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Cpn60 is Exclusively Localized Into Mitochondria of Rat Liver and Embryonic *Drosophila* Cells

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Abstract Several reports have claimed that the mitochondrial chaperonin cpn60, or a close homolog, is also present in some other subcellular compartments of the eukaryotic cell. Immunoelectron microscopy studies, using a polyclonal serum against cpn60, revealed that the protein is exclusively localized within the mitochondria of rat liver and embryonic *Drosophila* cells (SL2). Furthermore, no cpn60 immunoreactive material could be found within the nucleus of SL2 cells subjected to a 1 h 37°C heat-shock treatment. In contrast to these findings, immunoelectron microscopy studies, using a cpn60 monoclonal antibody, revealed mitochondrial and extramitochondrial (plasma membrane, nucleus) immunoreactive material in rat liver cells. Surprisingly, the monoclonal antibody also reacted with fixed proteins of the mature red blood cell. The monoclonal antibody, as well as cpn60 polyclonal sera, only recognize mitochondrial cpn60 in Western blots of liver proteins. Furthermore, none of the cpn60 antibodies used in this study recognized blotted proteins from rat red blood cells. Therefore, we suggest that the reported extramitochondrial localization of cpn60 in metazoan cells may be due to cross-reactivity of some of cpn60 antibodies with conformational epitopes also present in distantly related cpn60 protein homologs that are preserved during fixation procedures of the cells.

Key words: molecular chaperones, chaperonins, mitochondrial hsp60, subcellular localization of hsp60, heat-shock stress, Hsp60 antibodies

A final step in the biogenesis of proteins is their folding and assembly into macromolecular complexes. These activities are mediated by catalysts of protein folding and a set of helper proteins known as molecular chaperones [Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Yaffe et al., 1992; Jakob and Buchner, 1994]. Molecular chaperones have been identified in most of the subcellular compartments of the eukaryotic cell. Mitochondria from all organisms studied so far contained a 60 kDa heat-shock protein [Hutchinson et al., 1989; Jindal et al., 1989; McMullin and Hallberg, 1987; Mizzen et al., 1989; Picketts et al., 1989; Reading et al., 1989; Waldinger et al., 1988] that is immunologically, structurally, and functionally related to the prokaryotic GroEL and the chloroplast Rubisco subunit binding protein [Hendrix, 1979; McMullin and Hallberg, 1988; Mizzen et al., 1989; Picketts et al., 1989; Waldinger et al., 1988; Hemmingsen et al., 1988]. The 60 kDa heat-shock proteins are a subclass of molecular chaperones known as chaperonins (cpn60) [Hemmingsen et al., 1988; Ellis, 1990]. Chaperonins are abundant constitutive proteins that form a large homooligomer composed of one [Viitanen et al., 1992] or two stacked rings of seven subunits each [Hendrix, 1979; McMullin and Hallberg, 1988]. Recently, the crystal structure of prokaryotic GroEL has been resolved at a resolution of 2.8 Å [Braig et al., 1994]. Mitochondrial chaperonin is required for the folding and assembly of imported polypeptides within the matrix of the organelle [Cheng et al., 1989; Ostermann et al., 1989]. These activities require both ATP hydrolysis and the cooperation of the cochaperone cpn10 [Hartman et al., 1992a,b; Lubben et al., 1990; Höhfeld and Hartl, 1994; Hartl, 1994; Ellis, 1994], a homolog of the prokaryotic GroES [Chandrasekhar et al., 1986].

It has been widely accepted that cpn60 is localized in the mitochondria of the eukaryotic cell [Hutchinson et al., 1989; Jindal et al., 1989; McMullin and Hallberg, 1987; Mizzen et al., 1989; Picketts et al., 1989; Reading et al., 1989;

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Waldinger et al., 1988; Ellis 1990]. However, recent findings have identified cpn60 (or close homologs) in different subcellular structures. For instance, it has been localized at the plasma membrane of different cellular types [Fisch et al., 1990; Kaur et al., 1993; Jones et al., 1994], in secretory granules of mice pancreatic β-cells [Brudzynski et al., 1992], within the nuclei of a fish cell line [Sanders et al., 1994], and, finally, in the cytosol of plant cells [Grimm et al., 1991] and of a flagellated protozoan [Soltys and Gupta, 1994]. Although the biological roles of cpn60 are essential for cellular function, it is certainly intriguing to explain how this protein could be localized and how it could exert its biological functions in such different environments. Therefore, the purpose of this work was to study the subcellular compartmentation of cpn60 in rat liver and in embryonic *Drosophila* cells (SL2) by immunoelectron microscopy techniques. Furthermore, since cpn60 has been found in the nucleus of the cell after heat-shock [Sanders et al., 1994], we also studied its subcellular distribution in heat-shocked SL2 cells. Our findings support that cpn60 is exclusively localized into the mitochondria of metazoan cells, even under cellular stressful conditions.

