

Involvement of 70-kD Heat-Shock Proteins in Peroxisomal Import

Paul A. Walton,* Martin Wendland,‡ Suresh Subramani,‡ Richard A. Rachubinski,§ and William J. Welch||

*Department of Anatomy and Cell Biology, McGill University, Montreal, Canada H3A 2B2; ‡Department of Biology, University of California, San Diego, California 92037-0322; §Department of Anatomy and Cell Biology, University of Alberta, Edmonton, Canada T6G2H7; and ||Department of Medicine and Physiology, University of California, San Francisco, California 94143

Abstract. This report describes the involvement of 70-kD heat-shock proteins (hsp70) in the import of proteins into mammalian peroxisomes. Employing a microinjection-based assay (Walton, P. A., S. J. Gould, J. R. Feramisco, and S. Subramani. 1992. *Mol. Cell Biol.* 12:531-541), we demonstrate that proteins of the hsp70 family were associated with proteins being imported into the peroxisomal matrix. Import of peroxisomal proteins could be inhibited by coinjection of antibodies directed against the constitutive hsp70 proteins (hsp73). In a permeabilized-cell assay (Wendland and Subramani. 1993. *J. Cell Biol.* 120:675-685), antibodies directed against hsp70 proteins were shown to inhibit peroxisomal protein import. Inhi-

tion could be overcome by the addition of exogenous hsp70 proteins. Purified rat liver peroxisomes were shown to have associated hsp70 proteins. The amount of associated hsp70 was increased under conditions of peroxisomal proliferation. Furthermore, proteinase protection assays indicated that the hsp70 molecules were located on the outside of the peroxisomal membrane. Finally, the process of heat-shocking cells resulted in a considerable delay in the import of peroxisomal proteins. Taken together, these results indicate that heat-shock proteins of the cytoplasmic hsp70 family are involved in the import of peroxisomal proteins.

VIRTUALLY all proteins destined for the peroxisomal matrix and membrane are synthesized on free polyosomes in the cytoplasm (Goldman and Blobel, 1978; Robbi and Lazarow, 1978; Fujiki et al., 1984; Miura et al., 1984; Rachubinski et al., 1984; Roggenkamp et al., 1984) and are imported into the peroxisome posttranslationally (Goodman et al., 1984; Fujiki and Lazarow, 1985). Although two peroxisomal proteins, thiolase and sterol carrier protein 2, undergo proteolytic cleavage after import (Fujiki et al., 1985; Mori et al., 1991), most proteins are synthesized at their mature size. This differs from the mechanism of posttranslational import of proteins into the chloroplast (Mishkind and Scioli, 1988) and mitochondria (Attardi and Schatz, 1988; Hartl et al., 1989) where, in the main, the targeting signal resides as an amino-terminal leader sequence that is cleaved following import. Import of proteins into the matrix of the peroxisome is dependent upon the presence of a peroxisomal targeting signal in the amino acid sequence of the newly synthesized protein. Two forms of this peroxisomal targeting signal have been characterized: a COOH-terminal tripeptide with the sequence serine-lysine-leucine or a conservative variant thereof which has been found in many peroxisomal proteins (Gould et al., 1989; 1990), or an

amino-terminal leader sequence such as that found in peroxisomal thiolase (Swinkels et al., 1991). The degree to which peroxisomal proteins must be unfolded during translocation, and whether this process utilizes molecular "chaperones," is not known at present.

A family of proteins called the hsp70 heat-shock or stress proteins appear to be intimately involved in protein folding and transport in virtually all cell types (reviewed by Welch, 1991). Hsp70 proteins bind preferentially to denatured or unfolded proteins (Rothman, 1989; Palleros et al., 1991). Recent evidence has shown that constitutive members of the cytoplasmic hsp70 family are involved in translation by binding to and stabilizing nascent polypeptides as they emerge from the ribosome (Beckmann et al., 1990, 1992; Nelson et al., 1992). Post-translational transport of proteins across the membranes of the yeast endoplasmic reticulum (Chirico et al., 1988), mitochondria (Deshaies et al., 1988; Murakami et al., 1988; Sheffield et al., 1990), nucleus (Shi and Thomas, 1992), and lysosomes (Chiang et al., 1989) have all been reported to require the participation of members of the cytoplasmic hsp70 family.

Yet other members of the hsp70 family are also involved in the receipt of proteins as they arrive within the mitochondria (Grp 75, Ssc 1) and endoplasmic reticulum (BiP, Kar2). Mitochondrial hsp70 is required for protein import through the translocation contact sites and folding of the newly imported protein (Kang et al., 1990). The intra-organellar

Address all correspondence to Paul Albert Walton, Ph.D., Department of Anatomy and Cell Biology, McGill University, Montreal, Canada H3A 2B2.

forms of the hsp70 proteins appear to provide much the same function as does the cytoplasmic hsp70 proteins. Just as the cytoplasmic forms seem to retain newly synthesized proteins in an unfolded state until synthesis is complete, the intra-organellar forms seem to retard the folding of the imported protein until translocation is complete. The mitochondrial hsp70 protein contains an amino-terminal leader sequence which directs the import of this protein into the mitochondrial matrix (Craig et al., 1989). Another class of stress proteins, the hsp60 family, is homologous to the chloroplast Rubisco subunit-binding protein and the *Escherichia coli* heat-shock protein groEL (Hemmingsen et al., 1988). These proteins have cytoplasmic (TCP-1) (Frydman et al., 1992) and mitochondrially located members (Cheng et al., 1989; Osterman et al., 1989). These proteins are called "chaperonins" (Ellis, 1987) and directly participate in protein folding and assembly of oligomeric protein complexes (Koll et al., 1992). All of the members of the hsp60 and hsp70 families are ATP-binding proteins and their cycles of protein binding and release involve the hydrolysis of ATP.

The import of proteins into the mammalian peroxisome is an ATP-dependent process (Imanaka et al., 1987; Soto et al., 1993; Wendland and Subramani, 1993). The requirement for NEM-insensitive cytosolic factors in the process of peroxisomal import has recently been demonstrated (Wendland and Subramani, 1993). It is, however, presently unknown if members of the hsp70 family participate in the import of proteins into the peroxisomes or if hsp60-like proteins are involved in the assembly of protein oligomers.

