Antibodies Directed against the Peroxisomal Targeting Signal of Firefly Luciferase Recognize Multiple Mammalian Peroxisomal Proteins

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Abstract. We have previously shown that the peroxisomal targeting signal in firefly luciferase consists of the COOH-terminal three amino acids of the protein, serine-lysine-leucine (Gould, S. J., G.-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani, 1989. *J. Cell Biol.* 108:1657–1664). Antibodies were raised against a synthetic peptide that contained this tripeptide at its COOH terminus. Immunofluorescence and immunocryoelectron microscopy revealed that the anti-peptide antibodies specifically detected peroxisomes in mammalian cells. Further characterization revealed that the antibodies were primarily directed against the COOH-terminal three amino acids of the peptide. In Western blot experiments, the antibodies recognized 15–20 rat liver peroxisomal proteins, but reacted with only a few proteins from other subcellular compartments. These results provide independent immunological evidence that the peroxisomal targeting signal identified in firefly luciferase is present in many peroxisomal proteins.

PROTEIN transport into the peroxisome, ER, mitochondrion, or chloroplast requires the translocation of proteins across one or more biological membranes. The transport of proteins into these organelles is mediated by *cis*-acting targeting signals present within the imported proteins. Peroxisomal proteins have been shown to contain either a COOH-terminal peroxisomal targeting signal (PTS)[1] consisting of a tripeptide of conserved sequence (Gould et al., 1987, 1988, 1989; Miyazawa et al., 1989), or internal signals of undetermined nature (Small et al., 1987, 1988). There is no evidence that covalent modification of peroxisomal proteins occurs as a consequence of import (for a review of peroxisome biogenesis see Lazarow and Fujiki, 1985). In contrast, proteins that are transported into the ER, mitochondrion, or chloroplast generally contain nonconserved, NH_2-terminal targeting signals of variable length (usually 12–25 amino acids long) that are cleaved from the protein during or after import (for reviews see Walter and Lingappa, 1986; Douglas et al., 1986; Schmidt and Mishkind, 1986; Verner and Schatz, 1988).

We showed earlier that the minimal PTS in firefly luciferase is the COOH-terminal tripeptide serine-lysine-leucine. Mutagenesis of the PTS revealed that certain conservative amino acid changes could be made within the signal without altering its ability to act as a PTS. Specifically, the serine could be changed to alanine or cysteine and the lysine could be changed to arginine or histidine without affecting the activity of the targeting signal (Gould et al., 1989).

This report addresses the question of whether the type of PTS identified in firefly luciferase is a common feature of peroxisomal proteins. Antibodies were raised to a synthetic peptide containing one version of the minimal peroxisomal targeting signal (serine-lysine-leucine-COOH). Both immunofluorescence and immunocryoelectron microscopy were used to determine if the antibodies could differentiate between proteins of the peroxisomes and those of other cellular compartments. Western blot experiments were also performed to address the specificity of the antibodies for peroxisomal proteins and, in addition, to determine the diversity of peroxisomal proteins recognized by the antibodies.

Materials and Methods

Animals

Male Sprague-Dawley rats (170–220 g) were used in this study. Standard lab chow or standard lab chow supplemented with 0.5% gemfibrozil (wt/wt) was provided ad libitum for at least 10 d. Animals were fasted overnight before being sacrificed.

Peptides and Production of Antibodies

Synthetic peptides (peptide A, NH_2-CRYHLKPLQSKL-COOH; peptide B, NH_2-CRYHLKPLQ-COOH; peptide C, NH_2-CRYLAGPHGSKL-COOH; peptide D, NH_2-CRYLAGPHG-COOH) were obtained from the Agouron Institute (La Jolla, CA) both as free peptide and preconjugated to keyhole

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1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; PTS, peroxisomal targeting signal; SCP-2, sterol-carrier-protein-2.

limpet hemocyanin (KLH). The last nine amino acids of peptides A and C corresponded to the COOH-terminus of two peroxisomal proteins from rat, acyl-CoA oxidase and the hydratase-dehydrogenase bifunctional enzyme. Before injection with the peptide, each rabbit was bled to collect preimmune serum. A KLH conjugate of peptide A was injected into each of two rabbits. Approximately 8 wk after immunization, sera from the rabbits were tested by immunofluorescence and the serum from the animal with the highest titer was used for all subsequent experiments. Serum was used for the immunofluorescence experiments; for all others an IgG fraction was used.

