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Chaperonin polymers in archaea: The cytoskeleton of prokaryotes?

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Chaperonins are protein complexes that play a critical role in folding nascent polypeptides under normal conditions and refolding damaged proteins under stress conditions[1-4]. In all organisms these complexes are composed of evolutionarily conserved 60-kDa proteins arranged in double-ring structures with between 7 and 9 protein subunits per ring[5-7]. These double ring structures are assumed to be the functional units in vivo[1, 8, 9], although they have never been observed inside cells. Here we show that the purified chaperonin from the hyperthermophilic archaeon *Sulfolobus shibatae,* which is closely related to chaperonins in eukaryotes[10-12], has a double ring structure at low concentrations (0.1 mg/ml), but at more physiological concentrations, the rings stack end to end to form polymers. The polymers are stable at physiological temperatures (75 °C) and closely resemble structures observed inside unfixed *S. shibatae* cells. We suggest that in vivo chaperonin activity may be regulated by polymerization and that chaperonin polymers may act as a cytoskeleton-like structure in archaea and bacteria.

The *S. shibatae* chaperonin, known as the rosettasome[12], was purified at room temperature by chromatography and density gradient centrifugation and analyzed by transmission electron microscopy (TEM) (Fig. 1). At a concentration of 0.1 mg/ml the rosettasomes had a typical double-ring appearance[10] (Fig. 1A). At 0.5 mg/ml, however, many of the double rings were stacked, primarily end to end, to form short polymers (Fig. 1B), and at 1.0 mg/ml these polymers were longer and frequently aligned side by side to form bundles (Fig 1C). The polymers required Mg^{++}, indicated by their absence in 1.0 mg/ml samples that lacked $MgCl_2$ and KCl (Fig. 1D), their continued absence when KCl was added to these samples (Fig. 1E), and their reappearance when $MgCl_2$ was added (Fig. 1F). To determine if these polymers were stable at physiological temperatures for *S. shibatae,* which normally lives in geothermal hot springs[13], we exposed them to 75 °C for up to 3 h. The network of

branched polymers was stable at this temperature and micrographs were indistinguishable from those taken at room temperature (Fig. 2). This stability at physiological temperatures suggests that these polymers may exist in vivo.

It is known that the constituent proteins of the rosettasome (α and β subunits) are among the most abundant proteins in *S. shibatae* [12, 13]. To determine if their intracellular concentrations are conducive to polymer formation, we compared measured amounts of pure proteins with extracts from specific numbers of cells (Fig. 3) and used measurements from scanning electron micrographs of whole cells (n = 100) to determine cell volumes. We thereby calculated an intracellular rosettasome concentration of 24 to 28 mg/ml, depending on the method used for protein separation (see caption Fig. 3). Since Mg^{++} is also required for polymer formation, we determined its concentration in cells using inductive coupled plasma-atomic emission spectrometry (ICP-AES). We measured 1.03 mg Mg^{++}/g (cell dry weight), which corresponds to a concentration of 12.9 mM (assuming dry weight equals 30% wet weight and 1 g wet weight = 1 ml cell vol). The intracellular concentrations of both rosettasomes and Mg^{++} are conducive to polymer formation and polymers should therefore exist inside cells unless there are specific intracellular factors preventing their formation.

We looked for polymers in *S. shibatae* cells by TEM after removing their protein surface layer (S-layer) with detergent [14, 15] and staining them with uranyl acetate. We found structures remarkably similar to the in vitro polymers in many cells (e.g. Fig. 4 A-F). These intracellular polymers do not resemble known structures in *S. shibatae*, such as S-layer arrays[15-17] or chromatin[18-20], and while they do resemble actin and tubulin filaments[21], neither of these eukaryotic cytoskeletal proteins is present in *S. shibatae*. The average width of the intracellular polymers, 11 ± 1 nm, was nearly identical to that of the in vitro polymers, 10.7 ± 0.6, and both have a distinctive and similar periodic structure. Fourier analyses of this structure revealed periodicities of approx. 12, 7, and 5 nm in the intracellular polymers and 10, 8, 5, and 4 nm in the in vitro polymers. These periodicities are not

significantly different and can be attributed to features of the rosettasomes themselves or their orientation in the polymers. The similarity between the in vitro and in vivo polymers is evident in the direct comparison shown in Figure 4G. Therefore, as expected from the intracellular concentrations of rosettasome and Mg^{++}, we conclude that the archaeal chaperonin is polymeric in vivo.

