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Research Articles

Synthesis of a ubiquitously present new HSP60 family protein is enhanced by heat shock only in the Malpighian tubules of *Drosophila*

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Abstract. A homologue of the chaperonin protein of the HSP60 family has not been shown so far in *Drosophila*. Using an antibody specific to HSP60 family protein in Western blotting and immunocytochemistry, we showed that a 64-kDa polypeptide, homologous to the HSP60, is constitutively present in all tissues of *Drosophila melanogaster* throughout the life cycle from the freshly laid egg to all embryonic, larval and adult stages. A 64-kDa polypeptide reacting with the same antibody in Western blots is present in all species of *Drosophila* examined. Using Western blotting in conjunction with ^{35}S-methionine labeling of newly synthesized proteins and immuno-precipitation of the labeled proteins with HSP60-specific antibody, it was shown that synthesis of the 64-kDa homologue of HSP60 is appreciably increased by heat shock only in the Malpighian tubules, which are already known to lack the common HSPs.

Key words. Chaperonin; *Drosophila*; *groEL*; heat shock; heat shock proteins; HSP60; Malpighian tubules; TCP-1.

Cells of virtually every organism respond to sudden exposure to an elevated temperature (heat shock) by a transiently increased synthesis of a specific set of polypeptides, the heat shock proteins (HSP) or stress proteins, which help cells survive the stress damage[1–3]. This response, first discovered in *Drosophila*[4,5], is one of the most conserved biological responses, believed to be shared by all cells of all organisms. The HSPs are generally grouped into five major families according to their apparent molecular weights (in kDa), the HSP100, HSP90, HSP70, HSP60 and the low molecular weight (<30 kDa) HSP families. Members of the HSP90, HSP70 and the low molecular weight HSP families of *Drosophila* are very well characterized with respect to their genes, transcripts and polypeptides[1,2,6]. Surprisingly, a heat-inducible homologue of the HSP60 family has so far not been reported in *Drosophila*, although this family is known to be a highly conserved and essential chaperonin in cells not only under stress but even under normal physiological conditions[7,8].

An earlier study in our laboratory[9] revealed the heat shock response in the Malpighian tubules of *Drosophila* larvae, the osmo-regulatory and excretory organs attached to the midgut[10], to be unusual. In this larval tissue, none of the typical HSPs (83 kDa, 70 kDa, 28 kDa, 27 kDa, 23 kDa, 22 kDa) were induced by heat shock, but an entirely different set of polypeptides was induced. Among these, a polypeptide with an apparent mol. wt. of 64 kDa (erroneously estimated in that paper as 58 kDa) was found to be maximally induced by heat shock in the Malpighian tubules. Lakhotia and A. K. Singh[9] raised the possibility that the heat shock-induced 64-kDa polypeptide may be homologous to the HSP60 family of proteins of other organisms. Our present results, using ^{35}S-methionine labeling in conjunction with immuno-blotting using a specific antibody against HSP60 family proteins, confirm that this novel HSP seen in heat-shocked Malpighian tubules is indeed an HSP60 homologue. Interestingly, this polypeptide is found to be constitutively present in all tissues of *Drosophila*, beginning from the egg stage, although its synthesis is enhanced by heat shock only in the Malpighian tubules. This protein is also present in all species of *Drosophila* that were examined. Discovery of the HSP60 homologue in *D. melanogaster* will permit extensive genetic and molecular studies on this biologically important chaperonin.

Materials and methods

Fly strain and culture. Wild-type strains of *Drosophila melanogaster* (Oregon R$^+$), *D. ananassae*, *D. takahashi*, *D. bipectinata*, *D. malerkotliana* and *D. kikkawai* were used for these studies. The flies and larvae were reared on standard cornmeal-agar-yeast food at 22 ± 1 °C.

Preparation of protein samples, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immuno-blotting. Different organs (described in results) from larvae and adults of *D. melanogaster* were dissected in Poels' salt solution[11] (PSS) and transferred to fresh PSS in microfuge tubes. They were either heat-shocked at 37 °C for 30 min or were kept at 22 °C for 30 min as control. The heat-shocked and control tissues

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were labeled with ^{35}S-methionine (BRIT, Bombay, 200 μCi/ml; sp. act. 1000 Ci/mM) for 30 min at 37 °C or at 22 °C, respectively.

