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Localization of transforming growth factor-β1 in mitochondria of murine heart and liver

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Using both electron microscopic immunohistochemistry and cell fractionation techniques, we show that transforming growth factor-β1 (TGF-β1) is found in mitochondria of rat and mouse cardiac myocytes and rat hepatocytes. Four different polyclonal antibodies, raised against various epitopes encompassing the mature portion of the TGF-β1 molecule as well as the pro-region of its precursor, were used for the electron microscopy studies. The localization of TGF-β1 in mitochondria was confirmed by detection of the native peptide in mitochondria isolated from rat heart and liver; the majority of native TGF-β1 found in liver homogenates was recovered in highly pure mitochondrial fractions. The functional role of TGF-β in the mitochondrion is unknown at present.

Introduction

We have recently proposed (Sporn and Roberts, 1990) that transforming growth factor-β (TGF-β) may have significant regulatory functions within the interior of the cell, distinct from its many actions mediated by its external cell membrane receptor system (Massagué, 1990). In particular, the immunohistochemical localization of one or another of each of the three isoforms of TGF-β within highly specialized, terminally differentiated cells, such as cardiac myocytes and cerebrospinal sensory and motor neurons (Thompson *et al.*, 1988, 1989; Flanders *et al.*, 1991), suggests that new functions may yet be found for TGF-β.

One approach to solving the difficult problem of a structure in search of a function is to obtain further information about the local environment of such a structure. Here, we present new evidence indicating that TGF-β1 is found in mitochondria of rat and mouse cardiac myocytes and rat hepatocytes. The data have been obtained primarily by electron microscopic immunohistochemistry and have been extensively confirmed by cell fractionation studies, which show that native TGF-β1 can be recovered from isolated mitochondria, as measured in a sensitive and specific sandwich enzyme-linked immunosorbent assay (SELISA; Danielpour *et al.*, 1989).

Because of the somewhat unusual nature of the current findings, we are reporting them now, even though we have not yet defined a function for mitochondrial TGF-β. The localization of this regulatory molecule in a specialized organelle such as the mitochondrion indicates that there may be even further complexity to the already multifunctional actions of TGF-β (Massagué, 1990; Roberts and Sporn, 1990).

Results

Light microscopic immunohistochemistry of rat heart

We had previously noted a linear, punctate pattern of immunohistochemical staining of TGF-β1 in myocytes in paraffin sections of rat heart, suggestive of localization in mitochondria or contractile filaments (Thompson *et al.*, 1988). These studies used the immunoperoxidase technique coupled with the antibody LC 1-30, which reacts specifically with intracellular TGF-β1 (Flanders *et al.*, 1989). With the same antibody and higher power photomicrographs (Figure 1), we now show a striated banding pattern in longitudinal sections of myocytes (Figure 1A) and distinct peripheral staining in transverse sections of the same cells (Figure 1B). The pattern seen in transverse sections is particularly compatible with localization in mitochondria, many of which are adjacent to the cell mem-

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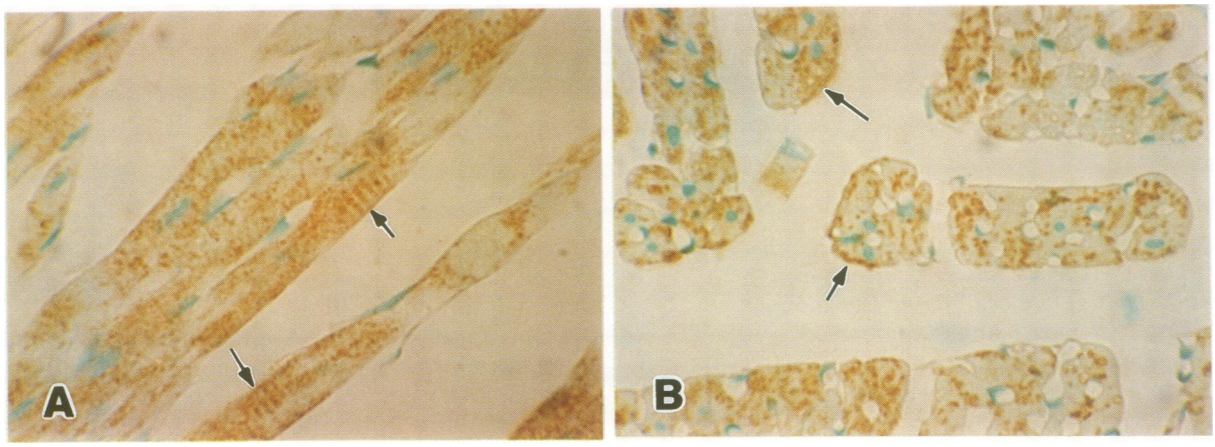


Figure 1. **Rat heart.** Immunocytochemical localization of TGF-β1 with antibody, LC 1–30. (A) Longitudinal section of muscle fibers showing transverse bands (arrows) positive for TGF-β1. (B) Transverse section with punctate staining, especially in peripheral locations (arrows), suggests presence of TGF-β1 in mitochondria. Myofibrils appear unstained. Fixation: 10% buffered formalin. ×575.

brane in cardiac myocytes. However, higher magnification, as provided by the electron microscope, is required for definitive resolution.

Electron microscopic immunohistochemistry of mouse and rat heart

We have used the metal mirror freezing technique (Phillips and Boyne, 1984) for cryofixation, followed by high-vacuum molecular distillation for removal of tissue water, to prepare samples for immunoelectron microscopy (Linner *et al.*, 1986). This method represents a fundamental advance in tissue preparation, making possible ultrastructural localization of soluble molecules without the problems of redistribution and loss that have limited the usefulness of conventional techniques.

