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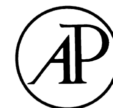

CYTOCHROME-C LOCALIZES IN SECRETORY GRANULES IN PANCREAS AND ANTERIOR PITUITARY

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We used quantitative immunogold electron microscopy to evaluate the subcellular distribution of cytochrome-c in normal rat tissues, employing a wide variety of monoclonal and polyclonal antibodies against mammalian cytochrome-c. Immunogold labeling of tissues embedded in the acrylic resin LR Gold shows highly specific labeling of mitochondria in all tissues examined, including adrenal gland, cerebellum, cerebral cortex, heart, kidney, liver, pituitary, pancreas, skeletal muscle, spleen and thyroid. In pancreatic acinar cells and anterior pituitary, however, there was also strong cytochrome-c reactivity in zymogen granules and growth hormone granules, respectively. In the pancreas, strong immunoreactivity is also detected in condensing vacuoles and in the acinar lumen. Immunocytochemical controls included (i) use of monoclonal antibodies to horse cytochrome-c which recognize an epitope not present in rat cytochrome-c, (ii) preadsorption of antibodies with purified cytochrome-c, and (iii) omission of the primary antibody. The indicated presence of cytochrome-c outside mitochondria in certain tissues under normal physiological conditions raises interesting questions concerning translocation mechanisms and the cellular functions of cytochrome-c. © 2001 Academic Press

KEYWORDS: cytochrome-c; mitochondria; zymogen granules; secretory granules; growth hormone; pancreas; pituitary; immunogold; LR Gold; electron microscopy.

INTRODUCTION

Cytochrome-c is a component of the mitochondrial respiratory chain and is thought to normally localize in the mitochondrial intermembrane space (Lehninger *et al*., 1993; Neupert, 1997). Recently, cytochrome-c has also been shown to have a functional role in the cytosol following apoptotic stimuli, wherein cytochrome-c is released from mitochondria and its relocalization to the cytosol leads to the activation of the caspase family of proteinases and the onset of apoptotic cell death (Liu *et al*., 1996; Reed, 1997; Green and Reed, 1998; Kroemer *et al*., 1998). In view of these two very different cellular roles, might cytochrome-c have functions in other extramitochondrial compartments under normal physiological conditions? An answer to this question would benefit from a detailed examination of the subcellular distribution of cytochrome-c in cells of diverse origin using high resolution immunoelectron microscopy. With the exception of two previous immunogold electron microscopy (EM) studies on myocardial (Krajewski *et al*., 1999) and in testes (Hess *et al*., 1993), and immunofluorescent localization studies in the *Drosophila* embryo (Varkey *et al*., 1999), detailed information on the subcellular distribution of cytochrome-c in diverse tissues is surprisingly lacking.

In the present report, we have examined the subcellular localization of cytochrome-c in a variety of rat tissues by immunogold electron microscopy of LR Gold sections using a number of well-characterized monoclonal and polyclonal antibodies raised against mitochondrial cytochrome-c.

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Although cytochrome-c is primarily localized in mitochondria in many tissues, significant cytochrome-c reactivity is detected at extra-mitochondrial sites in pancreas and pituitary. In the pancreas we find strong cytochrome-c reactivity within condensing vacuoles, zymogen granules and in the acinar lumen, and in the pituitary cytochrome-c reactivity is found within growth hormone granules. These findings suggest cytochrome-c may have as yet unrecognized extramitochondrial functions under normal physiological conditions in certain tissues. The various possibilities that could explain the results are discussed.

MATERIALS AND METHODS

Antibodies

The origin of various polyclonal and monoclonal antibodies to cytochrome-c is as follows. Affinity purified sheep antibody against cytochrome-c was from Bionostics (North York, Ontario, Canada). The preparation and characterization of mouse monoclonal antibodies 1E8, 6H2.B4 and 2G8.B6 against rat cytochrome-c, and 2.7D5 against pigeon cytochrome-c, was previously described (Goshorn *et al*., 1991; Minnerath *et al*., 1995; Mueller and Jemmerson, 1996; Jemmerson *et al*., 1999). 1E8, 2G8.B6 and 2.7D5 recognize an epitope around residue 62 on native mouse cytochrome-c (Jemmerson *et al*., 1999); 6H2.B4, which was not useful for EM immunolabeling, recognizes an epitope encompassing residues 60/62 (Goshorn *et al*., 1991). Mouse monoclonal antibodies 1G1.E9 and 2B5.F8 against horse cytochrome-c, which recognize an epitope encompassing residues 44/47 which is not present in rat cytochrome-c (Goshorn *et al*., 1991), were used as controls and showed no reaction in rat tissues.

