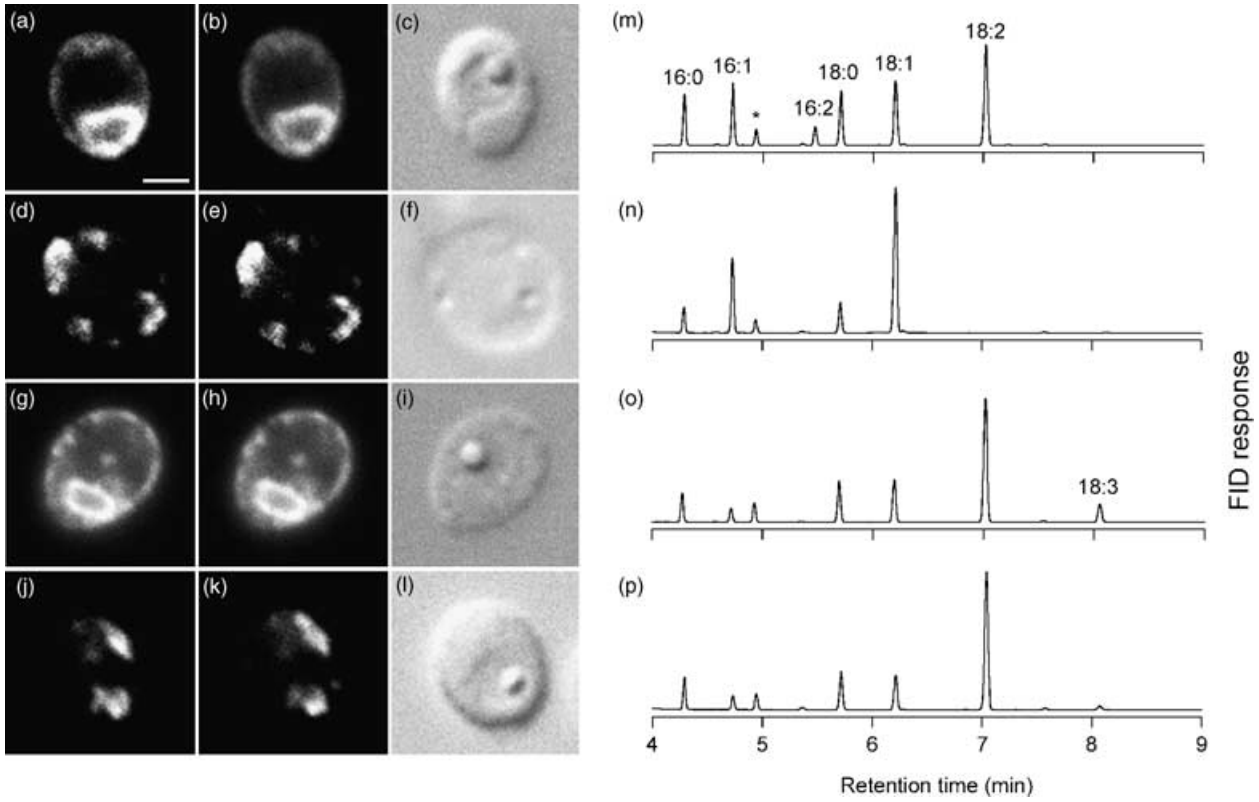

Figure 3. Localization and enzymatic activity of wild-type and C-terminal-truncated versions of FAD2 and FAD3 in *S. cerevisiae*.
(a–l) Yeast cells expressing either wild-type or C-terminal truncated versions of myc-tagged FAD2 or myc-FAD3 were grown overnight in galactose-containing medium followed by 2 h of incubation in glucose-containing medium to suppress the synthesis of the ectopically expressed protein(s). Cells expressing myc-FAD2 (a–c) and myc-FAD3 (g–i) also were co-expressing ss-GFP-HDEL, a marker protein for the yeast ER (Prinz *et al.*, 2000). All cells were fixed, processed for immunofluorescence microscopy with appropriate primary and secondary antibodies as described in the Experimental procedures, and then visualized using either fluorescence (a,b,d,e,g,h,j,k) or differential interference (c,f,i,l) microscopy. Bar in (a) = 2 μm. Shown are individual yeast cells (co)expressing either myc-FAD2 (a) and ss-GFP-HDEL (b), myc-FAD2 with its C-terminal 11 amino residues removed (d) along with immunofluorescence staining attributable to endogenous Tlg1p (a marker protein for the yeast Golgi; Holthuis *et al.*, 1998) in the same cell (f), myc-FAD3 (g) and ss-GFP-HDEL (h), or myc-FAD3 with its C-terminal 5 amino acids removed (j) along with immunofluorescence staining attributable to endogenous Tlg1p in the same cell (k).
(m–p) Yeast cells expressing either wild-type or C-terminal truncated versions of myc-tagged FAD2 or myc-FAD3 were grown overnight in galactose-containing medium either in the absence (m and n) or presence (o and p) of exogenous linoleic acid (18 : 2), the substrate for the FAD3 enzyme. Cells were then harvested, lipids were extracted, and fatty acid composition was determined by GC analysis of FAME; indicated on *y*-axis is flame ionization detector (FID) response. Labeled peaks correspond to methyl esters of palmitic (16 : 0), palmitoleic (16 : 1), stearic (18 : 0), oleic (18 : 1), linoleic (18 : 2), and linolenic (18 : 3) acids. The asterisk in (m) denotes the position of an internal standard (methyl heptadecanoate; 17 : 0). Shown are representative GC chromatograms (from four independent experiments) of FAME isolated from lipids of yeast cells expressing either myc-FAD2 (m), myc-FAD2 with its C-terminal 11 amino acids removed (n), myc-FAD3 (o), or myc-FAD3 with its C-terminal 5 amino acids removed.

amounts of linoleic acid (Figure 3n). The enzymatic activity of myc-FAD3 was also negatively affected when its C-terminus was truncated. Cells cultured in the presence of linoleic acid, the substrate for FAD3 enzyme, contained significantly more linolenic acid when expressing myc-FAD3 (4% of total fatty acids; Figure 3o) than when expressing myc-FAD3 without its C-terminal five amino acids (1% of total fatty acids; Figure 3p). These data indicate that the truncated versions of FAD2 and FAD3 were not as enzymatically active as their full-length counterparts because either they could not acquire electrons for catalytic activity (normally supplied by the ER electron transport chain) or deletion of their C-termini perturbed their structure to an extent that disrupted enzyme activity.

The C-termini of FAD2 and FAD3 are sufficient in localizing different GFP chimeric membrane proteins to the ER in plant cells

Our strategy for testing the role of the unique C-terminal residues in the ER localization of FAD2 and FAD3 in plant cells made use of the well-characterized reporter membrane proteins GFP-Cf9 (Benghezal *et al.*, 2000) and GFP-lysosomal membrane protein 1 (LAMP1) (Brandizzi *et al.*, 2002a), as well as onion epidermal cells serving as a model system for studying protein sorting in plant cells *in vivo* (Scott *et al.*, 1999). As shown schematically in Figure 4, GFP-Cf9 and GFP-LAMP1 are both type 1 (N_{in}–C_{out}) chimeric membrane proteins that consist of GFP fused to the

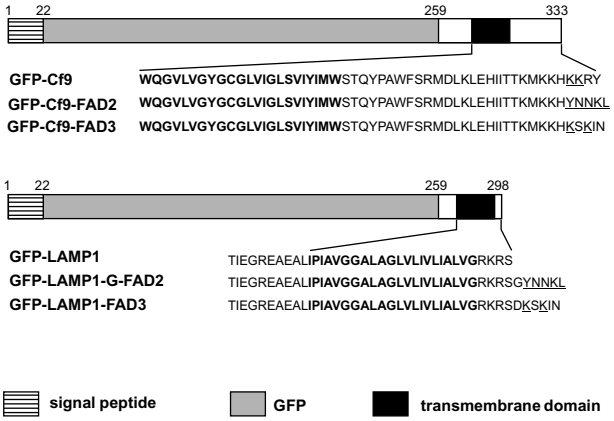

Figure 4. Schematic representation of selected GFP-Cf9 and GFP-LAMP1 chimeric proteins used in this study.
Chimeric proteins are drawn to scale and consisted of an N-terminal *Arabidopsis* chitinase signal peptide (residues 1–21), the GFP (residues 22–259), and C-terminal amino acid sequences from either Cf9 (residues 260–333) or LAMP1 (residues 260–298). In some cases, C-terminal Cf9 or LAMP1 sequences were modified with either FAD2 or FAD3 C-terminal sequences, including putative dilysine or aromatic amino acid-enriched ER retrieval motifs (underlined). Selected C-terminal sequences shown are indicated by the single-letter amino acid code, and the predicted membrane-spanning domain of Cf9 or LAMP1 is in bold.

