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Transport of Microinjected Proteins into Peroxisomes of Mammalian Cells: Inability of Zellweger Cell Lines To Import Proteins with the SKL Tripeptide Peroxisomal Targeting Signal

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Previous work has shown that the firefly (*Photinus pyralis*) luciferase contains a C-terminal peroxisomal targeting signal consisting of the tripeptide Ser-Lys-Leu. This report describes the microinjection of two proteins, (i) luciferase and (ii) albumin conjugated to a peptide ending in the sequence Ser-Lys-Leu, into mammalian cells grown in tissue culture. Following microinjection, incubation of the cells at 37°C resulted in peroxisomal transport of these exogenous proteins into catalase-containing vesicles. The translocation was both time and temperature dependent. The transport could be inhibited by coinjection of synthetic peptides bearing various peroxisomal targeting signal motifs. These proteins could be transported into peroxisomes in normal human fibroblast cell lines but not in cell lines derived from patients with Zellweger syndrome. These results demonstrate that microinjection of peroxisomal proteins yields an authentic in vivo system with which to study peroxisomal transport. Furthermore, these results reveal that the process of peroxisomal transport does not involve irreversible modification of the protein, that artificial hybrid substrates can be transported and used as tools to study peroxisomal transport, and that the defect in Zellweger syndrome is indeed the inability to transport proteins containing the Ser-Lys-Leu targeting signal into the peroxisomal lumen.

Peroxisomes are single-membrane-bound organelles found in almost all eukaryotic cells. The functions of this biochemically diverse organelle vary from one organism to another and are dependent upon cell type and environmental conditions (31). Though peroxisomes appear to be nonessential at the cellular level, it is clear that functional peroxisomes are required for normal human development. A class of autosomal recessive diseases known as peroxisomopathies (Zellweger syndrome, infantile Refsum's disease, etc.) is characterized by severe neurological, hepatic, and renal defects resulting in death within the first few months to years after birth (34). In cells from patients with these disorders, typical peroxisomes are absent though peroxisome ghosts can be detected (26, 27). These ghosts possess normal amounts of certain peroxisomal membrane proteins but lack peroxisomal matrix proteins, which are either absent altogether or present in the cytoplasm (17, 18, 28, 29, 32). Though none of the genes responsible for these human disorders have been identified, defects in any of at least eight different genetic loci may lead to these disorders (1, 21a). It has been suggested that the molecular basis of the Zellweger syndrome family of diseases may be the inability to transport matrix proteins into the peroxisome (26, 27). However, there has not yet been the demonstration of an import defect in these cells.

Proteins destined for the peroxisomes are synthesized on free polysomes in the cytoplasm (6, 8, 20, 22, 24, 25) and are transported into the peroxisome posttranslationally (5). Although at least two proteins, thiolase and acyl coenzyme A oxidase, undergo proteolytic processing after transport (7, 20, 21), most proteins are synthesized at their mature size. Transport of proteins into the peroxisome is dependent upon, among other factors, the presence of a peroxisomal targeting signal (PTS) on the newly synthesized protein. A C-terminal tripeptide PTS with the sequence serine-lysine-leucine-COOH (or a conservative variant) (9) was identified initially in firefly luciferase (11) and has subsequently been found in many peroxisomal proteins (9, 12). This signal is sufficient to direct proteins into peroxisomes in yeasts, plants, insects, and mammalian cells (2a, 10) and is present in peroxisomal proteins from each of these types of organisms.

Unfortunately, much less is known regarding the mechanism of peroxisomal protein translocation than is known about the targeting signals on the imported proteins. Transport of peroxisomal proteins appears to be a two-step process in vitro (14). The initial binding of protein to the organelle seems both energy and temperature independent, whereas the actual translocation event requires ATP and is temperature dependent (no transport at 0 to 4°C, import at 26°C). An ATPase activity (2) and a protein that binds ATP and has similarity to transport ATPases (15) have both been identified on the peroxisomal membrane.

Further elucidation of the mechanisms of peroxisomal protein import will require the use of in vitro systems for the study of this problem. Unfortunately, the fragility of purified peroxisomes, the lack of an easily identifiable hallmark for import (such as signal cleavage used to study translocation into the endoplasmic reticulum, mitochondria, and chloroplasts), and the inefficient import observed to date have combined to prevent the description of all but the most elementary aspects of peroxisomal protein import. As a first step toward the development of a reliable in vivo import system that offers many of the advantages of in vitro import assays, we have attempted to establish a microinjection system for the study of peroxisomal protein import. In this

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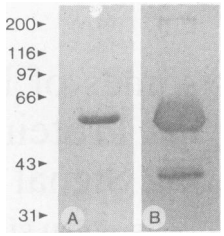


FIG. 1. SDS-polyacrylamide gel (A) and Western blot (B) of firefly luciferase. Each lane contained 10 μg of protein that was either stained with Coomassie blue or probed with anti-SKL antibodies. Numbers at left represent the positions of molecular weight markers (in kilodaltons).

study, we sought to elucidate features of peroxisomal protein transport in mammalian cells by monitoring the import of natural and chemically modified protein substrates into peroxisomes following microinjection.

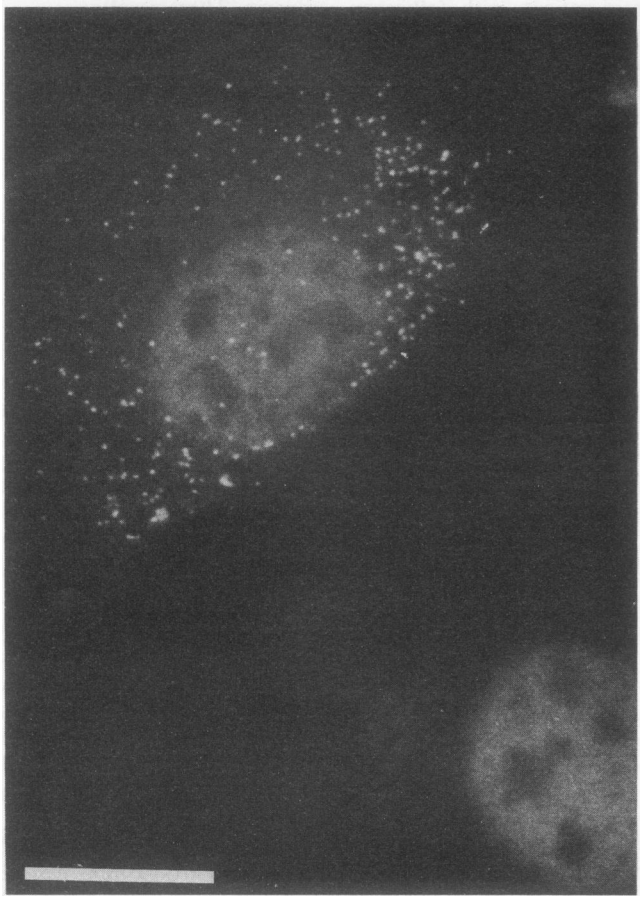


FIG. 2. Transport of microinjected luciferase into vesicles in BALB/c 3T3 cells. Cells were injected with approximately 10^5 molecules of luciferase and 2×10^4 molecules of nonspecific mouse IgG, incubated at 37°C for 18 h, and then processed for double-label indirect immunofluorescence. This assay employed rabbit antiluciferase and biotinylated anti-mouse IgG antibodies in the first step and rhodamine-conjugated anti-rabbit IgG antibodies and FITC-conjugated strepavidin in the second step. The injected cells were identified by the cytoplasmic FITC staining. The figure shows the punctate pattern of the rhodamine-stained luciferase following an 18-h incubation at 37°C. Bar, 10 μm.

