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Oilseed Isocitrate Lyases Lacking Their Essential Type 1 Peroxisomal Targeting Signal Are Piggybacked to Glyoxysomes

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Isocitrate lyase (IL) is an essential enzyme in the glyoxylate cycle, which is a pathway involved in the mobilization of stored lipids during postgerminative growth of oil-rich seedlings. We determined experimentally the necessary and sufficient peroxisome targeting signals (PTSs) for cottonseed, oilseed rape, and castor bean ILs in a well-characterized in vivo import system, namely, suspension-cultured tobacco (Bright Yellow) BY-2 cells. Results were obtained by comparing immunofluorescence localizations of wild-type and C-terminal–truncated proteins transiently expressed from cDNAs introduced by microprojectile bombardment. The tripeptides ARM–COOH (on cottonseed and castor bean ILs) and SRM–COOH (on oilseed rape IL) were necessary for targeting and actual import of these ILs into glyoxysomes, and ARM-COOH was sufficient for redirecting chloramphenicol acetyltransferase (CAT) from the cytosol into the glyoxysomes. Surprisingly, IL and CAT subunits without these tripeptides were still acquired by glyoxysomes, but only when wild-type IL or CAT–SKL subunits, respectively, were simultaneously expressed in the cells. These results reveal that targeting signal–depleted subunits are being piggybacked as multimers to glyoxysomes by association with subunits possessing a PTS1. Targeted multimers are then translocated through membrane pores or channels to the matrix as oligomers or as subunits before reoligomerization in the matrix.

INTRODUCTION

The post-translational targeting and import of proteins into peroxisomes appear to be unique when compared with the import of proteins into other organelles. Recent evidence suggests that protein subunits can gain access to the peroxisomal matrix even if they do not contain a peroxisome targeting signal (PTS). The most common PTS is a non-cleaved C-terminal SKL tripeptide motif, designated as a type 1 PTS (PTS1) (Gould et al., 1987; Subramani, 1993; Olsen and Harada, 1995). Another signal, designated PTS2, is a nanopeptide (R-L/I-X_5-H/Q-L) located within the N-terminal portion of peroxisomal-destined proteins (Osumi et al., 1991; Swinkels et al., 1991; de Hoop and Ab, 1992; Gietl, 1996). Subunits without a PTS seem to be acquired by peroxisomes by being carried or piggybacked with a subunit(s) possessing a PTS. For example, McNew and Goodman (1994) demonstrated that chloramphenicol acetyltransferase (CAT) subunits could be redirected or piggybacked from the cytosol of *Saccharomyces cerevisiae* and mammalian CV-1 cells into their respective peroxisomes by oligomerization with CAT subunits that possessed an appended C-terminal SKL. Glover et al. (1994) showed that thiolase subunits without a PTS2 were piggybacked from the cytosol into *S. cerevisiae* peroxisomes by dimerization with thiolase subunits possessing a PTS2.

These studies indicated that *S. cerevisiae* and mammalian peroxisomes have the ability to import multimeric proteins. In support of this contention, Walton et al. (1995) showed that 9-nm-diameter gold particles coated with SKL–COOH polypeptides conjugated to biotinylated human serum albumin were imported into peroxisomes of human fibroblast cells. Also, Häusler et al. (1996) demonstrated that the import of dihydrofolate reductase into trypanosome glycosomes (a variant of peroxisomes) was not inhibited by stabilization of the protein into a folded state. The authors concluded that glycosomes possess either a strong unfolding activity or an unusually large translocation channel. Collectively, these data suggest that the boundary membranes of mammalian, *S. cerevisiae*, and trypanosome peroxisomes contain large pores or channels through which multimeric proteins can be translocated into the peroxisome matrix (Rachubinski and Subramani, 1995; McNew and Goodman, 1996; Subramani, 1996).

Isocitrate lyase (IL) and malate synthase (MS) are enzymes unique to the glyoxylate cycle. This pathway is compartmentalized within specialized peroxisomes called glyoxysomes (Beevers, 1980), which have been identified in

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tissues of higher and lower plants, fungi, and some mammalian cells (Huang et al., 1983; Davis et al., 1989, 1990; Veenhuis and Harder, 1989). In higher plants, glyoxysomes are particularly prevalent in storage tissues of oilseeds (e.g., endosperm, cotyledons, and megagametophytes), where the glyoxylate cycle is centrally involved in the conversion of storage oils to carbohydrates during postgerminative growth (Beevers, 1980; Doman and Trelease, 1984).

All of the higher plant ILs that have been examined to date possess a putative PTS1, namely, either ARM–COOH or SRM–COOH (Beeching and Northcote, 1987; Comai et al., 1989; Turley et al., 1990; Janssen, 1995; Reynolds and Smith, 1995; Mullen and Gifford, 1997). However, a significant disparity exists in the literature concerning whether the PTS1 is necessary for the import of IL into plant peroxisomes. For example, the C-terminal 37 amino acid residues of oilseed rape IL (rIL) were shown to be necessary, and the four C-terminal residues (KSRM–COOH) were shown to be sufficient for import into leaf and root peroxisomes in transgenic Arabidopsis plants (Olsen et al., 1993). In concert, the C-terminal four residues (KARM–COOH) of cottonseed IL (cIL) were shown to be necessary for import into Chinese hamster ovary cells (Trelease et al., 1994). These results indicate that oilseed ILs were sorted to peroxisomes by the PTS1 pathway. In contrast, castor bean IL (cbIL) with 408 of its 576 amino acid residues removed from its C terminus was imported into isolated sunflower glyoxysomes (Behari and Baker, 1993). More recently, the same cbIL, but with only 19 of its C-terminal residues deleted, was expressed in *S. cerevisiae* and was still sorted to the peroxisome as evidenced by cofractionation with catalase in sucrose gradient fractions (Taylor et al., 1996). The results from these latter two studies suggest that cbIL is sorted to peroxisomes by a different import pathway than rIL or cIL.

In this study, we present evidence indicating that cIL, rIL, and cbIL are targeted to and imported into glyoxysomes by the PTS1 pathway in suspension-cultured tobacco (Bright Yellow) BY-2 cells. In addition, experimental results revealed that oilseed ILs and bacterial CAT can be imported into glyoxysomes via a piggyback mechanism. This strongly suggests that peroxisomal uptake of multimeric proteins occurs in plant cells.

RESULTS

Transiently Expressed cIL Is Targeted to BY-2 Glyoxysomes

Figure 1A shows the results of applying different dilutions of anti-IL IgGs to mock-transformed control cells bombarded with pRTL2/CAT, a plant expression vector containing CAT. At a concentration of 1:1000 (v/v) (Figure 1A, top), a majority (~70%) of the cells exhibited a punctate immunofluorescence pattern. These results indicated that organelles in most of the BY-2 cells possessed IL characteristic of glyoxysomes (Beevers, 1980; Huang et al., 1983). Background staining was a potential problem for distinguishing between transiently expressed and endogenous IL. It was overcome by applying anti-IL IgGs diluted 1:2000 (v/v) with PBS. The virtual absence of a punctate immunofluorescence pattern within the cells is shown at the bottom of Figure 1A.

To positively identify transiently expressed IL, cells were biolistically cotransformed with DNA encoding CAT and wild-type cIL (cILwt) and then double labeled with IgGs raised against CAT and cIL. Approximately 1 to 2% of the cells transiently expressed CAT (Figure 1B; easily observed by cytosolic BODIPY [Molecular Probes, Inc., Eugene, OR] staining), and of those, >90% also transiently expressed cILwt (Figure 1C, punctate rhodamine staining). To determine whether the introduced cILwt was localized to glyoxysomes, bombarded cells were double labeled with antibodies raised against cIL and cottonseed catalase. Figure 1D shows that the punctate cILwt pattern in one cell is superimposable on the punctate catalase pattern in the same cell (Figure 1E). Arrowheads indicate obvious examples of colocalizations. As expected, catalase staining also is apparent in all the neighboring cells (Figure 1E). Colocalization of cILwt with endogenous catalase indicates that cILwt was targeted to BY-2 glyoxysomes. The absence of BODIPY staining when cIL IgGs are omitted is evidence for authenticity of endogenous or introduced punctate IL immunofluorescence (Figure 1F).