Figure 2A shows that ultrathin control sections of mouse heart, prepared by the above method and then fixed with osmium vapor, have a well-preserved morphology; dense mitochondria with numerous cristae, Z-bands, and contractile filaments with dark and light banding patterns were all recognizable. Because of shrinkage, mitochondria were often surrounded by an electron-lucent halo. Three different antibodies, raised to peptides (32 amino acids or less) derived from separate regions of the linear TGF-β1 sequence, were then used to localize TGF-β1 within mitochondrial profiles in ultrathin sections of mouse heart. These were LC 1–30 (Figure 2C) as well as LC 78–109, and LC PRE 266–278 (not shown). The first two antibodies recognize epitopes on the mature portion of the TGF-β1 molecule, whereas the third recognizes a peptide sequence in the pro-region of the TGF-β1 precursor. Neither LC 1–30 nor LC 78–109 reacts with TGF-β2 by Western blot analysis (Flanders *et al.*, 1988, 1989), although there is slight cross-reactivity with TGF-β3 (Flanders unpublished data). LC PRE 266–278 does not cross-react with the precursor sequences of TGF-β2 or TGF-β3. Staining of sections with an antibody to α-actin gave the expected localization in contractile filaments (not shown). Omission of these primary antibodies eliminated all immunostaining (Figure 2B).

The similarity in the staining pattern obtained with antibodies to three discrete peptide sequences within the TGF-β1 molecule makes it highly unlikely that cross-reactivity with a peptide other than TGF-β is responsible for the mitochondrial immunolabeling. Although the internal fine structure of mitochondria is not well preserved in Figure 2, B and C (because of the multiple incubations with primary and secondary antibodies, as well as the frequent rinsings of the gold labeling procedure, which were not performed on the specimen shown in Figure 2A), the localization of label is clearly within mitochondrial profiles. Furthermore, preadsorption of antibody LC 1–30 with TGF-β1 coupled to Sepharose greatly suppressed immunostaining. Similar ultrathin preparations obtained from rat hearts, in addition to mouse hearts, and immunolabeled with LC 1–30 confirmed the mitochondrial localization of TGF-β1; almost no label was seen with this antibody in any other region of the cytoplasm of rat cardiac myocytes (not shown).

To test whether the mitochondrial localization was specific for the type 1 isoform, we also used antibodies to several unique epitopes in TGF-β2 and -3. An antibody to amino acids 50–75 of the mature TGF-β2 (Flanders *et al.,* 1990) showed only weak staining of rat heart mitochondria, whereas two antibodies, either to the mature or pro-regions of TGF-β3 (Flanders *et al.,* 1991), showed no staining of mouse heart sections (data not shown).

Electron microscopic immunohistochemistry of rat liver

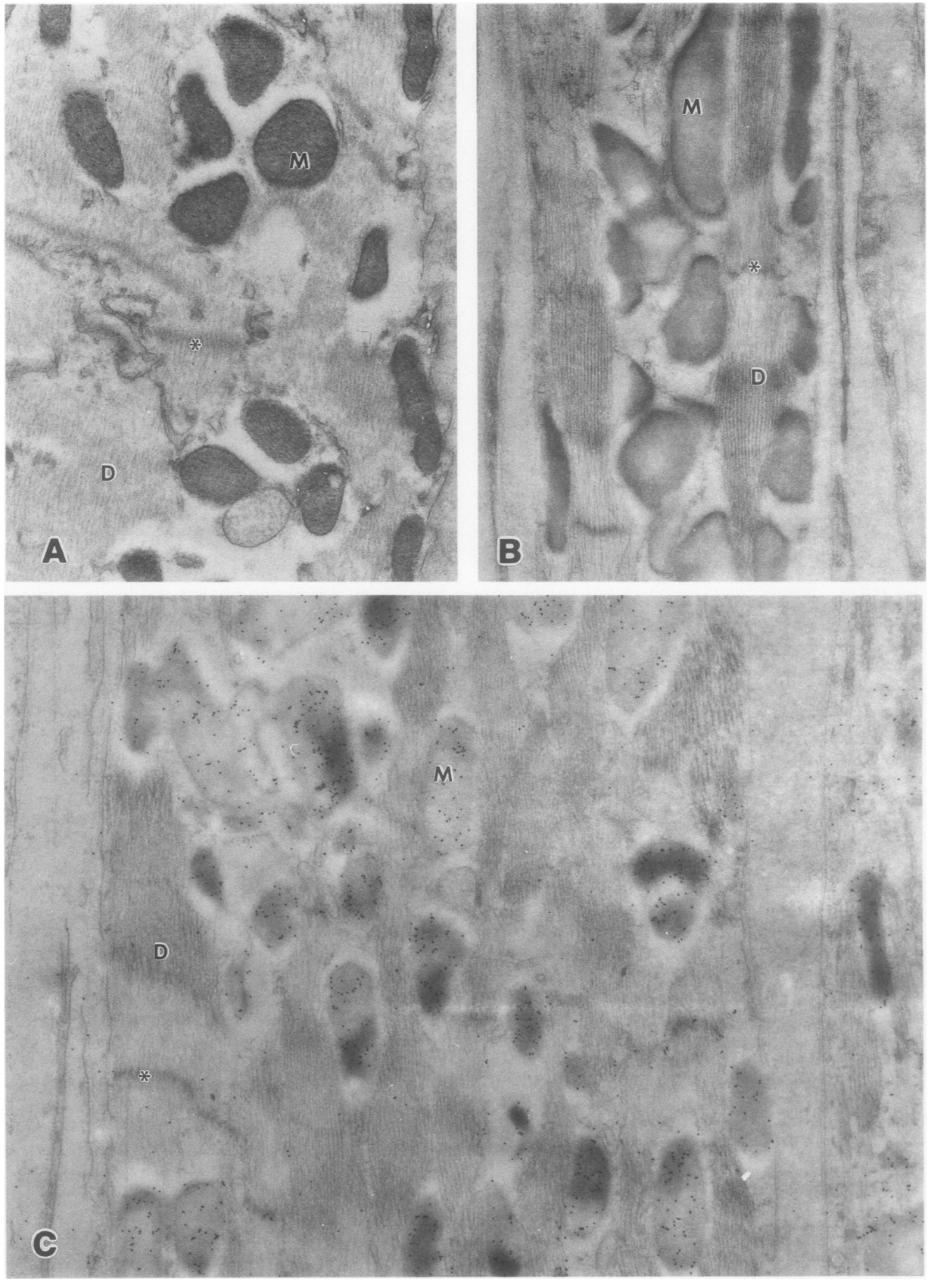
To determine whether TGF-β1 might be found in mitochondria in an organ other than the heart, we next studied rat liver, prepared as described above for mouse and rat heart (metal mirror cryofixation followed by high-vacuum removal of tissue water and osmium fixation). Immunolabeling was seen in mitochondria with antibody LC 1–30 (Figure 3), as well as with antibodies LC 50–75, 78–109, and LC PRE 266–278 (not shown). The staining observed with antibodies directed against several different regions of the TGF-β1 molecule again strongly indicates that the mitochondrial localization is not solely an artifact resulting from cross-reactivity with proteins other than TGF-β. In addition to immunolabeling of mitochondria in the liver, we observed prominent staining of other regions of the hepatocyte, particularly the smooth endoplasmic reticulum. We found this to be dependent on the fixation method used; when tissue was fixed with formalin instead of osmium, labeling was especially prominent over smooth endoplasmic reticulum (not shown). Inasmuch as the liver is believed to be a principal organ for the clearance of TGF-β from the body (Coffey *et al.,* 1987; Wakefield *et al.,* 1990), this finding is not surprising.

