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Dual Subcellular Distribution of Cytochrome b_5 in Plant, Cauliflower, Cells

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Subfractionation studies showed that cytochrome b_5 (cyt b5), which has been considered to be a typical ER protein, was localized in both the endoplasmic reticulum membrane (ER) and the outer membrane of mitochondria in cauliflower (*Brassica olracea*) cells and was a component of antimycin A–insensitive NADH–cytochrome *c* reductase system in both membranes. When cDNA for cauliflower cyt b5 was introduced into mammalian (COS-7) and yeast cells as well as into onion cells, the expressed cytochrome was localized both in the ER and mitochondria in those cells. On the other hand, rat and yeast cyt b5s were specifically localized in the ER membranes even in the onion cells. Mutation experiments showed that cauliflower cyt b5 carries information that targets it to the ER and mitochondria within the carboxy-terminal 10 amino acids, as in the case of rat and yeast cyt b5s, and that replacement of basic amino acids in this region of cauliflower cyt b5 with neutral or acidic ones resulted in its distribution only in the ER. Together with the established findings of the importance of basic amino acids in mitochondrial targeting signals, these results suggest that charged amino acids in the carboxy-terminal portion of cyt b5 determine its location in the cell, and that the same mechanism of signal recognition and of protein transport to organelles works in mammalian, plant, and yeast cells.

Key words: cytochrome b_5, dual subcellular localization, endoplasmic reticulum, mitochondria, targeting signal.

Abbreviations: cyt b5, cytochrome b_5; ER, endoplasmic reticulum; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride.

Correct targeting of nuclear-encoded protein within the eukaryotic cell is essential for its function and for the biogenesis of the various organelles. Protein targeted to the ER and mitochondrial compartments follows distinct pathways and different targeting signals and transport machineries (*1*). A majority of proteins targeted to the ER reach their destination through a co-translational mechanism that requires the association of the N-terminal signal sequence with a signal recognition particle (SRP) and with the translocation complex in the ER membrane (*2*). Mitochondrial protein transport, on the other hand, occurs through a post-translational mechanism and involves a complex series of interactions of the protein with various cytosolic factors (*3*). However, some ER proteins with large cytoplasmic domains and short membrane anchors escape the co-translational mechanism and are transported by the post-translational one (*4*).

Cytochrome b_5 (cyt b5) is a small heme protein associated with the ER membrane of animals, higher plants and yeast. It is composed of three domains: (i) the amino-terminal hydrophilic heme-containing domain, which participates in electron transferring functions; (ii) the hydrophobic transmembrane domain, which functions for insertion of the proteins into the membranes as "tail-anchored" proteins; and (iii) the carboxy-terminal short hydrophilic domain. Another type of cyt b5, OMb5, is found in the outer mitochondrial membrane of rat liver (*5*). It is about 70% identical in amino acid sequence and has the same domain structure as the ER counterpart (*6*). In a previous study, we obtained evidence that the carboxy-terminal 10 amino acid residues of rat cyt b5 and OMb5 contain sufficient information to transport the cytochromes to their correct organelles and that charged amino acids in this portion determine the destination organelle (*7–9*). The presence of a positively-charged amino acid in the carboxy-terminal portion targets the cytochrome more to mitochondria (*9*).

The amino acid sequences of cyt b5 from plants, including cauliflower (*10*), tobacco (*11*), and rice (*12*), have been determined by means of cDNA cloning. They share a common characteristic with OMb5, *i.e.*, they are rich in positively-charged amino acids in the carboxy-terminal polar portion: -LVVRQYTKKE for cauliflower, -FGIRFYTKQSSA for rice, and -VAIRIYTKSESA for tobacco, in contrast to -LMYRLYMAED for rat. The cytochrome, however, has been considered to be a typical ER membrane

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proteins and is reported to be transported to the ER in cDNA-transfected tobacco cells (*11*).

In the present study, we investigated whether cyt b5 is indeed transported to mitochondria as well as the ER in plant cells and whether the signaling and intracellular transport of cyt b5 to the ER membrane involve the same mechanism as that in mammalian cells. We obtained evidence that cyt b5 is localized to both the ER and the outer membrane of mitochondria in cauliflower cells, and that the principle for targeting of cyt b5 is common to mammalian, plant, and yeast cells.