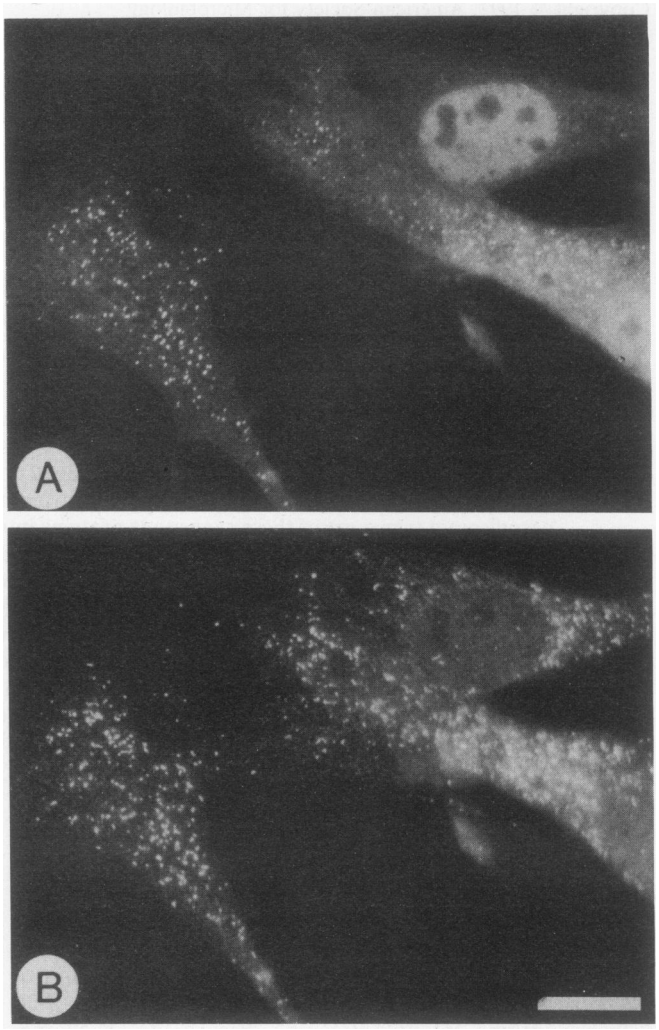


FIG. 3. Colocalization of microinjected luciferase (A) with endogenous catalase (B). Following injection of approximately 10^5 molecules of luciferase and incubation for 18 h at 37°C, cells were processed for double-label indirect immunofluorescence. This assay employed guinea pig antiluciferase and rabbit anticatalase antibodies in the first step and FITC-conjugated anti-guinea pig IgG and rhodamine-conjugated anti-rabbit IgG antibodies in the second step. Bar, 10 μm.

MATERIALS AND METHODS

Reagents. Luciferase from *Photinus pyralis* was purchased from Sigma (St. Louis, Mo.). Antibodies directed against luciferase, chloramphenicol acetyltransferase (CAT), and catalase were obtained as described previously (9). Rabbit polyclonal antibodies directed against the SKL peroxisomal targeting sequence were as previously described (13). Rabbit polyclonal antibodies directed against human serum albumin (HSA) were purchased from ICN (Costa Mesa, Calif.). Biotinylated goat anti-mouse antibodies and streptavidin-conjugated fluorescein isothiocyanate (FITC) were purchased from Amersham (Arlington Heights, Ill.). Rhodamine-conjugated goat anti-rabbit antibodies were obtained from Organon Technika-Cappel (Malvern, Pa.). Rhodamine-conjugated anti-rabbit, rhodamine-conjugated anti-guinea pig, and FITC-conjugated anti-guinea pig antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa.). Synthetic peptides were obtained from

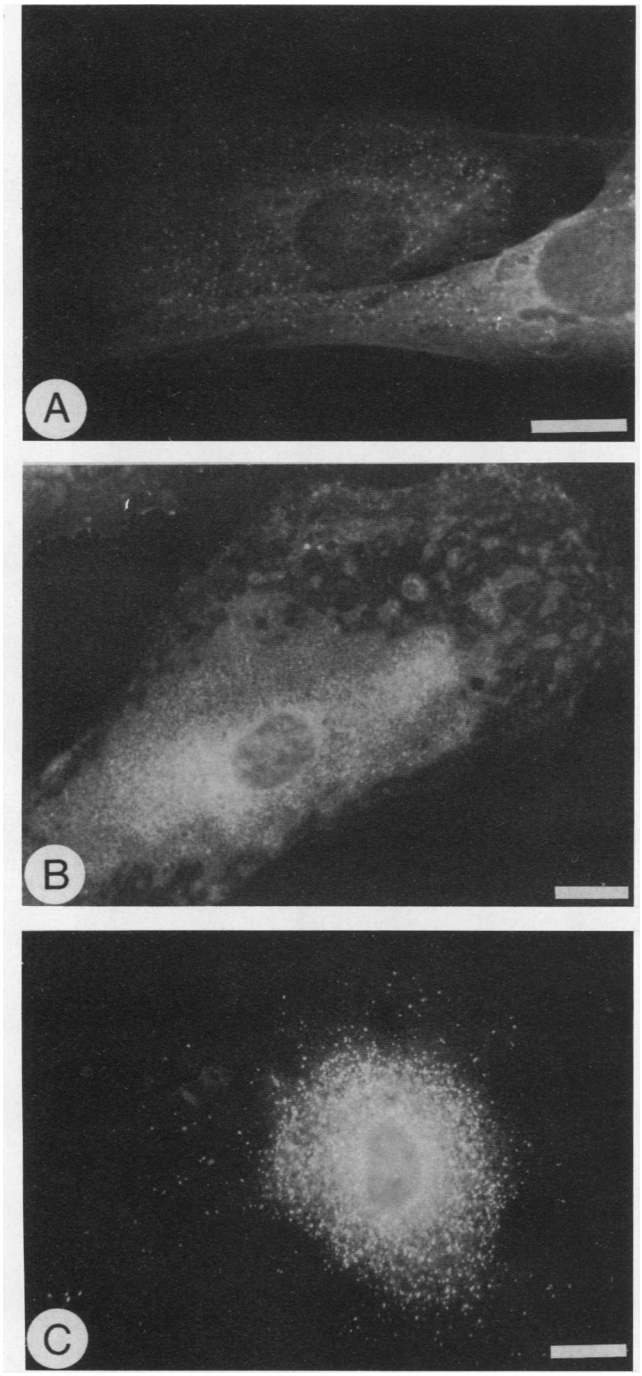


FIG. 4. Temperature dependence of transport of luciferase. Following injection, BALB/c 3T3 cells were incubated for 18 h at 20°C (A), 4°C (B), or 4°C followed by 24 h at 37°C (C) and were then processed for double-label indirect immunofluorescence. The figure shows the distribution of luciferase in these microinjected cells. Bar, 10 μm.

Agouron Institute or Multiple Peptide Systems (La Jolla, Calif.). *n*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS) was purchased from Pierce Chemical Company (Rockford, Ill.). Other reagents were purchased from the standard sources.