Subcellular Fractionations

Liver homogenates were fractionated as described by Leighton et al. (1968) except that preinjection of rats with Triton WR-1339 was omitted and the peroxisome-enriched fraction was washed only once. The peroxisome-enriched fraction (which also contained lysosomes, mitochondria, and microsomes) was then further separated by equilibrium density centrifugation on a steep linear Metrizamide (20–25% [wt/wt]) or Nycodenz (10–60% [wt/wt]) gradient (Bronfman et al., 1979; Hajra and Bishop, 1982). This peroxisome isolation procedure was improved by the incorporation of a reverse sucrose gradient in the Nycodenz or Metrizamide gradients. Specifically, a 5% final concentration of sucrose was added to the 10% (wt/vol) Nycodenz solution or a 3.3% final concentration of sucrose to a 20% (wt/vol) Metrizamide solution at the time of gradient formation. Addition of sucrose creates an essentially iso-osmolar environment throughout the gradient. This technique results in the isolation of peroxisomes that more closely resemble their in situ appearance (as determined by electron microscopy) and also substantially increases the yield of intact peroxisomes. The gradient was centrifuged (model OTD 75B; Sorvall Instruments Div., Newton, CT) using an ultra-vertical TV 850 rotor at 40,000 rpm for 50 min at 8°C. A total of 20–25 fractions were collected from the bottom of the centrifuge tube with a two-way needle. The mitochondrial and peroxisomal fractions of greatest purity (95–98%) were used for immunoblotting. Rat liver microsomes were prepared by differential centrifugation (de Duve et al., 1955), resulting in a microsomal and soluble fraction. All cell fractions were assayed for protein content and distribution of marker enzymes.

Isolation of Membranes

Purified peroxisomal fractions (95–98% pure) prepared by equilibrium density centrifugation were treated with sodium carbonate as described by Fujiki et al. (1982). The membranes isolated by this technique retain integral membrane proteins and exhibit a normal trilaminar appearance.

Assay of Marker Enzymes

The activities of catalase (a peroxisomal marker) and cytochrome oxidase (a mitochondrial marker) were measured according to Leighton et al. (1968) and Lazarow and de Duve (1973). Esterase (a microsomal marker) was measured according to Beaufay et al. (1974). Protein was determined by the method of Lowry et al. (1951) using BSA as a standard. Since Metrizamide interferes with the determination of protein, aliquots of the gradient samples were first precipitated in 10% TCA.

SDS Gel Electrophoresis and Immunoblotting

Proteins were separated on 9% or 10% polyacrylamide slab gels containing 0.1% SDS, by the method of Laemmli (1970). Electrophoresis was performed as 35 mA/gel. Protein samples were prepared in solubilization buffer (0.0625 M Tris/HCl, pH 6.8, 2% SDS, 5% beta-mercaptoethanol, 5% glycerol, and 0.005% bromophenol blue) and boiled for 2 min before loading onto the gel. The separated proteins were electrophoretically transferred to nitrocellulose paper in 20 mM Tris, 150 mM glycine and 20% methanol. The nitrocellulose was incubated for 2 min in PBS with 2% Tween-20, followed by incubation for 2 h at room temperature with an IgG fraction of the anti-peptide antibodies. After several washes, the nitrocellulose was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 60 min.

Immunofluorescence Microscopy

CV-1 cells plated on coverslips were fixed with 3% formaldehyde in PBS (pH 7.2) for 15 min. They were then permeabilized with 1% Triton X-100 for 5 min, after which they were washed extensively with PBS. The rabbit anti-peptide serum was applied at a dilution of 1:200. The cells were washed extensively again and incubated with a rhodamine-conjugate of a goat anti-rabbit IgG antibody. The cells were washed with PBS and mounted as described (Keller et al., 1987). In the peptide competition experiments, the peptides were preincubated with the antibody for 10 min before application to the cells.

The plasmid pSV2CAT-PMP20 was used to demonstrate that the anti-SKL antibody recognized peroxisomes in immunofluorescence experiments. This plasmid contains the last 12 amino acids of the *C. boidinii* peroxisome membrane-associated protein, PMP20 (Garrard et al., 1989), fused to the COOH terminus of chloramphenicol acetyltransferase (CAT), a cytosolic protein. The fusion protein (CAT-PMP20), when expressed in CV1 cells from the SV-40 early promoter in pSV2CAT-PMP20, is targeted to peroxisomes as judged by its co-localization with catalase in double indirect immunofluorescence experiments (Gould, S. J., G. -A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani, manuscript submitted for publication). For the double-labeling experiments described in this paper, the plasmid pSV2CAT-PMP20 was transfected into CV-1 cells and the cells were fixed 2 d after transfection. Double indirect-immunofluorescence was carried out essentially as described (Gould et al., manuscript submitted for publication) except that the rabbit antibody was the anti-PTS antibody (at a 1:200 dilution), not an anti-catalase antibody. Fluorescence microscopy was performed on a Zeiss photoscope II microscope.

Immunocryoelectron Microscopy

Small blocks of liver from rats were chopped in 3% formaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and immersed in the same fixative for 1 h. After washing in 0.1 M phosphate buffer (pH 7.2), the blocks were infused with 2.3 M sucrose for 30 min. Ultrathin sections were obtained by cryoultramicrotomy, as described by Tokuyasu (1980). Immunolabeling was performed essentially as described (Keller et al., 1986) except that the primary antibody was the anti-PTS antibody (1:200 dilution of the IgG fraction). After washing, the sections were treated with colloidal gold (6–8 nm in diameter) adducts of guinea pig antibodies to rabbit IgG. Immunolabeled frozen sections were osmicated and poststained with 1% aqueous uranyl acetate, dehydrated in a series of ethanol solutions to 95% ethanol, and infused in LR white acrylic resin (Polysciences Inc., Warrington, PA).

