In expanding pea leaves, over 95% of fatty acids (FA) synthesized in the plastid are exported for assembly of eukaryotic glycerolipids. It is often assumed that the major products of plastid FA synthesis (18:1 and 16:0) are first incorporated into 16:0/18:1 and 18:1/18:1 molecular species of phosphatidic acid (PA), which are then converted to phosphatidylcholine (PC), the major eukaryotic phospholipid and site of acyl desaturation. However, by labeling lipids of pea leaves with [14C]acetate, [14C]glycerol, and [14C]carbon dioxide, we demonstrate that acyl editing is an integral component of eukaryotic glycerolipid synthesis. First, no precursor-product relationship between PA and PC [14C]acyl chains was observed at very early time points. Second, analysis of PC molecular species at these early time points showed that >90% of newly synthesized [14C]18:1 and [14C]16:0 acyl groups were incorporated into PC alongside a previously synthesized unlabeled acyl group (18:2, 18:3, or 16:0). And third, [14C]glycerol labeling produced PC molecular species highly enriched with 18:2, 18:3, and 16:0 FA, and not 18:1, the major product of plastid fatty acid synthesis. In conclusion, we propose that most newly synthesized acyl groups are not immediately utilized for PA synthesis, but instead are incorporated directly into PC through an acyl editing mechanism that operates at both sn-1 and sn-2 positions. Additionally, the acyl groups removed by acyl editing are largely used for the net synthesis of PC through glycerol 3-phosphate acylation.

In plants acyl carrier protein (ACP)-dependent de novo fatty acid synthesis (FAS) is restricted to organelles (1). Essentially all acyl chains for membrane and storage lipid synthesis are produced in the plastid (2, 3). The initial products of FAS, 16- and 18-carbon fatty acids (FAs) esterified to ACP, are incorporated into glycerolipids by one of two routes: (i) acyl-ACP is used directly by acyltransferases of the “prokaryotic” pathway within the plastid, or (ii) the acyl-ACP thioester bond is hydrolyzed during acyl export from the plastid prior to FA reactivation and incorporation into glycerolipids by acyltransferases of the “eukaryotic” pathway outside the plastid. This two pathway mechanism for glycerolipid synthesis was first elucidated by radiotracer studies (4), and was confirmed genetically by analysis of Arabidopsis mutants (2, 5). The proportions of nascent FA (i.e. immediate products of FAS) incorporated into the eukaryotic and prokaryotic pathways vary widely among plants and different tissues within the same plant. The eukaryotic pathway predominates in non-photosynthetic tissues of all higher plants. In 18:3 plants, so called because they accumulate predominantly 18:3 and not 16:3 in their leaf galactolipids, 95% of the FA produced in the plastid is exported for assembly into glycerolipids by the eukaryotic pathway. Only phosphatidylglycerol (PG) is synthesized directly in the plastid from acyl-ACPs. This is in contrast to leaves of 16:3 plants which can also synthesize glycolipids by the prokaryotic pathway. Consequently, only about 60% of FA produced in the chloroplast by 16:3 plants is exported to the eukaryotic pathway (6). 18:3 plants, which include pea, make up about 88% of angiosperm species (7).

It is generally assumed that the major exported products of chloroplast FAS, namely 18:1 and 16:0 FA, are transferred to the outer envelope of the plastid where they are activated to acyl-CoAs by a long chain acyl-CoA synthetase (8, 9). The eukaryotic pathway for de novo PC synthesis then utilizes this pool of newly synthesized acyl-CoAs for sequential sn-1 and sn-2 acylations of glycerol 3-phosphate to produce 18:1/18:1 and 16:0/18:1 molecular species of phosphatidic acid (PA). PA is rapidly converted to phosphatidylcholine (PC) by the action of PA phosphatase and CDP-choline:1,2-diacyl-sn-glycerol choline-phosphotransferase (2). Desaturation of 18:1 to 18:2 and then 18:3 on PC produces the abundant polysaturated molecular species of PC (10, 11).

However, several lines of evidence suggest that this model may be inadequate and needs to take account of acyl editing. We define acyl editing, often also termed “remodeling” or “retailing,” as any process that exchanges acyl groups...
between polar lipids but which does not by itself result in the net synthesis of the polar lipids. Acyl editing has long been considered an important facet of phospholipid metabolism (12). Acyl editing relevant to this work can occur through two mechanisms. In plants acyl editing via a CoA:PC acyl exchange mechanism was demonstrated in microsomes isolated from developing seeds and was attributed to a reverse reaction of lysophosphatidylcholine acyltransferase (LPCAT) (13). LPCAT activity has also been described in isolated chloroplasts (14–16) as well as microsomes (17–20). Thus LPCAT allows for a mechanism for acyl editing, although the in vitro results do not indicate how prevalent the reaction might be in vivo. In this context, isolated pea chloroplasts incubated with [14C]acetate immediately label PC with newly synthesized FA through a channeled pool of acyl-CoA (21). Acyl editing involves hydrolysis of the phospholipid, such as PC to lyso-PC or even to glycerolphosphorylcholine (GPC), activation of the released free fatty acid and its reutilization for phospholipid synthesis from lyso-PC or GPC. Based on 18O labeling there is some indication that acyl chains esterified to bulk cellular PC are under a constant flux of acyl editing that proceeds wholly or in part through a hydrolytic deacylation-recacylation cycle (22).

