

Cytosol-dependent Peroxisomal Protein Import in a Permeabilized Cell System

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Abstract. Using streptolysin-O (SLO) we have developed a permeabilized cell system retaining the competence to import proteins into peroxisomes. We used luciferase and albumin conjugated with a peptide ending in the peroxisomal targeting sequence, SKL, to monitor the import of proteins into peroxisomes. After incubation with SLO-permeabilized cells, these exogenous proteins accumulated within catalase-containing vesicles. The import was strictly signal dependent and could be blocked by a 10-fold excess of a peptide containing the SKL-targeting signal, while a control peptide did not affect the import. Peroxisomal accumulation of proteins was time and temperature dependent and required ATP hydrolysis. Dissipation of the membrane potential did not alter the import

efficiency. GTP-hydrolyzing proteins were not required for peroxisomal protein targeting. Depletion of endogenous cytosol from permeabilized cells abolished the competence to import proteins into peroxisomes but import was reconstituted by the addition of external cytosol. We present evidence that cytosol contains factors with SKL-specific binding sites. The activity of cytosol is insensitive to *N*-ethylmaleimide (NEM) treatment, while the cells contain NEM-sensitive membrane-bound or associated proteins which are involved in the import machinery. The cytosol dependence and NEM-sensitivity of peroxisomal protein import should facilitate the purification of proteins involved in the import of proteins into peroxisomes.

THE posttranslational sorting of certain proteins synthesized on free cytoplasmic polysomes to peroxisomes represents one example of private protein-transport systems that serve to segregate proteins to subcellular compartments in eucaryotic cells. As is the case for protein targeting to all other subcellular compartments, the "right of entry" into the peroxisomal matrix is governed by at least two distinct types of peroxisomal targeting sequences (PTS)¹ (9, 32). One of these is a conserved, COOH-terminal tripeptide (PTS-1) which is found at the COOH terminus of ~30 peroxisomal proteins spanning the range of evolution from yeast to man (15–17, 20, 23, 34). This signal also serves as a general sorting signal for other microbodies such as glyoxysomes (20, 38) and glycosomes (13, 20) and has therefore been dubbed the microbody targeting signal (McTS) (20). The second PTS (PTS-2) was found to be within the amino-terminal cleaved leader sequence of the two peroxisomal 3-ketoacyl-CoA thiolases (24, 33). The signals that are presumed to grant entry of proteins into the membrane of peroxisomes have not yet been elucidated.

While our knowledge of the mechanism of protein import into peroxisomes has advanced as a consequence of the discovery of the sorting signals for matrix proteins and as the

result of genetic approaches to dissect the process (10–12, 18, 21, 36, 41), progress along the biochemical front has been somewhat slow. The details of the peroxisomal import machinery are of great interest because in human disorders such as the Zellweger syndrome (40) many peroxisomal matrix proteins are not transported faithfully into the organelle which exists instead in the form of membrane "ghosts" (27, 28, 39). The development of *in vitro* systems based on the import of radiolabeled proteins into purified rat liver (19, 23) or yeast peroxisomes (30, 31, 35) has revealed that the process is signal, energy, and temperature dependent. However, the fragility of purified peroxisomes and the absence of a reliable biochemical hallmark of peroxisomal protein import have hampered progress in the hands of all but a few investigators. Furthermore, these studies, based on the use of purified peroxisomes, are not readily extrapolatable to peroxisomes derived from human patients with peroxisomal disorders that seem to cripple the peroxisomal import machinery. To bypass these limitations, we recently described the development of an efficient and reliable *in vivo* import system based on microinjection of peroxisomal proteins into mammalian cells (39). We have now taken these studies to the next logical step by the development of a permeabilized cell system that retains the ability to transport proteins into the peroxisomal matrix in a signal-, time-, temperature-, and energy-dependent fashion. The use of this system reveals important new facts, not revealed by earlier studies, that should

1. *Abbreviations used in this paper:* HSA, human serum albumin; LDH, lactate dehydrogenase; NEM, *N*-ethylmaleimide; PTS, peroxisomal targeting sequences; SLO, streptolysin-O.

permit the biochemical fractionation of factors involved in peroxisomal protein import.

Materials and Methods

Reagents

Luciferase from *Photinus pyralis*, ATP γ S, GTP γ S, *N*-ethylmaleimide (NEM), *p*-phenylenediamine and the colorimetric diagnostic kit for determination of lactate dehydrogenase (LDH) activity were purchased from Sigma Chemical Co. (St. Louis, MO). Human serum albumin (HSA) and Mowiol were obtained from Calbiochem-Behring Corp. (San Diego, CA). Streptolysin-O (SLO) was purchased from Burroughs-Wellcome (Research Triangle Park, NC). Pronectin F was from Protein Polymer Technologies, Inc. (San Diego, CA). Rabbit reticulocyte lysate was purchased from Promega Corp. (Madison, WI). All primary antibodies for immunofluorescence were as previously described (15, 16, 39) and rhodamine- or FITC-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Synthetic peptides were obtained from Multiple Peptide Systems (La Jolla, CA). All other reagents were purchased from standard sources.

Cell Culture

CHO cells were grown in MEM supplemented with 10% FCS, penicillin, and streptomycin. Cultures were maintained in a humidified incubator with 5% CO₂.

Preparation of the HSA-SKL Conjugate

Cross-linking of a synthetic peptide with the sequence of NH₂-CRYHLKPLQSKL-COOH to HSA was performed as described (39). By comparing the migration of unmodified HSA and the modified HSA-SKL using SDS-PAGE it was estimated that ~5–8 peptides were attached to each HSA molecule.

Preparation of Cytosol

Nuclease-treated or untreated rabbit reticulocyte lysate as obtained from the manufacturer was diluted in transport buffer and used to reconstitute peroxisomal protein import in permeabilized cells. The protein concentration of reticulocyte lysate was ~50 mg/ml as determined by BioRad protein assay (BioRad Laboratories, Cambridge, MA). Maximum stimulation of peroxisomal protein import was observed when 25 mg/ml of reticulocyte lysate was used.

Brain cytosol was prepared as described previously (22) and was a generous gift of Dr. V. Malhotra (Univ of California, San Diego, CA). Brain cytosol had a protein concentration of ~10 mg/ml and showed maximum stimulation of peroxisomal protein import when used at 4 mg/ml.

For preparation of cytosol from cell culture, exponentially growing CHO cells were trypsinized from tissue culture plates. Trypsin was inactivated by the addition of 10 mM Tris/HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, and 0.7 mM Na₂HPO₄ (buffer A) containing 10% serum and the cells were collected by low speed centrifugation. The cells were washed two times in buffer A and once in 25 mM Tris/HCl, pH 7.4, 0.25 M sucrose (buffer B). The cell pellet was resuspended in 4 vols of buffer B (containing 1 μ g/ml each of aprotinin, leupeptin, and pepstatin and 1 mM PMSF) and was homogenized in a tight-fitting steel breaker by 5–10 strokes. The homogenate was centrifuged for 90 min at 100,000 *g* and the resulting supernatant was dialyzed against transport buffer (20 mM Hepes/KOH, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 5 mM magnesium acetate, 1 mM EGTA, 2 mM DTT and 1 μ g/ml each of aprotinin, leupeptin and pepstatin). The protein concentration was ~5 mg/ml and showed maximum stimulation of peroxisomal protein import at 2 mg/ml.

Permeabilization of Cells with SLO and Transport Assay

Two differing cell-permeabilization procedures were developed, allowing study of peroxisomal protein import when it is either independent or dependent on externally added cytosol.

