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HSP70 and HSP90 homologs are associated with tubulin in hetero-oligomeric complexes, cilia and the cortex of *Tetrahymena*

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SUMMARY

We show in the present study that homologs of hsp90 and hsp70 are induced by heat shocks in *Tetrahymena* and appear to form a high molecular mass complex (~700 kDa) with tubulin. Three members of the hsp70 family (hsp72, 73, and 78) and one member of the hsp90 family (hsp82) have been identified by immunological or by a combination of immunological and sequencing methods. The known components of the 700 kDa complex and the conditions under which it can be recovered suggest that it may be an induced protective assemblage rather than a normal processing intermediate. Immunoblotting and immunofluorescence studies suggest further that large amounts of hsp73 and lesser amounts of hsp82 are associated with mature microtubules in both cilia and the cortex in this cell type. Some site-specific localizations of the identified heat shock proteins were also noted in non-microtubular components of the cell cortex.

Key words: Chaperone, *Tetrahymena*, Tubulin

INTRODUCTION

What a cell is and what a cell does are predicated to a certain extent upon the structures it has or is capable of forming. Recent studies of microtubules in eukaryotic cells suggest that answers to some of the important questions about tubulin processing, assembly, and function lie in a more complete understanding of the interactions of these microtubule subunit proteins with associated proteins known as chaperones.

Early on, a mutant chaperone (TCP-1) in yeast was shown to result in the inability to assemble the mitotic apparatus (Ursic and Culbertson, 1991). Subsequently, TCP-1 was shown to be a component of an 800-950 kDa heteromeric ring complex in eukaryotic cells called CCT (Willison and Kubota, 1994) or TRiC (Frydman et al., 1992). Frydman et al. (1992) first demonstrated tubulin folding by CCT in vitro using guanidinium-Cl unfolded α- and β-tubulin, and Yaffe et al. (1992) did the same using tubulin translated in vitro. The product in both cases was folded and assembly-competent tubulins. These studies have been confirmed and extended by others (Fontalba et al., 1993; Gao et al., 1993; Melki et al., 1993; Paciucci, 1994).

Lewis et al. (1992) demonstrated that hsp70 may be associated with the CCT particle in mammalian cells, and others have shown that hsp70 binds directly to tubulin (Green and Liem, 1989; Sanchez et al., 1994). Recently, hsp70 has been found associated with the flagellar axonemes of *Chlamydomonas* (Bloch and Johnson, 1995). Less is known about the association of hsp90 with tubulin, but it has been shown convincingly that hsp90 is associated with microtubules in mammalian cells (Redmond et al., 1989; Czar et al., 1996).

There are at least ten different types of microtubule-based structures in the ciliated protozoan, *Tetrahymena thermophila* (Allen, 1967; Jaeckel-Williams, 1978; Williams and Bakowska, 1982). The more prominent ones are basal bodies, cilia, cortical ribbons, oral connectives, and nuclear spindles. The synthesis and assembly of a variety of topographically regulated microtubule systems can thus be studied in this single cell. Though tubulin gene expression has been studied in *Tetrahymena* (Gu et al., 1995), there appear to be no previous studies on the possible involvement of chaperones with tubulin in this ciliated protozoan.

We and others before us (Wilhelm et al., 1982; Hallberg et al., 1984) have observed that *Tetrahymena thermophila* responds to heat shocks by synthesizing massive amounts of proteins located at the 82 kDa and 73 kDa positions in one-dimensional polyacrylamide gels (Fig. 1). We suspected that these are the *Tetrahymena thermophila* equivalents of hsp90 and hsp70, respectively, and that both may be associated with tubulin in this cell type. Evidence in support of both propositions is presented here. Hsp82 and one or more of the hsp70-class members in *Tetrahymena* were found associated with both soluble tubulin and tubulin assembled into microtubules in cilia and in the cell cortex. Further study of tubulin-chaperone associations in *Tetrahymena* may shed light on the processing and assembly of tubulin in this cell type.

MATERIALS AND METHODS

Cells and cell fractions

The B strain of *Tetrahymena thermophila*, used throughout this study, was grown in the enriched proteose-peptone medium described by Nelsen et al. (1981). The cells were incubated with shaking at 30°C

and the heat shock temperature was 41°C. Two methods for isolating cortical cytoskeletons were used, both described by Williams and Honts (1995). In one method the cells are extracted in 1% Triton X-100 at low ionic strength, and in the other the cells are extracted with 1% Triton X-100 in 1.5 M KCl. Cilia were isolated by the method of Nozawa and Thompson (1971). Outer doublet microtubules were recovered from isolated cilia after detergent extraction and dialysis at low ionic strength according to the method of Gibbons (1965). Gerbil fibroma and mouse myeloma cells were used in some experiments. They were grown in DMEM-HG with 10% fetal bovine serum using standard procedures (Harlow and Lane, 1988).