MATERIALS AND METHODS

Cell Culture, Heat-Shock Treatment, and Measurement of Protein Synthesis Rates in Embryonic *Drosophila melanogaster* Cells

SL2 cells were plated in M3 medium supplemented with 10% fetal calf serum and grown at semiconfluence in 60 mm plates at 24°C. For the heat-shock treatment, plates were placed for 60 min in a culture chamber at 37°C [Alconada et al., 1994]. To measure rates of protein synthesis, 100 μCi of [^{35}S]methionine (1,200 Ci/mmol) were added to the incubation mixture and allowed to incorporate during 60 min at either 24°C or 37°C. Cells were pelleted, washed twice with PBS, and resuspended in 600 μl of a 20 mM phosphate buffer containing 150 mM NaCl, 1% Triton X-100, pH 7.0, and the following protease inhibitors: 2 μg/ml leupeptine, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Resuspended cells were freeze-thawed three times and centrifuged for 10 min at 100,000*g*.

Liver Homogenates, Isolation of Mitochondria, and Rat Red Blood Cells

Albino Wistar rats fed on standard laboratory chow were used for the experiments. One gram of rat liver was homogenized in 10 ml of 10 mM Tris-HCl, pH 7.4, containing 250 mM sucrose, 1 mM EGTA, and the following protease inhibitors: 100 μM benzamidine hydrochloride, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 100 μM benzethonium chloride, and 10 μM phenylmethylsulfonyl fluoride. Mitochondria were isolated in sucrose gradients [Cuezva et al., 1990].

Portal blood was obtained from female rats. Washed cells were finally resuspended in isotonic buffer (0.172N Tris-HCl, pH 7.6) to an approximate hematocrit of 50%. In order to obtain erythrocyte membrane and matrix fractions, aliquots of red blood cell suspensions were diluted 1:6 (v/v) with hypotonic buffer (Tris-HCl 20 mM, pH 7.6) and placed on ice for 30 min to achieve complete hemolysis. Samples were centrifuged at 20,000*g* for 40 min at 4°C. The matrix fraction was decanted and stored at −20°C. The membrane containing pellet was resuspended in hypotonic buffer and further centrifuged at 20,000*g* for 40 min at 4°C. This procedure was repeated until the sample became colorless. The final membrane pellet obtained was resuspended in hypotonic buffer containing 0.01% Triton X-100.

Western Blot Determination of Immunoreactive Proteins

Proteins from rat liver homogenates, isolated mitochondria and red blood cells, as well as from insect SL2 cells, were fractionated in SDS-12.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) [Izquierdo et al., 1990; Alconada et al., 1994]. Membranes were incubated with different antibodies as indicated in the figure legends. The antibodies used in this study were i) rabbit polyclonal antibody against hsp60 derived from *H. virescens* (SPA-805, StressGen, Victoria, B.C., Canada), ii) mouse monoclonal antibody against human recombinant hsp60 (clone LK-2) (SPA-807, *Stress*Gen), and iii) rabbit anti-DR1, a polyclonal serum raised against a synthetic peptide (NH2-VGLKAPGIIPRI-COOH) corresponding to the sequence region of the F1-ATPase α-subunit from rat liver mitochondria comprising residues 162 to 173 [Alconada et al., 1994]. This antibody has been previously shown to recognize insect and mammalian stress-proteins [Alconada et al., 1994]. The immunoreactive proteins were visualized using a goat-anti-rabbit (i and iii) or rabbit anti-mouse (ii) IgG peroxidase conjugate as previ-

ously described [Izquierdo et al., 1990]. An additional rabbit polyclonal serum against Synechococcus hsp-60 (SPA-804, *Stress*Gen) was also used. Protein concentrations were determined with the Bio-Rad protein assay.

Tissue Processing for EM

Small pieces of livers were fixed by immersion in freshly prepared 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M Sörensen phosphate buffer, pH 7.2, for 2 h at 4°C. Pelleted SL2 cells and other rat liver samples were fixed for 1 h with 1% glutaraldehyde in phosphate buffer saline (PBS) and 2% tannic acid. Tissue samples were rinsed in buffer and the free-aldehyde groups were quenched by immersion in 50 mM ammonium chloride in PBS (0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2) for 60 min at room temperature (two changes of 30 min each), rinsed in PBS, and, finally, processed for embedding in Lowicryl K4M (Chemische Wercke Lowi, Waldkraiburg, Germany) according to manufacturer's instructions. Gold interferential color ultrathin sections were collected in formvar/carbon-coated nickel grids. The grids were observed in a JEOL 1010 electron microscope under 80 kV accelerating voltage.

Immunocytochemical Localization of cpn-60

Grids containing thin sections of rat liver or SL2 cells were equilibrated for 5 min with PBS and then incubated with a 1/25 dilution in PBS of anti-hsp60 (either SPA-805 or SPA-807). Grids incubated with the monoclonal antibody (SPA-807) were further incubated for 45 min with a 50 μg/ml solution of rabbit antimouse IgG + IgM (DAKOPATTS A/8, Denmark) in PBS containing 0.1% Tween-20. After extensive rinses in PBS, grids were incubated with protein A complexed to gold particles (BioCell Research Laboratories, Cardiff, United Kingdom) of 14 nm, which were diluted to an OD_{525} of 0.4 in PBS containing 1% BSA, 0.1% Triton X-100, and 0.1% Tween-20. After 45 min of incubation with the protein A-gold, grids were washed twice in PBS and distilled water, and air-dried. Counterstaining was performed with 2% aqueous uranyl acetate (6 min) and 1% lead citrate (45 s). Several series of standard controls for immunocytochemical techniques were conducted in parallel to assess the specificity of the immunoreactive signals. The immunoreactivity of each antibody (number of gold particles/square micron) was calculated in electron micrographs taken at a magnification of 25,000. In the positive pictures (enlarged to a final magnification of 50,000) the number of gold particles per unit of area were counted.