To study peroxisomal protein import under conditions when it is independent from externally added cytosol, CHO cells were grown to subconfluent densities on coverslips. The coverslips were rinsed three times in

transport buffer, blotted to remove excess buffer, and inverted over a drop of transport buffer containing 0.2 U/ml SLO. The cells were permeabilized for 5 min at 32°C and the coverslips were rinsed 10 times in transport buffer to remove excess SLO. The coverslips were then inverted over a drop of transport buffer containing 50–100 μ g/ml of the HSA-SKL conjugate or 200–400 μ g/ml luciferase. To allow peroxisomal import, transport buffer had to be supplemented with an ATP-regenerating system consisting of 1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase. After incubation for up to 45 min at 37°C (humidified box floating in a waterbath), the coverslips were rinsed 10 times in transport buffer. The cells were fixed with 4% paraformaldehyde in PBS and subjected to indirect immunofluorescence to study the intracellular localization of HSA-SKL or luciferase.

To study peroxisomal protein import when it is dependent on externally added cytosol, CHO cells were grown on Pronectin F-coated coverslips to confluent density to ensure a stronger attachment of cells. The cells were permeabilized as described above, only that 2.0 U/ml of SLO was used and incubation with SLO was extended to 10 min. After permeabilization the coverslips were incubated for 15 min in 1 ml of cold transport buffer (4°C) to ensure sufficient leakage of internal cytosol. The coverslips were then incubated as described above with transport buffer containing HSA-SKL, an ATP-regenerating system and differing concentrations of cytosol.

Immunofluorescence Microscopy

Indirect immunofluorescence was performed to study the intracellular localization of various proteins. The cells were fixed by incubation in PBS containing 4% paraformaldehyde for 30 min, treated for 5 min with or without 1% Triton X-100 in PBS and washed with PBS. All antibody dilutions were in PBS and incubation was performed in a humidified chamber for 30 min each, followed by extensive rinsing of the coverslips. Finally the coverslips were mounted on microscope slides using Mowiol containing 0.1% *p*-phenylenediamine.

Quantitation of LDH Activity

Cells grown on coverslips were permeabilized with differing concentrations of SLO (0.2–2.0 U/ml), washed and scraped in transport buffer containing 0.5% Triton X-100. After centrifugation for 10 min at 12,000 *g* the supernatant was used as a cell extract to determine the remaining LDH activity. LDH activity was measured using a colorimetric diagnostic kit from Sigma Chemical Co. as described by the manufacturer. Relative activity units were determined and release of LDH from permeabilized cells was calculated from the LDH activity retained within the cells compared with that of non-permeabilized cells.

Treatment of Cytosol or Cells with NEM

Cytosol fractions were diluted with transport buffer to lower the DTT concentrations to 0.5 mM. Treatment of cytosol was performed with 7–14 mM NEM for 15 min at 4°C, followed by the addition of 14–28 mM DTT to quench excess NEM. Cells to be treated with NEM were permeabilized with SLO, rinsed in transport buffer devoid of DTT and incubated for 10 min in transport buffer containing 20 μ M NEM, followed by the addition of 2 mM DTT to quench excess NEM. In a control experiment cells were incubated with 200 μ M NEM in presence of 2 mM DTT.

Results

SLO Permeabilizes the Plasma Membrane but not the Peroxisomal Membrane of Mammalian Cells

To study the biochemistry of protein import into peroxisomes we developed a permeabilized cell system. Cells were permeabilized with SLO, a bacterial cytolysin which binds to cholesterol of the plasma membrane and forms pores 20–30 nm in diameter (3). The dimension of the pores allows the exchange of cytosolic components of up to 150 kD or even larger. CHO cells grown on coverslips were incubated for 5 min at 37°C with 0.2 U/ml SLO and excess SLO was washed away. Permeabilization and access to the cell interior was assayed by incubating the cells with anti-tubulin antibod-

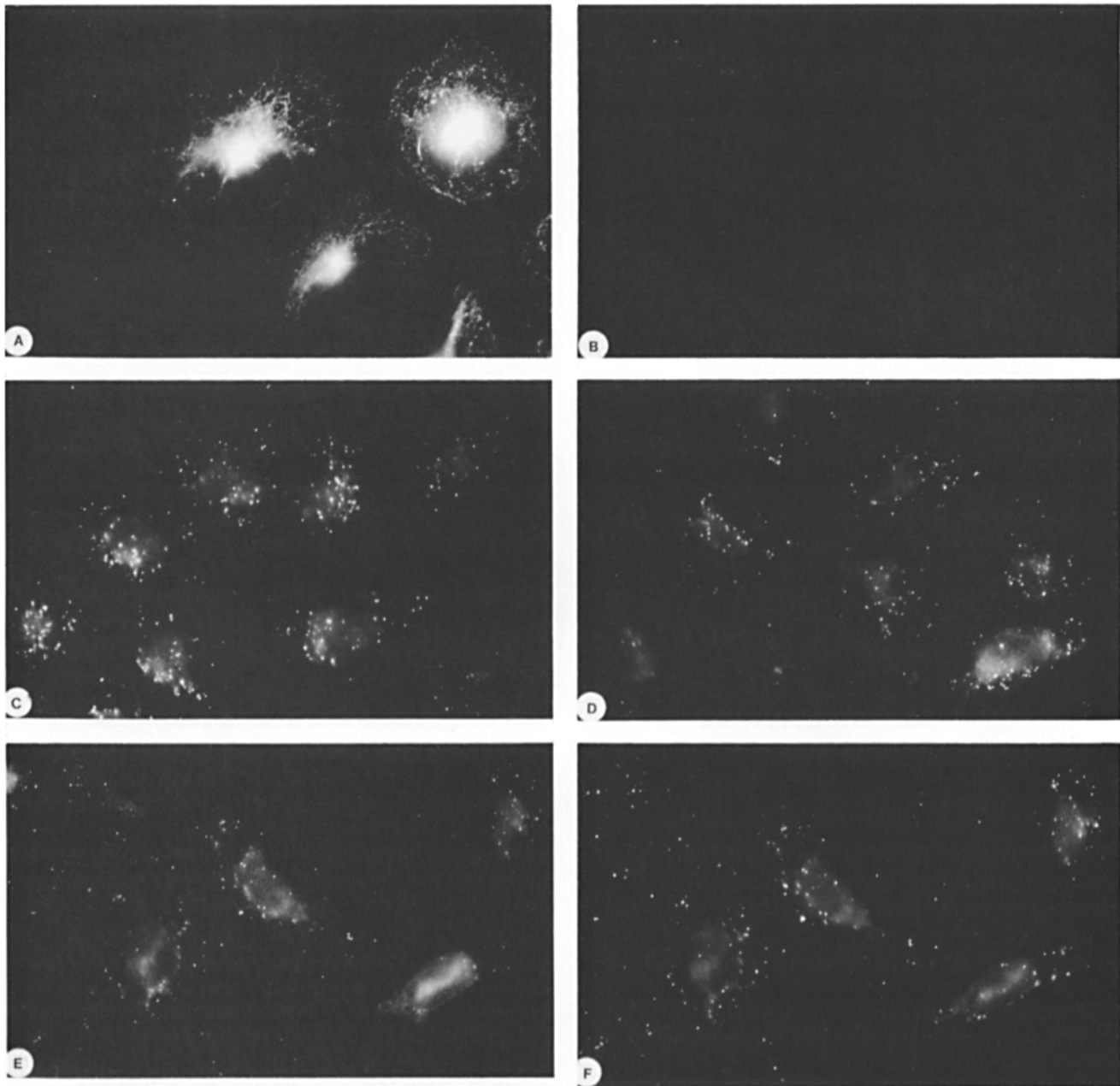


Figure 1. Permeabilization of CHO cells with SLO and reconstitution of peroxisomal protein import. CHO cells were grown on coverslips and treated with (A) or without (B) 0.2 U/ml SLO. The cells were fixed and incubated with rabbit anti-tubulin antibodies followed by rhodamine-coupled goat anti-rabbit antibodies. For reconstitution of peroxisomal protein import, SLO-permeabilized cells were incubated for 45 min at 37°C with HSA-SKL (C) or luciferase (D) in transport buffer supplemented by an ATP-regenerating system. The cells were then fixed, treated with Triton X-100, and processed for indirect immunofluorescence. Cells were first incubated with rabbit antibodies against HSA (C) or luciferase (D) and in the second step with goat anti-rabbit antibodies coupled to FITC. To analyze whether the punctate staining represented peroxisomal localization, SLO-permeabilized cells incubated with HSA-SKL were processed for double labeling indirect immunofluorescence. In the first step cells were incubated with guinea pig anti-HSA (E) and rabbit anti-catalase antibodies (F) and in the second step with goat anti-guinea pig antibodies coupled with FITC (E) and goat anti-rabbit antibodies coupled with rhodamine (F).