MATERIALS AND METHODS

Materials—Restriction enzymes and DNA modifying enzymes were purchased from Takara, Nippon Gene, and Toyobo. Primer DNA was from Hokkaido System Science Work Station. A QuickChange kit for site-directed mutation, Pfu DNA polymerase, and an expression vector pSG5 for mammalian cells were from Stratagene. Yeast expression vector YEp51 was a kind gift from Dr. H. Tanaka of the National Institute of Advanced Industrial Science and Technology. Dulbecco's modified Eagle's medium, fetal calf serum were from Nissui and Boehringer Mannheim, respectively. Peroxidase-conjugated and fluorescein isothiocyanate conjugated goat anti–rabbit IgG were from Cappel Products and EY Laboratory, respectively. Yeast extract, Peptone, and yeast extract without amino acid were from Difco. Zymolyase 100T and cellulase were from Seikagaku Kogyo and Yakult, respectively. BCA protein assay kit was from Pierce.

Preparation of Membrane Fractions from Cauliflower Inflorescences—Cauliflower inflorescences (*Brassica olracea*, L.) were purchased at the local market. Membrane fractions were prepared from cauliflower essentially as described (*13*). All procedures were carried out at 4°C. In a typical preparation, the florets were blended to a paste in a Waring blender with grinding buffer consisting of 0.3 M mannitol, 10 mM Hepes (pH 7.2), 1 mM EDTA, 0.05% (w/v) cysteine-HCl, 1 μM leupeptin, 1 μM pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The paste was then filtered through three layers of cheesecloth, and the filtrate was readjusted to pH 7.2 with KOH, then centrifuged to yield mitochondrial (1,000 ×*g*, 10 min; 6,000 ×*g*, 10 min) and microsomal (10,000 ×*g*, 10 min; 100,000 ×*g*, 60 min) pellets. The pellets were resuspended in a solution of 0.3 M mannitol, 10 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 10 mM KCl, 1 μM leupeptin, and 1 μM pepstatin.

Intact mitochondria were purified through a sucrose cushion (0.6 M sucrose, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 2 mM PMSF) by centrifugation at 47,000 ×*g* for 30 min. The mitochondrial pellet was resuspended in 3 volumes of 20 mM potassium phosphate buffer pH 7.2, containing 0.02% bovine serum albumin. Outer membrane was then purified according to Ito (*14*).

Construction of Cyt b5 Derivatives—cDNA for rat cyt b5 was obtained previously (*7*). cDNA for cauliflower and yeast cyt b5s were obtained by PCR, according to a published nucleotide sequence (*10*, *15*). The cDNA was inserted into pSG5, pUC18s35GFP, and YEp51 vectors for expression in mammalian, plant, and yeast cells, respectively.

To make Rb5C10, the carboxy-terminal 10 amino acid residues of cauliflower cyt b5 were fused by PCR with the amino-terminal portion of rat cyt b5 from which the carboxy-terminal 10 amino acids had been deleted. Site-directed mutations of the carboxy-terminal portion of cauliflower cyt b5 were done with the QuikChange kit.

Expression of Cyt b5 in COS-7 Cells and Cell Fractionation—DNA transfection into COS-7 cells and cell fractionation were carried out as described previously (*7*). After culture of the cells for 48 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, cells expressing the original and mutated cyt b5 were harvested and homogenized in ice-cold STE buffer (0.25 M sucrose, 20 mM Tris-HCl, 0.1 mM EDTA, 10 mg/ml leupeptin, and 10 μg/ml pepstatin, pH 8.0). The homogenate was centrifuged at 600 ×*g* for 5 min to precipitate the nucleus and unbroken cells, then the post-nuclear supernatant was successively centrifuged at 8,000 ×*g* for 8 min, 10,000 ×*g* for 8 min, and 200,000 ×*g* for 20 min, to obtain the mitochondrial, lysosomal, and microsomal fractions, respectively.

Expression of Cauliflower Cyt b5 in Plant Cells—Onion epidermal cell protoplasts were obtained by a modification of the method of Itai and Roth-Bejerano (*16*). The epidermal strips were suspended in 50 ml of a cell wall-digesting medium composed of 0.6 M mannitol, 3 mM Mes-Tris buffer, 1 mM $CaCl_2$, 0.5% (w/v) BSA, and 4% (w/v) cellulase, pH 5.4. The tissues were incubated at 30°C in the dark for 3 h. After filtration of the suspension, protoplasts were pelleted by centrifugation at 40 ×*g* for 5 min at 4°C.

DNA transfection was done by the PEG method. Plasmid DNA (5–20 μg in 10 μl of TE buffer) was mixed at room temperature with 6×10^5 protoplasts in 0.3 ml of preuptake solution (0.6 M mannitol, 50 mM sucrose, and 1 mM $CaCl_2$, pH 5.4) for 5 min. An equal volume of 40% PEG 4000 solution was added, and the sample was incubated at room temperature for 5 min. The sample was finally diluted with 4 ml of preuptake solution, then incubated in the dark at 25°C for 24 h.