The most direct line of evidence of acyl editing in plants comes from a careful analysis of the molecular species of monogalactosyldiacylglycerol and PC after labeling leaf disks of the 16:3 plant Brassica napus with carbon dioxide (23). [14C]Carbon dioxide labeling produced initial acyl-labeled species as expected for prokaryotic monogalactosyldiacylglycerol, namely dual labeled 16:0/16:1. By contrast for PC a high degree of scrambling between labeled and unlabeled acyl chains was noted. The authors concluded that there was continuous exchange of acyl groups between all molecular species of PC immediately after labeling and during the prolonged pulse-chase period of 48 h. In this report, we augment and extend these important observations and conclusions in several ways: 1) We perform rapid kinetic studies to more carefully address PC labeling and that of its precursors, PA and 1,2-diacyl-sn-glycerol (DAG). We address whether the initial incorporation of nascent fatty acids occurs via acyl editing, or if there is a rapid incorporation by de novo PC synthesis via glycerol-3-P (G3P) and DAG, which is followed by rapid acyl editing of PC. 2) We perform both total molecular species and stereochemical analyses on acyl-labeled PC using in vivo experiments with expanding pea leaves and seedlings. Pea is an 18:3 plant, so this complements the analysis done previously with the 16:3 plant B. napus (23). 3) We track molecular species of PC labeled in the glycerol backbone. When combined with the analysis of acyl labeling, this allows us to propose that sn-1 acyl editing is as important a component as sn-2 acyl editing. 4) As a control, we perform rapid labeling experiments in planta using carbon dioxide. The results parallel those obtained with excised tissue assays, indicating that there are no wound responses that compromise the metabolic conclusions obtained from excised tissue experiments.

From these studies we analyzed possible models by which newly synthesized FA are incorporated into eukaryotic lipids. The data presented in this report and from other studies do not allow us to unambiguously define one particular model, but do narrow down the possible routes in which nascent FA are incorporated into eukaryotic glycerolipids and suggest future directions to reexamine this important yet poorly understood area of plant lipid metabolism.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—All experiments were performed with Garden pea (Pisum sativum L. cv Little marvel) grown in soil/perlite/vermiculite (1:1:1) mixture at 22–25 °C under a day/night 8/16-h photoperiod of white light at 185–210 μmol m−2 s−1. Leaves, or in some cases, whole seedlings were harvested 8 days after sowing.

**Radiochemicals**—[1-14C]Acetic acid, sodium salt (specific activity 50 mCi/mmol), [U-14C]Glycerol (specific activity 150 mCi/mmol), and [14C]sodium bicarbonate (specific activity 50 mCi/mmol) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

**Leaf [14C]Acetate or [3-14C]Glycerol Labeling**—Leaf-labeling experiments used ~0.3 g fresh weight of pea leaf strips per assay, incubated in the light (180–220 μmol m−2 s−1) at 22–24 °C with reciprocal shaking in 5 ml of media containing 20 mM MES pH 5.5, 0.1× MS salts, and 0.01% Tween 20. Cut leaves were placed in media without radioisotope and precultured in ambient light for 5 min. Labeling was started by the addition of radioactive substrate and strong illumination. The reaction was quenched by transfer of leaves into isopropyl alcohol at 80 °C for 10 min. For labeling of acyl groups 250 μCi of [1-14C]acetic acid (1.0 mM) was used per replicate. For [14C]glycerol labeling of PC molecular species each incubation contained 47 μM [U-14C]glycerol (39 μCi). For [14C]glycerol time course the labeling media contained 25 μM [U-14C]glycerol (18 μCi), and the media was reused for consecutive 1, 3, 6, and 9 min time points. In vivo labeling with excised plant tissue can produce considerable variance between samples due to differences in development and in uptake of substrate. To minimize such variance each data point for total incorporation into lipids was normalized against the trend line for all time points to allow improved kinetic plots (Figs. 1 and 3).

**Seedling [14C]Carbon Dioxide Labeling**—Assays were conducted with 8-day potted pea seedlings in a closed 9.5 L glass desiccator under 250 μmol m−2 s−1 white light. [14C]CO2 was released by injection of 1 ml H2SO4 through the desiccator lid into a vial containing aqueous [14C]NaHCO3. The head space of the vial was quickly flushed with 30 ml of air, and the desiccator sealed for the desired time. Labeling was stopped by removal of the shoots at the base of the first leaves and quenching in 80 °C isopropyl alcohol. The assay used ~700 μCi substrate and ~40 seedlings. The seedlings from each labeling were split into two replicate samples (~20 seedlings each) for analysis. Although each labeling was nominally for 5 min, about 5 min was required to remove all the seedlings and quench, so the assay is reported as of 5–10 min duration.