Soluble tubulin-chaperone complexes were isolated from *Tetrahymena* cell lysates. The cells were first given a 30 minute heat shock at 41°C. Cell pellets containing 5×10^6 cells were then lysed by vortexing and incubating for 30 minutes at 0°C in 500 μl of a lysis buffer consisting of 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.5 mM PMSF, and 0.25 mM chloromercuriphenylsulfonic acid. The samples were clarified by centrifuging for 1 hour at 90,000 ***g*** in a Beckman L5-50B ultracentrifuge using an SW27 rotor. The clarified samples were loaded onto 10.2-40% sucrose gradients and centrifuged for 16 hours at 100,000 ***g*** in a Beckman SW40 rotor. Samples were removed from the top to the bottom of the gradient (850 μl each), dialyzed exhaustively against the above lysis buffer minus the detergents, concentrated by dialysis against 20% carbowax, and solubilized by adding an equal volume of 2× Laemmli sample buffer (Laemmli, 1970) and boiling for 2.5 minutes. Thyroglobulin (669 kDa) was loaded on a parallel gradient and the samples obtained were assayed for the presence of protein using the Bradford test (Bradford, 1976).

Antibodies

Monoclonal antibodies AC88, AC16, and AC10, directed against *Achyla* hsp90, were kindly provided by David O. Toft, Mayo Clinic. We raised polyclonal antibodies in rabbits directed against *Tetrahymena* hsp82 and hsp73 by injecting bands excised from one-dimensional polyacrylamide gels. Polyclonal antisera directed against *Tetrahymena* tubulin (Williams and Honts, 1987), epiplasm band C (Williams et al., 1987), and tetrin polypeptides (Williams et al., 1986) were obtained similarly, as reported in earlier studies.

The monoclonal antibody 15D3 recognizing *Tetrahymena* α-tubulin was a gift from Joe Frankel, Univ. of Iowa. Polyclonal antisera directed against surface antigens of *Tetrahymena* were provided by Paul Doerder, Cleveland State Univ., and the polyclonal anti-centrin 26/14-1 was a gift from Jeffrey Salisbury, Mayo Clinic. We obtained two monoclonal antibodies (9C10 and 4E3) directed against a 12 amino acid synthetic peptide made from the base sequence of the *Tetrahymena* hsp82 gene. We serendipitously obtained a clone, pBC1, that contains a substantial portion of the coding region of this gene while screening a *T. thermophila* cDNA library in another project. The insert is 1.4 kb long. Upon sequencing, we found that it codes for 437 amino acids and includes the stop codon (TGA) plus 138 bp of the trailer sequence. The inferred amino acid sequence shows 65.5% sequence identity with human hsp90 over the 441 amino acid overlap. We selected a likely antigenic region for peptide synthesis (K-N-K-P-L-W-M-R-K-P-E-E) using the GCG program PEPTIDESTRUCTURE. The peptide was synthesized by Bio-Synthesis, Inc. We then raised monoclonal antibodies against this peptide in mice using methods described earlier (Nelsen et al., 1994).

Electrophoresis

Cell pellets and pellets of cell fractions were solubilized in sample buffer and subjected to electrophoresis in either 6% or 8% SDS-polyacrylamide gels as described by Laemmli (1970). The molecular mass markers used were phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and trypsinogen (24 kDa). For two-dimensional gels, the samples were first separated in tube gels under non-equilibrium conditions as described by O'Farrell et al. (1977). The gels were removed from the tubes, equilibrated in sample buffers, then loaded on standard SDS-polyacrylamide slab gels for separation of the proteins in the second dimension. Proteins in the gels were visualized after staining with Coomassie Blue or by autoradiography. For autoradiography, the cells were first labeled by incubation in L-[^{35}S]methionine (1,000 Ci per mM, Amersham) at a final concentration of 2.5-5 μCi per 10^5 cells for 1 hour. Following electrophoresis, the gels were dried and exposed to Kodak XAR-5 film for 24 hours at −70°C using an intensifying screen.

Immunoblotting

Protein arrays were transferred from polyacrylamide gels to nitrocellulose membranes as described by Towbin et al. (1979), except that all solutions contained non-fat dry milk to reduce non-specific adsorption (Johnson et al., 1984). The positions of major bands, including marker proteins, were marked with a pencil after staining briefly in 0.5% Ponceau S in 1% acetic acid. The filters were treated with polyclonal antibodies for 2.5 hours at a 1:750 dilution, washed, then reacted with ^{125}I-conjugated sheep anti-rabbit IgG F(ab′)$_2$ fragment (10 μCi per μg, Amersham) at a final concentration of 0.166 μCi per ml for 1 hour. The filters were then washed, dried, and incubated against Kodak XAR-5 film at −70°C with an intensifying screen. Filters treated with monoclonal antibodies were processed similarly, using ^{125}I-conjugated sheep anti-mouse IgG F(ab′)$_2$ fragment (8 μCi per μg, Amersham) as the secondary probe. Monoclonal antibodies AC88, AC16, and AC10 were applied at a concentration of 5 μg IgG per ml. Monoclonal antibody 15D3, which is a hybridoma supernatant, was applied to the filters at a dilution of 1:20.