N-terminal *Arabidopsis* chitinase signal sequence. GFP-Cf9 also contains the C-terminal 74 amino acid residues of the tomato *Cladosporium fulvum*-9 (Cf9) disease resistance gene product, which includes a single TMD and a C-terminal dilysine motif (underlined, -KKRY-COOH) that confers localization of type 1 membrane proteins in the ER (Jones *et al.*, 1994 and Figure 5; compare fluorescence pattern attributable to GFP-Cf9 (panel a) in a transformed onion cell to the fluorescence pattern attributable to the well-characterized ER marker protein GFP-HDEL (panel c; Haseloff *et al.*, 1997)). Notably, disruption of the dilysine motif in GFP-Cf9 by replacement of the lysine residues with asparagine residues resulted in mislocalization of the fusion protein to the plasma membrane (compare fluorescence pattern of GFP-Cf9/KKΔNN (panel e) to the fluorescence pattern of the plasma membrane-bound fusion protein GFP-RhoGTPase (panel g; Bischoff *et al.*, 2000)). The GFP-LAMP1 fusion protein, on the other hand, contains the C-terminal 23-amino acid TMD and short cytosolic segment derived from the human LAMP1 (Fukuda *et al.*, 1988; Figure 4). This portion of LAMP1 efficiently targets the reporter fusion protein to the plasma membrane in tobacco (Brandizzi *et al.*, 2002a) and onion epidermal cells (Figure 5h). Additional evidence that GFP-LAMP1 and GFP-Cf9/KKΔNN were each localized to the plasma membrane and not, for instance, localized to the cell wall was provided when transformed cells were plasmolysed with 0.8 M mannitol; fluorescence attributable to either chimeric protein in a plasmolysed cell was internalized or pulled away from the cell wall (Figure 5j,k; data shown only for GFP-LAMP1).

To determine whether the C-terminal peptide sequences of FAD2 or FAD3 could functionally replace the dilysine motif of GFP-Cf9, the –KKRY sequence at the C-terminus of GFP-Cf9 was substituted with either the C-terminal FAD2 (-YNNKL) or FAD3 (-KSKIN) sequence (see Figure 4), and the subcellular location of each fusion protein investigated in onion cells. As shown in Figure 5, GFP-Cf9 containing either the FAD2 (panel l) or FAD3 (panel m) C-terminus localized to the ER. To next determine whether the C-terminus of FAD2 or FAD3 was sufficient for localizing a protein in the ER that was not normally found in this subcellular compartment, the FAD2 and FAD3 C-termini were appended to the C-terminus of GFP-LAMP1 (refer to Figure 4). Figure 5 (panels n and o) shows that the FAD3 C-terminal sequence -DKSKIN, but not the FAD2 C-terminal sequence -YNNKL, was sufficient for re-localizing the reporter protein from the plasma membrane to the ER. As previous experiments demonstrated that the functionality of a C-terminal ER retrieval signal is sensitive to the distance between the signal and the membrane (Cosson *et al.*, 1998; Teasdale and Jackson, 1996; Vincent *et al.*, 1998), we added a single glycine between the C-terminus of GFP-LAMP1 and the FAD2 sequence (-GYNNKL-COOH; see Figure 4). This modification resulted in localization of the reporter protein (GFP-LAMP1-G-FAD2) to the ER (Figure 5p), indicating that there is a minimum distance requirement (i.e. at least 5 resides from the membrane) for the FAD2 signal to operate in the context of the GFP-LAMP1 reporter protein. Notably, GFP-LAMP1 with a single glycine residue appended to its C-terminus did not target to the ER (Figure 5q), indicating that this modification alone did not confer any ER targeting information.

Figure 5(r–u) shows that the localization of GFP-Cf9-FAD2, GFP-Cf9-FAD3, GFP-LAMP1-G-FAD2 and GFP-LAMP1-FAD3 to ER was abolished when specific C-terminal amino acids residues predicted to function as part of the different putative ER retrieval motifs in either FAD2 or FAD3 (underlined in Table 1) were substituted with alanine residues. Specifically, replacement of either the tyrosine at the −5 position and leucine at the −1 position in FAD2 with alanines (i.e. -YNNKL to –ANNKA) or replacement of lysines at positions −5 and −3 in FAD3 with alanines (i.e. -KSKIN to –ASAIN) resulted in the accumulation of each modified reporter protein in the plasma membrane rather than the ER. Collectively, the data presented in Figure 5 demonstrate that the C-termini of both FAD2 and FAD3 contain molecular signals that are sufficient for localization of membrane proteins to the ER in plant cells.

A dominant-negative mutant of Arf1 GTPase affects the transient localization of GFP-TM23-G-FAD2 to the Golgi

Based upon our observations that FAD2 and FAD3 each contain peptide signals that are necessary and sufficient for

