

Evolutionary Conservation of a Microbody Targeting Signal That Targets Proteins to Peroxisomes, Glyoxysomes, and Glycosomes

Gilbert-André Keller,* Skaidrite Krisans,† Stephen J. Gould,† Jürg M. Sommer,§ Ching C. Wang,§ Wolfgang Schliebs,|| Wolfgang Kunau,|| Stuart Brody,† and Suresh Subramani†

* Department of Preclinical Pharmacology, Genentech Inc., South San Francisco, California 94080; † Department of Biology, San Diego State University, San Diego, California 92182; § Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143; || Institute of Physiological Chemistry, Ruhr University, Bochum, Germany; and † Department of Biology, University of California, San Diego, La Jolla, California 92093.

Abstract. Peroxisomes, glyoxysomes, glycosomes, and hydrogenosomes have each been classified as microbodies, i.e., subcellular organelles with an electron-dense matrix that is bound by a single membrane. We investigated whether these organelles might share a common evolutionary origin by asking if targeting signals used for translocation of proteins into these microbodies are related. A peroxisomal targeting signal (PTS) consisting of the COOH-terminal tripeptide serine-lysine-leucine-COOH has been identified in a number of peroxisomal proteins (Gould, S. J., G.-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. *J. Cell Biol.* 108:1657-1664). Antibodies raised to a peptide ending in this sequence (SKL-COOH) recognize a number of peroxisomal proteins. Immunoelectron microscopy experiments using this anti-

SKL antibody revealed the presence of proteins containing the PTS within glyoxysomes of cells from *Pichia pastoris*, germinating castor bean seeds, and *Neurospora crassa*, as well as within the glycosomes of *Trypanosoma brucei*. Western blot analysis of purified organelle fractions revealed the presence of many proteins containing this PTS in both glyoxysomes and glycosomes. These results indicate that at least one of the signals, and therefore the mechanism, for protein translocation into peroxisomes, glyoxysomes, and glycosomes has been conserved, lending support to a common evolutionary origin for these microbodies. Hydrogenosomes, the fourth type of microbody, did not contain proteins that cross-reacted with the anti-PTS antibody, suggesting that this organelle is unrelated to microbodies.

MICROBODIES are organelles characterized by an electron-dense matrix bound by a single membrane. Although different types of microbodies share common morphological features, they have traditionally been subcategorized into peroxisomes, glyoxysomes, glycosomes, and hydrogenosomes on the basis of differences in enzymatic activities present within these organelles. The peroxisomes, the most studied member of this group, contain hydrogen peroxide-generating oxidases, catalase, and the enzymes involved in the β -oxidation of long chain fatty acids (Lazarow and de Duve, 1976). The glyoxysomes, found in oil-rich tissues of seeds and certain microorganisms grown on long-chain fatty acids, contain, in addition to the fatty acid β -oxidation enzymes, the enzymes of the glyoxylate cycle, a Krebs cycle variant that plays an essential role in the conversion of lipids into carbohydrate (Breidenbach and Beevers, 1967; Trelease et al., 1971). The glycosomes, present only in Kinetoplastida, contain the first nine enzymes of glycolysis and glycerol metabolism as well as those of the fatty acid

β -oxidation pathway (Borst, 1986; 1989). The presence of the fatty acid β -oxidation enzymes in each of these microbodies has led to speculation that these organelles have a common evolutionary origin. Hydrogenosomes, found solely in certain amitochondrial ciliated protozoa, have been referred to as microbodies but differ from the others in that they are enclosed by a double membrane and lack catalase and the β -oxidation enzymes (Müller, 1980, 1988; Benchimol and De Souza, 1983).

We have previously demonstrated that the peroxisomal targeting signal in firefly luciferase consists of three amino acids Ser-Lys-Leu at the carboxy terminus of the protein. This tripeptide, or a conserved variant (serine, alanine, cysteine at the first position; lysine, histidine, arginine, at the second position; leucine at the COOH terminus), is both necessary for the peroxisomal sorting of luciferase and sufficient for directing normally cytosolic passenger proteins to peroxisomes (Gould et al., 1989). The targeting of luciferase to peroxisomes of mammalian, plant, insect, and yeast

cells (Gould et al., 1990a), the evolutionary conservation of the COOH-terminal peroxisomal targeting signal (PTS)¹ in peroxisomal proteins from various organisms (Gould et al., 1989), the immunological detection of the PTS in 15–20 rat liver peroxisomal proteins (Gould et al., 1990b), and the ability of the PTS to function in mammalian cells and in *S. cerevisiae* (Distel, B., S. J. Gould, T. Voorn-Brouwer, M. Van der Berg, H. Tabak, and S. Subramani, manuscript submitted for publication) argue strongly for the conservation of at least one mechanism of translocation of proteins into peroxisomes of evolutionary divergent organisms. However, the signals that sort proteins into the other microbodies are unknown (Borst, 1989). It was of interest, therefore, to determine whether the COOH-terminal tripeptide PTS Ser-Lys-Leu (SKL) is a ubiquitous topogenic signal for the targeting of proteins into glyoxysomes, glycosomes, and hydrogenosomes as well. If so, it would indicate a common evolutionary origin for each of these microbodies.

Materials and Methods

Growth Conditions, Subcellular Fractionation, and Enzyme Assays

Preparation of Rat Liver Samples. Maintenance and Gemfibrozil treatment of rats, as well as isolation of rat liver peroxisomes was as described in Gould et al. (1990b).

Preparation of *Pichia pastoris* Samples. Different subsets of peroxisomal proteins are induced in the yeast *P. pastoris* upon growth on different carbon sources. Wild-type *P. pastoris* cells were inoculated into SY medium (6.7 g yeast nitrogen base [YNB; Difco Laboratories Inc., Detroit, MI] and 0.5 g yeast extract [YE] per liter) supplemented with glycerol at a final concentration of 0.5% (SYG medium) and grown overnight. Cells were diluted 1:100 into fresh SYG medium and grown to an OD₅₉₅ of 0.7–1.0. At this time they were diluted 1:100 into either SYG medium, SYM medium (SY containing 0.5% methanol), SYE medium (SY containing 0.5% ethanol), or SYOL medium (SY containing 0.5% oleic acid, 0.05% Tween 40). Once the cultures reached an OD₅₉₅ of 0.5, they were processed for immunoelectron microscopy as described below. Extracts of cells grown under these conditions were made by vortexing cells with glass beads, spinning down the debris and freezing the supernatants at –70°C as described by Tschopp et al. (1987).

Preparation of *Neurospora crassa* Samples. Hyphae of *N. crassa* strain 74OR8-1a were grown in a shaker for 24 h in a medium containing 2% sucrose and then shifted to a medium containing 1 mM oleate-1% Tergitol as carbon source for an additional 12 h. Growth and culture conditions, cell homogenization, and preparation of crude extracts have been described before (Kionka and Kunau, 1985). Briefly, 10 ml of the crude extract was layered on top of a linear 30–60% sucrose gradient (volume 21 ml) and centrifuged for 90 min at 20,000 rpm (48,000 g) at 4°C using a vertical rotor in a centrifuge (SS90; Sorvall Instruments, Wilmington, DE). In addition to the previously described fractionation procedure (Kionka and Kunau, 1985), the protein in the fractions was monitored after centrifugation by passage through an ultraviolet analyzer (model UA-5 absorbance monitor; Isco, Lincoln, NE). The sucrose density was monitored by measurement of the refractive index of the fractions. Fumarase was used as a mitochondrial marker and assayed by an established procedure (Bergmeyer et al., 1974). Activities of catalase and the glyoxysomal enzyme isocitrate lyase were determined as described (Kionka and Kunau, 1985). Protein concentrations were measured as described by Bradford (1976) using BSA as standard.

Preparation of Castor Bean (*Ricinus communis* L.) Samples. Castor bean seeds were germinated without prior soaking in saturated vermiculite at 30°C, in the dark for 4 d. At this time glyoxysomes account for about 20% of the particulate material in the endosperm (Beever, 1979). The purified glyoxysomes were a generous gift from Irene Wainwright and Elma Gonzalez (University of California, Los Angeles, CA) and prepared as follows. Endosperm tissues were collected and glyoxysomes obtained by frac-

1. **Abbreviations used in this paper:** PTS, peroxisomal targeting signal; SKL, Ser-Lys-Leu.

tionation on sucrose gradients. The peak glyoxysome fractions which sediment at ~50% sucrose were combined, dialyzed overnight against 5 mM Tricine buffer, pH 7.5, and lyophilized before being used for Western blot analysis. The purity of the glyoxysomes was monitored as described by Gonzalez (1986).

Preparation of *Trypanosoma brucei* Samples. *T. brucei* was grown as described by Sommer et al. (1990). Glycosomes were isolated from the long slender bloodstream form of strain EATRO 110 and purified by centrifugation on a linear 1 to 2 M sucrose gradient (Sommer et al., 1990).