Import of cILwt into BY-2 Glyoxysomes

It was essential to show that the punctate immunofluorescence was localized to the matrix of the glyoxysomes and not a consequence of transiently expressed cILwt bound to the cytosolic side of glyoxysomal boundary membranes. We know from studies with mammalian cells that digitonin permeabilizes plasma membranes only and that Triton X-100 alone or in combination with digitonin (at a lower Triton X-100 concentration) permeabilizes all cellular membranes. We applied this knowledge to our studies with BY-2 cells.

When Triton X-100 was used alone to permeabilize all the cell membranes, as in Figure 1, it was used at a concentration of 0.3% (v/v); for mammalian cells, it was used alone at 1.0% (v/v) (e.g., Trelease et al., 1994). For the results presented in Figure 2, all of the cells were from the same batch of pRTL2/cILwt-bombarded, fixed, and pectolyase-treated cells. Figures 2A to 2D are positive controls illustrating our ability to use digitonin (25 μg/mL) plus Triton X-100 (0.05% v/v), or digitonin (25 μg/mL) alone, to differentially permeabilize all of the BY-2 cell membranes, or just their plasma membranes, to show the subcellular localization of endogenous glyoxysomal catalase and/or cytosolic tubulin. When the detergents were applied together (Figures 2A and 2C), catalase and tubulin were stained in the same cells, indicat-

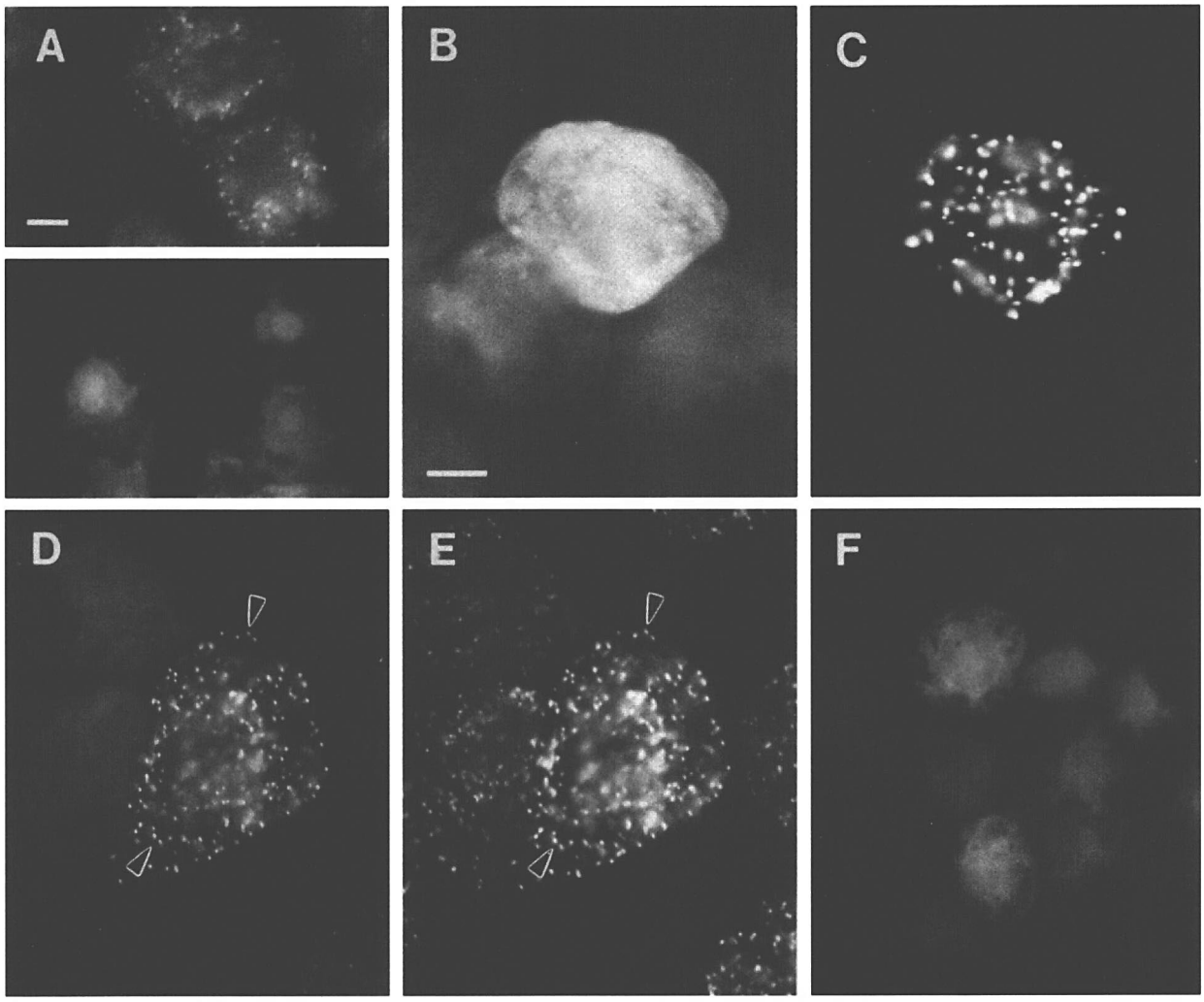

Figure 1. Immunofluorescence Microscopic Localization of cIL in BY-2 Glyoxysomes.

Cells were fixed in formaldehyde, treated with pectolyase Y-23 to partially digest the cell walls, permeabilized with 0.3% Triton X-100, and incubated with primary and secondary antibodies. Antibody concentrations were 1:1000 or 1:2000 (v/v) for anti-cIL IgGs, undiluted for CAT IgGs, 1:500 (v/v) for anti-catalase IgGs, 1:1000 (v/v) for anti–mouse and anti–rabbit BODIPY IgGs, and 1:1000 (v/v) for anti–rabbit rhodamine IgGs.

(A) BODIPY immunofluorescence of endogenous IL observed after applications of anti–cottonseed IL IgGs either at a dilution of 1:1000 (punctate pattern, top) or 1:2000 (no staining, bottom) in mock-transformed cells.

(B) and **(C)** Cells cotransformed with pRTL2/CAT and pRTL2/cILwt. In the same cell, cytosolic BODIPY immunofluorescence of CAT is evident in **(B)**, and punctate rhodamine immunofluorescence of cILwt is shown in **(C)**. Anti-cIL IgGs were at a dilution of 1:2000 (v/v).

(D) and **(E)** Colocalization of cILwt in one cell (BODIPY immunofluorescence) **(D),** with endogenous catalase (rhodamine immunofluorescence) in the same cell **(E)**. Note that surrounding cells in **(E)** also exhibit catalase staining of glyoxysomes. Arrowheads indicate obvious colocalizations. Anti-cIL IgGs were at a dilution of 1:2000 (v/v).

(F) Representative field of cells bombarded with pRTL2/cILwt. Anti-cIL IgGs were omitted. Cells were incubated in BODIPY-conjugated secondary IgGs.

Bars in **(A)** and **(B)** (for **[B]** to **[F]**) = 10 μm.

ing that both the plasma and glyoxysomal membranes were permeabilized. When only digitonin was applied (Figures 2B and 2D), catalase was not immunostained (Figure 2B), whereas in the same cells (Figure 2D), tubulin was immunostained, indicating that only the plasma membrane and not the glyoxysomal membrane was permeabilized.