RESULTS

Mitochondrial Localization of cpn60 in Rat Liver Cells

Immunoelectron microscopy of rat liver thin sections, using a rabbit polyclonal serum raised against moth cpn60 (SPA-805), revealed that the immunoreactive protein was almost exclusively localized into mitochondria (Fig. 1A,B and Table I). No immunoreactive material was detected in nuclei (Fig. 1A), peroxisomes (Fig. 1A), endoplasmic reticulum (Fig. 1A), and Golgi stalks (not shown). Furthermore, the antibodies did not recognize any protein in the microvilli or in the plasma membrane of the hepatocyte (Fig. 1B). Few gold particles revealing immunoreactive material were found free in the cytoplasm (Fig. 1A, arrowheads). In contrast to the previous identification of cpn60 immunoreactive material in the cytoplasm [Soltys and Gupta, 1994], gold particles revealing cpn60 in rat liver cytoplasm never appeared forming clusters (Fig. 1A). These results indicate that cpn60 is localized into mitochondria of rat liver. The few immunoreactive material detected in the cytoplasm could be accounted for by either of the following alternatives: i) recognition of a cytoplasmic cpn60 homolog, and/or ii) recognition of the precursor protein of the cpn60 subunit in transit to the mitochondrial compartment.

Cpn60 Is Not Found in the Nuclei of Heat-Shocked Cells

Immunoelectron microscopy of *Drosophila* SL2 cells grown at 24°C revealed that cpn60 is also exclusively localized into the mitochondria, i.e., no immunoreactive material was found in other subcellular structures (Fig. 2A,B). Since it has been described that cpn60 translocates into the nucleus of a fish cell line upon heat-shock treatment [Sanders et al., 1994], we studied the localization of this protein in SL2 cells subjected to a 37°C heat-shock treatment. Figure 3A shows the relative protein synthesis rates in *Drosophila* SL2 cells grown at 24°C and under heat-shock (37°C) conditions. The heat-shock stress promotes a significant increase in translation of the major heat-shock proteins, hsp 82 and hsp70, of *Drosophila* cells (Fig. 3A), in agreement with