This conclusion has important consequences for understanding rosettasome function and perhaps chaperonin function in general. If we maintain that the double ring is the functional chaperonin unit as is now believed[8,9], and the end of the rings and central cavities are the sites for recognition and binding of unfolded proteins[1, 2, 7], then polymerization may block these functional sites. Cells could then regulate chaperonin activity by regulating polymerization and chaperonin activity could therefore be changed, during heat shock for example, without *de novo* protein synthesis[22]. Alternatively, if the chaperonin double rings are primarily the building blocks for polymers, and the polymers themselves are the functional units, then the role of the double rings in heat shock [8, 23] and protein folding[24] may be quite different than previously thought. The polymers and polymer bundles may be the "chaperones" for protein folding and assembly by actively binding or passively sequestering folding intermediates of proteins. Hypothesizing such a cytoskeleton-like function for chaperonin polymers predicts possible interactions with other ring structures, such as the proteosome[25], other heat shock protein polymers, such as Hsp70[26], or other macromolecule such as RNA[27, 28].

While it is generally believed that prokaryotes lack a cytoskeleton, it has been argued that archaea must have some kind of cytoskeleton based on their morphology, their resistance to osmotic shock, and the gelling properties of their cytoplasm, although cytoskeletal proteins have not been identified[29, 30]. The abundance of chaperonins in archaea (4% of total protein in *S. shibatae*, higher in other species[31]) and in bacteria (1-7% of total protein[32]) is comparable to that of the major cytoskeletal proteins (tubulin and actin) in eukaryotes. We have demonstrated that an archaeal chaperonin forms polymers in vitro and that similar polymers are

present in vivo. Others have demonstrated that the bacterial chaperonin (GroEL), in conjunction with its co-chaperonin (GroES), also forms polymers in vitro[33] and that protein polymers are present in some bacteria that are immunologically cross reactive with antibodies against GroEL[34,35]. In addition, the archaeal chaperonins share nearly 40% amino acid sequence identity with a family of eukaryotic proteins known as TCP1's[10, 12, 36] that interact with tubulin and actin in vitro[37,5] and are essential for the normal development and function of the cytoskeleton in vivo[38-40]. These different lines of evidence support the hypothesis that chaperonins may have a cytoskeletal function in prokaryotes. In general, our findings provide a new perspective on the role of chaperonins in vivo.

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Figure Captions

Fig. 1: The *Sulfolobus shibatae* chaperonin (rosettasome) polymerizes at higher concentrations in the presence of Mg^{++}. In a buffer containing 20 mM HEPES (pH 7.5), 10 mM KCl, and 10 mM $MgCl_2$ (HKM buffer) double rings predominate at rosettasome concentrations of 0.1 mg/ml (A); rings and short chains were seen at 0.5 mg/ml (B); and long chains and bundles of polymers were present at 1.0 mg/ml (C). Chains and polymers did not form in 4.7 mM HEPES (pH 7.5) containing 24 μM EDTA & DTT (D); or when 10 mM KCl was added (E); but did form when 10 mM $MgCl_2$ was added (F).

Method: Rosettasomes were purified from *S. shibatae* cells grown at 75 °C in standard yeast extract medium[13]. Cells were opened by sonication in the presence of three volumes HKM buffer, extracts were treated with DNase at room temp. for 30 min and then centrifuged at 30,000 rpm for 30 min in 50.2 Ti rotor (Beckmann). The supernatant was applied to a DEAE-sepharose column equilibrated in HKM buffer and proteins were eluted in a 0 to 1 M NaCl gradient. Rosettasome containing fractions were determined by SDS-PAGE[10] and further purified by Mono-Q (Pharmacia). Protein concentration was determined by DC Protein Assay system (BioRad) using BSA as a standard. Different concentrations of freshly prepared protein samples (polymers nearly disappeared in samples stored at 4 °C for 1 week) were attached to lacy carbon grids with ultra thin formvar (Ladd Scientific), stained with 2% uranyl acetate for 3 min, air dried, and viewed in a Philips EM420T or CM30T with LAB6 filaments at 80 to 300 kV. No changes in the microstructure of samples was observed at the working resolution with electron doses of ~1 to 200 electrons/Å^2. Micrographs were taken within this dose range at defocuses of -200 to -800 nm with illumination-convergence angles of ~1 mR and scattering angle of 5 mR using a room temperature, double tilt, berylium stage. Micrographs were digitized using a flat bed, 10 bit, 1200 DPI scanner (Powerlook Pro,

UMAX) and data processing was done on Macintosh work stations with the programs NIH Image[41] and Adobe Photo Shop.