**General Methods**—Lipids were extracted from hot isopropyl alcohol quenched tissue with hexane/isopropyl alcohol (24)
after homogenization with a mortar and pestle. Chlorophyll was determined spectrophotometrically at 652 nm in 20% aqueous acetone (25). Radioactivity in the total lipid samples, eluted lipids or organic and aqueous phases recovered from transmethylation was quantified by liquid scintillation counting (Beckman Instrument Inc., Fullerton, CA), while radioactivity on TLC plates was visualized and quantified by electronic radiography (Packard Instrument Co., Meriden, CT). AgNO₃-TLC plates were prepared by impregnating Partisol® K6 silica gel 60 Å TLC plates (Whatman, Maidstone, UK) with 10% AgNO₃ in acetonitrile (w/v), drying in air and activating at gel 60 Å TLC plates (Whatman, Maidstone, UK) with 10% glycerol 3-phosphate standards, but not choline.

3.4. v/v/v/v), as sample radioactivity co-migrated with glycerol rated based on the number of double bonds by AgNO₃-TLC, development in chloroform/methanol (96:4, v/v), then fully in chloroform/methanol (99:1, v/v). The proportion of radioactivity in each band was determined by electronic autoradiography, then each band was eluted, the recovered lipids transmethylated, and the [¹⁴C]FAME analyzed by AgNO₃-TLC, as described above. To determine endogenous acyl groups from isolated PC molecular species, triheptadecanoin was added as an internal standard to each fraction during elution and prior to transmethylation and GC analysis of FAME. When necessary 1,2-diacyl-3-acetylglycerol fractions recovered from AgNO₃ TLC plates were further purified by reverse phase TLC on Partisol® KC18 silica gel 60 Å plates (Whatman) developed with methanol/acetonitrile/water (75:25:2, v/v/v).

**PC Acyl Group Stereochemistry**—PC was isolated as described above and digested with phospholipase A₂ (Crotalus atrox, Sigma) (28). Briefly, PC was dissolved in 1 ml of diethyl ether and 0.5 unit of PL A₂ in 0.1 ml of 50 mM Tris-HCl, pH 8.7, 5 mM CaCl₂. The reaction was mixed vigorously for 5 min then the ether was evaporated under N₂. To extract lipids 3.8 ml of chloroform/methanol (2:1, v/v) and 1 ml of 0.15 M acetic acid were added, the mixture vortexed, the chloroform phase collected and the aqueous phase back extracted with 2.5 ml of chloroform. Reaction products were separated on silica TLC plates developed with chloroform/methanol/acetic acid/water (50:30:8/4, v/v/v/v). Radioactivity in the free fatty acid and lyso-PC fractions was quantified by electronic autoradiography, then each product eluted and transmethylated by heating at 50 °C in 5% sulfuric acid in methanol for 30 min. Labeled and unlabeled FAME compositions were determined as described above.

**Lipid Molecular Species by Mass Spectrometry**—Pea leaf samples were analyzed by ESI-MS/MS by the Kansas Lipidomics Research Center. Extraction of lipids was conducted by their standard Arabidopsis leaf protocol. The data set from this analysis is available in supplemental Fig. S1.

**Net Rate of Fatty Acid Deposition**—The net rate of fatty acid synthesis by pea leaves was determined by harvesting 10 leaves from pea seedlings at the start and end of the light cycle for 3 days. After immediate weighing to determine fresh weight, FAME and chlorophyll contents were measured as described above. Triheptadecanoin was added during the lipid extraction as an internal standard, providing methyl heptadecanoate after transmethylation for FAME analysis by GC.

**RESULTS**

**Kinetics of Glycerolipid Labeling from [¹⁴C]Acetate**—Relationships between precursor and product pools in metabolic pathways are revealed by kinetic labeling experiments. The linear accumulation of a product, once reached, coincides with steady state labeling of all precursor pools. In this context [¹⁴C]acetate is ideal for the study of acyl lipid metabolism as it is rapidly taken up by excised leaf tissue and utilized for FAS, while the lipids are labeled in their acyl groups, with minimal head group labeling (21). The kinetics of [¹⁴C]acetate labeling of glycerolipids from rapidly expanding excised pea leaves over a period of 9 min is shown in Fig. 1. PC was the major radiolabeled glocycerolipid with over 65% of the label at all time points. Furthermore, steady state labeling was established very rapidly, with no detectable lag (Fig. 1A) and thus suggested that PC was...
a very early product of nascent FA incorporation into eukaryotic glycerolipids. A second independent 9-min time course gave similar results (data not shown). PE was labeled in a similar manner (Fig. 1C), with no obvious lag phase, but PC labeling was 15-fold greater than PE labeling despite endogenous PC levels only being twice that of PE (supplemental Fig. S1A).

Both the PA and the DAG pools were still filling after the onset of PC steady state labeling. The label in PA reached a maximum by 3 min whereas DAG labeling increased throughout the time course (Fig. 1B). Thus, although PA and DAG are intermediates in the de novo synthesis of PC via acylation of glycerol-3-P, these [14C]-acyl-labeled intermediates do not show a precursor-product relationship relative to PC labeling, and thus represent different glycerolipid pools. Of course, we cannot rule out that very small labeled PA and DAG pools might contribute to PC labeling in a precursor-product relationship, because experimentally, quenching in these kinetic assays limits the temporal resolution of the method. A statistical analysis suggested about 0.5 min of variance (95% CI) in rate extrapolations to zero time. In conclusion, we were not able to detect a [14C]acyl-labeled glycerolipid precursor pool to the labeling of PC.