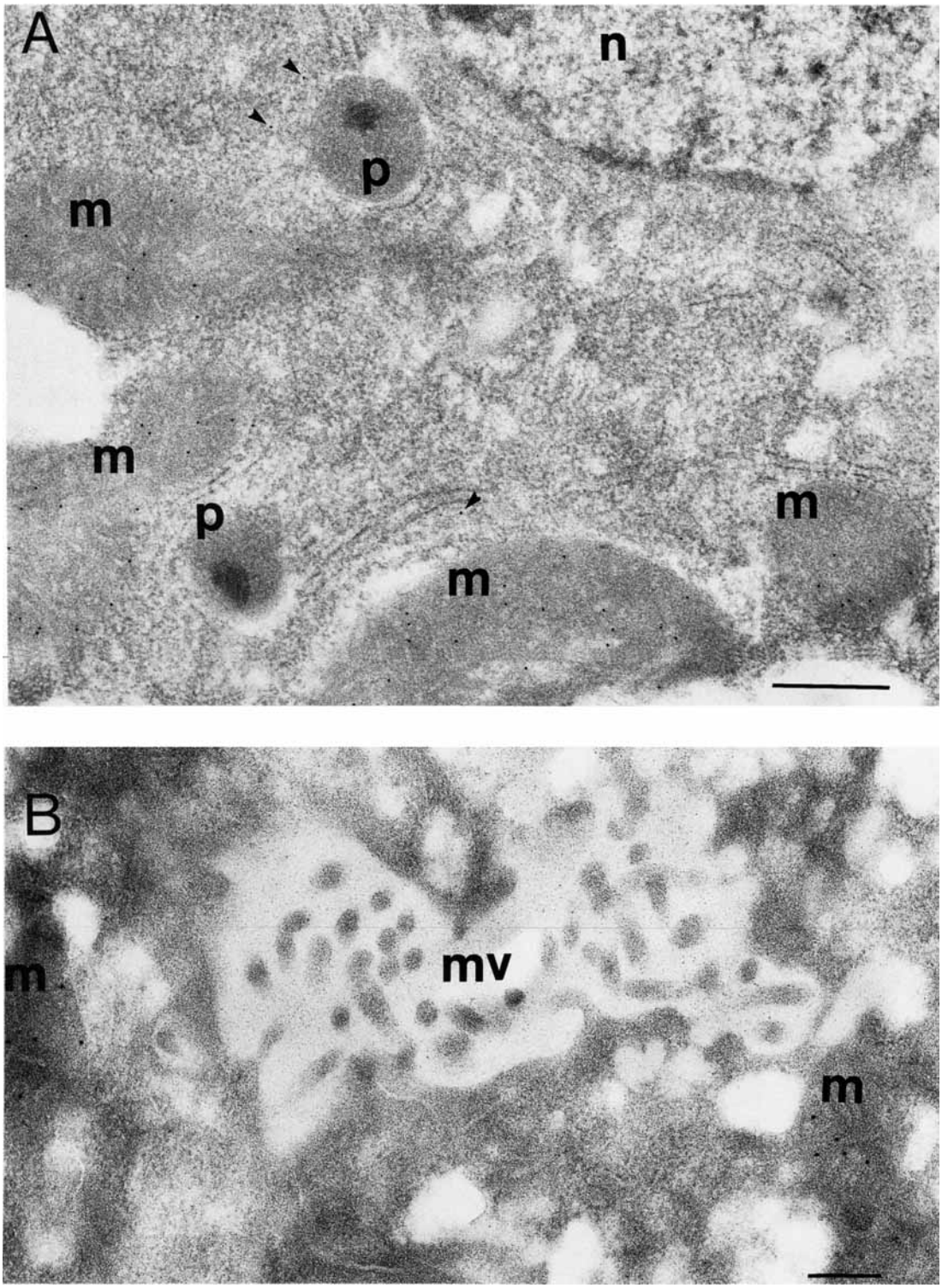

Fig. 1. Cpn60 is localized in mitochondria of rat liver cells. Small pieces of liver were fixed in 1% glutaraldehyde (**A**) and 4% paraformaldehyde (**B**). Thin sections were incubated with a 1/25 dilution of a rabbit polyclonal anti-hsp60 (SPA-805) and processed for immunoelectron microscopy as described under Materials and Methods. Gold particles decorated mitochondria (m). Few gold particles were found free in the cytoplasm (arrowheads). n, nucleus; p, peroxisome, mv, microvilli. Bars, 500 nm (A) and 200 nm (B).

TABLE I. Estimation of cpn60 Labeling Density in Rat Liver Cell Compartments and in Erythrocytes†

	Polyclonal antibody (SPA-805) (gold particles/μm^2)	Monoclonal antibody (SPA-807) (gold particles/μm^2)
Mitochondria	19.8 ± 2.7	5.5 ± 1.0**
Cytoplasm	0.6 ± 0.3	1.2 ± 0.2 (n.s.)
Nucleus	Undetectable	3.7 ± 0.6**
Erythrocytes	1.1 ± 0.2	20.2 ± 3.9*

†The results shown are means ± S.E.M.
*$P < 0.005$ and **$P < 0.0005$ when compared versus the polyclonal antibody by Student's t-test. n.s., non significant.

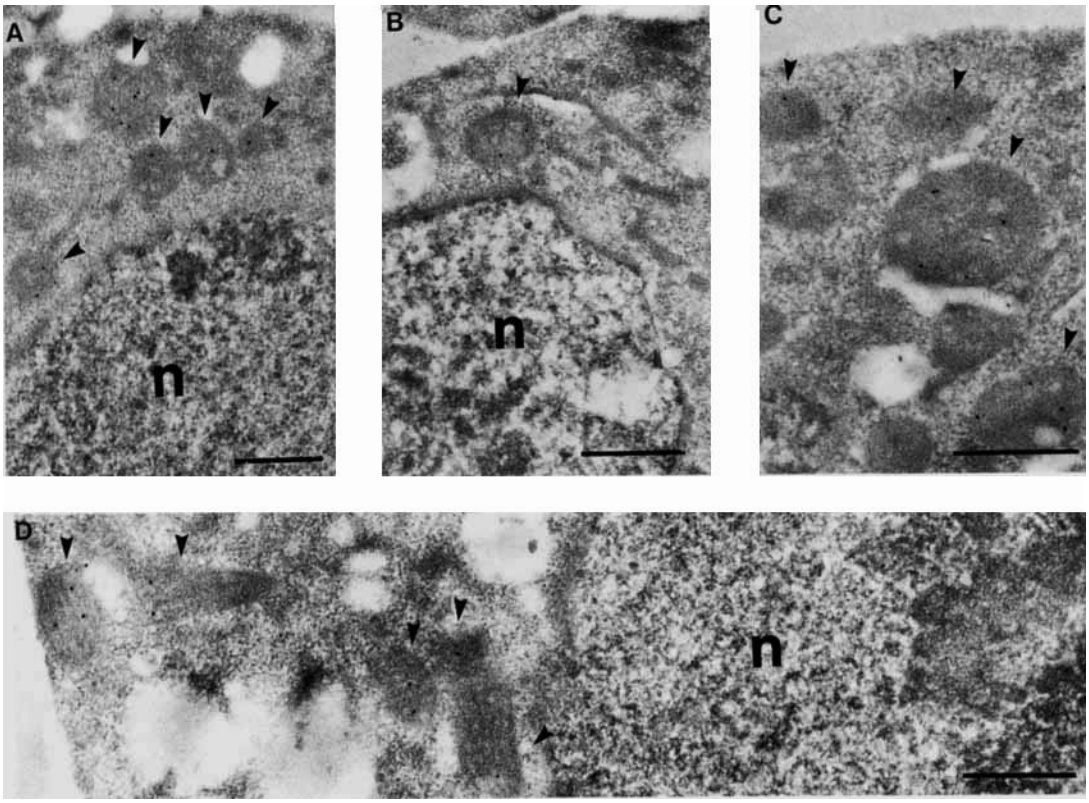

Fig. 2. Cpn60 is localized in mitochondria of embryonic *Drosophila* cells (SL2) even under cellular stressful conditions. Thin sections of SL2 cells grown at 24°C (**A,B**) or subjected to a 1 h 37°C heat-shock treatment (**C,D**) were incubated with a 1/25 dilution of a rabbit polyclonal anti-hsp60 (SPA-805) and processed for immunoelectron microscopy as described under Materials and Methods. Gold particles decorated mitochondria (*arrowheads*). *n*, nucleus. Bars, 500 nm.

