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Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells

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ABSTRACT **The intracellular localization of Cu,Zn superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) has been examined by immunofluorescence using four monoclonal anti-Cu,Zn superoxide dismutase antibodies raised against a recombinant human Cu,Zn superoxide dismutase derivative produced and purified from *Escherichia coli*. Colocalization with catalase, a peroxisomal matrix enzyme, was used to demonstrate the peroxisomal localization of Cu,Zn superoxide dismutase in human fibroblasts and hepatoma cells. In the fibroblasts of Zellweger syndrome patients, the enzyme is not transported to the peroxisomal ghosts but, like catalase, remains in the cytoplasm. In addition, immunocryoelectron microscopy of yeast cells expressing human Cu,Zn superoxide dismutase showed that the enzyme is translocated to the peroxisomes.**

Superoxide dismutases (SODs; superoxide:superoxide oxidoreductase, EC 1.15.1.1) are a family of enzymes unusual in that their substrate is an unstable and toxic free radical produced as a by-product of oxidative metabolism. These enzymes protect cells by catalyzing the dismutation of the superoxide radical to O_2 and H_2O_2 (1). In mammalian cells, three forms of SOD—Mn SOD, Cu,Zn SOD, and extracellular Cu,Zn SOD—have been identified. They are encoded by three separate genes and differ in their amino acid sequences and their localization (2–4). The human Cu,Zn SOD is a dimer of identical subunits, each consisting of 153 amino acids and containing one Cu and one Zn ion. Although usually described as a cytoplasmic enzyme, the subcellular localization of Cu,Zn SOD has been ascribed to various organelles. This view is based in part on cell fractionation experiments, in which the enzyme activity was found predominantly in the cytosol but also in the fraction corresponding to the mitochondrial intermembrane space (5) or with the lysosomal fraction (6). More recent investigations involving immunolocalization of the enzyme with a polyclonal antibody have reported the presence of Cu,Zn SOD in the cytoplasmic matrix, the nucleus, and the lysosomes of rat liver hepatocytes (7).

We suspected that Cu,Zn SOD might be a peroxisomal enzyme for the following reasons: (*i*) xanthine oxidase, which has recently been shown to be localized within peroxisomes (8, 9), generates toxic superoxide radicals as a reaction by-product, (*ii*) H_2O_2, a toxic product of superoxide dismutation, is removed by catalase, which is uniquely located in peroxisomes in mammals, (*iii*) catalase may require protection from superoxide radicals, which have been shown to inactivate it (10), and (*iv*) Cu,Zn SOD contains a tripeptide Ser-Arg-Leu close to the C terminus that might act as a peroxisomal targeting signal. We have therefore reexamined the cellular localization of the enzyme both in mammalian cells and in yeast expressing the human cDNA.

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MATERIAL AND METHODS

Monoclonal Antibodies. BALB/c mice were immunized with a Cu,Zn SOD/p31 fusion protein produced and purified from *Escherichia coli* (11). Spleen cells were fused with the mouse myeloma cell line P3x63 Ag.653 (12) and cells were plated in microtiter plates. Wells with cell growth were screened for SOD-specific antibodies by ELISA. The four monoclonal antibodies used in this study react with both native and denatured human Cu,Zn SOD and are designated CZSODF2, CZSODA3, CZSODA6, and CZSODA7. Antibody was purified from ascites fluid by ammonium sulfate precipitation followed by DEAE-Sepharose chromatography (13).