Cross-linking of peptides bearing the PTS to HSA. HSA, at a concentration of 10 mg/ml in phosphate-buffered saline (PBS), was incubated with 7.6 mM sulfo-MBS for 1 h at room temperature. Excess reagents were neutralized by the addition of Tris to 40 mM and separated from the MBS-linked proteins by Centricon filtration. The modified albumin was subsequently incubated with the synthetic peptide NH_2-CRYHLKPLQSKL-COOH overnight at 4°C. The cross-linked products were separated from the unreacted peptide, and the buffer was changed to PBS by Centricon filtration.

Cell culture. BALB/c 3T3 and HS 68 cells were obtained from the American Type Culture Collection. Zellweger syndrome fibroblasts GM00228 and GM04340 cells belonging to two different complementation groups were obtained from the Human Mutant Cell Culture Repository at Camden, N.J. BALB/c 3T3 cells, transfected with plasmid pSV2CAT-PMP-20 and expressing the peroxisomal CAT–PMP-20 fusion protein in a stable fashion, were prepared as previously described (9, 10). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. For microinjection, cells were plated on acid-washed glass coverslips.

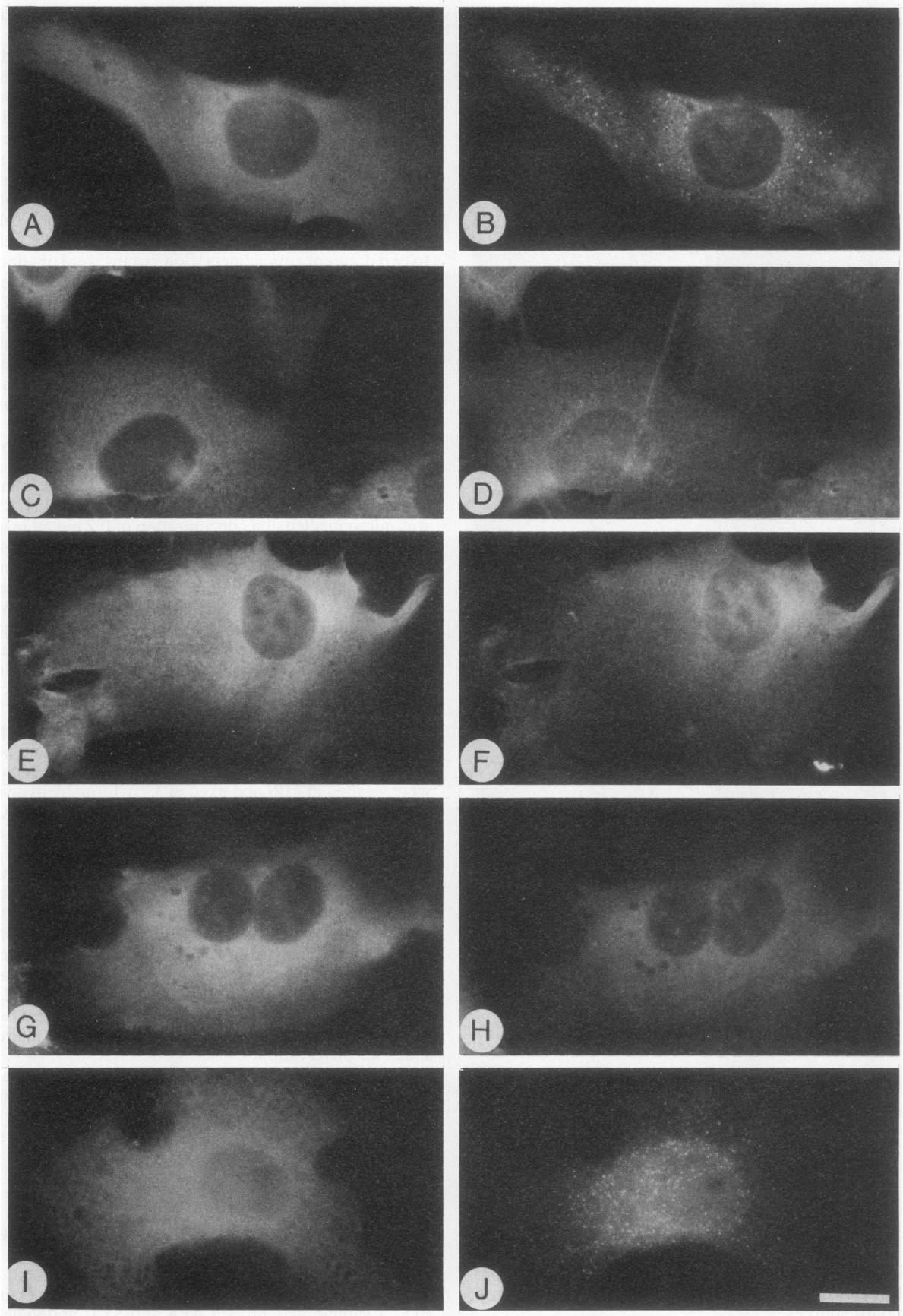
Microinjection and immunofluorescence microscopy. Cells were microinjected by using glass capillary needles as previously described (3) except that the needles were made by using a Kopf vertical pipette puller (model 720). Luciferase was microinjected at a concentration of 0.2 mg/ml in a buffer of 20 mM KPO_4 (pH 7.4), 100 mM KCl, and 40 mM potassium citrate. To facilitate identification of microinjected cells, mouse immunoglobulin G (IgG) (nonspecific) was coinjected at a concentration of 1 mg/ml. With an average injection volume of 5×10^{-14} liters, a molecular size of 62 kDa, and a concentration of 0.2 mg/ml, approximately 10^5 molecules of luciferase were injected per cell. In addition, injections included approximately 2×10^5 molecules of mouse IgG. HSA, containing the cross-linked PTS, was microinjected at a concentration of 0.5 mg/ml; approximately 2×10^5 molecules of albumin were injected per cell.

Following injection and incubation, the cells on coverslips were washed in PBS and fixed in 3.7% formaldehyde in PBS for 10 min. Cells were permeabilized with 1% Triton X-100 in PBS for 5 min and then washed with 0.1% Tween 20 in PBS (also used for subsequent washes). A mixture of rabbit antiluciferase or anti-HSA and biotinylated anti-mouse IgG antibodies (1:100 dilution) was applied to the coverslips in a humidified chamber at a 1:100 dilution and incubated for 30 min at room temperature. The cells were washed, a mixture of secondary reagents consisting of rhodamine-labeled goat anti-rabbit and FITC-labeled streptavidin (both at a 1:100 dilution) was applied to the coverslips, and the cells were incubated for 30 min. Cells were washed extensively and rinsed in H_2O, and coverslips were mounted on microscope slides for observation.

Fluorescence microscopy was performed with a Zeiss Axiophot Photomicroscope, using a 63× (1.3 numerical aperture) lens. Fluorescent images were recorded on Kodak T-Max 400 film which was pushed and developed one stop as instructed by the manufacturer.

RESULTS

Transport of microinjected luciferase into peroxisomes. Luciferase, purified from *P. pyralis*, migrated as a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fig. 1A). To ensure that the purified luciferase retained its PTS, a sample was probed with anti-SKL antibodies on a Western immunoblot (Fig. 1B). This protein was recognized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by the anti-SKL antibodies. These antibodies also recognize a second band with an apparent molecular size of 40 kDa; this band is presumed to be a product of proteolysis and is commonly observed in commercial preparations of the enzyme.

