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Peroxisomal Membrane Ghosts in Zellweger Syndrome—Aberrant Organelle Assembly

M. J. SANTOS, T. IMANAKA, H. SHIO, G. M. SMALL, P. B. LAZAROW

Peroxisomes are apparently missing in Zellweger syndrome; nevertheless, some of the integral membrane proteins of the organelle are present. Their distribution was studied by immunofluorescence microscopy. In control fibroblasts, peroxisomes appeared as small dots. In Zellweger fibroblasts, the peroxisomal membrane proteins were located in unusual empty membrane structures of larger size. These results suggest that the primary defect in this disease may be in the mechanism for import of matrix proteins.

ZELLWEGER SYNDROME IS A DISEASE in which an entire organelle, the peroxisome, appears to be missing, as first recognized by Goldfischer *et al.* (*1*). The peroxisome is nearly ubiquitous in eukaryotic cells and functions in fatty acid β-oxidation, plasmalogen biosynthesis, cellular respiration (H_2O_2-forming), gluconeogenesis, bile acid synthesis, and purine catabolism (*2*). This human genetic disorder, characterized by profound neurological impairment, metabolic disturbance, and neonatal death, has taught us much about peroxisome function (*3*) and promises to teach us more about peroxisome assembly. Some peroxisomal proteins are synthesized normally in Zellweger syndrome (*4*), but they are not assembled into peroxisomes. Many of these proteins are rapidly degraded, with the result that important soluble matrix enzymes (catalyzing β-oxidation) (*4*) and membrane-bound enzymes (catalyzing the initial steps in plasmalogen biosynthesis) (*4, 5*) are missing or seriously deficient, thus causing severe metabolic abnormalities (*3*). On the other hand, some peroxisomal enzymes (for example, catalase) are present in normal amounts in Zellweger cells but are located free in the cytosol (*4, 6, 7*).

These defects correlate with the known facts of peroxisome biogenesis. All peroxisomal proteins investigated thus far are synthesized on free polyribosomes and are assembled posttranslationally into preexisting peroxisomes (*8*). If the organelle is missing (*1*), newly made proteins will just diffuse through the cytosol, unable to enter a peroxisome. Under these circumstances, it is not surprising that many are degraded.

The autosomal recessive genetics of Zellweger syndrome indicates that a single mutation is responsible for the defects. One explanation would be that the mutation prevents the assembly of the peroxisomal membrane. In this case peroxisomal integral membrane proteins (PxIMPs) (*9*) should be absent, since those that have been studied are made on free polyribosomes (*10–12*) and are unlikely to be stable if not integrated into a membrane. Two other possibilities were suggested by the unexpected finding that several PxIMPs are present in normal amounts in Zellweger liver (*13*). The peroxisomal membranes could be assembled in Zellweger syndrome, but the import of matrix proteins is defective. This would result in empty (or nearly empty) membrane ghosts, which would not be recognizable by electron microscopy or cytochemistry. Alternatively, the PxIMPs, in the absence of peroxisomes, might have violated the rules of protein sorting and inserted into the wrong intracellular membrane(s).

To differentiate among these possibilities, we analyzed fibroblasts with a polyspecific antiserum against rat liver PxIMPs (polyspecific anti-PxIMPs) (*12*) (Fig. 1A, lane 1). This serum detected three human PxIMPs with masses of approximately 140, 69, and 53 kD in control cells (Fig. 1A, lanes 2 to 4). These PxIMPs were also present in Zellweger fibroblasts (lane 5), and they cosedimented in equilibrium density centrifugation of Zellweger fibroblast homogenates. However, the PxIMPs were found at an abnormally low density of 1.10 g/cm^3 (instead of the usual fibroblast peroxisome density of 1.17 g/cm^3)[lanes 6 and 7; further details in (*14*)]. Several other cell membranes also sedimented in the low-density region of the gradient where the PxIMPs were found (*14*). Therefore, the fractionation data are consistent with the possibility that the PxIMPs are present in aberrant peroxisomal membrane ghosts, but they do not exclude erroneous localization in lysosomes, the endoplasmic reticulum, or some other low-density organelle. Immunofluorescence studies were carried out to resolve this uncertainty.

The Rockefeller University, New York, NY 10021.