Preparation of *Trichomonas vaginalis* Samples. *T. vaginalis* was grown axenically at 37°C in Diamond's trypticase-yeast extract-maltose medium, pH 6.2 (Diamond, 1957), supplemented with 10% heat-inactivated horse serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml).

Gel Electrophoresis and Western Blot Analysis

SDS-PAGE (9–12.5%) of peroxisomal and glyoxysomal fractions was done according to Laemmli (1970). Proteins in gels were either stained with 0.25% Coomassie brilliant blue R250 in methanol/acetic acid/water (5:1:5) and destained in methanol/acetic acid/water (4:1:9) or electrophoretically transferred to nitrocellulose paper in a buffer containing 13 mM NaHCO₃/Na₂CO₃, 20% methanol, and 0.01% SDS (for *N. crassa* proteins) or in 20 mM Tris, 150 mM glycine, and 20% methanol (for rat liver or castor bean proteins). The nitrocellulose was then incubated for a few minutes in PBS with 2% Tween-20 and 5% BSA, followed by incubation with a 1:100 dilution of an IgG fraction of the anti-SKL antibody. Binding of the antibody was visualized by use of either alkaline phosphatase or horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at 30°C.

Western blots of the glycosomal fractions were done as follows. 10 µg of protein from purified glycosomes or from *T. brucei* cell lysates were separated by SDS-PAGE (10–15% acrylamide) and either stained with 0.1% Coomassie blue in 25% ethanol/8% acetic acid or transferred to nitrocellulose (Schleicher and Schuell Inc., Keene, NH) in buffer containing 15 mM CAPS (3-[cyclo-hexylamino]-l-propanesulfonic acid), pH 10.5, and 20% methanol at 400 mA for 90 min. The blot was incubated in TS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 5% (wt/vol) nonfat dry milk for at least 30 min before incubation with the primary antibody (1/200 anti-SKL antiserum diluted in TS and dry milk) for 2 h at room temperature. Following three rinses in TS, the blot was incubated with alkaline phosphatase conjugated goat anti-rabbit antibody (BioRad Laboratories, Richmond, CA) and bound antibodies were visualized in a buffer containing 0.1 M NaHCO₃, pH 9.8, 1 mM MgCl₂, 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, and 0.3 mg/ml nitroblue tetrazolium.

Cryoultramicrotomy and Immunolabeling

The different microorganisms were fixed by the addition of glutaraldehyde (0.5% final concentration) to the culture medium at the end of the log phase and stored for 24 h at 4°C. The cells were collected by centrifugation at 1,000 rpm, washed extensively in PBS, transferred to microtubes containing 10% melted gelatin in PBS, centrifuged in an Eppendorf microfuge (Brinkman Instruments, Inc., Westbury, NY) for 30 s, and kept on ice until the gelatin solidified. Blocks of 1 mm³ endosperm tissues of castor bean seeds were collected and fixed in 3% formaldehyde and 0.5% glutaraldehyde in 0.05 M cacodylate buffer.

Cryoultramicrotomy was performed as described by Tokuyasu (1973). Blocks of solidified gelatin containing the cells or endosperm tissues were infused with 2.3 M sucrose before being frozen in liquid nitrogen for thin sectioning. The characterization of the anti-PTS antibody has been described earlier (Gould et al., 1990b) and in the Results section. Immunolabeling and thin plastic embedding of the cryosections were performed as described by Keller et al. (1984). Briefly, cryosections of different microorganisms and castor bean endosperm were immunolabeled with an IgG fraction of the anti-SKL antibody at a concentration of 50 µg/ml for 10 min. After washing in PBS, the sections were treated with 10-nm colloidal gold adducts of affinity-purified goat antibodies to rabbit IgG (Keller et al., 1984). In some experiments, the 10-nm gold adducts were replaced by either immunoferritin or 5-nm gold adducts. The immunolabeled sections were treated by floating the grids on droplets of 1% reduced osmium, post-stained in 2% ethanolic uranyl acetate, and infused with LR white acrylic resin (London Resin Co., London, England) (Keller et al., 1984). After polymerization in a vacuum oven, the sections were observed without post-staining in a transmission electron microscope (CM12; Philips Electronic Instruments Inc., Mahwah, NJ) equipped with a 10-µm-diam objective aperture.

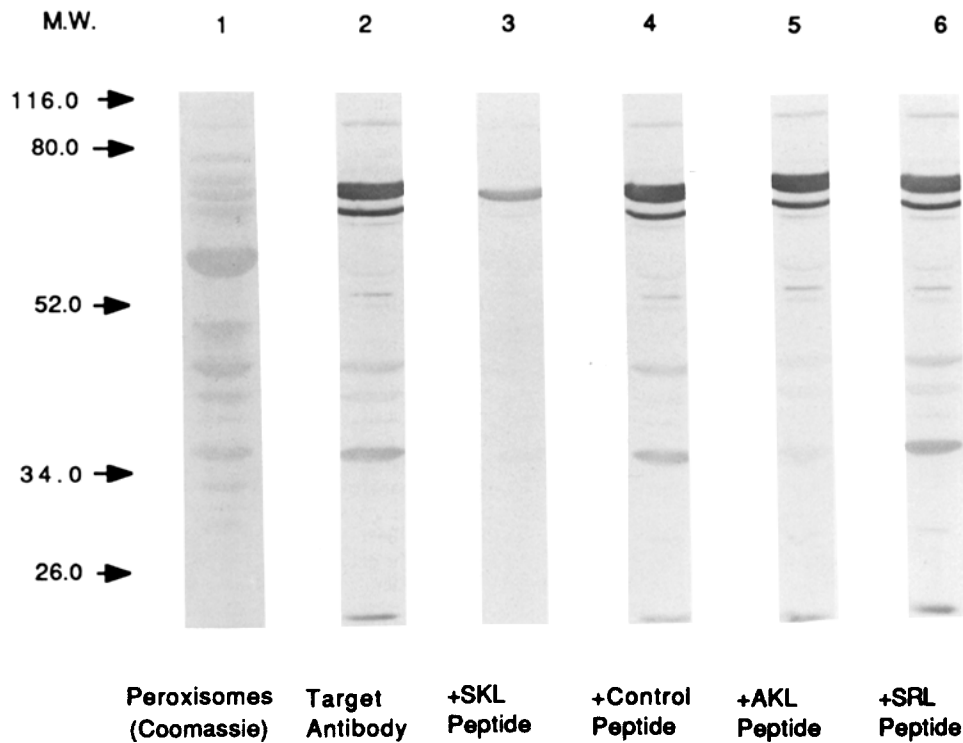


Figure 1. Specificity of the anti-SKL antibody. Proteins from purified rat liver peroxisomes (50 $\mu\text{g}/\text{lane}$) were subjected to SDS-PAGE, transferred to nitrocellulose, and strips corresponding to individual lanes were either used for Coomassie staining (lane 1) or for Western blots with the anti-SKL antibody (lanes 2–6). The antibody was either used directly (lane 2), or after preincubation for 30 min at room temperature with 10 $\mu\text{g}/\text{ml}$ SKL peptide (CRYHLK-PLQSKL, lane 3), 1,250 $\mu\text{g}/\text{ml}$ control peptide (CRYHLK-PLQ, lane 4), 1,250 $\mu\text{g}/\text{ml}$ AKL peptide (CRYHLKPLQ-AKL, lane 5) or 1,250 $\mu\text{g}/\text{ml}$ SRL peptide (CRYHLKPLQ-SRL, lane 6).

The recognition of the rat liver peroxisomal proteins by the antibody is competed only by the SKL-containing peptide and not by the other peptides even when they are used in 125-fold excess over the SKL peptide.

Results

Characterization of the Anti-SKL Antibody

We have previously characterized a rabbit antibody raised against a synthetic peptide ending in Ser-Lys-Leu-COOH ($\text{NH}_2\text{-CRYHLKPLQSKL-COOH}$), a form of the COOH-terminal tripeptide (PTS). The antibody appeared to be directed against the SKL-COOH segment of the peptide, specifically immunolabeled mammalian peroxisomes by immunofluorescence and immunocryoelectron microscopy, and bound to 15 to 20 rat liver peroxisomal proteins in Western blot experiments (Gould et al., 1990b). Further characterization of this antibody is presented in Fig. 1. Western blots of purified peroxisomal proteins again demonstrated that the anti-SKL antibody recognized a number of different peroxisomal proteins. Competition experiments had previously demonstrated that peptides containing SKL at their COOH terminus could block labeling of peroxisomal proteins in immunofluorescence experiments. Preincubation of such peptides with the anti-SKL antibody also blocked recognition of peroxisomal proteins in Western blot experiments (Fig. 1). Preincubation of the antibody with various peptides lacking this tripeptide or ending in the related sequences SRL (which functions as a PTS) did not affect the recognition of peroxisomal proteins by the antibody. A peptide ending in the sequence AKL did however compete to some extent although not as well as the SKL peptide (Fig. 1, lane 5). These results indicate that the antibody has much lower affinity for SRL or AKL than SKL and may in fact only recognize this form of the tripeptide PTS. Further Western blot experiments re-

vealed that the antibody did not recognize the SKL tripeptide when situated at internal locations in proteins (data not shown). Thus, the antibody appears to have a remarkable specificity for proteins containing SKL at their COOH termini.