ies. More than 90% of the cells permeabilized with SLO showed staining of the cytoskeleton with anti-tubulin antibodies (Fig. 1 A), while mock-treated cells showed no staining (Fig. 1 B). Furthermore, we confirmed that only the plasma membrane was permeabilized by SLO treatment, while the peroxisomal membrane remained intact. Staining of peroxisomal matrix proteins in SLO-permeabilized cells, as analyzed by anti-catalase or anti-SKL antibodies, was

negative and only possible after the cells were treated with the detergent Triton X-100 (not shown). Thus, under the conditions used, SLO treatment of CHO cells results in permeabilization of the plasma membrane allowing the entry of macromolecules to the cell interior, while the internal peroxisomal membranes remain intact as judged by their impermeability to antibodies that recognize peroxisomal matrix proteins.

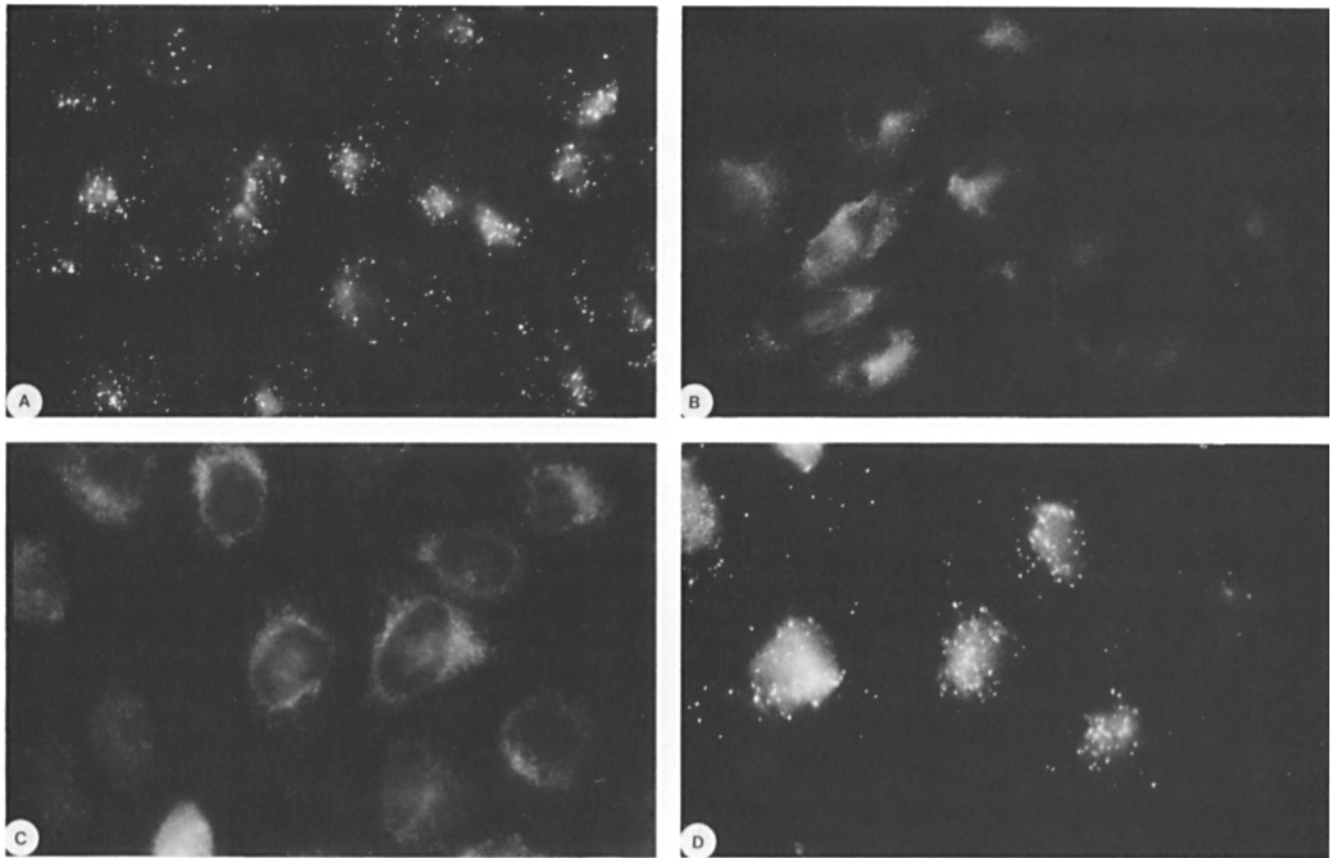


Figure 2. HSA-SKL is imported into the matrix of peroxisomes and import is strictly signal dependent. SLO-permeabilized cells were incubated with HSA-SKL as described in Fig. 1, fixed, and treated with (A) or without (B) Triton X-100 before processing for indirect immunofluorescence analysis. To study signal dependence of import, permeabilized cells were incubated with HSA-SKL in presence of a 50-fold excess of a 12-mer peptide (CRYHLKPLQSKL-COOH) ending in the SKL sequence (C) or a 50-fold excess of a control peptide lacking the SKL tripeptide (KKKKCHEDMFASDEEA-COOH) (D). Indirect immunofluorescence using rabbit anti-HSA antibodies was performed as described in Fig. 1.

Reconstitution of Peroxisomal Protein Import in SLO-permeabilized Cells

Previous studies have shown that cross-linking of a dodecameric peptide ending in the peroxisomal targeting sequence SKL-COOH, to HSA, creates an artificial substrate for the peroxisomal import machinery (39). We used this conjugate (HSA-SKL) in our present studies to monitor the import into peroxisomes.

CHO cells were grown on coverslips and permeabilized with SLO. The cells were subsequently incubated with HSA-SKL in transport buffer supplemented with an ATP-regenerating system. After incubation, the localization of HSA-SKL was analyzed by immunofluorescence. We observed an accumulation of HSA-SKL in vesicular structures resembling peroxisomes (Fig. 1 C). By colocalization with catalase these vesicular structures were identified as peroxisomes (Fig. 1, E and F).

To confirm that the HSA-SKL conjugate mimics the import of natural peroxisomal proteins, SLO-permeabilized cells were incubated with luciferase. Luciferase accumulated in vesicular structures (Fig. 1 D), as did HSA-SKL, that were identified as peroxisomes by colocalization with catalase (data not shown). Notably, efficient import of luciferase required a three- to fourfold higher molar concen-

tration compared with that of the HSA-SKL conjugate. The differing import efficiencies are probably due to the multiplicity of the SKL-targeting signal, which is present 5–8 times per HSA-SKL molecule whereas luciferase contains it only once.

The Observed Import Occurs into the Matrix of Peroxisomes and Is Signal Dependent

To address whether HSA-SKL was truly imported into the peroxisomal matrix or just binding to the peroxisomal surface, two sets of coverslips with CHO cells were permeabilized in parallel, incubated with HSA-SKL, and treated with or without Triton X-100 before processing for immunofluorescence (Fig. 2, A and B). The peroxisomal localization of HSA-SKL was only observed when the cells were treated with Triton X-100 (Fig. 2 A). This showed that HSA-SKL is imported into the matrix of peroxisomes, where it is inaccessible to antibodies unless the peroxisomal membrane is permeabilized. Furthermore, this result revealed that the membrane of peroxisomes remains impermeable to antibodies throughout the import assay.

