Import of microinjected proteins bearing the SKL peroxisomal targeting sequence into the peroxisomes of a human fibroblast cell line: evidence that virtually all peroxisomes are import-competent

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SUMMARY

Peroxisomes import virtually all of their membrane and matrix proteins post-translationally. It is presently unknown whether, in mammalian cells, their exits a pool of mature peroxisomes which have received their complement of proteins and are import-incompetent. Previous work has shown that fibroblasts are capable of importing microinjected peroxisomal proteins into peroxisomes. This report describes the import of a hybrid peroxisomal protein into virtually all peroxisomes of the microinjected cell. The peroxisomal import was uniform in both short and long incubations. Pretreatment of the cells with cycloheximide did not affect the import of the peroxisomal protein, nor was there any difference in the distribution of the imported protein. Sequential microinjection experiments demonstrated that peroxisomes that had imported luciferase were capable of importing another peroxisomal protein injected 24 hours later. These results suggest that, in fibroblasts, all peroxisomes have associated protein-import machinery; this evidence does not support the hypothesis that there exists a pool of import-incompetent peroxisomes.

Key words: peroxisomal biogenesis, peroxisomal targeting signal, protein translocation

INTRODUCTION

Peroxisomes are single-membrane-bound organelles found in almost all eukaryotic cells. These organelles contain enzymes of plasmalogen synthesis, sterol synthesis, fatty acid β-oxidation, and nitrogenous waste processing among many others (Tolbert, 1981; Van den Bosch, 1992). Classically defined as containing both a hydrogen peroxide-producing oxidase and catalase; peroxisomes are thought to provide an isolated environment in which the hydrogen peroxide, produced in a number of biological oxidations, can be eliminated by catalase before it can cause oxidative damage to other cellular components (Lazarow and Fujiki, 1985). The protein components of the peroxisomal membrane and matrix are synthesized on cytoplasmic ribosomes and imported into the organelle by post-translational mechanisms (Fujiki et al., 1984; Goldman and Blobel, 1978; Miura et al., 1984; Rachubinski et al., 1984; Robbi and Lazarow, 1978; Roggenkamp et al., 1984). It is widely believed that new peroxisomes, required in times of cellular growth, are formed from pre-existing peroxisomes. It is presently unknown whether the proliferation of the peroxisomes in mammalian cells occurs by budding and fission of pre-existing peroxisomes (Lazarow and Fujiki, 1985), or by the formation of a ‘peroxisome reticulum’ (Lazarow et al., 1980). Both of these methods of proliferation imply that there may exist a pool of import-competent peroxisomes and a pool of mature peroxisomes which may be import-negative. Initial experiments, carried out to address this question, found no difference in enzyme distribution between different peroxisomes based upon size or density (Leighton et al., 1969; Poole et al., 1970; Lazarow and De Duve, 1973). Indeed, the early studies found no evidence of a maturing of the peroxisome, over time spans of one week (Poole et al., 1970). However, recent studies have begun to demonstrate heterogeneity among peroxisomes. Cytochemical and immunocytochemical techniques have shown heterogeneous distribution of the peroxisomal oxidases of the regenerating rat liver (Le Her and Dubach, 1980; Angermüller and Fahimi, 1986, 1988; Baumgart et al., 1989; Roels and Cornelis, 1989). Serial electron microscopic sections of regenerating rat liver have shown the formation of a peroxisomal reticulum at early times and the subsequent return of single peroxisomes (Yamamoto and Fahimi, 1987a,b). While cytochemical staining demonstrated a uniform distribution for catalase, D-amino acid oxidase had a heterogeneous distribution within the peroxisomal reticulum. In addition, isolation of peroxisomes on density gradients has demonstrated pools of peroxisomes with different enzyme compositions (Flatmark et al., 1980; Goglia et al., 1989; Klucis et al., 1991; Heinemann and Just, 1992; Luers et al., 1993). In hepatocytes growing in tissue culture, peroxisomes isolated by density gradient centrifugation demonstrate two populations of differing density (Heinemann and Just, 1992). Pulse-chase experiments indicate that newly
synthesized acyl-CoA oxidase appears predominantly in the peroxisomes with the lower density, and is subsequently found in the higher density peroxisomes. Similar results have been reported for peroxisomes isolated from rat liver (Luers et al., 1993). In this study, the average diameter of the low density peroxisomes was less than that of the high density peroxisomes. The average diameter of both populations decreased in peroxisomes isolated from animals subjected to partial hepatectomy; this change is presumably a result of the peroxisomal proliferation induced by partial hepatectomy.