Immunoprecipitation

Tetrahymena cells were labeled with L-[^{35}S]methionine (1,000 Ci per mM; Amersham) in inorganic medium. They were starved for 45 minutes in 10 mM Tris-HCl, pH 7.4, at a density of 1×10^5 cells per ml, then concentrated 10-fold by centrifugation. Samples of the concentrate containing 1×10^5 cells were then removed to microcentrifuge tubes and incubated for 30 minutes at 41°C in the presence of 10 μCi of labeled methionine. Each sample of cells was pelleted and lysed by vortexing in 500 μl of lysis buffer (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.5 mM PMSF, and 0.25 mM chloromercuriphenylsulfonic acid). After 30 minutes at 5°C, the lysates were clarified by centrifugation and incubated with polyclonal antibodies at a dilution of 1:50 for 1 hour at 5°C. The antigen-antibody mixtures were added to 25 μl of washed (in TBS, pH 8.0, containing 1% Triton X-100 and 1 mg/ml BSA) Protein A-Sepharose beads, mixed, and incubated for 1 hour at 5°C. The beads were then washed 2× in the above buffer, once in TBS, once in 10 mM Tris-HCl, pH 6.8, and the protein was solubilized by boiling in 55 μl of Laemmli sample buffer and stored frozen. Following electrophoresis, the gels were dried and incubated with Kodak XAR-5 film for 24 hours at −70°C with an intensifying screen.

Immunofluorescence microscopy

Tetrahymena and myeloma cells were permeabilized and fixed in suspension in 50% ethanol containing 0.2% Triton X-100 (Nelsen et al., 1994). Gerbil fibroblasts were fixed and permeabilized similarly while attached to coverglasses. After fixation, the preparations were rinsed twice in Tris-buffered saline, pH 8.2, containing 0.1% bovine serum albumin (BSA) and suspended in primary antibody solution containing 1% BSA for 45 minutes. Polyclonal antisera were used at a dilution of 1:100, monoclonal antibody supernatants 9C10 and 4E3 were used at a dilution of 1:5, and the purified immunoglobulins (AC88, AC16, AC10) were used at a concentration of 3.6 μg/ml. The samples were washed in 0.1% BSA-TBS and suspended for 45 minutes in secondary antibody at a dilution of 1:100 in 1% BSA-TBS. FITC-conjugated goat anti-rabbit IgG (Sigma) was used following polyclonal antisera, and FITC-conjugated goat anti-mouse IgG

(Sigma) was used following the monoclonal antibodies. After staining, the preparations were washed in 0.l% BSA-TBS and observed with an Olympus BH2 fluorescence microscope equipped with epifluorescence. Photographs were taken using Kodak Tri-X Pan film, ASA 400.

RESULTS

Major heat-induced proteins in *Tetrahymena thermophila*

A one hour heat shock of 41°C induces extensive synthesis of relatively high molecular mass proteins that take the form of two major bands in one-dimensional gels of total cell *Tetrahymena* protein (Fig. 1). The top band is found at the 82 kDa position. This appears in two-dimensional gels to be a single protein displaying some charge heterogeneity (Fig. 2). The bottom induced band in one-dimensional gels (Fig. 1) shows an apparent molecular mass of 73 kDa, but appears in two-dimensional gels to consist of two components. These are labeled 72 and 73 in Fig. 2. A fourth heat shock protein with an apparent molecular mass of 78 kDa can be seen in both one-dimensional and two-dimensional gels (Figs 1, 2).

Antibodies and protein recognition

We obtained three monoclonal antibodies directed against *Achyla* hsp90 to see if any might recognize *Tetrahymena* hsp82 in immunoblots. Two were negative (AC88 and AC16) and one, AC10, was positive. The latter was reacted with total cell protein and with protein from cortical fractions of *Tetrahymena* (Fig. 3A). mAb AC10 reacted strongly with hsp82 in total protein from heat shocked cells (lane 1) and also in high-salt cortical cytoskeletons (lane 3), but very weakly with hsp82 in low-salt cortical cytoskeletons (lane 2). The second band (63 kDa) in lane 1 is believed to be a breakdown product of hsp82.

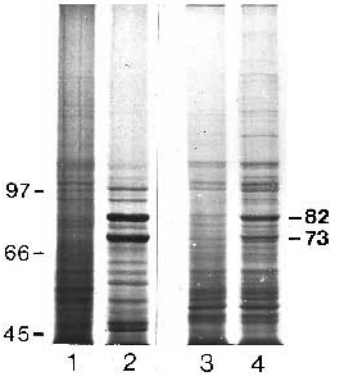

Fig. 1. The major heat-induced proteins of *Tetrahymena thermophila* are found in two prominent bands in SDS-polyacrylamide gels of total cell proteins separated in one dimension. Lane 2 shows induction by means of the pulse-incorporation of [^{35}S]methionine for 1 hour at a heat-shock temperature of 41°C. The pattern of incorporation over the same interval at the normal growing temperature of 30°C is shown in lane 1. The induction can also be seen by comparing Coomassie blue-stained gels of total cell protein from cells incubated for 1 hour at 41°C (lane 4) with those from cells kept at 30°C (lane 3). The gels were 6% acrylamide and the molecular mass standards were phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

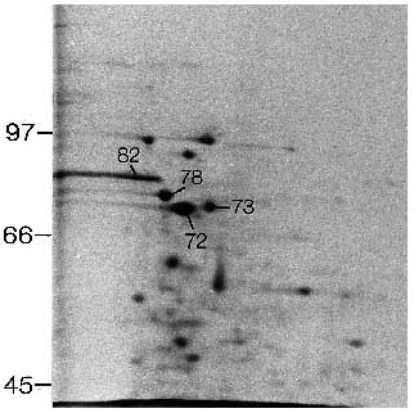

Fig. 2. Autoradiogram of a two-dimensional gel showing proteins synthesized by *T. thermophila* for 1 hour at the heat shock temperature (41°C). The proteins were separated in the first dimension (horizontal axis) in standard NEPHGE gels, and in the second by SDS-PAGE. The 82 kDa band seen in Fig. 1 is apparently a single protein, whereas the 73 kDa one-dimensional band is resolved into two components, labeled 72 and 73. There is also a prominently induced protein at 78 kDa which can also be seen in Fig. 1. Molecular mass markers as for Fig. 1.