Figures 2E to 2H illustrate results with the same group of cILwt-bombarded cells. Figures 2E and 2G show that cIL and tubulin were immunostained when both membranes were permeabilized. The low level of endogenous IL was not visualized in the neighboring nontransformed cells (Figure 2E) stained with anti-IL IgGs applied at a dilution of 1:2000 (v/v). When only

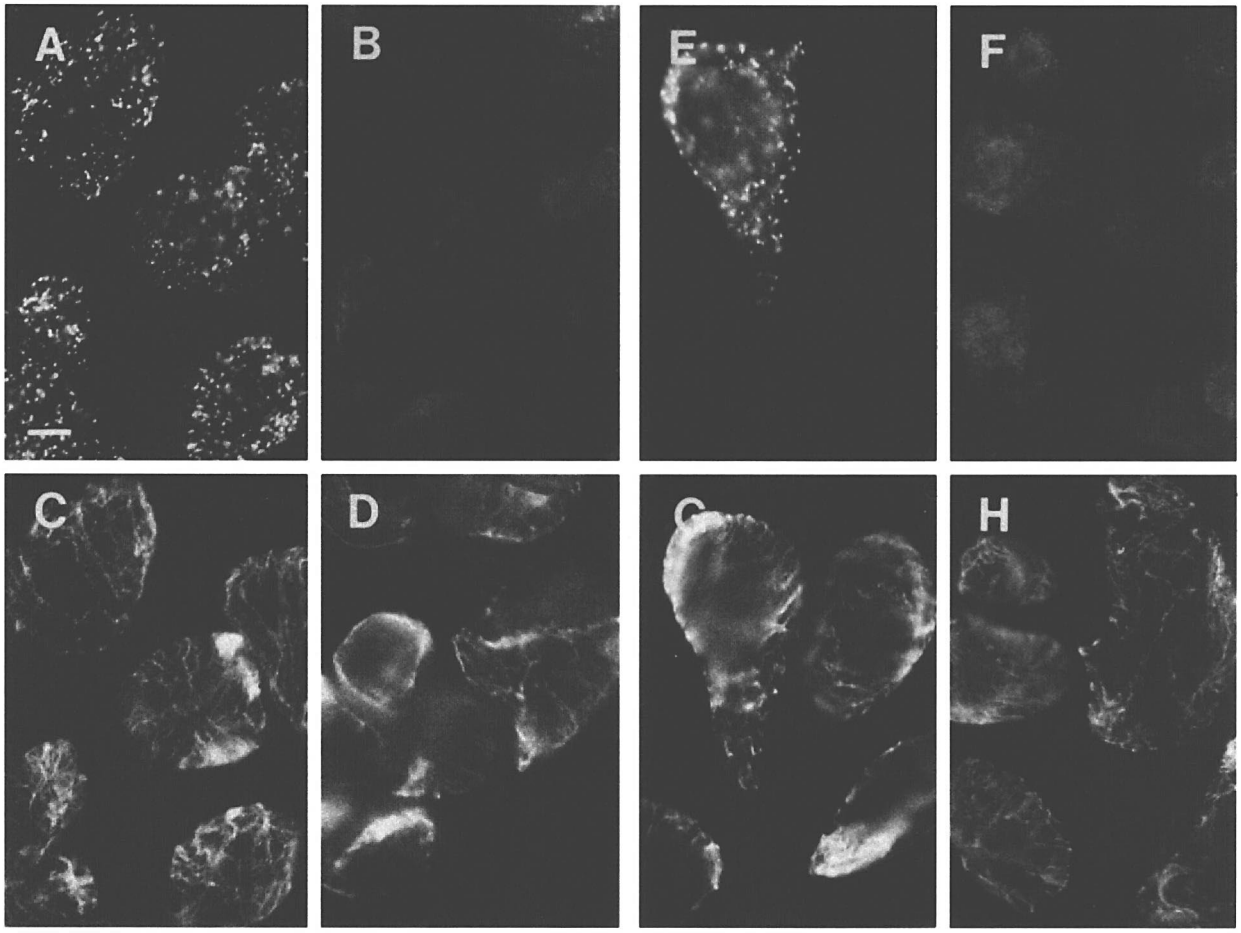

Figure 2. Immunofluorescence Patterns Demonstrate That cILwt Was Imported into BY-2 Glyoxysomes.

Cells bombarded with pRTL2/cILwt were fixed with formaldehyde and treated with pectolyase Y-23 and then incubated either in digitonin plus Triton X-100 or in digitonin alone to differentially permeabilize the plasma and glyoxysomal membranes. Antibodies raised against α-tubulin (1:500 [v/v]) were used to verify the permeabilization of the plasma membrane.

(A) Rhodamine immunofluorescence of endogenous catalase in glyoxysomes of cells incubated with digitonin (25 μg/mL) and Triton X-100 (0.05%).

(B) Representative image showing the lack of endogenous catalase staining (rhodamine immunofluorescence) in the same batch but with different cells than are shown in **(A)**, incubated with digitonin (25 μg/mL) alone.

(C) BODIPY immunofluorescence of α-tubulin in the cytosol of the same cells as shown in **(A)**.

(D) BODIPY immunofluorescence of α-tubulin in the cytosol of the same cells as shown in **(B)**.

(E) Rhodamine immunofluorescence of introduced cILwt in glyoxysomes (of one cell) in the same batch of cells used for **(A)** incubated with digitonin (25 μg/mL) and Triton X-100 (0.05%). Anti-cIL IgGs were at a dilution of 1:2000 (v/v).

(F) Representative image showing the lack of rhodamine immunofluorescence attributable to introduced cILwt in same batch of cells as shown in **(E)** incubated with digitonin (25 μg/mL) alone. Anti-cIL IgGs were at a dilution of 1:2000 (v/v).

(G) BODIPY immunofluorescence of α-tubulin in the cytosol of the same cells as shown in **(E)**.

(H) BODIPY immunofluorescence of α-tubulin in the cytosol of the same cells as shown in **(F)**.

Bar in **(A)** = 10 μm for **(A)** through **(H)**.

the plasma membrane was permeabilized (Figures 2F and 2H), transiently expressed IL was not stained (Figure 2F), whereas in the same cell (and others), tubulin was stained (Figure 2H), indicating that cILwt was not on the cytosolic side of the glyoxysomal boundary membrane but was imported into the matrix of these organelles (Figure 2E).

To ensure that the lack of fluorescence was not due to low transformation frequency, thousands of BY-2 cells on each slide were examined for 15 min, and the number of transiently expressing cells was noted. In the digitonin plus Triton X-100 experiments, we required a minimum of 10 cells exhibiting a punctate immunofluorescence pattern to be ob-

served before examining the slide in which digitonin was applied alone. No punctate immunofluorescence patterns were observed when the slides corresponding to the digitonin alone experiments were examined. These same parameters and observations were applied to results obtained in Figures 4 and 6.

C Termini of Oilseed ILs Are Necessary and Sufficient for Sorting to BY-2 Glyoxysomes

The results shown in Figure 3 demonstrate both the necessity of the C-terminal peptides to sort cIL, rIL, and cbIL to glyoxysomes and the sufficiency of the tripeptide ARM–COOH to import CAT into glyoxysomes. Figures 3A to 3F show immunofluorescence images stained with antibodies raised against cIL. Immunoblots from previous work show that the anti-cIL antibodies used in this study recognize IL from castor bean endosperm (Mullen, 1995). Furthermore, to ensure that anti-cIL IgGs recognized rIL and cbIL introduced into BY-2 cells, the cells were biolistically cotransformed with DNAs encoding CAT and either rILwt or cbILwt. Cells were double labeled with IgGs raised against CAT and cIL (1:2000 [v/v]). In every case, punctate immunofluorescence patterns attributable to rIL or cbIL staining (rhodamine) occurred concomitantly with cytosolic immunofluorescence due to CAT staining (BODIPY; data not shown).

Figure 3A shows a punctate staining pattern attributable to glyoxysome localization of introduced cILwt. Similar immunofluorescence patterns were observed for cells transiently