The Peroxisomes and Glyoxysomes of the Yeast *P. pastoris* Contain Proteins Ending in the Tripeptide PTS

Having established that the antibody was specific for at least one class of mammalian peroxisomal proteins, we used it as a probe to localize proteins ending in SKL-COOH in the microbodies of other eukaryotes. In the methylotrophic yeast *P. pastoris*, the development and metabolic functions of microbodies are modulated by growth conditions. *P. pastoris* normally contains only a few small microbodies per cell, but growth on oleic acid induces glyoxysome proliferation whereas growth on methanol induces the proliferation of peroxisomes (our unpublished observations). Cells grown on either glycerol, oleic acid, or methanol were fixed and cryosections of each sample were immunolabeled with the anti-SKL antibodies. Cells grown on glycerol contained one to three small microbodies (0.1–0.2 μm) per cell, usually in close proximity to the plasma membrane (Fig. 2A), whereas those induced by methanol were of large size (up to 1.5 μm) occupying a sizeable portion of the cytoplasm (Fig. 2B). Microbodies induced by oleate were organized in clusters of a couple to ten microbodies (0.1–0.3 μm) per cell profile (Fig. 2C). Independently of the culture conditions, the microbodies were the only organelles labeled with the anti-

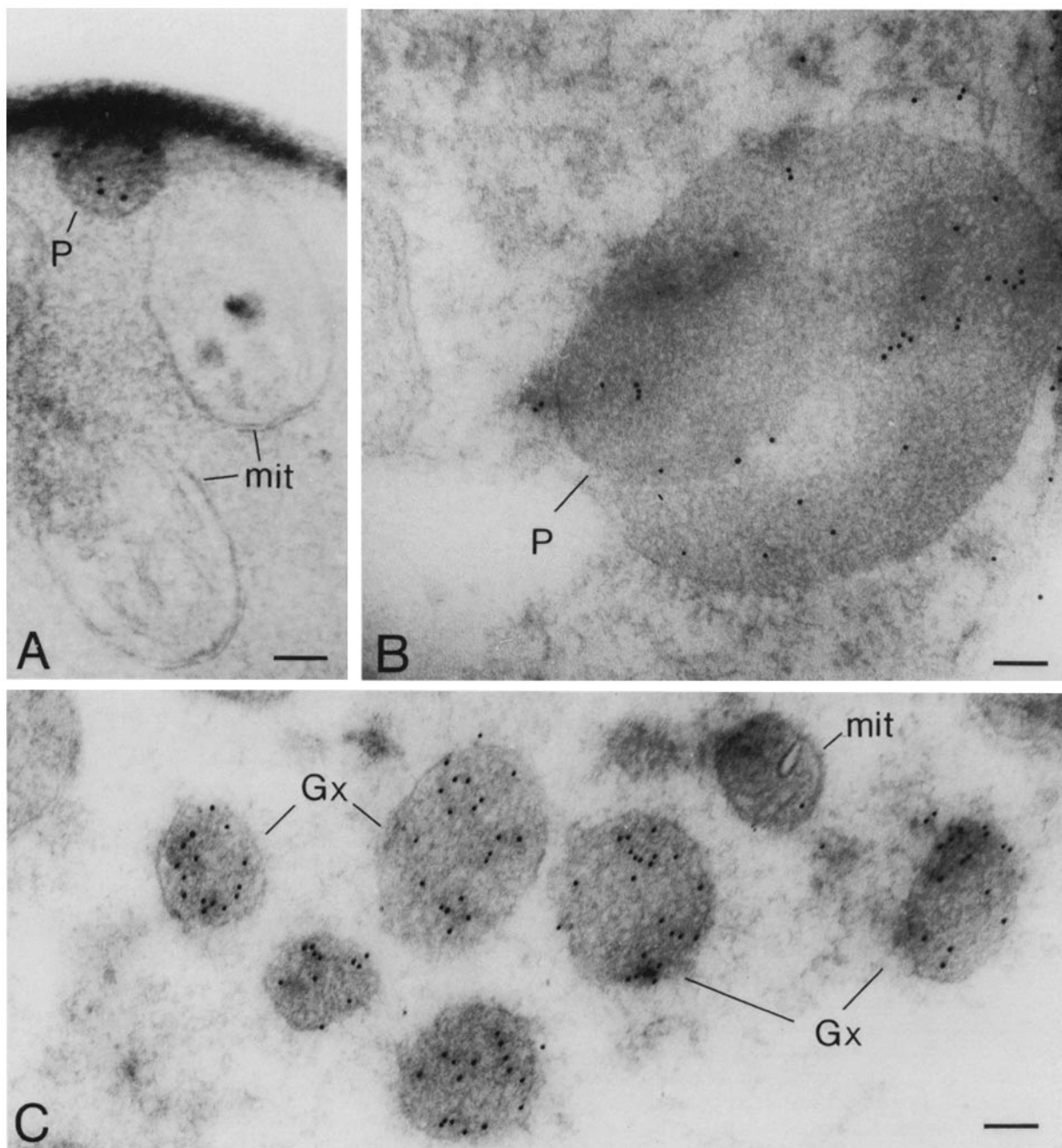


Figure 2. Cryosections of *P. pastoris* immunolabeled with the anti-SKL antibody. *P. pastoris* was grown on glycerol (A), methanol (B), and oleic acid (C). Note the difference in the organelles' number and size. Independently of the culture conditions, the peroxisomes in A and B, and the glyoxysomes in C are the only organelles immunolabeled. The mitochondria visible in the field are not labeled. See text for further explanation. P, peroxisome; Gx, glyoxysomes; mit, mitochondria. Bar, 0.1 μm .

SKL antibody and the peroxisomal immunolabeling was always restricted to the matrix of the organelles. Western blots of cell extracts made from cells grown on glycerol, ethanol, methanol, or oleate revealed several proteins (about six to seven) that were recognized by the antibody (data not shown).

Glyoxysomes of Castor Bean Endosperm Contain Proteins That Are Recognized by the Anti-SKL Antibody

We recently showed that luciferase was localized to the leaf

peroxisomes of transgenic tobacco plants expressing the insect enzyme (Gould et al., 1990a) and demonstrated by immunocryoelectron microscopy that leaf peroxisomes contained proteins ending in the tripeptide PTS (data not shown). Since it is established that peroxisomes derive from glyoxysomes during the germination and maturation of seedlings (Shopfer et al., 1976, 1983), it is likely that transitional glyoxysomes also contain proteins ending in SKL. In contrast to microbodies of other plant tissues, the microbodies in the endosperm of germinating castor beans do not undergo the transition from glyoxysomal to peroxisomal function.