previous findings [Yost et al., 1990]. Likewise, Western blotting of SL2 cells with an antibody that recognizes a constitutive p56 and inducible p71 hsp members of *Drosophila* cells [Alconada et al., 1994] indicated the induction of the stress response (Fig. 3B) in the batch of SL2 cells analyzed by immunoelectron microscopy techniques (Fig. 2C,D). The results obtained further revealed that under heat-shock conditions cpn60 is exclusively localized within the mitochondria (Fig. 2C,D). Interestingly, the heat-shock stress did not promote significant changes in the relative content of cpn60 in SL2 cells either when determined by Western blotting (Fig. 3C) or by immunoelectron microscopy techniques (Fig. 2), in agreement with the profile of synthesized proteins (Fig. 3A) [Yost et al., 1990].

An Artifactual Extramitochondrial Localization of cpn60?

The puzzling extramitochondrial localization of cpn60 [Fisch et al., 1990; Kaur et al., 1993; Jones et al., 1994; Brudzynski et al., 1992; Sanders et al., 1994; Grimm et al., 1991; Soltys and

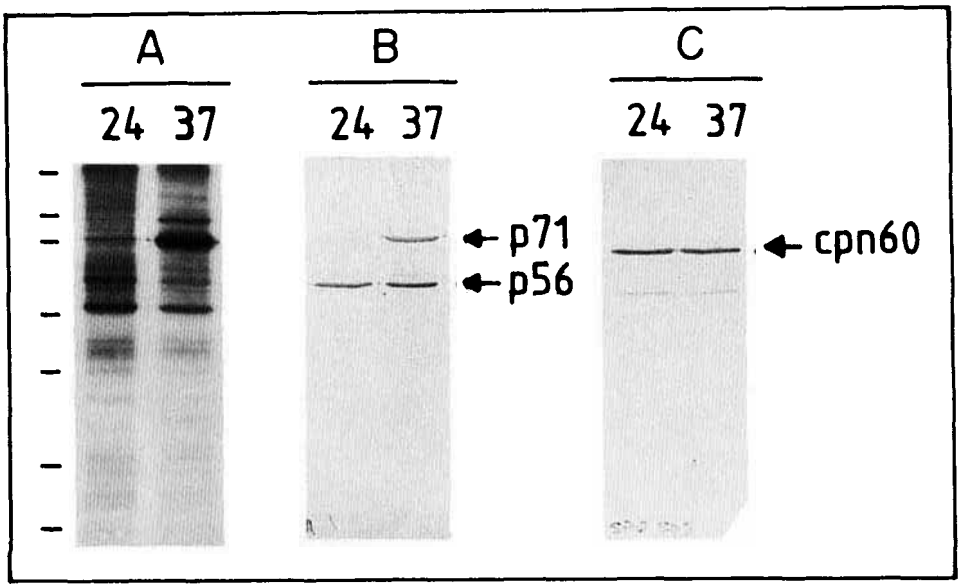

Fig. 3. Induction of heat-shock response in SL2 cells. Embryonic *Drosophila* cells were grown at 24°C (*lane 24*). Other cells were subjected to a 1 h 37°C heat-shock treatment (*lane 37*). After treatment, cellular proteins were fractionated in 12.5% SDS-PAGE and processed for fluorography ($\sim 10^5$ cpm/lane) (**A**), and for Western blotting with a rabbit polyclonal serum against DR-1 peptide (10 μg/lane) (**B**), or against moth hsp-60 (SPA-805) (10 μg/lane) (**C**). The migration of molecular mass markers is shown on the left (from top to bottom 200K, 97.4K, 69K, 46K, 30K, 21.5K, and 14.3K).

Gupta, 1994; Boog et al., 1992] prompted us to study its subcellular distribution in rat liver using a different antibody. A monoclonal antibody against human hsp60 (clone LK-2) [Boog et al., 1992] was used. The results obtained are summarized in Figure 4. The monoclonal antibody recognized immunoreactive proteins in the mitochondria (Fig. 4A). Interestingly, using the same tissue preparations as in Figure 1A, the immunoreactivity of this antibody within the mitochondria (Fig. 4A,C) was lower than that of the polyclonal serum (Fig. 1A and see also Table I). Surprisingly, the monoclonal antibody recognized immunoreactive material at the plasma membrane (Fig. 4A,B), microvilli (Fig. 4B), and within the nucleus (Fig. 4C) of the hepatocyte. Also, some peroxisomes revealed specific immunolabeling (Fig. 4A). Furthermore, rat red blood cells also stain heavily with this antibody (Fig. 4D, Table I).