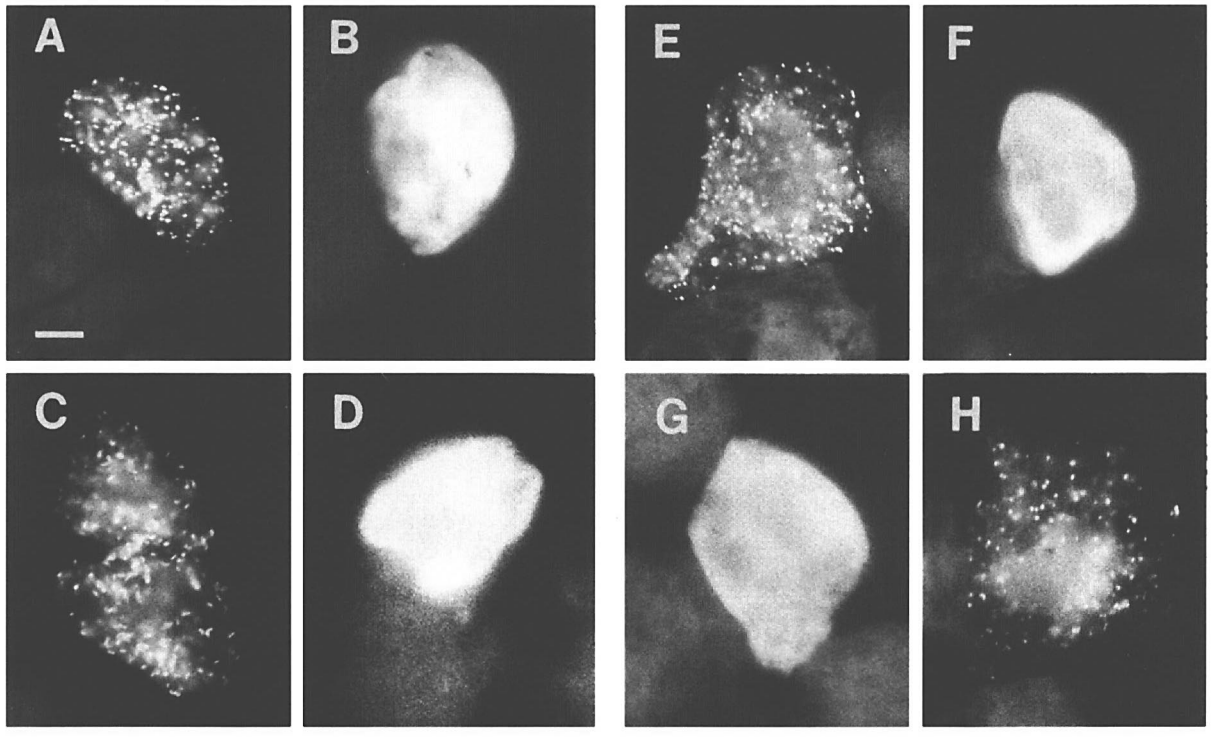

Figure 3. Immunofluorescence Localization of Wild-Type and C-Terminal Truncated Oilseed ILs and CAT or CAT with ARM Appended at the C Terminus.

Fixed and pectolyase Y-23–treated cells were permeabilized in 0.3% Triton X-100 and incubated in primary and secondary antibodies. Antibodies raised against cIL and CAT were applied at a dilution of 1:2000 (v/v) or were undiluted, respectively.
(A) Punctate BODIPY immunofluorescence of a pRTL2/cILwt-bombarded cell. Cells were incubated with anti-cIL IgGs.
(B) Cytosolic BODIPY immunofluorescence of a pRTL2/cIL-3–bombarded cell. Cells were incubated with anti-cIL IgGs.
(C) Punctate BODIPY immunofluorescence of a pMON316/rILwt-bombarded cell. Cells were incubated with anti-cIL IgGs.
(D) Cytosolic BODIPY immunofluorescence of a pMON316/rIL-37–bombarded cell. Cells were incubated with anti-cIL IgGs.
(E) Punctate BODIPY immunofluorescence of a pRTL2/cbILwt-bombarded cell. Cells were incubated with anti-cIL IgGs.
(F) Cytosolic BODIPY immunofluorescence of a pRTL2/cbIL-3–bombarded cell. Cells were incubated with anti-cIL IgGs.
(G) Cytosolic BODIPY immunofluorescence of a pRTL2/CAT-bombarded cell. Cells were incubated with anti-CAT IgGs.
(H) Punctate BODIPY immunofluorescence of a pRTL2/CAT–ARM–bombarded cell. Cells were incubated with anti-CAT IgGs.
Bar in **(A)** = 10 μm for **(A)** to **(H)**.

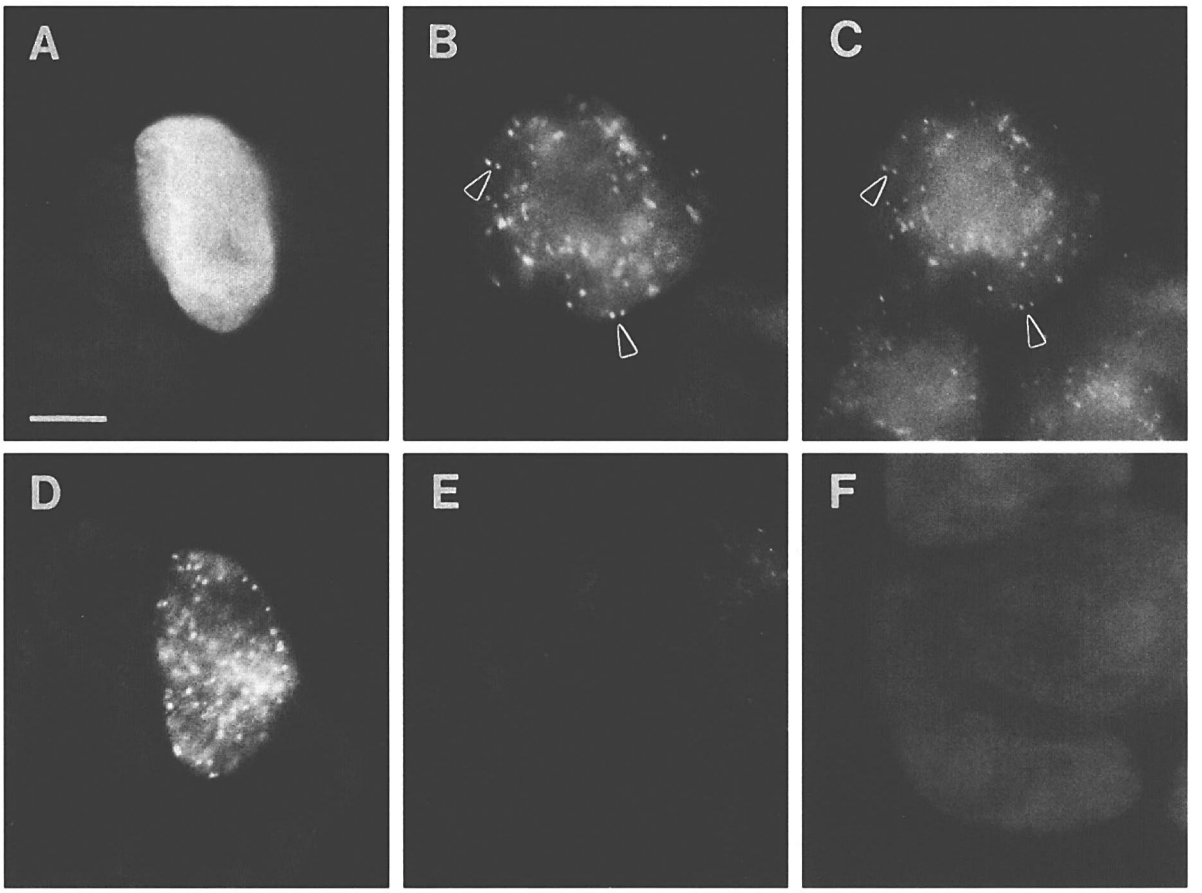

Figure 4. Immunofluorescence Localization of HA Epitope–Tagged cIL-3 Coexpressed with cILwt.

BY-2 cells were simultaneously bombarded with pRTL2/cILwt and pRTL2/HA–cIL-3 (unless otherwise noted), fixed with aldehyde, treated with pectolyase Y-23, permeabilized with 0.3% Triton X-100 (unless otherwise noted), and incubated with primary and secondary antibodies.
(A) Cytosolic Cy3 (indocarbocyanine) immunofluorescence attributable to the HA epitope on HA–cIL-3 in a cell transformed with pRTL2/HA–cIL-3. Anti-HA IgGs were at a dilution of 1:150 (v/v).
(B) and **(C)** Colocalization of Cy3 immunofluorescence attributable to HA–cIL-3 **(B)** and BODIPY immunofluorescence attributable to endogenous glyoxysomal catalase **(C)** in a cotransformed cell. Arrowheads indicate obvious colocalizations. Anti-HA IgGs were at a dilution of 1:150 (v/v); anti-catalase IgGs were at a dilution of 1:500 (v/v).
(D) and **(E)** Microscopic demonstration of the import of HA–cIL-3 into BY-2 glyoxysomes: punctate Cy3 immunofluorescence of HA–cIL-3 in a cotransformed cell permeabilized with 0.3% (v/v) Triton X-100 **(D)**. Anti-HA IgGs were at a dilution of 1:150 (v/v). **(E)** is a representative image of the same batch of cells as shown in **(D)**, showing the lack of punctate Cy3 immunofluorescence on the outside of the glyoxysomes. The cells in **(E)** were permeabilized with digitonin alone (25 μg/mL). Anti-HA IgGs were at a dilution of 1:150 (v/v).
(F) Absence of Cy3 immunofluorescence due to lack of HA IgG binding in cells mock transformed with pRTL2/cILwt.
Bar in **(A)** = 10 μm for **(A)** to **(F)**.

expressing rILwt (Figure 3C) and cbILwt (Figure 3E). rILwt and cbILwt were shown to be colocalized with endogenous catalase in glyoxysomes (data not shown), as was shown for cILwt (Figures 1D and 1E). C-terminal truncated versions of each IL, namely, cIL-3, rIL-37, and cbIL-3, however, accumulated in the cytosol (Figures 3B, 3D, and 3F).