Tissue preparation

Brown Norway rats (Charles River Labs, Wilmington, MA, U.S.A.) were anaesthetized with sodium pentobarbital and perfusion-fixed with freshly dissolved 4% paraformaldehyde in 100 mM sodium phosphate buffer, pH 7.4. Tissues were excised, cut into 1 mm cubes, washed with 0.1 M sucrose, 0.1 M maleate buffer, pH 6.0, and postfixed for 30 min in 1% uranyl acetate in the same buffer, followed by 4% uranyl acetate in 50% ethanol for 30 min. Serial dehydration was with ethanol to a final concentration of 100%. Postfixation using uranyl acetate enhances tissue preservation and tissues are well-preserved despite the omission of glutaraldehyde in the primary fixative. Postfixation with uranyl acetate has been used previously in immunocytochemistry (Berryman and Rodewald, 1990; Soltys and Gupta, 1996; Cechetto *et al*., 2000) and procedures for the embedding and sectioning of cells in LR Gold resin (Polysciences, Warrington, PA, U.S.A.) have been described (Soltys and Gupta, 1996).

Immunogold labeling

Antibody labeling of LR Gold sections was done as follows. Sections were preabsorbed at room temperature with 20% fetal calf serum in 0.1 M Tris-HCl, pH 7.5 (carrier buffer). Sections were then reacted with polyclonal or monoclonal antibody to cytochrome-c in carrier buffer for 1.5 h at 37°C in a humidified incubator. In antibody preadsorption controls, polyclonal antibody to cytochrome-c was reacted with purified cytochrome-c (Sigma Chemical Co., St Louis, MO, U.S.A.) at 50 μg/ml for 2 h at 37°C before application of the antibody-antigen complex to sections. Washing of sections was for 30 min with 5% bovine serum albumin in 0.1 M Tris-HCl, pH 7.5. Sections were reacted with a 1:4 dilution of goat anti-sheep 20 nanometer gold conjugate from British BioCell (Cedarlane Laboratories, Hornby, Ontario, Canada) or goat anti-mouse IgG 10 nanometer gold conjugate (Sigma). Washing of sections was with 0.05 M KCl in carrier buffer followed by H_2O. Sections were stained with 4% uranyl acetate in 25% ethanol (20 min), rinsed with 25% ethanol, then air dried. Sections were examined at 80 kV with a JEOL 1200 EX transmission electron microscope.

Quantitative analysis of immunogold labeling

Immunogold labeling intensities in different subcellular compartments were determined by direct planimetry and counting of gold particles per μm^2 using a Kontron MOP Videoplan (Carl Zeiss, Inc, Toronto, Ontario, Canada) (Cechetto *et al*., 2000). For the analysis of pancreatic and pituitary cells, twenty different cells were evaluated for each tissue from four labeling experiments. Values given for each subcellular compartment are the mean ± SEM (n=total number of organelles). Where gold particles were clustered and indicative of the reaction of more than one marker with a primary antibody molecule, they were scored as single particles. Labeling was considered significant at $P<0.05$.