In this study we sought to determine if heat-shock proteins of the cytoplasmic hsp70 family were involved in the process of peroxisomal protein import. Employing a microinjection-based assay (Walton et al., 1992a), human serum albumin conjugated to a peroxisomal targeting signal was used to study peroxisomal import in living cells. In addition, antibodies directed against cytoplasmic hsp70 proteins were coinjected with the hybrid peroxisomal proteins in order to determine their effect on the import of a protein from the cytoplasm to the peroxisomal matrix. Using a permeabilized-cell assay (Wendland and Subramani, 1993), the effects of exogenous heat-shock proteins on the import of proteins into the peroxisomes was examined. Finally, the association between hsp70 molecules and peroxisomes was studied using Western blotting and proteinase protection.

Materials and Methods

Reagents

Human serum albumin (HSA)¹ was purchased from Sigma Chem. Co. (St. Louis, MO). Sulfo-MBS and NHS-biotin were purchased from Pierce Chemical Company (Rockford, IL). HSA was conjugated to a 12-amino acid peptide (CRYHLKPLQSKL) to obtain the HSA-SKL hybrid protein, as described by Walton et al. (1992a). Biotinylated HSA containing the peroxisomal targeting signal serine-lysine-leucine (bHSA-SKL) was prepared as described previously (Walton et al., 1992a), except that NHS-biotin was included in the first incubation at a tenfold molar excess. Hsp73 was purified from bovine brain cytosol (Malhotra et al., 1989) as previously described (Welch and Feramisco, 1985). Antibodies directed against the cyto-

plasmic constitutive (73 kD) or inducible (72 kD) heat-shock proteins were as previously described, and included: (a) rabbit anti-hsp73 (Brown et al., 1993), (b) rat anti-hsp73 IB5 (Ohtsuka, K., B. Lokeshwar, and A. Lazlo, manuscript submitted for publication), (c) rabbit anti-hsp72 (Welch and Feramisco, 1984), (d) mouse anti-hsp72 C92 (Welch and Suhan, 1986), and (e) a mixture of mouse anti-hsp72 and anti-hsp73 (Riabowol et al., 1988). Antibodies directed against catalase were obtained from Calbiochem-Novabiochem (La Jolla, CA). Streptavidin-conjugated Texas red was purchased from Amersham (Oakville, Ontario). Fluorescently conjugated secondary antibodies and alkaline phosphatase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Proteinase K and Aprotinin were purchased from Boehringer (Laval, Quebec). Synthetic peptides were obtained from Agouron Institute or Multiple Peptide Systems (La Jolla, CA). Other reagents were purchased from the standard sources.

Cell Culture

The human fibroblast cell line, Hs68, was obtained from American Type Culture Collection (Rockville, MD). Cells were grown in DME supplemented with 10% fetal calf serum (GIBCO) BRL, Gaithersburg, MD). For microinjection, cells were plated on acid-washed glass coverslips. CHO cells were grown in DME supplemented with 5% fetal calf serum.

Microinjection and Immunofluorescence Microscopy

Cells were microinjected using glass capillary needles as previously described (Walton et al., 1992a). Biotinylated HSA-SKL was microinjected at a concentration of 0.5 mg/ml in a buffer of 20 mM KPO₄ (pH 7.4), 100 mM KCl. Where indicated, IgG's were coinjected at a concentration of 5 mg/ml. With an average injection volume of 5×10^{-14} l, $\sim 2 \times 10^5$ molecules of bHSA-SKL and 1×10^6 molecules of IgG were injected per cell.

Following microinjection, the incubation time was 2–16 h at 37°C. Where indicated in the figure legends, the cells were first permeabilized with digitonin (25 µg/ml) for 10 min followed by fixation and staining. This treatment results in the permeabilization of the plasma membrane while leaving the peroxisomal membrane intact (Wolvetang et al., 1984; 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 microscopy was performed with a Zeiss Axiophot Photomicroscope using a 63× (1.3 NA) lens. Fluorescent images were recorded using Kodak T-Max 400 film, pushed and developed one stop as per the manufacturer's instructions.

Permeabilized-cell Import Assay

CHO cells were grown on coverslips coated with Pronectin F (Protein Polymer Technologies Inc., San Diego, CA) to confluent density. The cells were permeabilized as described by Wendland and Subramani (1993) with 2.0 U/ml of streptolysin-O, followed by an incubation for 15 min in 1 ml of cold transport buffer (4°C) to insure sufficient leakage of endogenous cytoplasm. The coverslips were then incubated with transport buffer containing HSA-SKL (50–100 µg/ml), an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase) in the presence or absence of exogenous cytosol. To test the effects of exogenous rat anti-hsp73 antibodies and purified hsp73, these reagents were added to the transport buffer. After incubation for 45 min at 37°C, the coverslips were rinsed in transport buffer, the cells fixed with 4% paraformaldehyde in PBS, and subjected to indirect immunofluorescence to study the intracellular localization of HSA-SKL.

Western Blotting and Proteinase Protection Assay

Rat liver peroxisomes were purified and characterized as described previously (Bodnar and Rachubinski, 1991). For Western blotting, equal amounts of protein from normal and clofibrate-treated rat liver peroxisomes were loaded on SDS-PAGE gels. After transfer to nitrocellulose membranes (BioRad Labs., Hercules, CA), the blots were blocked with 5% skim milk powder and cut in order to specifically stain the regions of interest. The secondary antibodies were conjugated to alkaline phosphatase, and the color reagents were bromochloroindolyl phosphate and nitro blue tetrazolium (Harlow and Lane, 1988).

Proteinase K protection assays were performed as previously described (Miyazawa et al., 1989), except that 4 µg of proteinase K was added to 100 mg of rat liver peroxisomes. The incubation time was 30 min on ice. The

1. *Abbreviations used in this paper:* bHSA-SKL, biotinylated human serum albumin with a carboxy-terminal peroxisomal targeting signal serine lysine-leucine-COOH attached; HSA, human serum albumin.

proteinase was inactivated by the addition of 10 mM PMSF and Aprotinin (500 Kallikrein U/ml). The samples were immediately boiled in Laemmli sample buffer and applied to a 12% SDS-PAGE gel. Western blotting was performed as described above.