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**Acyl Compositions from [14C]Acetate Labeling of Lipids**—Fig. 2 shows the composition of labeled fatty acids from the [14C]acetate time course, for total lipids, PC, PA, DAG, PG, and PE. Oleate was the major product in total lipids (Fig. 2A), with saturates, mainly palmitate, decreasing from 32% at 1 min to about 24% for the remainder of the assay period. The reason for this decline is unknown. There was no detectable desaturation of 18:1 at the earliest time point but desaturation to 18:2 was detectable from 3 min onwards. PC had less saturates (11% falling to 8%) whereas PE had more saturates (54% falling to
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By the end of the time course desaturation produced 7% linoleate in PC (Fig. 2B). In contrast to PC and PE, PA (Fig. 2C) contained high levels of saturates (>-60%) throughout the time course and had an acyl composition with the closest match to PG. PA is also an intermediate of the prokaryotic glycerolipid synthesis pathway, and so the labeled PA pool is likely the precursor for prokaryotic PG labeling. In this context, close inspection of Fig. 1, B and C shows a kinetic precursor-product relationship between PA (label reaching a maximum at 3 min) and PG (lag phase ending at ~3 min), confirming this conclusion. DAG contained an intermediate level of saturates compared with PC and PE. The origin of the labeled DAG is not certain. It is probably largely eukaryotic in origin, and may arise in part from the reverse action of CDP-choline:DAG cholinephosphotransferase or phospholipase C on labeled PC, or from acyl groups edited from PC re-entering the eukaryotic de novo glycerolipid synthesis pathway. Any of these explanations is supported by the appearance of labeled linoleate in DAG by 9 min (Fig. 2D). Whatever its origin, the difference in labeled acyl group composition of PA and DAG compared with PC supports the conclusion from the kinetic data that they do not represent precursors of initial PC acyl labeling.

The Kinetics of Glycerolipid Labeling from [14C]Glycerol—[14C]Glycerol is rapidly taken up by pea leaves and incorporated into the glycerol backbone of glycerolipids. In addition, acyl groups also become labeled because glycerol 3-phosphate equilibrates with glycolytic precursors, leading to plastid acetyl-CoA production (29). To separately analyze the label from the backbone/head-group and the acyl chains, isolated lipid classes were transmethylated. Analysis of the aqueous fraction from PC by TLC indicated that the radioactivity was contained in the glycerol backbone and not the choline headgroup, as noted before (29). In marked contrast to acetate labeling, [14C]glycerol incorporation into lipids exhibited a lag (Fig. 3A). At the earliest time points, radioactivity in DAG was approximately equal to PC. We assume that the labeled PA includes a large contribution from the plastid component. However, chloroplast lipid assembly in 18:3-plants does not require PA conversion to DAG, so we also assume the labeled DAG is largely associated with extraplastidal membranes.

Two lines of argument support the notion that the biosynthesis of PC from labeled acetate (Fig. 1) and from labeled glycerol (Fig. 3) report different metabolic processes. First, for de novo PC synthesis the relative movement of label from PA to DAG to PC should be same for both glycerol and acyl group labeling strategies. However, at the earliest time points, it is clear that acetate acyl chain labeling produces PC >> DAG (Fig. 1A) whereas glycerol backbone labeling produces DAG ~ PC (Fig. 3A), suggesting separate metabolic processes. Second, when we analyze the different kinetics of acyl chain and glycerol labeling of PC and DAG from [14C]glycerol labeling (Fig. 3B), the amount of label in the acyl chains of DAG remained fairly constant (4–5%) while the acyl label in PC fell from 32% at 1 min to 12% at 9 min. The difference is explained by postulating two different pathways for PC synthesis. About 10% of the label from exogenous glycerol is rapidly utilized for de novo FAS and labeled acyl groups move rapidly to PC, and then to DAG, as described for Fig. 1. The remainder of the labeled glycerol moves through PA to DAG, which has a half-life for pool filling of about 3–4 min, and then to PC. Under these conditions the fraction of PC which is acyl-labeled is initially high but declines steadily, while this simple model predicts that the acyl-labeled fraction of DAG remains low and fairly constant. This is what is observed. Together the glycerol backbone and acyl chain labeling from [14C]glycerol and the [14C]acetate acyl group labeling provide evidence for a separate (acyl editing) pathway for newly synthesized FA to rapidly enter PC without de novo PC synthesis by the eukaryotic pathway.