Ovaries from adult flies of different species of *Drosophila* were dissected in PSS and directly processed for electrophoresis. In all cases, the protein samples were prepared and processed for SDS-PAGE as described earlier[12].

The electrophoresed polypeptides were transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany) by semi-dry blotting as described earlier[13]. For detecting the HSP60 family proteins, a rabbit polyclonal antibody against the HSP63 polypeptide of *Heliothis virescens* (SPA-805, StressGen, Canada), which specifically recognizes HSP60 family proteins from a wide range of species[14], was used at 1:1000 dilution as the primary antibody. The HSP70 was also detected in the same blots by using the 7Fb monoclonal antibody (kindly donated by Prof. S. Lindquist) as described earlier[15]. The primary antibodies were detected by appropriate HRP conjugated secondary antibodies (Sigma, USA, or Bangalore Genei, Bangalore) at the recommended dilutions. Immuno-blots containing ^{35}S-labeled proteins were fluorographed by dipping in toluene containing 22% 2,5-diphenyloxazole (PPO) for 3 min and drying for 1 h at room temperature, before exposing to X-ray film to detect radiolabeled newly synthesized polypeptides. The Western blots and the fluorograms were then compared.

Immuno-precipitation of HSP60 from Malpighian tubules of *D. melanogaster* larvae. Malpighian tubules from 60 larvae were kept as a control and from 60 were heat-shocked at 37 °C for 30 min, labeled with ^{35}S-methionine as above and dissolved by boiling in 40 μl of the SDS lysis buffer (50 mM Tris pH 7.5, 2% SDS)[16]. After 20-fold dilution with PBS (130 mM NaCl, 7 mM $Na_2HPO_4 \cdot 2H_2O$, 3 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.5) containing 2% BSA, 4 μl of the HSP60 antibody (SPA-805, StressGen) was added and the mix incubated overnight at 4 °C to immuno-precipitate HSP60. Immune complexes were collected on protein A-sepharose beads (Pharmacia) by slow rocking for 2 h at 4 °C. The beads were washed 3× in PBS, 0.5% Tween-20, and the immuno-precipitated proteins were recovered by boiling in the Laemmli[17] sample buffer. The ^{35}S-labeled and immuno-precipitated polypeptides were detected by fluorography following SDS-PAGE as above. Total ^{35}S-labeled proteins from control and heat-shocked Malpighian tubules were run in parallel in the same slab gel.

In situ immunostaining of *D. melanogaster* embryos, larval and adult tissues. Immunostaining of embryos was performed essentially as described by Patel[18]. Embryos were collected on sugar-agar plates for 1 h and allowed to age at 25 °C for different periods of time. Endogenous peroxidase activity in dechorionated, devitellinised and paraformaldehyde fixed embryos was quenched by incubating embryos in 70% methanol containing 0.3% H_2O_2 for 20 min with occasional mixing, following which the embryos were rinsed in 1 ml of methanol, rehydrated in a 1:1 mix of methanol:phosphate buffered saline (PBS) for 5 min and PBT [PBS containing 0.1% bovine serum albumin (BSA) and 0.1% Triton-X 100] for 3 × 10 min. Embryos were further washed in PBT for 1 h with five changes in between and blocked for 2 h in blocking solution (PBS containing 2% BSA and 10% normal goat serum) with gentle rotation. Embryos were then incubated overnight in HSP60 antibody SPA-805 (preadsorbed with fixed *Drosophila* larval heads) at a dilution of 1:50 in PBT. Following repeated washing in PBT over a period of 2 h, the embryos were incubated for 2 h in 0.5 ml of 1:1000 dilution of a preadsorbed goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Sigma). Incubation was followed by four washing of 30 min each in PBT and two washes of 5 min each in TBS (120 mM NaCl, 20 mM Tris pH 7.5). Embryos were then stained at room temperature in the dark with 0.5 ml of staining solution (100 mM Tris pH 7.5, 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride and 0.03% H_2O_2). Staining was monitored under a binocular microscope, and color was allowed to develop until the negative controls (processed in parallel but without incubation in the primary antibody) started getting a background color. The reaction was stopped by washing several times in PBS. Embryos were mounted in 80% glycerol buffered with PBS.