Expression of Cyt b5 in Yeast—Transformation of yeast (*Saccharomyces cerevisiae* strain BL2168) was performed basically according to Ito *et al.* (*17*). The transformed yeast cells were first cultured in leucine drop-out medium for 30 h, then in a medium containing 2% galactose in place of glucose for another 30 h. After Zymolyase treatment of the harvested cells, the cells were collected, resuspended in the lysis buffer (0.65 M sorbitol, 20 mM Tris-HCl pH 7.0, 1 μM leupeptin, and 1 μM pepstatin), then homogenized in a glass-Teflon homogenizer. After centrifugation at 1,500 ×*g* for 10 min, the post-nuclear supernatant was layered over a sucrose density gradient consisting of 0.5 ml of 2 M sucrose, 11 ml of a linear gradient from 0.7 to 1.8 M sucrose solution containing 20 mM Tris-HCl pH 7.0 and 1 μM leupeptin, 1 μM pepstatin, and 0.5 M sucrose solution, and centrifuged at 30,000 rpm for 3 h in an RPS-40T rotor (Hitachi). Fractions of 1 ml were collected from the top of the tube and designated as fraction numbers 1–14.

Immunofluorescence Microscopy—Immunofluorescence microscopy was carried out as described previously (*7*),

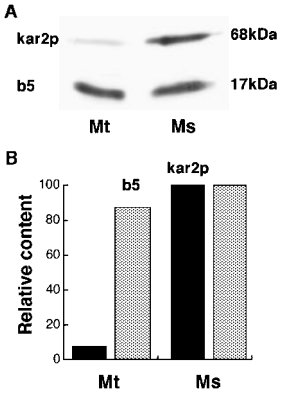

Fig. 1. **Distribution of cyt b5 between mitochondria and microsomes in cauliflower cell.** A: Mitochondrial (Mt) and microsomal (Ms) fractions were fractionated as described under "MATERIALS AND METHODS," and proteins were separated on SDS-PAGE. Anti-kar2p and anti-yeast cyt b5 were used as primary antibodies. The positions of protein molecular mass markers are shown on the right. B: The amounts of the corresponding proteins localized in Mt and Ms fractions were determined from immunoblotting shown in A. Cyt b5, lightly shaded bars; kar2p, densely shaded bars.

using rabbit anti–cyt b5 antibody and fluorescein isothiocyanate-conjugated goat anti–rabbit IgG for first and second antibodies, respectively.

Trypsin Treatment of Mitochondria and Microsomes—Microsomal and mitochondrial fractions were treated with 25 μg/mg proteins of trypsin at 0°C for 1 or 7 h in the presence of 0.1% Triton X-100. After addition of 2 mM PMSF to stop the reaction, the microsomal and mitochondrial fractions were centrifuged at 50,000 rpm for 20 min, and the pellets and supernatants were used for SDS-PAGE and immunoblotting.

Other Analytical Procedures—Antimycin-insensitive NADH–cytochrome *c* reductase activity was measured as described by Ito (*14*). Protein was determined by the BCA method with bovine serum albumin as a standard. Immunoblotting was done using antibodies against cyt b5 and the peroxidase-conjugated goat anti-rabbit IgG for the primary and secondary antibodies, respectively.

RESULTS

Dual Subcellular Localization of Cyt b5 in Cauliflower Cells—Intracellular localization of cyt b5 in cauliflower cells was studied using subcellular fractionation. Preparation of membrane fractions from cauliflower inflorescences was done essentially as described under "MATERIALS AND METHODS." Proteins of mitochondrial and microsomal fractions were separated on SDS-PAGE (Fig. 1). The anti–yeast cyt b5 antibody, which shows the same reaction characteristics as anti–cauliflower cyt b5 (data not shown), bound to a band of 17 kDa in both the microsomal and mitochondrial samples in about equal amounts. Considering the distribution pattern of kar2p, which is a marker protein of microsomes (*18*), the cyt b5–like protein found in mitochondrial fraction was not derived from contaminating microsomes and must be an integral component of mitochondria, though the cyt b5 has been considered to be localized only in the ER (*10*).

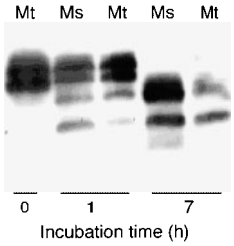

Fig. 2. **Trypsin treatment of mitochondrial and microsomal cyt b5.** Mitochondrial (Mt) and microsomal (Ms) fractions were first treated with 0.1% Triton X-100 at 0°C for 10 min, then trypsin was added to 25 μg/ml and incubation was continued for 1 or 7 h at 0°C as described under "MATERIALS AND METHODS." Digests were analyzed by SDS-PAGE and immmunoblotting.

