Transport of Microinjected Alcohol Oxidase from *Pichia pastoris* into Vesicles in Mammalian Cells: Involvement of the Peroxisomal Targeting Signal

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Abstract. This report describes the microinjection of a purified peroxisomal protein, alcohol oxidase, from *Pichia pastoris* into mammalian tissue culture cells and the subsequent transport of this protein into vesicular structures. Transport was into membrane-enclosed vesicles as judged by digitonin-permeabilization experiments. The transport was time and temperature dependent. Vesicles containing alcohol oxidase could be detected as long as 6 d after injection. Coinjection of synthetic peptides containing a consensus carboxy-terminal tripeptide peroxisomal targeting signal resulted in abolition of alcohol oxidase transport into vesicles in all cell lines examined. Double-label experiments indicated that, although some of the alcohol oxidase was transported into vesicles that contained other peroxisomal proteins, the bulk of the alcohol oxidase did not appear to be transported to preexisting peroxisomes. While the inhibition of transport of alcohol oxidase by peptides containing the peroxisomal targeting signal suggests a competition for some limiting component of the machinery involved in the sorting of proteins into peroxisomes, the organelles into which the majority of the protein is targeted appear to be unusual and distinct from endogenous peroxisomes by several criteria. Microinjected alcohol oxidase was transported into vesicles in normal fibroblasts and also in cell lines derived from patients with Zellweger syndrome, which are unable to transport proteins containing the ser-lys-leu-COOH peroxisomal targeting signal into peroxisomes (Walton et al., 1992). The implications of this result for the mechanism of peroxisomal protein transport are discussed.

ALCOHOL oxidase is a 630-kD octameric flavoprotein (Kato et al., 1976) that exists in a crystalloid form within the peroxisome (Sahm et al., 1975; Veenhuis et al., 1983). Made in methylotrophic yeasts (*Candida* sp., *Hansenula* sp., *Pichia* sp., *Torulopsis* sp.) in response to growth on methanol, this protein may account for up to 30% of the total cellular protein (Veenhuis et al., 1983; van der Klei et al., 1991). Cells from these organisms contain a large number of peroxisomes which may constitute 80% of the total cytoplasmic volume (Cregg et al., 1990). The peroxisomes principally contain alcohol oxidase, catalase, and dihydroxyacetone synthase (Rogenkamp et al., 1975; Douma et al., 1985; Goodman, 1985), with a core composed exclusively of crystalline alcohol oxidase (Veenhuis et al., 1978, 1981). Alcohol oxidase may account for approximately two-thirds of the total cellular protein in mutant cells overexpressing the protein from a vector (Roggenkamp et al., 1989). Synthesized on free polysomes in the cytoplasm (Lazarow and Fujiki, 1985; Roa and Blobel, 1983; Roggenkamp et al., 1984), the monomeric form of the enzyme is imported into the peroxisomes before octamerization and acquisition of catalytic activity (Goodman et al., 1984; Distel et al., 1987). Pulse-chase experiments indicate that the newly synthesized monomer has a half-life of about 20 min before its conversion to octamers (Goodman et al., 1984). However, peroxisomal transport is not a prerequisite for catalytic activity as recent experiments have demonstrated the presence of active alcohol oxidase in the cytoplasm of mutants of *Hansenula polymorpha* that lack peroxisomes (Cregg et al., 1990).

Alcohol oxidase from *H. polymorpha* is transported into peroxisomes when expressed in *Saccharomyces cerevisiae* (Distel et al., 1987), although it does not form octamers and is enzymatically inactive. However, when these cells were fused with *H. polymorpha* cells that did not express alcohol oxidase, active octameric enzyme was detected (van der Klei et al., 1989). These results imply the presence of additional components in *H. polymorpha* that are involved in the octamerization and activation of this enzyme. Alcohol oxidase introduced into protoplasts of *H. polymorpha* by liposome fusion failed to be transported into peroxisomes and was slowly degraded (Douma et al., 1990).

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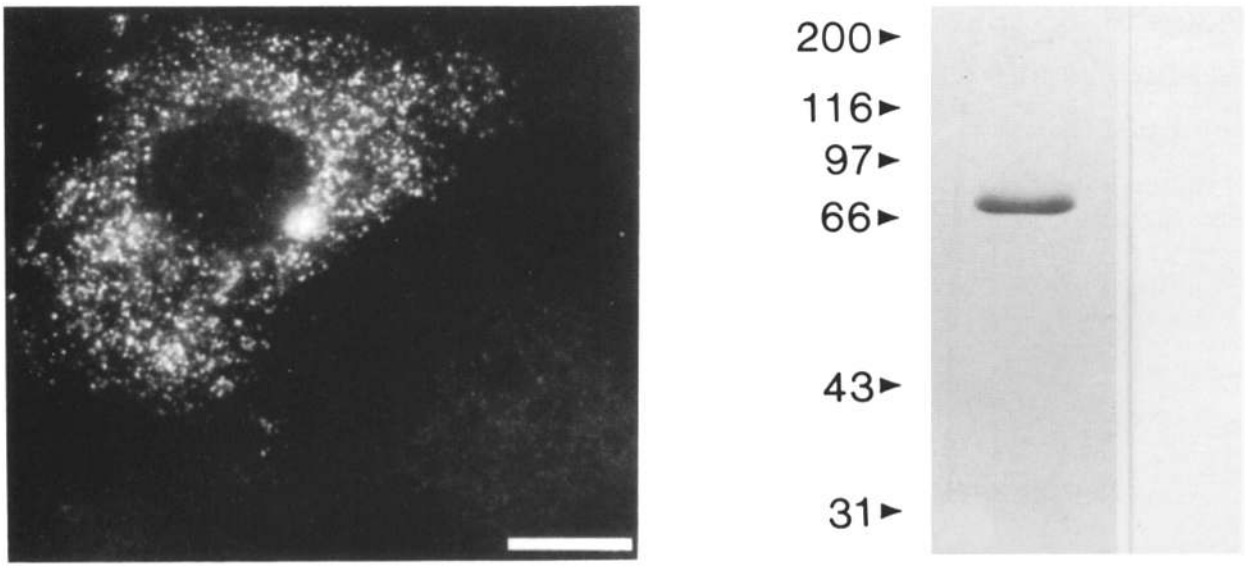

Figure 1. Transport of microinjected alcohol oxidase into vesicles in BALB/c 3T3 cells. Cells were injected with ~4.8 × 10^4 octamers of alcohol oxidase and 2 × 10^5 molecules of non-specific mouse IgG and subsequently incubated at 37°C for 18 h. This was followed by processing for double-label indirect immunofluorescence which used rabbit anti-alcohol oxidase and biotinylated anti-mouse IgG antibodies in the first step and rhodamine-conjugated anti-rabbit IgG antibodies and FITC-conjugated streptavidin in the second step. The injected cells were identified by the cytoplasmic FITC staining. (Left) Shows the punctate pattern of the rhodamine-stained alcohol oxidase following an 18-h incubation at 37°C. (Right) Shows an SDS-polyacrylamide gel containing 10 μg of alcohol oxidase, stained with Coomassie blue. Numbers at left represent the position of molecular weight markers (in kD). Bar, 10 μm.