Two polyclonal antibodies were also raised against the 82 kDa band excised from one-dimensional gels like those shown in Fig. 1. These antisera, called 82-1 and 82-2, reacted strongly with hsp82 in immunoblots of total protein and high-salt cortical cytoskeletons, as did mAb AC10, and also showed some degradation products in the total cell protein preparations.

One polyclonal antibody was raised against the 73 kDa band excised from one-dimensional gels of heat shocked *Tetrahymena* cells. This antiserum, called pAb 73, was reacted with total cell protein and with protein from cortical fractions of *Tetrahymena* (Fig. 3B). As seen in Fig. 3, lane 4, this antiserum recognized a major band at the 73 kDa position, a weaker band at the 78 kDa position, and some small peptides that are probably breakdown products of the other bands. The major band is likely composed of the two components seen in two-dimensional gels (72 and 73 kDa), because both would have been included in the band excised from one-dimensional gels. Recognition of hsp78 by this antiserum, however, suggests an immunological relationship with one or more of the others because this band was carefully avoided during excision of the 73 kDa antigen.

Interestingly, the three hsp70-class members are unequally distributed in the three cortical fractions presented in Fig. 3. Lane 5 shows that it is likely to be hsp73 alone that is recognized in the low-salt cortical fraction. Lane 6 suggests that hsp72 and hsp78 are found in the high-salt cortical fraction, and lane 7 shows that hsp73 alone is present in isolated cilia. These data suggest that at least some of the hsp70s are independently regulated topographically. In a separate experiment we showed that hsp73 remained associated with purified outer doublet microtubules isolated from cilia (data not presented).

The polyclonal antibodies, but not the monoclonal antibodies, also immunoprecipitated their respective target

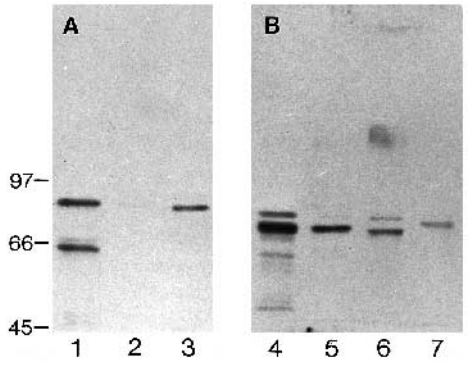

Fig. 3. Immunoblots of *Tetrahymena* protein preparations reacted with antibodies raised against heat shock proteins. (A) Blotted with mAb AC10, directed against the hsp90 of *Achyla*. It reacted in a major way with *Tetrahymena* hsp82 in both total cell extracts (lane 1) and high-salt cortical cytoskeletons (lane 3), and more weakly with hsp82 in low-salt cortical cytoskeletons (lane 2). The band at 63 kDa in lane 1 is believed to be a breakdown product of hsp82. (B) Blotted with pAb 73, raised against the 73 kDa band cut out of one-dimensional gels of heat-shocked *Tetrahymena* cell protein. It recognized hsps 72, 73, and 78 in total cell protein (lane 4), hsp73 in low-salt cortical cytoskeletons (lane 5), hsp72 and hsp78 in high-salt cortical cytoskeletons (lane 6), and hsp73 in isolated cilia (lane 7). Molecular mass markers as for Fig. 1.

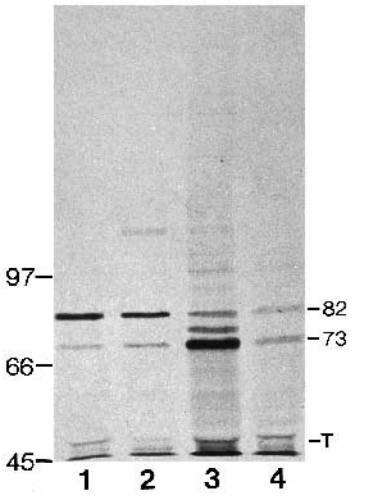

Fig. 4. Autoradiogram of [^{35}S]methionine-labeled *Tetrahymena* proteins immunoprecipitated from heat-shocked cells with antibodies directed against *Tetrahymena* proteins. pAb 82-1, pAb 82-2, pAb 73, and anti-tubulin were all raised in rabbits against *Tetrahymena* protein excised from gels. pAbs 82-1 and 82-2 immunoprecipitated hsp82 predominantly (lanes 1,2), but also brought down a small amount of band 73 and tubulin (T). pAb 73 immunoprecipitated hsps 72, 73, and 78 predominantly (lane 3), but also precipitated a significant amount of hsp82 (which it does not blot; cf Fig. 3), and some tubulin (T). Anti-tubulin immunoprecipitated tubulin (T, lane 4) together with detectable amounts of hsp82 and band 73 (hsp72 and/or hsp73). Molecular mass markers as for Fig. 1.

antigens from *Tetrahymena* cell lysates (Fig. 4). Lanes 1 and 2 (Fig. 4) show that pAbs 82-1 and 82-2, respectively, could be used to immunoprecipitate substantial amounts of hsp82. Lane 3 shows the recovery of substantial amounts of hsp72-73 and hsp78 from cell lysates with pAb 73.