Quantitation of TGF-β1 in isolated rat liver and heart mitochondria

To confirm that intracellular TGF-β1 detected by immunohistochemical techniques was in a native conformation in liver and heart mitochondria, we homogenized rat liver and heart each in mannitol-sucrose medium, fractionated them by differential centrifugation, and then assayed the resulting fractions for TGF-β content after acid-ethanol extraction. The purity of the mitochondrial fractions obtained from both liver and heart was monitored by standard electron microscopy, as shown in Figure 4, A and B; these preparations show well-preserved mitochondria and negligible contamination with other cellular organelles.

Three separate preparations of liver mitochondria were made; and amounts of TGF-β1 and TGF-β2 were measured in the whole homogenate, a crude nuclear fraction, the purified mitochondrial pellet, and a final supernatant fraction. The amounts of TGF-β1 or -2 in each fraction were determined with SELISAs specific for these isoforms (Danielpour *et al.,* 1989). These assays are each based on the binding of two different antibodies (one raised in rabbits, the other in turkeys) to distinct epitopes of the intact, native TGF-β1 or -2 molecules, which are used as the antigens for preparation of these highly specific antibodies. These assays do not recognize native TGF-β3, the heterodimer TGF-β1.2, or denatured TGF-β1 or -2; they are the most sensitive and specific methods currently available for the measurement of native TGF-β1 or -2. Unlike several bioassays currently used to assess TGF-β activity, these SELISA assays are not sensitive to the presence of other growth factors or mitogens present in the preparation. In addition, cytochrome oxidase (a mitochondrial marker; see Hogeboom and Schneider, 1955) and cholinesterase (a marker for endoplasmic reticulum; see Aldridge and Johnson, 1959) were also measured in the whole homogenate and in each fraction.

Figure 5 shows the averaged results obtained from three preparations of liver mitochondria. The majority of native TGF-β1 found in the original whole homogenate was recovered in the mitochondrial pellet (76% of the original total); this pellet also contained 80% of the original cytochrome oxidase activity, but only 10% of the recovered cholinesterase activity, confirming the purity of the isolated mitochondria observed by electron microscopy. The crude nuclear fraction contained 20% of the recovered TGF-β1, 14% of the recovered cytochrome oxidase, and only 3% of the cholinesterase. In contrast, the final supernatant fraction contained 87% of the recovered cholinesterase activity, but only 4% of the TGF-β1 and 6% of the cytochrome oxidase. Thus, there appears to be little native TGF-β1 in the supernatant fraction of rat liver; these data suggest that the epitopes detected in the smooth endoplasmic reticulum by immunoelectron microscopy, as described above, may represent partially degraded molecules that are in the process of being cleared by the liver (Coffey *et al.,* 1987; Wakefield *et al.,* 1990). The presence of TGF-β in the crude nuclear fraction is difficult to interpret, because this fraction is a heterogeneous mixture of un-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broken cells and large cell fragments as well as isolated nuclei. Thus, the majority of the TGF-β in the crude nuclear fraction is most likely associated with mitochondria found in unbroken cells (~14% of total cytochrome oxidase was recovered in the crude nuclei), although one cannot rule out the possibility that there is some nuclear localization of TGF-β. However, relatively few grains of immunogold were seen within nuclear profiles in the electron micrographs of rat liver. Assays for TGF-β2 failed to detect significant levels of this isoform in any of the prepared fractions or in the initial homogenate. These results are consistent with previous work that showed markedly lower levels of TGF-β2 than TGF-β1 in rat liver (Danielpour *et al.*, 1990).

