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An Evaluation of Hsp90 as a Mediator of Cortical Patterning in *Tetrahymena*

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ABSTRACT. This study asks two questions: 1) whether Hsp90 is involved in the regulation of cortical patterning in *Tetrahymena,* and 2) if it is, whether specific defects in this regulation can be attributed to functional insufficiency of the Hsp90 molecule. To address question 1, we compared the effects of a specific inhibitor of Hsp90, geldanamycin, on population growth and on development of the oral apparatus in two *Tetrahymena* species, *T. pyriformis* and *T. thermophila.* We observed that geldanamycin inhibits population growth in both species at very low concentrations, and that it has far more severe effects on oral patterning in *T. pyriformis* than in *T. thermophila.* These effects are parallel to those of high temperature in the same two species, and provide a tentative affirmative answer to the first question. To address question 2, we ascertained the base sequence of the genes that encode the Hsp90 molecules which are induced at high temperatures in both *Tetrahymena* species, as well as corresponding sequences in *Paramecium tetraurelia.* Extensive comparative analyses of the deduced amino acid sequences of the Hsp90 molecules of the two *Tetrahymena* species indicate that on the basis of what we currently know about Hsp90 both proteins are equally likely to be functional. Phylogenetic analyses of Hsp90 amino acid sequences indicate that the two *Tetrahymena* Hsp90 molecules have undergone a similar number of amino acid substitutions from their most recent common ancestor, with none of these corresponding to any known functionally critical region of the molecule. Thus there is no evidence that the Hsp90 molecule of *T. pyriformis* is functionally impaired; the flaw in the control of cortical patterning is more likely to be caused by defects in mechanism(s) that mediate the response to Hsp90, as would be expected from the ''Hsp90 capacitor'' model of Rutherford and Lindquist.

Key Words. Cortical development, geldanamycin, Hsp90, *Paramecium tetraurelia,* phylogenetic trees, stomatogenesis, *Tetrahymena pyriformis, Tetrahymena thermophila.*

MOLECULAR chaperones of the Hsp90 family are abundant and highly conserved proteins that are synthesized constitutively by eukaryotic cells and also are induced strongly by heat shocks (reviews: Csermely et al. 1998; Pearl and Prodromou 2000; Pratt 1998). Their in vivo function at normal growth temperatures is complex and by no means fully understood. It appears to involve the maintenance of certain regulatory proteins in reactive conformations in which they are competent to bind with their specific ligands, rather than a general promotion of assembly and folding of proteins (Nathan, Vos, and Lindquist 1997). This function has been best studied for steroid receptors (Pratt and Toft 1997). The Hsp90 molecular chaperones are also involved in the management of other classes of signaling proteins, including a variety of transcription factors and protein kinases (reviews: Csermely et al. 1998; Mayer and Bukau 1999).

Two distinct approaches have provided evidence that the Hsp90 chaperones are implicated in intracellular morphogenesis. In a cell biological approach, Hsp90 was found to be associated with centrosomes in *Drosophila* and mammalian cell lines and concentrated in the basal body region of developing *Drosophila* sperm (Lange et al. 2000). Interference with the function of Hsp90, either in pupal lethal trans-heterozygotes of loss-of-function mutant alleles of the unique Hsp90 family gene in *Drosophila, Hsp83*[1], or in *Drosophila* or mammalian cell lines treated with a specific Hsp90 inhibitor, geldanamycin, brings about abnormalities in mitotic divisions and in the partitioning of the centrosomal material (Lange et al. 2000). Other, viable, trans-heterozygotes of *Hsp83* mutations are male-sterile and defective in sperm production (Yue et al. 1999). Since spermatogenesis and spermiogenesis involve mobilization of microtubular arrays in extraordinarily complex ways, Yue et al. (1999) suggested that the *Drosophila* Hsp90 protein might be involved in regulation of microtubular assembly and/or function. However, these authors also found that only a small fraction of *Drosophila* Hsp90 binds to microtubules and the binding is not maintained through successive cycles of microtubule depolymerization and repolymerization. Similarly, Lange et al. (2000) reported that the centrosomal association of *Drosophila* Hsp90 can be separated from the centrioles, strongly suggesting that Hsp90 protein binds to non-microtubular components of the centrosome. These two recent studies thus leave the impression that *Drosophila* Hsp90 protein is involved in intracellular morphogenetic processes that depend upon microtubules even though this molecule does not bind directly to microtubules, or at most does so reversibly.

A second approach to the role of Hsp90 in morphogenesis is comparative and organismal. Unlike trans-heterozygotes, simple heterozygotes of *Drosophila Hsp83* loss-of-function mutations (i.e. *m/+)* are viable and fertile, but exhibit diverse morphological abnormalities, each in a low frequency. The abnormalities differ in different strains, and their expression is modifiable as judged by responses to selection experiments. Similar abnormalities were engendered in the same strains by exposure to geldanamycin, consistent with an Hsp90-mediated effect (Rutherford and Lindquist 1998). These observations led to a novel and intriguing hypothesis: that Hsp90 acts as a ''capacitor'' that helps to maintain the numerous components of the morphological phenotype in their normal ''wild-type'' state. The way this would work is that Hsp90 maintains regulatory molecules involved in biochemical pathways relevant to morphogenesis in metastable states that permit activation under appropriate circumstances (Nathan, Vos, and Lindquist 1997; Pratt 1998). Different inbred fly strains are likely to vary in the efficiency of different Hsp90-dependent pathways, each strain being suboptimal in one or another pathway. Involved in numerous such pathways, Hsp90 maintains even the suboptimal ones above a threshold of phenotypic adequacy. But if the con-

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[1] Different molecules of the Hsp90 family have different molecular weights, ranging from 82–94 kDa, but nonetheless are referred to collectively as ''Hsp90.'' In this paper, we refer to Hsp90 (first letter capitalized) as the entire class irrespective of molecular weight, and, for example, *T. thermophila* hsp82p (all small letters) as a particular protein in the Hsp90 class. Hence one and the same molecule may be referred to either as ''Hsp90'' to emphasize its class membership or (for example) ''hsp82p'' to emphasize its specific identity and molecular weight. This usage differs from the nomenclature rules for ciliates specified by Allen et al. (1998) only in the use of all-small letters to designate protein products of specific genes. The genes encoding these proteins are referred to by the molecular weights of their products, e.g. *Hsp83* of *Drosophila melanogaster.*

centration of Hsp90 is cut in half, the ''weak'' Hsp90-dependent pathways (different ones in different strains) sometimes will fall below that threshold and thereby reveal strain-specific morphological abnormalities, which can then be subjected to artificial or natural selection.

A key support for Rutherford and Lindquist's argument is their observation that the penetrance of the abnormalities observed in the *Drosophila Hsp83* heterozygotes is enhanced at high temperature. Conforming to its original characterization as a heat shock protein, synthesis and accumulation of Hsp90 family members are induced at high temperatures (review: Lindquist 1986). Though Hsp90 does not appear to prevent the thermal inactivation of proteins (Nathan, Vos, and Lindquist 1997), it instead facilitates the recovery of proteins that are heat-damaged (Freeman and Morimoto 1996; Nathan, Vos, and Lindquist 1997; Schneider et al. 1996) by mechanism(s) that are as yet unclear. Most pertinent, the principal Hsp90 family member in the yeast *Saccharomyces cerevisiae,* hsp82p is required at a higher concentration at high temperatures than at normal growth temperatures (Borkovich et al. 1989). Most temperature-sensitive point mutations encode a functionally defective hsp82p molecule whose impaired activity is sufficient to maintain viability of the yeast cells at permissive but not at restrictive temperatures (Nathan and Lindquist 1995). This implies that despite the accumulation of Hsp90 molecules at high temperature, the margin of safety for the function of these molecules is reduced under these conditions, and this in turn accounts for the enhanced penetrance of morphological abnormalities in *Drosophila Hsp83* mutant heterozygotes at high temperatures.

In *Tetrahymena thermophila,* synthesis of a heat shock protein with a molecular weight close to 82 kDa was strongly induced at supraoptimal temperatures (39 °C: Fung et al. 1995; 40 °C: Hallberg, Kraus, and Hallberg 1985; 41 °C: Williams and Nelsen 1997). We showed by immunoblots using a known anti-Hsp90 antibody that this protein, hsp82p, is a member of the Hsp90 family (Williams and Nelsen 1997). Abundant hsp82p is observed in cortical cytoskeletal preparations extracted at high salt concentrations (which lack microtubules), whereas little is found in immunoblots with proteins from cortical cytoskeletons extracted at low salt (which have abundant microtubules). At 41 °C, an association of hsp82p with tubulin at high temperature was nonetheless demonstrated by recovery of an immunoprecipitated hsp82p-hsp73p-tubulin complex from a sucrose density gradient (Williams and Nelsen 1997). At the cellular level, both monoclonal and polyclonal antibodies against hsp82p gave general cytoplasmic staining, but monoclonal antibodies raised against a 12-amino acid synthetic peptide made from a portion of the deduced hsp82p amino acid sequence also stained ciliary basal bodies more brightly than the cytoplasmic background (Williams and Nelsen 1997). These results suggest that the association of hsp82p with cortical microtubules is likely to be indirect at normal growth temperatures and becomes direct at sublethal high temperatures, when this protein becomes more abundant and enters complexes with soluble tubulin and with the induced Hsp70 proteins. This latter conclusion is consistent with the suggestion by Freeman and Morimoto (1996) that Hsp90 may cooperate with Hsp70 chaperones in protecting a wide variety of proteins in heat-stressed cells.

The observations summarized above, taken together, are consistent with the possibility that the *Tetrahymena* Hsp90 family member (hsp82p) might be involved in cortical patterning of microtubule-associated structures in this organism, analogous to its involvement in mitosis and spermiogenesis in other systems. If this is the case, the requirement will be most stringent at high temperatures, when the demands on this molecule for relatively nonspecific protection of cellular proteins are highest. Therefore, if abnormalities in cortical development at high temperatures differ greatly in different strains or species, this difference could be due to a molecular imperfection in the sensitive species, either in the hsp82p molecule itself or in a protein in a regulatory pathway with which it interacts.

In the preceding paper, we showed a major difference in the response to high temperature of two *Tetrahymena* species: *T. pyriformis* has a lower maximum growth temperature and exhibits dramatic abnormalities in cortical development when exposed to its maximum temperature, whereas *T. thermophila* has a much higher maximum growth temperature and expresses only minor abnormalities even at that high temperature (Frankel and Nelsen 2001). The cause might be that *T. pyriformis* has a debilitated Hsp90, which cannot function well at high temperatures, like the products of the yeast temperature-sensitive mutants described by Nathan and Lindquist (1995), or it might be that *T. pyriformis* has a suboptimal member of a Hsp-90 dependent regulatory pathway crucial for normal cortical development, as would be expected from the ''capacitor model'' (Rutherford and Lindquist 1998).