The previous studies did not directly resolve the question of whether their are two populations of peroxisomes in mammalian cells: a population capable of import, and perhaps a population of mature peroxisomes that are no longer import-competent. In the present study, we have made use of a microinjection-based assay of peroxisomal import in human cells to assess whether there exists a population of import-incompetent peroxisomes.

RESULTS

Microinjected bHSA-SKL is imported into virtually all peroxisomes

In order to determine whether there existed a population of peroxisomes that were incapable of importing proteins with the SKL peroxisomal targeting signal, the hybrid peroxisomal protein bHSA-SKL was microinjected into human fibroblasts and incubated for 16 hours. After this incubation, the cells were fixed and stained to assess the intracellular distribution of the bHSA-SKL, compared to that in the endogenous peroxisomes, as indicated by the presence of catalase. Confocal sections of microinjected cells indicated that virtually every peroxisome as defined by the presence of catalase (Fig. 1b,e) had imported bHSA-SKL (Fig. 1a,d) during the 16 hour incubation period. False-colour overlays of the two images showed that, in injected cells, the peroxisomes appeared yellow, indicating the colocalization of bHSA-SKL and catalase (Fig. 1c,f). In microinjected cells, few peroxisomes did not contain sufficient bHSA-SKL to render them visible by immunofluorescence. In uninjected cells, the peroxisomes appeared green in the absence of bHSA-SKL. Control experiments for the staining of bHSA-SKL and catalase (Fig. 2) indicated that the levels of non-specific staining and signal cross-over between the FITC and Texas Red channels were very low.

It seemed plausible that the homogeneous import of bHSA-SKL may have been due to the 16 hour incubation period; by this time the majority of the microinjected bHSA-SKL has been imported into peroxisomes. During this extended time, the peroxisomes may have had time to equilibrate and mask any heterogeneous distribution. To investigate whether this was the case, a shorter time point at four hours after microinjection was examined. Double-label immunofluorescence images of these injected cells demonstrated that, even at short time points, virtually all of the peroxisomes as defined by the presence of catalase (Fig. 3b,e) contained bHSA-SKL (Fig. 3a,d). False-colour overlays of the two images show that the peroxisomes have yellow cores, indicating the colocalization of bHSA-SKL and catalase (Fig. 3c,f). The false-colour overlays of the peroxisomes at this early time point were not as yellow as those images from later time points, presumably due to the relatively smaller amounts of bHSA-SKL imported.

Effects of cycloheximide on the peroxisomal import of bHSA-SKL

In order to determine whether the synthesis of new proteins was required for the import of bHSA-SKL, Hs68 cells were treated with cycloheximide (100 µg/ml) for 30 minutes prior to microinjection and during the subsequent 16 hour incubation. This level of cycloheximide has previously been shown to abolish the synthesis of heat-shock proteins in Hs68 cells following heat-shock (not shown). In order to assess whether treatment with cycloheximide limited the ability of the cells to import microinjected peroxisomal proteins, the cells were not digitonin-permeabilized prior to fixation and staining. The bHSA-SKL that remained unimported at the end of the incubation can be seen as diffuse cytoplasmic staining. The results (Fig. 4) indicate that the import of bHSA-SKL into peroxisomes was not noticeably limited or altered by the absence of

MATERIALS AND METHODS

Reagents

Biotinylated human serum albumin containing the peroxisomal targeting signal serine-lysine-leucine (bHSA-SKL) was prepared as described previously (Walton et al., 1994). Firefly luciferase was purchased from Sigma Chemical (St Louis, MO). Antibodies directed against human catalase were obtained from Calbiochem (La Jolla, CA) (this antibody also recognizes an unknown nucleolar antigen). Antibodies directed against firefly luciferase were as described previously (Walton et al., 1992a). Streptavidin-conjugated Texas Red was purchased from Amersham (Oakville, ON). Fluorescently conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Other reagents were purchased from the standard sources.