The signal in alcohol oxidase that targets the protein to peroxisomes is unknown. Although a consensus carboxy-terminal tripeptide peroxisomal targeting signal (PTS)[1] has recently been discovered in many peroxisomal proteins (Gould et al., 1989) such a sequence is absent from the carboxy terminus of alcohol oxidase from *P. pastoris* (Koutz et al., 1989) and *H. polymorpha* (Ledeboer et al., 1985). It is not known whether the three carboxy-terminal amino acids in alcohol oxidase (-ala-arg-phe-COOH) can act as a peroxisomal targeting signal.

The growth of methylotrophic yeasts on methanol induces the synthesis of alcohol oxidase as well as peroxisomal growth and proliferation. Peroxisomal proliferation in *H. polymorpha* occurs by pinching off small vesicles from mature peroxisomes which are filled with the required complement of enzymes (Veenhuis et al., 1978). While the precise signals for organelle growth and proliferation have not been identified, the overproduction of alcohol oxidase in *S. cerevisiae* (Distel et al., 1987) or in *H. polymorpha* (Distel et al., 1988; Roggenkamp et al., 1989) is sufficient for the induction of the growth of single-membrane-bound organelles that envelop a crystalline core of alcohol oxidase. Overproduction of alcohol oxidase alone in *H. polymorpha* induced growth of the peroxisomes, but did not trigger the proliferation of peroxisomes, nor did it result in the coordinate expression of the other enzymes of methanol assimilation.

The experiments described in this report were undertaken to determine whether this yeast protein could be recognized and transported into appropriate organelles in mammalian cells and whether alcohol oxidase could induce growth and/or proliferation of organelles.

1. *Abbreviation used in this paper*: PTS, peroxisomal targeting signal.

Materials and Methods

Reagents

Alcohol oxidase was kindly provided by the Philips Petroleum Company (Bartlesville, OK). Rabbit polyclonal antibodies directed against alcohol oxidase were a generous gift from Ben Distel and Henk Tabak (University of Amsterdam). Rabbit polyclonal antibodies directed against peroxisomal integral membrane proteins have been described earlier (Bodnar and Rachubinski, 1991). Other reagents were as described previously (Walton et al., 1992).

Cell Culture

BALB/c 3T3 and HS68 cells were obtained from American Type Culture Collection (Rockville, MD). GM00228 and GM04340; Zellweger patient fibroblasts belonging to two different complementation groups, were obtained from the Human Mutant Cell Culture Repository (Camden, NJ). BALB/c 3T3 cells transfected with the pSV2CAT-PMP-20 plasmid and expressing the peroxisomal CAT-PMP-20 fusion protein in a stable fashion were prepared as previously described (Gould et al., 1989, 1990*a*). Cells were grown in DME supplemented with 10% FCS. For microinjection, cells were plated on acid-washed glass coverslips.

Microinjection and Immunofluorescence Microscopy

Cells were microinjected using glass capillary needles as previously described (Walton et al., 1992). Alcohol oxidase was microinjected at a concentration of 1 mg/ml in a buffer of 20 mM KPO_4 (pH 7.4), 100 mM KCl, and 1.2% sucrose. To facilitate identification of microinjected cells, mouse IgG (non-specific) was co-injected at a concentration of 1 mg/ml. With an average injection volume of 5 × 10^{-14} liters, a molecular weight of 630 kD (octamer), and a concentration of 1 mg/ml, ~4.8 × 10^4 octamers of alcohol oxidase were injected per cell. In addition, injections included ~2 × 10^5 molecules of mouse IgG.

Analysis of the subcellular distribution of microinjected alcohol oxidase was performed as described previously (Walton et al., 1992).

Digitonin-permeabilization experiments were performed as previously described except that the reagents for immunofluorescence were diluted in PBS. After fixation in 3.7% formaldehyde the cells were permeabilized with 25 μg/ml digitonin for 10 min at room temperature. Digitonin, at this con-