Because peroxisomes have not been previously detected by immunofluorescence in fibroblasts, we first established their normal immunofluorescence pattern with an antiserum against catalase (Fig. 1C), which is the characteristic marker enzyme for the organelle. Peroxisomes appeared as many small fluorescent dots scattered throughout the cytoplasm in control fibroblasts (Fig. 2A). A similar pattern was observed with the polyspecific antiserum against PxIMPs (Fig. 2B) and with affinity-purified antibodies against the 69-kD IMP (Fig. 2C). Nothing was seen with the preimmune serum (Fig. 2D).

This peroxisomal immunofluorescence pattern was distinctively different from that of other organelles: the juxtanuclear Golgi apparatus (Fig. 3A), the lacy reticular network of endoplasmic reticulum (Fig. 3B), mitochondria (which were noticeably larger) (Fig. 3C), and lysosomes (which were extremely variable in size, number, and distribution among the normal fibroblasts) (Fig. 3, D and E).

We next applied these tools to Zellweger syndrome fibroblasts. The fluorescence patterns for lysosomes, mitochondria, Golgi apparatus, and endoplasmic reticulum were similar to those in control cells (Fig. 3). Therefore the Zellweger mutation does not affect the general cell architecture. However, the fluorescence patterns with antisera against peroxisomal proteins were markedly different. The polyspecific anti-PxIMPs (Fig. 2F) revealed structures that were fewer in number but considerably larger in size than normal peroxisomes. They were not restricted to any one region of the cytoplasm but often occurred in clusters near the nucleus. Their number varied in different cells. They were heterogeneous in size, a few of them being as small as normal peroxisomes. The affinity-purified anti-69IMP demonstrated a similar pattern and furthermore showed a ring-like fluorescence appearance, characteristic of a membrane localization (Fig. 2G). These large structures did not show immunofluorescence with the anti-catalase (Fig. 2E).

What are these unusual structures bearing the PxIMPs in Zellweger fibroblasts? These structures were reproducibly observed in Zellweger fibroblasts that had been fixed and permeabilized in various ways (Fig. 2), as well as in fibroblasts from another Zellweger patient (GM 228, not shown). The PxIMPs were not found randomly distributed among the cell membranes, which was a possible outcome in cells thought to lack peroxisomes. The fluorescence patterns demonstrated that these PxIMPs are absent from the plasma membrane, the nuclear membranes, the endoplasmic reticulum, and the Golgi apparatus.

Comparison of the fluorescence pattern of PxIMPs in Zellweger cells (Fig. 2, F and G), with the patterns of mitochondria and lysosomes (Fig. 3), shows some similarity, such that we could not exclude, on the basis of these results alone, the possibility that the PxIMPs might have been mislocalized into these organelles. However, the equilibrium density of Zellweger PxIMPs (1.10 g/cm^3) is much less than the density of mitochondria (1.14 g/cm^3) (*14*), and for this reason the PxIMPs cannot be in mitochondria.