Immunocytochemical localization of the HSP60 antibody in control and heat-shocked tissues (described in results) of *D. melanogaster* were carried out as described[15].

Results

As shown in figure 1, the HSP60 antibody specifically reacted in the Western blots with a 64-kDa polypeptide constitutively present in all the different *D. melanogaster* cell types tested: salivary glands, Malpighian tubules and brain ganglia from late 3rd instar larvae, and ovaries and Malpighian tubules from adult flies. An additional faint band moving slightly ahead of the

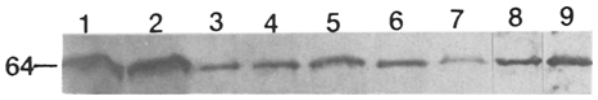

Figure 1. Constitutive expression of HSP64 protein. Western blot of control (lanes 1, 3, 5, 7, 8 and 9) and heat-shocked (lanes 2, 4 and 6) protein samples of larval Malpighian tubules (lanes 1 and 2), Malpighian tubules of adult male (lanes 3 and 4) and female flies (lanes 5 and 6), larval salivary glands (lane 7), larval brain ganglia (lane 8) and adult ovary (lane 9) probed with the HSP60 (SPA-805) antibody. Molecular weight (in kDa) is indicated at the side.

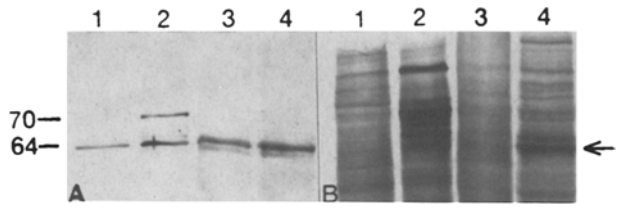

Figure 2. Induced synthesis of HSP64 in larval Malpighian tubules. (*A*) Western blot of control (lanes 1 and 3) and heat-shocked (lanes 2 and 4) protein samples of larval salivary glands (lanes 1 and 2) and Malpighian tubules (lanes 3 and 4) probed sequentially with the HSP60 (SPA-805) and HSP70 (7Fb) antibodies. (*B*) Fluorogram of the Western blot in (A). Molecular weights (in kDa) are indicated on left side. Arrow on right side indicates the heat shock-induced synthesis of HSP64 protein.

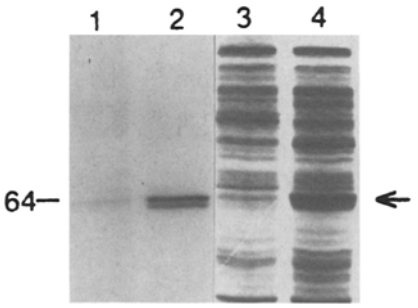

Figure 3. Immuno-precipitation of HSP64. ^{35}S-methionine-labeled cell lysates of larval Malpighian tubules were immuno-precipitated with HSP60 (SPA-805) antibody and then analyzed by SDS-PAGE and fluorography. Immuno-precipitated proteins from control (lane 1) and heat-shocked (lane 2) Malpighian tubules; total cellular proteins from control (lane 3) and heat-shocked (lane 4) Malpighian tubules. Molecular weight (in kDa) is indicated on left side. Arrow on right side indicates the heat shock-induced synthesis of HSP64 protein.

major 64-kDa band was also often seen (fig. 2). Western blots did not show any noticeable increase in the levels of either of these polypeptides after heat shock in any tissue, including the Malpighian tubules (figs 1 and 2). Since heat shock was known to enhance the synthesis of a 64-kDa polypeptide in Malpighian tubules[9,15], it was of interest to see if the 64-kDa polypeptide detected in the immunoblots and the ^{35}S-labeled 64-kDa band were identical. As shown in figure 2, a fluorogram of the Western blot revealed a strongly ^{35}S-methionine-labeled band (indicated by arrow) in the heat-shocked but not in the control Malpighian tubules at the same position as the antibody reacting band/s. As reported earlier[9,15], the control as well as heat-shocked samples of other tissues (brain ganglia and gut from larvae and brain ganglia, testes and ovaries from adult flies) did not show a strongly ^{35}S-methionine-labeled 64-kDa band (not shown). The complete absence of HSP70 in heat-shocked Malpighian tubules as shown in figure 2 (lane 4) is in accordance with the earlier reports[9,15].