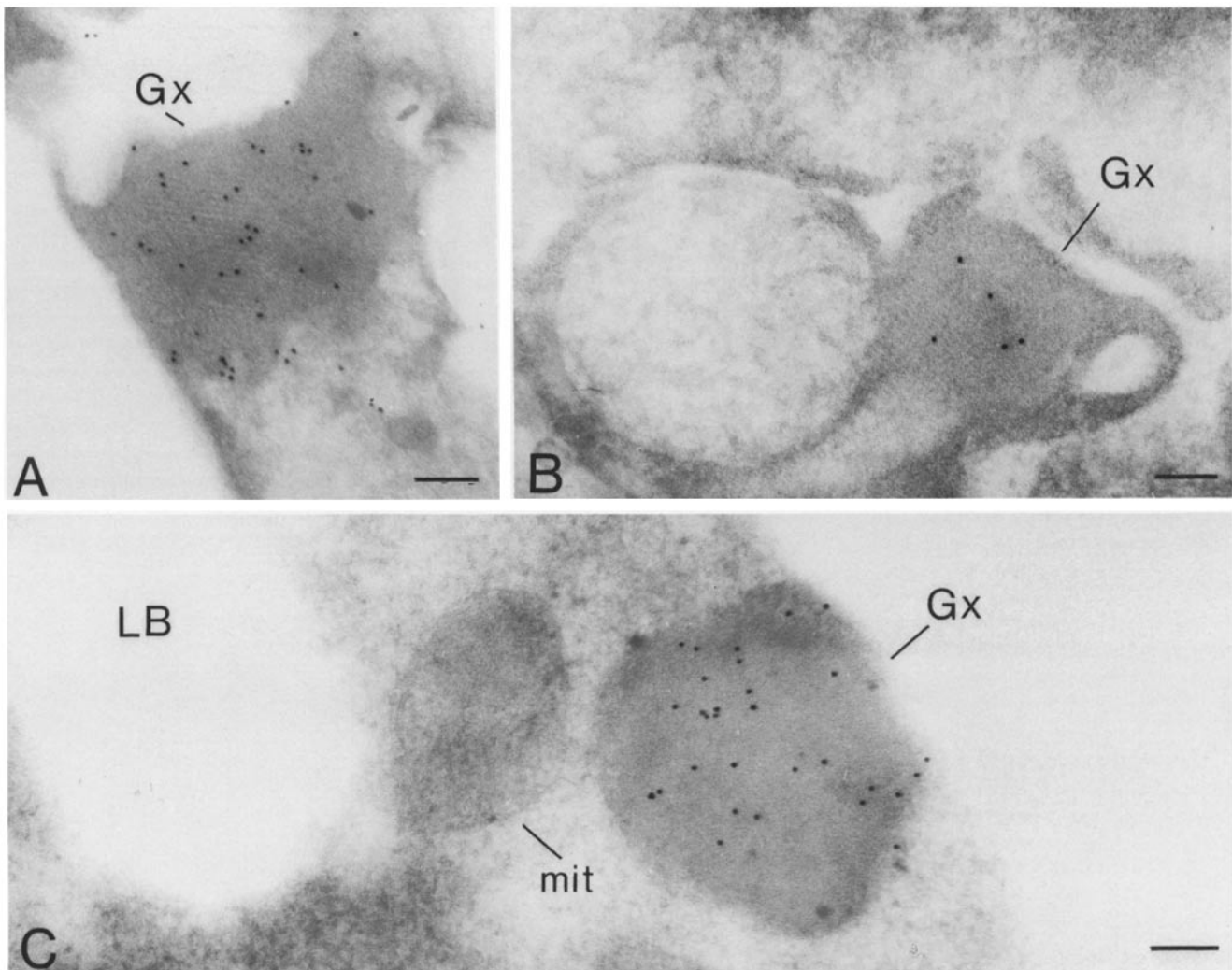


Figure 3. Cryosections of endosperm from a germinating castor bean seedling immunolabeled with the anti-SKL antibody. Specific labeling is observed over glyoxysomes (Gx) which display various shapes in this tissue (A, B, and C). Gx, glyoxysomes; LB, lipid bodies; mit, mitochondria. Bar, 0.1 μ m.

Thus, they provide a convenient system to determine the topogenic signal for true glyoxysomes. Immunolabeling with the anti-SKL antibody was performed on cryosections of endosperm tissue from *Ricinus communis* L. seeds. No appreciable labeling over the lipid bodies, the mitochondria, the vacuole, or the cell wall could be detected whereas a signal that varied from light to intense was observed over the matrix of the glyoxysomes (Fig. 3). Germinating castor bean glyoxysomes display polymorphic shapes (Fig. 3, A, B, and C) but are easily recognized as ovoid organelles in zone III of the endosperm where they are more numerous than in the other zones (Vigil, 1970). Further proof that the immunolabeling reflected the presence of glyoxysomal enzymes was obtained by Western gel analysis of a purified glyoxysomal fraction from castor bean endosperm. As shown in Fig. 4, at least 10 glyoxysomal proteins were recognized by the anti-SKL antibody.

Identification of Proteins Containing the Tripeptide PTS in Microbodies of *N. crassa*

The filamentous fungus *N. crassa* was used because biochemically distinct microbodies appear to exist within the

cell at the same time. The first, which is analogous to glyoxysomes, sediments at a density of 1.22 gm/ml in sucrose gradients and contains enzymes of the glyoxylate pathway (isocitrate lyase and malate synthase) and the fatty acid β -oxidation pathway but does not contain catalase. The second, believed to be a peroxisome, sediments at a density of 1.26 gm/ml. It contains uricase and a small proportion of the total catalase (Wanner and Theimer, 1982; Kionka and Kunau, 1985). In addition to peroxisomes and glyoxysomes, a third type of morphologically distinct organelle, the hexagonal crystal (Hoch and Maxwell, 1973), is present in *N. crassa*. The hexagonal crystal is thought to derive from one population of microbody (Allen, 1976). This organelle, which does not contain catalase, also sediments at a density of 1.26 mg/ml (Schliebs and Kunau, unpublished results).

N. crassa cells cultured in medium containing either glucose or oleic acid as carbon source were fixed during the logarithmic phase of growth and prepared for immunocyto-electron microscopy. In cells cultured in glucose minimal medium, microbodies appeared as spherical to elongated profiles which were relatively low in number. Their number increased when the cells were switched to a medium contain-

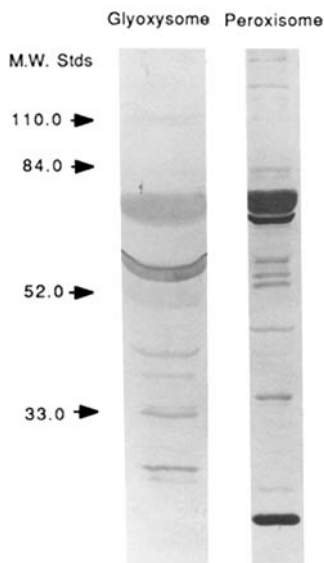


Figure 4. Western blot analysis of protein from purified glyoxysomal fractions using the anti-SKL antibody. Protein extracts (50–100 μ g/lane) from purified castor bean glyoxysomes or rat liver peroxisomes were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with the anti-SKL antibody.

ing oleic acid. The subcellular distribution of proteins ending in SKL is presented in Fig. 5. In addition to small microbodies (Fig. 5, *B* and *C*), the anti-SKL antibody labeled the matrix of the hexagonal crystals (Fig. 5 *A*).

Further characterization of the proteins was performed on purified cell fractions of *N. crassa* by Western blot analysis. Proteins (Fig. 6 *A*) from the mitochondrial (sucrose density of 1.19 g/ml) and glyoxysomal fractions (sucrose density of 1.21 g/ml), as well as the fraction enriched in hexagonal structures (density of 1.26 g/ml) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with the anti-PTS antibody. The antibody reacted specifically with four major microbody proteins with molecular masses of 68, 52, 42, and 35 kD (Fig. 6 *B*, lane 4). Only one predominant band at 21 kD was recognized by the antibody in the fraction enriched in hexagonal structures (Fig. 6 *B*, lane 2). This polypeptide, of unknown function, is a major protein of the hexagonal crystals (Schliebs and Kunau, unpublished data). In other experiments, proteins of the peroxisome fraction were also stained by the antibody (data not shown). As expected, the antibody did not react with any proteins of the mitochondrial fraction (Fig. 6 *B*, lane 6).

Proteins Containing the Tripeptide PTS Are Present in the Glycosomes of *T. brucei*

To determine whether glycosomal proteins also reacted with the anti-SKL antibody, a blood-stage form of *T. brucei* was cultured, fixed, and thin cryosections were immunolabeled with the anti-SKL antibodies. Gold particles were seen over membrane bound organelles with granular and slightly electron-dense material, typical of glycosomes (Fig. 7). No labeling was observed over any other sub-cellular organelles. Interestingly, whereas most glycosomes were labeled with numerous gold particles, some glycosomes were only weakly labeled at the periphery of the glycosomes or not at all. The few gold particles in the cytoplasm of *T. brucei* may represent glycosomal proteins in the process of being transported to the glycosomes.

Further evidence that glycosomal proteins contain the PTS was obtained by Western blots. Proteins from purified glyco-

somes were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with the anti-PTS antibodies (Fig. 8, lane 4). The antibodies recognized at least six protein bands present in the purified glycosomal fraction (75, 50, 47.5, 44.5, 38, and 29 kD).

Hydrogenosomes of the *Trichomonad* Flagellates Are not Recognized by the Anti-SKL Antibody

We also used the anti-SKL antibody to address the question of whether the hydrogenosomes found in *Trichomonad* flagellates and rumen ciliates are related to the other classes of microbodies. Cryosections of fixed *T. vaginalis* were immunolabeled with the anti-SKL antibody. Hydrogenosomes were easily recognized as spherical, elongated, or dumbbell-shaped organelles distributed in the cytoplasm. In contrast to the microbodies of the other microorganisms which were immunolabeled without exception, albeit with different intensities, the hydrogenosomes of neither *T. vaginalis* or *T. foetus* (Fig. 9) were labeled above the background of the immunolabeling technique even when more sensitive probes such as 5-nm gold adducts or immunoferritin were used as secondary antibodies (data not shown).

Discussion

The Tripeptide PTS Is a Common Feature of Microbody Matrix Proteins

Recent advances in our understanding of the signals that target proteins to peroxisomes have led to the identification of a tripeptide peroxisomal targeting signal (serine-lysine-leucine, or a conservative variant) located at the COOH-terminus of the majority of peroxisomal proteins (Gould et al., 1989). Antibodies which specifically detect at least one form of this PTS (serine-lysine-leucine-COOH) were found to react with a wide range of peroxisomal proteins from mammalian cells (Gould et al., 1990a). Though it has been suggested that the SKL tripeptide PTS can also function in diverse species such as yeast, plants, insects, and mammalian cells (Keller et al., 1987; Gould et al., 1990a; Distel, B., S. J. Gould, T. Voorn-Brouwer, M. Van der Berg, H. Tabak, and S. Subramani, manuscript submitted for publication) it had not yet been demonstrated that the anti-SKL antibody was capable of recognizing peroxisomal proteins in lower eukaryotes. Our finding that the anti-SKL antibodies detected peroxisomes in methanol-grown yeast (*P. pastoris*) and in plant leaf protoplasts (data not shown) demonstrates that this antibody can be used to detect peroxisomes in a wide range of organisms.