Luciferase was microinjected into BALB/c 3T3 cells. Injected cells were incubated overnight under normal growth conditions and processed for indirect immunofluorescence using antiluciferase antibodies. As shown in Fig. 2, the microinjected luciferase was present in vesicular structures very similar in appearance to peroxisomes. To confirm that these vesicles were indeed peroxisomes, double-label indirect immunofluorescence experiments were performed on injected cells. Costaining for luciferase and catalase (an endogenous peroxisomal protein) resulted in identical punctate patterns (Fig. 3).

The transport of luciferase was time dependent (data not shown, although similar to the results shown in Fig. 8). The protein began to appear within peroxisomes after 2 h at 37°C. The number of peroxisomes observed increased through 4 and 8 h and appeared to plateau by 18 h. Interestingly, the number of vesicles observed, but not their final size, increased with the amount of luciferase injected. The process appeared to be saturable because microinjection of high concentrations of luciferase (an order of magnitude greater than that used in Fig. 2) resulted in considerable cytoplasmic staining following overnight incubations (not shown). The addition of cycloheximide (100 μg/ml) had no effect on the import of luciferase into peroxisomes. The transport was temperature dependent and occurred in cells incubated for 18 h at 20°C (Fig. 4A) but not in cells incubated for 18 h at 4°C (Fig. 4B). In duplicate experiments, returning cells that had been incubated for 18 h at 4°C following injection to 37°C for 24 h resulted in peroxisomal transport of the microinjected protein (Fig. 4C).

Inhibition of peroxisomal transport by coinjection of peptides bearing a PTS. To confirm the requirement for the PTS in peroxisomal transport, synthetic peptides bearing the PTS SKL (NH_2-CRYHLKPLQSKL-COOH) were coinjected with luciferase at various concentrations, and the cells were incubated for 18 h at 37°C. The results (Fig. 5) indicated that this peptide did not appreciably diminish transport at a 10-fold (not shown) and a 20-fold (Fig. 5A and B) molar excess. At a 50-fold (Fig. 5C and D) or 100-fold (not shown) molar excess, the transport of luciferase into peroxisomes was abolished. Coinjection of other peptides bearing the related PTSs AKL (Fig. 5E and F) and SRL (Fig. 5G and H) at a 50-fold molar excess also resulted in the total inhibition in luciferase transport. A control peptide bearing the first nine amino acids of the SKL inhibitory peptide had no effect on transport at a 100-fold molar excess (Fig. 5I and J).

Transport of microinjected luciferase in human cells. The transport of peroxisomal proteins in three human cell lines was examined by microinjection of luciferase. The cell lines included a normal human fibroblast line, HS68 (Fig. 6A to C), and two cell lines derived from patients with Zellweger syndrome, GM00228 (Fig. 6D to F) and GM04340 (Fig. 6G to I). Cells from patients with Zellweger syndrome lack normal peroxisomes but do have peroxisome ghosts, a defective form of peroxisomes which contains peroxisomal membrane proteins but none of the peroxisomal matrix proteins such as catalase (26, 27). These cells test the fidelity of the microinjection system for the study of peroxisomal protein import. Immunofluorescence detection of endogenous catalase revealed the presence of peroxisomes in the normal fibroblast cells (Fig. 6A). Such punctate staining was absent in the two cell lines derived from patients with Zellweger syndrome (Fig. 6D and G). The normal fibroblast cells transported microinjected luciferase (Fig. 6B and C). This transport was into peroxisomes as determined by costaining for catalase (not shown). No transport of microinjected luciferase was observed in either of the Zellweger cell lines GM00228 (Fig. 6E and F) and GM04340 (Fig. 6H and I) after the 18-h incubation time.

Transport of chemically modified proteins bearing the PTS. To determine whether artificial substrates could be imported into peroxisomes, we created a protein-peptide conjugate in which dodecameric peptides ending in SKL-COOH were cross-linked to lysines in HSA. Approximately 5 to 15 such peptides were attached to each HSA molecule, as judged by an increase in apparent molecular weight on SDS-polyacrylamide gel electrophoresis (not shown). Because of the nature of the cross-linking chemistry, it is unlikely that a peptide could have attached to the C terminus of the protein. Therefore, the peptides must have been attached as side chains to lysine residues on the HSA molecule, and none could have been colinear with the protein. The HSA-SKL conjugate was microinjected into BALB/c 3T3 cells expressing the CAT–PMP-20 fusion protein, a known marker for mammalian peroxisomes (9, 10). Following 18 h of incubation, the HSA-SKL conjugate was observed in the peroxisomal compartment (Fig. 7). Import was time dependent (Fig. 8). The protein began to appear within peroxisomes after 2 h at 37°C (Fig. 8A and B). The number of such vesicles increased through 4 (Fig. 8C and D) and 6 (Fig. 8E and F) h, and translocation appeared to be complete by 18 h. Microinjection of unconjugated HSA into fibroblasts did not result in the import of this protein into the peroxisomal compartment after an incubation of 18 h (not shown). As a further control to ensure that the HSA-SKL conjugate behaved as expected for a peroxisomal protein, microinjection experiments were repeated with the Zellweger cell line GM00228. As expected, no import of the HSA-SKL conjugate occurred in this cell line (results not shown).

As an additional control, we sought to confirm that transport of HSA-SKL was into the lumen of the peroxisomes and not merely aggregation of the HSA-SKL molecules on the surface of the vesicles. To ascertain whether the HSA-SKL was transported into membrane-enclosed vesicles, cells microinjected with HSA-SKL and incubated for 18 h at 37°C were permeabilized with either digitonin or digitonin plus Triton X-100 (Fig. 9). Digitonin permeabilization of mammalian cells at a concentration of 25 μg/ml for 10 min has been demonstrated to allow access to the cytoplasmic

FIG. 5. Effects of coinjection of synthetic peptides bearing the PTS on the transport of microinjected luciferase. BALB/c 3T3 cells were injected with luciferase and the synthetic peptide NH_2-CRYHLKPLQSKL-COOH at a 20-fold (A and B) or 50-fold (C and D) molar excess or the synthetic peptide NH_2-CRYHLKPLQAKL-COOH (E and F) or NH_2-CRYHLKPLQSRL-COOH (G and H) at a 50-fold molar excess. The control peptide NH_2-CRYHLKPLQ-COOH was injected at a 100-fold molar excess (I and J). Following injection, BALB/c 3T3 cells were incubated for 18 h at 37°C and then processed for double-label indirect immunofluorescence. (A, C, E, G, and I) Injected cells as visualized by staining for coinjected mouse IgG; (B, D, F, H, and J) distribution of luciferase in these microinjected cells. Bar, 10 μm.