To study whether the import of HSA-SKL was signal dependent, we added a 50 molar excess of the free SKL peptide to the transport buffer. The import of HSA-SKL was completely abolished by the excess of the SKL peptide, while the

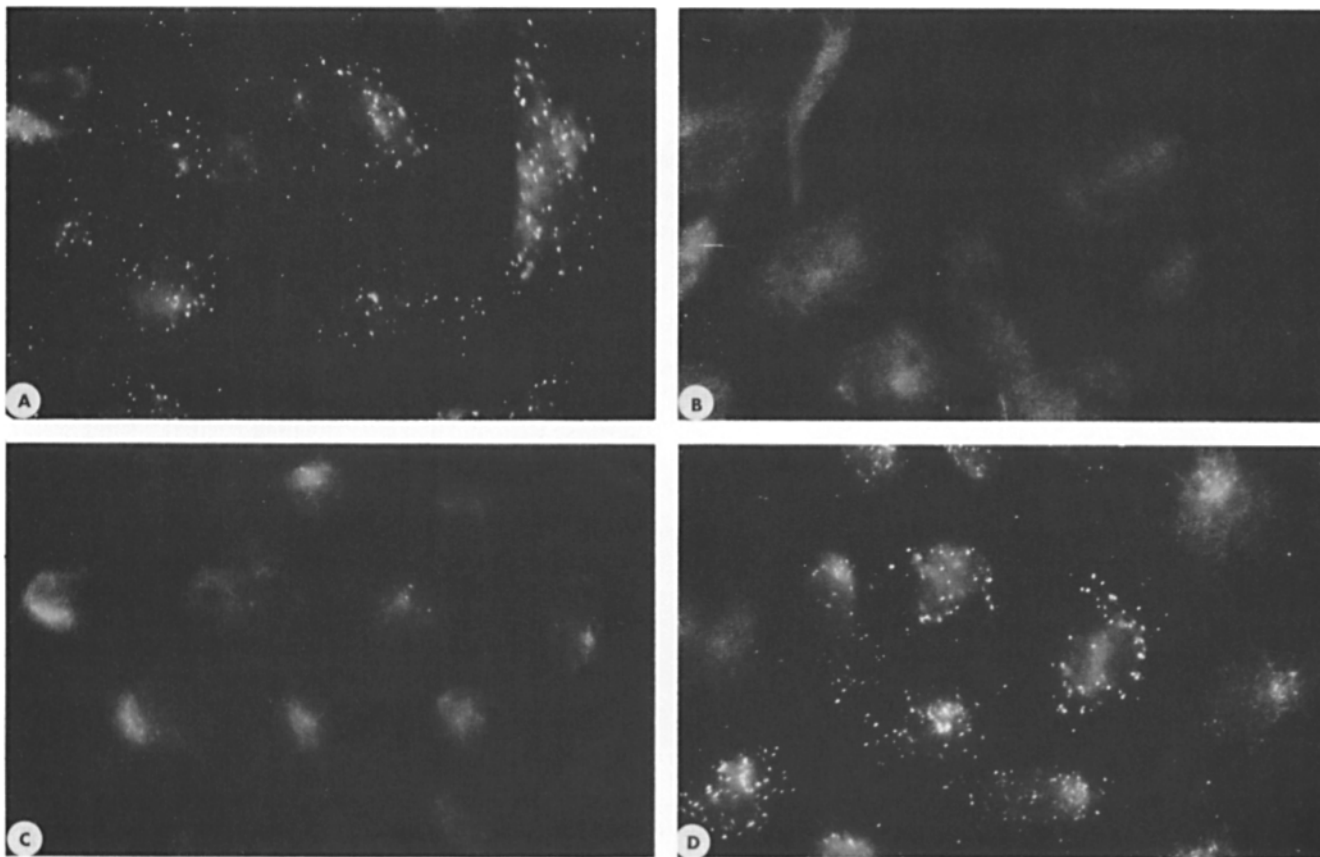


Figure 3. Peroxisomal protein import requires ATP hydrolysis. SLO-permeabilized cells were incubated with HSA-SKL in transport buffer supplemented by an ATP-regenerating system (A) or in absence of ATP (B). To see whether ATP hydrolysis is required, 1 mM ATP was replaced by 1 mM ATP γ S (C) or the transport buffer was supplemented in addition to the ATP-regenerating system with 100 μ M GTP γ S (D). Indirect immunofluorescence was performed as described in Fig. 1.

same excess of a control peptide lacking the SKL signal did not affect the import of HSA-SKL (Fig. 2, C and D). Testing different concentrations of the SKL peptide, we found that a 10-fold excess of the peptide was sufficient to block the import of HSA-SKL (not shown).

Peroxisomal Protein Import Requires Hydrolysis of ATP but no Membrane Potential

When SLO-permeabilized cells were incubated with HSA-SKL in transport buffer without ATP, the import of HSA-SKL dropped below the level of detection (compare Fig. 3, A and B). When ATP was replaced by ATP γ S no import was observed (Fig. 3 C), indicating a strict requirement for ATP hydrolysis during peroxisomal protein import.

For several transport pathways an involvement of GTP binding proteins has been shown (5, 25). The presence of GTP γ S (100 μ M) did not alter the efficiency of import of HSA-SKL into peroxisomes (Fig. 4 D). We conclude that GTP-hydrolyzing proteins are not involved in the import of proteins into peroxisomes.

Import of HSA-SKL was not altered in the presence of the ionophore carbonylcyanide-*m*-chlorophenylhydrazone (CCCP, 10 μ M) (Fig. 4, A and B), suggesting that a membrane potential is not required for import of proteins into peroxisomes.

Peroxisomal Protein Import Is Temperature and Time Dependent

No detectable import was observed when the cells were incubated with HSA-SKL at 4°C (Fig. 4 C). In comparing import at 22, 30, and 37°C, the most efficient accumulation of HSA-SKL in peroxisomes was at 37°C (not shown).

A study of the kinetics of the import of HSA-SKL into peroxisomes revealed detectable accumulation of HSA-SKL in peroxisomes after a lag-phase of \sim 20 min (Fig. 5, A-C). Import into peroxisomes continued for up to 60 min of incubation as reflected by the increasing signal strength (Fig. 5, D-F).

According to *in vitro* studies (19), peroxisomal protein import is initiated by a temperature-independent binding of proteins to peroxisomes. However, we did not detect HSA-SKL bound to peroxisomes after incubation at 4°C. This may be due to the inability to detect low amounts of HSA-SKL bound to peroxisomes by immunofluorescence. We assumed that a rate-limiting binding step may be identified by preincubating cells for 20 min at 4°C and then shifting them to 37°C. If significant binding takes place at 4°C, the kinetics of translocation may be accelerated after the temperature shift. However, following the temperature shift a lag-phase of \sim 20 min passed before a detectable amount of HSA-SKL was translocated into peroxisomes (not