The availability of an antibody that recognizes at least one form of the COOH-terminal tripeptide PTS has enabled us to ask whether the glyoxysomes, glycosomes, and hydrogenosomes use a similar form of targeting signal for the import of their proteins.

Glyoxysomes are a form of microbody found in germinating seeds and certain fungal species. Titus and Becker (1985) have provided evidence that in germinating seeds, glyoxysomes may convert to peroxisomes by the gradual replacement of glyoxysomal enzymes with peroxisomal enzymes in a continually intact microbody (Trelease et al., 1971). The reverse transition from peroxisomes to glyoxysomes in se-

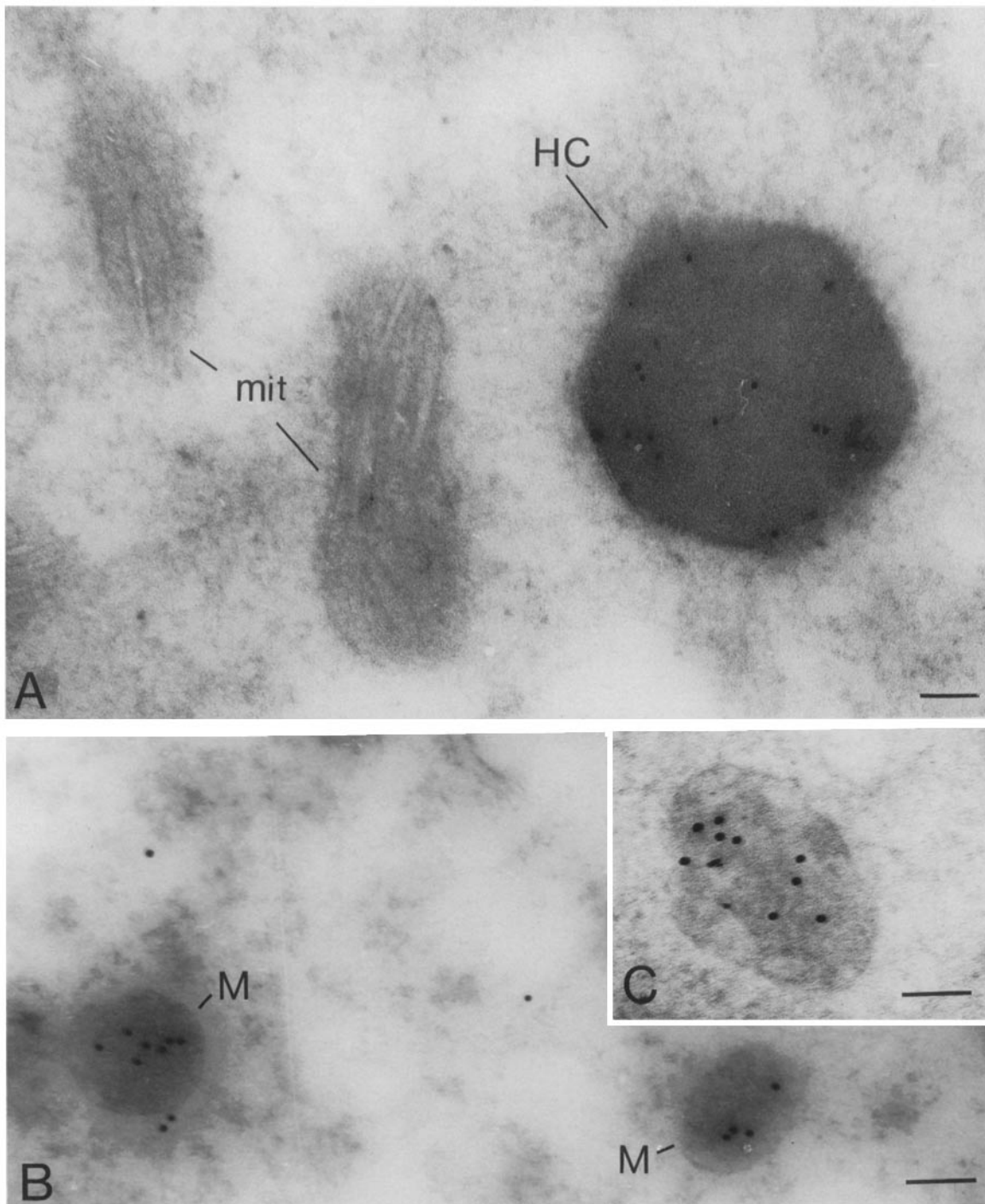


Figure 5. Cryosections of *N. crassa* immunolabeled with the anti-SKL antibody. (A) Specific labeling is exclusively observed in the hexagonal crystal (HC), and in B, in the matrix of two microbodies (M) visible in the field. (C) Higher magnification of a microbody showing that the labeling is associated with the matrix but not with the membrane of the organelle. HC, hexagonal crystal; mit, mitochondria; M, microbody. Bar, 0.1 μm .

nescent leaves has also been described and may occur in the same manner (De Bellis et al., 1990). Two models may explain this phenomenon. One possibility is that distinct forms of targeting signals and import mechanisms are used for glyoxysomal and peroxisomal proteins and that there is a change from one to another during this transition. Another possibility is that glyoxysomal and peroxisomal proteins use the same type of targeting signals and are imported by the same

mechanisms. Our findings that at least 10 (30–40%) of the 25–30 glyoxysomal matrix proteins (Gonzalez, 1990) are recognized by the anti-SKL antibody in glyoxysomes of castor bean endosperm and that these antibodies specifically detect only glyoxysomes in these cells imply that the tripeptide peroxisomal targeting signal is a feature of many glyoxysomal proteins. The use of the same targeting signal for glyoxysomes and peroxisomes argues strongly that the latter of the

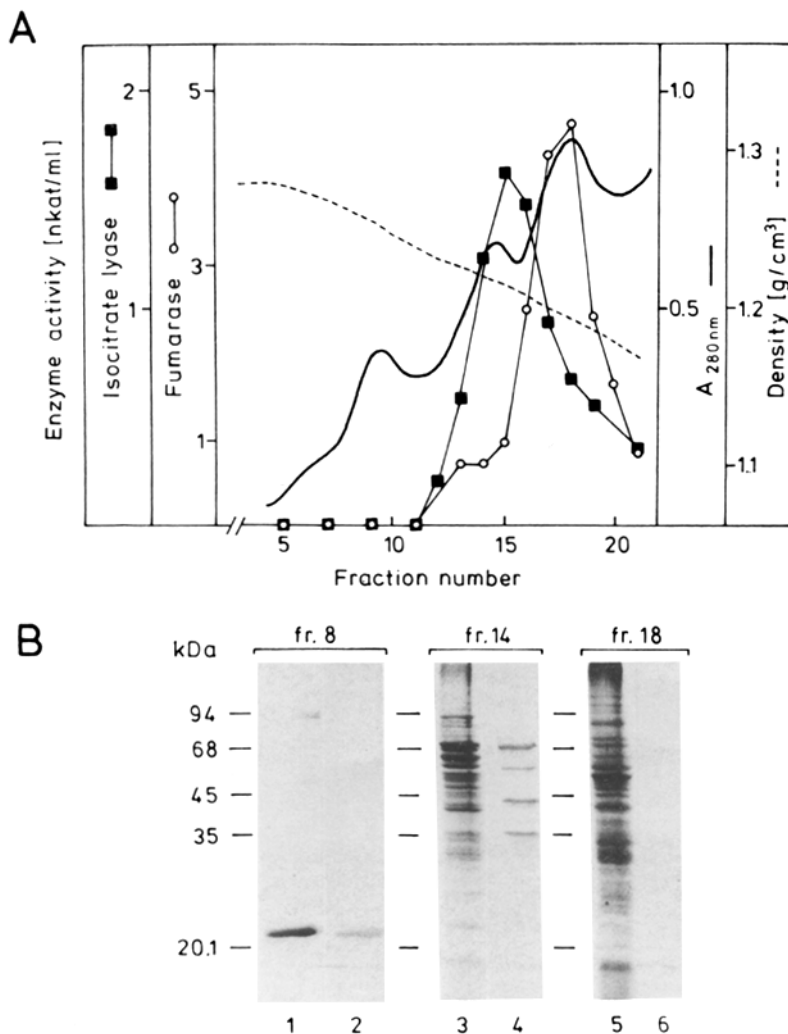


Figure 6. Recognition of the proteins in the glyoxysomes and hexagonal structures by the anti-SKL antibody. (A) Fractionation of the organelles of *N. crassa* by sucrose-density gradient centrifugation. Mitochondria and glyoxysomes were monitored using fumarase and isocitrate lyase, respectively, as markers. (B) Immunoblot analysis with anti-SKL antibodies of various organelle-enriched fractions. Hexagonal-shaped organelles (fraction 8), glyoxysome-like microbodies (fraction 14) and mitochondria (fraction 18) were identified by typical marker enzyme activities, relative protein amount (absorbance at 280 nm), and specific density. Proteins from each of these fractions (20 μ g/lane) were electrophoresed and stained with Coomassie blue (lanes 1, 3, and 5) or transferred to nitrocellulose and incubated with anti-SKL antiserum (lanes 2, 4, and 6).

two models is correct. Additional evidence supporting this view comes from the analysis of the sequences of glyoxysomal enzymes which revealed proteins ending in SKL, SRL, and ARL, all of which are forms of the consensus COOH-terminal PTS (Smith and Leaver, 1986; Volokita and Somerville, 1987; Comai et al., 1989). However, definitive proof that the consensus tripeptide PTS is necessary and sufficient for glyoxysomal targeting in plants will have to await further experimentation with transgenic plants or in vitro import systems.