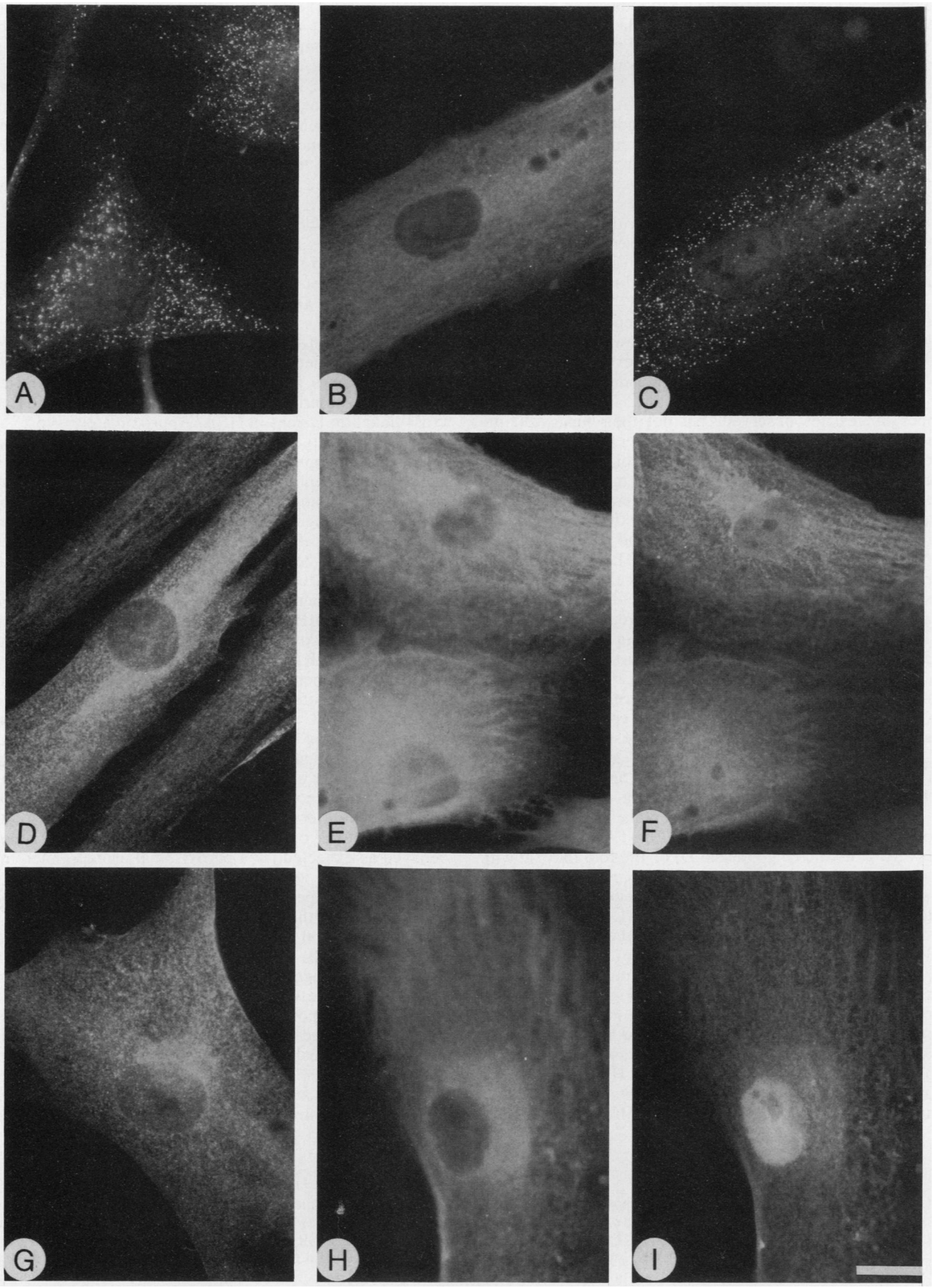
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FIG. 6. Transport of microinjected luciferase in human cell lines. Normal human fibroblasts (HS68) (A to C) and cell lines derived from patients with Zellweger syndrome, GM00228 (D to F) and GM4340 (G to I), were immunocytochemically stained for endogenous catalase (A, D, and G) or microinjected with luciferase and mouse IgG (B, C, E, F, H, and I). Following injection, cells were incubated for 18 h at 37°C and then processed for double-label indirect immunofluorescence. (B, E, and H) Injected cells as visualized by staining for coinjected mouse IgG; (C, F, and I) distribution of luciferase in these microinjected cells. Bar, 10 μm.

compartment while retaining the integrity of the intracellular membranes (33). After an incubation of 18 h at 37°C following injection of the HSA-SKL conjugate, cells permeabilized with digitonin demonstrated staining of the cytoplasmic mouse IgG (Fig. 9C), but the HSA-SKL was not visible (Fig. 9E). Permeabilization of identically injected and incubated cells with digitonin plus Triton X-100 revealed spherical HSA-SKL-containing structures (Fig. 9F) in injected cells (Fig. 9D). As an internal control, BALB/c 3T3 cells permeabilized with either digitonin or digitonin plus Triton X-100 were stained for endogenous catalase. In cells permeabilized with digitonin, the antibodies failed to reveal the peroxisomally located catalase (Fig. 9A) but showed the normally observed punctate pattern when permeabilized with digitonin plus Triton X-100 prior to staining (Fig. 9B). This finding demonstrates that the HSA-SKL was sequestered, like catalase, within the peroxisomal matrix.

DISCUSSION

Microinjection of purified proteins into mammalian cells has been an effective tool in the study of the structure of the cytoskeleton (3) and for probing the functions of key proteins (16), both in normal (23) and in transformed cells (4). The present report describes the transport of microinjected proteins bearing the PTS into peroxisomes of mammalian cells. The presence of the PTS SKL-COOH, either as a constituent of the purified firefly luciferase or as part of synthetic peptides cross-linked to HSA, was necessary and sufficient to direct this translocation in vivo. The retranslocation of luciferase unequivocally demonstrates that no irreversible alteration of the tripeptide PTS occurs upon import into peroxisomes and is in fact the first demonstration that a protein can undergo multiple translocations across the peroxisomal membrane. This type of protein translocation would not be possible for the majority of proteins destined for the endoplasmic reticulum, mitochondrion, or chloroplast, since these proteins lose their targeting signals by proteolytic cleavage upon import. However, retransport of a nuclear protein through the nuclear pore complex has been demonstrated for the catalytic subunit of cyclic AMP-dependent protein kinase following microinjection (19). This must obviously occur often as a method of transcriptional control and in the normal course of events, since the nucleus must reassemble after each cell division. Whether the ability of peroxisomal proteins to be retransported has any significance for peroxisomal or cellular function is not known.

The transport of microinjected peroxisomal proteins reflects many of the features of peroxisomal import observed in vivo. The colocalization of the microinjected proteins with endogenous peroxisomal proteins, transport into membrane-enclosed vesicles, the inhibition of transport by coinjection of peptides bearing the PTS, and the lack of transport of microinjected proteins into the peroxisomes of cells from Zellweger patients provide compelling arguments for the authenticity of import of microinjected proteins into the peroxisome. In addition, these results confirm that the defect in these cell lines from Zellweger patients is indeed the