Yeast Strains and Culture Conditions. *Saccharomyces cerevisiae* strain P017 [*Mata Leu2* (*cir°*)] (14) was transformed with plasmid pC1/1 PGAPHSODC6A,C111S (15) encoding expression of a thermostable human Cu,Zn SOD that has normal structure and activity (16). The pC1/1 PGAPHSODC6A, C111S shuttle vector contains the 2-μm yeast plasmid, pBR322, the defective yeast *Leu2* gene, and the human Cu,Zn SOD cDNA under the transcriptional control of the promoter for the yeast glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (15). Colonies were selected on plates lacking leucine, inoculated into 5-ml cultures of minimal dextrose medium lacking leucine, and grown to stationary phase at 30°C with aeration (17). Cultures (2 ml) were pelleted by centrifugation, washed once with oleic acid medium, and resuspended in 25 ml of oleic acid medium and grown 24 hr at 30°C with aeration. Sterile filtered oleic acid medium contained the following: yeast nitrogen base (6.7 g/liter) (Difco), yeast extract (0.5 g/liter) (Difco), oleic acid (1 ml/liter) (Sigma), Tween 80 (0.5 ml/liter) (Sigma), and 1 M $CuSO_4$ (100 ml/liter) (Cu,Zn SOD-producing strains).

Immunofluorescence Microscopy. Indirect immunofluorescence of Hep-G2 cells and fibroblasts from normal and Zellweger patients (obtained from D. Applegarth, British Columbia Children's Hospital) was performed as described (18). Primary monoclonal antibodies to human Cu,Zn SOD and rabbit antibodies to human catalase were used at 50 and 10 μg/ml, respectively. A monoclonal antibody to human Mn SOD (19), instead of the monoclonal antibodies to Cu,Zn SOD, was used as a control antibody. The secondary antibodies were rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG. Cells were photographed in the same plane of focus with a Leitz Aristoplan microscope using the appropriate filters for rhodamine and fluorescein.

Immunocryoelectron Microscopy. Cryoultramicrotomy, immunolabeling of cryosections, and thin plastic embedding of yeast cells were performed as described (20). The characterization of the rabbit antibody against the peroxisome targeting signal has been described elsewhere (21). The immunolabeled sections were observed without poststaining in a Philips CM12 transmission electron microscope equipped with a 10-μm-diameter objective aperture.

Abbreviation: SOD, superoxide dismutase.

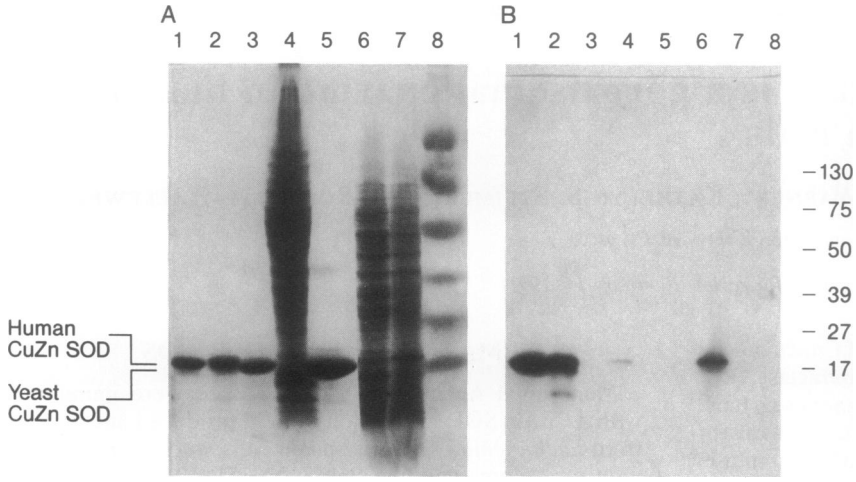

FIG. 1. Reactivity of monoclonal antibody CZSODF2 with purified Cu,Zn SODs and cell lysates. (*A*) Coomassie blue-stained SDS/polyacrylamide gel. Lanes: 1, 5 μg of recombinant human Cu,Zn SOD; 2, 3 μg of Cu,Zn SOD from human erythrocytes; 3, 5 μg of *S. cerevisiae* Cu,Zn SOD; 4, Hep-G2 cell homogenate; 5, 20 μg of *S. cerevisiae* Cu,Zn SOD; 6, cell lysate of yeast strain PO17, no plasmid; 7, cell lysate of yeast strain PO17 expressing plasmid-encoded human Cu,Zn SOD; 8, molecular weight markers ($M_r \times 10^{-3}$). (*B*) Immunoblots, with the monoclonal antibody against human Cu,Zn SOD, of the same protein samples as in *A* transferred to nitrocellulose paper. Staining was by the alkaline phosphatase-conjugated second antibody (goat anti-mouse IgG) method (33).