Results

Import of bHSA-SKL Involves the Association of Hsp70 Molecules with Peroxisomes

As has been demonstrated previously (Walton et al., 1992a), the microinjection of HSA-SKL into the cytoplasm of human fibroblast cells resulted in the import of immunologically detectable amounts of HSA-SKL into peroxisomes after ~8 h. In order to determine if import was occurring at earlier times and involved hsp70 molecules, a method of decreasing the high cytoplasmic levels of unimported bHSA-SKL and constitutive cytoplasmic hsp70 was required. If the cells were first permeabilized with digitonin, a treatment known to permeabilize the plasma membrane but not the peroxisomal membrane (Wolvetang et al., 1988; Walton et al., 1992a,b; Soto et al., 1993), most of the cytoplasmic molecules would diffuse away prior to fixation. Using this method, only bHSA-SKL which had been imported into peroxisomes would be fixed and visualized. The results demonstrate that microinjected bHSA-SKL could be visualized within vesicles at times as early as 2–4 h after microinjection (Fig. 1 a), and that these vesicles were peroxisomes because they contained endogenous catalase (Fig. 1 b).

To determine whether cytoplasmic hsp70 associated with bHSA-SKL during its import, double label indirect immunofluorescence of microinjected cells was performed. As demonstrated in Fig. 2, hsp70 molecules colocalized with the bHSA-SKL at these early time points. Employing two different antibodies directed against the cytoplasmic forms of hsp70: a mixture of mouse monoclonal antibodies (Fig. 2 b), or a rabbit polyclonal antibody (Fig. 2 d), vesicles in the process of accumulating bHSA-SKL also stained for proteins of the hsp70 family.

Inhibition of Peroxisomal Import by Coinjection of Antibodies Directed Against Constitutive Heat-Shock Proteins

To investigate if heat-shock proteins were involved in the import of proteins into the peroxisome, coinjection experiments involving the microinjection of bHSA-SKL and antibodies directed against the constitutive (hsp73) or inducible (hsp72) heat-shock proteins were performed. Although cells of primate origin express hsp72 under normal conditions, the amount is small when compared to the expression of hsp73. Microinjection of nonspecific mouse or rabbit IgG has previously been demonstrated to have no effect on the import of peroxisomal proteins (Walton et al., 1992a,b). Microinjection of nonspecific rat IgG at a concentration of 5 mg/ml did not inhibit the peroxisomal import of the coinjected bHSA-SKL (not shown). In contrast, the coinjection of antibodies directed against the constitutive hsp73 did result in the inhibition of import of bHSA-SKL into peroxisomes (Fig. 3, a and b). Under these conditions, the unimported bHSA-SKL could be observed as a diffuse staining in the cytoplasm of the injected cells. Coinjection of antibodies directed against the inducible hsp72 did not inhibit import (Fig. 3, c and d).

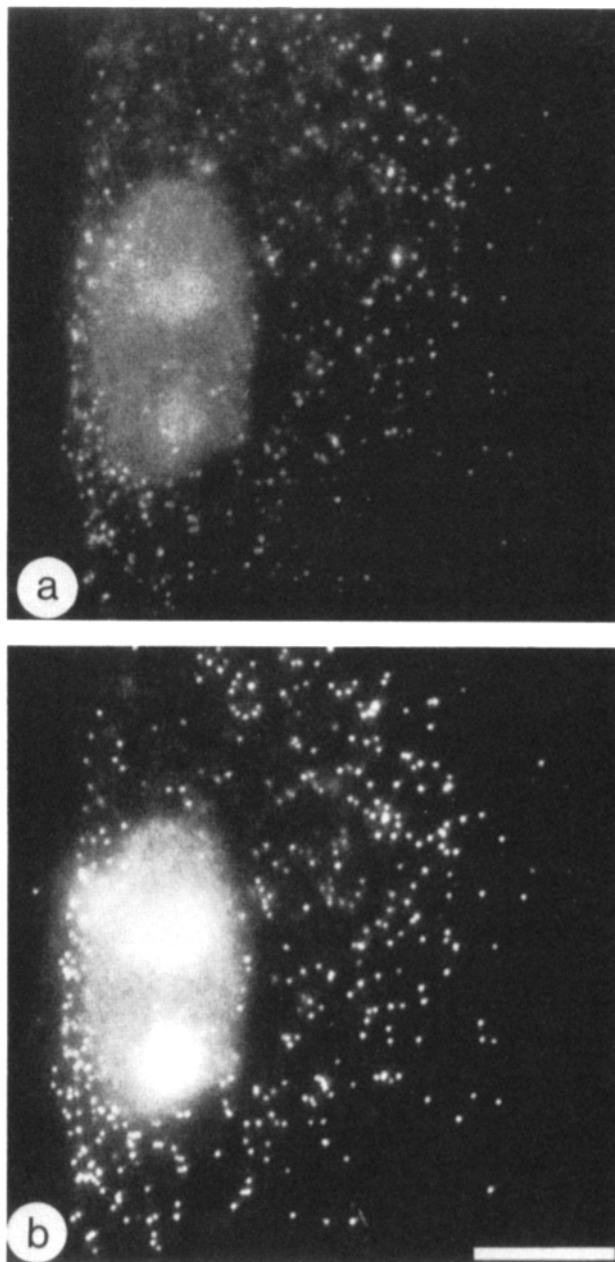


Figure 1. Import of bHSA-SKL into peroxisomes. Hs68 cells were microinjected with $\sim 2 \times 10^5$ molecules of bHSA-SKL and incubated for 4 h at 37°C. Cells were subsequently washed with PBS and permeabilized with digitonin (25 $\mu\text{g}/\text{ml}$) in PBS for 10 min. Following the digitonin treatment the cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and immunostained for bHSA-SKL and endogenous catalase. Figure shows the intracellular location bHSA-SKL (a) and catalase (b) in a microinjected cell. Bar, 5 μm .