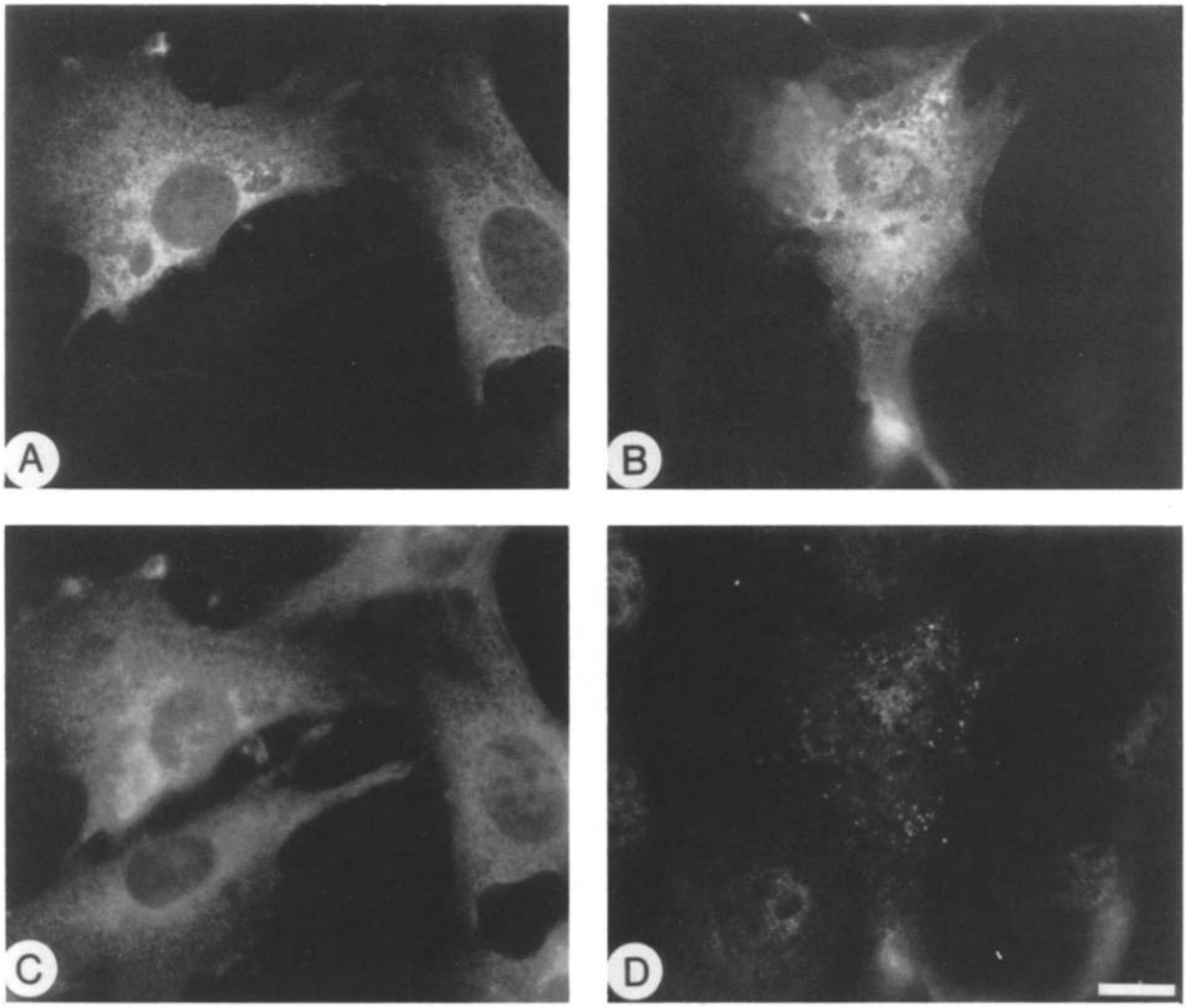

Figure 2. Transport of alcohol oxidase is into membrane-enclosed vesicles. BALB/c 3T3 cells were microinjected with alcohol oxidase and incubated 18 h at 37°C. Cells were fixed and permeabilized with either digitonin (25 μg/ml) (*A* and *C*) or digitonin plus Triton X-100 (0.05%) (*B* and *D*). Cells were then processed for double indirect immunofluorescence. The upper row (*A* and *B*) indicates injected cells as visualized by staining for coinjected mouse IgG. The lower row (*C* and *D*) shows the distribution of alcohol oxidase in these injected cells. Bar, 10 μm.

centration, has been shown to permeabilize the cholesterol-containing plasma membrane while maintaining peroxisomal membrane integrity (Wolvetang et al., 1990). To show that the digitonin did not attenuate the immunofluorescence signal, permeabilizations were performed with 25 μg/ml digitonin plus 0.05% Triton X-100.

Results

Transport of Microinjected Alcohol Oxidase

Alcohol oxidase, purified from *Pichia pastoris*, migrated as a single band on SDS-polyacrylamide gels (Fig. 1, *right*). The subcellular localization of the injected protein was monitored by indirect immunofluorescence of the injected cells. Alcohol oxidase was localized to vesicular structures within the mammalian cells. Uninjected cells did not contain such structures. Overnight (18 h) incubations at 37°C resulted in the induction of numerous alcohol oxidase containing vesicles of approximately the same size (Fig. 1, *left*). The number of vesicles, but not their final size, appeared to depend upon the amount of alcohol oxidase microinjected. This pattern persisted for at least 6-d postinjection.

Microinjected Alcohol Oxidase Is Transported to Membrane-bounded Vesicles

To ascertain whether the alcohol oxidase was transported into membrane-enclosed vesicles, cells microinjected with alcohol oxidase, and incubated 18 h at 37°C were permeabilized with either 25 μg/ml digitonin or digitonin plus 0.05% Triton X-100 (Fig. 2). Digitonin permeabilized the plasma membrane and allowed the visualization of the cytoplasmic mouse IgG and alcohol oxidase immediately following microinjection (not shown). When cells injected with alcohol oxidase and mouse IgG were incubated for 18 h at 37°C, permeabilized with digitonin and monitored by immunofluorescence, staining of the cytoplasmic mouse IgG was observed (Fig. 2 *a*) but the punctate staining for alcohol oxidase was no longer visible (Fig. 2 *c*). Permeabilization of identically injected and incubated cells with digitonin plus Triton