Peroxisomal Localization of Cu,Zn SOD in Human Hepatoma Cells. The specificity of purified monoclonal antibody CZSODF2 was first determined by Western blot (22). Fig. 1 shows that this monoclonal antibody specifically recognizes a M_r 19,000 protein band that comigrates with purified human Cu,Zn SOD (lanes 1 and 2) and with a protein from a Hep-G2 cell homogenate (lane 4). Double immunolabeling experiments with monoclonal antibody CZSODF2 against human Cu,Zn

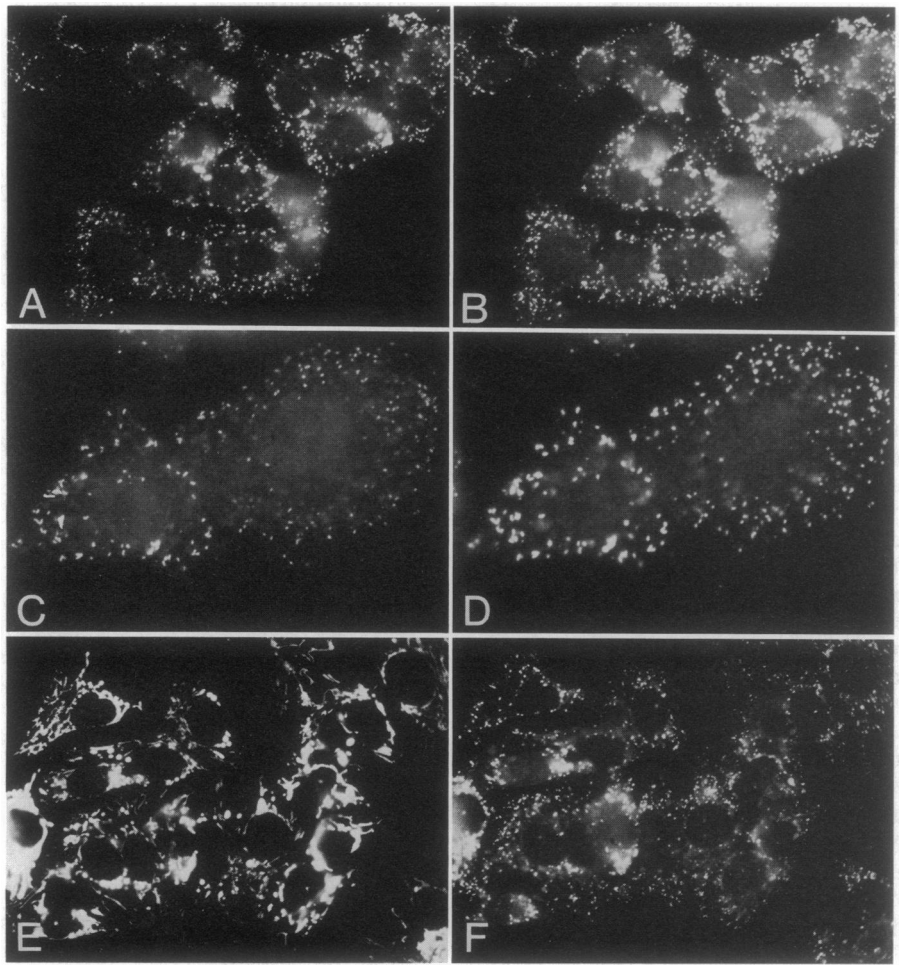

FIG. 2. (*A–D*) Peroxisomal localization of human Cu,Zn SOD by double immunofluorescence. Hep-G2 cells were simultaneously labeled for Cu,Zn SOD and catalase (*A–D*) and for Mn SOD (*E*) and catalase (*F*). The secondary antibodies were rhodamine-conjugated goat anti-rabbit IgG (for catalase) and fluorescein-conjugated goat anti-mouse IgG (for Cu,Zn and Mn SODs). (*A* and *B*) Field of Hep-G2 cells at low magnification. The punctate pattern obtained with the antibody against catalase, which marks the location of the peroxisomes, is superimposable to that of Cu,Zn SOD. The codistribution of Cu,Zn SOD (*C*) with catalase (*D*) can be better appreciated at a higher magnification. Cu,Zn SOD is detected only in the peroxisomes. The nucleus and the other cytoplasmic organelles are not labeled. A control experiment was performed with a monoclonal antibody against Mn SOD (*E*) and the rabbit anti-human catalase antibody (*F*). The anti-Mn SOD antibody labels the mitochondria intensely. Note that the distribution of Mn SOD is quite different from that obtained with the anti-Cu,Zn SOD antibody and is not superimposable on the distribution of catalase. (*A*, *B*, *E*, and *F*, ×490; *C* and *D*, ×1450.)