Figures 3G and 3H show that the C-terminal tripeptide on cbIL and cIL, that is, ARM–COOH, is sufficient for import of the passenger protein CAT into glyoxysomes. A punctate BODIPY pattern was observed in a cell transiently transformed with CAT–ARM (Figure 3H), whereas cytosolic accumulation of CAT without an appended tripeptide was evident in the transformed cell illustrated in Figure 3G. The punctate patterns attributable to CAT–ARM staining were colocalized with punctate endogenous catalase patterns, and CAT–ARM was shown to be imported into glyoxysomes via differential permeabilization experiments, such as those described in Figure 2 (data not shown).

Evidence for Import of Oligomerized Proteins into Glyoxysomes

Experiments were done to test the hypothesis that truncated oilseed ILs could be piggybacked by the wild-type ILs into glyoxysomes. cIL-3 was hemagglutinin (HA) epitope tagged at its N terminus to immunologically distinguish it from cILwt. Figure 4A shows with antibodies raised against HA the cytosolic accumulation of HA–cIL-3. Coexpression of HA–cIL-3 and cILwt resulted in a punctate immunofluorescence pattern with antibodies raised against HA (Figure 4B); the HA–cIL-3 colocalized with endogenous glyoxysomal catalase (Figure 4C). Arrowheads indicate clear examples of colocalizations. The weak cytosolic staining shown in Figures 4B and 4C is due to background fluorescence of Cy3 (indocarbocyanine) and BODIPY fluorochromes conjugated to secondary IgGs. Import of HA–cIL-3 into the glyoxysomes is demonstrated in Figures 4D and 4E. A punctate pattern was observed in Triton X-100–treated cells (0.3% v/v; Figure 4D), whereas a punctate pattern was not apparent in cells treated with digitonin (25 μg/mL; Figure 4E). Again, the weak cytosolic staining in Figures 4D and 4E is due to background fluorescence. Mock-transformed cells incubated with HA antibodies did not reveal any staining (Figure 4F). Thus, the results indicated that HA–cIL-3 was targeted to and translocated into glyoxysomes. Figure 5 shows immunofluorescence results of anti-IL antibody staining in cells coexpressing truncated and wild-type cIL, rIL, and cbIL. The truncated forms were not HA epitope tagged for these experiments. The lack of expected cytosolic staining from the truncated ILs (Figures 5A to 5C) indicates that the C-terminal truncated forms of rIL and cbIL also were imported into the glyoxysomes directed by the respective wild-type ILs.

Figure 6 demonstrates that piggybacking can occur with a bacterial protein that forms oligomers. When CAT–HA was transiently expressed, diffuse cytosolic staining was observed (Figure 6A). Coexpression of CAT–HA and CAT–SKL resulted in a punctate immunofluorescence pattern (Figure 6B), which colocalized with endogenous catalase (Figure 6C). Arrowheads indicate typical colocalizations. A single group of cobombarded cells was treated either with digitonin (25 μg/mL; Figure 6D) or with Triton X-100 (0.3% v/v; Figure 6E). The cytosolic staining shown in Figure 6D is indicative of CAT–HA, which was not imported into organelles. However, the punctate staining, with some cytosolic staining, that was observed in cells with all membranes permeabilized in 0.3% Triton X-100 (v/v) (Figure 6E) revealed that CAT–HA was directed into BY-2 glyoxysomes by CAT–SKL. Figure 6F represents the absence of immunostaining when the antibody raised against HA was omitted.

DISCUSSION

BY-2 Cells Possess Glyoxysomes

Peroxisomes in BY-2 cells were characterized previously as unspecialized peroxisomes, based on ultrastructural characteristics (0.1 to 0.3 μm in diameter and lack of internal structure), low specific activity of catalase in cell-free extracts, and growth of the nongreen cells on sucrose as the

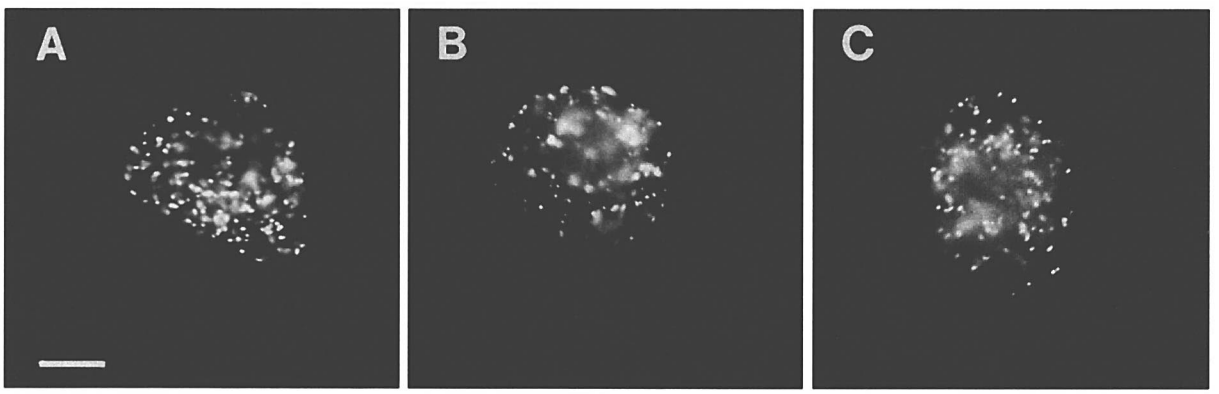

Figure 5. Immunofluorescence Localization of Three Different Oilseed ILs in Cells Cotransformed with Wild-Type and C-Terminal Truncated Forms.

Cells were cobombarded with DNAs encoding the wild-type and truncated versions of ILs from the same species, fixed with aldehyde, and treated with pectolyase Y-23. Cells were permeabilized with 0.3% Triton X-100. Antibodies raised against cIL were applied at a dilution of 1:2000 (v/v).

(A) Punctate BODIPY immunofluorescence in cells cobombarded with cILwt and cIL-3 multimers localized to BY-2 glyoxysomes.

(B) Punctate BODIPY immunofluorescence of rILwt and rIL-37 multimers localized to BY-2 glyoxysomes.

(C) Punctate BODIPY immunofluorescence of cbILwt and cbIL-3 multimers localized to BY-2 glyoxysomes.

Bar in **(A)** = 10 μm for **(A)** to **(C)**.