Either hypothesis predicts that exposure to geldanamycin, a specific inhibitor of Hsp90 (Grenert et al. 1997; Prodromou et al. 1997b; Stebbins et al. 1997), will mimic the differential effects of supraoptimal temperatures on the two *Tetrahymena* species, with a more drastic effect on *T. pyriformis* and a minor effect on *T. thermophila.* If this prediction is confirmed, then it becomes possible to begin to address the further question of what the defective interaction partner is by comparing the Hsp90 molecules of the two species. In this paper, we show, first, that the main prediction is indeed confirmed; second, that hsp82p is induced at high temperatures in *T. pyriformis* as it is in *T. thermophila,* and third, that a comparison of the predicted amino acid sequences of these proteins in the two species suggests that an unknown interaction partner(s) or component(s) of a signal-transduction chain associated with that interaction partner, and not hsp82p, is defective in *T. pyriformis.*

MATERIALS AND METHODS

Cells and culture conditions. The *T. pyriformis* and *T. thermophila* cells used in growth experiments, and for isolation of mRNA for RT-PCR cloning in *T. pyriformis,* were the same as those reported in the preceding publication (Frankel and Nelsen 2001). For *T. thermophila,* two closely related biological sources were used to ascertain the *HSP82* mRNA sequence: strain CU-428.1 (Table 1 of Cassidy-Hanley et al. 1997) of *T. thermophila,* in turn derived from inbred strain B1868 (18th generation of inbreeding of strain B, established in 1968) by MNNG mutagenesis followed by five generations of backcrossing to strain B1868 (Byrne, Brussard, and Bruns 1978) was used to obtain the 5′ end (bases 1 to 234, GenBank accession No. AF151113), whereas Strain B2086 (20th generation of inbreeding of strain B, established in 1986) was used to obtain bases 97 to 2339, extending to the 3′ end of the molecule (GenBank accession no. AF151114).

Tetrahymena stock cultures for experiments on population growth and development were maintained as described previously (Frankel and Nelsen 2001). In preparation for electrophoresis and immunoblotting, cells were maintained in 10-ml tube cultures in 1% proteose peptone plus 0.1% yeast extract (Difco, Becton-Dickinson, Franklin Lakes, NJ) and were transferred twice a week. Experiments on growth and development of *Tetrahymena* were all carried out in flask cultures of SPP-1% maintained in a reciprocal shaking bath as described previously (Frankel and Nelsen 2001). Growth preliminary to electropho-

resis and immunoblotting, and RNA isolation, was in an iron-chelate-enriched proteose-peptone-yeast extract-glucose (PPYGFe) medium described by Nelsen and Frankel (1981).

Geldanamycin, kindly provided by Dr. Jill Johnson of the Drug Synthesis and Chemistry Branch of the National Cancer Institute, was used from a 2 μg/μl stock solution dissolved in dimethyl sulfoxide (DMSO), which was stored at −20 °C.

Cytology. Monoclonal antibodies and immunostaining procedures were the same as in the preceding investigation (Frankel and Nelsen 2001). The Chatton-Lwoff silver impregation was done as described by Cole and Stuart (1999).

Electrophoresis and immunoblotting. In all experiments involving SDS-PAGE, the cells were washed and starved for 1 h in inorganic medium (4 mM K_2HPO, 1 mM KH_2PO, 39 mM NaCl, 1 mM $MgSO_4 \cdot 7H_2O$) prior to treatment. In the ^{35}S-methionine incorporation studies, 2×10^6 cells contained in 20 ml of inorganic medium were labeled by adding 50 μCi l-[^{35}S]methionine (1000 Ci per mM, Amersham, Pharmacia Biotech, Piscataway, NJ) and incubated for 1 h. Samples of *T. thermophila* were incubated at 30 °C and 41 °C, while samples of *T. pyriformis* were incubated at 28 °C and 33 or 34 °C. The cells from each sample were then harvested and solubilized in 400 μl Laemmli sample buffer. The proteins were separated in 6% gels according to standard procedure (Laemmli 1970). Each lane was loaded with 40 μl of sample buffer containing 30–35 μg protein. Following electrophoresis, the radiolabeled gels were dried, incubated from 6–12 h at −70 °C adjacent to Kodak XAR-5 film with an intensifying screen, then developed.

Immunoblots of *T. pyriformis* total cell protein were prepared by transferring unlabeled proteins separated in 6% polyacrylamide gels as described above to nitrocellulose filters (Towbin, Staehelin, and Gordon 1979) and staining with monoclonal antibodies directed against Hsp90 in *Achyla* (Riehl et al. 1985), kindly provided by David O. Toft of the Mayo Clinic. Monoclonal antibodies (Mab) AC88 and AC16 were negative for *Tetrahymena*, but AC10 was positive. Antibodies were reacted with the filters for 2 h at a concentration of 5 μg IgG per ml, washed, then treated with ^{125}I-conjugated sheep anti-mouse IgG $F(ab')_2$ fragment (8 μCi per μg, Amersham Pharmacia Biotech) at a final concentration of 0.166 μCi per ml for 1 h. The filters were then washed, dried, and incubated with Kodak XAR-5 film at −70 °C with an intensifying screen, and developed.

Cloning and sequence analyses. As reported earlier, we obtained a clone containing a 1.4-kb insert of the gene encoding *T. thermophila* Hsp90 (82 kDa) by screening a cDNA expression library made from mRNA isolated from strain B2086 with antibodies (Williams and Nelsen 1997). Upon sequencing by primer walking, we found that this clone codes for 437 amino acids, the stop codon TGA, and 138 bp of the 3′ UTR. It shows 65.5% amino-acid sequence identity with the comparable region in the gene encoding human Hsp90.

Using this clone as a probe, we obtained other clones from our library which, when sequenced, gave us all except the first 7 amino acids and the 5′ UTR of the gene encoding *T. thermophila* Hsp90. We then turned to PCR using a *T. thermophila* cDNA library made from strain CU-428, which is rich in full-length inserts (kindly provided by A. Turkewitz and D. Chilcoat, University of Chicago), using the vector primer T3 and the *T. thermophila HSP82* internal antisense primer 5′-TCGGTGATGGAAATGTATCT-3′ (amino acids # 48 to 54). The PCR products were purified and then cloned using the Invitrogen TOPO™ TA Cloning® kit (Invitrogen, Carlsbad, CA). Clones bearing inserts of the expected size were selected for sequencing (DNA Facility, University of Iowa Medical Center).

We used RT-PCR to obtain sequence information from the gene encoding *T. pyriformis* Hsp90. The mRNA was prepared from *T. pyriformis* GL using the Invitrogen Micro Fast Track™ 2.0 kit. The cells were grown at 28 °C to a density of 1.5×10^5 cells per ml, and given a 45-min heat shock (34 °C). The mRNA was isolated from 2.3×10^6 cells per sample (yield ~ 1 μg mRNA), and stored at −70 °C until use.

First strand cDNA was synthesized using oligo-dT primer and AMV reverse transcriptase (Invitrogen Cycle® kit). The application of PCR followed immediately using nine pairwise combinations of six primers, three in each direction. All gave PCR products of the predicted lengths. The primers were degenerate, designed using the sequences of conservative regions of Hsp90-encoding genes from other species, including *T. thermophila*, and the codon usage table for *T. thermophila* published by Wuitschick and Karrer (1999). The sense primers were 5′-ATGTCHYAAYAAGCYGAACA-3′, 5′-AARGAAATYTTYYTNAGAGAA-3′, and 5′-ATGATYGGTYAATTYGGTGT-3′; the antisense primers were 5′-TCARTCRACRTCTTCCAT-3′, 5′-CATRATTCTTTCCATRTTRGC-3′, and 5′-TCCATRATRAARACTCTTCT-3′. The PCR products were cloned as described above, and several of them were sequenced sufficiently to give and confirm the sequence of most of the coding region of the gene encoding *T. pyriformis* Hsp90.

We used 3′ RACE to confirm the sequence associated with the 3′ end of the coding region of the gene encoding *T. pyriformis* Hsp90, and to obtain 3′ UTR sequence information. This was done according to the procedure of Frohman (1990) using the standard dT17 and dT17-adapter primers, along with the internal primer 5′-GAAGGCTCAAGCTCTCAGAG-3′. 5′ RACE was less successful, but did confirm the sequence from amino acid 4 to 130. cDNA was prepared according to Frohman (1990) from mRNA using the antisense internal primer 5′-TGATGTTCGTCATCGT-3′. The cDNA was polyadenylated and subjected to two rounds of PCR. The first round made use of the dT17 and dT17-adapter primers together with the antisense internal primer 5′-ACACCGAATTGACCGATCAT-3′. The second round of PCR made use of the dT17-adapter primer and the internal antisense primer 5′-ATATCAGCACCACTGGAGAG-3′.

Paramecium tetraurelia (strain 51s) DNA was a gift from J. R. Preer, Indiana University. HSP90 DNA was amplified using primers: 5′-ACGTTCTACWSNAAYAARGA-3′ and 5′-CGCCTTCATDATNCKYTCCATRTTNGC-3′. The final product was 1651 bp, and had no introns. Two clones were sequenced on both strands by primer walking, and found to be slightly different at the DNA and amino acid levels.

Phylogenetic analysis. Amino acid sequences of all known eukaryotic Hsp90 genes were aligned with PIMA using maximal linkage, and the alignment was edited manually. Phylogenetic trees were inferred from 615 clearly homologous positions using distances and quartet puzzling. Distances were calculated with PUZZLE 4.0.1 (Strimmer and von Haesler 1996) using the JTT correction matrix, amino acid usage estimated from the data, and site-to-site rate variation modeled on a gamma distribution with 8 rate categories plus invariant sites, and the shape parameter estimated from the data. Trees were constructed using neighbor joining with BioNJ (Gascuel 1997) and Fitch-Margoliash using FITCH (Felsenstein 1993). One hundred bootstrap replicate trees were constructed in the same way using PUZZLEBOOT (shell script by M. Holder and A. Roger), except that the gamma shape parameter from the initial analysis was enforced on all bootstrap replicates. Quartet puzzling trees were constructed using the same parameters and 5000 puzzling steps.