Microinjected at a concentration of 5 mg/ml, the injected cells received $\sim 10^6$ molecules of IgG/cell.

Peroxisomal Import, Inhibited by Antibodies Directed Against Heat-Shock Proteins, Can be Restored by the Addition of Hsp73

Using a permeabilized cell system, it has been shown that

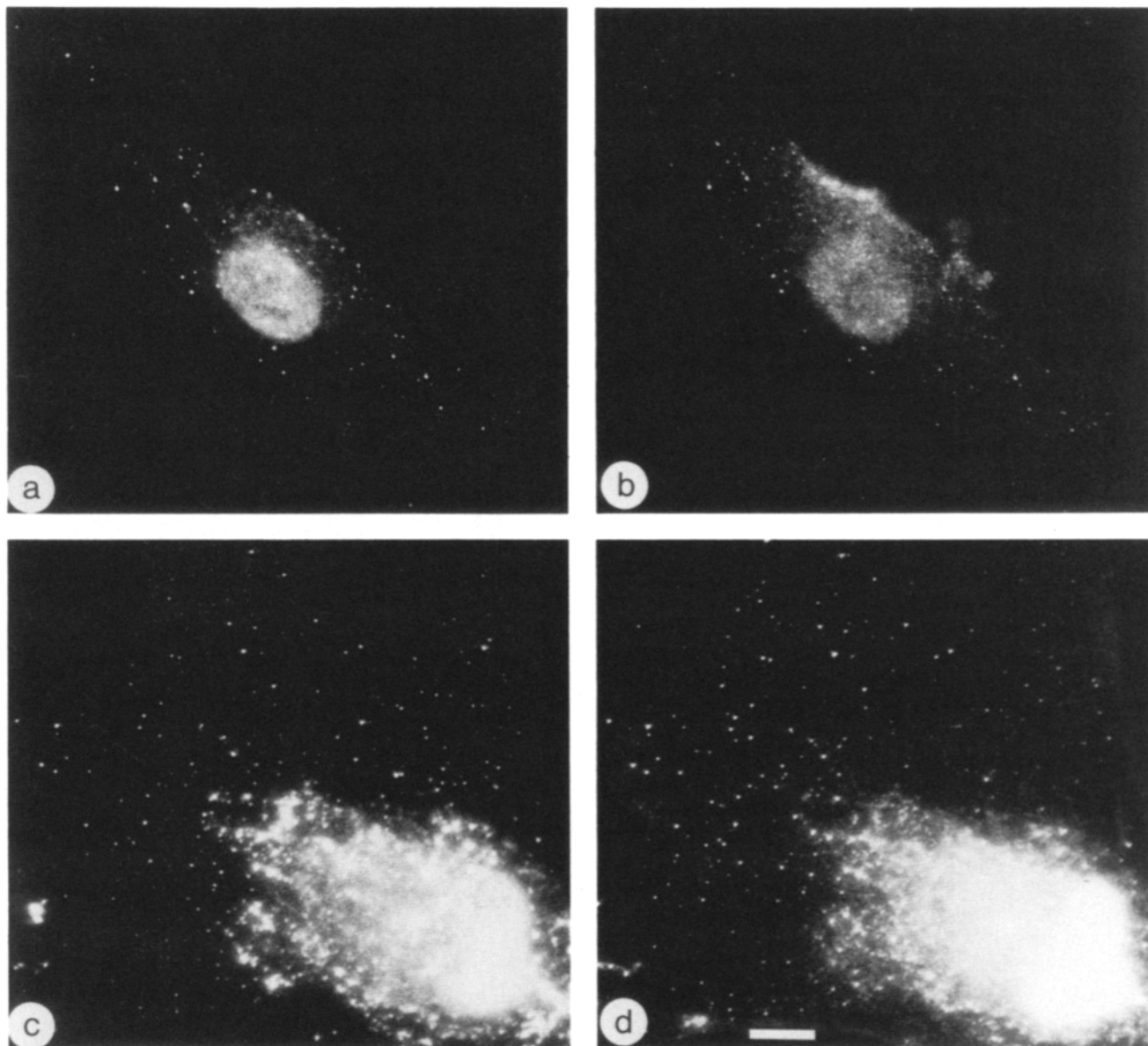


Figure 2. The peroxisomal association of bHSA-SKL and hsp70 molecules. Hs68 cells were microinjected with bHSA-SKL and incubated for 2 h (*a* and *b*) or 8 h (*c* and *d*) at 37°C. Cells were washed with PBS and permeabilized with digitonin (25 $\mu\text{g}/\text{ml}$) for 10 min. Following the digitonin treatment the cells were fixed, permeabilized, and immunostained for microinjected bHSA-SKL and endogenous hsp70 proteins. Figure shows the intracellular location of the microinjected bHSA-SKL (*a* and *c*) and hsp70 (*b* and *d*). Bar, 5 μm .

cytosolic factors are required in the process of peroxisomal import (Wendland and Subramani, 1993). CHO cells are permeabilized with streptolysin-O, a treatment which specifically perforates the plasma membrane but leaves the internal membranes intact. As a result, the endogenous cytoplasm is washed out of these cells. The permeabilized cells are incubated with HSA-SKL, an artificial substrate of peroxisomal import, in the presence or absence of exogenous cytosol. As shown in Fig. 4, whereas no import was observed in the absence of cytosol (Fig. 4 *a*), addition of cytosol in the form of rabbit reticulocyte lysate was necessary for peroxisomal import of HSA-SKL (Fig. 4 *b*). A Western blot of rabbit reticulocyte lysate showed that it is a rich source of hsp73 (data not shown). To determine if hsp73

was responsible for the stimulation of peroxisomal import, rabbit reticulocyte lysate was first incubated with antibodies directed against hsp73. Such a preincubation of rabbit reticulocyte lysate with anti-hsp73 antibodies now reduced the import of HSA-SKL into the peroxisomes (Fig. 4 *c*). Though greatly reduced, import was not completely abolished by the anti-hsp73 antibodies. This is similar to the results observed in Fig. 3, *a* and *b*, where microinjected anti-hsp73 greatly inhibited, but did not abolish, peroxisomal import in living cells. To confirm that the inhibition of import in the presence of anti-hsp73 antibodies was due to their interaction with hsp73, exogenous purified hsp73 was added to the cytosol/antibody mixture. As shown in Fig. 4 *d*, the activity of the cytosol/antibody mixture was restored to near control levels