The filamentous fungus *N. crassa* represents a rare case in which microbody enzyme activities appear to fractionate with different cell compartments. For example, peroxisomes and glyoxysomes exist within the same cell at the same time (Wanner and Theimer, 1982). Both types of microbodies were recognized by the anti-SKL antibody. Interestingly, the hexagonal crystals, a group of organelles morphologically related to the Woronin bodies, were also recognized by the anti-SKL antibodies. This is not too surprising considering that they may be derived from one of the microbody forms (Markham and Collinge, 1987). Western blot analysis showed that several distinct proteins are recognized in the three microbody fractions. Since very few peroxisomal or

glyoxysomal protein genes have been cloned in *N. crassa*, we are unable to identify the specific proteins ending in SKL.

Trypanosomes and other Kinetoplastida are unusual in the sense that they compartmentalize the enzymes of the glycolytic pathway (which are localized to the cytosol in all other eukaryotic cells) within a type of microbody called the glycosome (Opperdoes, 1987). Like peroxisomal proteins, glycosomal proteins are encoded by nuclear genes, synthesized in the cytosol and posttranslationally imported into the organelle (Borst, 1986). In recent years, two hypotheses have been presented to account for the transport of proteins to the glycosomes. The first was based on an analysis of the predicted three-dimensional structure for glycosomal and cytosolic forms of three glycolytic enzymes which revealed that in each case the glycosomal form contained two regions of high positive charge not present in the cytosolic forms. The two clusters of positively charged residues in the molecules, so-called "hot spots", were proposed to play an essential role in the import of the enzymes into the glycosomes (Wierenga et al., 1987). The second hypothesis was proposed by Swinkels et al. (1988), who determined that the glycolytic and the cytosolic forms of 3-phosphoglycerate kinase in Kinetoplastid *C. fasciculata* differ only by the pres-

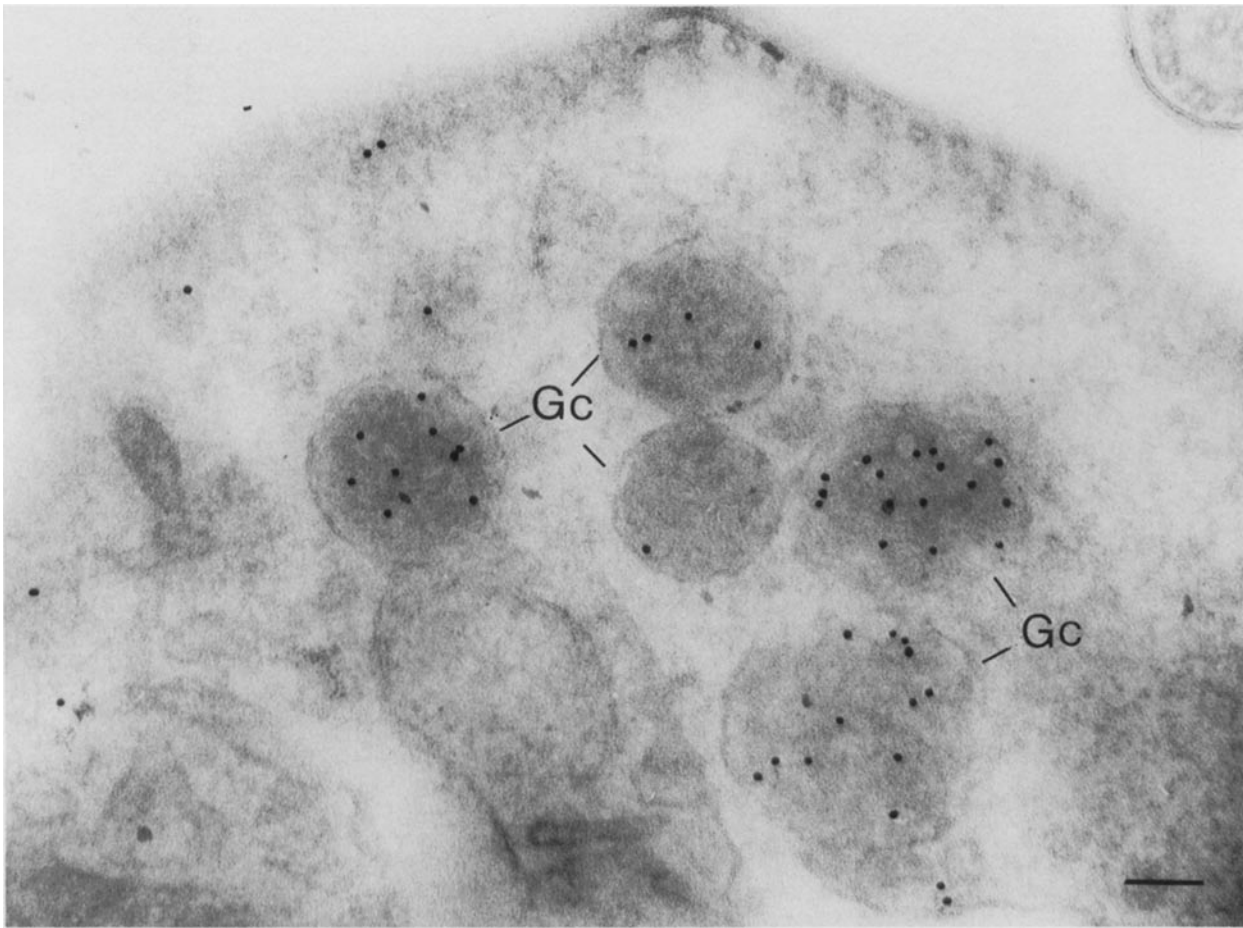


Figure 7. Cryosection of *T. brucei* immunolabeled with the anti-SKL antibody. The immunolabeling is mostly restricted to the matrix of the glycosomes (Gc). Note that not all the glycosomes are labeled with the same density. A few gold particles, which might represent proteins en route to the glycosomes, can be seen in the cytosol. Bar, 0.1 μm .

ence of a COOH-terminal extension of 38 amino acids in the glycosomal form. These authors concluded that the carboxy-terminal extensions, rather than the "hot spots," were involved in the routing and the transport of the enzyme into glycosomes. While the PTSs in luciferase and several other peroxo-

isomal proteins also reside at or near the COOH terminus, the consensus tripeptide PTS is absent from the COOH terminus of the glycosomal protein extension (Swinkels et al., 1988). Without excluding that "hot spots" or carboxy-terminal extensions are involved in targeting proteins to glycosomes, our results imply that six out of approximately 30 glycosomal proteins (~20%) are targeted to the glycosomes by means of the COOH-terminal tripeptide PTS. Our conclusion that the tripeptide PTS is used for the import of at least some proteins into glycosomes is supported by recent results which show that upon transformation of *T. brucei* with the genes encoding chloramphenicol acetyltransferase (CAT) or a CAT-SKL fusion protein, only the fusion protein was targeted to glycosomes (Fung and Clayton, 1991) and the finding that the glycosomal glyceraldehyde phosphate dehydrogenase and the glucose phosphate isomerase of *T. brucei* end in AKL and SHL, respectively (Michels et al., 1986; Marchand et al., 1989), each of which is a form of the tripeptide PTS (Gould et al., 1989).

It must also be emphasized that in all the organisms in which microbodies were recognized by the anti-SKL antibody, it was always the matrix and not the membrane of the organelles that was labeled. This suggests that the sorting of membrane proteins into these organelles occurs via some other, as yet unidentified, targeting signal(s).