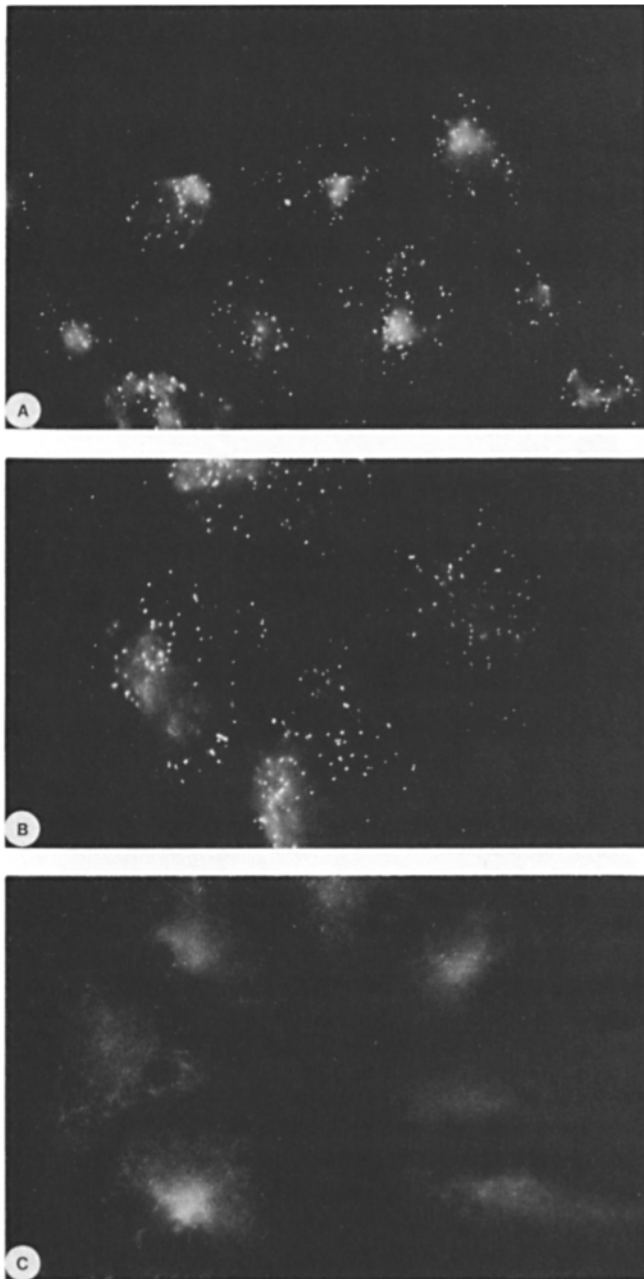


Figure 4. Peroxisomal protein import does not require a membrane potential and is temperature dependent. SLO-permeabilized cells incubated with HSA-SKL in absence (A) or presence of 10 μ M CCCP (B). (C) Cells incubated with HSA-SKL at 4°C instead of 37°C (A and B).

shown). This indicated that significant binding did not take place or that the binding sites were limited. In a further control, cells were preincubated for 20 min with HSA-SKL at 4 or 22°C. After extensive washing the cells were incubated for 45 min at 37°C in transport buffer containing the ATP-regenerating system but without addition of further HSA-SKL. Only a small amount of HSA-SKL was found in peroxisomes in the cells preincubated with HSA-SKL at 4°C, while the cells preincubated with HSA-SKL at 22°C translocated a significantly increased amount of HSA-SKL into peroxisomes (not shown). These results indicate that a

pretranslocation binding is enhanced at elevated temperatures.

Import of Proteins into Peroxisomes Depends on Cytosolic Factors

With the SLO concentrations used in our initial experiments, import of HSA-SKL was efficient without the addition of external cytosol and addition of external cytosol did not increase the import of HSA-SKL into peroxisomes (not shown). However, an insufficient release of internal cytosol may have masked the detection of cytosol dependence. The release of cytosolic components from SLO-permeabilized cells was assayed by measuring the activity of lactate dehydrogenase, a cytosolic marker enzyme of 135 kD. To our surprise we found that after treatment with 0.2 U/ml of SLO <10% of the LDH activity was released from the cells. Permeabilization of cells with 2.0 U/ml of SLO followed by an incubation in transport buffer for 15 min (see Materials and Methods) resulted in the loss of ~80% of the LDH activity. When cells permeabilized with the increased amount of SLO were incubated with HSA-SKL without the addition of external cytosol, the import into peroxisomes was reduced to non-detectable background levels in ~90% of the cells and some residual import was observed in 5–10% of the cells (Fig. 6 A). When external cytosol was added in form of rabbit reticulocyte lysate, import was reconstituted and accumulation of HSA-SKL in peroxisomes was found in >80% of the cells (Fig. 6 B). As with the reticulocyte lysate, import was stimulated by preparations of cytosol from bovine brain or CHO cells (shown in Fig. 6 C for bovine brain cytosol). However, BSA alone could not substitute for cytosol in the restoration of peroxisomal import (not shown). The import-stimulating activity in cytosol appears to be of protein nature, as the heating of the cytosolic extract at 95°C for 15 min destroyed its activity (Fig. 6 D).

Evidence for Cytosolic SKL-binding Sites

As shown in Fig. 2 the import of HSA-SKL can be blocked by the presence of free SKL peptide. This demonstrates that the import machinery is saturable and we propose that the SKL peptide blocks SKL-specific binding sites. To study the nature of these presumed binding sites we performed a two-step import assay. CHO cells were permeabilized with 0.2 U/ml SLO (import independent from external cytosol) and in the first step preincubated for 10 min at 22°C in transport buffer containing varying concentrations of the SKL peptide. After extensive washing the cells were incubated in the second step with HSA-SKL as under standard import conditions. We found that the initial incubation with the SKL-peptide completely blocked the import of HSA-SKL in the second step, when the peptide was used at a concentration >30 μ g/ml (Fig. 7 A). This showed that the import machinery could be blocked before the incubation with HSA-SKL. Furthermore, since extensive washing did not release the import block, this experiment provided even stronger evidence for the presence of saturable SKL-specific binding sites. Notably, the blocking effect of the SKL peptide was greatly reduced, when cells were preincubated with the SKL peptide at 4°C (not shown) as compared with the preincubation at 22°C.