To confirm that cyt b5–like protein found in the outer mitochondrial membrane is the same protein as microsomal cyt b5, trypsin digestion was done (Fig. 2). The mitochondria and microsomes were exposed to trypsin (2.5% of the amount of membrane proteins) in the presence of Triton X-100 at 0°C. After incubation for 1 or 7 h, PMSF was added to inactivate the protease. The digested fragments were detected by immunoblotting with anti–yeast cyt b5 antibody. The cyt b5–like protein in the outer membrane showed a similar peptide map to the microsomal cyt b5, though even at zero time the sample was slightly digested, this probably occurring during the processes of incubation and detection. The result suggests that cyt b5–like protein in the outer mitochondrial membrane is the same as or quite similar to microsomal cyt b5.

In rat liver, the rotenone- and antimycin-insensitive NADH–cytochrome *c* reductase activity associates with the outer mitochondrial membrane, and OMb5, a cyt b5–like hemoprotein, participates in this activity (*14*). To investigate the location of the cyt b5–like protein in the cauliflower mitochondria, the outer membrane was iso-

Table 1. **Localization of cauliflower cyt b5 to purified outer membrane of cauliflower mitochondria.**

Enzymes	Mitochondria		Outer mitochondrial membrane	
	Total activity (mu/g tissue)	Relative value	Total activity (mu/g tissue)	Relative value
Cyt oxidase	57.2	100	1.4	2.5
Antimycin-sensitive NADH cyt c reductase	18.4	100	<0.1	<0.5
Antimycin-insensitive NADH cyt c reductase	12.3	100	5.3	43
Cyt b5	–	100	–	65

Outer membrane of cauliflower cell was purified as described in "MATERIALS AND METHODS." Antimycin-insensitive NADH cyt c reductase activity is shown as marker for outer membrane of mitochondria. Antimycin-sensitive NADH cyt c reductase and cyt c oxidase activities are shown as markers for inner membrane of mitochondria.

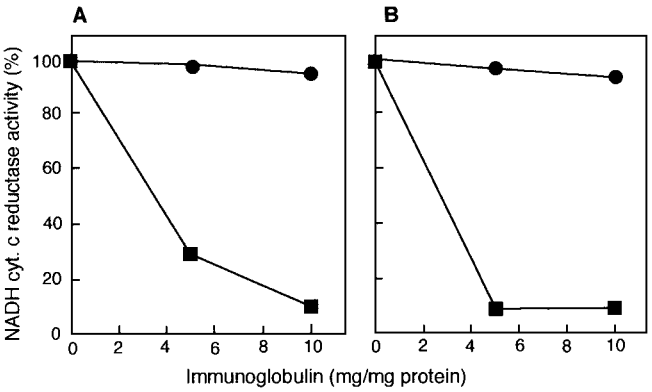

Fig. 3. **Inhibition of antimysin-insensitive NADH–cyt c reductase activities of mitochondria and microsomes by the antibody.** Mitochondria (A) and microsomes (B) were pre-incubated with anti–cyt b5 immunoglobulin or with control immunoglobulin in 0.1 M phosphate buffer (pH 7.5) for 10 min at 25°C, and then the reductase activity was assayed at 25°C by adding antimysin, cyt c, and NADH as described under "MATERIALS AND METHODS." The concentrations of mitochondria and microsomes in the reaction mixtures were each 0.04 mg of protein per ml. Control IgG, solid circle; anti–cyt b5 IgG, solid square.

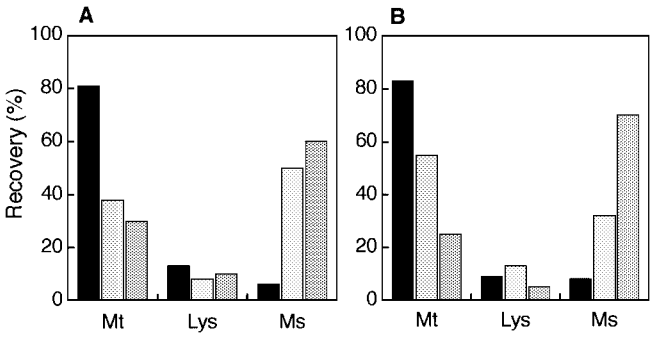

Fig. 4. **Subcellular distribution of rat and cauliflower cyt b5s expressed in onion epidermal protoplasts.** Subcellular distribution of the expressed cyt b5s (A, rat cyt b5; B, cauliflower cyt b5) and marker proteins in onion epidermal protoplast cells. Cells expressing cyt b5s were homogenized and the mitochondrial (Mt), lysosomal (Lys), and microsomal (Ms) fractions were fractionated, as described under "MATERIALS AND METHODS." The amounts of cyt b5s (lightly shaded bars) were measured by immunoblotting. The distribution of β-subunit of mitochondrial processing peptidase (black bars) and protein disulfide isomerase (densely shaded bars) are shown as markers for mitochondria and microsomes, respectively. The sum total values of the three fractions are given a value of 100%, and the bars indicate the percentage of the total that each represents.

lated as described under "MATERIALS AND METHODS." As seen in Table 1, the cyt b5–like protein exhibited the same distribution pattern as the antimycin-insensitive NADH–cytochrome *c* reductase activity, indicating localization of the cytochrome in the outer mitochondrial membranes and suggesting participation of the cytochrome in this activity of the membrane.