Results

Characterization of Anti-Peptide Antibodies that Recognize the Minimal Peroxisomal Targeting Signal

Rabbits were injected with a 12-amino acid-long peptide (peptide A, NH_2-CRYHLKPLQSKL-COOH) ending at the COOH terminus in the amino acids serine-lysine-leucine (the minimal PTS). Antibodies obtained from immunized rabbits were then used in immunofluorescence experiments with CV-1 monkey kidney cells. As expected, incubation of CV-1 cells with preimmune serum resulted in nonspecific staining of the cell (Fig. 1 *a*). In contrast, the immune serum labeled punctate structures within the cells, the morphology and distribution of which are characteristic of peroxisomes (Fig. 1 *b*). To confirm that these vesicles were indeed peroxisomes, double-labeling experiments were performed. CV-1 cells expressing the fusion protein CAT-PMP20, a known peroxisomal protein (Gould, S. J., G.-A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani, manuscript submitted for publication), were processed for immunofluorescence microscopy using both the anti-peptide serum (rabbit) and an anti-CAT monoclonal antibody (mouse) that reacts with the CAT-PMP20 fusion protein. The subcellular distribution of proteins recognized by the anti-peptide serum was superimposable with the distribution of the peroxisomal CAT-PMP20 protein (Fig. 1, *c* and *d*). This result demonstrates that the