RESULTS

Population growth in geldanamycin. *Tetrahymena pyriformis.* The effects of geldanamycin on population growth in *T.*

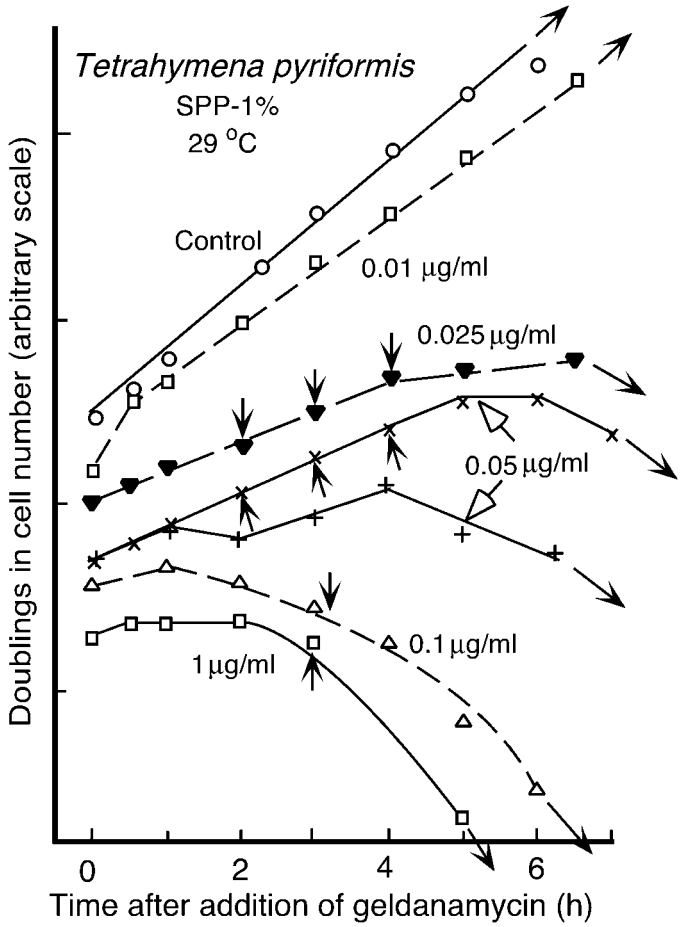

Fig. **1**. Effects of geldanamycin on population growth in *Tetrahymena pyriformis* maintained at 29 °C in SPP-1% medium. The abscissa represents time (h) after addition of geldanamycin in DMSO or of DMSO alone. The successively higher scale markings on the ordinate indicate successive doublings in cell number. Each curve is placed at an arbitrary position relative to the ordinate. The cell counts at 0 h (time of drug addition) were 43,000 cells/ml for **0.01 μg/ml** geldanamycin (in 0.0005% DMSO) (□); 38,000 cells/ml for **0.025 μg/ml** geldanamycin (in 0.00125% DMSO) (▼); 26,000 cells/ml (×) and 34,000 cells/ml (+) for **0.05 μg/ml** geldanamycin (both in 0.0025% DMSO); 15,000 cells/ml for **0.1 μg/ml** geldanamycin (in 0.005% DMSO) (△); 24,000 cells/ml for **1 μg/ml** geldanamycin (in 0.05% DMSO) (□), and 95,000 cells/ml for the **control** (0.025% DMSO) (○) (in a separate experiment, 0.05% DMSO was found to have no effect on population growth). Vertical arrows indicate times of sampling for immunostaining. Diagonal arrows to the right of the plots indicate whether the populations had grown to form dense cultures by about 24 h after addition of geldanamycin or of DMSO alone (↗) or whether the cells were few and unhealthy at that time (↘).

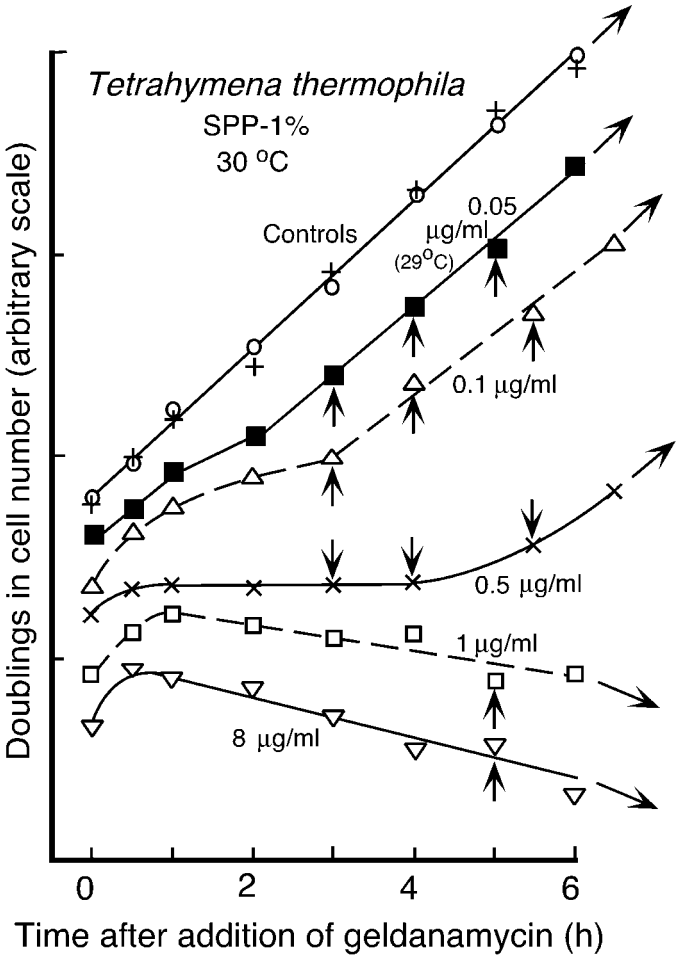

Fig. **2**. Effects of geldanamycin on population growth in *Tetrahymena thermophila* maintained at 30 °C in SPP-1% medium. The abscissa and ordinate are the same as in Fig. 1. The cell counts at 0 h (time of drug addition) were 83,000 cells/ml for **0.05 μg/ml** geldanamycin (in 0.0025% DMSO, at 29 °C) (■); 74,000 cells/ml for **0.1 μg/ml** geldanamycin (in 0.005% DMSO) (△); 76,000 cells/ml for **0.5 μg/ml** geldanamycin (in 0.025% DMSO) (×); 34,000 cells/ml for **1 μg/ml** geldanamycin (in 0.05% DMSO) (□); 39,000 cells/ml for **8 μg/ml** geldanamycin (in 0.4% DMSO)(▽) ; and 34,000 and 48,000 per ml for the **control** in 0.05% DMSO (+) and 0.4% DMSO (○), respectively. Vertical and diagonal arrows have the same meaning as in Fig. 1.

pyriformis were studied only at 29 °C, an optimal temperature for this species (Thormar 1959). At this temperature, the threshold for long-term inhibition of population growth, and eventual death, was 0.025 μg/ml (45 nM). However, at concentrations in the range of 0.025–0.05 μg/ml, growth continued for about 4 h after addition of the drug, with some variation in amount of increase (Fig. 1). At 0.1 μg/ml (180 nM) and 1 μg/ml (1.8 μM), cell number increased for only about 1 h before it began to decline.

As the geldanamycin stock was dissolved in DMSO, the addition of geldanamycin to the culture always involved the addition of DMSO as well. However, control experiments revealed that addition of DMSO alone at equivalent final concentrations had no effect on population growth.

Tetrahymena thermophila. The effects of geldanamycin on population growth in *T. thermophila* were studied at three temperatures: most extensively at 30 °C, a commonly used culture temperature for this species that is somewhat below the temperature optimum for vegetative growth; at 39.5 °C, which is near the upper limit of rapid exponential growth (see Fig. 4 of Frankel and Nelsen 2001); and at 22 °C, which is far below the temperature optimum.

The effect of geldanamycin at 0.05 and 0.1 μg/ml on population growth at 30 °C was weak and transient (Fig. 2). At 0.5 μg/ml, inhibition of cell multiplication was much more long lasting. At 1 μg/ml and above, exponential cell death was observed after an increase in the first hour following the addition of geldanamycin. The rate of decline in cell number was dependent upon cell density, becoming less at higher cell densities (not shown).

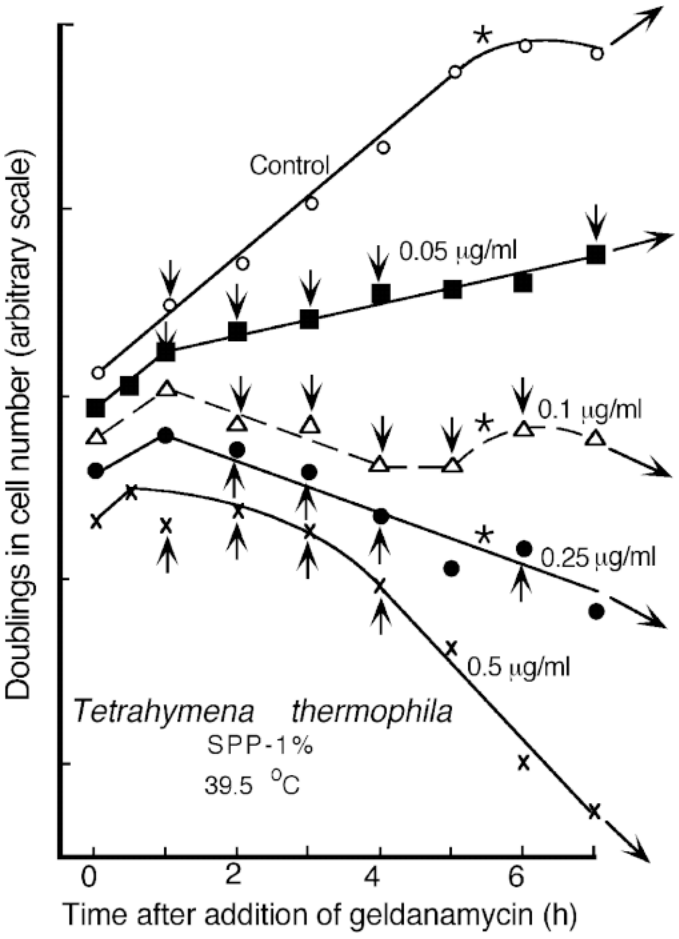

Fig. **3**. Effects of geldanamycin on population growth in *Tetrahymena thermophila* maintained at 39.5 °C in SPP-1% medium. The abscissa and ordinate are the same as in Fig. 1. The cell counts at 0 h (time of drug addition) are 53,000 cells/ml for **0.05 μg/ml** geldanamycin (in 0.0025% DMSO) (■), 29,000 cells/ml for **0.1 μg/ml** geldanamycin (in 0.005% DMSO) (△); 30,000 cells/ml for **0.25 μg/ml** geldanamycin (in 0.0125% DMSO) (●); 52,000 cells/ml for **0.5 μg/ml** geldanamycin (in 0.025% DMSO) (×); 33,000 cells/ml for a **control** with no additions (○) (in a separate experiment, 0.025% DMSO was found to have no effect on population growth). The star indicates a period of about one-half hour with no shaking. Vertical and diagonal arrows have the same meaning as in Fig. 1.

Table **1.** Diagnosis of stage-5 oral primordia of *Tetrahymena pyriformis* maintained in different concentrations of geldanamycin at 29 °C.

Conc. (μg/ml)	Expt.	h after drug add.	% With oral primordia[b]	Membranelles of st. 5 oral primordia[a]			
				Normal	Branch[c]	4+[d]	Abn.[e]
0	1[f]	—	50	99	0	0	1
0.025	2	3	48	97	0	0	3
		4	n.d.[g]	94	0	2	4
		5	n.d.	96	0	1	3
0.05	3	3	36	86	4	1	9
		4	n.d.	59	9	2	30
		5	n.d.	64	11	0	25
0.1	4	3	n.d.	12	35	22	31
	5	3.25	n.d.	12	27	17	44
	6	3.25	27	11	11	6	72
1.0	7	3	22	1	6	6	87[h]

[a] One hundred cells were sampled for each concentration and time point.
[b] Based on classification of 200 cells per sample.
[c] One of the three membranelles distally branched, otherwise normal.
[d] Four or more membranelles, each roughly normal in shape and orientation.
[e] All other abnormalities, including oral primordia with branched and extra membranelles if additional abnormalities are also present.
[f] Same as 29 °C control in Table 1 of Frankel and Nelsen (2001).
[g] n.d. = not determined.
[h] A true stage-5 configuration is not attained under these conditions.