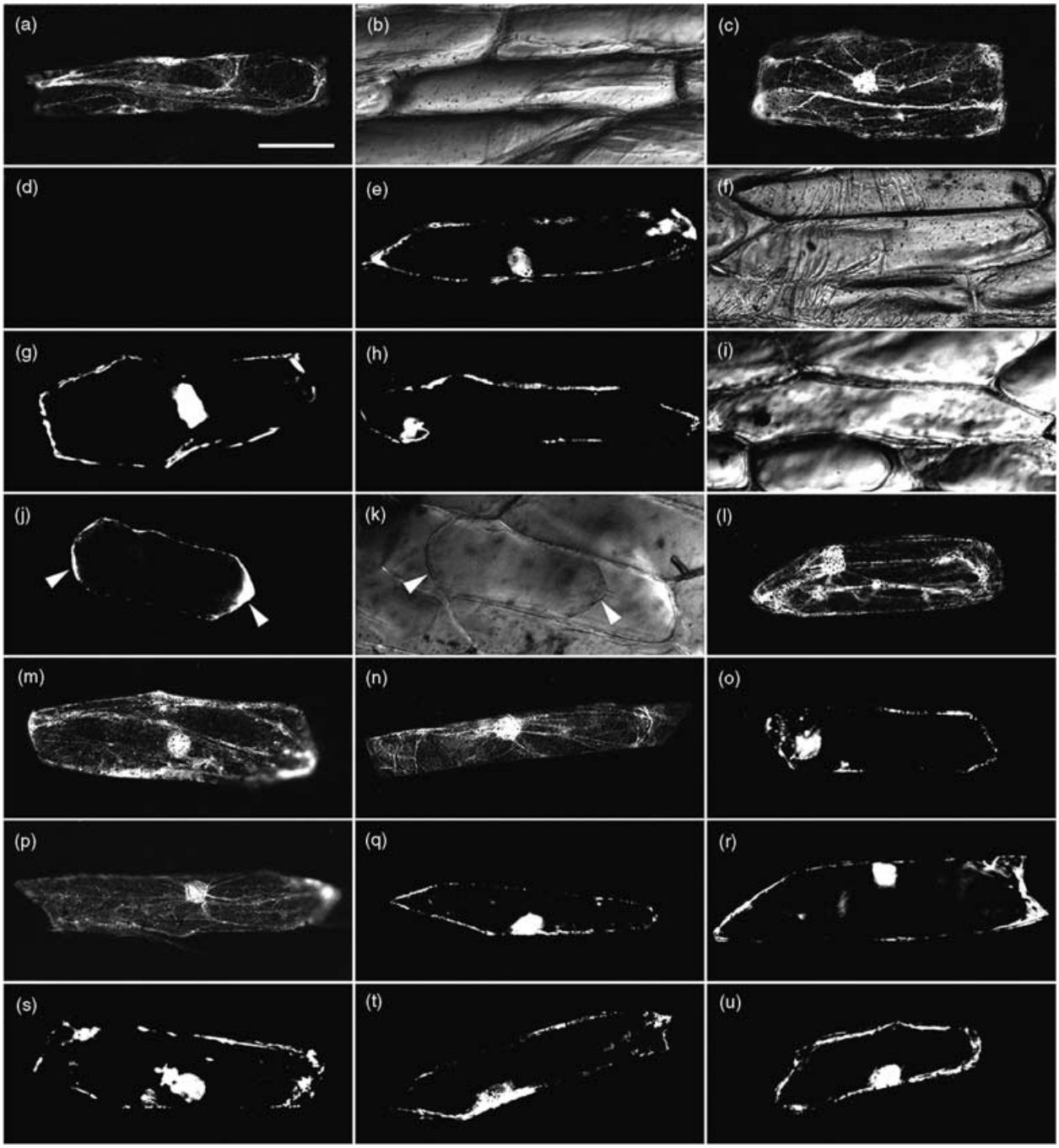

Figure 5. Localization of various GFP-Cf9 and GFP-LAMP1 fusion proteins in onion epidermal cells.
Onion epidermal peels were biolistically bombarded with DNA encoding different GFP chimeric proteins (or vector alone, d), incubated for 8 h in the dark, and then visualized using either fluorescence (a,c–e,g,h,j,l–u) or bright-field (b,f,i,k) microscopy. The transformed cell shown in panels (j and k) was plasmolysed with 0.8 M mannitol prior to viewing; solid arrows in (j and k) indicate the position of the plasma membrane detached from the cell wall. Bar in (a) = 100 μm. Shown are individual onion cells expressing either GFP-Cf9 (a,b), GFP-HDEL (c), mock transformed with vector DNA (pRTL2) alone (d), GFP-Cf9/KKΔNN (e,f), GFP-RhoGTPase (g) GFP-LAMP1 (h,i), GFP-Cf9/KKΔNN (j,k), GFP-Cf9-FAD2 (l), GFP-Cf9-FAD3 (m), GFP-LAMP1-FAD3 (n), GFP-LAMP1-FAD2 (o), GFP-LAMP1-G-FAD2 (p), GFP-LAMP1-G (q), GFP-Cf9-FAD2/YLΔAA (r), GFP-Cf9-FAD3/KKΔAA (s), GFP-LAMP1-G-FAD2/YLΔAA (t), or GFP-LAMP1-FAD3/KKΔAA (u).

localization to the ER, and also upon existing models of protein transport through the plant secretory pathway, our working hypothesis for the function of the FAD2 and FAD3 C-terminal sequences was that they act as retrieval signals to promote retrograde transport of escaped FADs from the Golgi back to the ER. This action would effectively maintain the steady-state localization of the proteins in the ER, despite transient localization to the Golgi. To determine

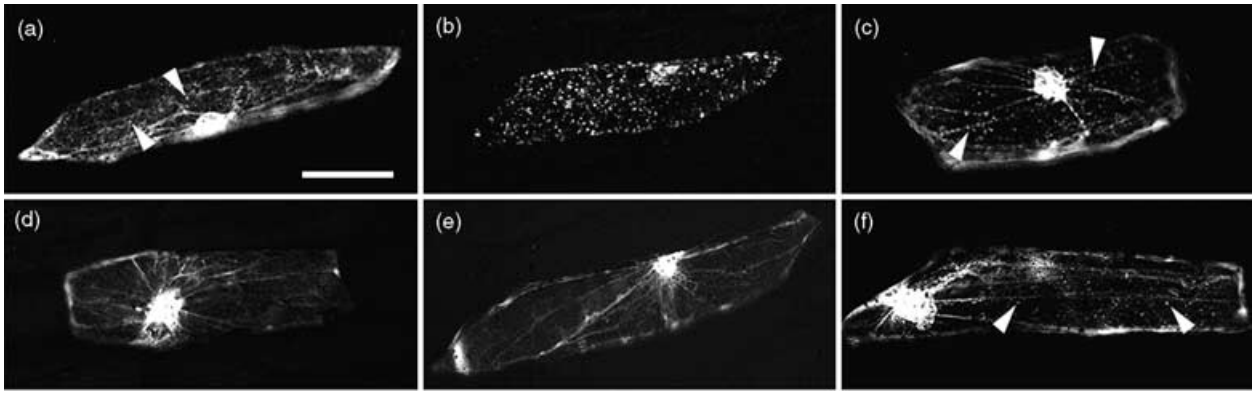

Figure 6. Dominant-negative effect of Arf1Q71L on the localization of Erd2-YFP and GFP-LAMP1-G-FAD2 in onion cells.
Onion epidermal peels were bombarded with DNA encoding GFP or YFP chimeric proteins (a–c) or co-bombarded with DNA encoding a GFP or YFP chimeric protein and either Arf1Q71L (d and e) or wild-type Arf1 (f). Bombarded peels were incubated in the dark for either 4 h (a,b,e,f) or 8 h (c and d) and then visualized using fluorescence microscopy. Erd2-YFP was expressed transiently for 8 h because no fluorescence attributable to this chimeric protein was detected at earlier time points (data not shown). Solid arrows in (a,c,f) indicate individual Golgi, and the bar in (a) = 100 μm. Shown are individual onion cells (co)expressing either GFP-LAMP1-G-FAD2 (a), GONST1-YFP (b), Erd2-YFP (c), Erd2-YFP and Arf1Q71L (d), GFP-LAMP1-G-FAD2 and Arf1Q71L (e), or GFP-LAMP1-G-FAD2 and wild-type Arf1 (f).

whether we could verify that FADs actually cycled between these two subcellular compartments, we took advantage of the observation that while GFP-LAMP1-G-FAD2 expressed for 8 h in onion epidermal cells was localized exclusively to the ER (Figure 5p), at earlier time points (e.g. 4 h), the fusion protein was found to be localized to both ER and numerous small punctate structures (Figure 6a). These GFP-LAMP1-G-FAD2-containing structures were also extremely mobile and often found to move along cytoplasmic strands (time-lapse data not shown). Based on their distinct morphology, distribution and dynamics the GFP-LAMP1-G-FAD2-containing punctate structures were presumed to be Golgi. Indeed, similar mobile punctate fluorescence structures were observed in onion cells expressing either the Golgi maker protein guanine nucleotide diphosphate (GDP)-mannose transporter fused to YFP (GONST1-yellow fluorescent protein (YFP); Baldwin *et al.*, 2001; Figure 6b) or the HDEL receptor, Erd2, fused to YFP (Erd2-YFP; Brandizzi *et al.*, 2002a; Figure 6c). This dual localization of Erd2-YFP to both Golgi and ER in onion cells is entirely consistent with the dynamic recycling of this chimeric protein between these two compartments in tobacco cells (Boevink *et al.*, 1998; Saint-Jore *et al.*, 2002).

To test whether the reporter proteins containing a FAD2 peptide signal were cycling between the ER and Golgi, we employed a dominant-negative mutant of *Arabidopsis* Arf1 (Arf1Q71L) that inhibits multiple steps in the secretory protein transport pathway, including disruption of vesicle trafficking between the ER and Golgi (Pepperkok *et al.*, 2000; Takeuchi *et al.*, 2002). Specifically, we tested whether blocking protein transport between the ER and Golgi with Arf1Q71L would alter the steady-state localization of co-expressed GFP-LAMP1-G-FAD2. Figure 6(d) demonstrates the dominant-negative nature of Arf1Q71L on the steady-state localization of Erd2-YFP in onion cells. Co-expression of Erd2-YFP and Arf1Q71L caused a change in the steady-state localization of the fusion protein from both the Golgi and ER to entirely the ER (compare Figure 6c,d). These results correspond in an identical manner with those obtained for Erd2-GFP and Arf1Q71L in tobacco suspension-cultured cells (Takeuchi *et al.*, 2002). When GFP-LAMP1-G-FAD2 was co-expressed with Arf1Q71L, the fusion protein, similar to Erd2-YFP, accumulated only in the ER rather than in both the Golgi and ER (Figure 6e; compare with Figure 6a), suggesting that the mutant effectively disrupted transport of GFP-LAMP1-G-FAD2 between the ER and Golgi. Control experiments with GFP-LAMP1-G-FAD2 co-expressed with either wild-type Arf1 (Figure 6f) or an empty vector (pRTL2; data now shown) did not result in a change in Golgi (or ER) localization of the fusion protein.