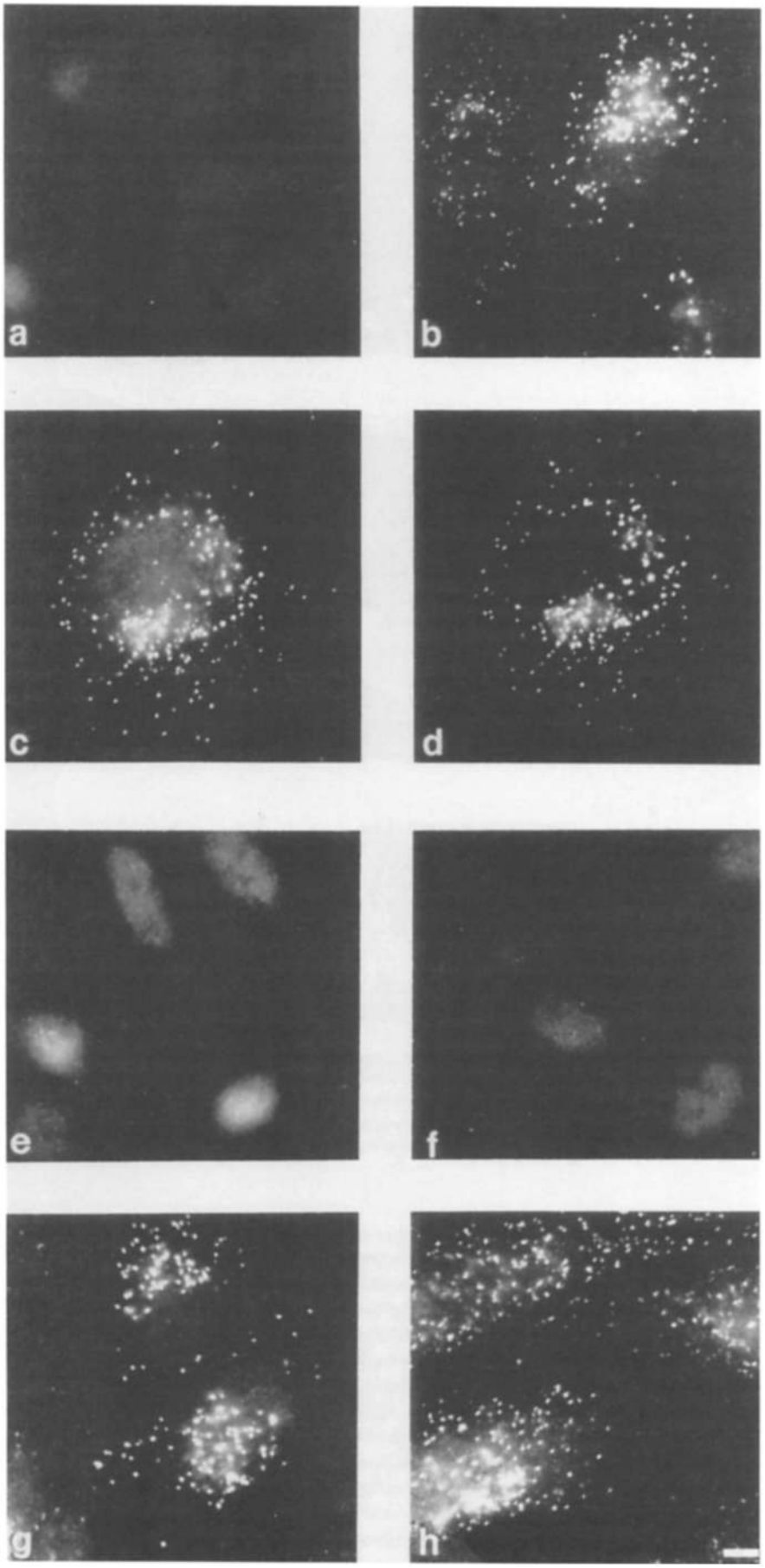

Figure 1. The anti-peptide antibody recognizes the tripeptide SKL and labels peroxisomes in indirect immunofluorescence experiments. CV-1 monkey kidney cells were used for indirect immunofluorescence using the following primary antibodies. (*a*) Preimmune rabbit antibody; (*b*, *c*, and *e–h*) rabbit anti-peptide antibody; and (*d*) mouse monoclonal against the CAT-PMP20 fusion protein which is peroxisomal (Gould, S. J., G.-A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani, manuscript submitted for publication). Secondary antibodies were rhodmaine-conjugated goat anti-rabbit antibody for all sections except for *d*, where fluorescein-conjugated goat anti-mouse IgG was used. Only the immune serum shows the punctate labeling pattern (compare *a* with *b*), which co-localizes with the distribution of a known peroxisomal protein (*c* and *d*). Competition experiments show that the anti-peptide antibody is directed against the minimal PTS (Ser-Lys-Leu-COOH). The primary anti-peptide antibody was competed with the following peptides before its use for indirect immunofluorescence. (*e*) Peptide A (CRYHLKPLQSKL), 2 μg/ml; (*f*) peptide C (CRYLAGPHGSKL), 2 μg/ml; (*g*) peptide B (CRYHLKPLQ), 200 μg/ml; (*h*) peptide D (CRYLAGPHG), 200 μg/ml. Competition for peroxisomal labeling (*e* and *f*) is seen only with peptides A and C which end in the tripeptide SKL, but not with peptides B and D which lack this PTS (*g* and *h*).