To confirm that the TGF-β1 associated with isolated mitochondria was not the result of nonspecific binding during cell disruption and fractionation, we homogenized rat livers in the presence of added ^{125}I-labeled TGF-β1, fractionated them, and determined the amount of radioactivity recovered in the mitochondrial pellet. Table 1 shows that only 10% of the added ^{125}I TGF-β1 was associated with the mitochondrial pellet, whereas nearly all of the labelled TGF-β was found in the supernatant; in contrast, 76% of the endogenous cellular TGF-β1 was found in the mitochondrial pellet, and only 4% was found in the supernatant. A similar distribution was seen with two different concentrations of ^{125}I TGF-β1. The amount of TGF-β adsorbed to mitochondria in these experiments is well below the endogeneous amounts measured by SELISA for isolated mitochondria. Thus, the isolated mitochondrial fraction from 7.5 g of liver was found to contain ~237 ng of endogenous TGF-β, whereas <0.1 ng of added ^{125}I TGF-β became bound to the mitochondrial pellet prepared from 7.5 g of liver (Table 1, Experiment 2).

It is well known that recovery of heart mitochondria during cell fractionation is less satisfactory than comparable results obtained with liver (Mela and Seitz, 1979). In spite of this, we found a strong correlation between the comparative recoveries of TGF-β1 and cytochrome oxidase during fractionation of a rat heart homogenate. Thus, the mitochondrial pellet contained 47% of the total TGF-β1 recovered in the whole homogenate, as well as 47% of the total cytochrome oxidase (data not shown). An additional 47% of the total TGF-β was recovered in the final supernatant, together with 38% of the total cytochrome oxidase, indicating in this case that all of the mitochondria were not recovered in the initial pellet.

The presence of TGF-β1 in rat heart mitochondria isolated by differential centrifugation of homogenates was further confirmed by immunoelectron microscopy. A pellet of isolated cardiac mitochondria was treated by the same cryofixation and molecular distillation procedure described above for whole tissue, fixed with osmium tetroxide, sectioned, treated with antibody LC 1–30, stained with immunogold, and then photographed with the electron microscope. A quantitative analysis of grain counts in electron photomicrographs from five separate fields was then made, as reported in Table 2; each micrograph contained between 9 and 11 isolated mitochondria. For each mitochondrion examined, the grain count per unit area was determined by dividing the number of grains counted by the area, measured in square micrometers. Areas of the electron micrographs free of any recognizable structures (the "background") served as controls. The results shown in Table 2 clearly indicate that TGF-β found in isolated mitochondrial pellets is intrinsically associated with mitochondria themselves, in preference to any amorphous extramitochondrial material that cosediments with these organelles. Thus, in spite of the numerous washings of the various organelles during cell fractionation, TGF-β remained associated with mitochondria, again suggesting that the mitochondrial localization of this peptide is not an artifact.

Discussion

The data presented here indicate that TGF-β1 is found in mammalian mitochondria. Although no single experiment reported herein inevitably leads to that conclusion, the totality of the data, obtained both by electron microscopy and cell fractionation of both heart and liver, using unique and different sets of analytical reagents for both sets of experiments, makes a compel-

Figure 2. **Mouse heart, electron photomicrograph.** Immunocytochemical localization of TGF-β1. Tissue was cryofixed, dried, fixed with osmium vapor, and embedded in Spurr resin. (A) Control, no treatment. Mitochondria (M) with well-preserved cristae, myofibrils with dense A bands (D) and Z bands (*). Staining: uranyl acetate, lead citrate. (B) Control, pretreatment with sodium periodate. No immunoreaction because of omission of primary antibody. There is loss of morphological detail in mitochondria (M). Staining: uranyl acetate. (C) Specific labeling of TGF-β1 in mitochondria (M) with LC 1–30 antibody. Staining: uranyl acetate. ×25 000.