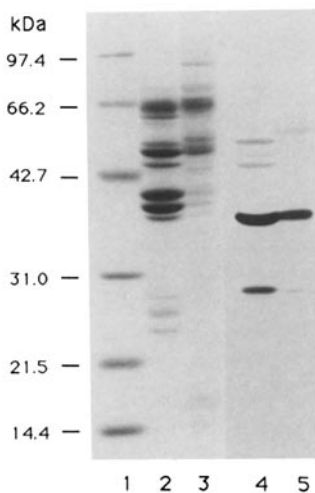


Figure 8. Western blot of glycosomes of *T. brucei*. Proteins (10 $\mu\text{g}/\text{lane}$) from purified glycosomal fractions (lanes 2 and 3) or from total extracts (lanes 3 and 5) were either stained with Coomassie blue (lanes 2 and 3) or used for Western blots (lanes 4 and 5) with the anti-SKL antibody. Molecular weight standards are shown (lane 1).

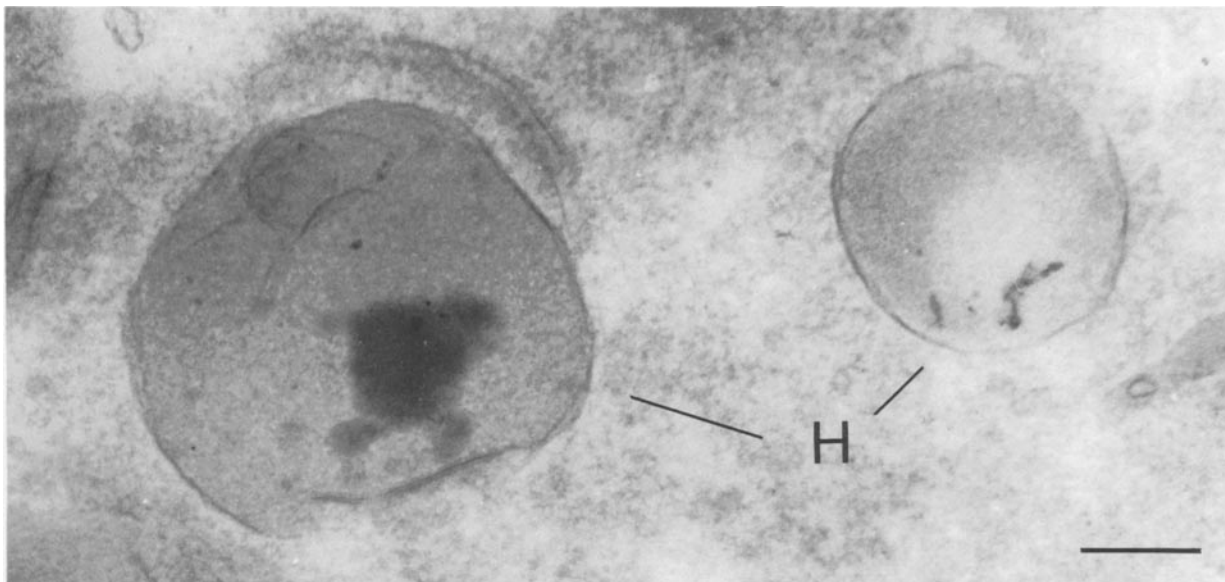


Figure 9. Cryosection of *T. vaginalis* immunolabeled with the anti-SKL antibody. In contrast to the microbodies of the other microorganisms, the hydrogenosomes (H) are not immunolabeled. Bar, 0.1 μm .

Hydrogenosomes Are not Related to Other Microbodies

In contrast to the immunolabeling results obtained with the microbodies of the different organisms we studied, we were unable to demonstrate the presence of proteins ending in SKL in the hydrogenosomes of *T. vaginalis*. Our data, in conjunction with the other evidence cited below, suggest that proteins are targeted to the hydrogenosomes by signal(s) different from the COOH-terminal SKL, but do not rule out the possibility that sequences similar to SKL, but unrecognizable by the anti-SKL antibody, may be present on the organelle proteins (as is the case for *Candida sp.*, see below). Additional lines of evidence supporting the idea that hydrogenosomes are unrelated to the microbodies include: (a) electron micrographs show that the hydrogenosomes of some anaerobic ciliated protozoa resemble mitochondria with invaginated and folded inner membranes (Finlay and Fenchel, 1989); (b) they share some common biochemical properties with mitochondria such as cyanide-insensitive superoxide dismutase (Cerkasovova et al., 1976); (c) DNA sequence analysis of the gene coding for the *T. vaginalis* hydrogenosomal ferredoxin predicts an amino terminal octapeptide which has properties typical of mitochondrial leader sequences (Johnson et al., 1990). These findings suggest that not only are the hydrogenosomes unrelated to other microbodies but that the hydrogenosome may be the anaerobic equivalent of the mitochondria.

Peroxisomal Targeting Signals of *Candida sp.*

The only other organism tested in which the anti-SKL antibody failed to yield any signal by immunocryoelectron microscopy was *Candida tropicalis* (data not shown). Unlike the conclusions drawn from our inability to label hydrogenosomes, whose relationship to peroxisomes, glyoxysomes, and glycosomes is tenuous at best, the absence of labeling in bona fide microbodies of *C. tropicalis* suggests that this organism may use different PTSs. The recent identification

of an SKL-like tripeptide, AKI (Nuttley et al., 1988), as the COOH-terminal PTS of the trifunctional enzyme of *C. tropicalis* (R. Rachubinski, personal communication), as well as the conservation of a similar sequence in other peroxisomal proteins of *Candida sp.* (e.g., AKL in *C. boidinii* PMP-20; Garrard and Goodman, 1989) argue that *Candida sp.*, like other eukaryotes, can use a different variant of the consensus tripeptide for protein translocation into peroxisomes. An analogous situation exists for signals that retain proteins in the ER. The COOH-terminal tetrapeptides KDEL, HDEL, and DDEL specify ER retention signals in mammalian cells, *S. cerevisiae* and *K. lactis*, respectively (Lewis et al., 1990).

Not only is the COOH-terminal tripeptide PTS of a different form in *C. tropicalis* proteins, but at least one *C. tropicalis* protein contains an altogether different type of PTS. In the case of the acyl-CoA oxidase from this yeast, Small et al. (1988) have described two internally located, ~ 100 -amino-acid regions which do not contain SKL motifs but function as PTSs. This suggests that a given organism may use more than one type of signal for sorting of proteins into microbodies.

Generality of Microbody Targeting Signals

In summary, the results presented here provide evidence that the transport of proteins to the different classes of microbodies depends upon similar specific sorting mechanisms that function in evolutionarily divergent organisms. They demonstrate that the targeting signal identified in firefly luciferase (Gould et al., 1989) not only specifies targeting to peroxisomes but more generally governs sorting of a substantial number of proteins to glyoxysomes and glycosomes. In each class of organelles, 20–40% of the Coomassie-stained proteins were recognized by the antibody. If this similarity in the protein sorting mechanism indicates a common ancestry, then the clear inference from the data presented here is that peroxisomes, glycosomes, and glyoxysomes share the same evolutionary origin. These findings, taken together with the morphological and enzymatic similarities between the three

microbodies, indicate that peroxisomes, glyoxysomes, and glycosomes may be much more alike than they are different from one another. Though the enzymatic differences between these three microbodies are important to keep in mind (as well as the possibility that targeting signals specific for either peroxisomes, glyoxysomes, or glycosomes may exist), the similarities between peroxisomes, glyoxysomes, and glycosomes suggest that they are essentially the same organelle. In this case, it may be more accurate to refer to the COOH-terminal tripeptide PTS as a general microbody targeting signal and to use the original term, the microbody (Rhodin, 1954), for this class of organelles, rather than peroxisomes, glyoxysomes, and glycosomes. We propose that any organelle that is bound by a single membrane and uses at least one general microbody targeting signal for import of its proteins (such as the COOH-terminal tripeptide microbody targeting signal) be referred to as microbodies.

We thank Dr. Robert P. Donaldson (George Washington University, Washington D.C.) for his advice on the germination of castor bean seeds, Dr. I. Wainwright and Dr. E. Gonzalez (University of California, Los Angeles, CA) for the gift of purified glyoxysomal fractions, Dr. G. Small (University of Florida, Gainesville, FL) for the gift of purified peroxisome fractions from *C. tropicalis*. We also thank Sarah Thompson for her technical help.

This work was supported in part by grants to S. Subramani from the March of Dimes Foundation (1081) and the National Institutes of Health (DK41737).

Received for publication 21 November 1990 and in revised form 26 April 1991.