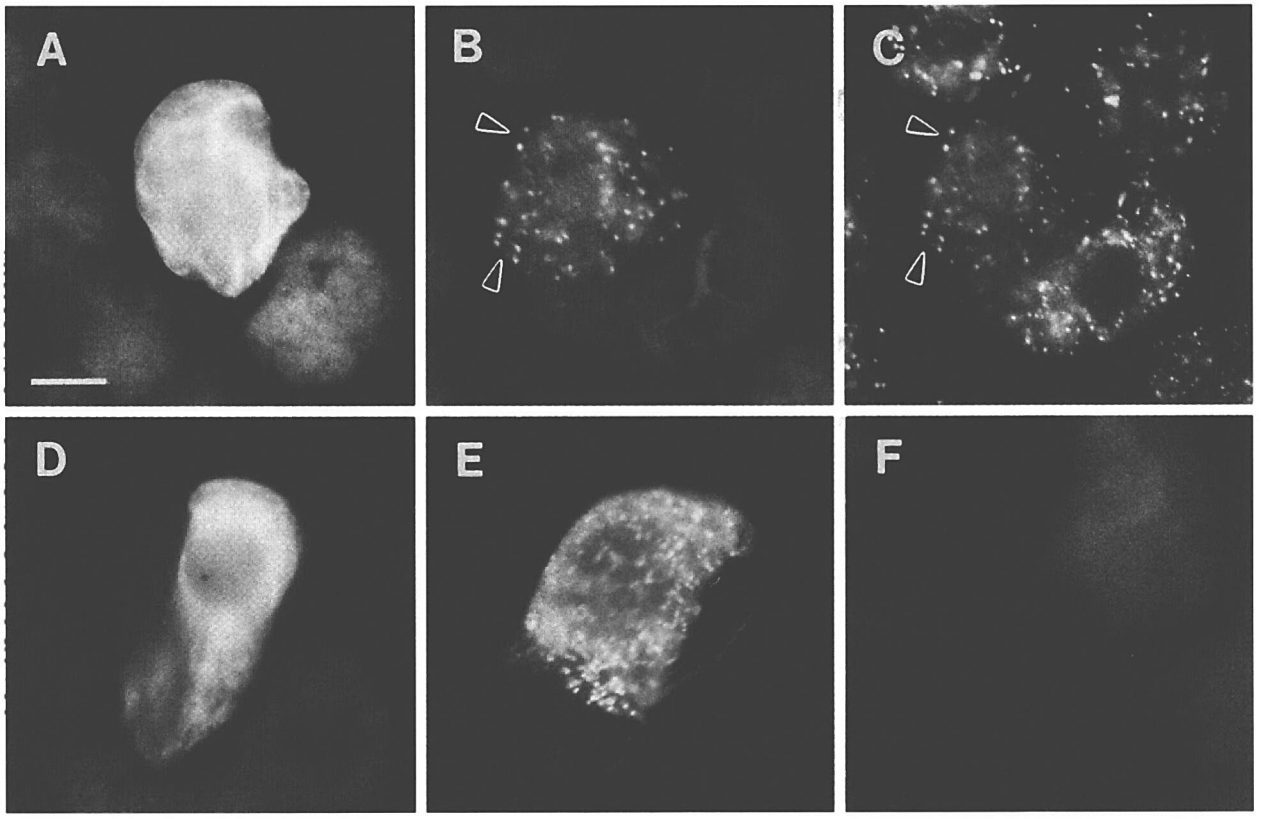

Figure 6. Immunofluorescence Localization of HA Epitope–Tagged CAT Coexpressed with CAT–SKL.

BY-2 cells were simultaneously bombarded with pRTL2/CAT–HA and pRTL2/CAT–SKL (unless otherwise noted), fixed with formaldehyde, treated with pectolyase Y-23, permeabilized with 0.3% Triton X-100 (unless otherwise noted), and incubated with primary and secondary antibodies.

(A) Cytosolic BODIPY immunofluorescence attributable to anti-HA IgGs binding to CAT–HA in one cotransformed cell. Anti-HA IgGs were at a dilution of 1:150 (v/v).

(B) and **(C)** Colocalization of BODIPY-stained CAT–HA (anti-HA IgGs) **(B)** with rhodamine immunofluorescence of endogenous glyoxysomal catalase **(C)** in a cotransformed cell. Arrowheads indicate obvious colocalizations.

(D) and **(E)** Demonstration of import of CAT–HA into BY-2 glyoxysomes. Cytosolic BODIPY immunofluorescence is attributable to CAT–HA in a cotransformed cell incubated with digitonin (25 μg/mL) **(D)**. Punctate and cytosolic BODIPY immunofluorescence of CAT–HA in a cotransformed cell incubated with Triton X-100 (0.3%) **(E)** is shown. Anti-HA IgGs were at a dilution of 1:150 (v/v).

(F) Representative image showing the absence of BODIPY immunofluorescence in cotransformed cells that were not incubated in anti-HA IgGs. Bar in **(A)** = 10 μm for **(A)** to **(F)**.

sole carbon source (Trelease et al., 1996a). The latter has added significance because sucrose in the growth medium was shown in cucumber suspension cells to be a catabolite repressor of genes expressing the two glyoxylate cycle enzymes IL and MS (Graham et al., 1994). Thus, we were surprised to detect punctate IL (and MS) immunofluorescence signals (Figure 1A, top) colocalized with endogenous catalase in a majority of the mock-transformed BY-2 cells. These characteristics indicated that the BY-2 peroxisomes are glyoxysomes (Beevers, 1979).

Evidence that the expression of IL was not a consequence of postbombardment trauma or incipient cell death was obtained from cells harvested directly from the growth flasks, that is, nonbombarded cells. Immunofluorescence detection of similar low levels of IL (and MS) were observed in the glyoxysomes (data not shown) of these cells. Therefore, it became important to distinguish endogenous IL from the introduced, transiently expressed IL. This was accomplished by diluting the anti-IL IgGs 2000 times; immunofluorescence attributable to endogenous IL staining was barely detectable (Figure 1A, bottom), yet introduced ILs were strongly stained in every case. Confirmation that the strongly stained cells were transiently transformed cells was achieved by bombarding cells simultaneously with DNA encoding wild-type ILs and CAT. Strong punctate IL immunofluorescence signals were observed in the same cells that were expressing

CAT (e.g., Figures 1B and 1C). Cotransfection of cultured mammalian cells with two genes consistently results in >95% of transiently transformed cells expressing both genes (Wigler et al., 1979; Kaufman, 1990). Coexpression of particle-bombarded β-glucuronidase and neomycin phosphotransferase II genes was reported by Mendel et al. (1989) for plant suspension-cultured cells. The ability to coexpress genes located on different plasmids in plant suspension-cultured cells is supported by our results. Hence, we are confident that interpretations of the results presented in this study were made from images of transiently transformed cells.

Oilseed ILs and CAT Are Imported into the Glyoxysomes

The punctate immunofluorescence images of introduced proteins indicate that the proteins were at least targeted to the glyoxysomes but do not show that the proteins actually were imported into the organelles. That is, the images could reflect proteins on the cytosolic side of the glyoxysomal boundary membrane. In cultured mammalian cells, an experimental design commonly used to demonstrate actual import into peroxisomes is to compare images after differential permeabilization of the plasma and peroxisomal membranes (Wolvetang et al., 1990; Rapp et al., 1993; Wendland and Subramani, 1993; Trelease et al., 1994). Specifically, Triton X-100 (1% [v/v] for mammalian cells and 0.3% [v/v] for BY-2 cells) permeabilizes all of the cellular membranes, whereas digitonin (25 μg/mL) selectively permeabilizes only the plasma membranes by forming digitonides with cholesterol located in the plasma membrane (Bangham and Horne, 1962). When the detergents are added together, the concentration of Triton X-100 is reduced (0.05%) because higher concentrations dissolve organelle membranes in both mammalian and BY-2 cells (this study). Actual import of an introduced protein into an organelle is demonstrated when a punctate immunofluorescence image observed with Triton X-100 treatment is abolished in cells treated only with digitonin. This indicates that the protein must be inside the peroxisome and not associated with the cytosolic side of the boundary membrane.

Cholesterol typically is present in relatively small quantities in the plasma membranes of plant cells, but other sterols, such as sitosterol and stigmasterol, are more common (Mudd, 1980). Because digitonin forms digitonides with sterols in general and not specifically with cholesterol (Gestetner et al., 1971; Takagi et al., 1982), we hypothesized that it could also selectively permeabilize plant cell plasma membranes. In cells treated with digitonin only, permeabilization of the plasma membrane to IgG molecules was demonstrated by the immunostaining of tubulin, a resident cytosolic protein (Figures 2D and 2H). Also, digitonin did not interfere with staining of endogenous catalase or introduced (punctate) clLwt (Figures 2A and 2E). Therefore, for subsequent experiments (Figures 4 and 6), Triton X-100 by itself at a concentration of 0.3% (v/v) was used to permeabilize all cellular membranes, and digitonin was used by itself to specifically permeabilize the plasma membrane. The results demonstrate that we can routinely demonstrate the import of introduced proteins into glyoxysomes. Recently, Hicks et al. (1996) reported on the in vitro import of proteins into nuclei of evacuolated BY-2 protoplasts. The plasma membrane but not the nuclear envelope was permeabilized by incubating the protoplasts in reduced high osmolarity medium (from 500 to 225 mM mannitol); the mechanism was not understood. This method was developed because the authors reported that digitonin or streptolysin did not permeabilize the evacuolated protoplasts; the reason for the ineffectiveness was not determined.