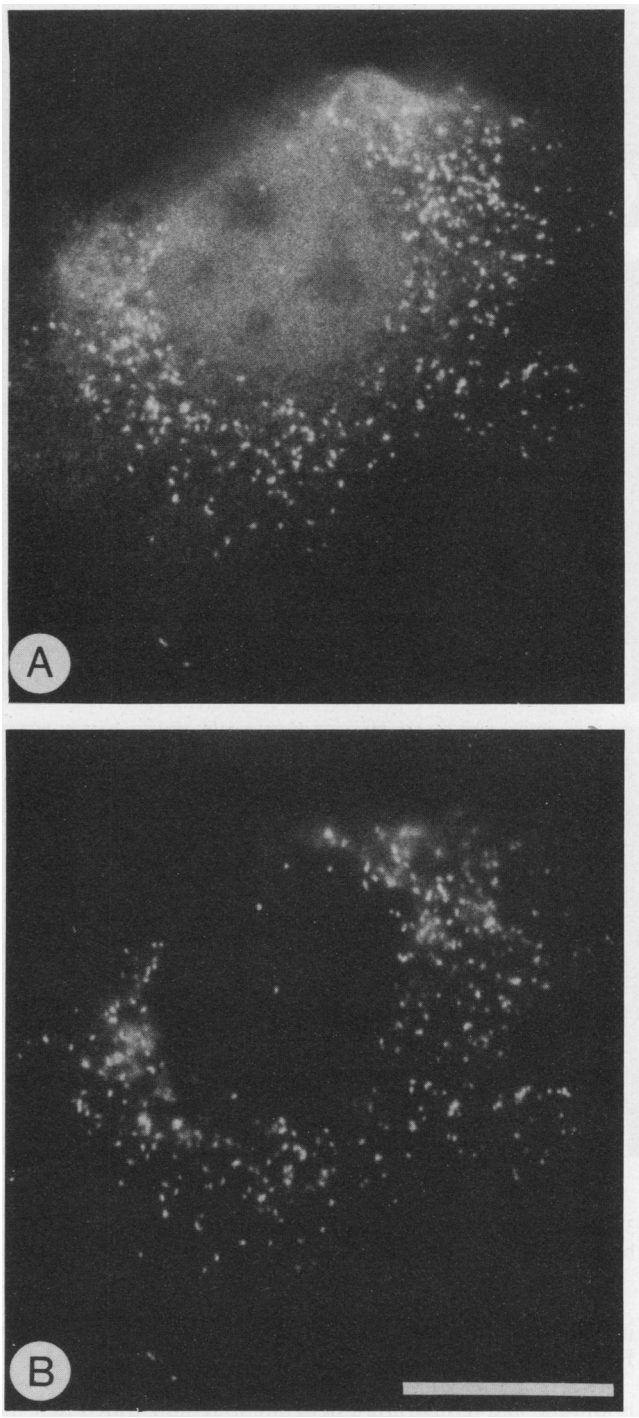

FIG. 7. Colocalization of microinjected HSA cross-linked with a synthetic peptide bearing the peroxisomal targeting signal (A) with the endogenous peroxisomal protein CAT–PMP-20 (B). Following injection, BALB/c 3T3 cells were incubated for 18 h at 37°C and then processed for double-label indirect immunofluorescence. Bar, 10 μm.

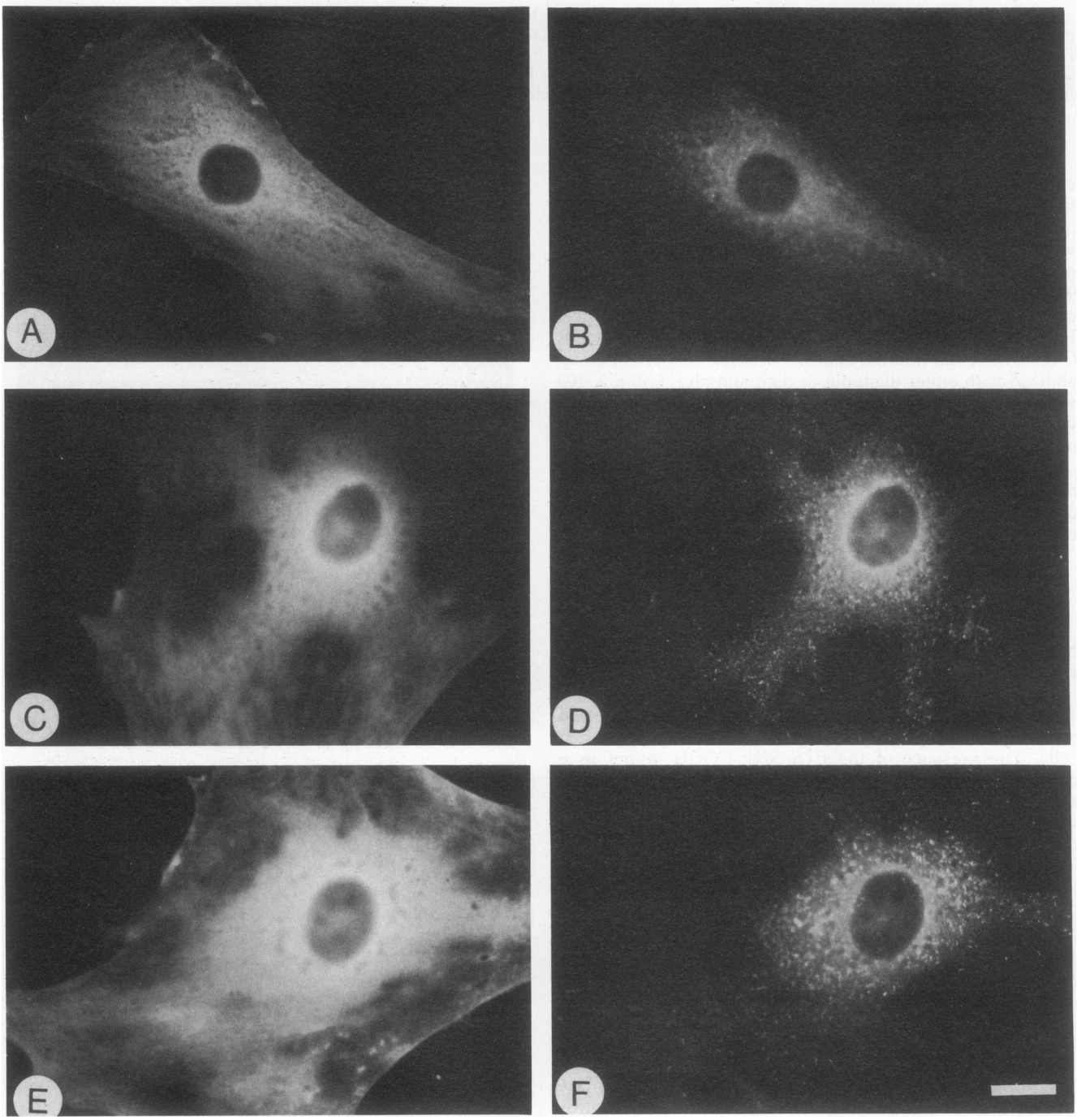

FIG. 8. Time course of transport of HSA-SKL into peroxisomes of BALB/c 3T3 cells. Following microinjection, cells were incubated at 37°C for 2 h (A), 4 h (B), or 6 h (C) and then processed for double-label indirect immunofluorescence. (A, C, and E) Injected cells as visualized by staining for coinjected mouse IgG; (B, D, and F) distribution of HSA-SKL in these microinjected cells. Bar, 10 μm.

inability to transport proteins containing the Ser-Lys-Leu PTS into the peroxisomal matrix.

The fact that import of the microinjected proteins was both time and temperature dependent demonstrated that the microinjection system faithfully reproduces the results obtained with in vitro systems (14, 30). The other features of peroxisomal protein import determined by using in vitro systems were that import occurred as a two-step process and that ATP was required (14). Since we did not observe any luciferase bound to vesicles after 18 h at 4°C (most likely because of the lack of sensitivity of our immunofluorescence assay) and could not manipulate the ATP levels in the cells (data not shown), we could not confirm these findings in our system.