We assumed that the import block may be caused by the

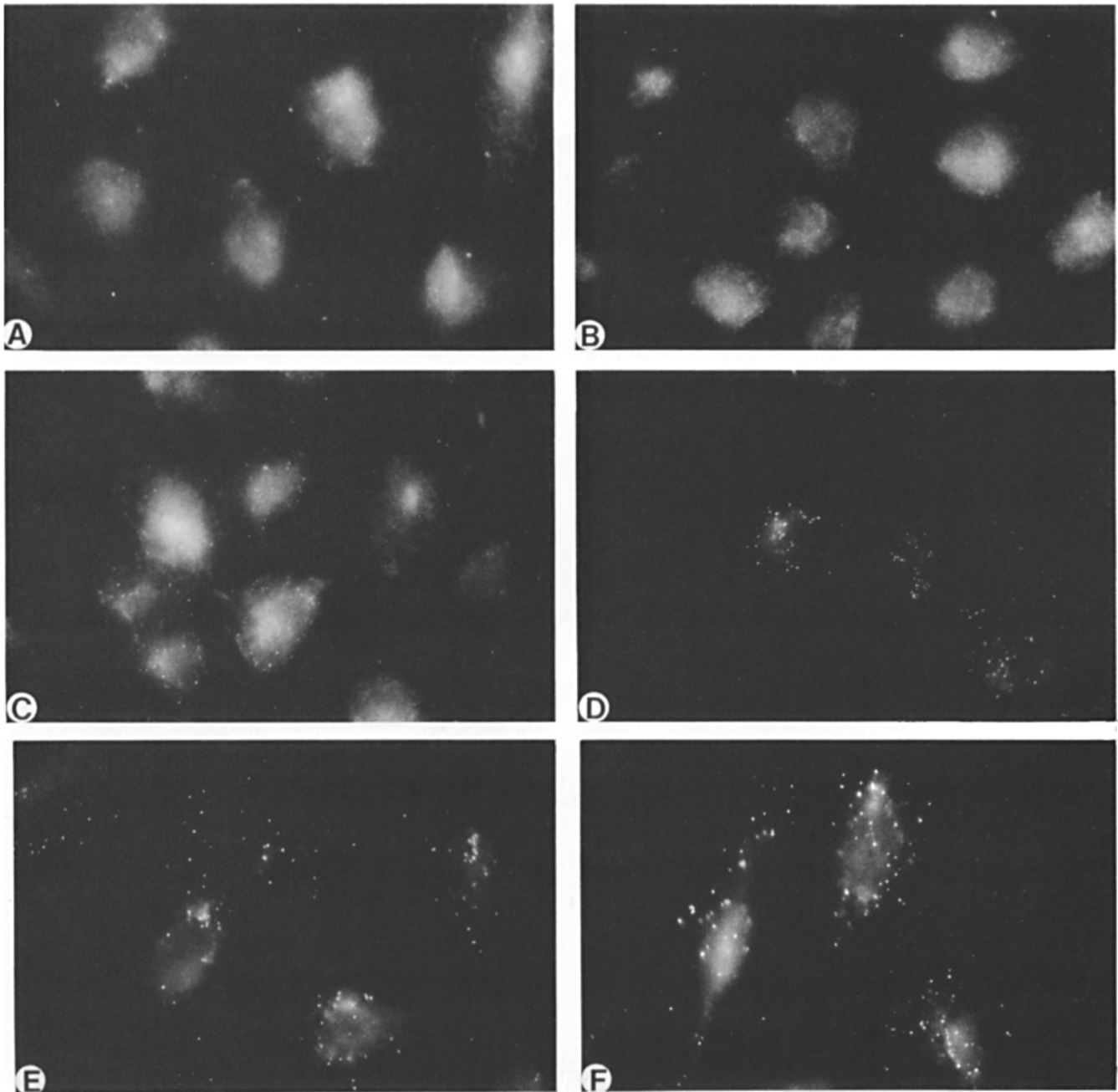


Figure 5. Time dependence of peroxisomal protein import. SLO-permeabilized cells were incubated with HSA-SKL for various periods of time. Localization of HSA-SKL is shown after 5 (A), 15 (B), 20 (C), 30 (D), 40 (E), and 60 (F) min.

binding of the SKL peptide to cytosolic binding sites and may be released by the addition of external cytosol. CHO cells were permeabilized with 0.2 U/ml SLO and preincubated in the first step with 25 $\mu\text{g/ml}$ of the SKL peptide. After washing, the cells were incubated in the presence or absence of external cytosol with HSA-SKL. As shown in Fig. 7 C, in absence of external cytosol the import of HSA-SKL was drastically reduced by the initial incubation with the SKL peptide. The addition of cytosol in the form of reticulocyte lysate, however, reconstituted import to near normal (Fig. 7 D). Thus, the import of HSA-SKL after the initial incubation with the SKL peptide became dependent on the addition of external cytosol. The cytosol dependence was not caused by a leakage of endogenous cytosol, since the

incubation of cells in the first step with 80 $\mu\text{g/ml}$ of a control peptide lacking the SKL-targeting signal followed by extensive washing did not affect the import of HSA-SKL (Fig. 7 B). We suggest that the SKL peptide binds to cytosolic binding sites and thereby blocks the import of HSA-SKL. The addition of external cytosol provides new binding sites which then allow the import of HSA-SKL into peroxisomes.

NEM-sensitive Factors Are Involved in the Import Machinery

NEM alkylates free thiol groups. It has been shown that a number of NEM-sensitive factors are involved in different intracellular transport pathways (1, 2, 8, 14). NEM treatment

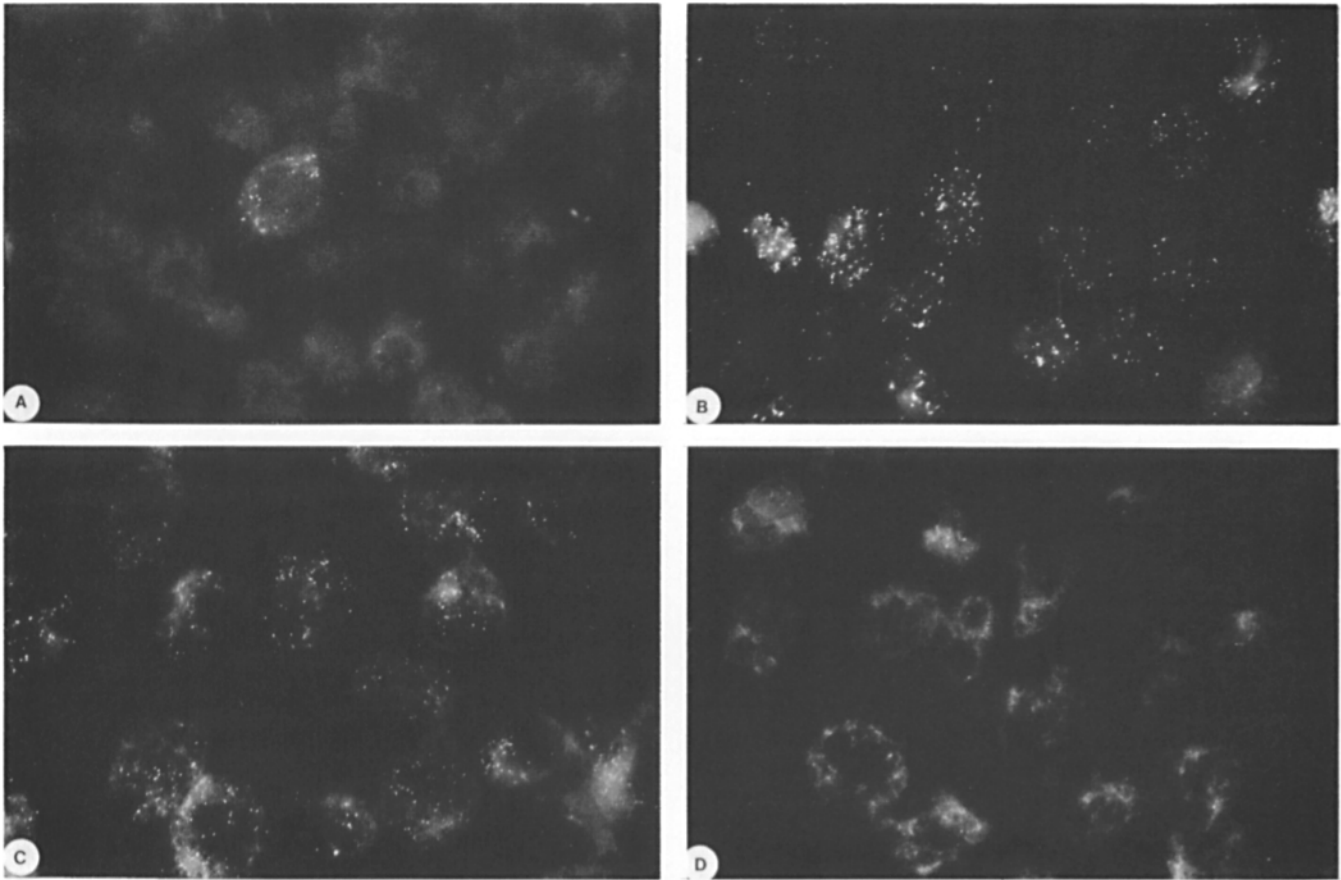


Figure 6. Cytosol dependence of peroxisomal protein import. CHO cells were permeabilized with 2.0 U/ml SLO and incubated with HSA-SKL in transport buffer without (A) or with (B) external cytosol in form of rabbit reticulocyte lysate. In (C) and (D) cells were incubated with HSA-SKL in presence of bovine brain cytosol which was intact (C) or heat inactivated (D). For heat-inactivation, cytosol was incubated for 15 min at 95°C and then centrifuged for 5 min at 12,000 *g* to remove aggregates of denatured protein.

(7–14 mM) of cytosol did not destroy its ability to stimulate peroxisomal protein import (compare Fig. 8, A and B). In contrast, when SLO-permeabilized cells were treated with as little as 20 μ M NEM, peroxisomal protein import was completely abolished (Fig. 8 C). Addition of external cytosol to NEM-treated cells did not reconstitute peroxisomal protein import (Fig. 8 D). To show that the lack of import was a specific result of thiol groups alkylated by NEM, we incubated cells with 0.2 mM NEM in the presence of 2 mM DTT. As expected these cells retained the ability to accumulate HSA-SKL in peroxisomes (Fig. 8 E).