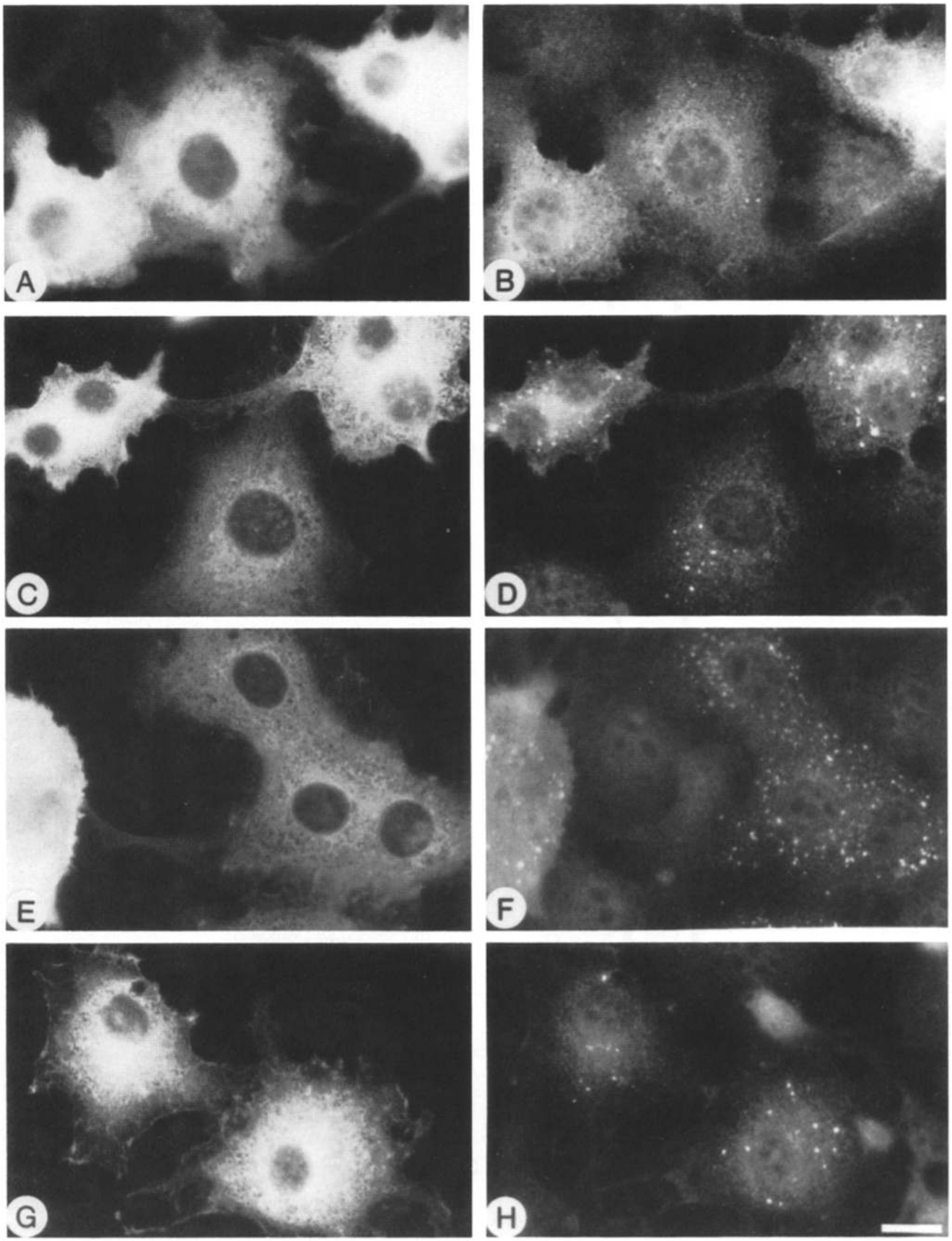

Figure 3. Time course and temperature dependence of transport of alcohol oxidase in BALB/c 3T3 cells. Cells were injected as described in Materials and Methods and incubated at 37°C for 20 min (*A* and *B*), 40 min (*C* and *D*), 60 min (*E* and *F*), or at 20°C for 60 min (*G* and *H*) followed by processing for double indirect immunofluorescence. The left column (*A*, *C*, *E*, and *G*) indicates the injected cells as visualized by staining for coinjected mouse IgG. The right column (*B*, *D*, *F*, and *H*) shows the distribution of alcohol oxidase in these microinjected cells. Bar, 10 μm.