Dimethylsulfoxide alone had no effect on the rate of population growth, even at a concentration as high as 0.4 μg/ml, the final concentration accompanying the highest concentration of geldanamycin used (8 μg/ml).

Population growth in *T. thermophila* was considerably more sensitive to geldanamycin at 39.5 °C than at 30 °C. At 39.5 °C, geldanamycin at 0.05 μg/ml permitted slow but continuous culture growth, whereas at 0.1 μg/ml and above cell numbers declined after an initial increase (Fig. 3). The decline was steep at 0.5 μg/ml.

The effects of geldanamycin at concentrations ranging from 0.05–1 μg/ml on *T. thermophila* maintained at 22 °C were similar to the effects of same concentrations at 30 °C (data not shown). The only difference was that cells did not recover from inhibition in 0.5 μg/ml at 22 °C as they did at 30 °C, indicating either a slightly greater sensitivity to geldanamycin at 22 °C or, perhaps more likely, experimental variation at a near-threshold concentration of the inhibitor.

Oral patterning in geldanamycin. *Tetrahymena pyriformis.* The normal development of the ciliary structures of the oral apparatus was described in the preceding paper (Frankel and Nelsen 2001). At 0.025 μg/ml or less, geldanamycin had only slight effects on the development of the oral ciliature (not shown). However, major effects were apparent starting at 4 h after addition at 0.05 μg/ml, and predominate at 0.1 μg/ml (Table 1). At these latter concentrations, the stage-1 anarchic field is normal (Fig. 4A) (for delineation of stages, see Fig. 6 of Frankel and Nelsen, 2001). Promembranelles form normally by alignment of basal body pairs during stage 3 (Fig. 4B); there are some membranellar dislocations in stage-4 oral primordia (Fig. 4C), but similar irregularities also are observed in untreated control cells (see Fig. 7 of Frankel and Nelsen 2001). However, in geldanamycin the abnormalities typically become more pronounced during stage 4 (Fig. 4D) rather than becoming attenuated as happens in the absence of the drug (Frankel and Nelsen 2001). These result in a great variety of configurations, ranging from slight abnormalities within membranelles (Fig. 4E) through branching of membranelles (Fig. 4F–H), sometimes combined with formation of small supernumerary membranelles (Fig. 4G), which may have varying orientations (Fig. 4H). The undulating membrane often develops normally (Fig. 4E), but sometimes ends up with minor (Fig. 4F) or major (Fig. 4H) gaps.

At 1 μg/ml, geldanamycin brings about more severe abnormalities. Although oral primordia still start out as normal oral fields (not shown), and promembranelles are formed from basal body pairs in the normal manner (Fig. 5A), the incipient membranelles always develop abnormally during stage 4 (Fig. 5B), and become thin and irregular (Fig. 5C, D). The undulating membrane is also abnormal, typically failing to become consolidated into a single (later double) row of basal bodies at a stage (marked by appearance of fission zone breaks in the cil-

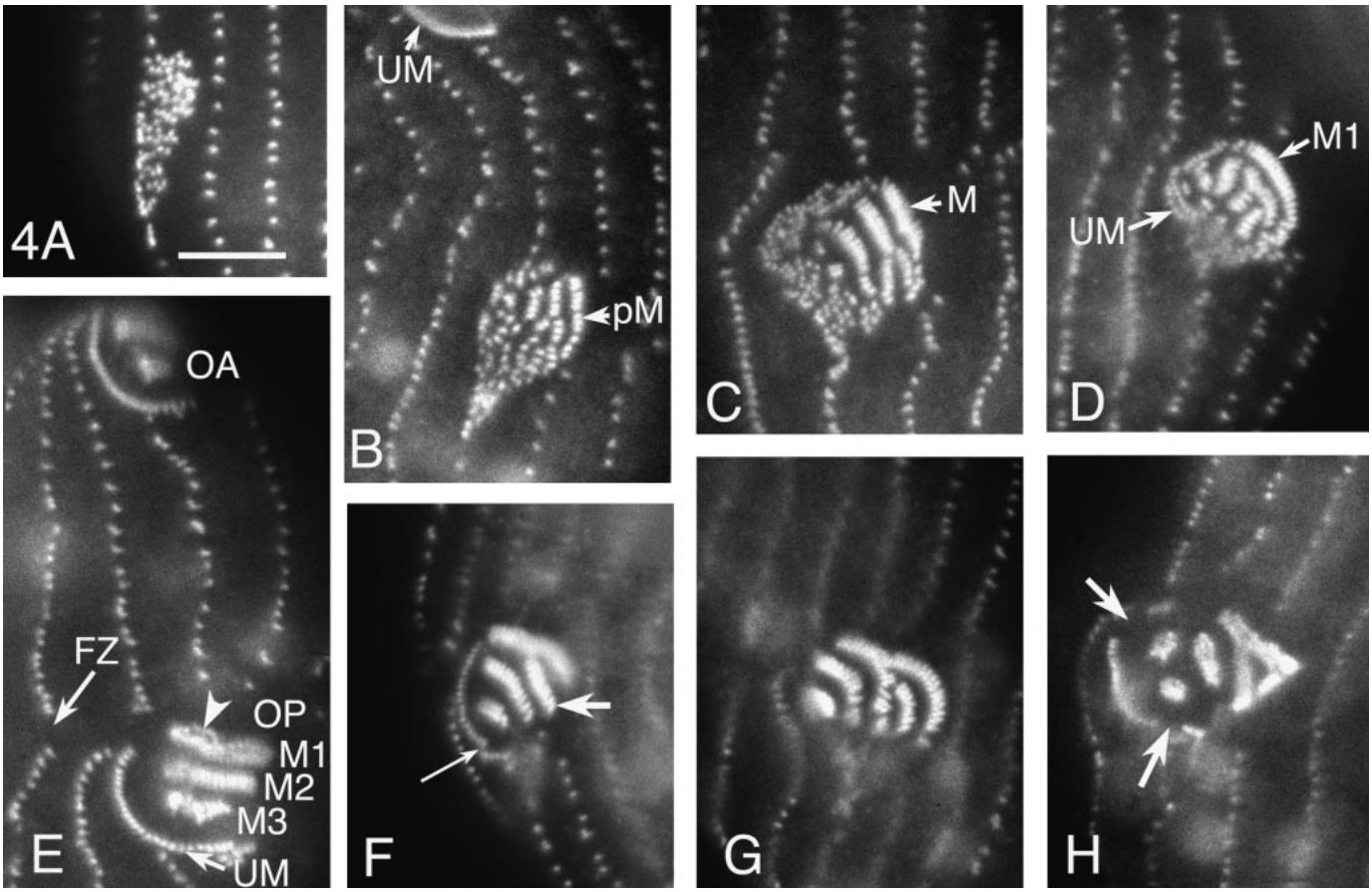

Fig. **4**. Images of ventral surfaces of *Tetrahymena pyriformis* cells exposed to geldanamycin at 0.05 μg/ml for 4 h (Table 1, Expt. 3) (**D, F**) or at 0.1 μg/ml for 3 1/4 h (Table 1, Expt. 6)(**A, B, C, E, G, H**) at 29 °C, and immunostained with 20H5 anti-centrin antibody. All of the images in this and the next two figures are aligned so that the anterior end of the cell is upward, and the cell's left corresponds to the viewer's right. All frames are printed at the same magnification. Bar = 10 μm. For an explanation of the stages, see Fig. 6 of Frankel and Nelsen (2001). **A.** Stage 1b. A normal oral field has formed. **B.** Stage 4a. Three promembranelles (pM) are present, with some short gaps. The undulating membrane (UM) of the anterior oral apparatus is visible near the top of the frame. **C.** Stage 4b. A third row of basal bodies has been added to each of the three membranelles (M) in the anterior portion of the oral primordium. Posteriorly, two promembranelles abut on the second membranelle. **D.** Stage 4b. The first membranelle (M1) is normally oriented, but the others have varying orientations. The undulating membrane (UM) is developing with overlapping files of basal bodies. **E.** Mid-stage 5. The oral primordium (OP) is nearly normal, with three well-developed membranelles (M1, M2, M3) and a normal undulating membrane (UM). However, the arrangement of ciliary rows within M1 is irregular (arrowhead). The fission zone (FZ) is well developed. The anterior oral apparatus (OA) is visible at the top of the frame. **F.** Mid-stage 5. An extra membranelle (thick arrow) is present between M1 and M2. There is a narrow gap (thin arrow) within the UM. **G.** Early stage 5. An oral primordium with severe membranellar dislocations. The undulating membrane is out of focus. **H.** Mid-stage 5. An extremely abnormal oral primordium, with branched and fragmented membranelles as well as large gaps within the UM (arrows).

iary rows) when this happens in less severely affected cells (e.g. Fig. 4E) and in controls. The oral primordia appear to have undergone an arrest and even partial resorption of oral development. There are very few dividers, and no increase in cell number (Fig. 1) at this concentration.

The orientation of the ciliary rows over the cell surface is generally normal in all concentrations of geldanamycin examined, although breaks and gaps were occasionally observed (Fig. 5B). The position and number of contractile vacuole pores are unaltered in *T. pyriformis* cells exposed to geldanamycin at 0.1 μg/ml (data not shown). Thus, while geldanamycin severely impairs development of new oral structures, it does not appear to affect large-scale cortical cytogeometry.

Oral development in DMSO alone, at a concentration of 0.05% (the same as is present with geldanamycin at 1 μg/ml) at 29 °C, was identical to that observed in a parallel control culture lacking DMSO (data not shown).

Oral development at 0.1 and 1 μg/ml of geldanamycin is taking place when cell number is starting to decline (Fig. 1). At 0.1 μg/ml, this development appears to go to completion with successful cell division, and some cells divide even at 1 μg/ml. As there is no increase in cell number at these concentrations, division of some cells must be compensated for by death of others.