Fig. 2: Comparison of the network of polymers at room temperatures and after a 3 h exposures to 75 °C (insert). In both heat treated and control samples bundles of polymers were present, consisting of double rings primarily stacked end to end (side views) and with some rings (end views) also visible.
Method: Rosettasome samples at 2 mg/ml were polymerized at room temperature in HKM buffer and exposed to 75 °C for 0, 1, 2, and 3 h in a programmable heating block (Hybaid, OmniGene). Samples removed from the heating block were immediately processed for TEM as described (Fig 1).

Fig. 3: Abundance of rosettasome in *S. shibatae* cells. The Coomassie-stained protein bands from 1.0 to 2.6 µg of rosettasome (Pure protein), total proteins from 1 to 4 x 10^8 cells (Cell extracts), and from a molecular mass standard (kDa) on a denaturing (SDS) polyacrylamide gel. The plot of the stained bands (area x intensity) vs known amounts of pure protein was used to calculate the amount of rosettasome in cells (insert).
Method: The concentration of *S. shibatae* cells in an actively growing culture was determined by direct counts on a haemocytometer (Neubauer). Volumes containing specific numbers of cells centrifuged at 10,000 RPM room temp. for 3 min (Hermle microfuge) and cell pellets were lysed by the addition of SDS-buffer (final conc. 10% glycerol, 5% ß-mercaptoethanol, 3% SDS, 65 mM Tris-HCl pH 6.8, 0.001% bromophenol blue). Extracts from different numbers of cells and samples containing varying amounts of pure rosettasome (see caption Fig. 1) were applied to a 10% denaturing polyacrylamide gel and proteins were separated by electrophoresis. After staining with Coomassie brilliant blue (R280), gels were scanned (Powerlook Pro scanner, UMAX) and the digitized areas and intensities of stain associated with rosettasome bands was determined. The linear plot derived from varying amounts of pure proteins was used to estimate the absolute amount of rosettasome in

cells. Concentrations were determined by correcting for cell volumes, which were calculated using the formula for a sphere and cell diameters measured from electron micrographs (n = 100). The rosettasome concentration of 24.4 mg/ml obtained using this procedure was corroborated (27.9 mg/ml) by two dimensional gel electrophoresis (data not shown).

Fig. 4: Transmission electron micrographs of unfixed *S. shibatae* cells with their protein surface layer removed. Whole cells (A, C, E) and magnified regions (B, D, F) show polymer structures (arrowheads). Comparison of in vitro and in vivo polymers (G).
***Methods*:** Cells (1 ml) in mid-log phase growth at 75 °C were removed from medium by centrifugation (30 sec. 12,000 rpm, table top centrifuge), washed and resuspended in 50 µl HKM buffer (Fig.1). Samples (8 µl) were placed on formvar lacy grids (Ladd Scientific) and 2 µl of 10% triton X100 was added. Triton X100 was removed by repeatedly washing in HKM buffer before a DNase (final. conc. 0.1 unit/µl, Promega) treatment for 10 min. Grids were washed again in HKM buffer, stained for 3 min in 2% uranyl acetate, and air dried. All solutions were 0.22 µm filtered. Transmission electron micrographs were done as described in Fig. 1. Fourier analysis was done on digitized images using the FFT implementation in NIH Image[42] and all dimensions are given for center to center measurements of specific features.