Participation of the cytochrome in the antimycin-insensitive NADH–cytochrome *c* reductase activity was confirmed by an antibody inhibition experiment (Fig. 3). The antibody blocked electron transfer from NADH to cytochrome *c* by up to 90% at an IgG to outer membrane proteins ratio of 0.7. The result indicates that cyt b5 is a component of the mitochondrial antimycin-insensitive NADH–cytochrome *c* reductase system, like the system in the ER in plant. These results suggest that cyt b5 exists as an intrinsic component of the mitochondrial outer membrane as well as the ER membrane and functions as an electron transfer component in these membranes, though the cytochrome has been considered to be a typical component of the ER membrane in plant cells.

Cauliflower Cyt b5 Is Transported to the ER and Mitochondria in Isolated Plant Cells—To confirm that a single protein is able to be transported to two organelles in plant cells, we investigated intracellular transport of cyt b5 expressed from a cDNA in plant cells. Since good cultured cells had not obtained from cauliflower, we used onion epidermal cells for isolation of protoplasts. The protoplasts were isolated as described under "MATERIALS AND METHODS," and were proved to be composed of active cells from the result of fluorescein diacetate staining (data not shown). The plasmids S35GFPcb5 and S35GFPRb5, which contained cauliflower and rat cyt b5 in a plant expression vector, respectively, were transfected into the protoplasts by PEG-mediated transformation, and the protoplasts were incubated for 24 h. The subcellular distribution of the protein was analyzed by immunoblotting with anti–cit b5 antibody after cell fractionation (Fig. 4). In this experiment, protein disulfide isomerase and mitochondrial processing peptidase were used as marker proteins for microsome and mitochondria, respectively. The distribution pattern of rat cyt b5 was essentially the same as that of the microsomal marker enzyme, while cauliflower cyt b5 was found to be present in both microsomes and mitochondria. The results indicate that cauliflower cyt b5 is indeed transported to both the ER and mitochondria in plant cells.

Intracellular Targeting of Cauliflower Cyt b5 in Mammalian and Yeast Cells—The above results also suggest that the protein transport mechanism is common and universal among eukaryotes. To confirm this, intracellular transport of cauliflower and rat cyt b5 was investigated with mammalian and yeast cells. As shown in Fig. 5, staining of mammalian COS-7 cells expressing cauliflower cyt b5 revealed a tubular network and a string-like structure around the nucleus, which are typical ER and mitochondrial patterns of fluorescence, suggesting that the protein is localized both in the ER and mitochondria, while the cells expressing rat cyt b5 showed an ER-type fluorescence pattern. When mutated protein, Rb5C10(cau), in which 10 amino acid residues of the carboxy-terminus of cauliflower cyt b5 were fused to the truncated rat cyt b5 lacking the corresponding 10 amino acids, was expressed, cells exhibited a dual fluorescent pattern, as shown in cauliflower cyt b5. These results were clearly confirmed by subcellular fractionation experiments (Fig. 5, D–F). The findings suggest that carboxy-terminal 10 amino acid residues of cauliflower cyt b5 carry information for intracellular location of the protein, like those of mammalian cyt b5 do (*7*).

We next investigated the subcellular distribution of cauliflower, rat and yeast cyt b5 in yeast cells. The yeast cells containing YEp51cb5, YEp51Rb5, or YEp51Cb5 plasmid were first cultured in 200 ml of leucine drop-out