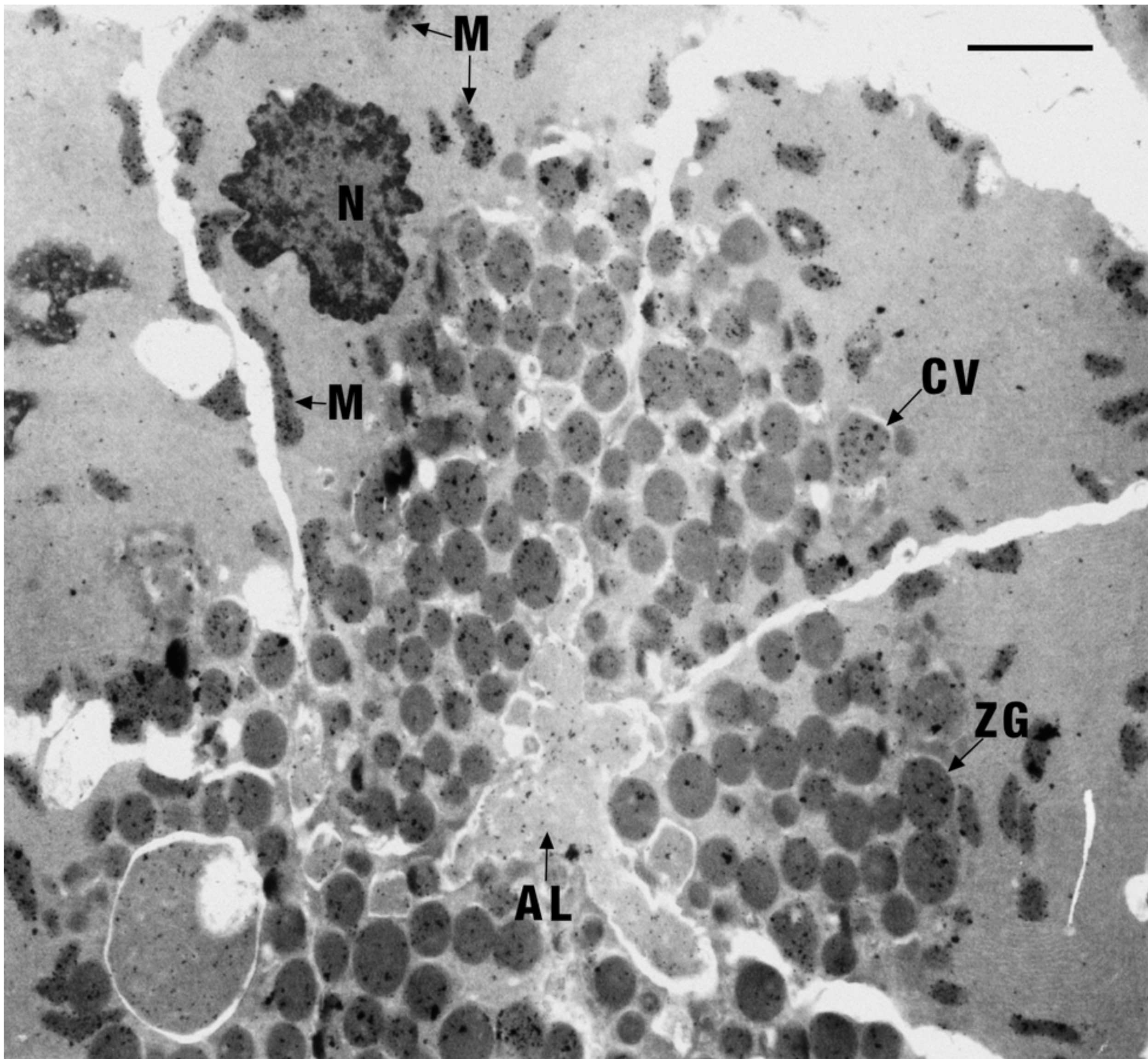

Fig. 1. Cytochrome-c subcellular localization in rat pancreatic acinar cells using immunogold electron microscopy. LR Gold sections were labeled with sheep polyclonal antibody to cytochrome-c followed by secondary antibody bound to 20 nm gold particles. M, mitochondrion; CV, condensing vacuole, ZG, zymogen granule; AL, acinar lumen; N, nucleus. Bar represents 2.0 μm.

RESULTS

Rat tissues embedded in the acrylic resin LR Gold were sectioned and then probed with cytochrome-c antibodies followed by immunogold markers. Similar results were obtained using both polyclonal and monoclonal antibodies against mammalian cytochrome-c. Figure 1 is a low magnification micrograph of acinar cells labeled with polyclonal antibody raised against rabbit cytochrome-c. Zymogen granules and condensing vacuoles have a distinctive size and morphology and are easily distinguished from mitochondria. Twenty nanometer gold particles were used to permit a general overview of labeling results in various compartments at low magnification. While the gold particles are detectable with the unaided eye at this magnification, the use of a low power magnifier is recommended. Strong cytochrome-c reactivity is found within mitochondria, condensing vacuoles, zymogen granules and in the acinar lumen. Figure 2 shows a higher magnification micrograph of an