To confirm that the antibody-reacting and ^{35}S-methionine-labeled co-migrating 64-kDa bands in the heat-shocked Malpighian tubules were identical, we used the HSP60 antibody to immuno-precipitate ^{35}S-labeled proteins from control and heat-shocked Malpighian tubules of *D. melanogaster*. Figure 3 shows that two closely spaced 64-kDa bands were immuno-precipitated in control as well as heat-shocked samples, but the ^{35}S-methionine uptake in both these polypeptides was considerably higher in the heat-shocked sample (lane 2) than in the control (lane 1); lanes 3 and 4 in this figure show the labeling profile of total proteins in equivalent numbers of control and heat-shocked Malpighian tubules. It is clear from a visual estimate of the density of labeled bands that heat shock caused a several-fold increase in the synthesis of HSP64 (lane 4). The relative labeling of the 64-kDa polypeptide in control and heat-shocked Malpighian tubules as a proportion of the total protein was of the same order as in the corresponding immuno-precipitated samples.

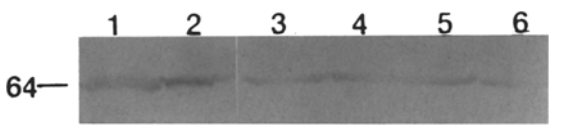

Figure 4. HSP64 is present in all species of *Drosophila*. Western blot of protein samples from ovaries of different species of *Drosophila* (*D. melanogaster*, lane 1; *D. ananassae*, lane 2; *D. takahashi*, lane 3; *D. bipectinata*, lane 4; *D. malerkotliana*, lane 5; and *D. kikkawai*, lane 6) challenged with the HSP60 (SPA-805) antibody: in all cases a positive band is seen at 64-kDa position.

Western blots of protein samples from ovaries of flies of different species of *Drosophila* revealed the constitutive presence of a 64-kDa polypeptide reacting with the HSP60 antibody in all cases (fig. 4).

Immunocytochemical staining with the HSP60 specific antibody (SPA-805) also showed the constitutive presence of this protein throughout development. Specificity of the antibody staining was confirmed by examining tissues processed in parallel which were not incubated with the primary antibody: none of them showed any appreciable staining (not shown). HSP60 was uniformly distributed in embryos during the entire course of embryogenesis (fig. 5A–C). Similarly, a uniform distribution of HSP60 was seen all along the gut, including the Malpighian tubules (fig. 5D), salivary glands (fig. 5E), imaginal disks and brain ganglia (not shown) of *D. melanogaster* larvae. The large polytenized cells of salivary glands, when examined at higher magnification, showed that HSP60 was present only in the cytoplasm, the nuclei being negative for antibody binding (fig. 5E). In the adults also, all the tissues that were examined (gonads, gut, brain ganglia etc.) showed the presence of the HSP60. In the ovaries, HSP60 was found in germarium, nurse cells, oocytes and follicle cells (fig. 5F). As in the case of salivary gland polytene cells, the large nurse cells also revealed the HSP60 to be present in the cytoplasm only. During the final stages of oogenesis,