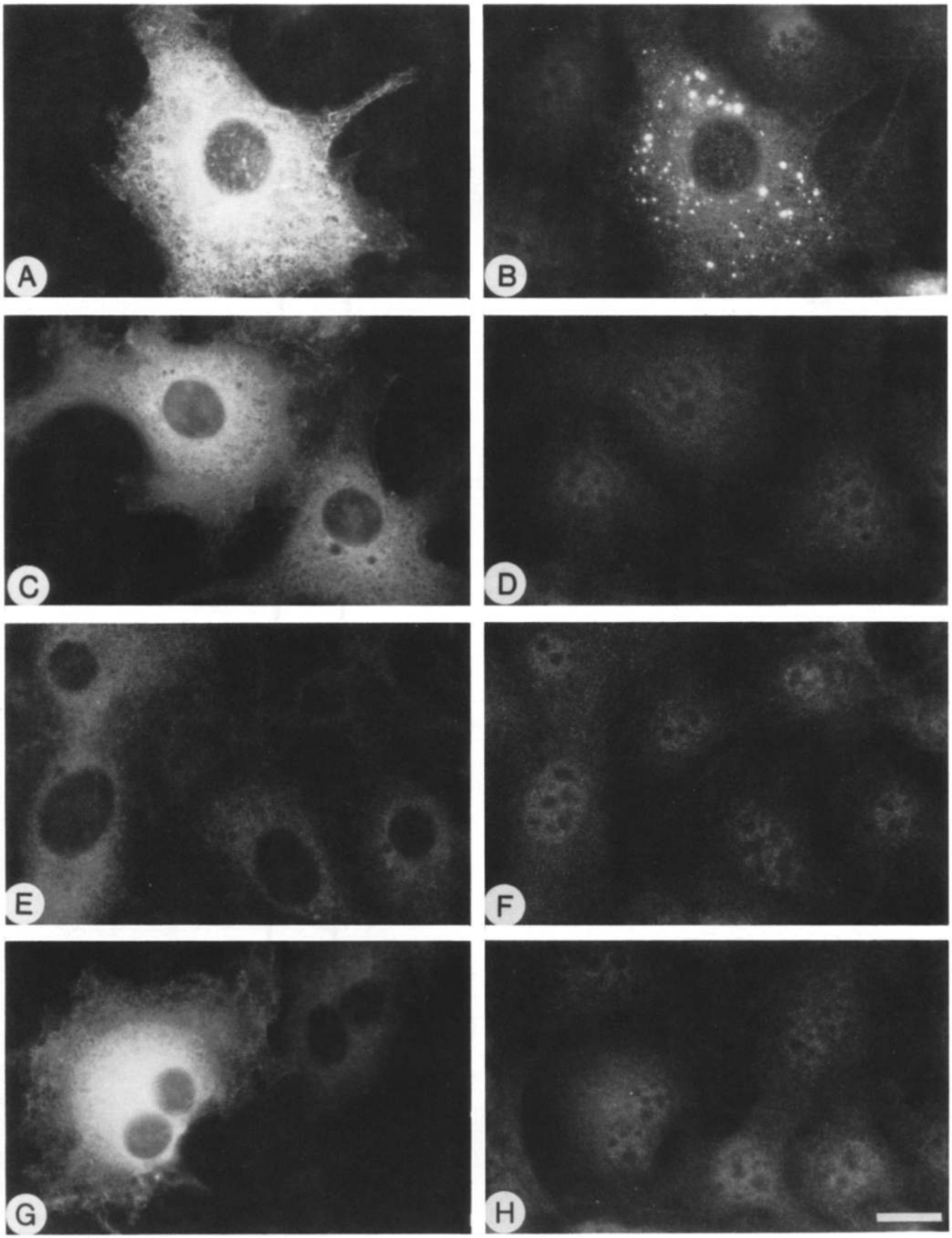

Figure 4. Effects of coinjection of synthetic peptides bearing the PTS on the transport of microinjected alcohol oxidase. BALB/c 3T3 cells were injected with alcohol oxidase and a 50-fold molar excess of control peptide (NH_2-CRYHLKPLQ-COOH) (*A* and *B*), or peptides bearing the PTS (NH_2-CRYHLKPLQAKL-COOH) (*C* and *D*), (NH_2-CRYSRLSHLSKL-COOH) (*E* and *F*), (NH_2-CRYHLKPLQSRL-COOH) (*G* and *H*). After injection, cells were incubated for 60 min at 37°C and then processed for double indirect immunofluorescence. (*A*, *C*, *E*, and *G*) Indicates injected cells as visualized by staining for coinjected mouse IgG. (*B*, *D*, *F*, and *H*) Shows the distribution of alcohol oxidase in these injected cells. Bar, 10 μm.

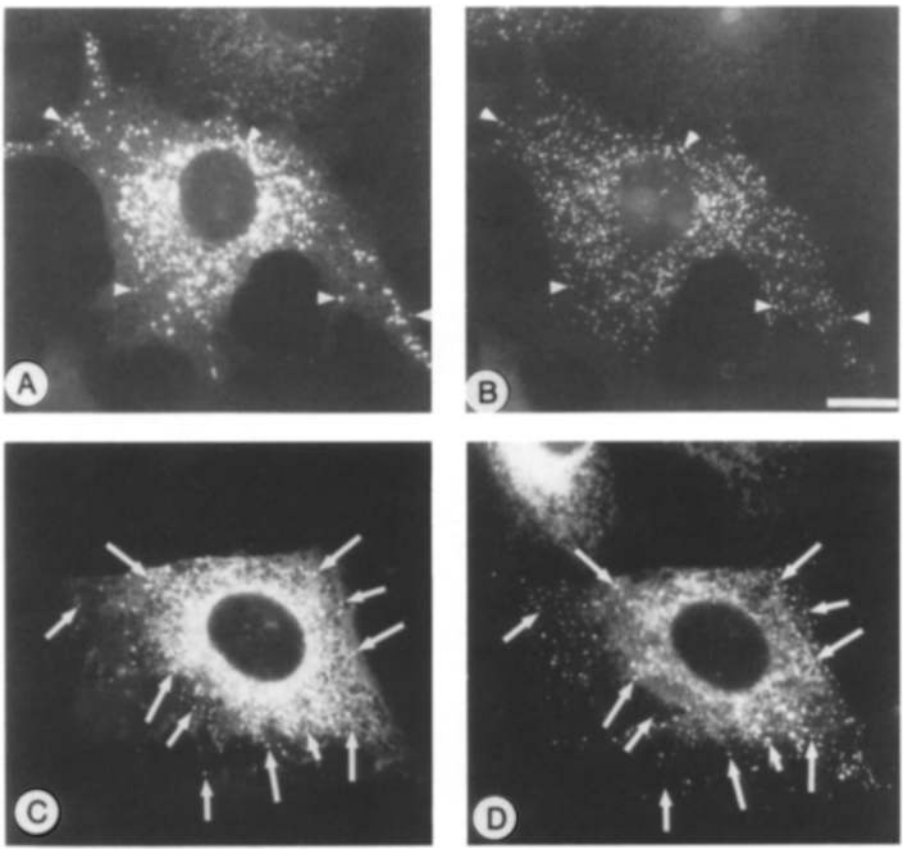

Figure 5. Localization of alcohol oxidase and other endogenous peroxisomal proteins. Microinjected alcohol oxidase (*A* and *C*) and peroxisomally located CAT-PMP-20 (*B*) and peroxisomal integral membrane proteins (*D*) in BALB/c 3T3 cells, and BALB/c 3T3 cells transfected with plasmid pSV2CAT-PMP-20. After injection, cells were incubated for 16 h at 37°C (*A* and *B*), or 3 h at 37°C (*C* and *D*), and then processed for double indirect immunofluorescence. Arrows indicate vesicles that costained for both alcohol oxidase and endogenous peroxisomal proteins. Determination of costaining vesicles involved the printing of large-format photographs of each micrograph at identical magnification. Coalignment was deduced by triangulation from fixed reference points on each photograph. Bar, 10 μm.

X-100, followed by indirect immunofluorescence, revealed punctate structures containing alcohol oxidase (Fig. 2 *d*) in injected cells (Fig. 2 *b*). In control experiments, BALB/c 3T3 cells permeabilized with digitonin alone failed to reveal the peroxisomally located catalase, but showed the normally observed punctate pattern when permeabilized with digitonin plus Triton X-100 before immunocytochemical staining (Walton et al., 1992).

Time and Temperature Dependence of Transport

The transport of alcohol oxidase was time and temperature dependent (Fig. 3, *a–h*). No vesicles were observed immediately following microinjection of alcohol oxidase (not shown). A few vesicles appeared after incubations of 20 min at 37°C (Fig. 3, *a* and *b*) and the size and number grew through 40 min (Fig. 3, *c* and *d*) and 60 min (Fig. 3, *e* and *f*). Transport was inhibited by incubation at 20°C (Fig. 3, *g* and *h*). No punctate staining was observed in 60-min incubations at 4°C (not shown).

Peptides Containing the Tripeptide PTS Inhibit Transport of Alcohol Oxidase

To determine if the transport of alcohol oxidase used components of the mammalian peroxisomal transport system, the effect of synthetic peptides shown to be inhibitory to the transport of microinjected luciferase (Walton et al., 1992) was studied. These peptides mimic the carboxy-terminal tripeptide peroxisomal targeting signal described by Gould et al. (1990*b*) and were not identical to the carboxy terminus of alcohol oxidase from *P. pastoris*. Three synthetic peptides bearing the peroxisomal targeting signals NH_3-CRYHLKPLQAKL-COOH (Fig. 4, *c* and *d*), NH_3-CRYSRLSHLSKL-COOH (Fig. 4, *e* and *f*), and NH_3-CRYHLKPLQSRL-COOH (Fig. 4, *g* and *h*) were injected at a 50-fold molar excess with the alcohol oxidase. As can be seen in Fig. 4, these coinjected peptides abolished transport of alcohol oxidase in 60-min incubations. In long-term incubations (18 h), coinjection of these inhibitory peptides blocked alcohol oxidase transport in BALB/c 3T3 cells (not shown). A control peptide bearing the first nine amino acids of the AKL and SRL inhibitory peptides (NH_3-CRYHLKPLQ-COOH) had no effect on transport either at a 50-fold (Fig. 4, *a* and *b*) or at a 100-fold molar excess (not shown).