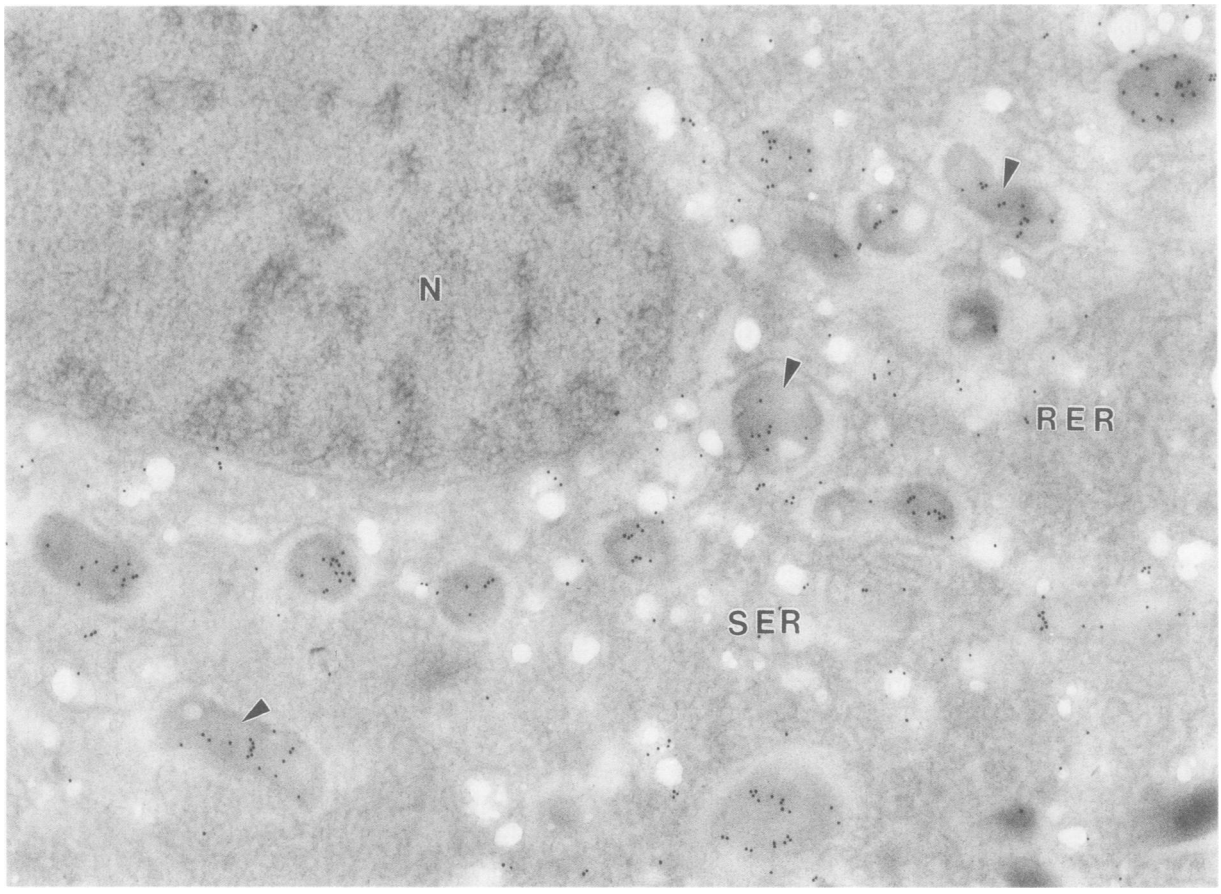

Figure 3. **Rat liver, electron photomicrograph.** Immunocytochemical localization of TGF-β1 with LC 1–30 antibody. Gold grains are localized predominantly over mitochondria (arrowheads). Smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), and nucleus (N) show little labeling. Tissue preparation as described in Figure 2. The immunohistochemical technique causes loss of the glycogen, resulting in empty spaces throughout the SER. Staining: uranyl acetate. ×35 000.

ling case that the results obtained are not artifactual. The metal mirror freezing technique, followed by slow and controlled molecular distillation of tissue water, is one of the best available methods for localization of diffusible macromolecules by electron microscopy (Linner *et al.,* 1986). Likewise, cytochrome oxidase is an established marker for monitoring mitochondrial recovery during cell fractionation procedures and has been used for this purpose for many years (Hogeboom and Schneider, 1955). For immunolocalization of TGF-β by electron microscopy, we have obtained similar results with four unique antibodies directed against different epitopes in the linear TGF-β1 sequence, making it highly unlikely that the staining of mitochondria by immunogold represents cross-reactivity with another protein. We have previously shown by Western blot analysis that antibodies LC1–30, 50–75, and 78–109 do not react with dimeric TGF-β2 (Flanders *et al.,* 1988, 1989), although there is slight cross-reactivity with TGF-β3 (Flanders, unpublished data). The antibody against the TGF-β1 precursor (LC PRE 266–278) does not cross-react with the precursor sequences of either TGF-β2 or TGF-β3. For chemical determination of TGF-β1 and -2 in isolated mitochondria, we have used the most sensitive and specific method available, which can distinguish between native TGF-β1 and -2 (Danielpour *et al.,* 1989).

We should also emphasize that, although we have detected epitopes of TGF-β1 in mitochondria of the heart and liver with the antibodies described in the present study, this does not imply that the mitochondrion is the sole intracellular location of the TGF-β1 structure. Thus, in rat heart fixed with osmium, we can immunolocalize the epitope corresponding to amino acids 50–75 of TGF-β1 in the contractile filaments of myocytes (data not shown). It is entirely possible that different epitopes of the