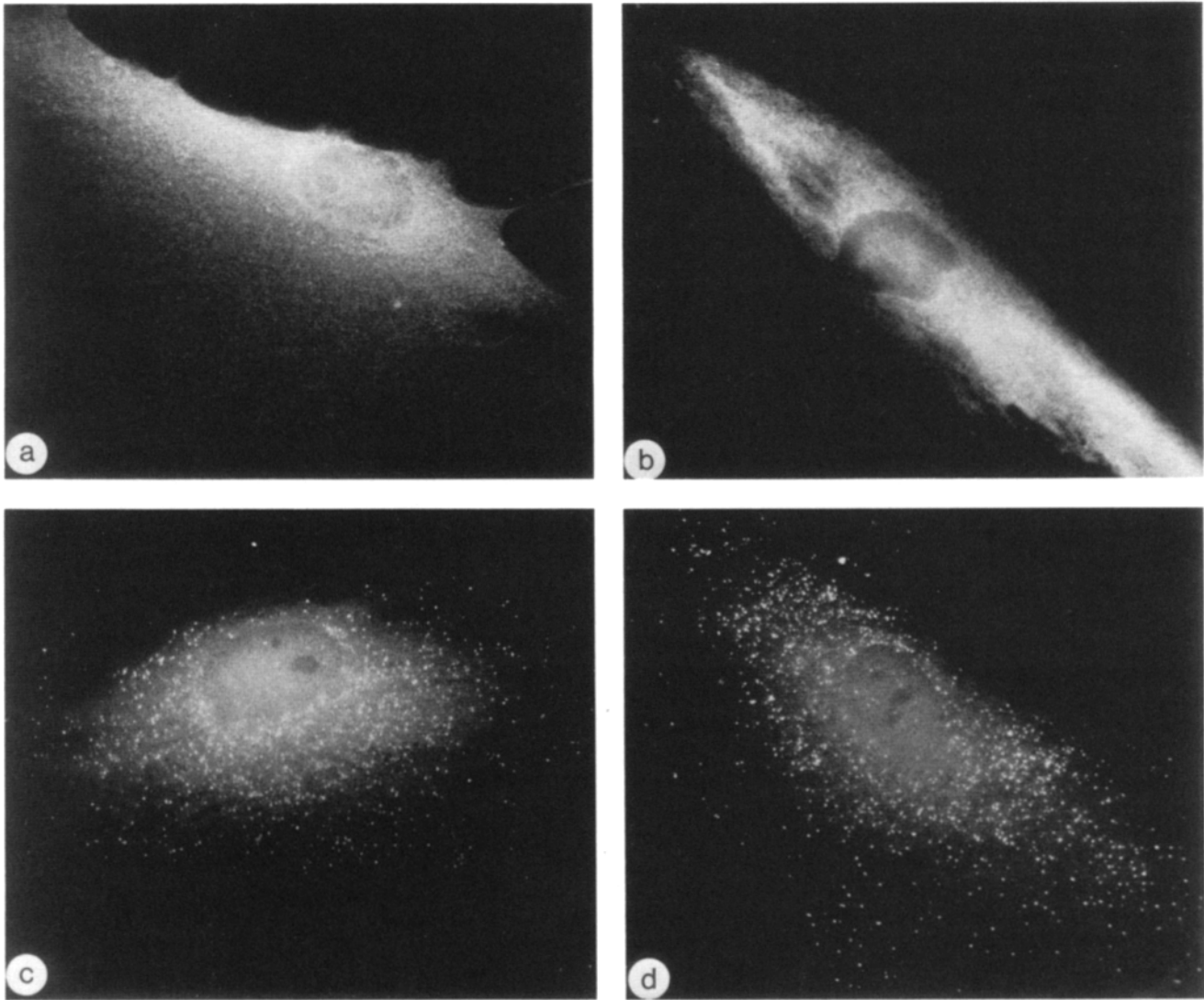


Figure 3. Effects of coinjection of antibodies directed against the constitutive (73 kD) or inducible (72 kD) heat-shock proteins on the peroxisomal import of microinjected bHSA-SKL. Hs68 cells were microinjected with bHSA-SKL and either: rabbit α -hsp73 polyclonal antibodies Q-11 (a), rat α -hsp73 monoclonal antibodies IB5 (b), rabbit α -hsp72 polyclonal antibodies (c), or mouse α -hsp72 monoclonal antibodies C92 (d). Cells were subsequently incubated at 37°C for 16 h and processed for double label indirect immunofluorescence. Microinjected cells were identified by staining for the coinjected IgG (not shown). Figure shows the intracellular location of the microinjected bHSA-SKL. Bar, 5 μ m.

by the addition of hsp73. These results indicate that hsp73 is likely to be one of the soluble factors required for peroxisomal protein import.

Heat-Shock Proteins Are Associated with Isolated Rat Liver Peroxisomes

In order to determine if heat-shock proteins were associated with peroxisomes under in vivo conditions, we isolated peroxisomes from normal rats and rats treated with clofibrate, a drug which causes peroxisomal proliferation. Western blots of purified peroxisomes from normal rat liver showed that small amounts of hsp70 copurified with peroxisomes (Fig. 5 a, lane 1). Rats treated with clofibrate expressed higher than normal levels of thiolase, an enzyme of the β -oxidation pathway, in the purified peroxisomes (Fig. 5

b, lanes 1 and 2). Significantly higher levels of hsp70 copurified with these peroxisomes (Fig. 5 a, lane 2). Each lane in each panel had the same amount of total peroxisomal protein loaded. Characterization of the purified peroxisomes indicated that levels of contamination with nonperoxisomal proteins were very low (Bodnar and Rachubinski, 1991).