Characterization of the FAD2 ER retrieval signal through functional analysis and mutagenesis

As the FAD2 C-terminal sequence represented a new type of ER retrieval signal in plants and because there is much less known about this particular type of signal in comparison to the dilysine ER retrieval motif present in FAD3, we examined the functional aspects of the FAD2 peptide signal in greater detail. Toward this end, we used the reporter protein GFP-LAMP1-G-FAD2, which contains the minimal FAD2 sequence that is sufficient for ER localization, to replace individual or multiple amino acids within the FAD2–YNNKL-COOH sequence with a variety of other residues and then evaluated their effects on subcellular localization of the resulting reporter proteins. The results of these experiments are presented in Table 2. As discussed previously, replacement of the Y and L residues at the −5 and −1

Table 2 Characterization of the *Arabidopsis* FAD2 ER retrieval motif

C-terminal sequence	ER localization
–RKRSGYNNKL	+
–RKRSGANNKA	−
–RKRSGYAAAL	−
–RKRSGFNNKL	+
–RKRSGINNKL	+
–RKRSGVNNKL	+
–RKRSGANNKL	−
–RKRSGQNNKL	−
–RKRSGYANKL	−
–RKRSGYRNKL	+
–RKRSGYLNKL	+
–RKRSGYSNKL	−
–RKRSGYNAKL	−
–RKRSGYNKKL	+
–RKRSGYNLKL	+
–RKRSGYNSKL	−
–RKRSGYNNAL	−
–RKRSGYNNRL	+
–RKRSGYNNDL	+
–RKRSGYNNEL	+
–RKRSGYNNQL	−
–RKRSGYNNKA	−
–RKRSGYNNKF	+
–RKRSGYNNKS	−
–RKRSGYNNKQ	−
–RKRSGYNNKV	+
–RKRSGYNNKT	−
–RKRSGYSNKF	+
–RKRSGYNSKF	+
–RKRSGYNKL	−
–RKRSGYKL	−
–RKRSGYNNKLG	−
–RKRSGILHKL*	+
–RKRSGYHSDF*	+

Various amino acid mutations were introduced into the C-terminus of the chimeric protein GFP-TM23-G-FAD2 (fusion junction: -RKRSG/YNNKL-COOH) and the resulting constructs expressed transiently for 8 h in onion epidermal cells. Localization either to the ER (+) or to some other subcellular location, namely the plasma membrane (−), was assessed using fluorescence microscopy and was based on the similarity in fluorescence patterns to marker proteins localized to either the ER (GFP-TM23-G-FAD2 and GFP-HDEL) or plasma membrane (GFP-TM23-G-FAD2 YLΔAA and GFP-RhoGTPase). Specific amino acid substitutions in the FAD2 C-terminal –YNNKL sequence are underlined. Results shown for each construct are representative of >50 transformed cells from at least two independent biolistic bombardment experiments. GFP-TM23-G reporter protein sequences marked with an asterisk were derived from the C-terminus of either cytochrome P450 (-ILHKL) or Pex10p (-YHSDF; refer also to Table 3).

positions with alanine residues abolished ER localization of the resulting GFP-LAMP1-G-FAD2 reporter protein (Figure 5t). In a similar manner, replacement of either the intervening –NNK- residues in the FAD2 motif with alanines or substitution of each amino acid individually within the sequence with an alanine residue abolished targeting to the ER (Table 2). Collectively, these results indicated that each amino acid position within the FAD2 sequence contributes information to the ER retrieval signal.

Comparison of the FAD2 and diverged FAD2 protein sequences presented in Table 1 (along with other members of each enzyme family; data not shown) provided insight into the functional redundancy that might exist at each amino acid position within the peptide motif. Indeed, replacement of the Y and L residues at the −5 and −1 positions of the signal with other large hydrophobic amino acids (e.g. F, I, or V) preserved ER localization of the reporter protein, while replacement of these residues with either smaller hydrophobic residues (A) or hydrophilic residues (Q, S, or T) disrupted ER targeting (Table 2). Similar comparisons of FAD2 and diverged FAD2 proteins suggested that the −2 position of the peptide signal must be occupied by a charged amino acid. In agreement with this premise, substitution of the K at the −2 position of the FAD2 signal with either R, D, or E preserved ER targeting, whereas replacement with A or Q disrupted targeting. The −4 position of the FAD2 signal is occupied by many different types of amino acids in various FAD2 sequences (see Table 1), indicating that this position tolerates substantial variability in amino acid side chain structure. Indeed, the N at this position of the FAD2 sequence could be functionally replaced with either R or L, but surprisingly, substitution of the N with S disrupted ER targeting. This mutant peptide sequence (-YSNKL) was almost identical to the C-terminal sequence of tung (*Aleurites fordii*) FAD2 (-YSNKF), with the exception that the mutant FAD2 peptide contained an L rather than an F at the −1 position. This difference suggested that F might contribute more targeting information than L at the −1 position, and that this larger contribution might be important when other residues in the signal are less functional (e.g. S at the −2 position). Indeed, replacement of the L in the mutant FAD2 signal -YSNKL with F (resulting in -YSNKF) restored ER localization (Table 2). A similar phenomenon was observed at the −3 position, where replacement of the N with S (-YNSKL) abolished ER targeting, but substitution of the L in this mutant with an F (-YNSKF) restored targeting to the ER.

In addition to making individual amino acid substitutions within the FAD2 signal, we observed that deletion of either one or two residues within the signal (-YNKL or -YKL), or addition of an extra glycine residue at the end of the signal (-YNNKLG) disrupted localization of the reporter protein to the ER (Table 2). Collectively, the results presented in Table 2 define the physiochemical requirements of amino acids at each position within the signal and reveal a sequence motif consisting of Φ-X-X-K/R/D/E-Φ-COOH, where -Φ- are large hydrophobic amino acid residues. In addition, our results define the minimum length and positioning of the FAD2 signal with respect to the C-terminus

and indicate that specific combinations of residues, including those at positions -X-, may be required within the signal depending on the types of amino acids present at each position.

Other Arabidopsis proteins contain C-terminal sequences similar to the FAD2 ER retrieval motif

We next utilized a bioinformatics approach to analyze the frequency of occurrence of the C-terminal ER retrieval motif of FAD2 in other proteins from *Arabidopsis*. Specifically, we used the PATMATCH program (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) to search the *Arabidopsis* genome for open-reading frames (ORFs) $\geq$100 codons in length and which encoded proteins with the C-terminal sequences that contained amino acid residues at the -5 through -1 positions that were shown to be functional in the context of GFP-LAMP1-G-FAD2; -[FYWILV]-[X]-[X]-[KRDE]-[FYWILV]-COOH (Table 2). Next, we selected only those candidate proteins that contained a TMD and were predicted or known to be located in the ER. Using these criteria, we identified 47 proteins that are listed in Table 3 and included FAD2 (Δ^{12}-oleate desaturase), 13 members of the cytochrome P450 monooxygenase family (Durst and Nelson, 1995; Durst and O'Keefe, 1995), serine palmitoyltransferase (Tamura *et al.*, 2001), ribophorin (Fu *et al.*, 2000), two isoforms of phospholipid:diacylglycerol acyltransferase (Dahlquist *et al.*, 2000), three isoforms of dehydrodolicyl diphosphate synthase (DDPS; Cunillera *et al.*, 2000), and the peroxisomal biogenesis factor Pex10p (Schumann *et al.*, 2003). Notably, fusion of the C-terminal peptide sequences of either cytochrome P450 (-ILHKL) or Pex10p (-YHSDF) to the GFP-LAMP1-G reporter protein resulted in localization to the ER (Table 2). Collectively, these data suggest that, in addition to FAD2, a number of other proteins in *Arabidopsis* utilize a C-terminal Φ-X-X-K/R/D/E-Φ sequence motif as an ER retrieval signal.