Cytosolic complexes of tubulin and heat shock proteins in Tetrahymena

It was observed in the experiment presented in Fig. 4 that tubulin was also present in the immunoprecipitates recovered with antibodies directed against *Tetrahymena* heat shock proteins. Further, it appeared that some hsp73 was coprecipitated with hsp82 by pAbs 82-1 and 82-2 (lanes 1,2 in Fig. 4), and that some hsp82 was coprecipitated with the hsp70s when pAb 73 was used (lane 3). There are two possible explanations for this result. Either the polyclonal antibodies are acting non-specifically, or hetero-oligomeric complexes exist within the cell and are being recovered.

Hetero-oligomeric complex formation is suggested by the fact that immunoblotting revealed none of the cross-reactivity implied by the alternative hypothesis of non-specificity. We sought to confirm this interpretation by seeing if hsps would coprecipitate with tubulin using anti-tubulin serum. As seen in lane 4, Fig. 4, bands at 73 kDa and 82 kDa were seen in addition to tubulin when heat shocked *Tetrahymena* cells were immunoprecipitated with a polyclonal antibody raised against *Tetrahymena* tubulin (Williams and Honts, 1987). The experiment was controlled by immunoprecipitating lysates from heat-shocked *Tetrahymena* cells using polyclonal antisera directed against other *Tetrahymena* antigens, and with Protein A-Sepharose beads alone. We tested anti-H3 surface antigen, anti-T surface antigen, anti-centrin, anti-tetrin, and anti-epiplasm band C. In no case was heat shock protein coprecipitated along with the corresponding antigen or by Protein A beads alone. The immunological data therefore suggest that there is an in vivo association between two or more high molecular mass heat shock proteins and tubulin in *Tetrahymena*.

We sought to confirm this conclusion using very different procedures. We undertook to isolate the putative tubulin-hsp complexes directly by continuous sucrose density gradient centrifugation. The results, presented in Fig. 5, confirm the in vivo occurrence of such complexes. The cells were first heat shocked (41°C) for 30 minutes. They were then lysed, clarified by centrifugation, and loaded onto a 10.2-40% sucrose gradient and centrifuged at 100,000 ***g*** for 16 hours. To get some idea of size, we centrifuged thyroglobulin (~669 kDa) in a parallel gradient and recovered it in fraction #3. Coincidentally, this was the fraction that contained the most tubulin and hsp82 (Fig. 5), and also hsp73 (not shown).

The gradient was unloaded from top (fraction 1) to bottom (fraction 15). The samples were then solubilized with equivalent volumes of 2× SDS Laemmli buffer and loaded into the lanes of an 8% SDS-polyacrylamide gel. The gel was then transferred to a nitrocellulose membrane, blotted with mAb AC10 (hsp82) and mAb 15D3 (α-tubulin), alone and in combination, and reacted with ^{125}I-anti-mouse immunoglobulin. It can be seen in Fig. 5 that both proteins were concentrated in lane 3, with some spread to either side in each case. This confirms the existence of tubulin-hsp complexes in *Tetrahymena* cells that was suggested by the immunoprecipitation experiments.

Fluorescence microscopy

Indirect immunofluorescence microscopy with anti-hsp anti-

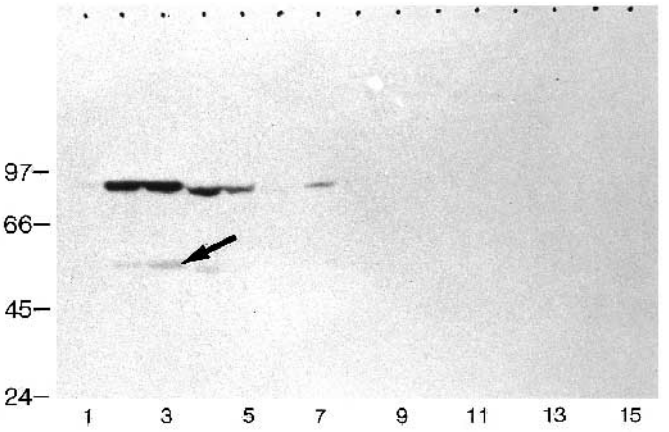

Fig. 5. Autoradiogram of a panel of 15 fractions from a continuous sucrose gradient run in an 8% SDS-polyacrylamine gel and immunoblotted with mAb AC10 to detect hsp82 and mAb 15D3 to detect α-tubulin. Both tubulin (arrow) and hsp82 were predominantly in fraction 3, with lesser amounts in fractions 2 and 4. Hsp82 can also be seen in fractions 5 and 7. The cells were heat-shocked for 30 minutes at 41°C, lysed, clarified, loaded onto a 10.2-40% sucrose gradient, and centrifuged for 16 hours at 100,000 ***g***. The gradient was unloaded from top (fraction 1) to bottom (fraction 15). A parallel gradient was loaded with thyroglobulin and centrifuged similarly. The thyroglobulin (669 kDa) was found in fraction 3, indicating a molecular mass for the tubulin-hsp82 complex of about this magnitude. Molecular mass markers as for Fig. 1, plus trypsinogen (24 kDa).

bodies was used to visualize the intracellular distributions of the *Tetrahymena* high molecular mass heat shock proteins. All antibodies stained the endoplasm intensely, indicating that large quantities of these proteins were present throughout the cell. This is illustrated in Fig. 6B using a polyclonal antibody directed against the 73 kDa band from one-dimensional gels. A similar result was obtained with pAb 82-1, pAb 82-2, mAb AC10, and mAb 9C10. The intense staining often made it difficult to see the cortical staining, but this could be seen in favorable specimens (Figs 6C,D), and was easily verified in broken cells.