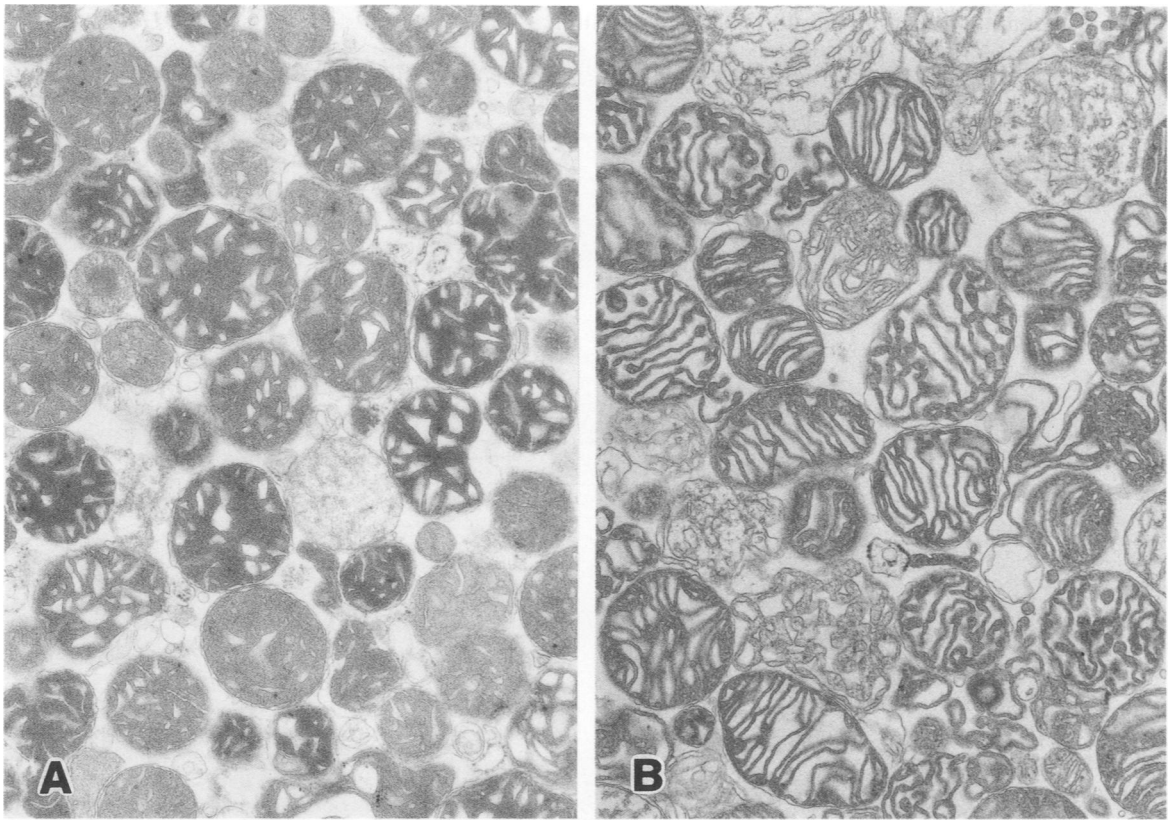

Figure 4. **Electron photomicrographs of isolated mitochondria obtained by homogenization and differential centrifugation of tissue.** (A) Rat heart. (B) Rat liver. Both preparations show well-preserved mitochondria and lack of contamination by other cell organelles. Fixation: glutaraldehyde, osmium tetroxide. Staining: uranyl acetate, lead citrate. ×20 000.

TGF-β molecule may be exposed within different regions of the interior of the cell—just as we and others have shown that if one compares the extracellular versus the intracellular staining of TGF-β1, then one finds that different epitopes of the N-terminus of TGF-β1 are immunoreactive (Flanders *et al.,* 1989).

The function of TGF-β in the mitochondrion remains to be determined, and it would be premature to speculate on any specific function at present. It is clear that mitochondrial functions, including their oxidative metabolism and their capacity to regulate the movement of calcium ions, as well as their protein synthesis and DNA replication, must be integrated in some way with the functions of the rest of the cell. Indeed, it has recently been shown that the Bcl-2 protooncogene is localized to the inner mitochondrial membrane and is involved in regulating programmed cell death in lymphoid cell lines (Hockenbery *et al.,* 1990). However, at present we cannot ascribe any special function to TGF-β in the mitochondrion. Mitochondrial DNA does not code for TGF-β, nor is there any apparent relationship between TGF-β and molecules that regulate trafficking in and out of the mitochondrion. Because it has been shown that the 135-kDa binding protein that is associated with latent TGF-β has several binding sites for calcium ion (Dählback *et al.,* 1990; Kanzaki *et al.,* 1990), it is possible that there may be a role for TGF-β in regulating calcium ion function in the mitochondrion; further studies will be required to evaluate this.

The history of TGF-β research during the past 10 years indicates that the scope, complexity, and multifunctionality of TGF-β has always been underestimated (Roberts and Sporn, 1990; Sporn and Roberts, 1990). Thus, it is entirely possible that there may be several roles for TGF-β in the mitochondrion, some of which relate to events that occur over a relatively long time span and others that might involve regulatory processes with a cycle of ≤1 min. Just as TGF-β acting at its external cell membrane receptor appears to be involved in coupling a cell to its external environment, TGF-β in the mitochondrion may likewise have a role in coupling the

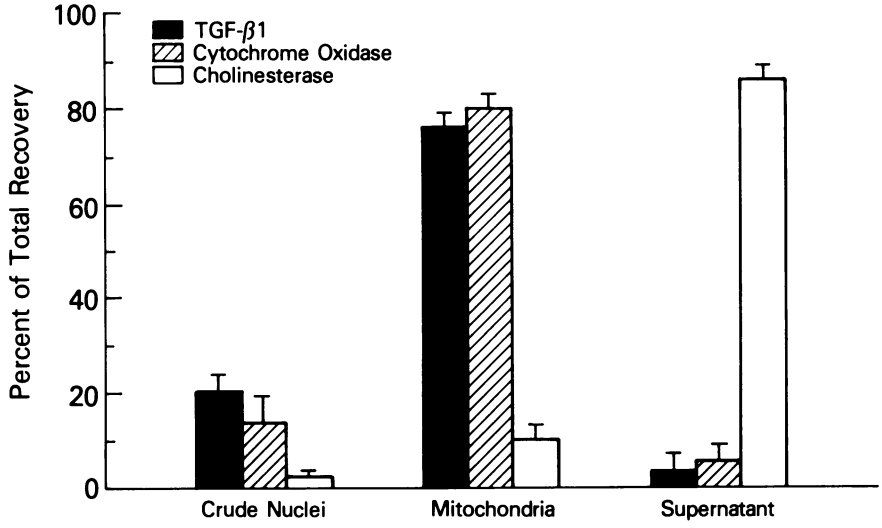

***Figure 5.* Recoveries of TGF-β1, cytochrome oxidase, and cholinesterase in subcellular fractions of rat liver homogenates.** Liver was fractionated and assayed as described in Materials and methods. Measured values in the initial homogenates were as follows: TGF-β1, 39.5 ± 6.8 ng/g wet weight of liver; cytochrome oxidase, 1240 ± 180 U/g; and cholinesterase, 1.08 ± 0.02 U/g (units for cytochrome oxidase and cholinesterase are defined in Sporn *et al.*, 1962). Values shown in the figure are the averages obtained from three separate preparations of rat liver.

activities of this organelle with those of the rest of the cell. The definitive localization of TGF-β in the mitochondrion that we have shown here now makes this an important new problem in cell regulation.