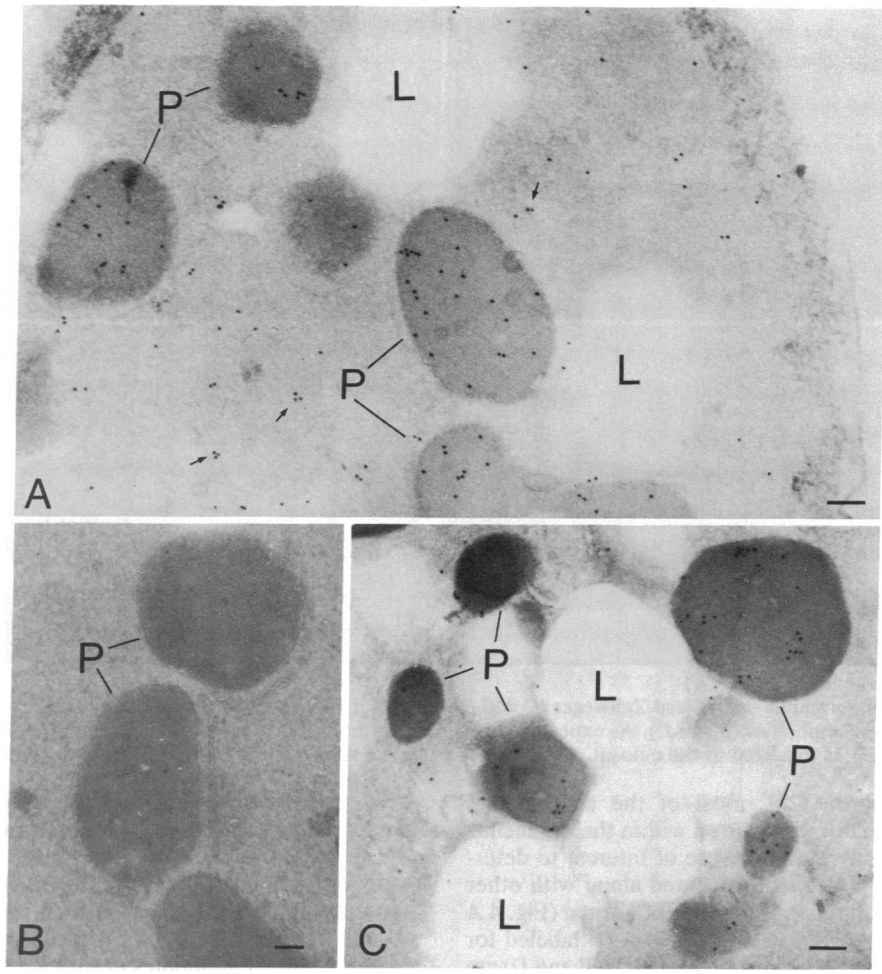

FIG. 3. Immunocryoelectron microscopy of yeast showing the peroxisomal localization of human Cu,Zn SOD in transformed cells. (*A*) *S. cerevisiae* cells transformed with a plasmid encoding expression of human Cu,Zn SOD were grown on a medium containing oleic acid to induce peroxisome proliferation. Human Cu,Zn SOD is localized mainly within membrane-bound organelles although the enzyme can also be seen in the cytoplasm (arrows). Cytoplasmic labeling may represent the site of enzyme synthesis and protein transport to the peroxisomes. (*B*) Control cells without the plasmid expressing human Cu,Zn SOD grown on oleic acid medium. The membrane-bound organelles are not labeled for human Cu,Zn SOD. (*C*) Same cells as in *A* but immunolabeled with a rabbit antibody raised against the peroxisome targeting signal (Ser-Lys-Leu at the C terminus) (21, 26). L, lipid droplet; P, peroxisomes. (Bars = 0.1 μm.)

SOD and a rabbit anti-human catalase antibody were carried out on Hep-G2 cells. At low magnification, the immunolabeling pattern for Cu,Zn SOD (Fig. 2*A*) is indistinguishable from that for catalase (Fig. 2*B*). Careful examination of the immunofluorescence patterns at higher magnification reveals that the vesicular structures labeled by the anti-Cu,Zn SOD and anti-catalase antibodies are superimposable (Fig. 2 *C* and *D*). Since catalase is a classical peroxisomal marker, these results indicate that Cu,Zn SOD is predominantly concentrated within the peroxisomes in human hepatoma cells. In addition, a monoclonal antibody against human Mn SOD (19) was substituted in the same immunolabeling protocol. The anti-Mn SOD monoclonal antibody gave the typical vermiculate immunolabeling pattern of the mitochondrial compartment (Fig. 2*E*), which is totally