Tetrahymena thermophila. The effects of geldanamycin on oral development are much less severe in *T. thermophila* than they are in *T. pyriformis.* At 30 °C, abnormalities in oral development of *T. thermophila* appear only at a concentration of geldanamycin (1 μg/ml) at which cell number is steadily declining (Table 2, Fig. 2). The proportion of cells exhibiting abnormalities at this concentration varies considerably from experiment to experiment (Table 2). Even when fairly common, the abnormalities are mild. Early stages of membranelle development are typically normal, with occasional irregularities near the distal ends of promembranelles (Fig. 6A). The abnormalities that persist are basically of four kinds: (a) dislocations of basal

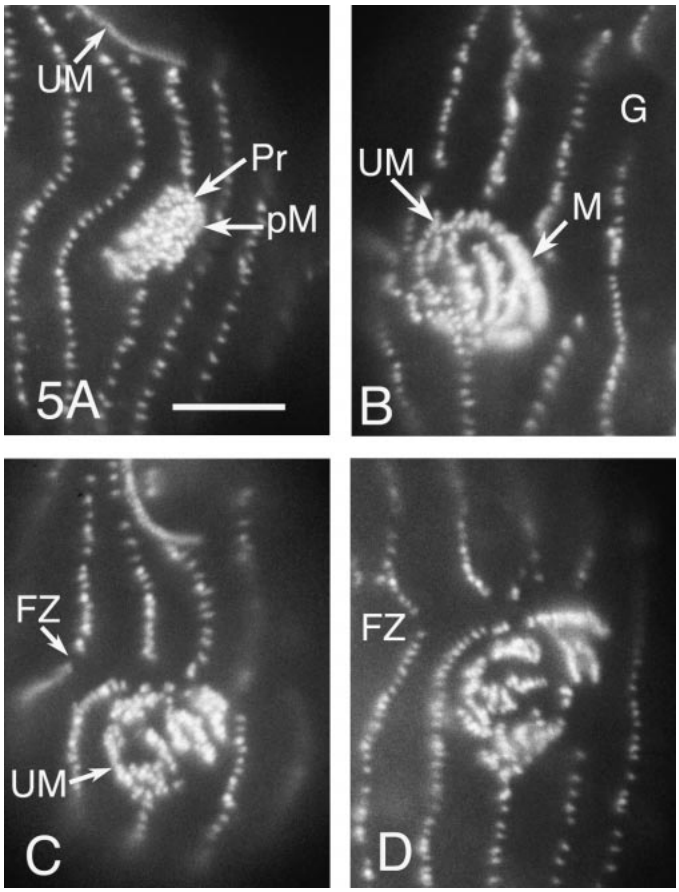

Fig. **5**. Images of ventral surfaces of *Tetrahymena pyriformis* cells exposed to geldanamycin at 1 μg/ml for 3 h at 29 °C (Table 1, Expt. 7) and immunostained with 4F9 anti-basal body antibody. All frames are printed at the same magnification. Bar = 10 μm. **A.** End of stage 2. The oral primordium consists of tightly clustered pairs (Pr) of basal bodies, which are just beginning to align into promembranelles (pM) at the outer (left) edge of the oral primordium. The undulating membrane (UM) of the anterior oral area is seen at the top of this frame and of frame C. **B.** Stage 4b. Two membranelles (M) are well formed though somewhat irregular. The outermost (first) one is branched. The undulating membrane (UM) is not well organized. Some ciliary rows have gaps (G) at unusual latitudes. **C.** Aberrant stage 5. A fission zone (FZ) is present, but the oral primordium is arrested in its development and extremely irregular. Only one portion of the undulating membrane (UM) is properly aligned. **D.** An even more aberrant stage-5 oral primordium. A fission zone (FZ) forms nonetheless.

body rows within oral membranelles, which usually are subtle (Fig. 6B) but sometimes are more pronounced (Fig. 6C), (b) persistence of a branch or small supernumerary membranelle fragment near the distal end of the first membranelle during stage 4 (Fig. 6D), resulting in overt branching (Fig. 6E) or distal irregularities (Fig. 6F) of mature membranelles, (c) occasional persistence of a fourth membranelle (Fig. 6H), and (d) occasional incomplete differentiation of the undulating membrane (Fig. 6G). Examination of cells simultaneously decorated by the anti-centrin antibody and stained by DAPI indicates that both macronuclei and micronuclei divide normally (not shown) in 1 μg/ml of geldanamycin.

At higher concentrations of geldanamycin, up to 8μg/ml (Fig. 2), very little oral development was seen, and no scoring was attempted.

At 39.5 °C, *T. thermophila* cells are far more sensitive to geldanamycin than at 30 °C, yet the same parallel exists between effects of this drug on culture growth and on oral development. At 0.05 μg/ml, at which growth continues, and at 0.1 μg/ml, at which growth for the most part ceases (Fig. 3), oral development is frequent and almost invariably normal (Table 3). Abnormalities in oral development become common only at 0.25 μg/ml, a concentration at which there is a fairly steady decline in cell number (Fig. 3) and a reduced frequency of oral development (Table 3). These abnormalities are evident in only a minority of the oral primordia, and resemble those observed at a 4-fold higher concentration of geldanamycin at 30 °C. At 0.5 μg/ml, virtually no oral development was observed at 39.5 °C (Table 3) in samples fixed before the precipitous decline in cell numbers (Fig. 3).

Maintenance of *T. thermophila* cells at 22 °C allowed for better long-term survival in geldanamycin than at higher temperatures; after 23 h in the drug at 1 μg/ml (a concentration that allowed little if any increase in cell number) about 20% of the oral primordia were abnormal. The range of abnormalities was similar to that observed at the effective concentrations at the other temperatures.

Detection of an Hsp90 protein in *Tetrahymena pyriformis*. It was previously shown that the Hsp90 class member in *T. thermophila* has an apparent molecular weight of 82 kDa (Williams and Nelsen 1997). A protein of slightly lower apparent molecular weight (80 kDa) was induced by growth at high temperature (33 °C) in *T. pyriformis* (Fig. 7); even more of this protein is made at 34 °C (Fig. 8). This protein reacts with a monoclonal antibody specific to Hsp90, AC10 (Riehl et al. 1985) (Fig. 8), identifying it as a bona fide member of the Hsp90 family.

Cloning and sequence analysis of *Tetrahymena* and *Paramecium* Hsp90. *Tetrahymena thermophila.* The cDNA of the genes encoding *T. thermophila* Hsp90 [GenBank accession numbers AF151113 and AF151114] was cloned by a combination of colony screening and PCR of a cDNA library (see Materials and Methods). The base composition within the ORF is 43% GC, contrasting with only 15% GC in the 5′ and 3′ untranslated regions, in agreement with expectations based on other sequences from *T. thermophila* (Wuitschick and Karrer 1999). Codon usage in this coding sequence conforms to expectations for a highly expressed *T. thermophila* protein (Wuitschick and Karrer 1999). Conceptual translation of this coding sequence yields a protein made up of 706 amino acids, with a molecular weight of 81,759 Daltons, agreeing closely with the apparent molecular weight of the actual protein (Fig. 7). Hence we will informally call the gene *TtHSP82* and the corresponding protein *Tt*hsp82p (the technically correct designations are *HSP82* and Hsp82p respectively, but the *Tt* prefix distinguishes them clearly from the *T. pyriformis* genes and proteins to be encountered below while the all-small-letter protein designation distinguishes the specific gene product from the Hsp90 class to which it belongs). A Southern blot (not shown) supports the inference that *T. thermophila* has a single gene encoding a member of the Hsp90 family. As we have not ascertained the genomic sequence, we do not know whether the gene contains introns.

Sequence comparisons indicate that *TtHSP82* is an ''ordinary'' member of the gene family encoding Hsp90 proteins in that it lacks exceptional characteristics. Its conceptually translated product shares 60% to 70% identity with other amino acid sequences of the Hsp90 family, for example 68% with the hsp90p of the *Arabidopsis thaliana,* 61% with the hsp82p of *Saccharomyces cerevisiae,* and 64% with human hsp90p-α. Regions of the molecule known to be highly conserved in other organisms are also conserved in *T. thermophila.* For example,

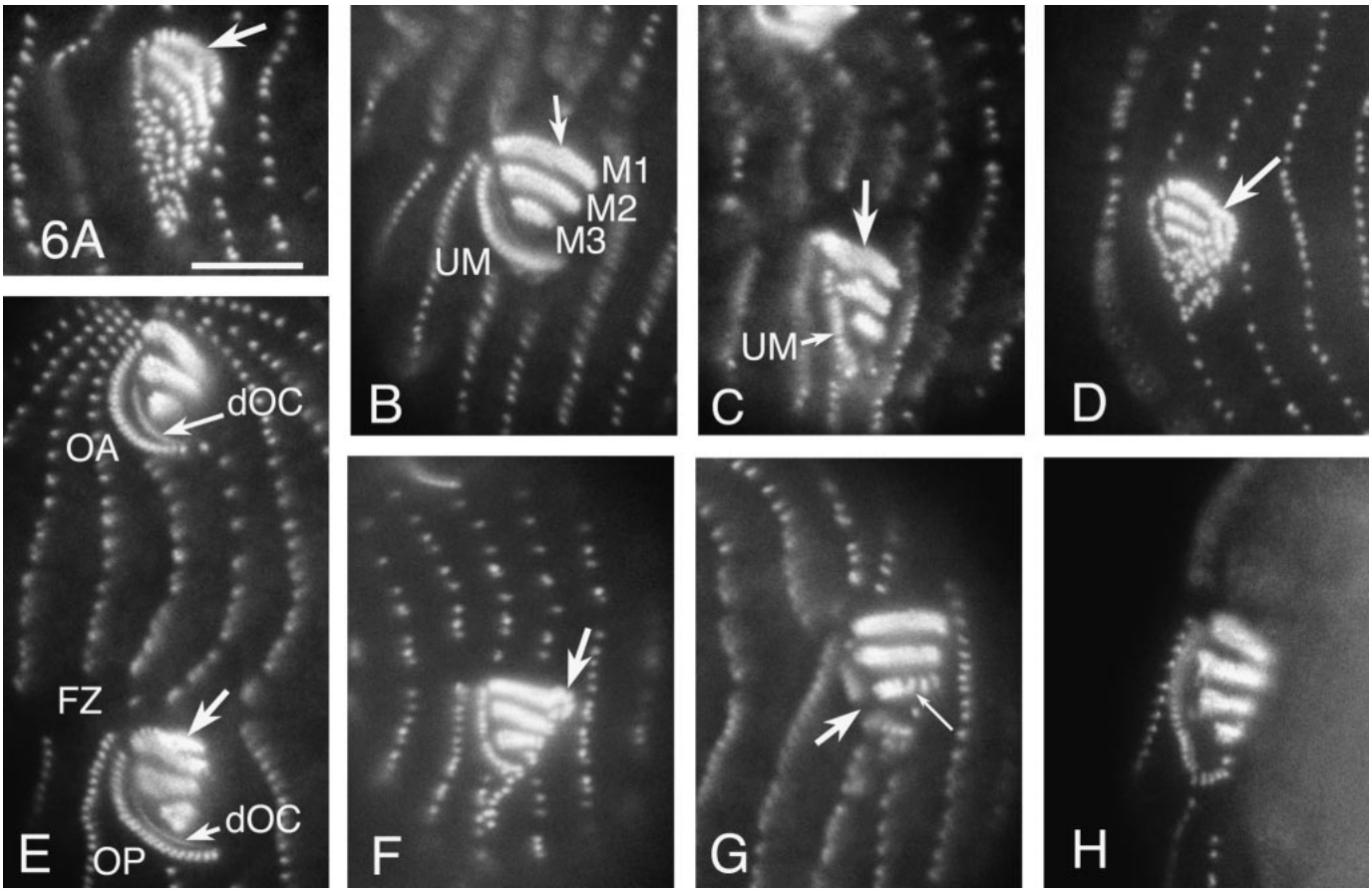

Fig. **6**. Images of ventral surfaces of *Tetrahymena thermophila* cells exposed to geldanamycin at 1 μg/ml for 5 h at 30 °C [Table 2, Expt. 5 (**D, F**); Table 2, Expt. 6 (**A, B, C, E, G**)] or for 23 h at 22 °C (**H**) and immunostained with 4F9 anti-basal body antibody (**D, F)** or with 20H5 anti-centrin antibody (**A, B, C, E, G, H).** All frames are printed at the same magnification. Bar = 10 μm. **A.** Stage 4a. The oral primordium is normal except for a small promembranelle fragment wedged between the first and second promembranelle (arrow). **B.** Mid-stage 5. Three membranelles (M1, M2, M3) and an undulating membrane (UM) are all normal except for a minimal dislocation of ciliary rows within M1 (arrow). **C.** Early stage 5. A more obvious example of membranellar dislocation (arrow) with an under-developed undulating membrane (UM). **D.** Stage 4b. An oral primordium that is normal except for a distal branch in the first membranelle (arrow), **E.** Mid-stage 5. The distal branch in the first membranelle is maintained as membranelle development is completed. Developing oral crescents (dOC) are visible in the OP and in the anterior oral area (OA). The fission zone (FZ) is well developed. **F.** Mid-stage 5. The oral primordium is normal except for some irregularity (arrow) at the distal end of the first membranelle. **G.** Mid-stage 5. A wide gap (thick arrow) interrupts an under-developed UM; also, columns of basal bodies are unusually spread out within the third membranelle (thin arrow). **H.** Late stage 5. An oral primordium with four membranelles.