Materials and methods

Animals

Sprague-Dawley rats and NIH Swiss mice were used. They were killed either by decapitation or by intraperitoneal injection of phenobarbital. Liver or heart was rapidly removed and either fixed immediately in 10% buffered formalin for light microscopic examination or cryofixed for electron microscopic studies.

Antibodies

Polyclonal antibodies were made in rabbits to synthetic peptides corresponding to various amino acids of the mature and precursor forms of TGF-β1. Antibodies include LC 1–30 (Flanders *et al.*, 1989), corresponding to the amino-terminal 30 amino acids of mature TGF-β1, and antibodies LC 50–75 and LC 78–109 (Flanders *et al.*, 1988), corresponding to amino acids 50–75 and 78–109 of the mature growth factor, respectively. Antibody LC PRE 266–278 was raised against a synthetic peptide consisting of amino acids 266–278 present in the pro-region of the precursor of TGF-β1 (Wakefield *et al.*, 1988). IgG was isolated from serum by elution from Protein A Sepharose (Goeding, 1978).

Immunohistochemistry

Tissue samples were fixed for 24 h in 10% buffered formalin, postfixed in Bouin's solution, dehydrated through a graded series of alcohol solutions, and embedded in paraffin. Sections of 5-μm thickness were deparaffinized and subjected to an immunohistochemical procedure to visualize the localization of TGF-β1. The following schedule was used: 1) 0.3% hydrogen peroxide in methanol to block endogenous peroxidase (30 min); 2) 1 mg/ml hyaluronidase (Sigma Chemical, St. Louis, MO), buffered to pH 5.5 with 0.1 M sodium acetate in 0.15 M NaCl (30 min); 3) 10% goat serum to block nonspecific protein binding (45 min); 4) 20 μg/ml anti-TGF-β1, 4°C, overnight; 5) affinity-purified goat anti-rabbit IgG diluted 1:40 in buffer (1 h); 6) rabbit peroxidase-antiperoxidase diluted 1:100 in buffer (1 h); and 7) 0.5 mg/ml diaminobenzidine (Sigma Chemical) in 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-buffered saline (0.05 M Tris buffer pH 7.2, 0.15 M NaCl) containing 0.1% H_2O_2 (5 min). The buffer solution used for rinsing between each step consisted of 0.01 M Tris-buffered saline, 0.1% normal goat serum, and merthiolate at a final concentration of 0.01%. Controls were done either by replacing the anti-TGF-β1 rabbit IgG with purified nonimmune rabbit IgG, by omitting the primary antibody, or by replacing the LC 1–30 antibody with IgG that had been preincubated with TGF-β1-Sepharose, a procedure that removes antibodies that are specific for TGF-β1 (Heine *et al.*, 1987). Normal affinity-purified goat anti-rabbit IgG and peroxidase-antiperoxidase were obtained form Cooper Biomedical (Malvern, PA). Counterstaining was done with methyl green.

Table 1. Recovery of added TGF-β1 in subcellular fractions of liver

	Cytochrome oxidase (U/g wet wt.)	^{125}I TGF-β (cpm)
Experiment 1		
Crude nuclei	43	38 000
Mitochondria	970	54 000
Supernatant	109	430 000
Experiment 2		
Crude nuclei	84	2 000
Mitochondria	840	5 000
Supernatant	128	42 000

A perfused rat liver (6.5 g in Experiment 1 and 7.5 g in Experiment 2) was homogenized with 586 000 cpm (3 ng) of ^{125}I-labeled TGF-β1 in Experiment 1 or 53 000 cpm (0.28 ng) of ^{125}I-labeled TGF-β1 in Experiment 2. Mitochondria were then isolated as previously described, and the amount of ^{125}I TGF-β1 in each subcellular fraction was determined. A unit of cytochrome oxidase is defined in Sporn et al., 1962.

Table 2. Quantitative immunoelectron microscopy of TGF-β1 in isolated rat heart mitochondria

Photograph number	Sample	Mean grain count per square micrometer	*n*	SE	Probability, 2-tailed
1	Mitochondria	10.24	9	1.08	<0.001
	Background	2.76	8	0.51	
2	Mitochondria	8.81	9	0.99	<0.001
	Background	2.30	10	0.26	
3	Mitochondria	15.41	10	1.58	<0.001
	Background	2.15	7	0.30	
4	Mitochondria	7.65	10	1.23	<0.002
	Background	2.38	4	0.27	
5	Mitochondria	7.73	11	0.51	<0.006
	Background	1.91	3	0.42	

Isolated mitochondria were prepared for microscopy as described for whole tissue. Method A was used for immunolocalization of TGF-β1, using antibody LC 1-30. Non-parametric Kruksal-Wallis and Wilcoxon rank sum tests were used for statistical analysis.