Thus it appears that NEM-sensitive membrane-bound or associated proteins are involved in the peroxisomal import machinery. In contrast to many other intracellular transport steps that require cytosolic factors which are NEM sensitive, cytosolic factors for peroxisomal protein targeting are insensitive to NEM at the concentration used.

Discussion

The use of permeabilized cell systems has greatly facilitated the reconstitution of a variety of protein trafficking steps such as transfer from the ER to the Golgi complex (4, 6, 26), transport between Golgi cisternae (29), vacuolar transport (37), or nuclear transport (1, 2). The development of such a system for the transport of proteins into the peroxisomal matrix represents a significant advance that should allow the

elucidation of a variety of parameters relevant to the mechanism of peroxisomal protein import.

The permeabilization conditions we have used selectively permeabilize the plasma membrane while peroxisomal membranes remain intact. This was concluded from the observation that peroxisomal membranes in SLO-treated cells remained impermeable to antibodies against peroxisomal matrix proteins and labeling of matrix proteins required the use of Triton X-100. SLO-permeabilized cells retain the ability to import proteins into preexisting peroxisomes. Several features of the observed import are in accordance with earlier observations obtained using *in vitro* approaches (19, 23, 29, 30, 31, 35) or the microinjection system (39). These features underscore the authenticity of our permeabilized cell system and rule out artifactual aggregation or binding to the surface of peroxisomes in our system. The import of the marker proteins was strictly signal dependent and they were translocated into the matrix of catalase-containing vesicles. Furthermore, translocation was time and temperature dependent and required ATP hydrolysis. GTP would not substitute for ATP and a membrane potential was not necessary for peroxisomal import.

The time, temperature, and energy dependence for the translocation of proteins into peroxisomes indicate that it is an active transport process.

The inhibition of import caused by the presence of free SKL peptide during the import assay demonstrates the signal

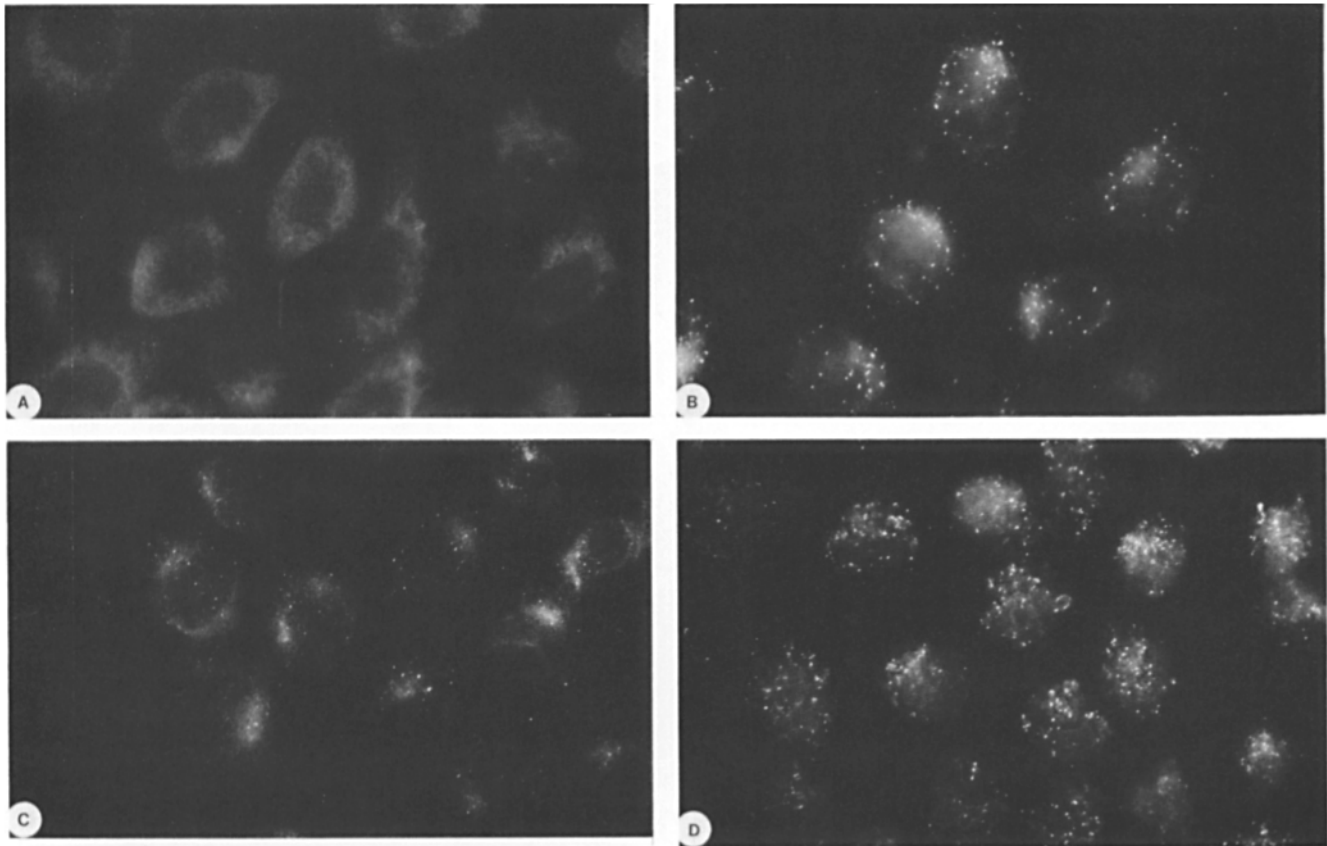


Figure 7. A two-step import assay provides evidence for cytosolic SKL-binding sites. Cells were permeabilized with 0.2 U/ml SLO and in the first step incubated for 10 min in transport buffer containing 30 $\mu\text{g/ml}$ of the SKL peptide (A) or 80 $\mu\text{g/ml}$ of a control peptide lacking the SKL targeting sequence (B). The cells were washed extensively and were subsequently incubated for 45 min with transport buffer containing HSA-SKL and an ATP-regenerating system. (C and D) Cells were permeabilized with 0.2 U/ml SLO, incubated for 10 min in transport buffer containing 25 $\mu\text{g/ml}$ of the SKL peptide, washed extensively and were then incubated for 45 min in absence (C) or presence of rabbit reticulocyte lysate (D) with HSA-SKL under standard import conditions.

dependence of the import and also shows that the import machinery is saturable. It is unlikely that the inhibition of import by SKL peptide can be attributed to saturation of the peroxisomal matrix with peptide. As shown in Fig. 7, after import was inhibited with SKL peptide, the simple addition of cytosol restored the import of HSA-SKL into peroxisomes.

The translocation of proteins into peroxisomes is supposed to be initiated by a binding of protein to the peroxisomal surface. According to *in vitro* studies (19) this binding is temperature and energy independent. Analyzing the intracellular distribution of HSA-SKL after incubation with permeabilized cells, we were able to detect its accumulation in the peroxisomal matrix but unable to discern its distribution before translocation. Therefore, our conclusions on pretranslocation stages are indirect. The strongest indication for a binding step preceding the translocation event comes from the observation that preincubation of cells with the SKL peptide blocks import of HSA-SKL (Fig. 7). Three observations indicate this binding is enhanced at elevated temperature.

First, in the two-step import assay the blocking effect of the SKL peptide was significantly reduced if the cells were preincubated with the SKL peptide at 4°C as compared with 22°C. Second, when cells were incubated with HSA-SKL at

4°C and then shifted to 37°C, the lag-phase after which an identifiable amount of HSA-SKL was translocated into peroxisomes was not reduced. Finally, cells preincubated with HSA-SKL at 4°C, washed and incubated at 37°C without further addition of HSA-SKL accumulated only small amounts of HSA-SKL in peroxisomes. In contrast, cells incubated in the same way, only that the preincubation with HSA-SKL was at 22°C, translocated a significantly increased amount of HSA-SKL into peroxisomes.