The Hsp70 Proteins Associated with Peroxisomes Are Accessible to Exogenous Proteinases

To determine whether the hsp70 molecules isolated in association with rat liver peroxisomes were on the exterior surface of the peroxisome or in an interior location, proteinase protection assays were undertaken. In this experiment, freshly isolated peroxisomes were employed to eliminate the possibility that freezing and thawing might result in leaky peroxi-

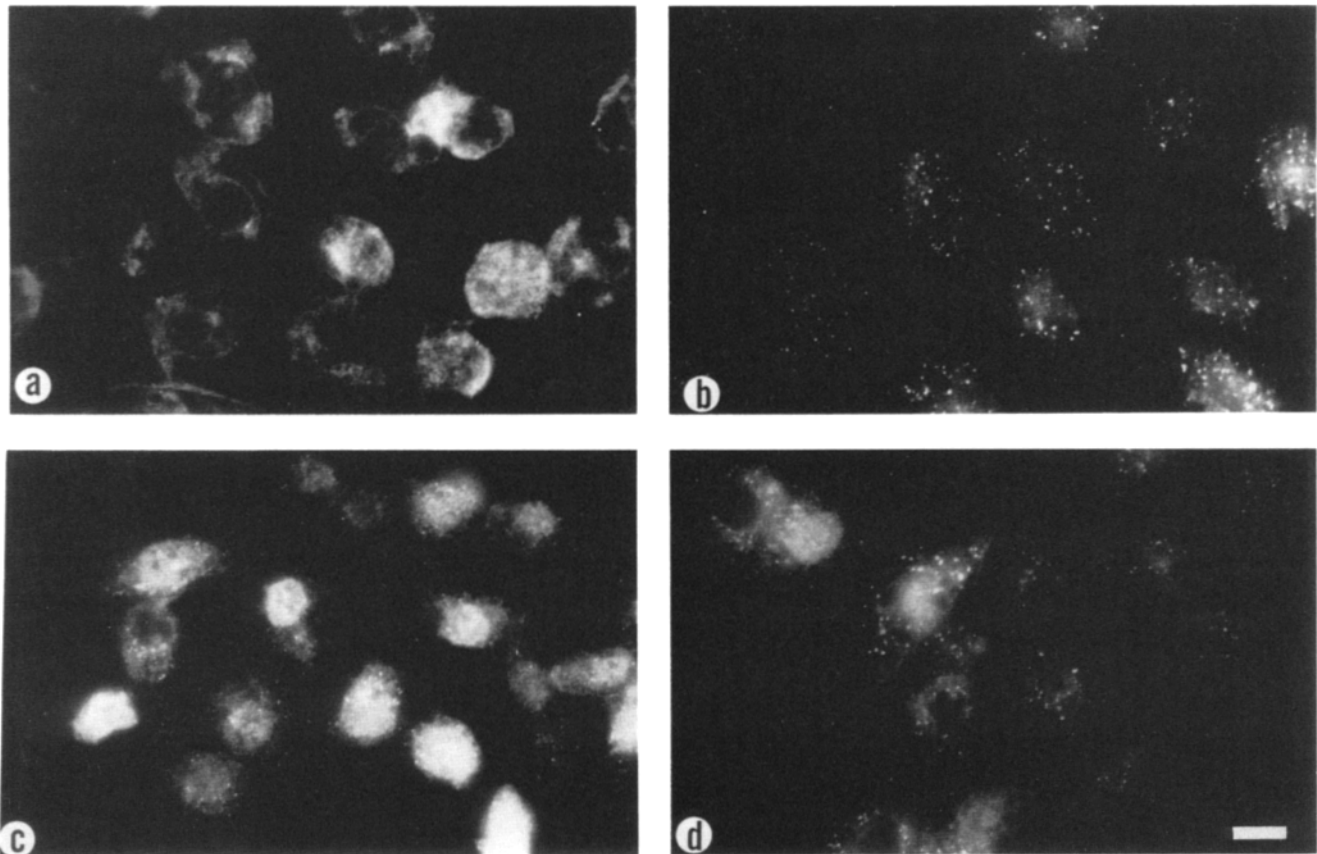


Figure 4. Inhibition of peroxisomal import by antibodies directed against hsp73 in a permeabilized-cell assay, and the recovery of that import by the addition of exogenous hsp73. CHO cells were permeabilized with streptolysin-O and incubated with HSA-SKL in the absence (a), or presence of exogenous cytosol in the form of rabbit reticulocyte lysate (b-d). Rabbit reticulocyte lysate was used in its native form (b), or preincubated with rat α -hsp73 monoclonal antibodies IB5 (c and d). Purified hsp73 was added to the antibody-treated cytosol (d), to determine its effect on import. After incubation at 37°C for 45 min, the cells were washed, fixed, and stained for the intracellular location of the HSA-SKL. Bar, 5 μ m.

somes. However, identical results were obtained using previously frozen peroxisomes. By virtue of its location within the peroxisomal matrix, thiolase was resistant to proteinase K degradation in the absence of detergents, but was subject to proteolysis in the presence of 1% Triton X-100 (Fig. 6, middle). However, antibodies directed against peroxisomal integral membrane proteins (Bodnar and Rachubinski, 1991) detected a new band with an apparent molecular mass of 29 kD following proteinase K treatment in the absence or presence of detergents. This 29-kD protein is assumed to be a proteolytic fragment of a higher molecular mass peroxisomal membrane protein, although its native identity could not be determined. Proteinase K protection assays against purified peroxisomes demonstrated that the immunodetectable hsp70 was subject to proteolysis in the absence of exogenous detergents (Fig. 6, top). This result indicates that the hsp70 molecules are located on the exterior surface of the peroxisomal membrane.