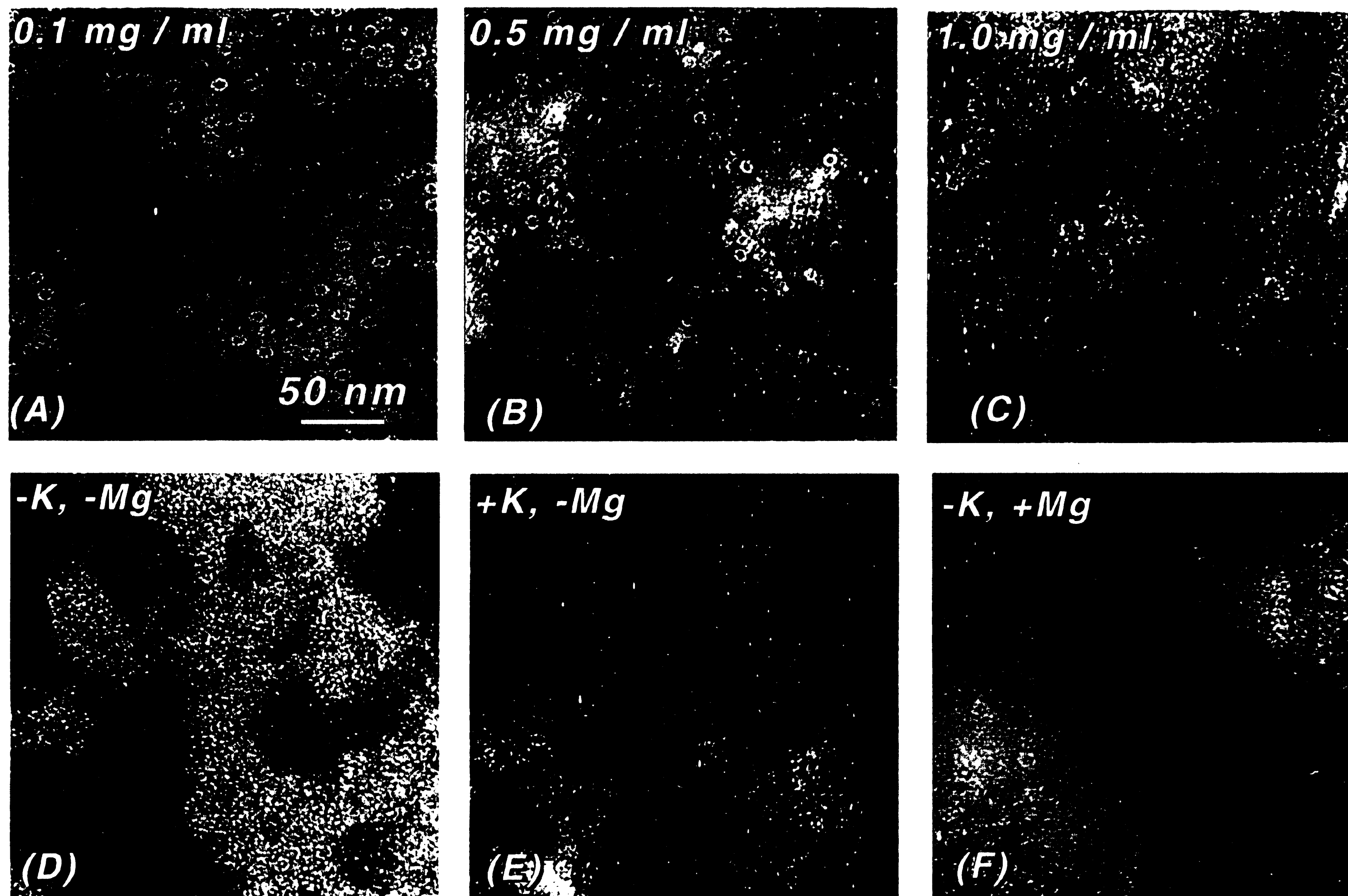