Since we are unable to discern pretranslocation stages our observations are not necessarily in conflict with the findings of Imanaka et al. (19), who observed efficient binding to peroxisomes at low temperatures. One possible explanation is that the binding to cytoplasmic factors is enhanced at elevated temperatures, while the following step, the binding to the peroxisomal surface is temperature independent in permeabilized cells. Furthermore, since the blocking effect of the SKL peptide was independent of the presence of ATP (not shown), we suggest that the binding step is energy independent, which is in accordance with the observations of Imanaka et al. (19).

Bellion and Goodman (7) observed that the treatment of *Candida boidinii* cells with the ionophore CCCP prevented the import of alcohol oxidase into peroxisomes and its subsequent octamerization. However, since the treatment of cells

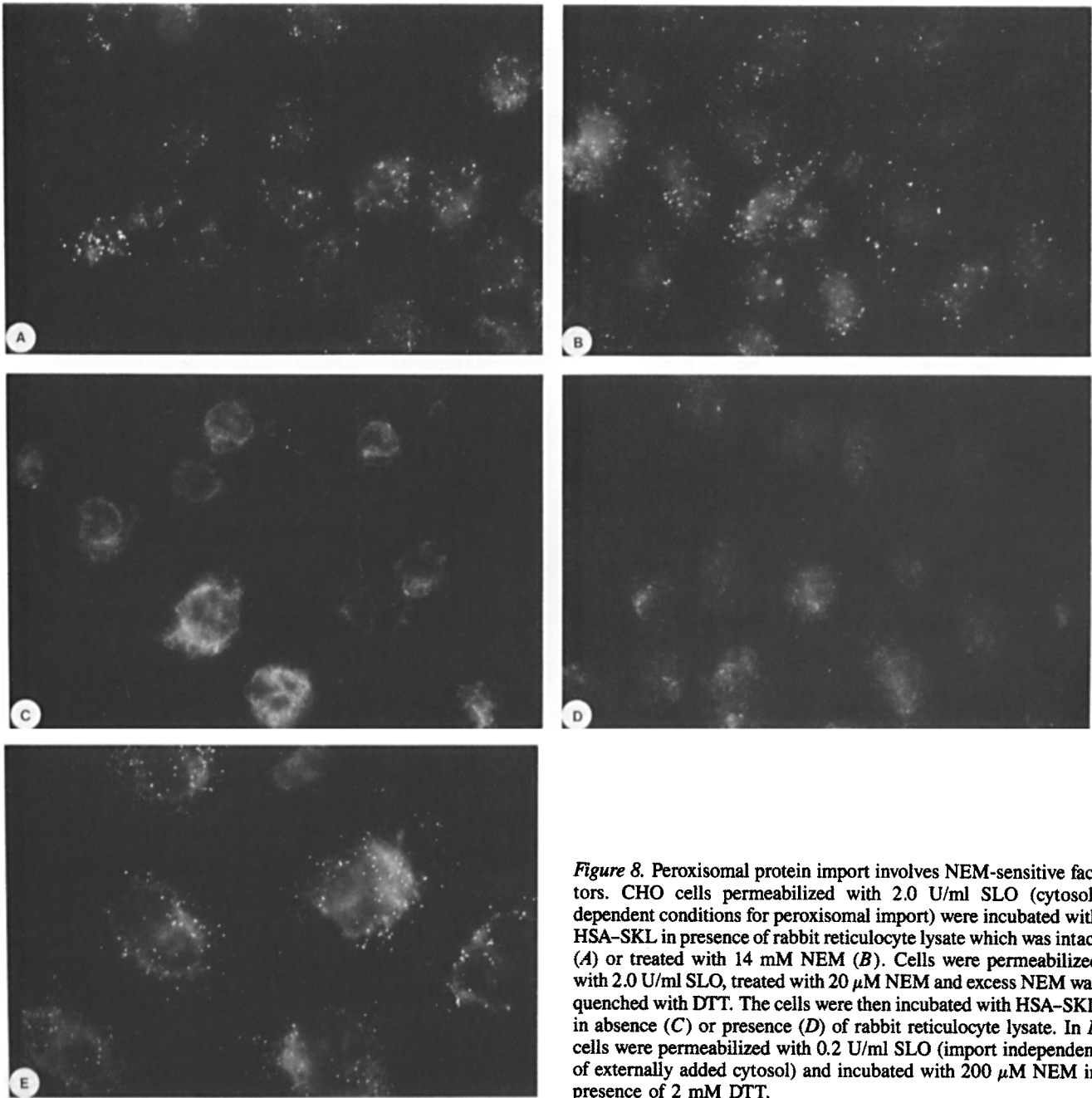


Figure 8. Peroxisomal protein import involves NEM-sensitive factors. CHO cells permeabilized with 2.0 U/ml SLO (cytosol-dependent conditions for peroxisomal import) were incubated with HSA-SKL in presence of rabbit reticulocyte lysate which was intact (A) or treated with 14 mM NEM (B). Cells were permeabilized with 2.0 U/ml SLO, treated with 20 μ M NEM and excess NEM was quenched with DTT. The cells were then incubated with HSA-SKL in absence (C) or presence (D) of rabbit reticulocyte lysate. In E cells were permeabilized with 0.2 U/ml SLO (import independent of externally added cytosol) and incubated with 200 μ M NEM in presence of 2 mM DTT.

also resulted in a significant drop in intracellular ATP concentrations it remained open whether the blocked import was due to the disruption of a membrane potential across the peroxisomal membrane or due to the lack of ATP. Our permeabilized cell system and the *in vitro* system using purified peroxisomes (19) show that a membrane potential is not required for peroxisomal protein import.

A major breakthrough in our studies is the demonstration of cytosol dependence for peroxisomal protein import, a feature which has not been identified with the *in vitro* import assay (19, 23, 29, 30, 31, 35) or the microinjection system (39). The inability of heat-treated cytosol to support peroxisomal protein import suggests that proteins rather than small molecules are being supplied by the cytosol. The involvement of cytosolic factors for import in the *in vitro* sys-

tem (19) may have been masked by the addition of rabbit reticulocyte lysate along with the translated protein, at a concentration sufficient to stimulate peroxisomal import. Cytoplasmic factors are potential candidates for PTS receptors (SKL-binding proteins) and further work is in progress to address this interesting question. Very promising in this regard are the observations of the two-step import assay. In cells retaining endogenous cytosol, peroxisomal protein import can be blocked by an incubation with the SKL-peptide before the incubation with HSA-SKL. Reconstitution of peroxisomal protein import depends on the addition of external cytosol. In our model the SKL peptide binds to cytosolic SKL-binding sites and by saturation blocks peroxisomal protein import. Providing new binding sites by the addition of external cytosol releases the import block. An alternative ex-

planation is that cytosol provides activities necessary to unfold HSA-SKL or luciferase before they are imported into the peroxisomal matrix.

Another feature, not identified so far, is that the import machinery involves NEM-sensitive factors. Our results indicate that these NEM-sensitive factors are membrane or membrane-associated proteins.

In conclusion, we have established a novel permeabilized cell system to study peroxisomal protein import overcoming some of the limitations encountered when purified peroxisomes are used. The identification of cytosol dependence for peroxisomal protein import is novel and promising for the search of PTS receptors as well as other proteins (such as chaperonins) necessary for protein import into peroxisomes. Furthermore, it will be of interest to identify the membrane or membrane-associated NEM-sensitive factors which are apparently involved in the translocation event. Another advantage of our system is the ability to study peroxisomal protein import in cell lines from Zellweger patients, which have been shown to have defects in the peroxisomal import machinery (27, 28, 39). We hope to gain insights into the underlying molecular defects in these human disorders by the application of the permeabilized cell system described here.

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