different from the punctate pattern characteristic of peroxisome labeling (Fig. 2*F*). This shows that the peroxisomal localization of Cu,Zn SOD was not due to cross-reactivity of the secondary antibody (the fluorescein-labeled goat anti-mouse IgG) with a peroxisomal protein. All four anti-human Cu,Zn SOD monoclonal antibodies labeled the peroxisomes in single immunolabeling experiments (data not shown).

Human Cu,Zn SOD Is Targeted to the Peroxisomes of the Yeast *S. cerevisiae*. To ensure that the results obtained with Hep-G2 cells were not due to cross-reactivity of the anti-Cu,Zn SOD monoclonal antibodies with an epitope(s) present on a peroxisomal protein unrelated to Cu,Zn SOD, we used transformed yeast cells expressing the human enzyme (15). Expression of the human enzyme in transformed, but not in control, cells was confirmed by Western blot analysis (Fig. 1, lanes 6 and 7). The antibodies to the human protein did not recognize yeast Cu,Zn SOD (lanes 3 and 5). *S. cerevisiae* contains few visible peroxisomes when grown under normal conditions (24), but peroxisomes multiply upon growth on oleic acid (25). Immunocryoelectron microscopy of yeast cells grown in oleic acid medium showed that the human enzyme was concentrated in the matrix of small subcellular organelles surrounded by a single membrane (Fig. 3*A*). Morphologically similar organelles were detected in the cytoplasm of wild-type cells but, as expected, they were not labeled with the anti-human Cu,Zn SOD antibody (Fig. 3*B*). Immunolabeling with antibodies to the peroxisomal targeting signal confirmed that the organelles containing the human enzyme were indeed peroxisomes (Fig. 3*C*). These antibodies recognize multiple peroxisomal proteins in mammalian cells and eukaryotic microorganisms including *S. cerevisiae* (21, 26).