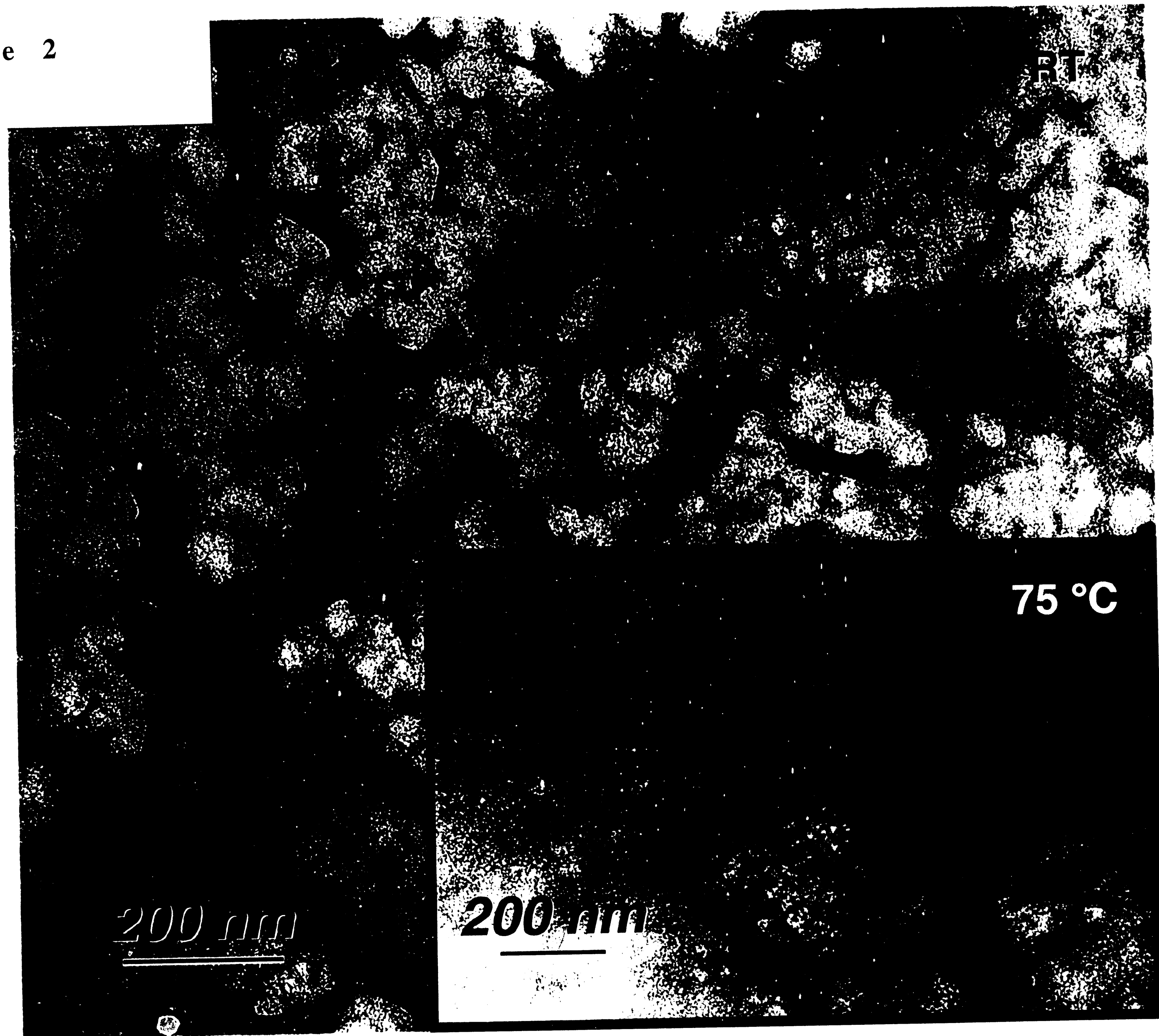