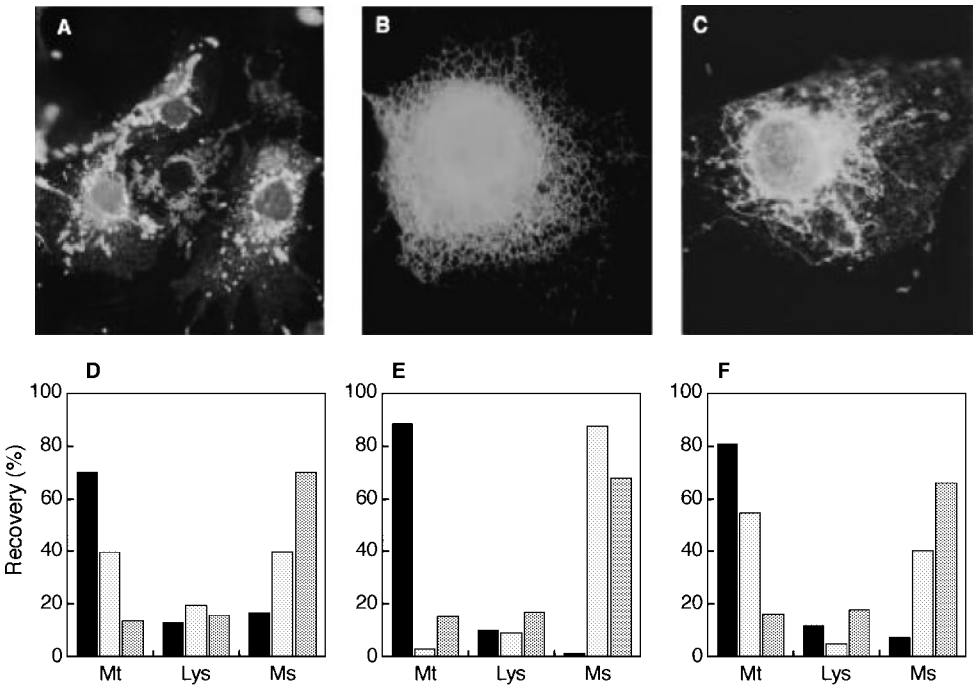

Fig. 5. **Subcellular distribution of cauliflower and rat b5s and Rb5C10(cau) expressed in COS-7 cells.** A–C: Indirect immunofluorescence microscopy of COS-7 cells expressing cyt b5s [A, cauliflower cyt b5; B, rat cyt b5; and C, Rb5C10(cau)]. Immunofluorescence staining was carried out as described under "MATERIALS AND METHODS." Anti–yeast cyt b5 antibody was used as the primary antibody of the staining for (A) and anti–rat cyt b5 antibody for (B) and (C). D–F: Subcellular distribution of the expressed cyt b5s [D, cauliflower cyt b5; E, rat cyt b5; and F, Rb5C10(cau)] and marker proteins in COS cells. Cells expressing cyt b5s were homogenized and the mitochondrial (Mt), lysosomal (Lys), microsomal (Ms) fractions were fractionated as described under "MATERIALS AND METHODS." The amounts of cyt b5s (lightly shaded bars) were measured by immunoblotting. The distributions of monoamine oxidase protein (black bars) and NADPH-cytochrome *c* reductase (densely shaded bars) are shown as markers for mitochondria and microsomes, respectively. Percent recovery of cyt b5s and marker proteins in each fraction are shown as described in the legend to Fig. 4.

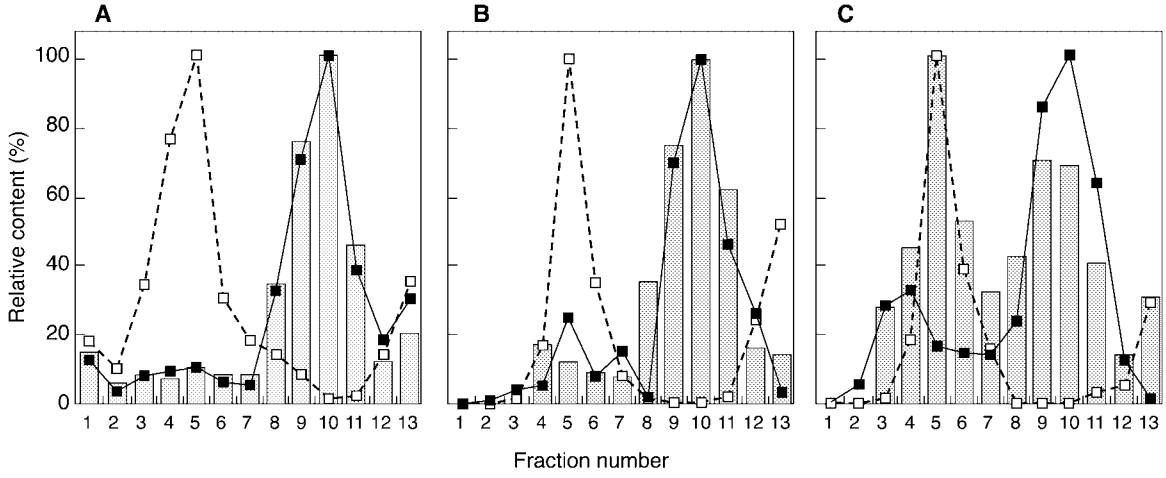

Fig. 6. **Subcellular distribution of yeast, rat, and cauliflower cyt b5s expressed in yeast cells.** Plasmids containing cDNA of each cyt b5 were transfected into yeast cells by the PEG method. After incubation for 60 h, the cells were harvested, homogenized and subfractionated by density gradient centrifugation as described under "MATERIALS AND METHODS." A: Cells expressing yeast cyt b5. B: Cells expressing rat cyt b5. C: Cells expressing cauliflower cyt b5. The amounts of expressed proteins (lightly shaded bars) were measured by immunoblotting. The distributions of β-subunit of mitochonrial processing peptidase (broken lines) and dolicol phosphate mannose synthetase (—) are shown as markers for mitochondria and microsomes, respectively.