Discussion

Insertion of FADs into ER membranes

Nearly all of the membrane proteins localized in the various subcellular compartments of the secretory pathway are co-translationally inserted into the ER, a process that is strictly dependent upon the protein's first hydrophobic TMD serving as an N-terminal signal sequence anchor and the SRP/Sec61p translocon machinery. It is apparent from the results presented in this study that FAD2 and FAD3 are also inserted into the ER via a conventional co-translational SRP-mediated mechanism. For instance, we demonstrated that both proteins were efficiently integrated into microsomal membranes only when their mRNAs were translated in the presence of microsomes. In addition, we showed that protease digestion of the α subunit of the SRP receptor on the cytoplasmic surface of the ER membranes inhibited FAD2 and FAD3 integration. While these data for the co-translational insertion of FAD2 and FAD3 into the ER were somewhat expected based on current paradigms of membrane protein biogenesis (for pertinent examples of plant proteins, see Abel *et al.*, 2002; Beaudoin *et al.*, 2000), they contradict results from a previous study whereby the mRNA for another FAD enzyme, rat liver stearyl-Co desaturase (SCD), was found to be associated primarily with free polyribosomes rather than membrane-bound polyribosomes. Based on these data, it was hypothesized that the enzyme targeted post-translationally to the ER (Thiede and Strittmatter, 1985). However, in this previous study, neither ER membrane binding by SCD was examined nor was the possibility addressed that the population of ribosomes that were classified as cytosolic or 'free' ribosomes included those released from ER membranes during extraction (Walter and Blobel, 1983). Thus, it is plausible that SCD (like FAD2 and FAD3) is synthesized primarily on ER membrane-bound polysomes and inserted into the ER co-translationally. Indeed, we found that rat SCD, like plant FAD2 and FAD3, was more efficiently integrated into the ER when its mRNA was translated in the presence of microsomes than when SCD protein was incubated with microsomes post-translationally (data not shown).

FAD2 and FAD3 possess different ER retrieval signals at their C-termini

Based on the predicted membrane topology of FAD2 and FAD3 (Dyer and Mullen, 2001; Shanklin *et al.*, 1994), which includes both their N- and C-termini facing the cytosol, and given the fact that FAD2 and FAD3 proteins contain sequences resembling known cytosolically exposed ER retrieval motifs (Table 1), our working hypothesis for the steady-state localization of FADs in the ER was that they were retrieved from post-ER compartments by these distinct C-terminal peptide sequences and the COPI-mediated retrograde vesicle transport pathway. Direct evidence in support of the first portion of this model was obtained by demonstrating that the C-terminal pentapeptide sequences of FAD2 (-YNNKL) and FAD3 (-KSKIN) were both necessary and sufficient for localizing a variety of native and reporter protein constructs to the ER in either plant or yeast cells. Our targeting studies also revealed that the FAD2 C-terminal –YNNKL peptide sequence, similar to the dilysine peptide motif (Benghezal *et al.*, 2000), mediated localization to the ER by acting as a retrieval motif and not involved in static retention. Indirect evidence in support of this premise was that GFP-LAMP1-G-FAD2 was localized to both ER and Golgi in onion cells at 4 h after biolistic bombardment, but was localized exclusively to ER at 8 h (Figure 6). These data

Table 3 List of selected *Arabidopsis* proteins containing a putative C-terminal aromatic amino acid-enriched ER retrieval motif

Annotation	TAIR ID	C-terminal sequence
ATP-Binding Cassette (ABC) transporter family protein	At5g61690	–TRPPSPNALGHKSLRQGVRGIVIDL
Ca^{2+}-dependent protein kinase	At5g04870	–MQKGSITGGPVKMGLEKSFSIALKL
Copper amino oxidase	At2g42490	–LETKESEVKEVVAPKALQTGLLSKL
Cycloartenol synthase	At3g45130	–YIHRQVLFSLTSLIIFASYFLFRRY
Cytochrome P450	At2g17060	–SYTHAPFPAATTFPQHGAHLIIRKL
Cytochrome P450	At2g24180	–EAPGLTSHRKHPLHLVPVNVINRKL
Cytochrome P450	At3g13720	–AEEDTIINFPTVHMKNKLPIWIKRI
Cytochrome P450	At3g14610	–SYVHAPQTVMTTRPQFGAHLILHKL
Cytochrome P450	At3g14630	–SYVHSPYRVFTIHPQCGAHLILHKL
Cytochrome P450	At3g14660	–SYVHAPYTVLTTHPQFGAPLILHKL
Cytochrome P450	At3g14640	–SYVHAPQTVVTIHPQFGAHLILRKL
Cytochrome P450	At3g14650	–SYVHAPYTVFTIHPQFGAPLIMHKL
Cytochrome P450	At3g14690	–SYVHAPYTVITIHPQFGAQLIMHKL
Cytochrome P450	At1g57750	–HKVEPIPSILLRMKHGLKVTVTKKI
Cytochrome P450	At5g04660	–IDFAGKLEFTVVMKNPLRAMVKPRI
Cytochrome P450	At3g10570	–IDFAGKLEFTVVMKKPLRAMVKPRV
Cytochrome P450	At5g04630	–VDFTGKLVFAVVMKNPLRARVKARV
Dehydrodolichyl diphosphate synthase	At5g58782	–DFDKQKFIEALVSYQRRDRRFGSRL
Dehydrodolichyl diphosphate synthase	At2g23410	–DFDKDKLLEALASYQRRERRFGCRV
Dehydrodolichyl diphosphate synthase	At5g58780	–DFDKDKLLEALVSYQRRERRFGCRV
Disease resistant protein	At3g25020	–SYKPEWLVCLVKSRNPLRSFFGFEY
Δ^{12}-oleate desaturase (FAD2)	At3g12120	–AKECIYVEPDREGDKKGVYWYNNKL
Fatty acid elongase 1	At2g15090	–LRDVKPSANSPWEDCMDRYPVEIDI
Fatty acid elongase 1	At4g34510	–LRNVEPSVNNPWEHCIHRYPVKIDL
GDSL motif lipase/hydrolase	At2g19050	–NMVVAKAAYAGLITSPYSLSWLARL
Glucose-6-phosphate translocator	At1g21870	–PRTPRTPRNKMELIPLVNDKLESKI
Glycosyl transferase family	At1g52400	–QKVSGKWYSEFLKPQFPTSKLREEL
GTPase activator protein	At5g41940	–MAGHLDVWKLLDDAHDLVVNLHDKI
Late embryonic abundant protein	At5g60530	–TPSLFSPTCRLCRFKPQEESLSADI
Leucin-rich repeat TM protein kinase	At2g45340	–THESSNQRPSMEDVIQELNNLAADY
Long-chain fatty acid CoA ligase	At1g64400	–PQLLKYYQKEIDEMYKKNREVQLRV
Membrane trafficking factor	At4g04210	–PKPLTDLTQTIEEAGLANSVVLQKF
Na^{+} inorganic phosphate co-transporter	At2g38060	–APLCNRRTSLLDPKRSPWTLYCLEL
Nitrate transporter	At3g25260	–LELFYLVLCVLTLNLFNYIFWAKRY
Pex10p	At2g26350	–EKQECPLCRTPNTHSSLVCLYHSDF
PC diacylglycerol acyltransferase	At3g44830	–SGQEIGGDRIYSDVMRMSERISIKL
PC diacylglycerol acyltransferase	At5g13640	–NGSDIGHDQVHSGIFEWSERIDLKL
Proline-rich protein	At5g19810	–PPPSKYGRVYSPPPPGKSWLWFLKL
Protein phosphatase 2C protein	At3g51370	–TNQVSSVKGPPLSIRGGGMTFPKKI
Putative Na^{+}/H^{+} antiporter	At2g01980	–DEEDEDEGIVVRIDSPSKIVFRNDL
Receptor kinase	At3g21930	–LSRSCNFRYELYPFISPKGSYYTKF
Ribophorin I	At1g76400	–IASQQQKIIALRQEIEDLLEFIDEI
Really Interesting New Gene (RING) finger protein	At4g00305	–WIQDDKMTCPLCRTPIVPDFYFFRL
Serine palmitoyltransferase	At3g48790	–IKYFTAAPKKQEEKNGNTSKFKLRI
Subtilisin-like serine protease	At1g66210	–TWADNEGHNVRIPLSVRTRVFNFKI
Subtilisin-like serine protease	At5g59120	–SSANLIWSDGTHNVRSPIVVYTSDY
Long fatty acid condensing enzyme	At2g16280	–LNNVKPSVSSPWEHCIDRYPVKLDF

Arabidopsis membrane protein candidates whose C-termini contain an aromatic amino acid-enriched ER retrieval motif (-FYWILV-X-X-KRDE-FYWILV-COOH; underlined) according to the residues shown to be functional in the context of the FAD2 C-terminal reporter protein (see Table 2) were identified using the PATMATCH program. Predicted TMDs (not shown) were identified using the TMHMM program (version 2.0).

suggest that at early time points following bombardment, when transient protein expression levels are high (Daniell, 1997; Okada *et al*., 1986), the retrieval machinery was saturated and, thus, a portion of reporter protein accumulated in the Golgi as well as in the ER. However, at later time points, when transient protein expression levels had decreased, GFP-LAMP1-G-FAD2 was more effectively retrieved to the ER and, therefore, the reporter protein did not exhibit steady-state localization to the Golgi. We also found no indication that GFP-LAMP1-G-FAD2 was localized to post-Golgi compartments such as the vacuole or plasma membrane at even later time points following

bombardment, e.g. 12–20 h, suggesting that those reporter proteins that had accumulated in the Golgi at 4 h were recycled back to the ER.