Cell culture

The human fibroblast cell line, Hs68, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in calf serum (Gibco). For microinjection, cells were plated on acid-washed glass coverslips.

Microinjection and immunofluorescence microscopy

Cells were microinjected using glass capillary needles as previously described (Walton et al., 1992a). Following microinjection, the cells were incubated for the indicated times at 37 °C. Where indicated in the figure legends, the cells were first permeabilized with digitonin (25 µg/ml) for 10 minutes, followed by fixation and staining. This treatment results in the permeabilization of the plasma membrane while leaving the peroxisomal membrane intact (Wolvetang et al., 1990; Walton et al., 1992a,b). Cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 in PBS and immunostained as previously described (Walton et al., 1992a), except that the biotinylated-HSA-SKL was detected using streptavidin-conjugated Texas Red. Fluorescence confocal microscopy was performed with a Zeiss LSM 420 using a 63× (1.3 NA) lens. Identical x,y,z-axis optical sections were made of the Texas Red (bHSA-SKL) and FITC (catalase) fluorescence. False-colour overlays of the two sections were prepared at the time of image collection. Digitized images were stored and printed without further modification using a Kodak XLS 8300 digital printer.
Import-competent peroxisomes newly synthesized proteins. The level of immunofluorescence observed in control cells (Fig. 4a) was indistinguishable from that in cycloheximide-treated cells (Fig. 4b). In cells that had been digitonin-permeabilized to release the unimported bHSA-SKL (Fig. 5), the import appeared to be very similar between control cells (Fig. 5a) and cycloheximide-treated cells (Fig. 5b). In both cases cycloheximide had no observable effect on peroxisomal import; the number of vesicles that were catalase-positive but did not contain bHSA-SKL was very few. In addition, the level of immunofluorescent staining for the endogenous catalase was indistinguishable between the cycloheximide-treated and the control cells (Figs 4 and 5, green channel).

Sequential import of microinjected peroxisomal proteins

We sought to determine whether peroxisomes, having imported one microinjected protein, could subsequently import a second microinjected protein into the same compartment. Luciferase, which has been previously demonstrated to be imported into endogenous peroxisomes (Walton et al., 1992a; Soto et al., 1993), was microinjected initially, and bHSA-SKL was microinjected 24 hours later. After a subsequent 16 hour incubation, the cells were fixed and stained as described in the figure legend. Double-label indirect immunofluorescence of the microinjected cells indicated that the vesicles containing bHSA-SKL (Fig. 6a) also contained luciferase (Fig. 6b). False-colour overlays (c,f) indicate vesicles that contain catalase (green), bHSA-SKL (red), or both (yellow). Bar, 5 µm.

**Fig. 1.** Colocalization of microinjected bHSA-SKL and endogenous catalase. Hs68 cells were microinjected with bHSA-SKL and incubated for 16 hours at 37°C. Cells were subsequently washed with PBS and permeabilized with digitonin (25 µg/ml) in PBS for 10 minutes. Following the digitonin treatment the cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and immunostained for bHSA-SKL and endogenous catalase. Staining consisted of rabbit anti-catalase as the primary reagent and FITC-conjugated donkey anti-rabbit antibodies and streptavidin-conjugated Texas Red as secondary reagents. Identical confocal microscopic sections were scanned for both fluorochromes. The figure shows the intracellular location of bHSA-SKL (a,d) and catalase (b,e) as greyscale images in microinjected cells. False-colour overlays (c,f) indicate vesicles that contain catalase (green), bHSA-SKL (red), or both (yellow). Bar, 5 µm.
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The import of bHSA-SKL into catalase-positive vesicles indicates that, at the earliest time points measurable by the microinjection assay, virtually all of the peroxisomes are capable of import. However, it remained possible that the peroxisomes were re-equilibrating and that initial differences were unobserved. It is beyond the capabilities of the present study to address the earliest events of peroxisomal import, and as such we have limited our study to the demonstration that there does not exist a population of peroxisomes that have lost their capacity for protein import. Import in the presence of cycloheximide demonstrates that, prior to microinjection, each peroxisome has associated protein-import machinery, and that the synthesis of new proteins is not required to render the peroxisomes import-competent. The absence of a compartment that contains bHSA-SKL, but not catalase, in the presence of cycloheximide appears to indicate that there is no proliferation of peroxisomes as a result of the microinjection and import of bHSA-SKL. As the immunofluorescent signal for catalase did not diminish during the experimental period, it is not believed that the peroxisomes are dividing; such a proliferation in the presence of cycloheximide would result in the dilution of the endogenous peroxisomal proteins. In addition to the use of catalase as a peroxisomal marker, a form of ‘pulse-chase’ experiment indicated that a second protein microinjected into the cytosol of Hs68 cells would be imported into the identical compartment occupied by a previously microinjected protein. This result demonstrates that the import of peroxisomal proteins does not result in the formation of a mature and import-incompetent peroxisome, at least over the time scale of 24 hours.