69 of the 72 otherwise invariant residues in the highly conserved N-terminal ATP-binding portion of the Hsp90 molecule, crystallized from human (Stebbins et al. 1997) and yeast (Prodromou et al. 1997b) sources, are conserved in the inferred *T. thermophila* homologue. More critically, the two specific amino acids known to be indispensable for ATP binding (Obermann et al. 1998; Panerotou et al. 1998) are conserved in *Tt*hsp82p, as are all but one of the 11 additional amino acids known to undergo temperature-sensitive mutation in yeast (Kimura, Matsumoto, and Yahara 1994; Nathan and Lindquist, 1995) as well as six of the seven amino acids known to mutate to Hsp90-based enhancers of *sevenless* (Cutforth and Rubin 1994) and suppressors of Raf signaling (van der Straten et al. 1997) in *Drosophila melanogaster*.

The carboxy-terminus of the putative *Tt*hsp82p, MEDVD, differs only in one conservative substitution from the otherwise universal Hsp90 MEEVD carboxy-terminus, a region known to be vital for binding to proteins associated with Hsp90 (Ramsey et al. 2000). In contrast, the sequence of the extremely variable amino-terminal end of the molecule is unique in *T. thermophila* up to the highly conserved glutamic acid residue found at position 6 in the *Tt*hsp82p sequence.

Tetrahymena pyriformis. By use of RT-PCR with degenerate primers, supplemented with 3′- and 5′-RACE (see Materials and Methods), we obtained the entire coding sequence of the gene encoding the Hsp90 molecule of *T. pyriformis*, except for 10 bases at the 5′ end (GenBank accession # AF287229). The nucleotide sequence of this gene (*TpHSP81)* has a 92% identity with the corresponding sequence of *TtHSP82*. Conceptual translation of the *TpHSP81* gene yields an amino acid sequence of 703 amino acids (assuming that the four uncertain amino acids at the amino-terminal end are the same as in the corresponding molecule of *T. thermophila*) with a molecular weight of 81,343 Daltons. This is slightly higher than anticipated from migration of the protein in acrylamide gels (see Fig.7). We call this deduced acid sequence *Tp*hsp81p for ease of comparison with the *Tt*hsp82p of *T. thermophila.*

Comparison of T. thermophila and T. pyriformis Hsp90 sequences. When the amino acid sequences of the deduced hsp82/81 proteins of the two *Tetrahymena* species are com-

Table **2.** Diagnosis of stage-5 oral primordia of *Tetrahymena thermophila* maintained in different concentrations of geldanamycin at 29 or 30 °C.

Conc. (μg/ml)	Expt.	h after drug add.	Membranelles of st. 5 oral primordia[a]			
			Normal	Branch	4+	Abn.
0	1[b]	—	99	0	0	1
0.05	2	3	(98)[c]	0	0	(2)
		5	100	0	0	0
0.1	3	3	97	0	0	3
		4	97	0	0	3
0.5	3	4	97	0	0	3
		5.5	98	1	0	1
	4	5	(98)[c]	0	0	(2)
1.0	5	5	(52)[d]	(25)	(3)	(19)
	6	5	79	12	0	9
	7	3.5	97	0	0	3
		5	88	1	5	6

[a] One hundred cells were sampled for each concentration and time point, except as indicated. Designations under this heading have the same meaning as in Table 1.
[b] Same as 30.0 °C experiment in Table 2 of Frankel and Nelsen (2001).
[c] Based on classification of 50 cells, doubled for comparability.
[d] Based on classification of 63 cells, adjusted to percentages for comparability.

pared, amino acids at three positions in *Tt*hsp82p (D235, K236, and N697), are absent from *Tp*hsp81p; the remainder of the amino acid sequence is 95% identical in the two species, with 24 conservative and 10 nonconservative amino acid differences, most of which occur at positions that are generally variable (Fig. 9). All of the highly conserved sites of the ATP binding region of the molecule as well as the sites defined by known mutations in *Saccharomyces* and *Drosophila* are occupied by the same amino acids in *Tp*hsp81 as in *Tt*hsp82p, with two exceptions. These are the highly conserved T and V residues at positions 162 and 163 in *Tt*hsp82p, which are replaced by A and I in *Tp*hsp81p. These residues are located in a β-sheet [S6 in the human hsp90p (Stebbins et al. 1997), f in *S. cerevisiae* hsp82p (Prodromou et al. 1997b)] at the edge of the ''molecular clamp'' (Prodromou et al. 1997b) that makes up the ATP- and

Table **3.** Diagnosis of stage-5 oral primordia of *Tetrahymena thermophila* maintained in different concentrations of geldanamycin at 39.5 °C.

Conc. (μg/ml)	Expt.	h after drug add.	Percent with oral primordia[b]	Membranelles of st. 5 oral primordia[a]			
				Normal	Branch	4+	Abn.
0	1[c]	—	52	98	0	0	2
0.05	2	4	45	99	0	0	1
		7	n.d.	100	0	0	0
0.1	3	4	48	97	1	1	1
		5	n.d.	99	0	0	1
		6	n.d.	98	0	0	2
0.25	3	4	21	10	0	0	0
		6	68	90	1	5	4
0.5	2	4[d]	3[e]	—	—	—	—

[a] Actual counts are given, totaling 100 except in the 4-h sample at 0.25 μg/ml. Designations under this heading have the same meaning as in Table 1.
[b] Based on classification of 200 cells per sample.
[c] Same as 39.5 °C experiment in Table 2 of Frankel and Nelsen (2001).
[d] Six-h sample not taken due to low cell density at that time.
[e] All but one of these are stage-1 (anarchic field) oral primordia.

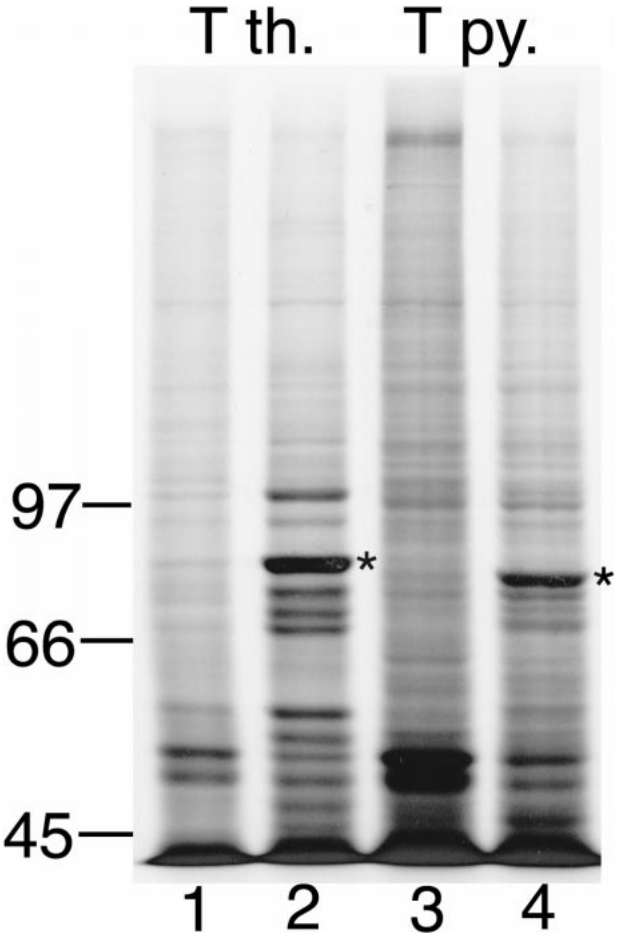

Fig. **7**. Heat-induced synthesis of Hsp90 in *Tetrahymena thermophila* (lane 2, asterisk) and *T. pyriformis* (lane 4, asterisk) shown by ^{35}S-methionine incorporation into cell protein during a one-hour interval at the respective heat shock temperatures of the two species (41 °C for *T. thermophila*, and 33 °C for *T. pyriformis*). The level of incorporation at near-optimal temperatures over the same interval is shown for *T. thermophila* in lane 1 (30 °C) and for *T. pyriformis* in lane 3 (28 °C). The Hsp90 class member has an apparent molecular weight in polyacrylamide gels of 82 kDa for *T. thermophila* and 80 kDa for *T. pyriformis.* The molecular weight markers are phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and the gel is 6% in acrylamide.

geldanamycin-binding region of this molecule (Prodromou et al. 1997a). The V → I replacement is conservative, whereas the T → A is not, although this same position is replaced by an F in both *Paramecium tetraurelia* Hsp90 sequences (see below), making the biological importance of conservation of a threonine residue at position 162 questionable. The comparison of amino acid sequences thus provides no convincing positive evidence in support of the hypothesis that there is a functional difference between the Hsp90 family molecules in the two *Tetrahymena* species.