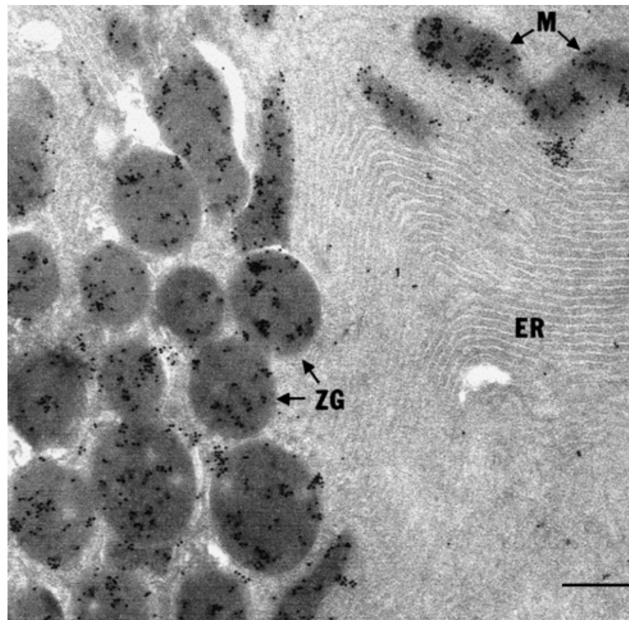

Fig. 2. High magnification micrograph of cytochrome c reactivity in mitochondria and zymogen granules of pancreatic acinar cells. Sheep polyclonal antibody used. Only background labeling is observed in endoplasmic reticulum. M, mitochondrion; ZG, zymogen granule; ER, endoplasmic reticulum. Bar represents 0.5 μm.

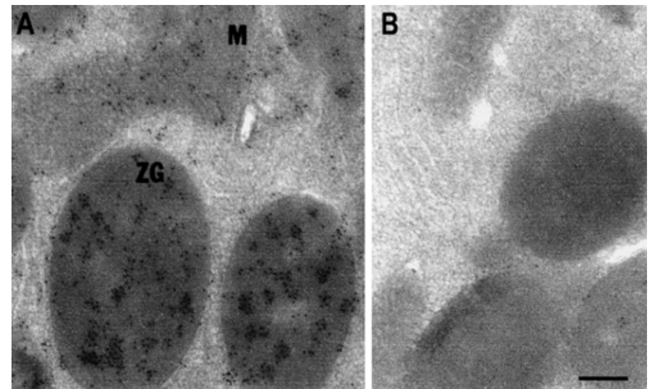

Fig. 3. Cytochrome-c subcellular localization in rat pancreatic acinar cells. (A) Immunogold labeling using 1E8 monoclonal antibody to mouse cytochrome c. Reactivity is present in both zymogen granules (ZG) and mitochondria (M). (B) Immunocytochemical negative control using 1G1.E9 monoclonal antibody with specificity for horse cytochrome-c. Secondary antibody was bound to 10 nm gold particles. Bar represents 0.25 μm.

acinar cell. While zymogen granules and mitochondria are intensely labeled, there is little reactivity in the ER and cytosol.

The same subcellular localization of cytochrome-c was also obtained using three monoclonal antibodies against mouse cytochrome-c. Figure 3A is monoclonal antibody labeling of acinar cells using 1E8 monoclonal antibody, showing strong labeling of zymogen granules and mitochondria. Similar results were obtained with the 2.7D5 and 2G8.B6 monoclonal antibodies (not shown). The epitope specificities of these monoclonal antibodies are known. The 1E8, 2G8.B6 and 2.7D5 monoclonals recognize an epitope around residue 62 on mouse cytochrome-c which is also present in rat and bovine cytochrome-c (Jemmerson *et al*., 1999). Mouse monoclonal antibodies 1G1.E9 and 2B5.F8 against horse cytochrome-c recognize an epitope encompassing residues 44/47 which is not present in rat cytochrome-c (Goshorn *et al*., 1991) and were used as negative controls. Figure 3B is such an immunocytochemical control showing negligible background labeling using 1G1.E9 antibody to horse cytochrome-c. Similar background labeling was also obtained in control experiments using preadsorption of polyclonal cytochrome-c antibody with purified cytochrome-c before application to