Cu,Zn SOD Is Cytoplasmic in the Fibroblasts of Zellweger Syndrome Patients. Zellweger syndrome is due to an autosomal recessive mutation that causes incomplete assembly of peroxisomes (27). Although the peroxisomal membranes are

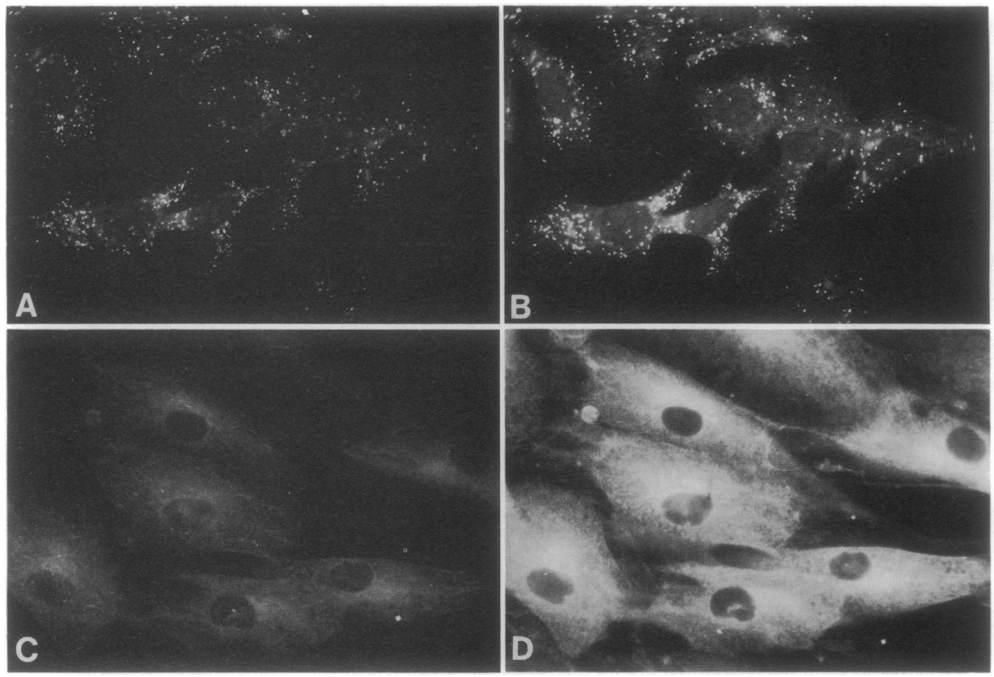

FIG. 4. Fibroblasts of normal (*A* and *B*) and Zellweger (*C* and *D*) patients immunolabeled with the anti-Cu,Zn SOD monoclonal antibody (*A* and *C*) and anti-catalase antibodies (*B* and *D*). As expected on the basis of the deficient peroxisome assembly in Zellweger patients, Cu,Zn SOD (*C*), like catalase (*D*), is localized to the cytosol, whereas it is peroxisomal in normal fibroblasts (*A* and *B*). (×490.)

present in the cytoplasm (28), most of the peroxisomal enzymes that are normally transported within the organelles remain in the cytosol. It was therefore of interest to determine whether Cu,Zn SOD was mislocated along with other peroxisomal matrix proteins. Fibroblasts of normal (Fig. 4 *A* and *B*) and Zellweger patients (Fig. 4 *C* and *D*) labeled for Cu,Zn SOD (Fig. 4 *A* and *C*) and catalase (Fig. 4 *B* and *D*) are shown in Fig. 4. The immunolabeling shows that Cu,Zn SOD is also a peroxisomal enzyme in normal human fibroblasts. In contrast, both enzymes were localized throughout the cytoplasm in Zellweger fibroblasts.

DISCUSSION

We have demonstrated the peroxisomal localization of Cu,Zn SOD by showing that the enzyme (*i*) colocalized with catalase in the peroxisomes of Hep-G2 cells and normal fibroblasts, (*ii*) is targeted to peroxisomes in *S. cerevisiae* transformants expressing human Cu,Zn SOD but is not present in control cells, and (*iii*) is localized with catalase in the cytoplasm of fibroblasts from Zellweger patients.