Paramecium tetraurelia. Conceptual translation of the two Hsp90 sequences cloned from *Paramecium tetraurelia* yields amino acid sequences corresponding to the residues between # 31 and # 585 of *Tt*hsp82. The two *Paramecium* Hsp90 amino acid sequences (GenBank accession # AF287230 and AF287231) are highly similar to each other, with only six differences. Each of these *Paramecium* sequences shows 80% identity to the amino acid sequence of the corresponding re-

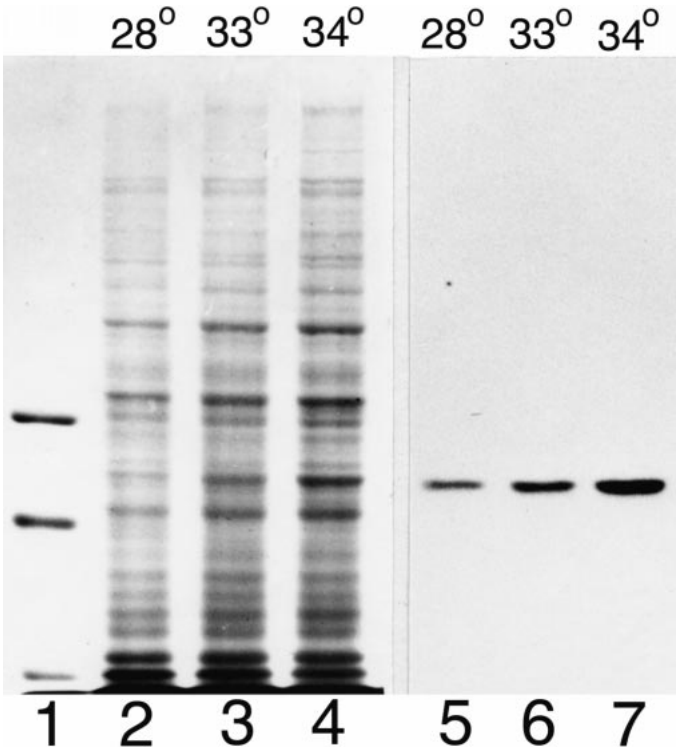

Fig. **8**. An immunoblot showing recognition of the heat-induced 80 kDa protein in *Tetrahymena pyriformis* by monoclonal antibody AC10 directed against Hsp90 in *Achyla* (Riehl et al. 1985). Total cell protein from cells grown at the optimum temperature (28 °C) is resolved by SDS-PAGE in lane 2 and blotted with mAb AC10 in lane 5. An increased amount of the *T. pyriformis* Hsp90 family member was synthesized during a one-hour interval at 33 °C (lanes 3, 6), and a still greater amount was detected after 1 h at 34 °C (lanes 4, 7). The gel was 6% in acrylamide, and the molecular weight markers (lane 1) are those listed in Fig. 7.

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                    1111222222222222333444455556666 6
                    5677746662222333446880560046334478999
                    7235692353689056690132431250285699047
T.thermophila       KVDANSTVNEEDEEDKDEVETQETLISSTKAYSSQKN
T.pyriformis        RTNNDHATTVDEDD--DDEDSLDVMLIASSSTTTKN-
Paramecium 1        SVDNN-FVDVDDENAE-DEETLEVMIIGTKST.....
Paramecium 2        SIDNN-FVDVDDENAE-DEETLEVMIIGTKAT.....
Eimeria             PTDAN-TVKEDEEKEGDKEETLDVMFSDSKEIDEEAD
Plasmodium          TADTN-TVKEEEEKEDEKTETLEVMFTDSDEYEEEAD
Homo                PSNQE-TV-ELKGEE-DKEDTLEIFFTSDKKTDDELD
Arabidopsis         KGDTN-TV-EE--K--DEEENLDVIFSTTKKTD-E-A
Trypanosoma         QDDVN-TVSEEAAK--EDEDTLDLIFSTHQSYD-ANT
Saccharomyces       PTKEE-TVLEEDD-D-KEEEDTLDVAFSTIEEYDEAEP
                    cncncnncnnccccc--ccnccncccccccncccccn-
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Fig. **9**. Individual amino acid differences between *Tetrahymena thermophila* and *Tetrahymena pyriformis* Hsp90s aligned to homologous positions of other eukaryotes. Positions are numbered in vertical columns at the top according to the *T. thermophila* amino acid numbering sequence (e.g. ''57'' in the left-most column designates the 57th amino acid from the amino terminal end of the deduced *Tt*hsp82p). Shading indicates conserved amino acids, with sites with over 80% identity in black and those with over 80% similarity in gray. Below, the conservative (c) versus non-conservative (n) substitutions between the *Tetrahymena* species are indicated.

gions of *Tt*hsp82 and *Tp*hsp81. The invariant and critical residues noted above are similarly conserved in the *Paramecium* and *Tetrahymena* sequences. Apart from two T → S substitutions at highly conserved or functionally important positions (one in *Paramecium* and one in *Tetrahymena*), the only noteworthy difference is at position 162 in the *Tt*hsp82 sequence, where, as noted above, a generally conserved threonine is replaced by alanine in *Tp*hsp81 and by phenylalanine in both *P. tetraurelia* sequences.

An alignment of known Hsp90 amino acid sequences with those of *T. thermophila*, *T. pyriformis*, and *P. tetraurelia* can be obtained upon request from P. Keeling (pkeeling@interchange.ubc.ca).

Phylogenetic analysis of Hsp90. The *Paramecium* Hsp90 sequences can be used to help deduce the evolution of the two *Tetrahymena* sequences by providing a closely related, but nevertheless external, reference point. Of the 32 positions that differ between *Tt*hsp82 and *Tp*hsp81 in the region in which *P. tetraurelia* sequences are available (Fig. 9), the number of positions at which *P. tetraurelia* Hsp90–1p is identical to *Tt*hsp82p (12) is nearly the same as the number of positions at which *P. tetraurelia* Hsp90–1p is identical to *Tp*hsp81p (11) (all three Hsp90 sequences are different at the remaining nine positions). This suggests that neither of the *Tetrahymena* Hsp90 sequences is excessively divergent. This impression is strongly confirmed by a phylogenetic tree of Hsp90 sequences, which includes the known ciliate Hsp90 sequences nested among the sequences of cytosolic Hsp90 molecules of other eukaryotes (Fig. 10). This tree shows the expected grouping of the ciliate Hsp90 sequences together with the apicomplexan sequences within the alveolate clade, the expected fairly deep branching between the *Tetrahymena* and *Paramecium* sequences, and, most pertinent to the present study, roughly equal lengths of the branches leading to the two *Tetrahymena* species. Hence, it is reasonable to conclude that the Hsp90 molecules of both *Tetrahymena* species have undergone similar divergence from their most recent common ancestor. Altogether, then, the *T. pyriformis* Hsp90 is similar to that of *T. thermophila* both at the level of individual residues of functional importance, and in overall level of conservation, suggesting that the Hsp90 amino acid sequence of *T. pyriformis* is no less functional than that of *T. thermophila*.

DISCUSSION

Effects of geldanamycin on two *Tetrahymena* species. Geldanamycin is a member of a family of antibiotics that is known to inhibit Hsp90 function (Whitesell et al. 1994) by specific binding to the ATP-binding pocket in the N-terminal domain of the Hsp90 molecule (Grenert et al. 1997; Stebbins et al. 1997). This drug has been regarded as highly specific in its effect on Hsp90 (review: Pratt 1998), the only reservation being that it might also affect other molecules that are structurally closely related to Hsp90, such as type-II DNA topoisomerases (Grenert et al. 1997).

There are three arguments for application of these generalizations concerning action of geldanamycin in yeast and mammalian cells to *Tetrahymena* as well. First, the concentrations of geldanamycin sufficient to inhibit cell multiplication, especially in *T. pyriformis*, are extremely low and similar to those reported earlier for mammalian cells (McIlwrath, Brunton, and Brown 1996; Uehara et al. 1986). Second, the residues in the N-terminal domain of the Hsp90 molecule critical for geldanamycin- (and ATP-) binding are highly conserved in the *T. thermophila* hsp82p and the *T. pyriformis* hsp81p. Third, the effects of geldanamycin on patterning of the oral apparatus are quite unlike those observed earlier with inhibitors of macromolecular synthesis, notably cycloheximide (Frankel 1969).

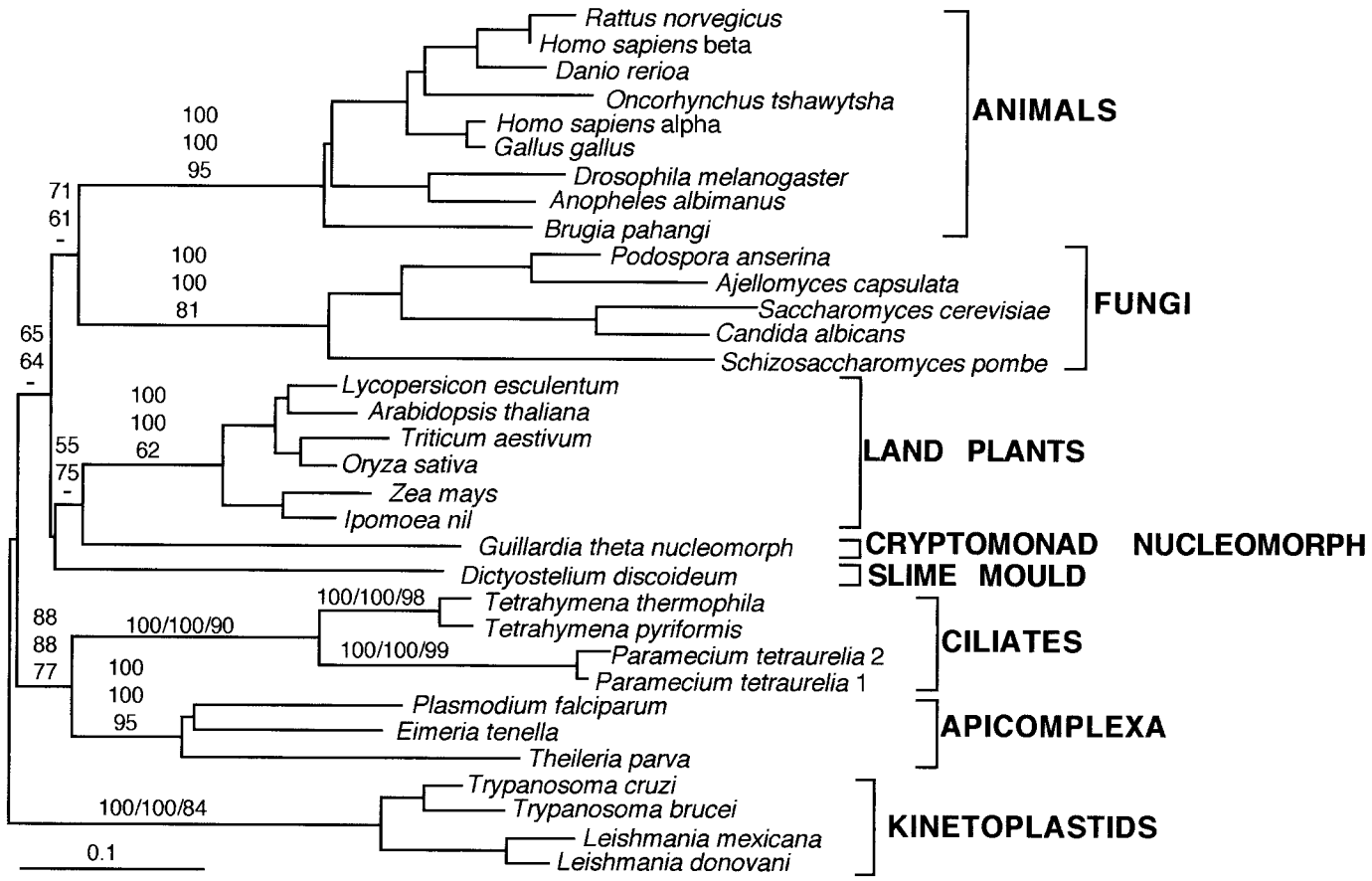

Fig. **10**. Phylogeny of Hsp90. Neighbor-joining tree of gamma-corrected distances. Numbers at each node correspond to: gamma-corrected neighbor-joining bootstraps (top line), gamma-corrected Fitch-Margoliash bootstraps (center line), and percent of quartet puzzling steps (bottom line). Scale bar represents 0.1 (corrected) substitutions per site.