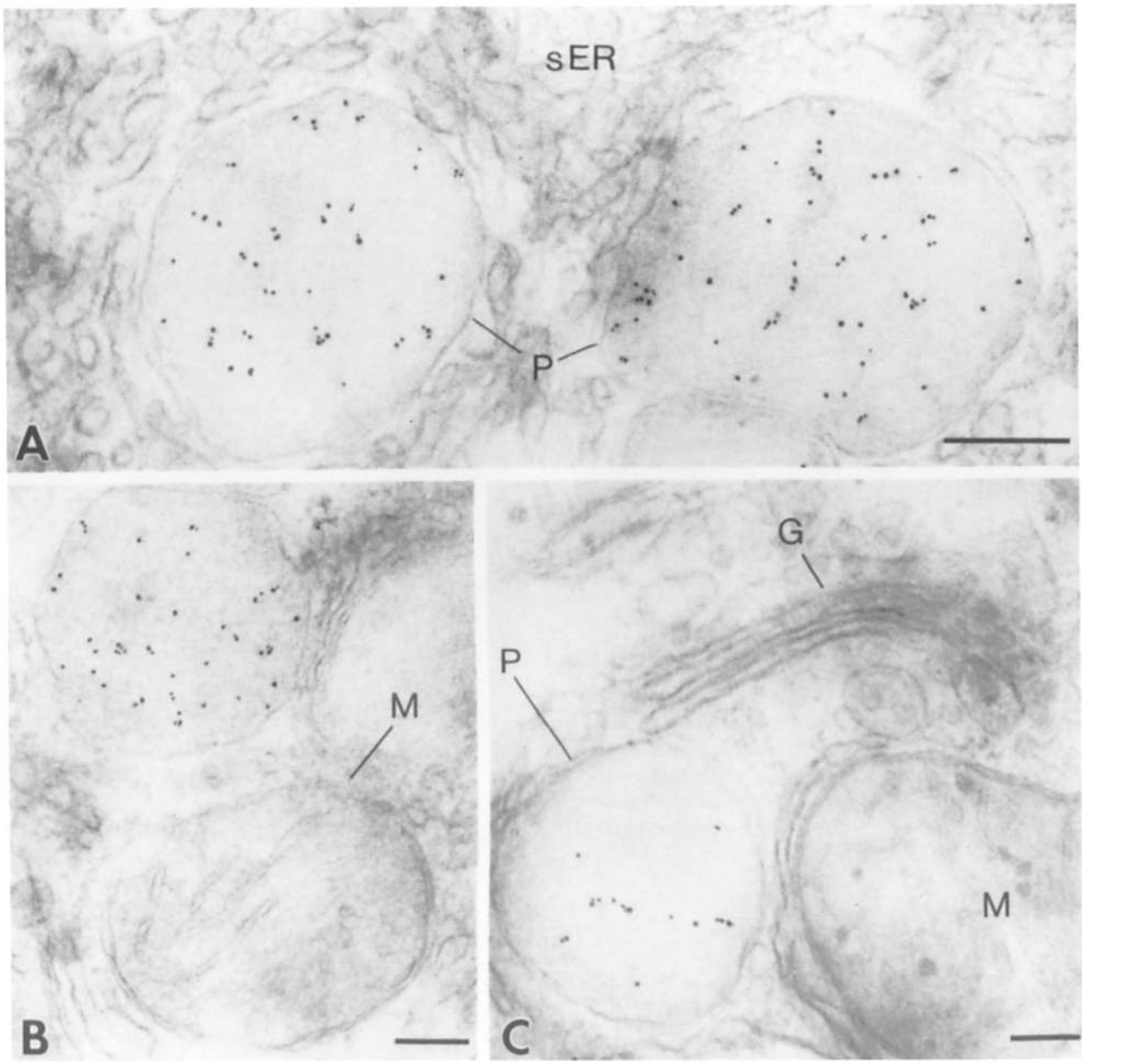

Figure 2. The anti-PTS antibodies recognize peroxisomes by immunocryoelectron microscopy. Rat liver sections were prepared for immunocryoelectron microscopy as described in Materials and Methods. Electron-dense gold particles represent the distribution of proteins in the cell recognized by the anti-PTS antibodies. The gold particles specifically decorate the peroxisomes of the cell and do not label other organelles such as the smooth ER (*A*), the mitochondria (*B* and *C*), or the Golgi apparatus (*C*).

vesicular structures recognized by the antiserum are peroxisomes.

Synthetic peptides were used in competition experiments to determine which amino acids of the peptide the anti-serum was directed against. Four different synthetic peptides were used as competitors: peptide A, the peptide used for immunization (NH_2-CRYHLKPLQSKL-COOH); peptide B, identical to peptide A except that it did not contain the COOH-terminal three amino acids (NH_2-CRYHLKPLQ-COOH); peptide C, which, like peptide A, ended in serine-lysine-leucine (NH_2-CRYLAGPHGSKL-COOH); and peptide D, identical to peptide C except that it did not contain the COOH-terminal serine-lysine-leucine (NH_2-CRYLAG-PHG-COOH). The anti-peptide serum was incubated separately with each peptide before use for immunofluorescent staining of CV-1 cells. Preincubation with peptides A or C at a concentration of 2 μg/ml completely abolished the ability of the serum to label peroxisomes (Fig. 1, *e* and *f*). However, incubation of the serum with peptides B or D at the same concentration (2 μg/ml), or even at 100-fold greater concentration (200 μg/ml), did not affect its ability to label peroxisomes (Fig. 1, *g* and *h*). The presence of the COOH-terminal tripeptide serine-lysine-leucine in peptides which did compete for antibody binding (A and C), was the only difference between them and peptides B and D, which did not. Thus, detection of peroxisomal proteins by the immune serum was mediated by its recognition of the sequence serine-lysine-leucine-COOH, the minimal PTS.

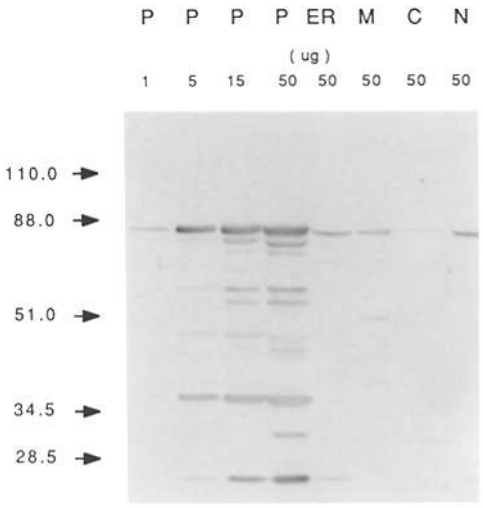

Figure 3. Western blot of subcellular fractions of rat liver cells using the anti-PTS antibodies. *P*, peroxisomes; *ER*, endoplasmic reticulum (microsomes); *M*, mitochondria; *C*, cytosol; *N*, nuclei. Numbers at the top of the gel refer to the micrograms of protein loaded in the lane below the numbers. The numbers and arrows at the left edge of the figure represent molecular mass markers (in kilodaltons). Note that the anti-PTS antibodies recognize many peroxisomal proteins but only a few proteins in other subcellular fractions.

Anti-PTS Antibodies Recognize Peroxisomes by Electron Microscopy

Immunocryoelectron microscopy experiments provided additional confirmation that the anti-PTS antibodies specifically detected peroxisomal proteins. Portions of rat livers were frozen, sectioned, and incubated with anti-PTS antibodies. Secondary antibodies (conjugated with gold particles) were used to visualize the subcellular distribution of proteins recognized by the antibodies. As evidenced by the distribution of electron-dense gold particles, the antibodies labeled the peroxisomes of the cells (Fig. 2). The specificity of this antibody for peroxisomal proteins was demonstrated by the fact that neither the ER nor other organelles were labeled above background levels (Fig. 2). Significantly, the antibody recognized proteins in the peroxisome matrix but no labeling of the peroxisome membranes was observed.