These results show a surprising contrast in the subcellular localization of cpn60 immunoreactive material in eukaryotic cells depending upon the antibody used for the study. Whereas a polyclonal serum exclusively localized cpn60 into mitochondria of both mammalian and insect cells (Figs. 1 and 2), in agreement with previous findings [Hutchinson et al., 1989; Jindal et al., 1989; McMullin and Hallberg, 1987; Mizzen et al., 1989; Picketts et al., 1989; Reading et al., 1989; Waldinger et al., 1988], a monoclonal antibody reveals immunoreactive material in mature red blood cells and other subcellular structures of the hepatocyte (Fig. 4). We suggest that the results obtained with the monoclonal antibody cannot be taken as indicative of the localization of cpn60 (or even close homologs) in such compartments for the following reasons:

First, the polyclonal antibody (SPA-805) has a higher titer in immunoelectron microscopy experiments than the monoclonal one (SPA-807), i.e., the number of gold particles/square micron of mitochondrial section is higher (compare Fig. 1 vs. Fig. 4; and see Table I). Consequently, if cpn60 is present in other compartments it should be expected that the polyclonal antibody will recognize the protein at those locations. However, the polyclonal antibody does not recognize extramitochondrial cpn60, with the exception of the low labeling of rat liver cytoplasm above discussed (Figs. 1 and 2, Table I).

Second, it could be argued that cpn60 has a different conformation, and therefore, may expose different epitopes, depending upon the cellular compartment where it might be present. In this situation, Mab SPA-807 would recognize the putative cpn60 conformation present at extramitochondrial localizations, while SPA-805 would not. However, this is not very likely considering the much wider recognition spectrum

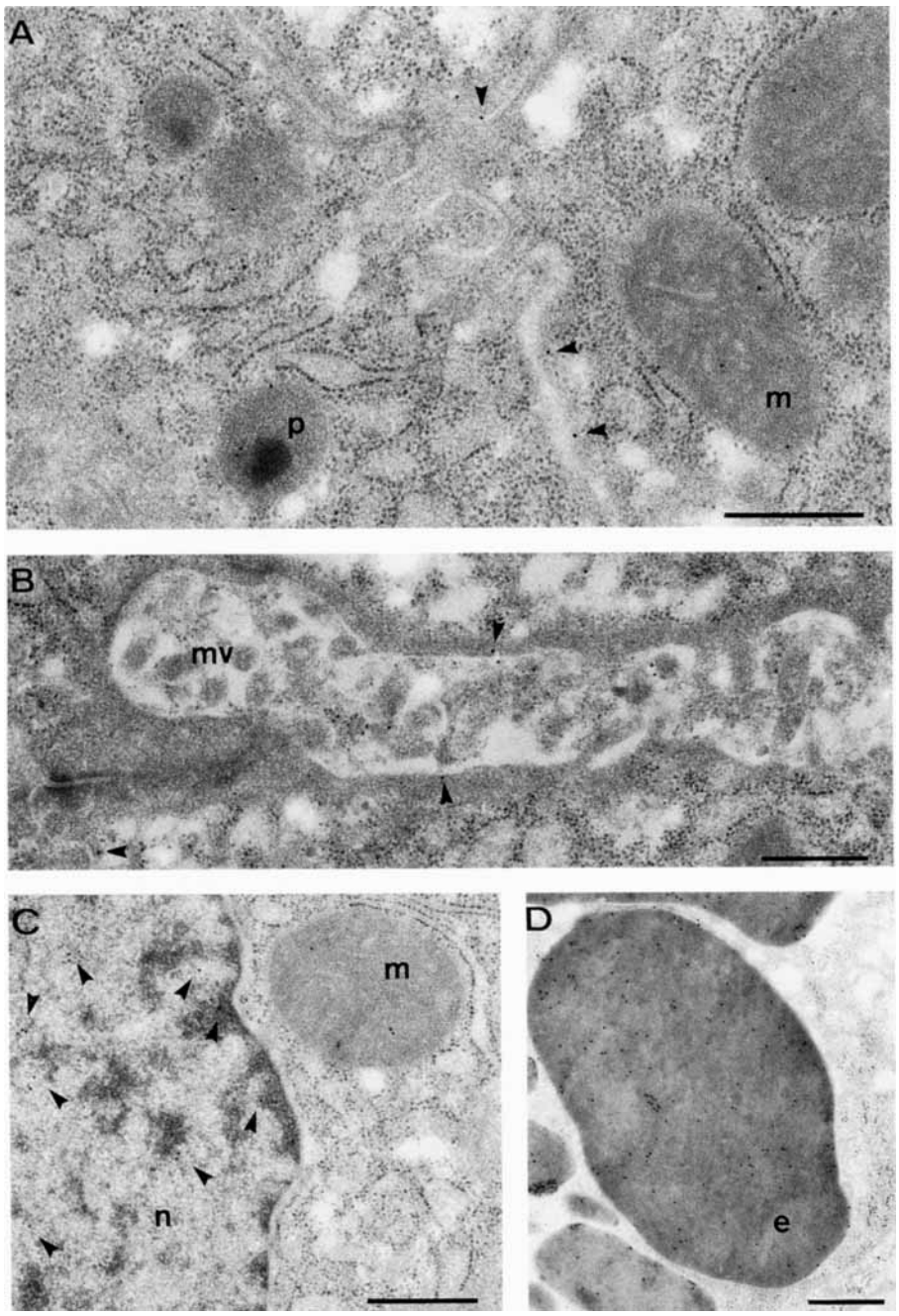

Fig. 4. A monoclonal antibody against cpn60 apparently reveals a different subcellular compartmentation of the protein. Thin sections were incubated with a 1/25 dilution of a monoclonal antibody (clone LK-2) against human cpn60 (SPA-807) and processed for immunoelectron microscopy as described in Materials and Methods. Gold particles decorated mitochondria (m in **A** and **C**), certain peroxisomes (p in A), plasma membrane (arrowheads in A and **B**), the microvilli (mv in B), and the nucleus (arrowheads in **C**) of the hepatocyte. In addition, the matrix of red blood cells (e in **D**) found within the liver thin sections contained significant gold-immunolabeling. Bars, 500 nm.