Cu,Zn SOD is widely described as a cytoplasmic enzyme, although the published literature regarding the subcellular localization of the enzyme in mammalian cells is somewhat contradictory. Geller and Winge (6) presented data indicating that the enzyme cosegregated with lysosomal enzymes in cell fractionation experiments. Indeed, our own experiments using conventional cell fractionation showed that the majority of the enzyme activity was associated with the fraction containing the lysosomes (data not shown). It is well documented that portions of several peroxisomal matrix proteins are routinely found in other cell fractions, even when using the most moderate methods of cell disruption (29). We suspect that Cu,Zn SOD is released from peroxisomes under the conditions used for isolation and purification of the organelles. More recently, using an immunoelectron microscopy approach, Chang *et al.* (7) found Cu,Zn SOD in virtually all subcellular compartments of rat hepatocytes, with the exception of the endoplasmic reticulum and the Golgi apparatus. Although the lysosomes contained the highest concentration, most of the enzyme was located in the cytoplasmic and nuclear matrices. One important difference between these studies and our own is that we have used monoclonal antibodies raised against a recombinant protein rather than a polyclonal antibody raised against the protein purified from tissue. Unlike polyclonal antibodies, monoclonal antibodies cannot contain antibodies against proteins contaminating the immunizing antigen preparation. The multiple intracellular localizations of the enzyme observed by Chang *et al.* (7) in liver cells may be related to the nature of their polyclonal antibody preparation.

Because our results conflict with the published literature regarding the localization of Cu,Zn SOD (5–7), we were careful not to rely on a single experimental cell type and to include extensive controls in our experiments. The most important of the control experiments was the use of yeast cells expressing or not expressing human Cu,Zn SOD. Since the difference between these two yeast strains is limited to human Cu,Zn SOD and just a few other plasmid-encoded proteins, the localization of human Cu,Zn SOD to peroxisomes and the absence of any signal in wild-type cells is almost conclusive evidence for a peroxisomal localization of the enzyme. The localization of Cu,Zn SOD in peroxisomes also makes considerable biological sense, for the reasons listed at the end of the Introduction. Conversely, the multiple intracellular localizations of Cu,Zn SOD reported in the literature require the presence of multiple targeting signals in the sequence of the protein to sort it to such diverse cellular compartments. This is inconsistent with what is currently known about targeting and translocation of proteins into the various cellular organelles.

One major class of targeting signal identified in peroxisomal proteins consists of a conserved tripeptide (Ser, Ala, or Cys at the first position; Lys, His, or Arg at the second position; Leu at the third position) (30). Usually situated at the extreme C terminus of several peroxisomal proteins, the tripeptide can be identified at internal locations in others. The C-terminal portion of human catalase that can act as a peroxisomal targeting signal contains the tripeptide Ser-His-Leu 10 amino acids from the C terminus (31). Therefore, we

plan to determine whether the enzyme is sorted to the peroxisomes by the tripeptide Ser-Arg-Leu at amino acid positions 142–144, 9 residues before the C terminus, or by another as yet unidentified signal. The targeting and transport of the human enzyme into the peroxisomes of *S. cerevisiae* transformants is consistent with recent findings showing that the sorting of peroxisomal proteins has been conserved throughout eukaryotic evolution (21, 26).

What are the biological implications of a peroxisomal localization for Cu,Zn SOD? First, an important function of peroxisomes appears to be antioxidant defense by removal of H_2O_2 and superoxide. By analogy, the mitochondrion, which also generates superoxide radicals, contains Mn SOD. Thus, it seems evident that compartmentalization of these processes results in containment of potentially damaging forms of oxygen. Second, Mn SOD and Cu,Zn SOD are the only two intracellular SODs, which suggests that the majority of the SOD activity resides in the mitochondria and the peroxisomes. We therefore suspect that in addition to electron transfer proteins in mitochondria (31) and xanthine oxidase in peroxisomes (8, 9), other enzymes that produce superoxide will be identified in these organelles. Conversely, it should be advantageous to confine proteins that are rapidly inactivated by superoxide (23, 32) to the cytoplasm. Both immunofluorescence and immunocryoelectron microscopy indicate that a fraction of Cu,Zn SOD is present in the cytoplasm (Fig. 2*E* and Fig. 4 *A* and *C*). Therefore, it seems probable that there is also some SOD activity in the cytoplasm due to SODs in transit to the mitochondria or peroxisomes (see Fig. 3*A*).

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