The staining of basal bodies was most clearly seen in cells reacted with mAb 9C10 (Fig. 6D) and mAb 4E3 (not shown). These monoclonal antibodies were raised against a 12 amino acid synthetic peptide prepared using base sequence information from the *Tetrahymena* hsp82 gene as described in the Materials and Methods section. Both antibodies stained the cells very much like pAbs 82-1 and 82-2 but gave a clearer indication of the association with basal bodies.

DISCUSSION

The synthesis of hsp82 is quantitatively dominant in the heat shock response of *Tetrahymena thermophila* (Wilhelm et al., 1982; this report). Hsp82 appears to be a single polypeptide in two-dimensional polyacrylamide gels (Fig. 2), and Southern blots of genomic DNA probed with pBC1 described above

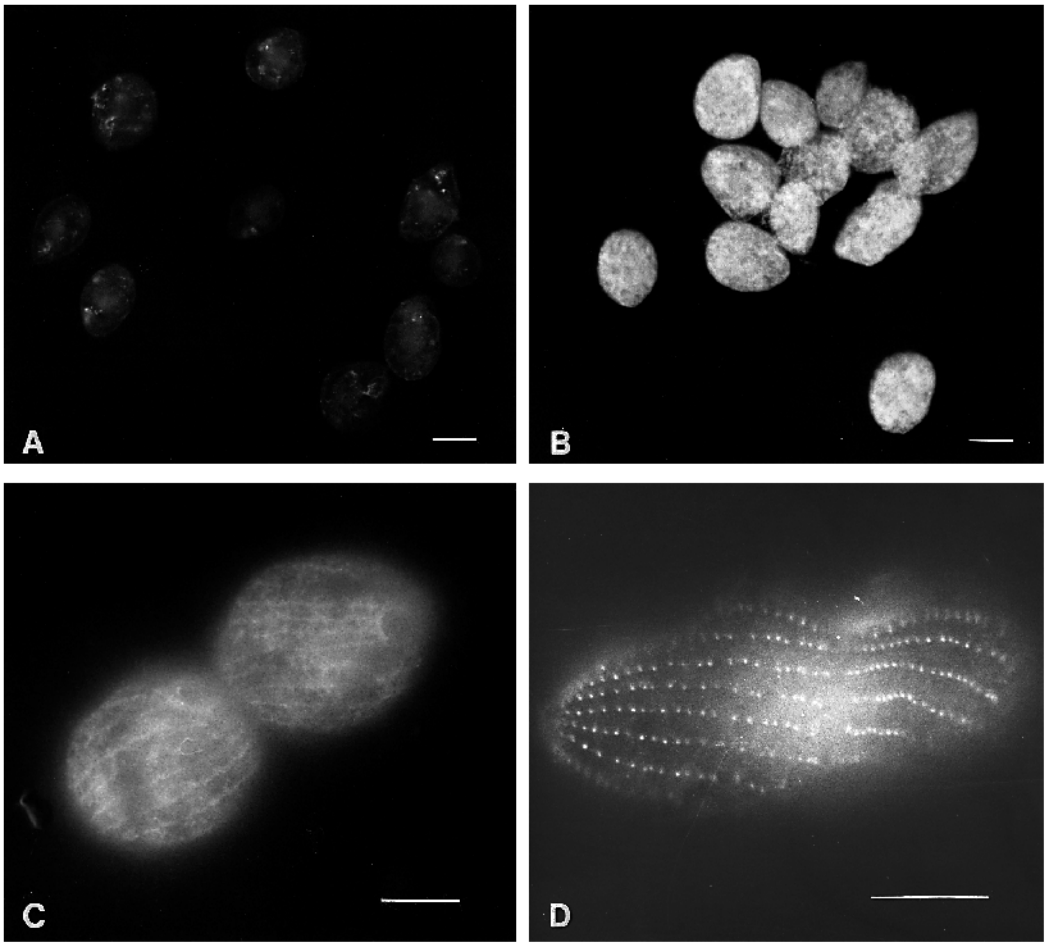

Fig. 6. Indirect immunofluorescence staining of *Tetrahymena thermophila* with antibodies raised against *Tetrahymena* heat shock proteins. (A) Cells stained with pre-immune serum from the rabbit used in raising pAb 73, followed by FITC-conjugated anti-rabbit IgG. Each cell shows a few small fluorescing granules, and the oral apparatus typically showed weak fluorescence. Bar, 20 μm. (B) Cells stained with pAb 73 and FITC-conjugated anti-rabbit immunoglobulin. All cells showed brightly stained endoplasm, as seen here. Bar, 20 μm. (C) Higher magnification of a dividing cell stained with pAb 73 and FITC-conjugated anti-rabbit IgG. The longitudinal rows of cilia can be distinguished within the cortex, though individual ciliary units are not obvious. Bar, 10 μm. (D) Dividing cell stained with mAb 9C10 (directed against *Tetrahymena* hsp82) and FITC-conjugated anti-mouse IgG. The individual basal bodies in the ciliary rows are brightly stained. Bar, 10 μm.