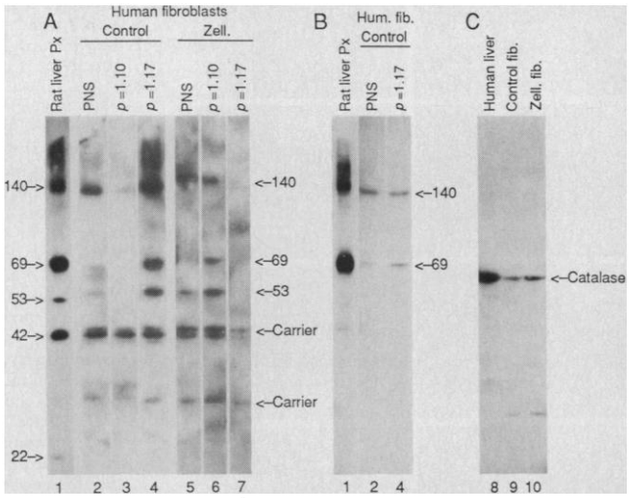

Fig. 1. Immunoblots of peroxisomal proteins in human fibroblasts. (**A**) Polyspecific antiserum detecting the 140-, 69-, and 53-kD PxIMPs. Fibroblast postnuclear supernatants (PNS) were fractionated in Nycodenz gradients (*14*). Membranes were prepared by the carbonate procedure (*19*) with yeast mitochondria as carrier (*14*) from 400 μg of PNS protein (lanes 2 and 5), from the peak fraction of normal peroxisomes (density 1.17 g/cm^3, lane 4), and from a fraction of density 1.10 g/cm^3 that contains microsomes, lysosomes, and other organelles (lanes 3 and 6). Membranes were likewise prepared from the 1.17 g/cm^3 density fraction of the Zellweger fibroblast gradient (lane 7), although no peroxisomal enzymes were detected there. Membranes from 10 μg of purified rat liver peroxisomes were included as a control (lane 1). Immunoblotting was performed (*13*) with antiserum 328 against rat liver PxIMPs (*12*), five of which are visible in lane 1 (masses in kilodaltons). These are integral membrane proteins by the criterion of not being extracted with carbonate (*19*). The human PxIMPs have slightly different mobilities than the corresponding rat proteins. Detection of the PxIMPs in the fibroblast PNS was somewhat variable, while in purified peroxisomes they were reproducibly observed. Lanes 1 to 4 and 5 to 7 are from different gels. There was some nonspecific binding to yeast mitochondrial proteins ("carrier") that was also seen with the carrier alone (not shown). (**B**) Affinity-purified antibodies against the 69-kD PxIMP. Sample preparation as in (A). Specific antibodies also recognized the 140-kD PxIMP (likely a dimer) and the 42-kD rat PxIMP (lane 1) but no other peroxisomal proteins (*12*). (**C**) Anti-catalase. Homogenates of human liver (50 μg of protein, lane 8) and fibroblasts (100 μg). Rabbit antiserum 330 was raised against bovine liver catalase in a fashion similar to that described (*10*).

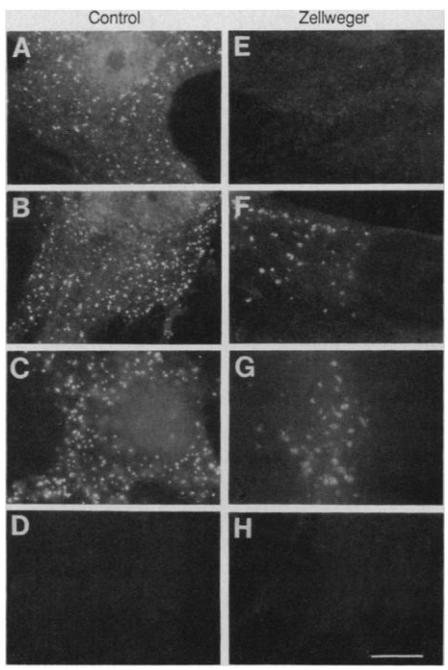

Fig. 2. Immunofluorescence localization of peroxisomal proteins in control (IMR-90) and Zellweger (GM 4340) fibroblasts. (**A** and **E**) Anti-catalase. (**B** and **F**) Anti-PxIMPs. (**C** and **G**) Affinity-purified anti-69IMP. (**D** and **H**) Preimmune serum. Fibroblasts (Mutant Cell Repository) were grown (*7*) on cover slips and were fixed with 4% paraformaldehyde in 0.1*M* cacodylate buffer (*p*H 7.4). They were washed with 100 m*M* phosphate (*p*H 7.4) and 0.15*M* NaCl [phosphate-buffered saline (PBS)], permeabilized with 100% methanol, blocked with 1% bovine serum albumin in PBS, and incubated with antisera for 2 hours. After washing, they were incubated with fluorescein isothiocyanate (FITC)–conjugated to goat anti-rabbit immunoglobulin G (IgG) (Boehringer Mannheim) for 45 minutes, washed, and mounted in 90% glycerol in 100 m*M* tris-HCl (*p*H 9). For (C) and (G), the cells were fixed for 30 minutes in 2% paraformaldehyde, 75 m*M* lysine, and 10 m*M* sodium periodate (PLP fixative) (*20*), washed, and permeabilized with 0.05% saponin in 0.1% albumin-PBS. Nikon Labophot epifluorescence microscopy. Bar, 10 μm. Similar fluorescence patterns were observed with different fixation (4% paraformaldehyde, PLP, 0.05% glutaraldehyde, or 100% methanol) and permeabilization (100% methanol, 0.05% saponin, or 1% Triton X-100) conditions.