Anti-PTS Antibodies Recognize Multiple Peroxisomal Proteins

The specificity of the anti-PTS antibodies was also tested in Western blot experiments. Purified peroxisomal, ER (microsomal), mitochondrial, cytosolic, and nuclear proteins from rat liver cells were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with the anti-PTS antibody. The antibodies recognized at least 16 proteins present in the purified peroxisomal fraction (Fig. 3; *P*, 50 μg) but reacted with only a small number of proteins present in the ER (*ER*, 50 μg), mitochondrion (*M*, 50 μg), cytosol (*C*, 50 μg), or nucleus (*N*, 50 μg). The two bands present in all lanes (molecular masses of ~80 and 26 kD, respectively) were due to unavoidable contamination of nonperoxisomal fractions with peroxisomes (~1–4% contamination; compare the lanes of *ER* [50 μg], *M* [50 μg], *C* [50 μg], and *N* [50 μg] with lane *P* [1 μg]). Nevertheless, the serum did react weakly with a few proteins unique to ER, mitochondrial, cytosolic, or nuclear fractions that could not be explained by peroxisomal contamination.

The reactivity of the anti-PTS antibody towards peroxisomal membrane proteins was determined by additional Western blot experiments. Purified peroxisomes from rat livers were subjected to high pH (11.5) sodium carbonate treatment to strip all nonintegral proteins from the peroxisome membrane (Fujiki et al., 1982). Soluble and membrane fractions of peroxisomes were then used in Western blot experiments. The three lanes on the left side of Fig. 4 are Western blot profiles of whole (*W*), soluble (*S*), and membrane (*M*) proteins from purified peroxisomes. We used 50 μg of whole peroxisomal proteins, 45 μg of soluble proteins, and 5 μg of membrane proteins in this experiment to mimic the approximate content of soluble and membrane proteins in the peroxisomes. The protein composition of the membrane fraction (as determined by analysis of Coomassie-stained protein gels) closely matched that of previous reports (Fujiki et al., 1982) and, as expected, differed significantly from that of the soluble fraction. It is apparent that most proteins recognized by the antibodies were from the soluble fraction. The antibodies recognized several proteins

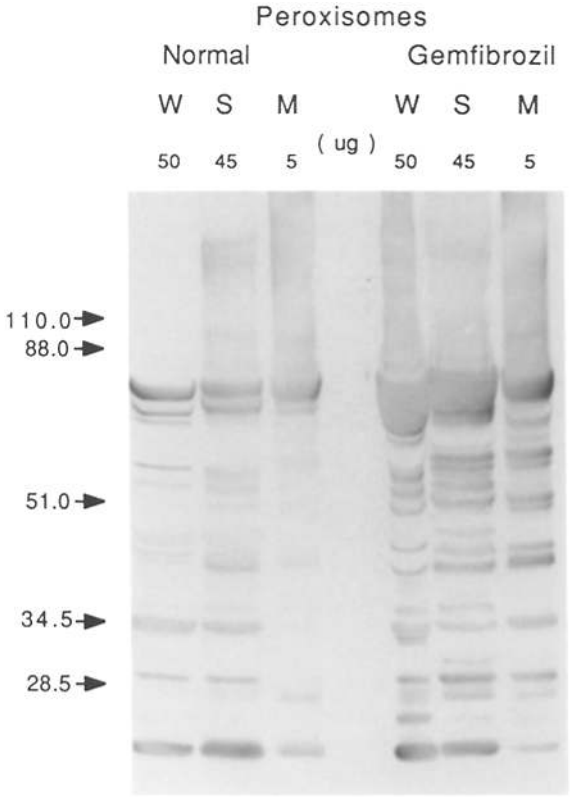

Figure 4. Western blot of whole (*W*), soluble (*S*), and membrane (*M*) fractions of rat liver peroxisomes using the anti-PTS antibodies. Peroxisomes were obtained from either normal rats (left three lanes) or from rats fed a diet containing the hypolipidemic drug Gemfibrozil (right three lanes). Numbers at the top refer to the amount of protein loaded in each lane (in micrograms). The numbers and arrows at the left edge of the figure represent molecular mass markers (in kilodaltons).

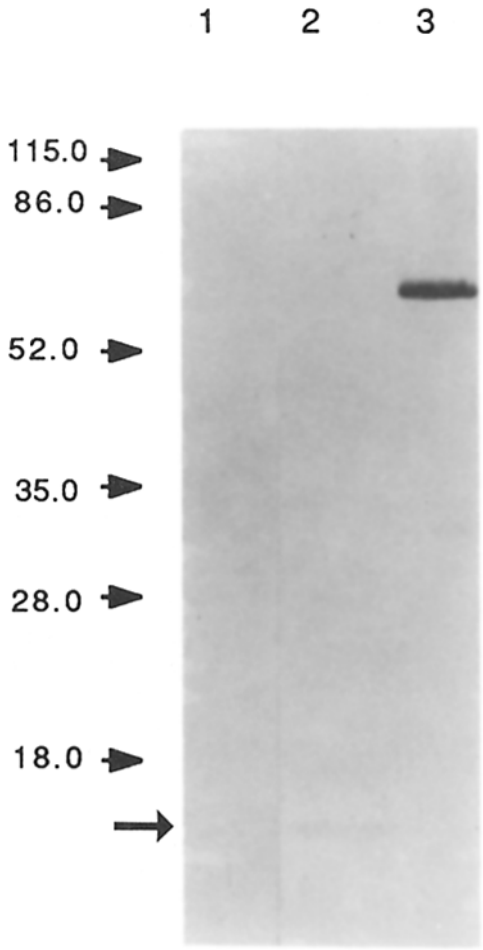

Figure 5. The anti-PTS antibody recognizes other pure peroxisomal proteins known to contain the PTS. Western blot of purified catalase, SCP-2, and luciferase using the anti-PTS antibody. The antibody (which was made against the COOH-terminal peptide sequence of a different peroxisomal protein, acyl CoA oxidase) does not detect catalase (lane *1*), but does detect SCP-2 (lane *2*, 13.5 kD, see *arrow*), and luciferase (lane *3*, 60 kD). The arrows and numbers at the left edge of the figure represent molecular mass markers (in kilodaltons).