suggest that there is a single copy of the hsp82 gene in *Tetrahymena* (data not presented). There are several reasons in addition to size and heat-induced synthesis to conclude that *Tetrahymena* hsp82 is a member of the hsp90-class of chaperones. First, this polypeptide reacts strongly with mAb AC10. This antibody, provided by David O. Toft, is one of several hsp90 antibodies raised against the 88 kDa heat shock protein from the water mold *Achyla ambisexualis*. A widely used antibody of this series is AC88, and its preparation is described by Riehl et al. (1985). AC10 has not appeared in previous publications, but is known to recognize hsp90-class members in *Achyla*, chicken, and humans (D. O. Toft, personal communication). AC88, unlike AC10, does not recognize the *Tetrahymena* protein.

Also in support of the identity of *Tetrahymena* hsp82, we found the polyclonal antibodies that we raised against the *Tetrahymena* protein recognized a 90 kDa protein in mouse myeloma cells (unpublished observations). Finally, the inferred amino acid sequence from the 1.4 kb pBC1 insert showed close to 65% sequence identity with the corresponding region (~437 amino acids) from several hsp90-class members, including hsp90 from humans. The monoclonal antibodies (9C10 and 4E3) that we raised against a synthetic peptide representing a portion of this sequence did not blot *Tetrahymena* protein, but did stain both *Tetrahymena* (Fig. 5) and fibroblasts (unpublished observations) in a manner similar to pAbs 82-1 and 82-2. We tentatively conclude that there is a single hsp82 gene in *Tetrahymena* that specifies an 82 kDa protein that is a member of the hsp90 class of chaperones.

A second major response of *Tetrahymena thermophila* to 41°C was the elevated synthesis of proteins that ran in a band at about the 73 kDa position in one-dimensional gels (Fig. 1). This band included two proteins, designated hsp72 and hsp73, as indicated by two-dimensional gel electrophoresis (Fig. 2). The former is slightly smaller and more acidic than the latter. A third induced protein with an apparent molecular mass of 78 kDa could be seen in both types of gels. This protein appears to be immunologically related to either hsp72, hsp73, or both. It was well-separated from the 73 kDa band that was excised and used to raise pAb 73 in rabbits, yet immunoblotted consistently with this antiserum. We have no information at present on the degree of similarity that these proteins may have with hsp70-class members of other species, other than size and the heat-shock response itself. *Tetrahymena* hsp78 may be related to BiP/GRP78, known in other systems to be involved in translocating proteins into the lumen of the endoplasmic reticulum (Vogel et al., 1985). *Tetrahymena* produces large amounts of secretion product, and the secretory pathway is beginning to be understood in this cell type (Chilcoat et al., 1996).

The polyclonal antisera that we raised in rabbits specifically immunoblotted the gel bands that were used as antigens (Fig. 3). In addition, pAb 73 blotted hsp78, as indicated above. These antisera also effectively immunoprecipitated the corresponding heat shock proteins from *Tetrahymena* cell lysates prepared from heat shocked cells. It can be seen in Fig. 4 that substantial quantities of hsp82 were recovered using pAbs 82-1 and 82-2, and that hsps72, 73 and 78 were effectively immunoprecipitated by pAb 73.

The immunoprecipitates obtained from these cells further suggested the possibility that at least some of these heat shock proteins may exist within the cell in association with each other and with tubulin. This is because the anti-hsp82 antibodies precipitated tubulin and hsp72 (and/or hsp73) along with hsp82 (Fig. 4, lanes 1,2), and pAb 73 precipitated tubulin and hsp82 along with hsps72, 73 and 78 (Fig. 4, lane 3). Of course, this might also be due to non-specific precipitation by these antibodies. However, no evidence for cross-reactivity was seen in any of the immunoblots. We tested this further by immunoprecipitating with tubulin and examined the precipitate in one-dimensional gels. As seen in Fig. 4, lane 4, the precipitate contained an 82 kDa band and a 73 kDa band along with tubulin.

It was considered possible that all three types of antisera, or possibly Protein A-Sepharose alone, might be bringing down the three antigens coordinately but non-specifically simply because of the large quantities of these proteins present in the cells under these conditions. Accordingly, we precipitated with several other types of polyclonal antisera (surface antigen, centrin, tetrin, and membrane skeleton) and with Protein A beads alone. In no case was precipitation of either tubulin or heat shock proteins observed. This strongly suggests that tubulin and the high molecular mass heat shock proteins were coprecipitated because they are associated with each other in *Tetrahymena* cells.