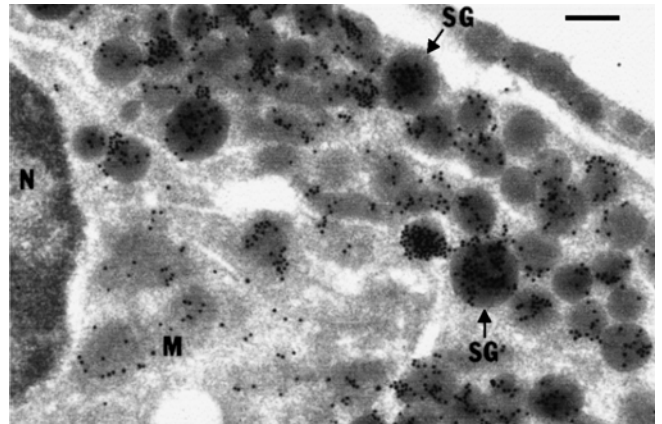

Fig. 4. Cytochrome-c subcellular localization in rat anterior pituitary using immunogold electron microscopy. Rabbit polyclonal antibody to cytochrome-c and secondary antibody bound to 20 nm gold particles were used. SG, growth hormone secretory granule; M, mitochondrion; N, nucleus. Bar represents 0.25 μm.

sections or omission of the primary antibodies (not shown). Thus, in pancreatic acinar cells both polyclonal and monoclonal antibodies against mammalian cytochrome-c show highly specific immunogold labeling in mitochondria, condensing vacuoles, zymogen granules, and in the acinar lumen.

Evidence for the presence of a cytochrome-c-related protein in secretory granules was also obtained in growth hormone secretory cells of the anterior pituitary. Figure 4 shows labeling of a growth hormone secretory cell using polyclonal antibody to cytochrome-c. The identity of these

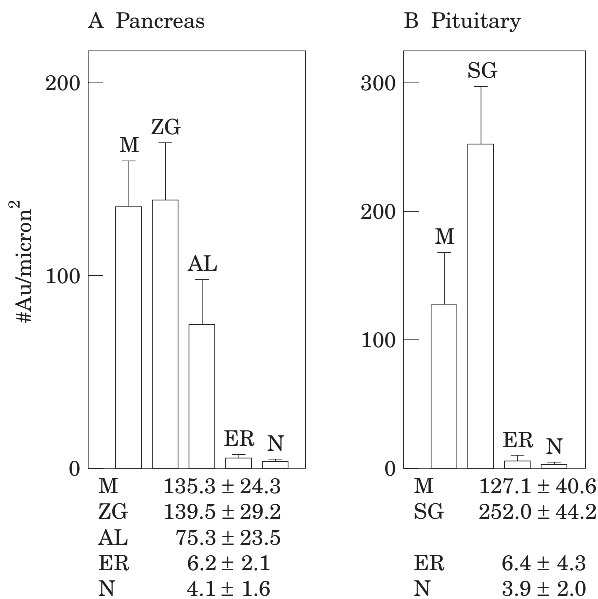

Fig. 5. Quantitative analysis of cytochrome-c immunogold labeling. (A) Pancreatic acinar cells. Mitochondria, M (n=98), zymogen granules, ZG (n=221); acinar lumen, AL (n=39); endoplasmic reticulum, ER (n=67); nucleus, N (n=14). (B) Pituitary growth hormone cells. Mitochondria, M (n=102); secretory granules, SG (n=201); endoplasmic reticulum, ER (n=11); nucleus, N (n=13). The mean values of the number of gold (Au) markers per $\mu m^2 \pm$ SEM are tabulated. $P<0.05$. Immunogold labeling procedures as described in Figure 1.

secretory granules was established in separate experiments using antibody to growth hormone (not shown) (Cechetto *et al*., 2000). As seen, there is strong cytochrome-c reactivity in both mitochondria and secretory granules. The low reactivity observed in ER, cytosol and in the nucleus is near background levels. Other tissues examined were the adrenal gland, kidney, cerebral cortex, cerebellum, heart, thyroid, liver, skeletal muscle, heart muscle and spleen. While EM labeling of mitochondria in these tisuses was also highly specific, there was no evidence for the presence of cytochrome-c reactivity at extramitochondrial sites (not shown).

The results of quantitative immunocytochemical analysis of pancreatic and pituitary sections is shown in Figures 5A and B, respectively. In pancreas (Fig. 5A), the intensity of cytochrome-c labeling in zymogen granules is similar to that in mitochondria ($m=139 \pm 29$ vs 135 ± 24 gold particles/μm^2 respectively) and in condensing vacuoles (not shown), while reactivity in the acinar lumen was reduced ($m=75 \pm 23$ gold particles/μm^2). In growth hormone secretory cells Fig. 5B), cytochrome-c labeling intensity in secretory granules is nearly two-fold higher than in mitochondria ($m=252 \pm 44$ vs 127 ± 41 gold particles/μm^2 respectively). In both pancreas and pituitary, the low labeling observed in other compartments, including the ER and nucleus (4–6 particles/μm^2), was similar to the background labeling of sections in regions without cells.