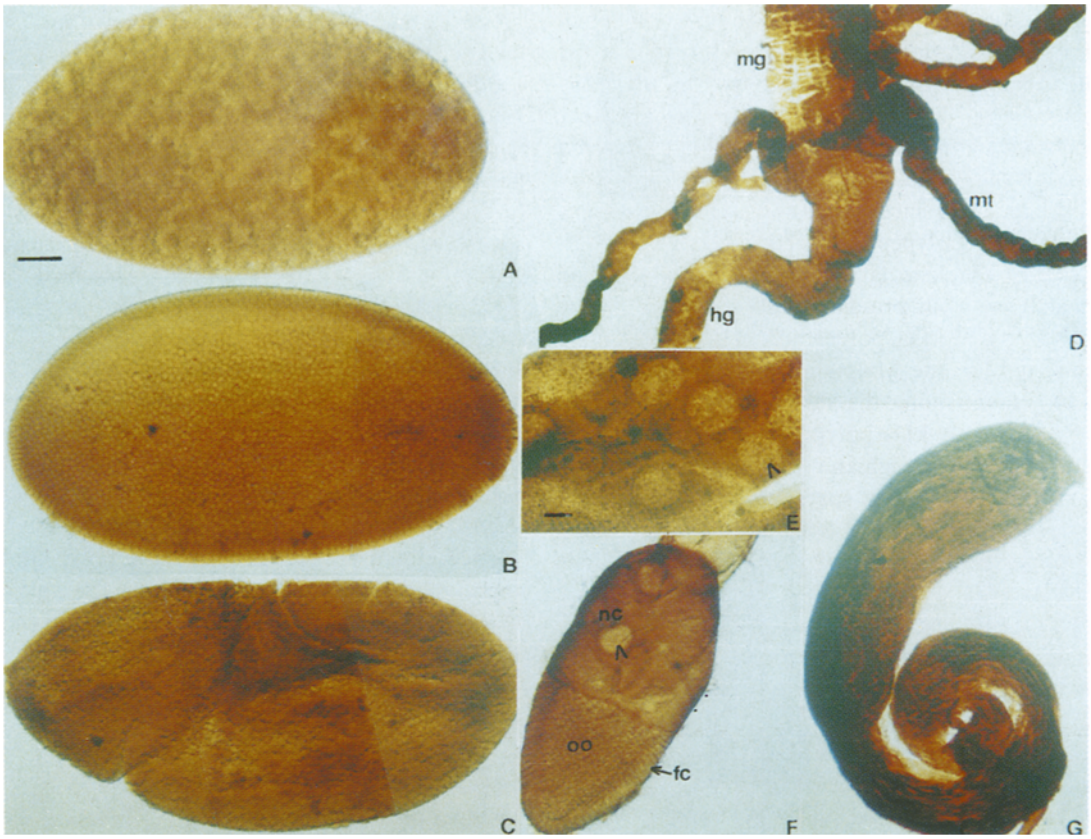

Figure 5. HSP60 is constitutively present in all cell types of *D. melanogaster*. In situ immunostaining of embryos (pre-blastoderm, *A*; cellular blastoderm, *B*; and gastrulation stage, *C*), late 3rd instar larval gut with associated Malpighian tubules (*D*), larval salivary gland (*E*), adult ovarian follicle (*F*) and testis (*G*) with the HSP60 antibody. Note the general staining of all cell types in all stages; the nuclei (arrowheads) of salivary gland cells (*E*) and of nurse cells (*F*) are clearly unstained. fc = follicle cells, hg = hind gut, mg = midgut, mt = Malpighian tubules, nc = nurse cells, oo = oocyte. Scale bars represent 50 μm; all figures, except (*E*), are at same magnification as (*A*).

when the nurse cells break down, the concentration of HSP60 appeared to be much higher at the anterior end of the oocyte, suggesting a transfer of this protein from the nurse cells to the oocyte (not shown). HSP60 was uniformly distributed in the mature unfertilized egg as it was in young embryos. All organs of the male reproductive system also showed uniform staining with the HSP60 antibody (only testis is shown in fig. 5G). Heat shock did not have any effect on antibody staining in any of the larval or adult tissues examined (not shown).