Though certain features of peroxisomal protein import may always be refractory to study with in vivo systems, others are not. The inhibition caused by coinjection of the synthetic peptides that contain any of the three different

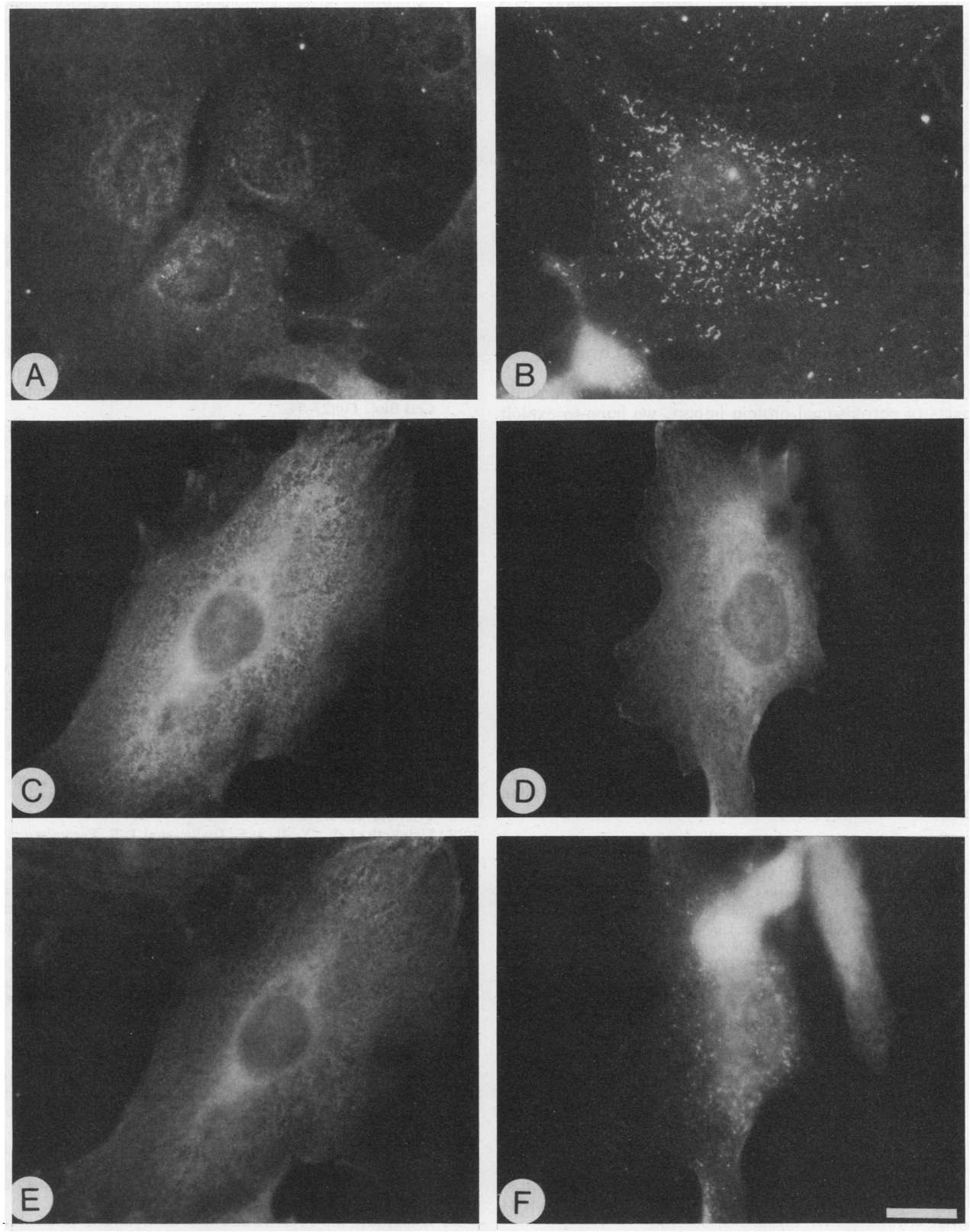

FIG. 9. Transport of HSA-SKL is into membrane-enclosed vesicles. BALB/c 3T3 cells were microinjected with HSA-SKL and incubated for 18 h at 37°C (C to F). Cells were fixed and permeabilized with either digitonin (25 μg/ml) (A, C, and E) or digitonin plus Triton X-100 (0.05%) (B, D, and F). Cells were then processed for double-label indirect immunofluorescence. (A and B) Staining of the peroxisomally located catalase in BALB/c 3T3 cells; (C and D) injected cells as visualized by staining for coinjected mouse IgG; (E and F) distribution of HSA-SKL in these injected cells. Bar, 10 μm.

forms (SKL, SRL, AKL) of the C-terminal tripeptide PTS (9) is the first demonstration that the peroxisomal import process is saturable and suggests that there is a single class of PTS receptors capable of recognizing several distinct class I PTSs. Also, though the transport of proteins into the peroxisomes required the PTS, it did not require that the targeting signal be located at the carboxy terminus of a linear polypeptide. Because of the chemistry used for attachment of SKL-containing peptides to the HSA, such a configuration would have been impossible. In addition, the fact that 5 to 15 peptides were coupled to each HSA molecule indicates that, as with mitochondrial protein import, the peroxisomal protein import apparatus is able to tolerate branched polypeptides. Coinjection of a 50-fold excess of synthetic peptide bearing the PTS resulted in only partial inhibition of HSA-SKL transport (results not shown). It appears that the additional PTS sequences present on the HSA-SKL molecule increase its chances of being recognized by a receptor and transported into the peroxisome.

Given that the microinjection system can be used to study aspects of peroxisomal protein import, we hope to exploit this system to answer some of the many remaining questions regarding this process. The observations that we have made should be helpful in the eventual development of reliable in vitro systems for the study of peroxisomal protein transport. In addition, the microinjection system provides the means to assay for peroxisomal protein import in primary cell lines derived from patients with peroxisomal disorders, circumventing the need for established cell lines. Thus, it should be possible to test whether such lines are deficient in the import of proteins with different classes of PTSs and to establish whether all complementation groups of Zellweger syndrome patients have identical import deficiency phenotypes.

ACKNOWLEDGMENTS

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REFERENCES

1. **Brul, S., A. Westerveld, A. Strijland, R. J. A. Wanders, A. W. Schram, H. S. A. Heymans, R. B. H. Schutgens, H. van den Bosch, and J. M. Tager.** 1988. Genetic heterogeneity in the cerebrohepatorenal (Zellweger) syndrome and other inherited disorders with a generalized impairment of peroxisomal functions. J. Clin. Invest. **81**:1710–1715.
2. **Del Valle, R., U. Soto, C. Necochea, and F. Leighton.** 1988. Detection of an ATPase activity in rat liver peroxisomes. Biochem. Biophys. Res. Commun. **156**:1353–1359.

2a. **Distel, B., et al.** Submitted for publication.