in the membrane fraction, but most appeared to be residual soluble proteins remaining in the membrane preparation. This was surprising considering that the overall protein composition of the soluble and membrane fractions were dramatically different on Coomassie-stained gels. The fact that only trace amounts of soluble peroxisomal proteins are sufficient to give a strong signal on Western blots of peroxisomal membrane fractions indicates that the affinity of the antibodies for these proteins is quite high. Some unique bands were present in the membrane fraction, but none corresponded to any previously identified peroxisomal membrane proteins. Also, some of these bands were not visible in blots of total peroxisomal proteins, indicating that they may have been degradation products. These results extend those obtained by immunocryoelectron microscopy and indicate that the anti-PTS antibodies recognize many soluble peroxisomal proteins, but few, if any, peroxisomal membrane proteins.

We repeated these experiments with liver peroxisomes from rats that had been fed the hypolipidemic drug gemfibrozil. This drug causes a marked increase in the number of the peroxisomes of liver cells (Gray and de la Iglesia, 1984). Concomitant with this increase in peroxisome number there is an increase in the amount of peroxisomal proteins within the cell and a change in the relative abundance of several peroxisomal proteins. Comparison of total rat liver proteins detected by the antibodies from normal (Fig. 4, lane *W* on left) and drug-fed (lane *W* on right) rats revealed that the antibodies recognized additional proteins in drug-fed rats. This result is consistent with previous reports on the peroxisome-inducing effects of hypolipidemic drugs (Lalwani et al., 1983). Though proteins comprising the membrane and soluble fractions were quite different on Coomassie-stained gels, the patterns of bands on the Western blots in each fraction were again almost identical.

We also tested whether the anti-PTS antibodies could detect known peroxisomal proteins, in this case luciferase, sterol-carrier-protein-2 (SCP-2) or catalase. Luciferase contains the tripeptide PTS serine-lysine-leucine at its COOH terminus, and SCP-2, recently shown to be peroxisomal in rat liver cells (Keller et al., 1989), ends in a form of the PTS, alanine-lysine-leucine (Morris et al., 1988). However, purified catalase lacks its COOH-terminal 20–30 amino acids due to proteolysis (Furuta et al., 1986) and its PTS (possibly the tripeptide serine-histidine-leucine at positions 516–518 of the 526 amino acid protein) is contained within that region of the protein (Gould et al., 1988). Both luciferase and SCP-2 contain the tripeptide PTS and were detected by the anti-PTS antibodies (Fig. 5, lanes *2* and *3*, respectively) whereas catalase, the protein lacking the consensus PTS sequence, was not (Fig. 5, lane *1*).

Discussion

We previously identified the minimal PTS in firefly luciferase and showed that it consists of the COOH-terminal tripeptide serine-lysine-leucine (Gould et al., 1989). An important question raised by this earlier observation was whether other peroxisomal proteins also contained a similar type of targeting signal. The type of PTS identified in luciferase is present in several of the peroxisomal proteins for which sequences are available (Gould et al., 1988, 1989). Data in this paper provide independent immunological evidence that the PTS is indeed conserved in many mammalian peroxisomal proteins.

Immunofluorescence and immunocryoelectron microscopy experiments demonstrated that immune serum raised against a peptide ending in the PTS specifically detected peroxisomes, presumably because it was directed against the PTS and thus recognized many peroxisomal proteins. Two sets of experiments demonstrated that the antibodies were directed against the COOH-terminal three amino acids of the peptide. First, two different peptides containing the COOH-terminal PTS sequence competed efficiently for labeling of peroxisomes by anti-serum (in immunofluorescence experiments) whereas the same peptides lacking serine-lysine-leucine at their COOH termini were unable to compete. Second, Western blots of purified peroxisomal proteins (luciferase, SCP-2, catalase) revealed that the antibodies only recognized proteins that contained the PTS sequence (luciferase and SCP-2). It should be noted that the peptide used as antigen contained the same COOH-terminal nine amino acids as rat acyl-CoA oxidase. The ability of the anti-peptide antibody to recognize firefly luciferase and rat SCP-2, as well as many unidentified peroxisomal proteins, supports our conclusion

that the antibodies are directed against the PTS, the feature common to each protein.

The specificity of the antibodies for the sequence serine-lysine-leucine-COOH was not altogether unexpected. Before injection into rabbits, the antigenic peptide (NH_2-CRYHLK-PLQSKL-COOH) was coupled via its NH_2-terminal cysteine residue to the carrier protein keyhole limpet hemocyanin. Thus, the COOH-terminal portion of the peptide should have been more exposed and accessible than the NH_2-terminal portion of the peptide. As a result, it was expected that resulting antibodies would more likely be directed against the COOH terminus of the peptide.