medium for 30 h, then in medium containing 2% galactose in place of glucose for another 30 h. The subcellular distribution of cyt b5 expressed in the yeast cells was analyzed by sucrose density gradient centrifugation of the lysates, followed by immunochemical quantitation, using immunoblotting as described in "MATERIALS AND METHODS." Figure 6 shows the subcellular distribution of cyt b5 and of dolichol phosphate mannose synthase (Dpm1P) and α subunit of yeast mitochondria processing peptidase (yMPPα) as markers for ER and mitochondria, respectively. The pattern of distribution of yeast cyt b5 was the same as that of Dpm1P (Fig. 6A). The same result was obtained with rat cyt b5 (Fig. 6B). In cells expressing cauliflower cyt b5, however, both the ER and mitochondria were found to possess the protein, suggesting that cauliflower cyt b5 was transported into the ER and mitochondria in yeast cells. Taken together, these observations indicate that the transport mechanism of cyt b5 is common and universal among eukaryotes.

Charged Amino Acids in the Carboxy-Terminal Portion of Cauliflower Cyt b5 Are Key for Signal Function—Our previous study on the rat cyt b5 and OMb5 provided evi-

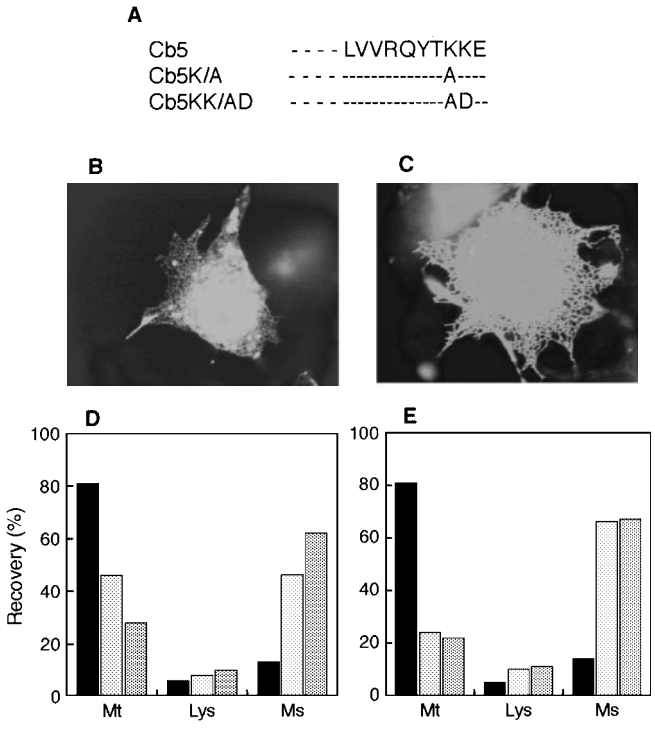

Fig. 7. **Subcellular distribution of cauliflower cyt b5 and its derivatives with site-directed mutations at the carboxy terminus.** A: Construction of cauliflower cyt b5 derivatives with site-directed mutations at the carboxy terminus. B and C: Indirect immunofluorescence microscopy of COS-7 cells expressing mutant cyt b5s (B, cells expressing Cb5K/A; C, cells expressing Cb5KK/AD). Staining was carried out as described under "MATERIALS AND METHODS." Anti–rat cyt b5 antibody was used as primary antibody. D and E, subcellular distribution of mutant cyt b5s expressed (D, Cb5K/A; E, Cb5KK/AD) and marker proteins in COS cells. Cell fractionation and the determination of the cyt b5 and marker proteins were performed as described under "MATERIALS AND METHODS." Percent recovery of cyt b5s and marker proteins in each fraction is shown as described in the legend to Fig. 4.

dence that charged amino acids in the carboxy-terminal signal portion of these proteins are key for signal function (*9*). Cauliflower cyt b5 is the type of protein that targets to both ER and mitochondria, and it contains three positive charges, Arg-128, Lys-132, Lys-133, and one negative charge, Glu-134, at the carboxy-terminal end. To determine whether the charged amino acids at the carboxy terminus of cauliflower cyt b5 have the same signaling function, two amino acids, Lys-132 and Lys-133, were replaced with alanine or aspartic acid by site-directed mutagenesis (Fig. 7A). In the cells expressing the mutant protein, cb5K/A, both the mitochondria and ER were stained and the ER was more strongly stained (Fig. 7B). The cells expressing cb5KK/AD showed only a reticular staining pattern that is characteristic of the ER in immunofluorescence microscopy (Fig. 7C). Essentially the same results were obtained in the subcellular fractionation studies (Fig. 7, D and E). Thus, both of the basic residues are important for the targeting function of the cytochrome to mitochondria.