Additional evidence that the FAD2 C-terminal peptide -YNNKL serves as an ER retrieval signal was obtained using transient expression experiments with Arf1Q71L, a dominant negative mutant of Arf1 known to disrupt retrograde vesicle transport between the Golgi and ER (Pepperkok *et al.*, 2000; Takeuchi *et al.*, 2002). Specifically, we demonstrated that Arf1Q71L prevented GFP-LAMP1-G-FAD2, as well as the control fusion protein YFP-Erd2 (known to recycle between the Golgi and ER; Brandizzi *et al.*, 2002a), from being localized to the Golgi at early time points following bombardment (Figure 6). While at first glance these data seem to contradict the expected Golgi localization of GFP-LAMP1-G-FAD2 in the presence of an Arf1 mutant that disrupts primarily Golgi-to-ER transport, they are in agreement with data from a recent study showing that Arf1Q71L has multiple effects on membrane protein traffic between the ER and Golgi in plant cells (Takeuchi *et al.*, 2002). The authors of this study proposed that if folding of a fluorescent fusion protein was sufficiently slow, then the secondary effects of the Arf1 mutant, including ER-to-Golgi anterograde defects caused by the lack of Golgi-derived components necessary for ER budding resulted in the protein being 'stuck' in the ER. While we have no solid data on the folding kinetics for nascent FAD proteins, this explanation provides a reasonable account for our observations with Arf1Q71L and GFP-LAMP1-G-FAD2 (and YFP-Erd2). What now remains are more detailed analyses of FAD2 (and FAD3) constitutive recycling between the ER and Golgi using other approaches such as high-resolution, time-lapse microscopy described recently by Brandizzi *et al.* (2002b) and/or more traditional biochemical approaches, e.g. detection of Golgi-specific sugar modifications.

Nature of the FAD2 ER localization signal

While the dilysine-type ER retrieval motif present in FAD3 has been functionally characterized in at least one other plant ER membrane protein (tomato Cf9; Benghezal *et al.*, 2000), and even more well characterized in mammalian ER membrane proteins (Jackson *et al.*, 1990; Nilsson *et al.*, 1989; Teasdale and Jackson, 1996; Vincent *et al.*, 1998; Zerangue *et al.*, 2001), much less is known about the molecular properties of the FAD2-like aromatic ER retrieval motif in any organism. This motif was initially identified by Cosson *et al.* (1998) using the δ-COP subunit as bait to isolate interacting proteins in a yeast two-hybrid experiment. The interacting sequence identified by this screen was demonstrated to be sufficient in mediating COPI-dependent ER retrieval of membrane-bound reporter proteins in both yeast and mammalian cells and closely resembled a previously characterized tyrosine-containing ER retrieval motif (Mallabiabarrena *et al.*, 1992, 1995).

Our comprehensive characterization of the FAD2 peptide signal confirms and extends previous studies of the aromatic ER retrieval motif (Cosson *et al.*, 1998; Mallabiabarrena *et al.*, 1992, 1995) in several ways. First, alignments of FAD2 and diverged FAD2 enzyme sequences provided significant insight into the potential degeneracy of the peptide signal, which was tested and confirmed experimentally. We showed that the first and last amino acid positions of the signal must be occupied by large, hydrophobic amino acids, that the penultimate position must be occupied by a charged residue, and that the remaining two amino acid positions were largely variable, although specific amino acid combinations of residues might be required. For example, substitution of either asparagine residues at the −4 or −3 positions of the FAD2 signal (underlined, -YNNKL) with a serine residue abolished targeting unless the leucine at the −1 position was also substituted with phenylalanine (e.g. -YSNKF or –YNSKF). These data demonstrate both additive contributions of amino acids during decoding of the peptide signal and illustrates that an aromatic (phenylalanine) residue contributes more significant targeting information than other large hydrophobic (leucine) residues when present at the −1 position. For this reason, we refer to the peptide signal on FAD2 as an aromatic ER retrieval motif.

We also showed that spacing of the FAD2 peptide signal relative to the membrane plays an important role in the functionality of this ER retrieval motif. Differences in the subcellular location of the reporter proteins GFP-LAMP1-FAD2 and GFP-LAMP1-G-FAD2 provided direct evidence that a minimum of five amino acid residues are required between the C-terminal-most residue of the TMD and the hydrophobic residue at the −5 position in the peptide signal. These data are consistent with the minimum spacing requirements between the membrane and either the aromatic amino acid-enriched motif described by Cosson *et al.* (1998) or the dilysine motif (Teasdale and Jackson, 1996; Vincent *et al.*, 1998). Another important feature of the FAD2 aromatic amino acid-enriched ER retrieval motif was that it only appeared to function at the extreme C-terminus, as addition of a glycine to the C-terminus of GFP-LAMP1-G-FAD2 resulted in the reporter protein accumulating at the plasma membrane (Table 2). It is important to note, however, that several other putative plant ER membrane proteins contain aromatic amino acid-enriched motifs a few amino acids upstream from their C-termini. Whether these peptide signals function in ER localization within the context of the native proteins, i.e. whether other amino acid sequences adjacent to the aromatic ER motif in these proteins allow the signal to function upstream of the C-terminus, is currently being investigated.

Presence of the aromatic ER retrieval motif in other putative ER-localized plant membrane proteins

The results from our bioinformatics search presented in Table 3 revealed that a number of proteins encoded by the *Arabidopsis* genome contained C-terminal sequences similar to the aromatic ER retrieval motif described here for FAD2. These data are important because the majority of plant ER membrane proteins do not possess a prototypic dilysine motif (Denecke, 2003; Gomord *et al*., 1999) and, while we concede that some of the proteins identified in our screen have not been experimentally proven to be resident ER membrane proteins, many others have, and our identification of a putative aromatic ER retrieval motif now provides a working model for how they are localized in the ER. For instance, our data that some members of the cytochrome P450 family may be localized to the ER by a C-terminal aromatic ER retrieval motif extends the results of previous studies of mammalian P450s in which an unknown ER retrieval signal was shown to be located in the cytosolic-facing, C-terminal portion of the enzyme (Szczesna-Skorupa *et al*., 1995, 2000). Another notable candidate identified in our screen of *Arabidopsis* proteins containing a putative C-terminal aromatic ER retrieval motif was the peroxisomal biogenesis factor, Pex10p. While well known to be an integral membrane protein in yeast and mammalian peroxisomes (Purdue and Lazarow, 2001), disruption of this gene in *Arabidopsis* effects many aspects of seed development, including the aberrant formation of ER-derived vesicles involved in the biogenesis of peroxisomes, lipid bodies, and protein bodies (Schumann *et al*., 2003). The existence of a putative aromatic ER retrieval motif on Pex10p now provides an explanation for how this protein might be recycled back from post-ER compartments to carry out its function in vesicle formation at the ER. In support of this premise, we demonstrated that the C-terminal sequence of Pex10p (-YHSDF), as well as the C-terminal sequence –ILHKL found on three isoforms of cytochrome P450, were sufficient in localizing the reporter proteins GFP-LAMP1 (Table 2) or GFP-Cf9 (data not shown) to the ER. Collectively, these data suggest that the aromatic ER retrieval motif described here plays an important role in the biogenesis of many different plant ER membrane proteins, and also suggest new avenues of research for studying the variety of cellular processes in which these proteins participate.