DISCUSSION

The results of our immunogold EM labeling studies show that cytochrome-c immunoreactivity in many rat tissues is exclusively localized in mitochondria, as would be expected given the mitochondrial respiratory chain function of this protein (Lehninger *et al*., 1993; Reed, 1997). However, we found cytochrome-c reactivity in the anterior pituitary is also localized within growth hormone granules, and in the pancreas high levels of reactivity are found in condensing vacuoles, zymogen granules and in the acinar lumen. The possibility that the extramitochondrial labeling could be nonspecific or due to cross-reactivity with some other antigen is unlikely because a wide variety of monoclonal and polyclonal antibodies with different epitope specificities all gave similar localization results. These antibodies have been previously well-characterized to react exclusively with cytochrome-c (see above). Further, various controls including the use of monoclonal antibodies to horse cytochrome-c that recognize epitopes not present in rat cytochrome-c, preadsorption of the antibodies with cytochrome-c and omission of the primary antibody, all show no labeling of either mitochondria or secretory granules and indicate the labeling is specific. Taken together, these results strongly suggest that the only protein with which the antibodies react is mitochondrial cytochrome-c.

In contrast to the strong cytochrome-c reactivity observed in secretory granules in pancreas and pituitary, cytochrome-c reactivity in ER and Golgi in these tissues was near background levels and different from the strong labeling of ER and Golgi typically seen for secretory proteins (unpublished results). A similar lack of labeling of ER and Golgi has been reported for several other mitochondrial proteins reported to be present in secretory granules, including Hsp60 (Brudzysnki *et al*., 1992*a*,*b*; Cechetto *et al*., 2000), P32 protein (gC1q-R) (Soltys and Gupta, 2000*b*) and tumor necrosis factor receptor-associated protein 1/TRAP-1 (Cechetto and Gupta, 2000). These observations indicate either that the levels of these proteins in transit through the ER and Golgi compartments is

exceedingly low or that they may be directly imported into secretory vesicles. Although in the present study cytochrome-c labeling intensities in secretory granules did not appear to be related to either granule volume or distribution, previous work on mitochondrial Hsp60 localization in insulin secretory granules showed Hsp60 is localized in mature but not in immature granules (Brudzynski *et al*., 1992*a*,*b*), strongly reinforcing the concept of direct import into secretory granules.

If cytochrome-c is directly imported into secretory vesicles, because cytochrome-c is not detected in the cytosol under normal physiological conditions then import may be occuring directly from mitochondria or from specialized regions of ER associated with mitochondria (Soltys and Gupta, 2000*a*). It should be noted that examples of close apposition of mitochondrial and zymogen granule membranes with concomitant deformation of mitochondria can be observed (Figs 2 and 3). A few other possibilities for how mitochondrial proteins may reach extramitochondrial destinations have been discussed elsewhere (Soltys and Gupta, 1999*b*, 2000*a*).

There have not been any previous EM immunocytochemical studies on the distribution of cytochrome-c in a wide variety of animal tissues. Two immunogold EM studies dealt only with myocardial cells (Krajewski *et al*., 1999) and testes (Hess *et al*., 1993). While cytochrome-c reactivity in myocardial cells was found restricted to mitochondria (Krajewski *et al*., 1999), cytochrome-c reactivity in testes, very interestingly, was also observed in chromatoid bodies (Hess *et al*., 1993) which are large condensed structures thought to function in the storage of mRNAs and which interact with the Golgi during the formation of the acrosomic system (Toppari *et al*., 1991). In the *Drosophila* embryo, immunofluorescence imaging has shown punctate mitochondrial-like labeling (Varkey *et al*., 1999) but ultrastructural studies have not been done to confirm that only mitochondria are labeled.