3. **Feramisco, J. R.** 1979. Microinjection of fluorescently labeled α-actinin into living fibroblasts. Proc. Natl. Acad. Sci. USA **76**:3967–3971.
4. **Feramisco, J. R., R. Clark, G. Wong, N. Arnheim, R. Milley, and F. McCormick.** 1985. Transient reversion of *ras* oncogene-induced cell transformation by antibodies specific for amino acid 12 of *ras* protein. Nature (London) **314**:639–642.
5. **Fujiki, Y., and P. B. Lazarow.** 1985. Post-translational import of fatty acyl-CoA oxidase and catalase into peroxisomes of rat liver *in vitro*. J. Biol. Chem. **260**:5603–5609.
6. **Fujiki, Y., R. A. Rachubinski, and P. B. Lazarow.** 1984. Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes. Proc. Natl. Acad. Sci. USA **81**:7127–7131.
7. **Fujiki, Y., R. A. Rachubinski, R. M. Mortensen, and P. B. Lazarow.** 1985. Synthesis of 3-ketoacyl-CoA thiolase of rat liver peroxisomes on free polysomes as a larger precursor. Induction of thiolase mRNA activity by clofibrate. Biochem. J. **226**:697–704.
8. **Goldman, B. M., and G. Blobel.** 1978. Biogenesis of peroxisomes: intracellular site of synthesis of catalase and uricase. Proc. Natl. Acad. Sci. USA **75**:5066–5070.
9. **Gould, S. J., G.-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani.** 1989. A conserved tripeptide sorts proteins to peroxisomes. J. Cell Biol. **108**:1657–1664.
10. **Gould, S. J., G.-A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani.** 1990. Peroxisomal import is conserved between yeast, plants, insects, and mammals. EMBO J. **9**:85–90.
11. **Gould, S. J., G.-A. Keller, and S. Subramani.** 1987. Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. J. Cell Biol. **105**:2923–2931.
12. **Gould, S. J., G.-A. Keller, and S. Subramani.** 1988. Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. J. Cell Biol. **107**:897–905.
13. **Gould, S. J., S. Krisans, G.-A. Keller, and S. Subramani.** 1990. Antibodies directed against the targeting signal of firefly luciferase recognise multiple mammalian peroxisomal proteins. J. Cell Biol. **110**:27–34.
14. **Imanaka, T., G. M. Small, and P. B. Lazarow.** 1987. Translocation of acyl-CoA oxidase into peroxisomes requires ATP hydrolysis but not a membrane potential. J. Cell Biol. **105**:2915–2922.
15. **Kamijo, K., S. Taketani, S. Yokota, T. Osumi, and T. Hashimoto.** 1990. The 70-kDa peroxisomal membrane protein is a member of the Mdr (P-glycoprotein)-related ATP-binding protein superfamily. J. Biol. Chem. **265**:4534–4540.
16. **Lamb, N. J. C., A. Fernandez, M. A. Conti, R. Adelstein, D. B. Glass, W. J. Welch, and J. R. Feramisco.** 1988. Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. J. Cell Biol. **106**:1955–1971.
17. **Lazarow, P. B., V. Black, H. Shio, Y. Fujiki, A. K. Hajra, N. S. Datta, B. S. Bangaru, and J. Dancis.** 1985. Zellweger syndrome: biochemical and morphological studies on two patients treated with clofibrate. Pediatr. Res. **19**:1356–1364.
18. **Lazarow, P. B., Y. Fujiki, G. M. Small, P. Watkins, and H. Moser.** 1986. Presence of the peroxisomal 22-kDa integral membrane protein in the liver of a person lacking recognisable peroxisomes (Zellweger syndrome). Proc. Natl. Acad. Sci. USA **83**:9193–9196.
19. **Meinkoth, J. L., Y. Ji, S. S. Taylor, and J. R. Feramisco.** 1990. Dynamics of the cyclic AMP-dependent protein kinase distribution in living cells. Proc. Natl. Acad. Sci. USA **87**:9595–9599.
20. **Miura, S., M. Mori, M. Takiguchi, M. Tatibana, S. Furuta, S. Miyazawa, and T. Hashimoto.** 1984. Biosynthesis and intracellular transport of enzymes of peroxisomal beta-oxidation. J. Biol. Chem. **259**:6397–6402.
21. **Miyazawa, S., T. Osumi, T. Hashimoto, K. Ohno, S. Miura, and Y. Fujiki.** 1989. Peroxisome targeting signal of rat liver acyl-CoA oxidase resides at the carboxy terminus. Mol. Cell. Biol. **9**:83–91.

21a. **Moser, A.** Personal communication.

22. **Rachubinski, R. A., Y. Fujiki, R. M. Mortensen, and P. B. Lazarow.** 1984. Acyl-CoA oxidase and hydratase-dehydrogenase, two enzymes of the peroxisomal beta-oxidation system, are synthesized on free polysomes of clofibrate-treated rat liver. J. Cell Biol. **99**:2241–2246.
23. **Riabowol, K. T., R. J. Vosatka, E. B. Ziff, N. J. Lamb, and J. R. Feramisco.** 1988. Microinjection of *fos*-specific antibodies blocks DNA synthesis in fibroblast cells. Mol. Cell. Biol. **8**:1670–1676.
24. **Robbi, M., and P. B. Lazarow.** 1978. Synthesis of catalase in two cell-free protein-synthesizing systems and in rat liver. Proc. Natl. Acad. Sci. USA **75**:4344–4348.
25. **Roggenkamp, R., Z. Janowicz, B. Stanikowski, and C. P. Hollenberg.** 1984. Biosynthesis and regulation of peroxisomal methanol oxidase from the methylotrophic yeast *Hansenula polymorpha*. Mol. Gen. Genet. **194**:489–493.

26. **Santos, M. J., T. Imanaka, H. Shio, and P. B. Lazarow.** 1988. Peroxisomal integral membrane proteins in control and Zellweger fibroblasts. J. Biol. Chem. **263**:10502–10509.
27. **Santos, M. J., T. Imanaka, H. Shio, G. M. Small, and P. B. Lazarow.** 1988. Peroxisomal membrane ghosts in Zellweger syndrome—aberrant organelle assembly. Science **239**:1536–1538.
28. **Santos, M. J., J. M. Ojeda, J. Garrido, and F. Leighton.** 1985. Peroxisomal organisation in normal and cerebrohepatorenal (Zellweger) syndrome fibroblasts. Proc. Natl. Acad. Sci. USA **82**:6556–6560.
29. **Small, G. M., M. J. Santos, T. Imanaka, A. Poulos, D. M. Danks, H. W. Moser, and P. B. Lazarow.** 1988. Peroxisomal integral membrane proteins are present in livers of patients with Zellweger syndrome, infantile Refsum's disease and X-linked adrenoleukodystrophy. J. Inherited Metab. Dis. **11**:358–371.
30. **Small, G. M., L. J. Szabo, and P. B. Lazarow.** 1988. Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes. EMBO J. **7**:1167–1173.
31. **Tolbert, N. E.** 1981. Metabolic pathways in peroxisomes and glyoxysomes. Annu. Rev. Biochem. **50**:133–157.
32. **Wanders, R. J. A., M. Kos, B. Roest, A. J. Meijer, G. Schrakamp, H. S. A. Heymans, W. H. H. Tegelaers, H. van den Bosch, R. B. H. Schutgens, and J. M. Tager.** 1984. Activity of peroxisomal enzymes and intracellular distribution of catalase in Zellweger syndrome. Biochem. Biophys. Res. Commun. **123**:1054–1061.
33. **Wolvetang, E. J., J. M. Tager, and R. J. A. Wanders.** 1990. Latency of the peroxisomal enzyme acyl-coA:dihydroxyacetonephosphate acyltransferase in digitonin-permeabilized fibroblasts: the effect of ATP and ATPase inhibitors. Biochem. Biophys. Res. Commun. **170**:1135–1143.
34. **Zellweger, H.** 1988. Peroxisomes and peroxisomal disorders. Ala. J. Med. Sci. **25**:54–58.