Glycerolipid Molecular Species in Expanding Pea Leaves—Molecular species of PC from expanding pea leaves were analyzed by GC of FAMEs following AgNO3-TLC separation of the corresponding 1,2-diacyl-3-acetylglycerols. The data are shown in Fig. 4A, and are very similar to a published analysis of PC molecular species from pea leaf microsomes as determined by reverse-phase HPLC (20). In addition pea leaf PC molecular species from expanding leaves were determined by ESI-MS/MS.
The major fatty acids of pea leaf PC are 18:2 (44%), 18:3 (31%), 16:0 (18%), 18:0 (4.2%), and 18:1 (2.6%). The major molecular species that we observe are the expected combinations of these fatty acids, given that saturates are confined to the sn-1 position. Oleic (18:1) and palmitic (16:0) are the major products of chloroplast FAS (Fig. 2). We note that if these nascent FA were incorporated via de novo PC synthesis without any contribution from acyl editing then 18:1/18:1-PC would be the most abundant initial species formed. However this species represents less than 1% of the total PC (MM, Fig. 4A). Likewise, the PA molecular species contain only trace amounts of 16:0/18:1 (C34:1) and 18:1/18:1 (C36:2) based on ESI-MS/MS (Fig. 4C). The majority of the C36:2 PA (and PC) species will be 18:0/18:3 (34:3). PA may include biosynthetic pools, pools derived from lipid signaling via phospholipase D (30) and DAG kinase, and the putative PA pool for delivery of eukaryotic molecular species to the plastid for galactolipid synthesis (31). PA also contains a small amount of 34:6, which is presumed to be 18:3/16:3, and may therefore indicate a plastid contribution.

Analysis of Molecular Species of PC from [14C]Acetate Labeling—To examine the initial molecular species of PC produced from incorporation of nascent FA, pea leaves were labeled with [14C]acetate for 5 min. This time was a compromise between allowing enough labeling of PC for the analysis, yet keeping the amount of labeled 18:2 to a minimum (Fig. 2B). Each labeled molecular species, analyzed as the 1,2-diacyl-3-acetylelglycerol derivative, was also analyzed for labeled fatty acid composition. Fig. 5A and B show the distribution of PC molecular species by mass and [14C]acyl group, respectively. If acyl chains enter eukaryotic phospholipid synthesis via glycerol 3-phosphate acylation without acyl editing the predicted initial molecular species of PC would be 16:0/18:1 and 18:1/18:1 (S/M and M/M, respectively). Each will be dual-labeled with newly synthesized FA, and if the saturates are 10% of the total label (Fig. 2B) then the S/M to M/M labeling ratio will be ~1:4. This does not occur. Also, the S/M molecular species was only labeled with oleate; the corresponding palmitate was unlabeled. The MM molecular species which would be predicted to contain 80% of the initial label actually represented only 6.5% of all the labeled molecular species. It is unclear if this species contains one or both acyl groups labeled. The data in Fig. 5B indicate that all other labeled PC molecular species contain one labeled acyl group (18:1 or 16:0/18:0) together with an unlabeled acyl group (18:2, 18:3, or 16:0/18:0). Thus >90% of the molecular species of [14C]PC contain one newly synthesized (labeled) FA in the same molecule as a previously synthesized (unlabeled) FA. This finding strongly suggests acyl editing as a mechanism of PC synthesis with nascent FA.

Analysis of Molecular Species of PC Synthesized de Novo from [14C]Glycerol—Because acyl editing only exchanges acyl groups it does not result in net synthesis of phospholipid. However, in order for a leaf to grow net (i.e. de novo) PC synthesis is required. To determine the PC molecular species and hence ascertain which acyl chains are involved in de novo eukaryotic glycerolipid synthesis, pea leaves were incubated with [14C]glycerol. The PC glycerol backbone will be labeled regardless of whether the acyl chains are nascent FA or from acyl editing. Fig. 5C shows the distribution of label among PC molecular species.
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molecular species after 5 min of [14C]glycerol labeling. The data are corrected to give only the glycerol backbone labeling by subtracting the small amount of acyl labeling (Fig. 3B) that occurs from [14C]glycerol. The PC molecular species distribution obtained with glycerol backbone labeling (Fig. 5C) is quite different from that for acetate acyl labeling (Fig. 5B), but closely resembles the endogenous mass distribution (Fig. 5A), with the exception of a significant reduction in 18:2-containing molecular species and an increase in 18:3-containing species. The major initial PC molecular species predicted from incorporation of only nascent FAs, 18:1/18:1, was less than 0.2% of labeled species. The total 18:1 content of the glycerol-labeled PC is 5%, some of which may derive from nascent FA. We know that at this time point the ratio of unsaturates to saturates in PC from nascent FA is about 10:1 (Fig. 2B) and that there are negligible polyunsaturates. Thus we infer that at this point there is a 5.5% contribution of newly synthesized FA to de novo PC synthesis, or, conversely, that the bulk (>95%) of the acyl groups in PC molecular species labeled via glycerol backbone come from acyl editing. Fig. 5D, which describes PC molecular species analysis from [14C]carbon dioxide labeling, will be described in a later section.

Stereochemistry of PC Acylation—Molecular species of PC can be defined by acyl composition and/or by stereochemistry. To determine the stereospecific incorporation of newly synthesized FA, PC isolated from 5-min incubations of pea leaves with [14C]acetate were digested with phospholipase A2 and the reaction products analyzed (Fig. 6). 62% of the label was incorporated at the sn-2 position and 38% at the sn-1 position. Saturates were predominately found in the sn-1 position, indicating that the PLA_2 digestion was sn-2-specific. Although de novo PC synthesis requires equimolar acylations at the sn-1 and sn-2 position we cannot assume that this will be the case for the 16:0 and glycerol moiety only (label in acyl chains has been subtracted). Results expressed as mean ± S.E. (triplicate determinations). D, 5–10 min [14C]CO_2 acyl chain-labeled PC molecular species. Results expressed as mean ± S.E. (quadruplicate determinations).
18:1 labeled FA if they are mixing with an endogenous acyl-CoA pool containing 18:2 and 18:3, because we do not know the acyl transferase specificity at each position. Previous workers have reported either a similar sn-1 to sn-2 distribution of acyl label, for PC from 30 min of acetate labeling of spinach leaves (32), or, using a 2-h acetate labeling of leek leaves, a 2:1 to 3:2 enrichment in the sn-2 position (33, 34). The unequal stereochemical labeling complements the conclusion that nascent FAs are incorporated into PC along side previously synthesized FA.