Experimental procedures

Recombinant DNA procedures and reagents

Standard recombinant DNA procedures were performed as described by Sambrook *et al*. (1989). Molecular biology reagents were purchased either from New England BioLabs (Beverly, MA, USA), Promega (Madison, WI, USA), or Perkin-Elmer (Perkin-Elmer Biosystems, Mississauga, Canada). Synthetic oligonucleotides were synthesized by either Invitrogen (Frederick, MD, USA) or University of Guelph Laboratory Services (Guelph, Canada), DNA was isolated using Qiagen reagents (Qiagen, Mississauga, Canada), and dye-terminated cycle sequencing was used with an Applied Biosystems Model 377 automated sequencer (Perkin-Elmer Biosystems) to verify that additional mutations were not introduced during amplification or cloning procedures. Mutagenesis was carried out using appropriate complementary forward and reverse mutagenic primers and the QuikChange™ site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Complete details on the sequences of all oligonucleotide primers used in this study are available from the authors upon request. PCR was performed with either a Hybaid PCR Express thermal cycler (Continental Laboratory Products, San Diego, CA, USA) or a GeneAMP PCR System 2400 (Perkin-Elmer Biosystems).

Plasmid construction

The vectors pSPUTK (Falcone and Andrews, 1991) and pSPUTK-*Bgl*II were used for *in vitro* membrane insertion experiments. Both plasmids contain the SP6 promoter, the high-efficiency β-globin 5′ untranslated region (UTR), and a Kozak's initiation site (Kozak, 1992) for efficient translation in a reticulocyte lysate. pSPUTK-*Bgl*II was constructed by digesting pSPUTK with *Eco*RV and *Hpa*I, blunting both ends with T4 DNA polymerase, and then re-ligating. This removed approximately 60 base pairs of sequence, including a *Bgl*II site that would have interfered with cloning into another *Bgl*II site at the 5′ end of the multiple cloning region. Construction of pSPUTK/FAD2, pSPUTK-*Bgl*II/FAD3, and pSPUTK-*Bgl*II/SCD was carried out as follows. DNA sequences encoding either *Arabidopsis* FAD2, *Brassica* FAD3, or rat liver SCD were PCR-amplified from pKS/mycFAD2 (Dyer and Mullen, 2001), pRTL2/myc-FAD3 (Dyer and Mullen, 2001), and YEp352/wt-ratSCD (provided by Edgar Cahoon, Donald Danforth Plant Science Center; Shanklin *et al*., 1994), respectively, using appropriate forward and reverse mutagenic primers that added a Kozak's initiation site to each cDNA as well as unique restriction sites in the 5′ and 3′ UTRs. All PCR products were gel-purified and subcloned into the yeast expression plasmid pYES2.1 TOPO (URA, 2μ; Invitrogen). FAD2, FAD3, and SCD ORFs were then excised from pYES2.1 TOPO using the appropriate restriction enzymes and transferred to similarly digested, dephosphorylated pSPUTK or pSPUTK-*Bgl*II. The construction of pSP/CytoB_5 (Janiak *et al*., 1994), containing the rat liver Cb_5 ORF, and pGEM/svBPI3 (Falcone and Andrews, 1991), containing the bovine pre-prolactin ORF, has been described previously.

Plasmids encoding C-terminal truncated versions of FAD2 and FAD3 were constructed using PCR with either pKS/myc-FAD2 or pRTL2/myc-FAD3 as template DNA. Individual PCR reactions also included an appropriate forward primer and reverse mutagenic primer that introduced a stop codon at either the −11 position in the C-terminus of FAD2 (underlined, -KKGVYWYNNKL-COOH) or the −5 position in FAD3 (underlined, -KSKIN-COOH). PCR products were then gel-purified and subcloned into pYES2.1 TOPO. The construction of pRS425/GFP-ER (LEU, 2μ), which encodes GFP with an N-terminal signal sequence and a C-terminal ER retention signal (ss-GFP-HDEL), under control of the MET25 promoter, was generated by excising the *Kpn*I–*Sac*I fragment from pWP1055 (provided by Tom Rapoport, Harvard Medical School; Prinz *et al*., 2000), treating with T4 DNA polymerase to form blunt ends, and then ligating into *Sma*I-digested pRS425.

Construction of pRTL2/GFP-Cf9 and pRTL2/GFP-Cf9 KKΔNN was carried out by ligating the *Bam*HI fragment from pCBJ98 and pCBJ99 (provided by David Jones, Australian National University; Benghezal *et al.*, 2000) into *Bam*HI-digested pRTL2. pRTL2 is a plant expression vector containing the cauliflower mosaic virus 35S promoter and terminator, and a tobacco etch potyvirus leader sequence for enhanced translation (Restrepo *et al.*, 1990). The construction of pRTL2/GFP-LAMP1 was constructed in the following manner. First, the cassette vector, pRTL2/GFP-*Xba*IΔCf9, was generated by introducing an in-frame *Xba*I site at the sequences coding for the GFP and Cf9 junction in pRTL2/GFP-Cf9. Digestion of pRTL2/GFP-*Xba*IΔCf9 with *Xba*I resulted in the removal of sequences encoding the Cf9 C-terminus and, thereby, allowed for the subsequent ligation to the 3′ end of the GFP ORF of two sets of oligonucleotides possessing *Xba*I complementary ends and encoding -SST- linker, the factor X cleavage site (-IEGREAE-), an alanine linker, and a portion of the human LAMP1 C-terminus, including the LAMP1 23-amino acid TMD (-LIPIAVGGALAGLVLIVLIAYLVG-) and immediately adjacent C-terminal four amino acid residues (-RKRS-COOH). The design of this synthetic DNA fragment was based on an identical GFP-LAMP1 construct (termed TM23) previously reported by Brandizzi *et al.* (2002a). Plasmids coding for GFP-Cf9 and GFP-LAMP1 mutants with altered amino acid residues at the C-terminus of either Cf9 or LAMP1 were constructed using site-directed mutagenesis.

pRTL2/ARF1 was generated by amplifying the ARF1 ORF from pUC18/AtARF1GFP (provided by Akihiko Nakano, RIKEN, Japan; Takeuchi *et al.*, 2002) with a forward primer that corresponded to sequences, including the ARF1 initiation codon (and *Nco*I site) and a reverse mutagenic primer that introduced a stop codon and 3′ *Bam*HI restriction site at the ARF1-GFP sequence junction. The resulting PCR products were digested with *Nco*I and *Bam*HI and ligated into *Nco*I–*Bam*HI-digested pRTL2, yielding pRTL2/ARF1. pRTL2/ARF1Q71L was generated using site-directed mutagenesis with pRTL2/ARF1 as template DNA and complementary forward and reverse primers that introduced the point mutation (Q71L; a glutamic acid at position 71 changed to a leucine) in the ARF1 ORF and resulting in the protein being GTP restricted (Pepperkok *et al.*, 2000).

The construction of pBIN/mGFP5-HDEL (provided by Jim Haseloff, University of Cambridge; Haseloff *et al.*, 1997), pGONST1-YFP (provided by Paul Dupree, University of Cambridge; Baldwin *et al.*, 2001), pVKH16-EN6/Erd2-YFP (provided by Nadine Paris, Université de Rouen; Brandizzi *et al.*, 2002a) and pTG/AtROP6Q64L (provided by Arthur Molendijk, Max-Plank Institute; Bischoff *et al.*, 2000) have been described elsewhere.