Figure 1

Figure 2



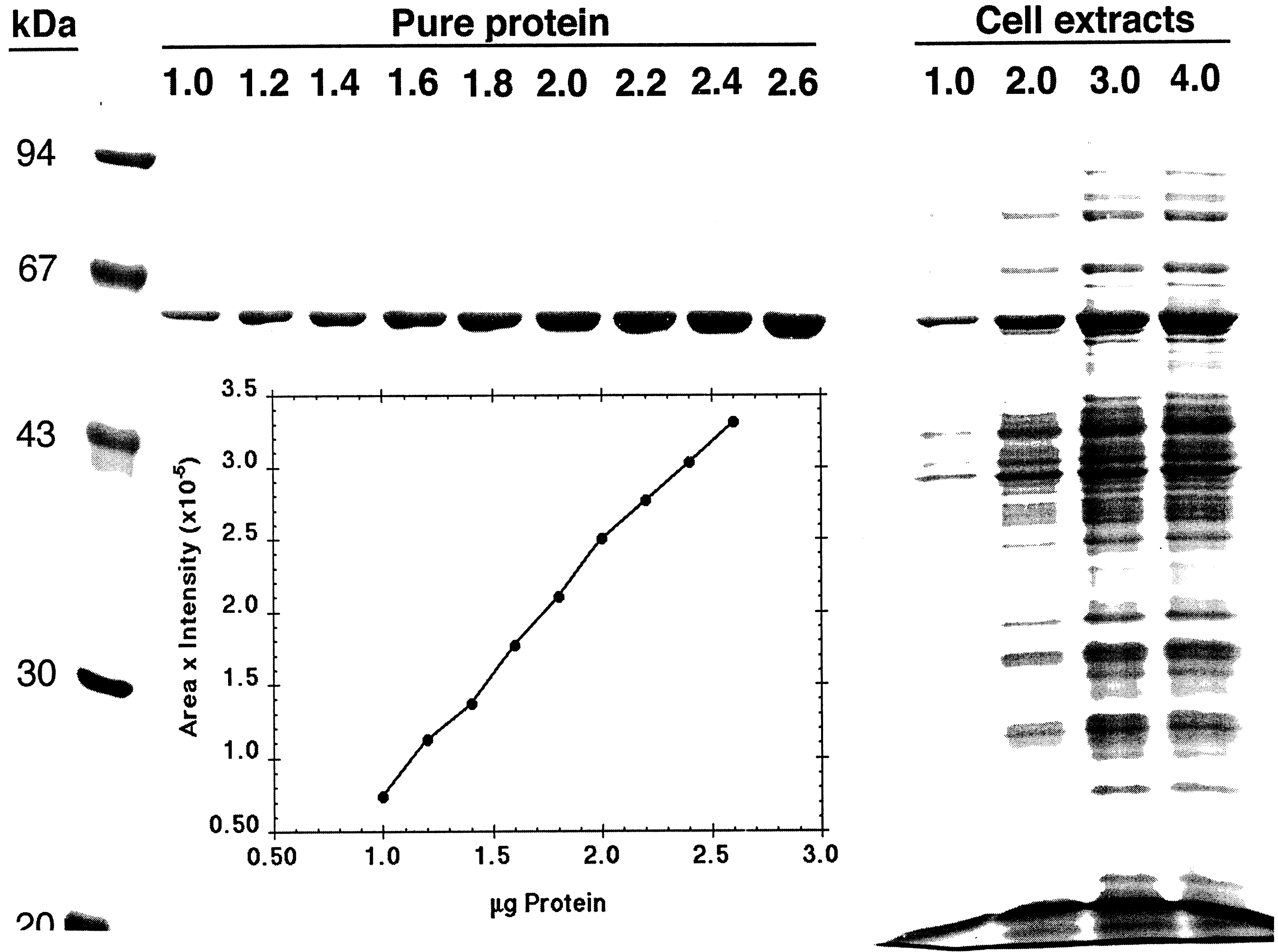


Figure 3