**Unlabeled FA Composition of Endogenous PC, LPC, and Various Labeled PCs**—From Fig. 5B we have estimated the composition of the unlabeled FAs that are associated with the nascent, [14C]acetate-labeled FAs. The calculated values are shown in Fig. 7, along with the acyl composition of endogenous PC (calculated from Fig. 4A) and of endogenous lyso-PC (obtained by ESI-MS/MS; supplemental Fig. 51K). To a first approximation all three profiles are similar. The unlabeled, paired acyl groups from [14C]acetate-labeled PC have a slightly reduced 18:2 and increased saturates compared with endogenous PC. The unlabeled fatty acids must have had an inverted stereochemical distribution to that of the labeled FA measured in Fig. 6; that is, 62% sn-1 and 38% sn-2. The fact that the FA profile of lyso-PC was similar to that of total PC allows us to propose that the endogenous lyso-PC pool is produced by approximately equal sn-1 and sn-2 deacylations, whether catalyzed by hydrolysis or by PC acyl exchange with CoA. Thus the appropriate lyso-PC pool is available for both sn-1 or sn-2 acylations with newly synthesized FA. The unequal stereochemical acylation with nascent FA found in PC (Fig. 6) therefore may arise from sn-2 acyl migration in lyso-PC to the more thermodynamically stable sn-1 position, or possibly a higher reacylation of the sn-1 position with the unlabeled FA that have been previously removed by acyl editing. It is also possible that the bulk lyso-PC pool has little to do with the incorporation of nascent FA. Fig. 7 also shows the FA composition of molecular species of PC labeled by [14C]glycerol (calculated from Fig. 5C).

**[14C]Carbon Dioxide Labeling**—The use of excised tissue facilitates rapid kinetic analysis. However, incubation of excised leaf tissue might cause a wound response that could alter metabolism. In particular, lipase activities may be rapidly and transiently induced that could alter phospholipid metabolism. To control for this possibility whole pea seedlings were labeled for 5–10 min with [14C]CO2. Label is rapidly incorporated into both the acyl chains and glycerol backbone of lipids. Fig. 8 compares the distribution of acyl group labeling among lipid classes for [14C]CO2 labeling of whole seedlings and [14C]acetate labeling of cut leaves at approximately equivalent assay times. Both labeling experiments indicated that PC is the major recipient of newly synthesized FA and that the relative acyl group labeling among other lipid classes is similar. Additionally, molecular species of PC were analyzed for acyl chain labeling from whole seedlings with [14C]CO2 (Fig. 5D). This distribution should be compared with that from [14C]acetate labeling of cut leaves (Fig. 5B). The slightly longer time of assay (~10 min including seedling removal and quenching) for [14C]carbon dioxide compared with [14C]acetate labeling allowed slightly greater desaturation of 18:1 to 18:2. However, it is clear that the pattern of nascent FA acyl labeling from [14C]CO2 matches that of [14C]acetate. We conclude that the relative proportions of individual lipids and initial PC molecular species labeled in incubations of excised leaves was not due to a change in lipid metabolism caused by a wound response but is indicative of the *in planta* lipid metabolism.

**DISCUSSION**

Largely through analyses of the kinetics, molecular species and stereochemistry of the immediately labeled products of eukaryotic glycerolipid synthesis we demonstrate that, in rapidly expanding pea leaves, 1) there was no detectable precursor-product relationship for PA and DAG in the incorporation of nascent FA into PC, 2) nascent FA were incorporated into PC...
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FIGURE 9. Four possible models describing pathways and fluxes for the incorporation of nascent FA, exported from the chloroplast immediately after de novo FAS, into PC. For simplicity, the models are set up not to depend on enzyme specificity, and show only PC. The mechanisms for removal of fatty acids from PC and provision of acyl-CoA are not defined, while the width of the arrows gives an indication of relative flux. The gray box represents the chloroplast; G3P, glycerol 3-phosphate; FFA, free fatty acid. Model 1 shows a description lacking any acyl editing. Models 2 and 3 are considered the best representations for the acyl editing that accompanies in the incorporation of nascent FA into PC, but a definitive choice of the most correct model is not yet possible.

more rapidly than the de novo synthesis of PC from glycerol, suggesting two systems for the incorporation of acyl groups into PC, (3) greater than 90% of nascent FA were incorporated into PC molecules in combination with an endogenous FA, (4) greater than 95% of de novo synthesized PC was esterified by recycled acyl groups, (5) acyl editing can take place at both the sn-1 and sn-2 positions, and (6) that pea leaves contained an endogenous lyso-PC pool that could be involved in acyl editing. Experiments with carbon dioxide labeling of whole seedlings demonstrated that the metabolism seen in excised leaves is not due to a wound response.