of polyclonal antibodies as compared to Mabs. Besides, mitochondrial cpn60 is recognized by SPA-807 in Western blots of denatured proteins (Fig. 5), what suggests that the monoclonal antibody does not require a native conformation of cpn60 to recognize the chaperonine epitope.

Third, an additional result against an extramitochondrial localization of cpn60 is provided by the labeling of the matrix of mature red blood cells by the monoclonal antibody (Fig. 4). It has been reported that erythrocytes, as expected for cells devoid of organelles, do not contain hsp60,

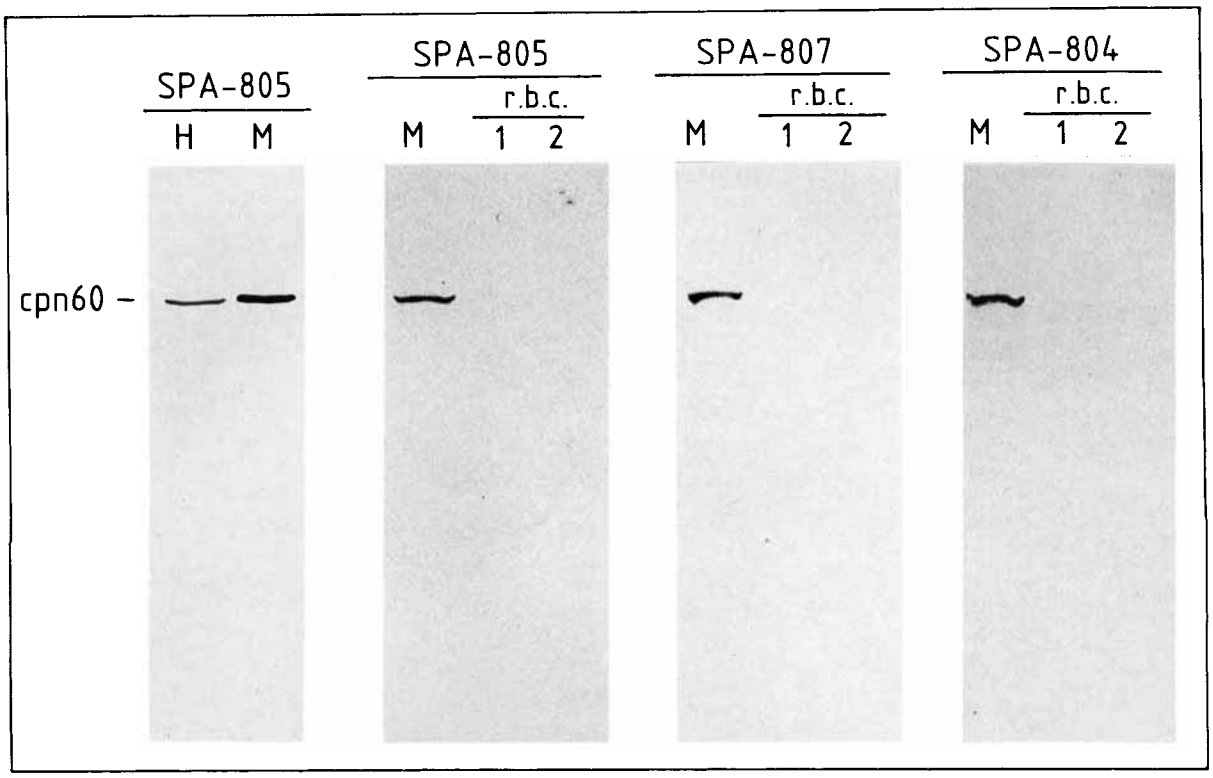

Fig. 5. No cpn60 immunoreactive protein could be detected in Western-blots of rat red blood cells. Proteins from rat liver homogenates (*lane H*) (60 μg) and isolated mitochondria (*lane M*) (25 or 50 μg), and the matrix (*lane 1*) (50 μg) and membrane (*lane 2*) (50 μg) fraction or rat red blood cells (r.b.c.) were fractionated in 12.5% SDS-PAGE. After transfer to PVDF membranes, the membranes were incubated with different antibodies against cpn60. SPA-805, polyclonal rabbit serum against moth cpn60; SPA-807, monoclonal antibody against human cpn60; SPA-804, polyclonal rabbit serum against *Synechococcus* cpn60. The immunoreactivity of cpn60 significantly increases in isolated mitochondria when compared to rat liver homogenates. Similar findings were obtained with Mab SPA-807 (not shown).

whereas they do contain hsp70 members [Gromov and Celis, 1991]. Interestingly, in Western blot experiments of fractionated proteins from rat red blood cells and isolated mitochondria, this antibody (SPA-807), and some others, recognized mitochondrial cpn60, but none of them recognized immunoreactive proteins in the membrane and matrix fraction of the erythrocyte (Fig. 5). Therefore, these findings support the idea that the immunoreactive protein(s) recognized by the monoclonal antibody in immunoelectron microscopy experiments is not cpn60, but some other protein(s).