Oilseed ILs Are Acquired as Oligomers by the PTS1 Pathway

One important aspect of this research was to determine whether oilseed ILs were sorted to plant peroxisomes in the same in vivo system by the PTS1 pathway. We chose these ILs because of a discrepancy in the literature concerning the PTS responsible for the targeting/import of rIL and cbIL (Behari and Baker, 1993; Olsen et al., 1993). The observed differences between rIL and cbIL could be explained by an actual difference in the PTSs as a consequence of the origin of the enzymes, that is, in triploid senescent endosperm (cbIL) versus diploid persistent cotyledons (rIL) (Trelease et al., 1994). Alternatively, the discrepancy may be due to the evaluation of results with different experimental import systems, namely, in vitro versus in vivo systems.

Our hypothesis that all oilseed ILs were targeted/imported into glyoxysomes via the PTS1 pathway was based on the knowledge that all three ILs exhibited an 85% amino acid sequence identity (Turley et al., 1990) and that all possess conserved SKL motifs. The results of our studies clearly indicate that the C terminus of all three plant ILs was necessary for import into BY-2 glyoxysomes (Figure 3). When three amino acid residues were removed from the C terminus of clL or cbIL, or 37 amino acid residues from rIL, the transiently expressed ILs accumulated in the cytosol (Figures 3B, 3D, and 3F). Furthermore, the tripeptide ARM, located at the C terminus of clL and cbIL, was sufficient to reroute CAT from the cytosol to the glyoxysomes when the tripeptide was appended to the C terminus of CAT (Figures 3G and 3H). We did not obtain any evidence for piggybacking of introduced truncated ILs by endogenous tobacco IL. clL-3 and cbIL-3 were allowed to express in BY-2 cells for 40 hr (instead of the normal 20 hr), and punctate patterns were not observed (data not shown). We assumed that the 40-hr expression period would be sufficient time for the translocation machinery to import enough IL for us to observe, by our methods, a punctate pattern interspersed with cytosolic immunofluorescence. Thus, we believe that clL-3 and cbIL-3 likely did not piggyback into glyoxysomes because they did not interact with endogenous tobacco IL.

We showed previously that the efficacy of BY-2 cells was equivalent to transgenic plants for studying peroxisomal protein targeting (Banjoko and Trelease, 1995; Trelease et al., 1996a, 1996b). For example, the C-terminal tetrapeptide KSRM of rIL, fused to CAT, was equally sufficient for targeting this passenger protein to both transgenic Arabidopsis peroxisomes (Olsen et al., 1993) and BY-2 glyoxysomes (Trelease et al., 1996a). Our results are in direct contrast with those of Baker and co-workers concerning the targeting/import signal of cbIL. A possibility is that the truncated cbILs (e.g., cbIL-408 [Behari and Baker, 1993] and cbIL-19 [Taylor et al., 1996]) acquired some import competence due to conformational changes of the truncated proteins. Piggybacking of truncated forms by wild-type subunits cannot be considered because neither wild-type forms of IL nor any other peroxisomal protein would be involved in the in vitro import experiments (Behari and Baker, 1993). Moreover, as discussed above, it seems that low levels of endogenous IL were not capable of piggybacking large amounts of introduced IL into cells (Figures 3 and 4). Otherwise, it is difficult to discuss reasons for this discrepancy because neither the necessity nor the sufficiency of any other portion of cbIL has been reported (Behari and Baker, 1993; Marrison et al., 1993; Onyeocha et al., 1993; Taylor et al., 1996).

Given the interpretations of our results that indicated sorting via the PTS1 pathway, it was of interest to learn whether IL subunits oligomerized before or during translocation into the glyoxysomes. The native form of eukaryotic ILs is a tetramer (~260 kD) composed of identical subunits (~62 kD) (Vanni et al., 1990). Thiolase was interpreted to be imported as a dimer into *S. cerevisiae* peroxisomes (Glover et al., 1994), and CAT was interpreted to be imported as a trimer into *S. cerevisiae* and mammalian peroxisomes (McNew and Goodman, 1994), based on the demonstrated ability of a signal-depleted subunit(s) to piggyback with an unmodified subunit(s) into the organelles. The results of our experiments (Figure 4) indicated that the HA–cIL-3 subunit was imported into BY-2 glyoxysomes only when the wild-type subunits were coexpressed in the same cells. The truncated subunit was distinguished from the wild-type subunit by the appended HA epitope, that is, the punctate immunofluorescence after the application of anti-HA IgGs could only be attributable to the cIL-3 (Figures 4B and 4D). Virtually the same results were obtained with CAT, that is, it was imported into the glyoxysomes only when a subunit of CAT with an appended PTS1 (CAT–SKL) was simultaneously expressed in the cells (Figure 6). Our results also indicated that truncated forms of rIL and cbIL were imported into glyoxysomes only when the wild-type subunits were coexpressed in the cells (Figure 5). In these experiments, the truncated subunits were not distinguishable from the wild-type forms, but the lack of cytosolic immunofluorescence concomitant with the punctate immunofluorescence was a strong indication that all of the truncated forms were imported into glyoxysomes. Other evidence came from numbers of immunofluorescent cells on slides. Typically, ~1% of the cells are transformed (normally 15 to 20 transformants per slide), and of these, >90% typically coexpress proteins. In these experiments, if the truncated ILs were not targeted to glyoxysomes, then a sufficient number of cells per slide were examined to have identified cytosolic immunofluorescence. Such cells were not observed.

The most straightforward interpretation of these results is that the truncated forms of the oilseed ILs and CAT are piggybacked as oligomers into the glyoxysomes by the respective wild-type forms. Even though IL functions catalytically as a tetramer and CAT as a trimer, our data do not allow us to predict the size of the oligomer involved in the piggybacking mechanism. We cannot state unequivocally that the proteins examined in our study were even translocated through the membranes as oligomers, regardless of the conclusions put forward by others (Glover et al., 1994; McNew and Goodman, 1994, 1996; Rachubinski and Subramani, 1995; Walton et al., 1995; Mullen and Trelease, 1996; Subramani, 1996). For instance, once the putative IL tetramer has engaged the translocation machinery, each of the subunits may be disassembled and imported separately into the glyoxysomal matrix. Regardless of the translocation mechanism used, IL oligomers of some size certainly were formed in the cytosol and sorted to glyoxysomes; a subunit(s) lacking an essential PTS1 was then imported into the matrix of the organelle. It is possible that large pores or channels exist in the boundary membrane, as postulated for all types of peroxisomes (Subramani, 1996). There is no direct evidence as yet, however, to support this hypothesis.

METHODS

Cell Culture

Tobacco (*Nicotiana tabacum* cv Bright Yellow 2) (BY-2) cell suspension cultures were maintained as described by Banjoko and Trelease (1995). Cells were grown in Murashige and Skoog (Sigma) medium, gently agitated on an orbital shaker at 25°C in the dark, and subcultured weekly.

Plasmid Constructions

All DNA manipulations and polymerase chain reaction (PCR) conditions were as previously described in Trelease et al. (1996b). The cDNA encoding the wild-type cottonseed isocitrate lyase (clLwt) (Turley et al., 1990) was excised from pGEM/clLwt by full digestion with XbaI and partial digestion with NcoI. The 1900-bp NcoI-XbaI fragment containing clLwt was ligated into the plant expression vector pRTL2 (Restrepo et al., 1990), which was digested previously with NcoI and XbaI, to create pRTL2/clLwt. The constructs for wild-type (pMON316/rlLwt) and C-terminal minus 37–amino acid residue (pMON316/rlL-37) oilseed rape ILs (rILs) were provided by J. Harada (University of California at Davis) (Olsen et al., 1993), and the wild-type (pRok8/cbILwt) castor bean IL (cbIL) was sent to us by A. Baker (University of Leeds, Leeds, UK) (Behari and Baker, 1993). The cDNA

Table 1. Sequence of PCR Primers and Oligonucleotides Used to Construct Mutant ILs and CATs