The existence of tubulin-chaperone complexes in vivo was confirmed by sucrose density gradient fractionation. *Tetrahymena* cells were heat-treated (41°C) for 30 minutes, lysed, and the lysate was clarified by ultracentrifugation. The clarified lysate was then loaded on a 10.2-40% continuous sucrose gradient and centrifuged overnight at 100,000 ***g***. The fractions were recovered, solubilized in Laemmli buffer, and the proteins identified after electrophoresis in an 8% polyacrylamide gel by immunoblotting with mAb AC10 (recognizes *Tetrahymena* hsp82) and mAb 15D3 (anti-α-tubulin). Both hsp82 and α-tubulin were most highly concentrated in fraction 3, with lesser amounts present in fractions to either side but absent from the two ends of the gradient (Fig. 5). The cosedimentation of tubulin and hsp82 supports the conclusion that tubulin and hsp82 are associated with each other in the cytosol. A similar result was obtained when gradient fractions were analyzed by immunoblotting with pAb 73 and a polyclonal anti-tubulin antibody (not presented). Together with the immunoprecipitation results, these data support the conclusion that there are hetero-oligomeric complexes containing tubulin, hsp82, and one or more hsp70-class members in the cytosol of heat-shocked *Tetrahymena thermophila*.

It has been shown in a number of laboratories that the 800-950 kDa chaperone complex known as TRiC (Frydman et al., 1992) or CCT (Willison and Kubota, 1994) participates in the folding of tubulin in vitro and releases it in an assembly-competent form (Yaffe et al., 1992; Frydman et al., 1992; Gao et al., 1993; Melki et al., 1993; Fontalba et al., 1993; Tian et al., 1996). The CCT complex has a double ring structure and is in general similar to the chaperonins of eubacteria and mitochondria. It is unlikely that the 700 kDa tubulin-chaperone complex we have isolated from *Tetrahymena* is related to this normal processing intermediate, however, for a number of reasons. First of all, hsp90 is a major component of the *Tetrahymena* complex, and there are no previous reports of hsp90 occurring in association with CCT complexes. Additionally, the complete in vitro folding pathway of β-tubulin has recently

been worked out (Tian et al., 1996), and hsp70 and hsp90 are not among those proteins known to be involved. Finally, we were only able to recover the 700 kDa complex from heat-treated cells in the present study. Together, these considerations suggest the alternative possibility that the hsp70 and hsp82 proteins induced by heat in *Tetrahymena* may associate with partially unfolded tubulin subunits during the heat shock thereby protecting them against further denaturation or degradation that might result from prolonged exposure to high temperature. Further studies will be required to determine the precise role played by the 700 kDa tubulin-chaperone complexes found in heat-shocked *Tetrahymena* cells.

Our immunoblots have revealed that *Tetrahymena* high molecular mass heat shock proteins are also present in mature cilia and in the cortical cytoskeleton. This was found in both untreated (30°C) and heat-shocked cells (all lanes in Fig. 3 are from cells grown at 30°C). The pAb 73 serum recognized hsp73 in isolated cilia, and hsps 72, 73 and 78 in cortical preparations (Fig. 3). The mAb AC10 recognized hsp82 in cortical preparations, as seen in Fig. 3. In addition, we found that the 82 kDa band was visible after long exposures in western blots of mAb AC10 and pAb 82-1 against cilia. The fluorescence patterns seen in *Tetrahymena* cells with these antibodies offered partial confirmation of these observations. In addition to a strong endoplasmic staining (Fig. 6B), the presence of these high molecular mass heat shock proteins could be detected in the cortex with all antibodies used here under favorable conditions, again at both 30°C and 41°C. The best images were obtained with pAb 73 (Fig. 6C) and mAb 9C10 (Fig. 6D). The latter shows that hsp82 is in the basal bodies, or in something closely associated with basal bodies. Cilia, on the other hand, were rarely seen in immunofluorescence preparations using these antibodies. Occasionally, however, oral cilia were stained in cells treated with the polyclonal antibodies directed against *Tetrahymena* hsp82.

The association of *Tetrahymena* hsp73 and hsp82 with ciliary microtubules specifically was established by blotting purified outer doublet protein with pAb 73 and pAb 82-1. The association of hsp73 with cortical microtubules can be inferred from the different responses of the two types of cytoskeletal preparations. The high-salt preparation has no microtubules (Williams and Honts, 1995) and blotted at the 72 kDa and 78 kDa positions with pAb 73, whereas the low-salt cytoskeletal preparation containing abundant microtubules (Williams and Honts, 1995), blotted at the 73 kDa position with this antiserum (Fig. 3). The low-salt cytoskeletal preparation gave a weak reaction at the hsp82 kDa position with mAb AC10 (Fig. 3) and pAb 82-1. We tentatively conclude that mature ciliary and cortical microtubules are associated with significant amounts of hsp73 and with lesser amounts of hsp82. The site-specific distribution of some of the heat shock proteins studied here is also of interest. In particular, large amounts of hsp82, 72, and 78 were found in unknown components of high salt cortical residues, a fraction that is devoid of microtubules and of hsp73.

It was reported earlier that hsp70 is present in the flagella of *Chlamydomonas* (Bloch and Johnson, 1995). This is consistent with our finding hsp73 in the cilia of *Tetrahymena*. We are not aware of a report other than ours of hsp90-class proteins in eukaryotic cilia or flagella. Both hsp70 (Green and Liem, 1989; Ahmad et al., 1990) and hsp90 (Redmond et al., 1989; Czar et al., 1996) have been reported to be intimately associated with microtubules in mammalian cells. The authors of these reports have suggested that these chaperones may play a direct role in the in vivo assembly and/or functioning of cytoplasmic and mitotic microtubules. It would appear that the same suggestion can be made for the more stable microtubules of cilia, flagella, and the ciliate cortex.

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