These results lead us to conclude that the major pathway for the incorporation of newly synthesized FA into eukaryotic glycerolipids is largely through acyl editing of PC and that de novo synthesis of eukaryotic glycerolipids primarily utilizes acyl chains recycled from acyl editing. We will discuss these results in terms of the possible mechanisms and flux models (Fig. 9) that can be proposed to drive acyl editing.

18:1/18:1 and 16:0/18:1 Are Not Initial Molecular Species of Eukaryotic Glycerolipid Synthesis.—The classical scheme for eukaryotic phospholipid synthesis in leaves (2, 11), as summarized in Fig. 9 (Model 1) predicts the biosynthesis of dual-labeled 16:0/18:1 and 18:1/18:1 PA, DAG and then PC species sequentially. However, our kinetic analysis failed to detect precursor-product relationships between PA, DAG, and PC pools for acyl group labeling (Figs. 1 and 2). Furthermore, we have not been able to observe the expected, dual-labeled 16:0/18:1 and 18:1/18:1 PC species. Based on estimates of FAS fluxes and endogenous PA and PC molecular species pool sizes (see supplemental Note 1), labeled 16:0/18:1 and 18:1/18:1 molecular species of PC should have been easily detected by our analysis if Model 1 was correct. Additionally, glycerol labeling of de novo synthesized PC revealed mostly esterification with endogenous saturates and polyunsaturates, and not newly synthesized saturates and oleate. Taken together these three lines of evidence strongly suggest that 16:0/18:1 and 18:1/18:1 molecular species are not the first products of nascent FA incorporation into eukaryotic glycerolipids and that Model 1 is incorrect.

Most Newly Synthesized FAs Enter PC by sn-1 and sn-2 Acyl Editing, whereas de Novo PC Synthesis Primarily Uses Recycled Acyl Groups.—We have shown that the majority of newly synthesized (labeled) FAs in pea leaves are immediately incorporated into PC molecular species where >90% are paired with endogenous (unlabeled) FAs. A similar observation of >60% incorporation of nascent FA paired with endogenous FA is also reported by Williams et al. (23) for B. napus leaves 1 h after a pulse of carbon dioxide labeling. Because we have used rapidly expanding leaves from an 18:3 plant, whereas Williams et al. (23) used mature leaves from a 16:3 plant, it appears that the observation may be a general one. The result implies some form of acyl editing. Either the nascent FA are used directly in an acyl editing process (Fig. 9, Model 2), or acyl groups are released from endogenous lipids and mix with the nascent FA prior to completing the acyl editing cycle and/or acylation of glycerol-3-P for de novo PC synthesis (Fig. 9, Models 3 and 4). Models 2–4 will be discussed in more detail later. Whatever the acyl editing mechanism, net (i.e. de novo) phospholipid synthesis from glycerol-3-P is required for the leaf to grow. Glycerol labeling showed that de novo PC synthesis utilized mainly endogenous palmitate (+ stearate), linoleate, and linolenate and not newly synthesized FA. Thus the FA used for de novo PC synthesis must be largely (>95%) recycled from acyl editing, and, almost certainly, largely from PC. The fact that saturated FAs are major acyl groups released by acyl editing suggests that sn-1 hydrolysis or CoA:PC sn-1 acyl exchange contributes in a major way to this process. This is confirmed by the results from the acetate labeling experiments shown in Fig. 5B and Fig. 6, namely that >90% of the molecular species contain only one labeled acyl group and 40% of the labeled acyl groups are at the sn-1 position. For Models 2 and 3, this implies that a sn-1 acyl editing process for incorporation of nascent FA into PC represents 30–40% of the net flux. This conclusion from our in vivo labeling represents a substantial biochemical activity largely overlooked or underrepresented by previous in vitro analyses of PC acyl metabolism (13, 16) and in vivo labeling studies (23).

Proposed Models for Acyl Editing.—Questions then arise as to the pathways, fluxes and mechanisms for the metabolism of PC synthesis utilizing nascent FA from the chloroplast. The puzzle
is complex because of the large number of unknowns, and our current data do not completely resolve the models proposed. However, it is instructive to review possible models to highlight the unknowns requiring resolution. Three models are considered which might accommodate a flux of x mol of nascent FA from the chloroplast, producing 0.5x mol of net PC synthesis (Fig. 9, Models 2–4). For simplicity, the models were initially set up not to depend on enzyme specificity, and show only PC. The mechanisms for removal of fatty acids from PC and provision of acyl-CoA are not defined. They may be via phospholipase action with activation of the released free fatty acid by an acyl-CoA synthetase, or via direct CoA:PC acyl exchange.