Western blots of purified peroxisomal proteins (probed with the antibodies) revealed that at least 16 different peroxisomal proteins could be detected by the antibodies. In contrast, only a few proteins were detected by the antibodies in the ER, mitochondrial, cytosolic, or nuclear fractions and the signal observed in these cases was quite weak. Assuming that the proteins detected on the Western blots were sufficiently abundant to be visible on the Coomassie-stained gel of peroxisomal proteins (all but a few of the reactive bands did correspond to a band on the Coomassie-stained gel), ~44% of the 39 peroxisomal proteins on the gels were recognized by the antibody. The large number of peroxisomal proteins detected by the anti-PTS antibodies demonstrates that the type of PTS identified in luciferase is a feature of many peroxisomal proteins.

There are several plausible explanations for why the anti-PTS antibodies failed to detect all peroxisomal proteins. Two possibilities for which examples already exist are (*a*) that some proteins may undergo proteolysis, lose the tripeptide PTS, and no longer be detected by the antibodies, and (*b*) that certain peroxisomal proteins may contain a different type of PTS not related to the tripeptide PTS of luciferase. It has been reported that purified catalase commonly lacks its COOH-terminal 20 amino acids (Furuta et al., 1986). Because peroxisomal targeting activity has been identified within the COOH-terminal 27 amino acids of this protein (Gould et al., 1988), it is likely that mature catalase has lost its PTS. As for the second possibility, Small et al. (1988) have identified two regions of the yeast (*Candida tropicalis*) acyl-CoA oxidase that bear no relation to the PTS identified in luciferase yet appear to act as peroxisomal targeting signals.

Another important possibility is that the anti-PTS antibodies used in this report may, in fact, recognize only a subset of the forms of the tripeptide PTS. As mentioned earlier, we have demonstrated that several conservative amino acid substitutions within the PTS of luciferase had no discernible effect on peroxisomal targeting (serine changed to alanine or cysteine; lysine changed to arginine or histidine; Gould et al., 1989). The anti-PTS antibodies described in this paper were raised against a peptide that contained only one of the nine possible permutations of the PTS, serine-lysine-leucine. It is possible that antibodies directed against different forms of the tripeptide PTS would recognize a different subset of peroxisomal proteins. We are currently attempting to raise antibodies that recognize each of the nine known permutations of the tripeptide PTS.

Additional experiments revealed that the anti-PTS antibody detected soluble peroxisomal proteins very well but recognized peroxisomal membrane proteins poorly, if at all. Though a few bands were observed only on blots of peroxisomal membrane proteins, none corresponded to any membrane proteins visible on the Coomassie-stained gel. In contrast, most soluble peroxisomal proteins detected by the antibodies corresponded to proteins on the Coomassie-stained gels. Furthermore, the immunoelectron microscopy experiments with the anti-PTS antibody revealed labeling of the peroxisome matrix and not of the membrane. These observations raise the interesting possibility that peroxisomal membrane proteins may use a different type of PTS from that of soluble peroxisomal proteins. If true, the use of different targeting signals by membrane and soluble peroxisomal proteins might explain certain results obtained in the study of cells from human patients with Zellweger's Syndrome, a genetic disorder resulting in peroxisomal abnormalities. Specifically, cells from these patients appear to be incapable of importing peroxisomal matrix proteins into the organelle, but are still able to assemble certain peroxisomal membrane proteins into peroxisome-like "ghosts" (Suzuki et al., 1987; Santos et al., 1988*a*,*b*). Since sequences are not yet available for mammalian peroxisomal membrane proteins, we do not know whether they contain SKL or some other peroxisomal membrane targeting signal.

The anti-PTS antibodies we have raised should be useful for a number of purposes. We have described their application as a general probe for detection of many peroxisomal proteins. Thus, they should be of use as general markers for peroxisomes and peroxisomal proteins. Preliminary experiments already show that they do indeed recognize peroxisomes from diverse species. The antibodies may also be capable of identifying clones encoding peroxisomal proteins in cDNA expression libraries and be applicable as a diagnostic tool for detecting disorders that affect peroxisomal proteins, such as Zellweger's syndrome. Perhaps the most interesting possibility is the potential use of the antibodies as antigens for production of anti-idiotypic antibodies. Anti-idiotypic antibodies raised against anti-PTS antibodies may be capable of detecting the putative receptor for the PTS. A similar strategy using anti-idiotypic antibodies has already been exploited by Pain et al. (1988) to identify a receptor for the chloroplast targeting signal. Considering that no information is available regarding the components of the peroxisomal translocation machinery, identification of a receptor for the PTS would be an important step towards elucidation of the mechanism of protein transport into the peroxisome.

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Note Added in Proof: The recognition of all except for two of the peroxisomal proteins by the anti-PTS antibody in Western blots was abolished by preincubation of the antibody with a peptide ending in the sequence SKL (peptide A) but not by a peptide lacking the SKL sequence (peptide B), demonstrating that the antibody does indeed recognize the PTS in many peroxisomal proteins.

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