Heat-Shock Retards Peroxisomal Import

Cells subjected to heat-shock undergo a redistribution of their hsp70 molecules. We sought to determine if heat-shock would inhibit the import of microinjected bHSA-SKL into peroxisomes. Hs68 cells, microinjected with bHSA-SKL,

were incubated at 44°C for 2 h and then returned to 37°C for the remainder of the incubation period. This treatment markedly increased the expression of the inducible hsp72 (results not shown). Unlike control cells, incubated at 37°C, the heat-shocked cells showed no detectable import of bHSA-SKL into the peroxisomes after 12 h (Fig. 7 a). The bHSA-SKL remained detectable in the cytoplasm. By 16 h the heat-shocked cells had begun to regain a normal mor-

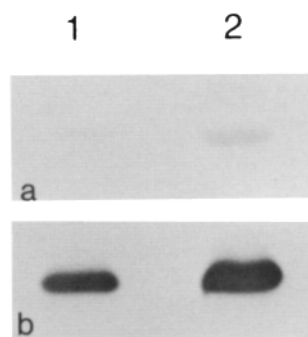


Figure 5. Purified rat liver peroxisomes have associated heat-shock proteins. Western blots of purified peroxisomes from normal (lane 1) and clofibrate-treated rat liver (lane 2). a, which contained 24 μ g of peroxisomal proteins in lanes 1 and 2, was probed with antibodies directed against hsp70. b, which contained 8 μ g of peroxisomal proteins in lanes 1 and 2, was probed with antibodies directed against thiolase.

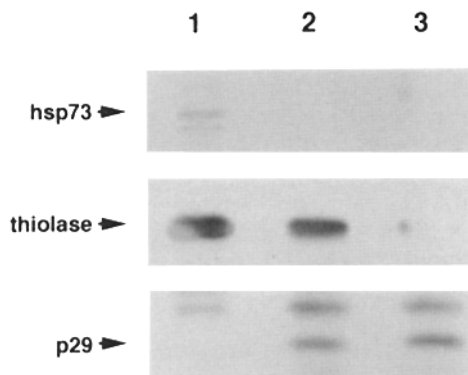


Figure 6. Effects of proteinase K on the peroxisomally associated hsp73 molecules. Purified rat liver peroxisomes (100 μ g) were incubated on ice for 30 min in the absence (lane 1) or presence of 4 μ g proteinase K in the absence (lane 2) or presence of 1% Triton X-100 (lane 3). The samples were subsequently treated with proteinase inhibitors and Western blotted using antibodies directed against hsp73 (top), thiolase (middle), or peroxisomal integral membrane proteins (bottom).

phology and bHSA-SKL could be detected in peroxisomes (Fig. 7b). These results indicate that cells subjected to a 2-h period of heat-shock have a compromised peroxisomal import capacity, resulting in at least an 8-h delay in peroxisomal import.

Discussion

The involvement of cytoplasmic hsp70 proteins in the post-translational transport of proteins to their appropriate sub-cellular compartment has been demonstrated for many organelles. It is believed that this interaction between hsp70 and imported proteins functions to prevent misfolding or aggregation. Hence, the protein is maintained in a import-competent state until the entire polypeptide chain is synthesized or translocated. In addition, proteins of the hsp70 family may be present within the matrix of the organelle to receive the imported proteins (Kang et al., 1990; Mizzen et al., 1991), delaying folding until all of the required information is present.

In the present study, cytoplasmic hsp70 molecules were shown by double label indirect immunofluorescence in close association with proteins destined for import into the peroxisomal matrix. In uninjected cells, little or no staining of hsp70 was visible, indicating that the steady state peroxisome did not normally have associated with it sufficient hsp70 to render it visible by immunofluorescence (results not shown). In addition, purified peroxisomes were found to have hsp70 molecules associated with their exterior surface. Interestingly, the amount of heat-shock proteins associated with purified peroxisomes increased under conditions of increased import of peroxisomal proteins. In order to determine whether hsp70 proteins might be directly involved in the import of proteins into the peroxisome, we coinjected a hybrid peroxisomal protein with antibodies directed against the constitutive or inducible forms of the hsp70 protein family. Microinjection of antibodies directed against hsp70 proteins have been demonstrated to inhibit the migration of these proteins to the nucleolus following heat-shock (Brown et al.,

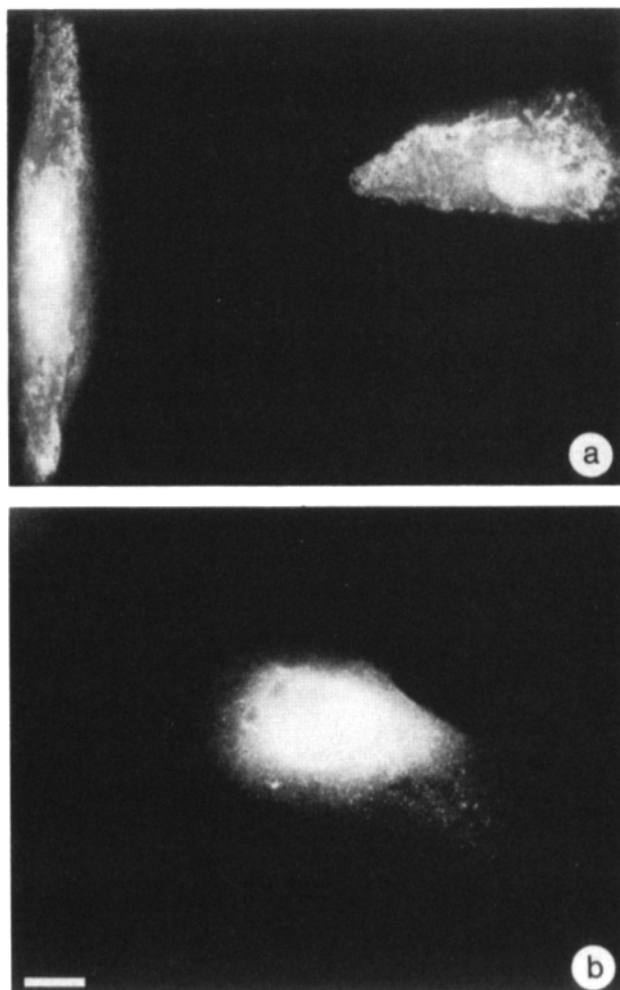


Figure 7. Effects of heat-shock on the peroxisomal import of bHSA-SKL. Hs68 cells, microinjected with bHSA-SKL, incubated at 44°C for 2 h. Cells were subsequently incubated at 37°C for 12 h (a) or 16 h (b), followed by fixing and staining. Microinjected cells were identified by staining for the coinjected nonspecific rat IgG (not shown). Figure shows the subcellular location of the microinjected bHSA-SKL. Bar, 5 μ m.