Some, but not all, of the Microinjected Alcohol Oxidase Is Transported to Preexisting Peroxisomes

To determine if the microinjected alcohol oxidase was transported into the matrix of peroxisomes, further injections into BALB/c 3T3 cells expressing the CAT-PMP-20 fusion protein in a peroxisomal location were undertaken. The expression and peroxisomal transport of this fusion protein, containing the last 12 amino acids of the *C. boidinii* PMP-20 gene fused to the carboxy terminus of chloramphenicol acetyltransferase in transfected CV-1 cells has been described previously (Gould et al., 1989, 1990*a*). In the present study the CAT-PMP-20 protein colocalized with endogenous catalase to the peroxisomes as judged by double indirect immunofluorescence (not shown). The results (Fig. 5, *a* and *b*) demonstrate that the majority of vesicles containing alcohol oxidase were distinct from those containing the CAT-PMP-20 fusion protein. They differed in both size and location

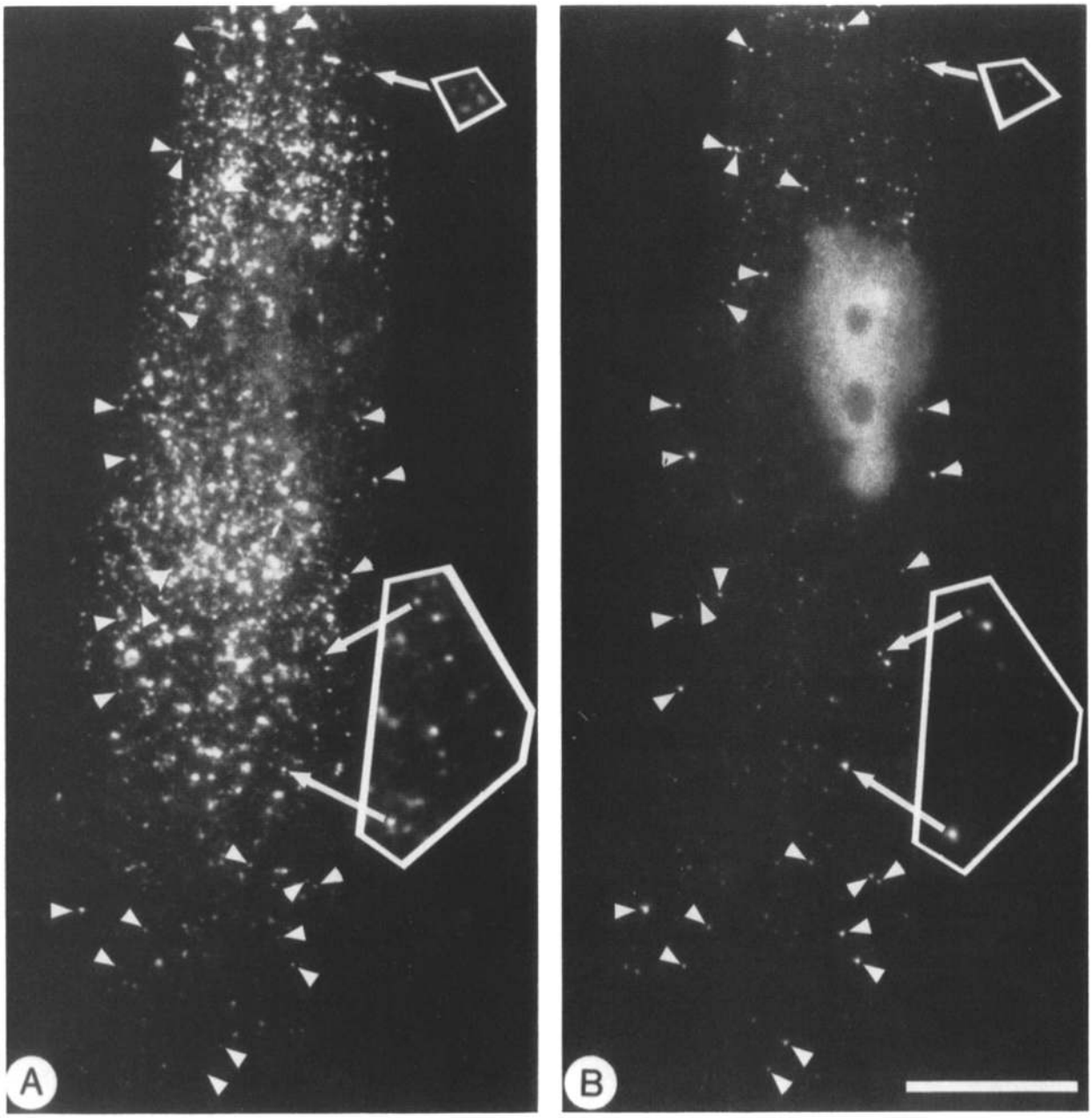

Figure 6. Localization of coinjected alcohol oxidase and luciferase. Cells microinjected with alcohol oxidase (*A*) and luciferase (*B*) were incubated for 16 h at 37°C and then processed for double indirect immunofluorescence. Arrows indicate vesicles that costained for both alcohol oxidase and luciferase. Inserts are approximately twofold magnifications. Determination of costaining vesicles involved the printing of large-format photographs of each micrograph at identical magnification. Coalignment was deduced by triangulation from fixed reference points on each photograph. Bar, 10 μm.

when compared to endogenous peroxisomes. However, several vesicles (~1% of the total) costained for both alcohol oxidase and CAT-PMP-20, as judged by both vesicle size and triangulation from fixed reference points. When microinjected alcohol oxidase was costained with antibodies directed against peroxisomal integral membrane proteins (Fig. 5, *c* and *d*) similar results were obtained; a small fraction of the vesicles containing the alcohol oxidase colocalized with vesicles that stained for peroxisomal integral membrane proteins.