DISCUSSION

The presence of cpn60 in the mitochondria, the nuclei, and the plasma membrane is difficult to accomodate with our present knowledge about protein sorting pathways and protein function. In the first place, it implies a sophisticated cellular biology for the translation of cpn60 mRNA, so that the chaperonin could be sorted to different cell compartments, i.e., following the secretory and post-translational sorting pathways for its membrane and mitochondrial localization, respectively. On the other hand, nuclear localization of cpn60 [Sanders et al., 1994] implies that the translation of the chaperonin mRNA could be initiated after the coding region of the N-terminal mitochondrial targeting sequence of the chaperonin precursor [Jindal et al., 1989; Mizzen et al., 1989; Picketts et al., 1989; Reading et al., 1989] or, alternatively, proteolytic maturation of the cpn60 precursor protein occurs in the cell cytoplasm. The latter is a less likely situation since it has been shown that the mitochondrial hsp60 precursor protein [Mizzen et al., 1989], as well as other mitochondrial precursors [Anderson, 1981], accumulate in the cytoplasm of cells treated with a K^+ ionophore. In any case, the reported evidence is that in all organisms studied so far, cpn60 is synthesized with a mitochondrial targeting pre-sequence for import into mitochondria [Jindal et al., 1989;

Mizzen et al., 1989; Picketts et al., 1989; Reading et al., 1989; Cheng et al., 1990].

Furthermore, a recent report has provided in vitro evidence that folding of the cytosolic isoform of malate dehydrogenase (MDH) is unaffected by the presence of cpn60, cpn10, and Mg-ATP, whereas folding of the mitochondrial MDH isoform is markedly increased [Staniforth et al., 1994]. A similar observation has been reported for the folding of the cytosolic and mitochondrial aspartate aminotransferase isozymes [Mattingly et al., 1995]. These findings suggest that the folding of homologous proteins located in different cellular environments is assisted by different chaperones [Mattingly et al., 1993], thus arguing against a functional role for cpn60 at extramitochondrial localizations. Likewise, an extramitochondrial localization of cpn60 suggests that the chaperonin could perform its biological function in environments with quite different polarity. This is quite surprising because it is expected that differences in the polarity of the media will promote changes in protein ultrastructure, affecting the binding of regulatory molecules [Hartman et al., 1992a,b; Lubben et al., 1990; Höhfeld and Hartl, 1994; Hartl, 1994; Ellis, 1994] and, therefore, chaperonin function.

Recently, we have provided evidence that cpn60 members are distantly related homologous proteins to the V- and F-type ATPases [Alconada et al., 1994]. Furthermore, a statistically significant sequence similarity [Flores et al., in preparation] and immunological cross-reactivity [Alconada et al., 1994] have been reported between these protein families (ATPases and chaperonins) and the molecular chaperones of the 70 kDa family (see Fig. 3B). Although it is conceivable that the extramitochondrial localization of cpn60 (or close homologs) [Fisch et al., 1990; Kaur et al., 1993; Jones et al., 1994; Brudzynski et al., 1992; Sanders et al., 1994; Grimm et al., 1991; Soltys and Gupta, 1994; Boog et al., 1992] could be due to species and cell type differences, our findings suggest that such unexpected localizations for the protein could be related with the recognition by cpn60 antibodies of conserved epitopes also present in some of these distantly related ATP-binding protein families [Alconada et al., 1994; Flores et al., in preparation]. In this regard, the epitope recognized by Mab SPA-807 is located between residues 383 and 419 of the human hsp60 sequence [Boog et al., 1992], a sequence region that is conserved in hsp70 members (comprised between residues 179 and 215 of the human 70kDa heat-shock protein sequence [Flores et al., in preparation]). This may explain the labeling of the matrix of red blood cells with cpn60 antibodies (Fig. 4 and Table I). As indicated above, hsp70 but not hsp60 members have been described to be present in erythrocytes [Gromov and Celis, 1991]. Conceivably, this epitope may be preserved during fixation procedures of the liver (Fig. 4), but lost during SDS-PAGE procedures (Fig. 5). Interestingly, this is supported by previous findings using antibodies raised against a synthetic peptide (DR-2) derived from that region of the rat liver F1-ATPase α-subunit sequence [Alconada et al., 1994]. This antiserum did not recognize blotted proteins, but did react in affinity chromatography experiments with a mitochondrial p74 contained in soluble cell extracts [Alconada et al., 1994].

In conclusion, our findings indicate that cpn60 is exclusively localized into mitochondria of metazoan cells (Figs. 1 and 2), even under cellular stressful conditions (Fig. 2). Therefore, we suggest that the identification of cpn60 immunoreactive material outside the mitochondria is related with the identification of distantly related cpn60 homologs and/or artifacts of the experimental approach.

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