Mutant	Description	Primer Sequence (5′ to 3′)
cIL-3	Internal (cIL) forward primer	GGCAGTTCATAACGTTAGC
	Internal (pGEM vector) reverse primer	AACAGCTATGACCATG
	Mutagenic forward primer	TCTGGTTGTGGCTAAGTAGAGAATGTAAGAC
	Mutagenic reverse primer	CATTCTCTACTTAGCCACAACCAGATTGCC
cbIL-3	Internal (cbIL) forward primer	GGAGCTGAACTTCAAGCTACTGAGG
	Mutagenic reverse primer	CGCCATCTAGAGCTCTCCTACATCCTTTACTTGGCAACCACC
HA–cIL-3	Coding strand	CATGGGGTACCCATACGACGTGCCAGACTACGCCGG
HA–cIL-3	Noncoding strand	CATGCCGGCGTAGTCTGGCACGTCGTATGGGTACCC
CAT–SKL and CAT–ARM	Internal (*CAT*) forward primer	TTCACCATGGGCAAATAT
CAT–SKL	Mutagenic reverse primer	CGCCATCTAGAATAACTGCCTTATAATTTTGACGCCCCGCCC
CAT–ARM	Mutagenic reverse primer	CGCCATCTAGAATAACTGCCTTACATACGGGCCGCCCCGCCC

encoding cbILwt was subcloned into a modified version of pRTL2 (pRTL2ΔN/S). pRTL2ΔN/S was constructed by digesting pRTL2 with NcoI and SacI, blunting both ends with T4 DNA polymerase, and then religating. This destroyed the NcoI and SacI sites (ΔN/S) and also removed sequences that would have coded for additional amino acids at the N terminus of cbILwt. cbILwt DNA was excised from pRok8 using XbaI and SacI. The XbaI-SacI fragment was modified using T4 DNA polymerase and then blunt-end ligated into the SmaI site of pRTL2ΔN/S to create pRTL2/cbILwt.

Mutant ILs were synthesized via PCR mutagenesis. Primer sequences are listed in Table 1. All PCR products were ligated into the pCRII vector by TA cloning (Invitrogen Corp., San Diego, CA) before subcloning into pRTL2. pRTL2/cIL-3 was constructed using overlap extension PCR mutagenesis (Trelease et al., 1994) that changed the codon for alanine in the C-terminal ARM to a stop codon. The fragment containing the cIL-3 mutation was excised from pCRII with BstEII and XbaI and ligated into pRTL2/cILwt digested with BstEII and XbaI. To construct pRTL2/cbIL-3, the reverse primer produced mutated sequences that changed the DNA encoding the alanine of the C-terminal ARM to a stop codon, and it also introduced a unique XbaI site that was not complementary to the template. Thus, when the PCR product was synthesized, it contained an XbaI site 3′ of the stop codon. The cbIL-3 fragment was excised from pCRII with NheI and XbaI and ligated into pRTL2/cbILwt digested with NheI and XbaI.

pRTL2/HA–cIL-3 was constructed in the following manner. Complementary oligonucleotide sequences (Table 1) encoding a translation start site (AUG), glycine linkers, and a hemagglutinin (HA) epitope (underlined, MGYPYDVPDYAG; Kolodziej and Young, 1991) were annealed and phosphorylated with T4 polynucleotide kinase. Annealed oligonucleotides with overhanging ends were ligated into a NcoI site of partially digested pRTL2/cIL-3. To confirm that the sequences encoding the HA epitope tag were inserted at the 5′ NcoI site of cIL-3 open reading frame (CCATGG) and not at the internal NcoI site, plasmid DNA from selected transformants was sequenced. pRTL2/HA–cIL-3 encodes cIL-3 with an HA epitope tag at the N terminus of the protein.

pRTL2/CAT was created by excising the sequence encoding chloramphenicol acetyltransferase (CAT) from pCAMVCN (Pharmacia Biotechnology) with HincII and then ligating the fragment into pRTL2ΔN/S restricted with SmaI. pRTL2/CAT–SKL and pRTL2/CAT–ARM were produced using PCR primers (Table 1) that modified sequences in the 3′ untranslated region of *CAT* DNA to generate CAT with an appended SKL or ARM C-terminal tripeptide, a stop codon, and an XbaI site. The PCR product was subcloned into pCRII, excised with NcoI and XbaI, and ligated into pRTL2/CAT restricted with NcoI/XbaI. pMON316/CAT–HA was constructed by excising an EcoRI fragment from pCAT18-HAΔ (provided by J. Goodman, University of Texas, Dallas) containing sequences encoding two copies of the HA epitope appended to the extreme 3′ end of the CAT open reading frame. The EcoRI fragment was ligated into pMON316/CAT, provided by J. Harada (Olsen et al., 1993), which was digested previously with EcoRI.

The correct sequence of all constructs was confirmed from nucleotide sequence analyses performed on an Applied Biosystems 377 automated DNA sequencer at the Arizona State University Bioresources Facility.

Microprojectile Bombardment of BY-2 Cells and Immunofluorescence Microscopy

BY-2 cells were harvested 4 days after subculture by centrifugation and resuspended in an equal volume of 2 × transformation buffer (2 × growth media without 2,4-D, and with 250 mM sorbitol and 250 mM mannitol) as described previously (Banjoko and Trelease, 1995). Cells were spread in a thin layer over filter papers moistened with 1 × transformation buffer and allowed to equilibrate for 1 hr (Trelease et al., 1996a). M-17 tungsten particles (Bio-Rad) were coated with DNA as described previously (Banjoko and Trelease, 1995), except that a total of 10 μg of DNA was precipitated on 4.5 mg of tungsten particles. For piggybacking experiments, transient cotransformations were performed as described above, except that 5 μg of each DNA construct was precipitated on M-17 tungsten particles. After microprojectile bombardment, cells were left in unwrapped dishes for 20 hr. Cells were then scraped from the filter paper, except from the center, where most of the tungsten particles were deposited, and incubated for 1 hr in formaldehyde fixative (4%) prepared in 1 × growth media minus 2,4-D (Banjoko and Trelease, 1995). Cells were washed three times in 1 × PBS (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) and incubated at 30°C with 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) in 1 × PBS for 2 hr. Plasma and organellar membranes were permeabilized by incubating cells for 15 min at room temperature in 0.3% (v/v) Triton X-100 (T 8787; Sigma), as previously described (Banjoko and Trelease, 1995).

For experiments designed to demonstrate import of transiently expressed proteins into glyoxysomes, harvested BY-2 cells were resuspended in an equal volume of 2 × growth media (minus 2,4-D) and spread on filter papers moistened with 1 × growth media (minus 2,4-D). Twenty hours after microprojectile bombardment, cells were

incubated for 3 min in formaldehyde fixative (2% in 50 mM potassium phosphate, pH 7.2), the fixative solution was removed, and cells were incubated in fresh fixative for an additional 25 min before washing in 1 × PBS. The plasma membrane and organelle membranes were differentially permeabilized by incubating cells for 15 min in digitonin (25 μg/mL) (D 1407; Sigma), in a mixture of digitonin (25 μg/mL) and Triton X-100 (0.05% v/v), or in Triton X-100 (0.3% v/v) only.

For studies with nontransformed BY-2 cells, cells harvested 4 days after subculture were collected by centrifugation and resuspended in 4% formaldehyde in growth media (minus 2,4-D). After incubation in fixative for 1 hr, cells were washed three times in PBS, incubated in pectolyase Y-23 as described above, and permeabilized for 15 min in 0.3% Triton X-100.

Applications of primary and fluorescent dye–conjugated secondary antibodies were performed as described previously (Banjoko and Trelease, 1995; Trelease et al., 1996a). Antibodies raised in rabbits (anti-cIL [Doman and Trelease, 1985], anti-catalase [Kunce et al., 1988], and anti-malate synthase [Trelease et al., 1987]) were affinity purified on protein A columns, according to Kunce et al. (1988), and used at concentrations of 1:2000, 1:500, and 1:500 (v/v), respectively. Other antibody concentrations were as follows: undiluted anti-CAT monoclonal antibody (hybridoma medium; gift from S. Subramani, University of California at San Diego, La Jolla); anti-α-tubulin monoclonal antibody at 1:500 (Amersham Corp.); anti-HA monoclonal antibody at 1:150 (12CA5; Boehringer Mannheim); anti-mouse BODIPY at 1:1000 (Molecular Probes Inc., Eugene OR); anti-rabbit BODIPY at 1:1000 (Molecular Probes Inc.); anti-rabbit rhodamine at 1:1000 (Jackson ImmunoResearch Laboratories, West Grove, PA); anti-mouse Cy3 at 1:1000 (Jackson ImmunoResearch Laboratories). Photographs were taken with 400 ASA black and white print film, using a Zeiss Axiovert 100 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

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