1993), and to increase the cells' thermolability (Riabowol et al., 1988). The use of both microinjection and permeabilized-cell techniques described in the present report were initiated in order to resolve the effects of antibodies directed against hsp70 proteins on peroxisomal import, distinct from those effects on protein translation in general. For example, as proteins synthesized on free ribosomes are associated with members of the hsp70 family (Beckman et al., 1990), microinjection of antibodies directed against hsp70 proteins might be expected to have an adverse effect on protein synthesis. Consequently, we chose microinjection of substrates for import to distinguish between effects of anti-hsp70 antibodies on synthesis and import of proteins destined for organelles. Using this technique, microinjection of antibodies directed against hsp73 resulted in the inhibition, but not the abolition, of peroxisomal import. In human cells grown in tissue culture, a small amount of hsp72 is expressed under normal conditions (Welch et al., 1983). The small amount of import remaining in the presence of anti-hsp73 antibodies

indicates that hsp72 may function in a limited way to facilitate import of the coinjected bHSA-SKL.

Complementing the microinjection experiments, strong evidence for the involvement of hsp70 proteins in peroxisomal import was obtained from studies using permeabilized cells. The activity of exogenous cytosol in stimulating peroxisomal import is greatly reduced after preincubation with anti-hsp73 antibodies. That the inhibition of such activity was a result of the reduction of hsp70 levels was confirmed by the restoration of import that accompanied the addition of purified hsp73 to the antibody-treated cytosol. Restoration of import, however, was not entirely complete. One possible explanation is that the antibody not only blocks or inhibits hsp73, but also indirectly inhibits other unidentified cofactors that interact with hsp73 to stimulate peroxisomal import. Support for the view that hsp73 interacts with others proteins is provided by the observation that exogenous hsp73 in the absence of other cytosolic factors does not support peroxisomal import (data not shown). This indicates that hsp73 stimulates import in conjunction with other unidentified soluble factors.

Heat-shock of cells resulted in the temporal inhibition of import of proteins into the peroxisome. During the time following heat-shock, hsp70 proteins are recruited to perform a number of other tasks. One of the immediate effects of heat-shock is the relocalization of hsp70 proteins to the nucleolus of the cell (Welch and Feramisco, 1984). It is not known if the function of hsp70 proteins in the nucleolus is to refold damaged ribosomal components or to facilitate their removal. Although it seemed possible that the heat-shock proteins were binding to the hybrid proteins in a nonspecific manner, and thus could be titrated away by the microinjection of other hybrid proteins, coinjection of HSA bearing a control peptide at a fourfold molar excess did not distinguishably inhibit import of bHSA-SKL. The results presented in Fig. 7 appear to indicate that substrates for post-translational import draw their chaperones from a common cytoplasmic hsp70 pool, and that under conditions of cellular stress insufficient hsp70 proteins may be available for import. However, it is possible that an alternative explanation for the inhibition of peroxisomal import may be a decrease in cellular ATP levels, which have been observed in *Tetrahymena* cells following heat-shock (Findly et al., 1983).

ATP has been shown to be required for import into the peroxisomes of yeast (Bellion and Goodman, 1987) and mammalian cells (Imanaka et al., 1987; Soto et al., 1993; Wendland and Subramani, 1993). Although proteins with ATPase activity have been identified on the surface of the peroxisome (Douma et al., 1987; Del Valle et al., 1988; Kamijo et al., 1990; Mosser et al., 1993), they have not been shown to be involved in the import of proteins into the peroxisomal matrix. One possible role for ATP in the import of proteins destined for the mitochondria has been demonstrated to be the maintenance of proteins in a "transport-competent" state (reviewed by Neupert et al., 1990). Proteins destined for posttranslational import, bound to hsp70 molecules, would require ATP hydrolysis in the cycle of dissociation and import. Thus the binding and hydrolysis of ATP by hsp70 molecules involved in the import of peroxisomal proteins may be the basis of the observed ATP requirement.

In addition to the involvement of cytoplasmic hsp70 in the

process of protein import, other organelles have a distinct internal pool of hsp70-like proteins (Mizzen et al., 1991; Brodsky et al., 1993). To date, there has been no demonstration of a distinct hsp70 protein constitutively targeted to the peroxisome. Evidence presented in this report (Fig. 6) indicates that, for purified rat liver peroxisomes, all of the hsp70 molecules recognized by antibodies directed against the cytoplasmic hsp70 proteins are found on the outside of the peroxisomal membrane. Whether the peroxisome contains hsp70-like (Grp75) and hsp60-like (GroEL or TCP-1) chaperone proteins is unknown at this time. Such complexes are involved in the folding and assembly of multimeric proteins (Osterman et al., 1989; Frydman et al., 1992; Koll et al., 1992; Langer et al., 1992), and as such would be predicted to have homologues involved in the assembly of multimeric peroxisomal proteins.

Just what is the role of cytoplasmic hsp70 proteins in the process of peroxisomal import? The role likely would appear to differ from that required for import into the mitochondrial matrix, which involves a protein in an extended conformation traversing a channel. At present, there is no evidence suggesting that peroxisomal proteins require extensive unfolding prior to import. Indeed, in previous reports (Walton et al., 1992a,b; Soto et al., 1993; Wendland and Subramani, 1993) and in this present report, prefolded proteins microinjected into the cytoplasm or incubated with permeabilized cells have been imported into peroxisomes. In their role as molecular chaperones, hsp70 molecules have never been demonstrated to unfold proteins as part of their function. Two models can be envisioned for the role of hsp70 molecules in stimulating peroxisomal protein import. In the first model, hsp70 molecules interact directly with the peroxisomal targeting signal and inhibit its folding to allow subsequent access of the signal to the peroxisomal targeting signal receptor and translocation machinery. In this model, hsp70 molecules do not unfold the transported protein or its peroxisomal targeting signal, but simply stabilize the peroxisomal targeting signal when it is exposed during thermal fluctuations of the protein structure. In an alternative model, hsp70 mediates its stimulatory functions without interacting directly with the transported protein. Our data favor, but do not yet prove definitively, the first model.

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