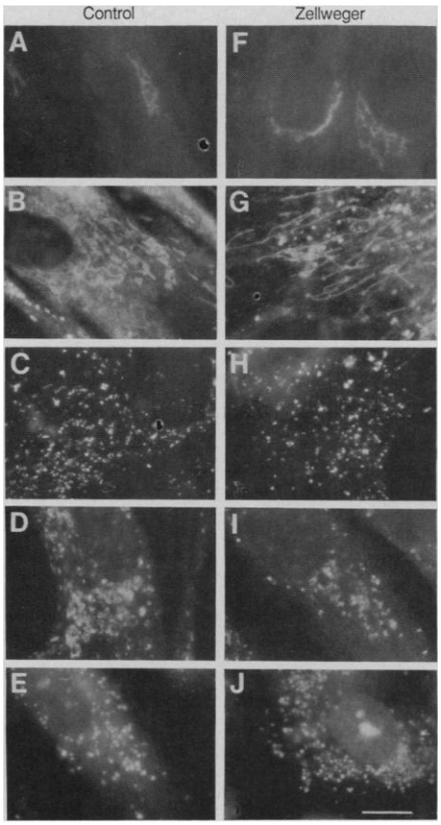

Fig. 3. Fluorescence visualization of organelles in control and Zellweger fibroblasts. (**A** and **F**) Golgi apparatus immunofluorescence with an antiserum against a Golgi IMP (*21*). (**B** and **G**) Endoplasmic reticulum fluorescence with 3,3′-dihexyloxacarbocyanine iodide (*22*). (**C** and **H**) Mitochondrial immunofluorescence with an antiserum against human mitochondrial α-propionyl-coenzyme A carboxylase (*23*). (**D** and **I**) Lysosomal immunofluorescence with an antiserum against a lysosomal IMP (*24*). (**E** and **J**) Lysosomes stained in vivo with acridine orange (*25*). The cells were fixed with PLP (A) and (F) or 4% paraformaldehyde (B to D) and (G to I). Bar, 10 μm.

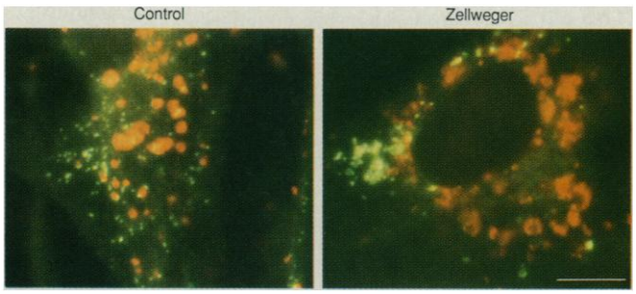

Fig. 4. Simultaneous localization of peroxisomal and lysosomal antigens with double immunofluorescence. Control and Zellweger fibroblasts were fixed with PLP and incubated with a mixture of antisera; rabbit anti-PxIMPs and mouse monoclonal antibodies against lysosomes (*26*). They were subsequently incubated with a mixture of labeled antisera: FITC (green) conjugated to goat anti-rabbit IgG and rhodamine (red) conjugated to goat anti-mouse IgG. Bar, 10 μm.

Localization in lysosomes was excluded by double immunofluorescence, which demonstrated that the PxIMPs were mainly in structures other than lysosomes (Fig. 4). These results do not exclude the possible presence of a small amount of PxIMPs inside lysosomes, consistent with the known autophagic turnover of peroxisomes (*15*) and the report by Arias *et al.* (*16*) that Zellweger fibroblasts contained a few residual peroxisomes, many of which appeared to be within phagolysosomes.

These results demonstrate the presence of abnormal peroxisomal membranes in Zellweger syndrome fibroblasts. These membranes can have little, if any, content because previous investigations demonstrated that peroxisomal matrix proteins are either deficient or found in the cell sap (*1, 3–7*). This is confirmed here by a different method, the immunofluorescence localization of catalase, which was not seen in particles in the Zellweger cells (Fig. 2E) (*17*). Thus Zellweger fibroblasts contain peroxisomal ghosts.

This result suggests that the primary defect in Zellweger syndrome is an inability to import proteins into peroxisomes. The defect could be in the membrane translocation machinery or in a cytosolic factor. Specific possibilities are the putative membrane receptor (*12*) or the protein that utilizes adenosine triphosphate during import (*18*).

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