The origin of cytochrome-c found in secretory granules is presently not clear. Cytochrome-c is synthesized in the cytosol as apocytochrome c. Although mitochondrial targeting of cytochrome-c depends on internal sequences (Wang *et al*., 1996), insertion into and passage across the outer membrane appears to be lipid-mediated and unassisted by any outer membrane protein (Neupert, 1997). Following entry into the mitochondrial intermembrane space, apocytochrome-c is covalently modified to contain heme by cytochrome-c heme lyase. Following covalent modification and final folding, cytochrome-c is thought to remain localized within the intermembrane space (Neupert, 1997). An important question will be to determine whether the extramitochondrial cytochrome-c reactivity detected in our localization studies results from apocytochrome-c imported directly into the secretory pathway in pancreatic and pituitary cells, or apocytochrome-c is initially imported into mitochondria, covalently modified and folded, then exported from mitochondria to these destinations. Although further studies are required to clarify this important point, it should be noted that the monoclonal antibodies used in the present study do not react with apocytochrome-c (Goshorn *et al*., 1991; Jemmerson *et al*., 1999), suggesting indirectly that cytochrome-c found in secretory granules comes from mitochondria.

The presence of cytochrome-c immunoreactivity in zymogen granules and growth hormone granules adds to the growing evidence that a variety of mitochondrial proteins may be shared in other subcellular compartments under normal physiological conditions. Examples include P32 protein (gC1q-R) (Soltys and Gupta, 2000*b*), tumor necrosis factor receptor-associated protein 1/TRAP-1 (Cechetto and Gupta 2000), Hsp60/Cpn60 (Koga *et al*., 1989; Brudzynski *et al*., 1992*a*,*b*; Kaur *et al*., 1993; Xu *et al*., 1993, 1994; Vélez-Granell *et al*., 1994; Soltys and Gupta, 1996, 1997; Khan *et al*., 1998; Cechetto *et al*., 2000), Hsp10/Cpn10 (Cavanagh and Morton, 1994; Vélez-Granell *et al*., 1994), mitochondrial Hsp70 (Dahlseid *et al.*, 1994; Singh *et al.*, 1997) and plasma membrane fatty acid binding protein, which is the mitochondrial isoform of aspartate aminotransferase (Isola *et al*., 1997). It is highly remarkable that, similar to cytochrom-c, several of these proteins have also been found in secretory granules, including P32 protein (gC1q-R) (Soltys and Gupta, 2000*b*), tumor necrosis factor receptor-associated protein 1/TRAP-1 (Cechetto *et al.*, 2000), Hsp60/Cpn60 (Brudzynski *et al.*, 1992*a*, *b*; Vélez-Granell *et al.*, 1994; Cechetto *et al*., 2000) and Hsp10/Cpn10 (Velez-Granell *et al*., 1994). These findings of various unrelated mitochondrial proteins in the secretory pathway point to the likelihood of a novel translocation pathway involving mitochondria (Soltys and Gupta, 1999*b*, 2000), and suggest additional mitochondrial proteins will be identified with similar subcellular distributions. Furthermore, these findings may also suggest a new level of complexity by which mitochondria can modulate secretion. A functional involvement of mitochondria in secretion has been well demonstrated in the

case of insulin secretion, where mitochondrially-generated chemical signals (rather than exported proteins) are thought to play direct roles in insulin secretion (Maechler *et al*., 1997; Parkkila *et al*., 1998). The general topic of mitochondrial proteins which localize and function outside mitochondria under normal physiological conditions has been reviewed elsewhere (Soltys and Gupta, 1999*a*,*b*, 2000*a*).

It is interesting to note that several mitochondrial proteins found in the secretory pathway appear to be extracellular signaling proteins, including Hsp60/Cpn60 (Kirby *et al*., 1995; Kol *et al*., 2000; Ohashi *et al*., 2000) and Hsp10/Cpn10 (Cavanagh and Morton, 1994; Meghji *et al*., 1997). As an alternative to a role for cytochrome-c in secretory granule biogenesis and secretion in pancreas and pituitary, it is possible that secreted cytochrome-c may have cell signaling or other extracellular functions.

It is presently thought that cytochrome-c is localized only in mitochondria under normal physiological conditions. The release of cytochrome-c from mitochondria into the cytosol during apoptosis plays a central role in the cascade of events leading to cell death (Green and Reed, 1998; Kroemer *et al*., 1998). Our present findings suggest cytochrome-c under normal physiological conditions is also found in large amounts in specific extramitochondrial compartments, excluding the cytosol. How cytochrome-c reaches these extramitochondrial sites is presently not known. It will be of interest to evaluate whether the translocation of cytochrome-c to these extramitochondrial sites is perturbed during apoptosis, concomitant with cytochrome-c release into the cytosol. Identifying the functional role of cytochrome-c outside mitochondria in normal tissues is also of great interest.

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