Immunoelectron microscopy

Tissues were cryofixed on a life cell CV 100 cryofixation unit (kindly provided by LifeCell, The Woodlands, TX) according to a method described previously (Linner *et al.*, 1986; Livesey *et al.*, 1989). After freezing, the tissue blocks were transferred to Nunc tubes (Cryomed, Mt. Clements, MI) and stored in liquid nitrogen until further processed. To obtain optimal antigen preservation, a high-vacuum technique for removal of amorphous phase tissue water without ice crystal damage was employed (Linner *et al.*, 1986). This method permits the drying of unfixed specimens in vacuo at a temperature range from −190°C to room temperature, followed by fixation with osmium tetroxide vapor and embedding in a low-viscosity epon resin (Spurr, 1969). The whole process was carried out by either Advanced Biotechniques (Columbia, MD), or LifeCell.

For immunolabeling we employed two methods, one involving biotin-streptavidin linkage (method A) and the other a direct labeling procedure (method B). For both methods, ultrathin sections of gold interference were collected on Formvar-coated nickel grids and air dried. The sections were immersed in a saturated aqueous solution of sodium metaperiodate for 5 min to oxidize lower oxides of osmium to osmium tetroxide.

Method A. 1) inhibition of endogenous biotin by incubating in streptavidin (15 min), followed by biotin (15 min) (Biotin blocking kit, Vector Laboratories, Burlingame, CA); 2) blocking of nonspecific labeling with 5% bovine serum albumin (BSA), 5% ovalbumin, 10% normal goat serum in phosphate-buffered saline (PBS), 30 min; 3) incubation with the primary antibody, concentration 22 or 220 μg/ml, 4°C, overnight; 4) incubation with biotinylated goat anti-rabbit IgG, 60 min; and 5) incubation with streptavidin/10 nm colloidal gold, 45 min. The wash solution used between each incubation was PBS. Primary and secondary antibodies were diluted in 1% BSA, 1% ovalbumin, 2% normal goat serum in PBS. After a final rinse in distilled water, the sections were stained for 5 min in saturated aqueous uranyl acetate.

Method B. Steps 1–4 were carried out in similar fashion as described for method A; 5) the primary antibodies were visualized by use of goat anti-rabbit IgG coupled to 10 nm gold particles (Janssen Biotech, Olen, Belgium) as a secondary antibody, 60 min; 6) fixation with 2% glutaraldehyde in PBS, 15 min; and 7) counterstaining with saturated aqueous solution of uranyl acetate, 5 min. The antibodies were diluted in 0.1% BSA, 1% normal goat serum in 0.01 M Tris-buffered saline. The wash solution consisted of PBS.

Isolation of heart mitochondria

Adult female rats were killed, and the hearts were removed and rinsed in PBS. These hearts were then minced and homogenized in buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) pH 7.4, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 0.2% wt/vol BSA (buffer A). The homogenate was passed through a 75-μm (465-mesh) Nitex filter to remove connective tissue. After a centrifugation at 500 × *g* for 10 min to remove nuclei and cellular debris, the mitochondria were isolated by centrifugation at 10 000 × *g* for 10 min. The resulting pellet was washed several times by resuspending it in buffer A and centrifuging at 10 000 × *g* for 10 min. The purity of these mitochondria was determined by examination with the electron microscope. These mitochondria were then used for statistical analysis of immunostaining of TGF-β and for quantitation of TGF-β content.

Isolation of liver mitochondria

Adult female rats were killed, and the livers were perfused with cold PBS. The livers were then removed, minced, and homogenized in buffer A. An aliquot of this homogenate was removed and used to determine the starting amounts of marker enzymes and TGF-β. Mitochondria were isolated by differential centrifugation as described (Johnson and Lardy, 1967; Nedergaard and Cannon, 1979). This procedure yielded three fractions: 1) a crude nuclear pellet, 2) a mitochondrial pellet, and 3) a supernatant. For each of these fractions, the recovery of mitochondria, determined by assaying cytochrome oxidase (Cooperstein and Lazarow, 1951; Hogeboom and Schneider, 1955), and the recovery of endoplasmic reticulum, determined by assaying cholinesterase

(Aldridge and Johnson, 1959; Dietz *et al.,* 1973), was calculated. The purity of the mitochondrial pellet was also determined by electron microscopic examination.

Quantitation of TGF-β in tissue fractions

Each of the samples obtained during the fractionation of the rat livers was assayed for TGF-β content. To do this, we first divided each fraction in half. Radiolabeled carrier TGF-β (~10 000 dpm of ^{125}I-labeled TGF-β1) was added to one-half of each fraction, allowing the calculation of TGF-β recovered during sample preparation. All of the samples were then extracted with four volumes of acid-ethanol prepared as described (Roberts *et al.,* 1980) and incubated overnight at 4°C with stirring. The precipitate was removed by centrifugation at 10 000 × *g* for 20 min, and the resulting supernatants were dialyzed against 4 mM HCl. The dialyzed samples were lyophilized until dry and then resuspended in 1–2 ml of 20 mM HCl. These samples were then incubated overnight at 4°C on a rocking platform to dissolve the TGF-β; any insoluble material was removed by centrifuging for 5 min at 16 000 × *g*. The volume of the resulting supernatant was determined and the recovery of the ^{125}I TGF-β was calculated. The concentration of TGF-β in each of the nonradioactive samples was then determined by the use of the TGF-β SELISA (Danielpour *et al.,* 1989). The amount of TGF-β in each of the tissue fractions was calculated from the concentration of TGF-β determined by SELISA and the recovery of the radiolabeled carrier TGF-β. Cytochrome C and the reagents for measuring cholinesterase (diagnostic kit 421-10) were purchased from Sigma Chemical. TGF-β1 was iodinated as previously described (Frolik *et al.,* 1984) to a specific activity of 2 μCi/pmol.

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