DISCUSSION

The present study clearly demonstrated that, cyt b5 is localized to both the ER and outer membrane of mitochondria in cauliflower cells, though it has been considered to be a typical integral membrane protein of the ER in various plant cells (*e.g.*, Refs. *10* and *11*), and that a common mechanism for targeting of cyt b5 operates in mammalian, plant, and yeast cells.

Examples of the same gene products targeted to the ER and outer mitochondrial membrane are becoming more common. NADH–cyt b5 reductase (*19*, *20*), aldehyde dehydrogenase (*21*), glutathione S-transferase (*22*, *23*), and the proto-oncogene product Bcl-2 (*24*, *25*) have been proved to localized in both membranes. The present study enrolled cauliflower, probably plant, cyt b5 as a new member of these membrane proteins. Our data, especially from mutation experiments, have shown that the proteins are transported to both organelles through specific interaction with organelle proteins, not through a non-specific hydrophobic one.

We showed in the previous study of mammalian cells that intracellular transport of two isoforms of cyt b5 (cyt b5 in the ER, and outer membrane cyt b5 (OMb5) in the outer mitochondrial membrane) to the ER and mitochondria involves competing pathways and is controlled by the charged amino acids at the carboxy terminus. Introduction of a positively charged residue into the carboxy terminus of cyt b5 altered the intracellular location of this protein from the ER to the mitochondria, while replacement of positively charged amino acids in this portion of OMb5 with neutral or acidic ones resulted in transport of the mutant protein to the ER. Mutants B5D134A and OMbK144A, in which the acidic and basic amino acids, respectively, in the carboxy terminus were replaced with neutral ones, exhibited dual distribution to the ER and mitochondria.

Bcl-2 was also reported to locate in the ER and nuclear membranes as well as in mitochondria (*24*). The protein has two basic amino acids, His and Lys, located just after the transmembrane segment at the carboxy-terminal end (*26*), and these residues could function as the targeting signal for mitochondrial transport.

The carboxy-terminal 10 amino acid residues of cauliflower cyt b5 include three positively charged amino acid residues, and replacing these residues with neutral ones resulted in the transport of the mutant protein, Cb5KK/AA, to the ER, suggesting that the positively charged amino acids play an important role in the mitochondrial targeting of cauliflower cyt b5. It is likely that the targeting of cauliflower cyt b5 to the mitochondria occurs by a similar mechanism involving the recognition of positively charged amino acids at the carboxyl terminus of OMb5 and Bcl-2, and their intracellular transport to mitochondria.

Although the signals for post-translational targeting of proteins to the ER remain unknown, positively charged amino acids in either the amino- or carboxy-terminal portion direct proteins to mitochondria. Such function of the residues in cauliflower cyt b5 is probably insufficient for the signal to mitochondria and some portion of the protein may leak out of the transport apparatus so that the protein is transported to or associated with the ER membrane.

We also demonstrated that rat, cauliflower, and yeast cyt b5s were transported to the same destinations in a heterologous system. Cauliflower cyt b5 showed dual subcellular distribution to the ER and mitochondria in mammalian COS cells, as in plant cells, while rat and yeast cyt b5s were localized only in the ER even in the plant cells. The results indicate that the same mechanism of signal recognition and protein transport to organelles operates in mammalian, plant, and yeast cells, though we have no data for subcellular distribution of yeast cyt b5 in plant cells.

In mammalian cells, cyt b5 and OMb5 are a component of the NADH-cytochrome *c* reductase system in the ER and outer mitochondrial membrane, respectively (*14*). The former is involved in fatty acid desaturation and drug and steroid hydroxylation systems, while a known function of the latter is the reduction of monodehydroascorbate ascorbate to ascorbate (*27*). These cytochromes receive an electron from a common electron donor, NADH–cytochrome b_5 reductase, and transfer it to distinct acceptors. In plant cells, the same cytochrome is present in both membranes and participates in the electron transfer systems involving NADH–cytochrome *c* reductase activity. Each system seems to have specific and distinct functions and regulation mechanisms, though little information is available on physiological electron acceptors for cyt b5 in plants.

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