Model 2 describes a situation whereby nascent FA are channeled to lyso-PC. Their incorporation allows just one labeled FA per re-synthesized PC molecule, as observed experimentally. The endogenous FA released from PC is concomitantly used in the de novo synthesis of PC. The endogenous FA may enter a general acyl-CoA pool, and the model does not rule out other sites of phospholipid acyl editing also supplying this pool. Model 2 implies acyl transfer reactions for nascent FA incorporation and de novo PC synthesis may be spatially distinct metabolic processes. The model cannot accommodate the catabolism of PC to GPC, because the acylation of GPC by the nascent, labeled FA will lead to two labeled acyl groups per PC molecule. As the endogenous, unlabeled acyl distribution in PC molecular species from acetate labeling closely matches the endogenous PC acyl distribution (Fig. 7), the lyso-PC and the FA pools produced by PC editing must have the similar acyl profiles. This is confirmed by the endogenous LPC analysis. The fact that both lyso-PC and unlabeled FA in PC/FA*-PC have compositions identical to the bulk PC does not absolutely require sn-1 and sn-2 deacylations to be on an equimolar basis. However, the phospholipase/acyl exchange activities (and any lyso-PC isomerization) will control the sn-1 and sn-2 distributions observed in acetate-labeled acyl groups. Thus Model 2 can accommodate the excess sn-2 acylation noted in acetate labeling. Model 2 requires an explanation for the reduction in 18:2 FA content for glycerol-labeled PC, by, for example, proposing a preference for the transfer of 18:2-containing acylglycerol moieties to the chloroplast for galactolipid synthesis (29).

Model 3 describes a situation whereby the nascent FAs are directed to a general acyl-CoA pool, along with FAs from PC acyl editing. Thus the pairing of labeled and unlabeled acyl groups in PC is caused by a high flux of acyl editing, which greatly dilutes out nascent FA in the common acyl-CoA pool. In this model it is possible to propose that PC editing stops at lyso-PC or instead goes through two deacylation steps to produce GPC. However, because GPC-dependent acyltransferases have not been reported in plants, we will focus on the lyso-PC example, as shown in Fig. 9 (Model 3). An analysis of the molecular species of PC labeled from acetate showed that >90% had single acyl group labeling. Without any substrate specificity constraints, this requires that >90% of the flux of acyl groups in the acyl-CoA pool originate from unlabeled fatty acids (supplemental Fig. S2). Consistent with this observation, analysis of the molecular species of PC with glycerol backbone labeling required that >95% of the flux for de novo PC synthesis come from acyl editing. With this model the sn-1 and sn-2 distributions for nascent FA do not have to fit a 1:1 ratio. Also, it is easy to explain the deficit of 18:2 acyl groups in the de novo PC labeling with glycerol. We can simply assert that there is some acyl-CoA selectivity in the de novo synthesis at either sn-1 or sn-2 acylation. A moderate change in the de novo synthesis pathway acyl composition will have little effect on the endogenous acyl group composition measured by acetate labeling. Of course, the simplicity of this explanation does not prove it.

In Models 2 and 3 the acyl editing cycle (phospholipid deacylation and reacylation) is distinct from de novo phospholipid synthesis. It is, however, possible to have de novo phospholipid synthesis be part of the acyl editing cycle (Fig. 9, Model 4). This requires PC to be completely degraded to glycerol or glycerol-3-P, and choline or choline-P. In this model there is only one pathway for the synthesis of PC, and if the PC turnover rate is high relative to nascent FA production, much of the labeling data is consistent with this model. However, a comparison of labeling kinetics for DAG and PC using either acetate or glycerol substrates strongly suggested that we were dealing with two distinct metabolic processes for PC synthesis. This is inconsistent with Model 4, and on this basis, we rule it out as a dominant pathway of leaf cytosolic glycerolipid synthesis.

In this discussion we have arrived at the conclusion that one of a pair of simple models (Fig. 9, Models 2 and 3), offers the best description of cytosolic glycerolipid synthesis in pea leaves. The models present quantitatively very different magnitudes for the fluxes involved in acyl editing, and give different views on how such acyl editing integrates with the incorporation into PC of nascent FA exported from the chloroplast. Currently, we cannot categorically rule out either Model 2 or Model 3. Furthermore, it is always possible to build more complexity into models, including substrate specificity and the possibility of some hybrid combination. Future oxygen-18 and acyl-CoA pool labeling studies may yield pertinent information on the mechanism of acyl editing: lipolytic or through CoA:PC acyl exchange. Enzymology with isolated chloroplasts and microsomes may yield useful information on transacylation and de novo acyl transferase mechanisms and selectivity. Such studies must take into account the very substantial sn-1 component identified for acyl editing. Furthermore, endogenous PC is 2-fold higher than PE, but acyl labeling of PC from acetate (and also from glycerol) is 15-fold greater. This is despite the total leaf lyso-PE pool being approximately equivalent to lyso-PC (supplemental Fig. S1B). Thus, there is 7.5- or 15-fold preference for incorporation into PC via acyl editing that will have to be taken into account in defining any mechanism. A complete understanding may require the study of metabolism in KO mutants. However, with so many genes annotated as lipases, acyl hydrolases, and transacylases (35), most with unknown functions, this may not be a facile approach even to this tricky question. Defining the mechanisms of the incorporation of nascent fatty acids into glycerolipids will help our understanding of lipid turnover and be relevant to research areas with more practical applications such as the integration of seed triacylglycerol synthesis with phospholipid turnover and the effects of biotic and abiotic stresses on phospholipid metabolism.
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