When alcohol oxidase was coinjected with luciferase (Fig. 6, *a* and *b*) the proteins appeared to be transported both to identical and distinct vesicles. The luciferase was transported to peroxisomes as demonstrated by colocalization with the endogenous catalase (Walton et al., 1992). These results indicated that at least a fraction of the microinjected alcohol oxidase was transported to vesicles containing peroxisomal proteins, but there were also vesicles containing alcohol oxidase or luciferase alone. Coinjection of alcohol oxidase and luciferase resulted in the transport of both proteins without apparent competition at the levels used.

Transport of Microinjected Alcohol Oxidase in Human Cells

The transport of alcohol oxidase in various human cell lines was examined by microinjection. The cell lines included a

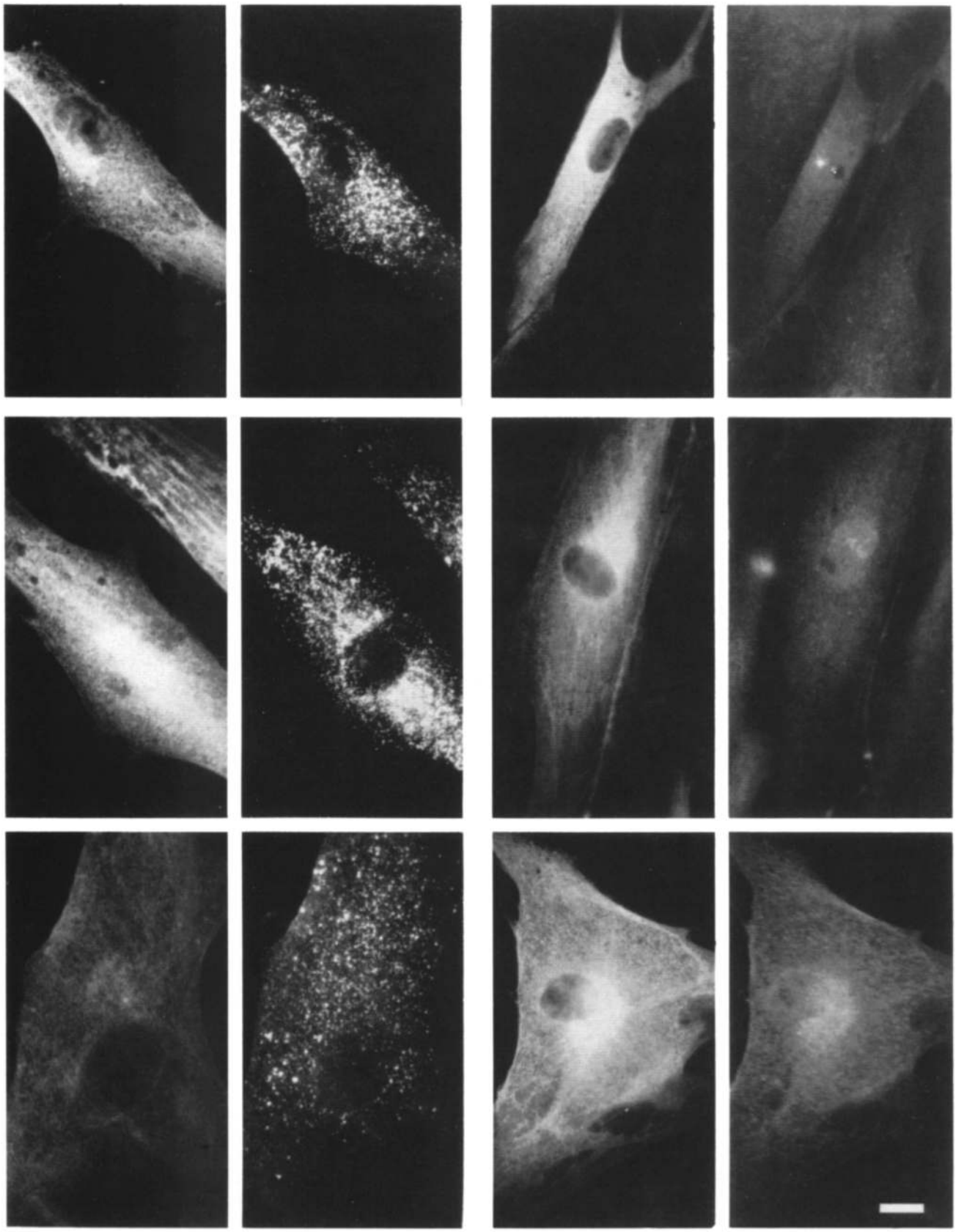

Figure 7. Transport of microinjected alcohol oxidase in human cell lines. Normal human fibroblasts (HS68) (*top row*) and cell lines derived from patients with Zellweger syndrome GM00228 (*second row*) and GM04340 (*third row*), were injected with alcohol oxidase (first and second columns), or alcohol oxidase plus a 100-fold molar excess of a peptide bearing the PTS (NH_2-CRYHLKPLQAKL-COOH) (third and fourth columns). After injection the cells were incubated for 16 h at 37°C and then processed for double indirect immunofluorescence. Columns 1 and 3 indicate injected cells as visualized by staining for coinjected mouse IgG. Columns 2 and 4 show the distribution of alcohol oxidase in these injected cells. Bar, 10 μm.

normal human fibroblast cell line (HS68), and two cell lines derived from patients with lethal abnormalities in peroxisomal transport. The cell lines GM00228 and GM04340 come from patients with Zellweger syndrome. These two cell lines were unable to transport microinjected proteins ending in the SKL tripeptide PTS into their peroxisomes (Walton et al., 1992). As can be seen in Fig. 7 (first and second columns) all three cell lines imported extensive amounts of alcohol oxidase in overnight (18 h) incubations. This transport uses some components of the peroxisomal transport machinery. Coinjection of the inhibitory peptide (NH_2-CRYHLKPLQAKL-COOH) bearing the PTS at a 100-fold molar excess abolished transport in all of the cell lines (Fig. 7, third and fourth columns).

Discussion

This report describes the transport of microinjected alcohol oxidase into vesicles in mammalian cells. The inability to detect alcohol oxidase-containing vesicles in cells permeabilized with digitonin confirms that the protein is on the inside of the vesicles, and not residing untransported on the surface or as aggregates in the cytoplasm.

The vesicles containing alcohol oxidase appear to grow to a definite size upon overnight incubations. Like the peroxisomes seen in yeast (Goodman et al., 1984; Distel et al., 1988; Roggenkamp et al., 1989; Cregg et al., 1990), the final diameter of these vesicles is 0.5–1.0 μm. It is not known if there is a mechanism which limits the size of the vesicles, although they are much larger than that observed with endogenous peroxisomes.