In vitro *membrane insertion experiments*

Salt-extracted canine pancreatic ER membranes (microsomes) were prepared as previously described by Walter and Blobel (1983). One equivalent of microsomes was defined as 100 fmol of SRP receptor α-subunit as measured by protein blotting (Andrews *et al.*, 1989). Plasmids were transcribed *in vitro* using SP6 polymerase (MBI Fermentas Inc., Burlington, Canada). RNAs were translated in the presence or absence of 2 equivalents of microsomes (Perara and Lingappa, 1985) using rabbit reticulocyte lysate, and proteins were labeled with ^{35}S-methionine (Perkin-Elmer Biosystems-NEN). After a 1-h incubation at 24°C, translation reactions were halted by adding cycloheximide (Sigma–Aldrich Ltd., Oakville, Ont., Canada) to a final concentration of 1 mM. In some experiments, microsomes were pretreated with 5 μg ml^{-1} sequencing grade trypsin (Roche Diagonistic, Laval, Canada) on ice for 30 min, and then the tryspin was inactivated with phenylmethylsulfonyl fluoride (PMSF) before incubation in reticulocyte lyaste translation reactions (Andrews *et al.*, 1989). For post-translational insertion assays, 2 equivalents of microsomes were added to cycloheximide-terminated translation reactions and incubated for 1 h at 24°C. Co- and post-translation reactions were layered onto a sucrose cushion (0.5 M sucrose, 1 mM DTT, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.5)) and centrifuged at 100 000 ***g*** for 10 min at 4°C in an ultracentrifuge (TL100, Beckman, Mississauga, Canada) using a TLA100 rotor (Beckman). The supernatant, containing soluble proteins, was then divided into two equal fractions, representing the top (T) and the middle (M) fractions. The middle fraction comprises the bulk of the sucrose cushion. The microsome-containing pellet (named the bottom (B) fraction) was re-suspended in 1% (w/v) SDS, 10 mM Tris–HCl (pH 9.5) and heated at 70°C for 10 min. An equivalent amount of each fraction corresponding to the original translation reaction was analyzed by SDS–PAGE using a Tris-Tricine buffer system (Schägger and Jagow, 1987). Proteinase K treatments were performed as described by Falcone *et al.* (1999). Microsome-associated proteins (collected in the bottom fraction, see above) were re-suspended in 10 mM Tris–HCl (pH 7.5), 2.5 mM $MgCl_2$, 1 mM DTT, and then proteinase K (Sigma–Aldrich Ltd.) was added to each sample at a final concentration of 0.4, 0.1, or 0.01 μg ml^{-1} and incubated for 15 min at 0°C. Proteolysis was terminated by the addition of 1 mM PMSF (Sigma–Aldrich Ltd.). SDS–PAGE loading buffer heated to 90°C was added to each sample, and samples were analyzed by SDS–PAGE. Labeled proteins were visualized and quantified using a Molecular Dynamics STORM PHOSPHORIMAGER with allied software (version 5.0; Amersham Biosciences, Baie d'Urfé, Canada).

Membrane integration was determined by washing pelleted microsomes with alkaline sodium carbonate as previously described by Andrews *et al.* (1992). Briefly, membranes collected in the bottom fraction (see above) were re-suspended in 75 μl of 100 mM Na_2CO_3 (pH 11.5), and after 30 min at 0°C, the sample was layered onto a 0.5 M sucrose cushion and centrifuged at 100 000 ***g*** for 10 min. Integral membrane proteins in the pellet fraction (P) and soluble (lumenal) and peripheral membrane proteins in the gradient (S) were collected and analyzed by SDS–PAGE as above.

Transient transformations of onion epidermal cells

Onions were purchased at a local greengrocer (Guelph, Canada), and inner epidermal layers were peeled, cut into approximately 2–3-cm^2 pieces, and placed outward side down onto the surface of a 100 mm × 15 mm Petri dish containing 4.3 g l^{-1} Murashige-Skoog (MS) salt mixture (pH 5.7; Life Technologies Inc., Burlington, Canada), 30 g l^{-1} sucrose, solidified with 4% (w/v) agar (Scott *et al.*, 1999). Precipitation of 10 μg of plasmid DNA (or 5 μg of each plasmid for co-expression studies) onto M-17 tungsten particles and biolistic transformations of onion peels using a Biolistic Particle Delivery System-1000/He (Bio-Rad Laboratories, Hercules, CA, USA) were carried out as previously described by Banjoko and Trelease (1995). Following bombardment, Petri dishes were sealed with parafilm and incubated for 2–20 h (see Figure legends for details) at 25°C in the dark. Peels were then mounted onto glass slides without a cover slip, and GFP signals were analyzed via fluorescence microscopy. Plasmolysis of onion cells was carried out by incubating bombarded peels 5 min prior to viewing by microscopy with 0.8 M mannitol (prepared in the MS salt and vitamin mixture described above).

Yeast growth conditions, transformations, extraction and analysis of lipids, and processing for indirect immunofluorescence microscopy

The yeast strain MMYO11α (McCammon *et al.*, 1990) was used in all studies. Plasmids were transfected into yeast cells using the lithium acetate method of Gietz and Woods (1994), and transformants were maintained at 30°C on synthetic dextrose (SD) [2% (w/v) dextrose and 0.67% (w/v) yeast nitrogen base without amino acids] plates containing appropriate auxotrophic supplements. Liquid culturing of transformed cells in either synthetic dextrose medium or synthetic galactose (SGal) [2% (w/v) galactose and 0.67% (w/v) yeast nitrogen base without amino acids] medium with appropriate amino acid supplements plus or minus 0.1% (v/v) linoleic acid was performed as described by Dyer *et al.* (2002). All cells were cultured at 30°C and then processed, depending on the experiment (see Figure legends for details), either at mid-log or late-log phase.

The enzymatic activity of various fatty acid desaturases ectopically expressed in yeast cells was evaluated by gas chromatography (GC) analysis of yeast fatty acid composition, as described previously by Dyer *et al.* (2002). Briefly, yeast cells were harvested at late-log phase by centrifugation, cells were converted to spheroplasts by enzymatic digestion of cell walls, and total cellular lipids were extracted with chloroform/methanol. Fatty acid methyl esters (FAME) were prepared using sodium methoxide, and then FAME were separated, identified, and quantified using a Hewlett-Packard GC/FID/MSD system (model 5890 series II) with CHEMSTATION software (Agilent, Palo Alto, CA, USA). Methyl heptadecanoate was included as an internal standard.

Yeast cells were processed for indirect immunofluorescence microscopy as described previously by Pringle *et al.* (1991), with a few modifications. Briefly, cells expressing *GAL*1-controlled myc-tagged, wild-type or modified (C-terminal truncation) FAD2 or FAD3 genes were grown overnight in SGal selective medium to reach OD_{600} of 2–3 (pulse conditions), centrifuged, re-suspended in SD selective medium, and grown for another 2 h (chase conditions). Growth in glucose-containing medium shuts down the expression of GAL-induced genes and avoids the contribution of newly synthesized proteins to the immunofluorescence pattern. Cells expressing wild-type myc-FAD2 or myc-FAD3 also co-expressed the ER marker protein ss-GFP-HDEL. All cells were then centrifuged and fixed with 4% (w/v) formaldehyde in SD medium for 1 h, washed three times with B-buffer (1 mM KPO_4, 1.2 M sorbitol (pH 7.5)), and then re-suspended to an OD_{600} of 5. To a 1-ml aliquot of concentrated cells was added 2 μl of 2-mercaptoethanol followed by 10 μl of 10 mg ml^{-1} zymolase 20T (ICN, Mississauga, Canada) in B-buffer. Cells were then incubated for 20 min at 37°C on a roller, centrifuged, and washed two times with B-buffer. Aliquots of the resulting spheroplasts were applied to individual reaction wells on glass slides (F.G.R. Steinmentz Inc., Surrey, Canada) previously treated with a 0.1% (v/v) poly-lysine solution (Sigma-Aldrich Ltd.) to allow cell attachment. After 30 min, 0.1% (v/v) NP-40 in B-buffer was then added to the spheroplasts for 15 min in a moist chamber. Residual detergent was removed by aspiration, and spheroplasts were washed once with B-buffer and then two times with phosphate-buffer saline (PBS) solution containing 0.1% (w/v) BSA. Permeabilized spheroplasts were then incubated for 1 h with primary IgGs, washed several times in PBS, and incubated for an additional 1 h with appropriate dye-conjugated secondary antibodies. Stained cells were then washed in PBS and sealed with a cover slip and nail polish. Mouse antimyc IgGs (clone 9E10) and goat antimouse or antirabbit cyanine (Cy2 or Cy3)-conjugated secondary IgGs were from Berkeley Antibody Company (Richmond, CA, USA) and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), respectively. Antibodies against recombinant *S. cerevisiae* Tlg1p, a marker protein for the yeast Golgi apparatus were raised in rabbits.

Microscopy

Fluorescent, bright-field, and differential interference contrast (DIC) images of cells were all acquired using a Zeiss Axioskop 2 MOT epifluorescence microscope (Carl Zeiss, Toronto, Canada) with either a Zeiss 10× Plan A objective lens for onion epidermal cells or a Zeiss 100× Plan Apochromat oil-immersion objective for yeast cells. Image capture was performed using a Retiga 1300 charge-coupled device camera (Qimaging, Burnaby, Canada) and NORTHERN ECLIPSE 5.0 software (Empix Imaging Inc., Mississauga, Canada). All fluorescence images of cells shown in individual figures were obtained using identical acquisition settings and processing variables and are representatives of >50 independent (transient) transformations from at least two independent biolistic bombardment experiments. Figure compositions were generated using ADOBE PHOTOSHOP 6.0 (Adobe Systems Inc., San Jose, CA, USA).

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