The conclusions presented in this study differ from those results obtained from regenerating rat liver. After partial hepatectomy, electron micrographic studies have demonstrated heterogeneous distributions of peroxisomal enzymes (Le Her and Dubach, 1980; Angermüller and Fahimi, 1986, 1988; Yamamoto and Fahimi, 1987b; Baumgart et al., 1989; Roels
and Cornelis, 1989) and the transient appearance of a ‘peroxisome reticulum’ (Yamamoto and Fahimi, 1987b). We have never observed a structure that could be a peroxisome reticulum in microinjected cells, although it is possible that the immunofluorescent micrographs in our report have insufficient resolution to distinguish such a peroxisomal structure. Peroxisomes isolated from regenerating rat liver could be divided on density gradients into two populations (Luers et al., 1993). The average diameter of the low density, import-competent peroxisomes was less than that of the high density import-incompetent peroxisomes. These populations appeared to reach an equilibrium after approximately 90 minutes, and as such it is possible that it may have been undetected in our microinjection assay. In addition, Heinemann and Just (1992) have reported that, in cultured hepatocytes, peroxisomes exist as two populations of differing density, the lighter of which appeared to be import-competent. Visual inspection of the results presented in Figs 1, 3, 4, and 5 indicated that there did not appear to be a distinction in import capabilities between large and small peroxisomes. It is possible that there are differences

Fig. 3. Colocalization of microinjected bHSA-SKL and endogenous catalase after 4 hour incubations. Hs68 cells were microinjected with bHSA-SKL and incubated for 4 hours at 37°C. Cells were subsequently washed with PBS and permeabilized with digitonin (25 µg/ml) in PBS for 10 minutes. Following the digitonin treatment the cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and immunostained for bHSA-SKL and endogenous catalase. Identical confocal microscopic sections were scanned for both fluorochromes. The figure shows the intracellular location of bHSA-SKL (a,d) and catalase (b,e) as greyscale images in a microinjected cell. False-colour overlay (c,f) indicates vesicles that contain catalase (green), bHSA-SKL (red), or both (yellow). Bar, 5 µm.
between isolated peroxisomes and microinjection into living cells that may explain these discrepancies.

In yeast, the biogenesis of peroxisomes appears to take place in two distinct stages (reviewed by Subramani, 1993). There is a growth of the individual organelle and a proliferation of the numbers of organelles. In the methylotrophic yeast *Hansenuila polymorpha*, the peroxisome appears to initially undergo a period of growth, followed by fission and proliferation (Veenhuis et al., 1979). However, in *Candida boidinii*, proliferation of the peroxisomes precedes matrix protein import and growth of the organelles (Veenhuis and Goodman, 1990). The processes of growth and proliferation can be uncoupled, and can take place in the absence of the other function. Growth can take place in the absence of proliferation in *Saccharomyces cerevisiae* or *H. polymorpha* overexpressing alcohol oxidase (Distel et al., 1988; Godecke et al., 1989). Conversely, proliferation can take place in the absence of growth in mutants of *Pichia pastoris* (McCollum et al., 1993; Sprong and
Import-competent peroxisomes (Subramani, 1993). Indeed, in human cells from patients affected with Zellweger syndrome the peroxisomal ‘ghosts’ are able to proliferate without engaging in the import of matrix proteins (Santos et al., 1988). Our results demonstrate that in cultured normal human fibroblasts, peroxisomes do not lose their capacity for the import of SKL-targeted proteins. These results imply that for these cells, under these conditions, growth precedes proliferation and that the two stages of peroxisomal biogenesis are not necessarily coupled.

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