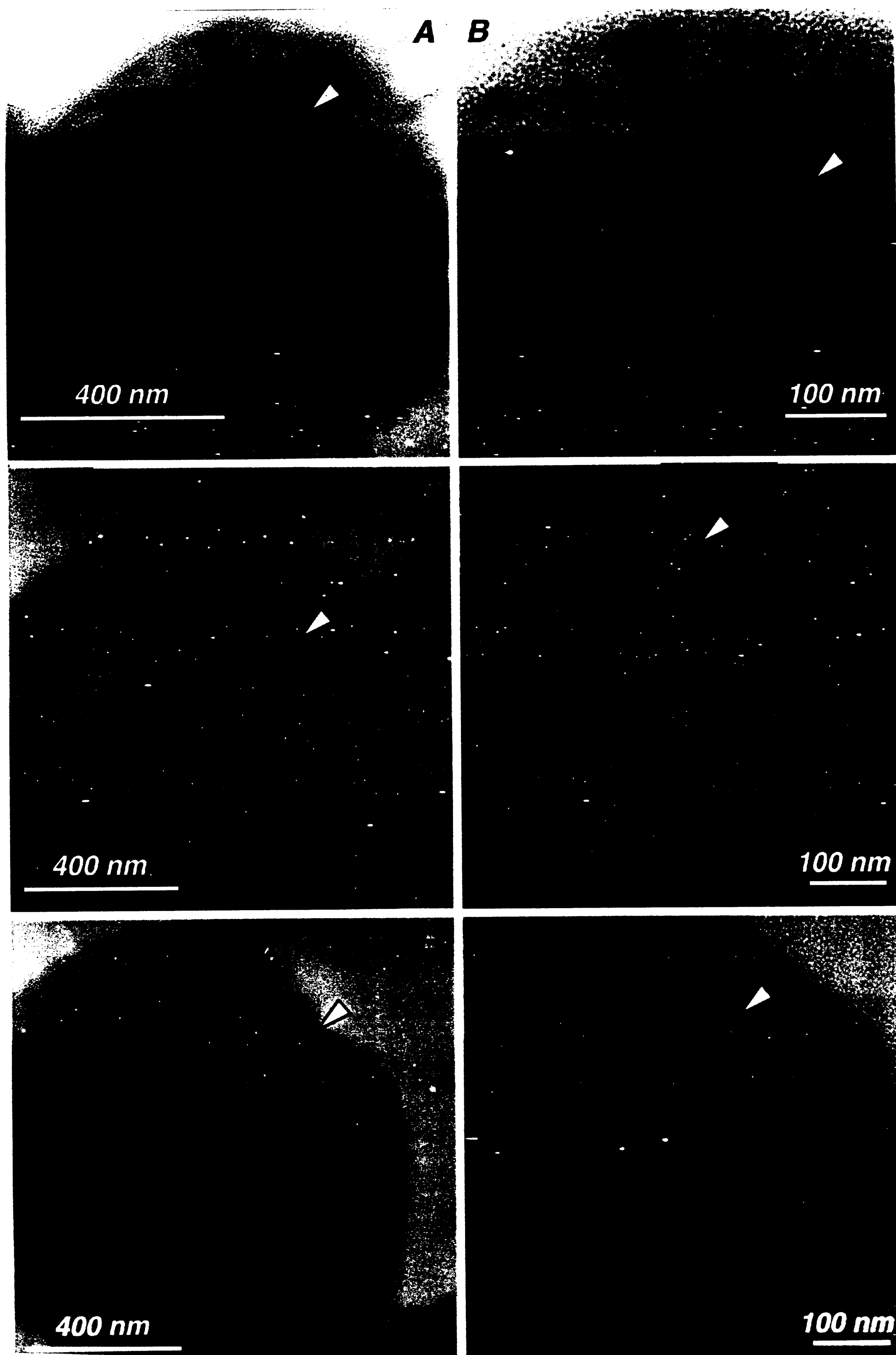


Figure 4

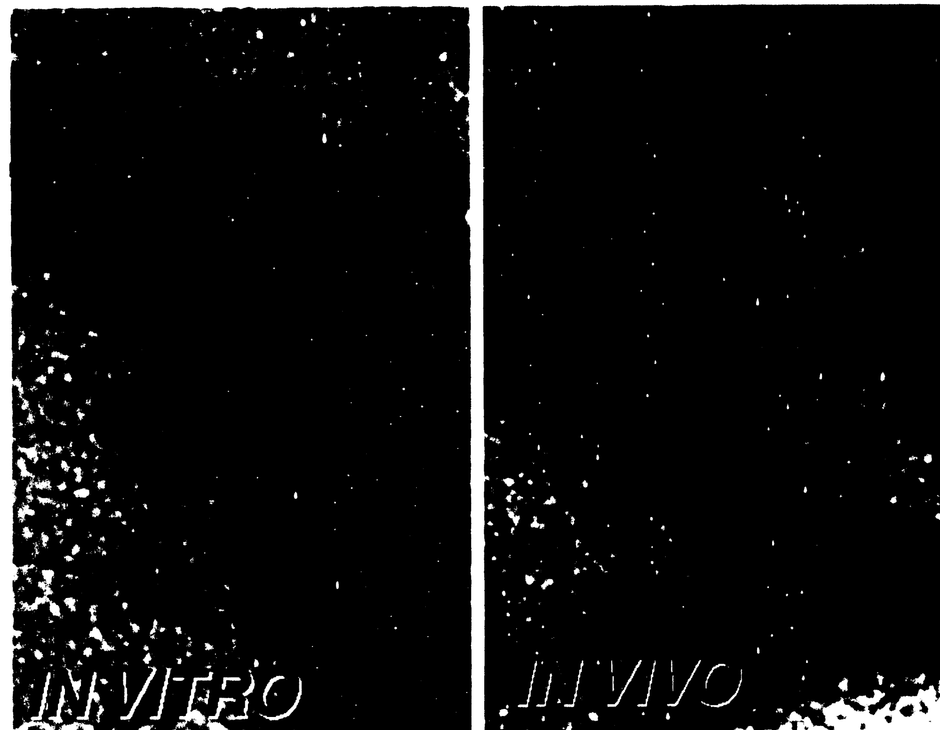


Figure 4G