The similarity with the effect of colchicine (Nelsen 1970) may be accounted for by an indirect effect of geldanamycin on biological processes mediated by microtubules, much as the mitotic apparatus is affected both by colchicine and by geldanamycin (Lange et al. 2000).

A comparison of the responses of the two *Tetrahymena* species to geldanamycin is complicated by interactions with growth temperatures, in particular supraoptimal temperatures. Nonetheless, since *T. pyriformis* grown at its optimal temperature is somewhat more sensitive to geldanamycin than is *T. thermophila* grown at a heat shock temperature (compare Fig. 1 and 3), we conclude that cell multiplication is probably more severely affected by this drug in *T. pyriformis* than in *T. thermophila*.

In both *Tetrahymena* species, there is an initial increase in cell number during the first hour (or more) after addition of the drug. Such an initial increase is commonly observed with other inhibitors (e.g. actinomycin D: Frankel 1965). It probably is a consequence of delays in penetration and action of the drug combined with an intrinsic insensitivity to interference after the ''physiological transition point'' for cell division (Rasmussen and Zeuthen 1962) and the corresponding ''stabilization point'' for the development of new oral structures (Frankel 1962), both of which immediately precede the onset of cytokinesis. Following this initial increase, growth may either continue or else cells may start dying; at some concentrations, growth of *T. pyriformis* continues for a few hours before death begins (Fig. 1). This alternative ''growth or death'' response is not peculiar to geldanamycin, as it was also observed in both species at supraoptimal temperatures (Frankel and Nelsen 2001).

The specific cause of division arrest and lethality in geldanamycin is unknown; Hsp90 manages many, albeit not all, cellular regulatory proteins (Csermely et al. 1998; Nathan, Vos, and Lindquist 1997). Which proteins essential for cell division in ciliates are the ones most critically dependent upon Hsp90 function is unknown. Cell division kinases, well characterized in *Paramecium* (Tang, Adl, and Berger 1997; Tang, Pelech, and Berger 1994, 1995; Zhang and Berger 1999) and active in *Tetrahymena* as well (Zhang et al. unpubl.), might be among the critical targets.

Abnormalities in oral development appear in both *Tetrahymena* species at concentrations of geldanamycin that are ultimately lethal. However, the severity and frequency of these abnormalities differ greatly in the two species. This difference is broadly parallel to a comparable difference observed at high temperatures. In *T. pyriformis,* the patterning of the newly formed oral ciliature is drastically abnormal in almost all cells at 33 °C (Frankel 1964; Frankel and Nelsen 2001); it is similarly abnormal in geldanamycin in the range of 0.1—1.0 μg/ml at 29 °C. In *T. thermophila,* patterning of oral structures is mildly abnormal in some cells in the range of 40.5–41.5 °C and in a narrow window of geldanamycin concentrations just below concentrations that prevent all oral development. Even under the most stressful conditions, namely geldanamycin added to *T. thermophila* at a temperature (39.5 °C) just below that which can bring about oral abnormality on its own, there is no en-

hancement of that abnormality. These results largely confirm the prediction, made before the study began, that exposure to geldanamycin would mimic the effects of high temperatures, thereby suggesting that molecular interactions mediated by Hsp90 might be involved in regulating cortical patterning in this ciliate.

This conclusion, however, requires some qualification. Although the fully developed membranellar abnormalities elicited in *T. pyriformis* by geldanamycin, especially at 1 μg/ml, are as extreme, bizarre, and widespread as any observed at high temperature, the abnormalities become evident at a somewhat later stage of oral development in the drug than at 33 °C. In addition, the global patterning of other cortical elements, including the ciliary rows of the body surface, is more perturbed at the high temperature than in geldanamycin. It appears, therefore, that in *T. pyriformis* the developmental processes affected by geldanamycin comprise a subset of those affected by high temperature and are centered on the orientation and completion of the emerging membranelles. In *T. thermophila,* oral primordia in which membranelles are beginning to form appear less abnormal in geldanamycin (at 29 °C) than they do at high temperature, and the predominant abnormalities are somewhat different under the two conditions [compare Fig. 9 in Frankel and Nelsen (2001) to Fig. 6 in this paper].

In conclusion, there is a general breakdown of morphogenetic regulation in *T. pyriformis* near its relatively moderate high temperature limit, and a part of this breakdown could be due to failure of protein-protein interactions mediated by Hsp90. These interactions may be presumed to work more effectively in *T. thermophila,* at least relative to the interactions that are required for the successful accomplishment of cell division in this species.

Does *T. pyriformis* possess a debilitated Hsp90 molecule? If the *Tetrahymena* Hsp90 molecules are directly involved in regulation of spatial patterning in the ciliate cortex, and if that regulation is defective in *T. pyriformis,* then we might expect to find a molecular basis for such defects either in the *T. pyriformis* Hsp90 molecule, or in its reaction partner or the molecular pathways dependent upon that reaction partner.

Only the Hsp90 component is accessible at this time. We have obtained the complete amino acid sequence of the Hsp90 molecule of *T. thermophila* (*Tt*hsp82p) and a virtually complete sequence for *T. pyriformis (Tp*hsp81p). The deduced molecular weight of the *Tt*hsp82p amino acid sequence exactly matches that of the immunologically identified Hsp90 molecule of this species, whereas the molecular weight of the deduced *Tp*hsp81p sequence is slightly greater than the 80 kDa anticipated from the *T. pyriformis* immunoblots. We have no explanation for the disparity in *T. pyriformis.* Both analyses of the proteins in two-dimensional gels (Williams and Nelsen 1997) and of DNA in Southern blots indicate that only a single Hsp90 species exists in *T. thermophila;* the same is likely to be true for *T. pyriformis.*

We know of three ways of assessing the hypothesis that the defective biological response of *T. pyriformis* to high temperature is due to a debilitated Hsp90 molecule. One is to compare the amino acid sequences of the Hsp90 molecules, with the expectation that *T. pyriformis* (but not *T. thermophila)* has undergone alterations in highly conserved amino acids, notably ones known to have been mutated to temperature-sensitive alleles in yeast (Nathan and Lindquist 1995). A second way is to reconstruct the evolutionary changes in the Hsp90 molecules of the two *Tetrahymena* species from their most recent common ancestor, in the expectation that the sexual *T. thermophila* will have conserved most of the Hsp90 sequence of the most recent common ancestor, whereas the asexual *T. pyriformis* will have suffered extensive and random alterations in its Hsp90 molecule. A third method is to swap the genes encoding the Hsp90 molecules between species, in the expectation that the Hsp90 molecule of each of the two species will generate at least some of the temperature responses characteristic of that species when it replaces the Hsp90 of the other species.

We have carried out the first two comparisons, with fairly clear results. For the first, we are limited by current ignorance of the full structure and function of the Hsp90 molecule. Nonetheless, based on what we do know the Hsp90s of both *Tetrahymena* species are impressively conservative. They share virtually all of the conserved amino acids in the well understood N-terminal portion of the molecule, including those that are essential for its ATPase activity (Obermann et al. 1998; Panerotou et al. 1998). They also share the extreme C-terminus, which is known to interact with all of the Hsp90-associated molecules tested thus far (Ramsey et al. 2000). Finally, they share the wild-type amino acids at the conserved sites subject to temperature-sensitive mutation. Overall, the substitutions observed in both *Tetrahymena* Hsp90s generally occur at positions that also vary in other organisms, including the closely related *Paramecium.* Thus, within the limitation of our current incomplete understanding of the structure-function relations of the Hsp90 molecule, the first expectation of the hypothesis that *T. pyriformis* has a partially disabled Hsp90 molecule has not been met.

Neither has the second. A phylogenetic analysis, using the *Paramecium* sequences as the closest outgroup, reveals that the Hsp90 amino acid sequences of the two *Tetrahymena* species are similarly diverged from their most recent common ancestor. If differences in the Hsp90 sequences of the two *Tetrahymena* species were due to genetic drift, they drifted to a similar degree despite the circumstance that *T. thermophila* is sexual and probably a vigorous outbreeder in nature (Doerder et al. 1995) whereas *T. pyriformis* is amicronucleate and hence asexual, although it may have become so rather recently (Nanney et al. 1998).

The third test, the *TpHSP82–TtHSP81* gene swap, has not yet been accomplished. Currently it would be easier to accomplish this from *T. pyriformis* to *T. thermophila,* using the gene substitution technology well established in the latter species (Hai, Gaertig, and Gorovsky 1999). Our prediction based on the outcome of the other two tests is that the *Tp*hsp81p would function normally when substituted for *Tt*hsp82p in *T. thermophila,* maintaining the physiological and morphogenetic responses to geldanamycin and to high temperature characteristic of that species. If this were to be the outcome, then we would have to identify the defective reaction partner(s) or pathways in which these partners function to account for the parallel differences in responses to high temperature and geldanamycin documented in this and the preceding papers. The existence of such defective reaction partners or pathways also would be predicted by application of the ''Hsp-90 capacitor'' model of Rutherford and Lindquist (1998).

Hsp90 phylogeny in ciliates. The Hsp90 phylogenetic tree (Fig. 10) displays a fairly conventional overall topology. Several major eukaryotic lineages, notably the land plants, fungi, animals, and kinetoplastids are recovered, consistent with other phylogenies (e.g. Cavalier-Smith, 1993; Keeling et al. 1999; Philippe and Adoutte 1998; Wainwright et al. 1993). This phylogeny also places the apicomplexans and ciliates as sister groups, consistent with both rRNA and other protein-based phylogenies. Within the ciliates, *Paramecium* and *Tetrahymena* show a fairly deep phyletic divergence, again consistent with phylogenies based on rRNA [small subunit: Greenwood, Sogin, and Lynn (1991); Strüder-Kypke et al. (2000); large subunit: Baroin-Tourancheau et al. (1995)]. Altogether, Hsp90 phylog-

eny appears to be relatively robust and informative, suggesting that this molecule may be a useful marker for eukaryotic evolution.

The divergence between the amino acid sequences of the Hsp90 molecules of the two *Tetrahymena* species is surprisingly large: the Hsp90 molecules of these two morphologically virtually indistinguishable species differ more than does human Hsp90α from chicken Hsp90. Naturally, other proteins show greater or lesser degrees of conservation, but for the most part these two *Tetrahymena* species are fairly divergent at the molecular level (review: Frankel 1999). Thus, *Tetrahymena* appears to support the notion that ''the morphological designs of the ciliates are far more conservative than the molecules that compose them'' (Nanney et al. 1998). The substantial difference between the Hsp90 molecules of these two species therefore probably bespeaks a relatively old phyletic divergence rather than any major evolutionary instability in the molecule itself.

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