Discussion

Members of the HSP60 family proteins, also known as chaperonin[8,19,20], are highly conserved[7,8], are constitutively present in all organisms and have essential functions in protein folding[7,21–23]. In view of their known widespread presence as well as their essential functions, it is rather surprising that so far a homologue of HSP60 was not described in *Drosophila*. An earlier report from our laboratory[9] suggested that in view of its apparent molecular weight, the major heat-inducible polypeptide in Malpighian tubules of *Drosophila* could be an HSP60 homologue. Our present results unambiguously confirmed this. It was seen that a 64-kDa polypeptide in all the tissue types of *Drosophila* reacted with an HSP60 family protein-specific antibody[14]. We have also used an antibody against the HSP60 of *Anabaena* (SPA-804 of StressGen) and found that this too specifically cross-reacted in Western blots with the 64-kDa polypeptide of *Drosophila* (data not shown). The widespread constitutive presence of the HSP60 homologue in *Drosophila* was further confirmed by immunocytochemical localization: all cell types throughout development were found to contain easily detectable amounts of this protein. This is in agreement with the known presence of HSP60 in all cell types of other organisms. Also in agreement with the properties of HSP60 was the finding that in *Drosophila* cells this protein is absent from nuclei[8,19–21]. Thus, an HSP60 homologue is constitutively present in all cell types of *D. melanogaster*. Furthermore, Western blots of other species of *Drosophila* showed that this protein is conserved in the genus.

Fluorograms of the Western blots of ^{35}S-methionine-labeled proteins in our present study suggested that the heat shock-induced 64-kDa polypeptide in the Malpighian tubules co-migrated with the HSP60 antibody reactive band/s. Immuno-precipitation of ^{35}S-labeled proteins with the HSP60 antibody unambiguously confirmed their identity, since synthesis of the immuno-precipitated 64-kDa polypeptide/s was increased several-fold after heat shock to larval Malpighian tubules. As was noted earlier[9], our present results also showed that synthesis of the 64-kDa polypeptide was increased by heat shock only in the Malpighian tubules. Therefore, we conclude that among the various tissues examined, the HSP60 family 64-kDa polypeptide is heat shock-inducible only in the Malpighian tubules. At this time it is not clear if the two closely spaced bands identified by the antibody represent two different HSP60 homologues[24] or post-translationally modified forms of the same HSP60.

It is rather surprising that although synthesis of the 64-kDa polypeptide/s was considerably enhanced following heat shock to Malpighian tubules, the total amount, as revealed by the Western blotting and in situ immunostaining of cells, was not correspondingly increased beyond the levels seen in controls. It is possible that along with increased synthesis of the HSP64 in heat-shocked Malpighian tubules, there is also a greater turnover, so that the steady-state levels of this protein do not appreciably increase; this needs further analysis.

It may be noted that a Tcp-1-related polypeptide was earlier reported in *D. melanogaster*[25]. Since Tcp-1 is a conserved cytosolic chaperonin in eukaryotes[26,27] and shows a weak homology with the HSP60 family[27–30] and since the molecular weight of Tcp-1 is in the range characteristic of the HSP60 family (60–65 kDa), it remains possible that the HSP64 identified by us and the Tcp-1 identified by Ursic and Ganetzky[25] are identical. However, since the antibody (SPA-805) used in the present study is against a moth 63-kDa mitochondrial polypeptide sharing a high degree of homology to both the GroEL protein of *E. coli* and to eukaryotic chaperonin[14], and is known to cross-react specifically with HSP60/GroEL family proteins only, in all organisms, we consider it unlikely that the 64-kDa HSP identified in our study is the same as the Tcp-1 protein. Moreover, the Tcp-1 protein is not heat-inducible[31] and, as in other eukaryotes[32], it is to be expected that *Drosophila* will also have distinct HSP60 and Tcp-1 homologues.

Existence of a pattern of induction of heat shock proteins specific for Malpighian tubules in all species of *Drosophila* as well as in several other insects[33,34] suggests a functional significance. Chemicals like sodium cacodylate that mimic the heat shock effect have also been seen to maximally induce the synthesis of the HSP64 in the Malpighian tubules, without inducing the other typical HSPs (unpubl.). It is likely that the special osmo-regulatory and nitrogenous waste excretory functions of the Malpighian tubules[10] impose certain constraints on the cells so that their response to heat shock is unusual[35]. Further studies are needed to understand the biological significance of this intriguing situation. Identification of an HSP60 family polypeptide in *Drosophila* would provide a good handle for detailed genetic analysis of this ubiquitous protein. Cloning of the gene for HSP64 of *Drosophila* (in progress in our laboratory) will permit answers to the question of the significance and regulation of the specific induction of this protein by heat shock in the Malpighian tubules only.

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