References

- Allen, E. D. 1976. Development of crystalline inclusions ("ergosterol crystals") in *Neurospora crassa*. *Protoplasma*. 90:297-306.
- Beevers, H. 1979. Microbodies in higher plants. *Annu. Rev. Plant Physiol.* 30:159-197.
- Benchimol, M., and W. De Souza. 1983. Fine structure and cytochemistry of the hydrogenosomes of *Tritrichomonas foetus*. *J. Protozool.* 30:422-425.
- Bergmeyer, H. U., K. Wehn, and M. Grassl. 1974. In *Methoden der enzymatischen Analyse*. H. U. Bergmeyer, editor. 3rd edition. Verlag Chemie, Weinheim, Germany. 479-480.
- Borst, P. 1986. How proteins get into the microbodies (peroxisomes, glyoxysomes, glycosomes). *Biochimica Biophys. Acta*. 866:179-203.
- Borst, P. 1989. Peroxisome biogenesis revisited. *Biochimica Biophys. Acta*. 1008:1-13.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Briedenbach, R. W., and H. Beevers. 1967. Association of the glyoxylate cycle enzymes in a novel subcellular particle from castor bean endosperm. *Biochem. Biophys. Res. Commun.* 27:462-469.
- Cavalier-Smith, T. 1987. The simultaneous symbiotic origin of mitochondria, chloroplasts and microbodies. *Ann. New York Acad. Sci.* 503:55-71.
- Cerkasova, A., J. Cerkasov, J. Kulda, and J. Reischig. 1976. Circular DNA and cardiolipin in hydrogenosomes, microbody-like organelles in *Trichomonas*. *Folia Parasitol.* 23:33-37.
- Comai, L., C. S. Baden, and J. J. Harada. 1989. Deduced sequence of a malate synthase polypeptide encoded by a subclass of the gene family. *J. Biol. Chem.* 264:2778-2782.
- De Bellis, L., P. Picciarelli, L. Pistelli, and A. Alpi. 1990. Localization of glyoxylate-cycle marker enzymes in peroxisomes of senescent leaves and green cotyledons. *Planta*. 180:435-439.
- Diamond, L. S. 1957. The establishment of various trichomonads of animals and man in axenic cultures. *J. Parasitol.* 43:488-490.
- Finlay, B. J., and T. Fenchel. 1989. Hydrogenosomes in some anaerobic protozoa resemble mitochondria. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 65:311-314.
- Fung, K., and C. Clayton. 1991. Recognition of a peroxisomal tripeptide entry signal by the glycosomes of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 45:261-264.
- Garrard, L. J., and J. M. Goodman. 1989. Two genes encode the major membrane-associated protein of methanol-induced peroxisomes from *Candida boidinii*. *J. Biol. Chem.* 264:13929-13937.
- Gonzalez, E. 1986. Glycoproteins in the matrix of glyoxysomes in endosperm of castor bean seedling. *Plant Physiol.* 80:950-955.
- Gonzalez, E. 1990. Expression and localization of malate synthase during maturation and desiccation of castor bean seeds. *Protoplasma*. 154:53-58.
- Gould, S. J., G.-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108:1657-1664.
- Gould, S. J., G.-A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani. 1990a. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:85-90.
- Gould, S. J., S. Krisans, G.-A. Keller, and S. Subramani. 1990b. Antibodies directed against the peroxisomal targeting signal of firefly luciferase recognize multiple mammalian peroxisomal proteins. *J. Cell Biol.* 110:27-34.
- Johnson, P. J., C. E. D'Oliveira, T. E. Gorrel, and M. Müller. 1990. Molecular analysis of the hydrogenosomal ferredoxin of the anaerobic protist *Trichomonas vaginalis*. *Proc. Natl. Acad. Sci. USA*. 87:6097-6101.
- Hoch, H. C., and D. P. Maxwell. 1973. Proteinaceous hexagonal inclusions in hyphae of *Whetzelinia sclerotiorum* and *Neurospora crassa*. *Can. J. Microbiol.* 20:1029-1035.
- Keller, G.-A., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1984. An improved procedure for immunoelectron microscopy: ultrathin plastic embedding of immunolabeled frozen sections. *Proc. Natl. Acad. Sci. USA*. 81:5744-5747.
- Kionka, C., and W. H. Kunau. 1985. Inducible β -oxidation pathway in *Neurospora crassa*. *J. Bacteriol.* 161:153-157.
- Laemli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Ann. Rev. Cell Biol.* 1:489-540.
- Lewis, M. J., D. J. Sweet, and H. R. B. Pelham. 1990. The *ERD2* gene determines the specificity of the luminal ER protein retention signal. *Cell*. 61:1359-1363.
- Marchand, M., U. Kooystra, R. K. Wierenga, A. M. Lambeir, J. Van Beeumen, F. R. Opperdoes, and P. A. M. Michels. 1989. Glucosephosphate isomerase from *Trypanosoma brucei*. Cloning and characterization of the gene and analysis of the enzyme. *Eur. J. Biochem.* 184:455-464.
- Markham, P., and A. J. Collinge. 1987. Woronin bodies of filamentous fungi. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 46:1-11.
- Michels, P. A. M., A. Poliszczak, K. A. Osinga, O. Misset, J. Van Beeumen, R. K. Wierenga, P. Borst, and F. R. Opperdoes. 1986. Two tandemly linked identical genes code for the glycosomal glyceraldehyde phosphate dehydrogenase in *Trypanosoma brucei*. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1049-1056.
- Müller, M. 1980. The hydrogenosome. *Symp. Soc. Gen. Microbiol.* 30:127-142.
- Müller, M. 1988. Energy metabolism of protozoa without mitochondria. *Ann. Rev. Microbiol.* 42:465-488.
- Nuttley, W. M., J. D. Aitchison, and R. A. Rachubinski. 1988. cDNA cloning and primary structure determination of the peroxisomal trifunctional enzyme hydratase-dehydrogenase-epimerase from the yeast *Candida tropicalis* pK233. *Gene*. 69:171-180.
- Opperdoes, F. R. 1987. Compartmentalization of carbohydrate metabolism in trypanosomes. *Ann. Rev. Microbiol.* 41:127-151.
- Rhodin, J. 1954. Correlation of ultrastructural organization and function in normal and experimentally changed proximal and convoluted tubule cells of the mouse kidney. Ph.D. thesis, Aktiebolaget Godvil, Stockholm.
- Schopfer, D., D. Bajracharya, R. Bergfeld, and H. Falk. 1976. Phytochrome-mediated transformation of glyoxysomes into peroxisomes in the cotyledons of mustard (*Sinapis alba* L.) seedlings. *Planta*. 133:73-80.
- Schopfer, P., and K. Apel. 1983. Intracellular photomorphogenesis. In *Encyclopedia of Plant Physiology*, New Series. W. Schopshire, Jr. and H. Mohr, eds. Springer-Verlag, Berlin, Germany, pp. 258-288.
- Small, G. M., L. J. Szabo, and P. B. Lazarow. 1988. Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1167-1173.
- Smith, S. M., and C. J. Leaver. 1986. Glyoxysomal malate synthase of cucumber: Molecular cloning of a cDNA and regulation of the enzyme synthesis during germination. *Plant Physiol.* 81:762-767.
- Sommer, J. M., J. A. Thissen, M. Parsons, and C. C. Wang. 1990. Characterization of an in vitro assay for import of 3-phosphoglycerate kinase into the glycosomes of *Trypanosoma brucei*. *Mol. Cell. Biol.* 10:4545-4554.
- Swinkels, B. W., R. Evers, and P. Borst. 1988. The topogenic signal of the glycosomal (microbody) phosphoglycerate kinase of *Crithidia fasciculata* resides in a carboxy-terminal extension. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1159-1165.
- Titus, D. E., and W. M. Becker. 1985. Investigation of the glyoxysome-peroxisome transition in germinating cucumber cotyledons using double-label immunoelectron microscopy. *J. Cell Biol.* 101:1288-1299.
- Tokuyasu, K. T. 1973. A technique for ultramicrotomy of cell suspensions and tissues. *J. Cell Biol.* 57:551-565.
- Treleace, R. N., W. M. Becker, P. J. Gruber, and E. H. Newcomb. 1971. Microbodies (glyoxysomes and peroxisomes) in cucumber cotyledons. Correlative biochemical and ultrastructural study in light- and dark-grown seedlings. *Plant Physiol.* 48:461-475.

- Tschopp, J. F., P. F. Brust, J. M. Cregg, C. A. Stillman, and T. R. Gingeras. 1987. Expression of the *lacZ* gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res.* 15:3859-3876.
- Veenhuis, M., M. Mateblowski, W. H. Kunau, and W. Harder. 1987. Proliferation of microbodies in *Saccharomyces cerevisiae*. *Yeast.* 3:77-84.
- Vigil, E. L. 1970. Cytochemical and developmental changes in microbodies (glyoxysomes) and related organelles of castor bean endosperm. *J. Cell Biol.* 46:435-454.
- Volokita, M., and C. R. Somerville. 1987. The primary structure of spinach glycolate oxidase deduced from the DNA sequence of a cDNA clone. *J. Biol. Chem.* 262:15825-15828.
- Wanner, G., and R. R. Theimer. 1982. Two types of microbodies in *Neurospora crassa*. *Annu. New York Acad. Sci.* 386:269-284.
- Wierenga, R. K., B. M. Swinkels, P. A. Michels, K. Osinga, O. Misset, J. Van Beeumen, W. C. Gibson, J. P. Postma, P. Borst, and F. R. Opperdoes. 1987. Common elements on the surface of glycolytic enzymes from *Trypanosoma brucei* may serve as topogenic signals for import into glycosomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:215-221.