Several lines of evidence suggest that in mammalian cells the transport of alcohol oxidase is into peroxisomes. First, a subset of the alcohol-oxidase–containing vesicles clearly colocalizes with vesicles containing two other bona fide peroxisomal matrix proteins, luciferase and CAT-PMP20 (Figs. 5 and 6). Some of the alcohol oxidase–containing vesicles also colocalize with vesicles containing peroxisomal membrane proteins (Fig. 5). Second, the complete inhibition of transport of alcohol oxidase into vesicles by peptides containing the tripeptide PTS but not by control peptides lacking this PTS is a strong argument for the peroxisomal nature of the vesicles. Finally, the time and temperature dependence of alcohol oxidase transport into vesicles, as well as the saturability of the transport process, are all reminiscent of peroxisomal protein import in microinjected cells (Walton et al., 1992).

The temperature dependence is indicative of an energy-requiring process. Demonstration of a requirement for ATP by depleting endogenous ATP supplies was not possible in this in vivo system. We did not observe any alcohol oxidase bound to vesicles after 60 min at 4°C. Therefore, we could not confirm the results of Imanaka et al. (1987) in which they demonstrated binding of acyl-CoA oxidase to rat liver peroxisomes, without transport, at 0°C. The transport of alcohol oxidase was approximately eightfold faster than that observed for microinjected luciferase (Walton et al., 1992). Although this may reflect a different ability to detect the proteins, it may also indicate a difference in transport between luciferase and alcohol oxidase.

Although, as stated above, some of the alcohol oxidase was transported into vesicles harboring other known peroxisomal proteins, the majority of the alcohol oxidase–containing vesicles did not contain other peroxisomal proteins. It seemed possible that the import of such large amounts of alcohol oxidase masked the endogenous peroxisomal proteins from immunological detection. In yeast, alcohol oxidase assembles into large crystalline arrays following import (Veenhuis et al., 1978, 1981). However, it seems inconceivable that the preexisting peroxisomal proteins were being entombed by the recently imported alcohol oxidase, as costaining with antibodies directed against peroxisomal integral membrane proteins failed to demonstrate a significantly greater number of costained vesicles. In uninjected cells these antibodies directed against peroxisomal integral membrane proteins appear to stain the endoplasmic reticulum. However, within 1–2 min after injection of alcohol oxidase into normal fibroblast and Zellweger cell lines, the reticular pattern of staining is diminished and many punctate vesicles appear. These vesicles persist for at least 16-h postinjection. This antibody does not recognize alcohol oxidase on Western blots, nor is the alcohol oxidase in punctate vesicles immediately after microinjection. Bodnar and Rachubinski (1991) have used this antibody to demonstrate that a 50 kD peroxisomal integral membrane protein is synthesized on membrane-bound polysomes of the ER. We hypothesize that we have observed the recruitment of a protein constituent of the ER into vesicles following microinjection of alcohol oxidase. Further experiments are in progress to characterize this intriguing observation in greater detail.

The nature of the majority of vesicles containing transported alcohol oxidase is an enigma. The alcohol oxidase–containing vesicles did not costain with antibodies directed against proteins of the lysosomal, rough ER or Golgi compartments (results not shown). Furthermore, the absence of any KFERQ-like sequences (Dice et al., 1987; Koutz et al., 1989) in alcohol oxidase, the absence of any effect on the vesicles by chloroquine and the long-term stability of the vesicles argue that the vesicles are not lysosomes. In view of the evidence that transport of alcohol oxidase into vesicles is completely inhibited by PTS peptides and in the absence of any reason to think that the vesicles comprise some other subcellular compartment, we conclude that the alcohol oxidase–containing vesicles are either novel peroxisomes or a new peroxisome-like compartment.

The existence of vesicles containing only a subpopulation of peroxisomal enzymes is not unprecedented. In *Neurospora crassa*, there exist vesicles that contain peroxisomal proteins but not catalase (Wanner and Theimer, 1982; Kionka and Kunau, 1985). Several yeasts display heterogeneities in their microbody population. When *H. polymorpha*, *C. boidinii*, or *C. utilis* were shifted from media containing ammonium sulfate to those containing methylamine as the nitrogen source, the newly synthesized amine oxidase was transported to a distinct subset of microbodies (Veenhuis et al., 1989). In *H. polymorpha*, overexpression of alcohol oxidase in a nonmethylotrophic state results in its transport to vesicles that lack the other enzymes of methanol assimilation (Distel et al., 1988). Synthesis of large amounts of alcohol oxidase does not result in the expression of other peroxisomal proteins (Roggenkamp et al., 1989). Similarly, expression of dihydroxyacetone synthase from *H. polymorpha* in *S. cerevisiae* induces growth but not proliferation of peroxisomes (Godecke et al., 1989). Similarly, microinjec-

tion of alcohol oxidase into mammalian cells results in the induction of new vesicles and the translocation of the alcohol oxidase into peroxisome-like vesicles, but does not involve the expression and transport of other peroxisomal proteins into these same vesicles.

The ability of Zellweger syndrome fibroblasts to transport alcohol oxidase but not catalase, luciferase or albumin conjugated to peptides bearing the carboxy-terminal tripeptide PTS (Walton et al., 1992) is reminiscent of the transport of another matrix protein, peroxisomal thiolase, into peroxisomes in some of these patients (Balfe et al., 1990). Recent evidence from our laboratory has shown that thiolase contains a new amino-terminal PTS distinct from the carboxy-terminal tripeptide PTS (Swinkels et al., 1991). Based on this analogy, and the lack of evidence for the existence of an SKL-like PTS in alcohol oxidase (Hansen et al., 1990), we suggest that alcohol oxidase, like thiolase, is likely to contain a different PTS than luciferase. Import of some proteins destined for the mitochondria is proposed to be mediated through two different targeting signals that bind to two distinct receptors. Translocation then occurs by interaction of these receptors with a general insertion protein located in the outer mitochondrial membrane (Pfanner and Neupert, 1990). The intriguing result that peptides containing the tripeptide PTS inhibit transport of alcohol oxidase into peroxisome-like vesicles would then imply that although alcohol oxidase and luciferase contain different PTS's they share a common downstream component of the recognition and/or translocation machinery.

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