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COCHAPERONINS ARE HISTONE-BINDING PROTEINS

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Cochaperonins (cpn10) assist chaperonins (cpn60) in mediating folding of polypeptide substrates in an ATP-dependent reaction. Moreover, they have been shown to be secretory products of living cells and to perform discrete biological activities without the need to interact with cpn60. Here, we have investigated the possible existence of cellular cpn10 binding sites that could mediate such activities. For this purpose, we performed binding studies with iodinated cpn10 on whole cells and on electrophoretically separated eukaryotic cell lysates. The former studies yielded negative results, whereas in the latter binding to several proteins was detected. These proteins were identified as being histones. Binding was observed to all core histones (H2A, H2B, H3 and H4) and, although weaker, to the linker histone H1 as well. These results show that cpn10 are histone-binding proteins.

Molecular chaperones are proteins that bind to and stabilize conformers of other proteins thereby facilitating their correct fate *in vivo* (1). Many chaperones have been termed heat-shock proteins because their production is greatly enhanced during the cellular response to elevated temperatures (2).

One of the most studied families of molecular chaperones is the chaperonin (cpn) complex which consists of a cpn of 55-65 kDa subunit size (cpn60) that exists as a 14-subunit oligomer with double toroid appearance in the electron microscope (3,4) and of a heptameric co-chaperonin of 10 kDa (cpn10) subunit size (3). These two proteins cooperatively mediate ATP-dependent protein folding (5-7) and constitute a highly conserved family of proteins present in bacterial cytosol, mitochondria and chloroplasts. More recent work has shown, however, that cpn10 is able to induce discrete biological activities that are apparently unrelated to its role in polypeptide folding without the need to interact with cpn60 (8). Moreover, both prokaryotic and eukaryotic cpn10 have been shown to be secretory products (8-10). Altogether these results suggest the possibility that cpn10 can interact with specific binding sites different from cpn60. In order to investigate this possibility we sought cellular target structures for cpn10 different from cpn60.

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In the present work we have performed binding studies with iodinated cpn10 on whole cells and on electrophoretically separated eukaryotic cell lysates and found them to interact with cellular targets that were identified as histones.

MATERIALS AND METHODS

Cells and reagents. Thymocytes were obtained from minced thymuses isolated from Lewis rats (120-150 grams, Charles River, Monza, Italy). Recombinant *Mycobacterium tuberculosis* cpn10 and human cpn10 (11) were obtained by high-level expression of their corresponding cDNA in *Escherichia coli* and subsequent purification to homogeneity from bacterial cell lysates by a single step reversed-phase HPLC (G. Legname et al., manuscript in preparation). Human serum was obtained from a healthy donor and mouse serum from a Balb/c mouse (Charles River). Purified calf thymus histones (H1, H2A, H2B, H3, H4) and GroES were obtained from Boehringer Mannheim (Germany), ribonuclease A from Merck (Darmstadt, Germany) and lysozyme from Sigma (St. Louis, MO, USA). All other chemicals and reagents used were of the highest grade obtainable.

Preparation of [^{125}I]cpn10. Recombinant *M.tuberculosis* cpn10, human cpn10 or GroES were dissolved in phosphate-buffered saline (PBS) and iodinated with carrier-free Na[^{125}I] (DuPont NEN, Cologno Monzese, Milano, Italy) at room temperature for 15 min using the Iodogen reagent (Bio-Rad, Richmond, CA, USA). Free [^{125}I] was removed on PD10 desalting columns (Bio-Rad, Richmond, CA, USA) equilibrated with PBS-0.5% bovine serum albumin (BSA). The radiospecific activity of the [^{125}I]cpn10 was 30-50 μCi/μg of protein as determined by trichloroacetic acid precipitation of unfractionated [^{125}I] cpn10.

Electrophoretic methods. SDS-PAGE was carried out on 15% acrylamide gels according to Laemmli (12) with samples that had been boiled in 0.0625M Tris-HCl, pH 6.8 with 2% SDS, 10% glycerol and 5% 2-mercaptoethanol. The proteins were transferred to nitrocellulose membranes by electroblotting for 2 hours at 250mA or overnight at 25mA. During electrophoresis and transfer gels were cooled at 4°C. Membranes were then blocked with 3% BSA in PBS for 1 hour at room temperature.

Ligand blotting. Blocked membranes were incubated with [^{125}I] cpn10 ($2x10^6$ cpm/lane, 10^{-10}M) in PBS, 0.1% BSA at room temperature under constant agitation. After 4 hours membranes were extensively washed with PBS, 0.2% Tween 20, 0.1% BSA and exposed overnight at -80°C on a XAR Kodak film.

Solid-phase binding assay. 96-well microtiter plates (EIA plates, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0.5 μg/well of purified histones in 0.1M carbonate buffer, pH 9.6. After saturation with PBS, 1% BSA for 1 hour at 37°C, plates were incubated with [^{125}I]*M.tuberculosis* cpn10 ($8.7x10^{-9}$M in 100 μl PBS, 0.1% BSA) for 4 hours at room temperature in the absence or presence of 50 μg/ml of soluble histones or lysozyme. At the end of the incubation plates were washed 4 times with 0.2% Tween 20, 0.1% BSA and 100 μl/well of 1M NaOH were then added. After 1 hour at room temperature samples were harvested and radioactivity was measured in an LKB γ-counter.

RESULTS

Identification of binding sites for *M.tuberculosis* cpn10. Initial binding experiments performed with [^{125}I]*M.tuberculosis* cpn10 on whole cells (mouse spleen cells, K562 erythroleukemia cells and P19 teratocarcinoma cells) of different origin had yielded negative results (data not shown). On the other hand, binding sites were detected when the ligand was tested on cell lysates. The binding of [^{125}I]*M.tuberculosis* cpn10 to cell lysates was determined in ligand blotting experiments. The corresponding autoradiography obtained in an experiment performed on a rat thymocyte lysate is shown in Fig. 1. [^{125}I]*M.tuberculosis* cpn10 identified several bands of Mr 10-17kDa and, more weakly, a band of Mr ~ 33kDa. A similar pattern of bands was identified on all other eukaryotic cell lysates tested (rat splenocytes, rat

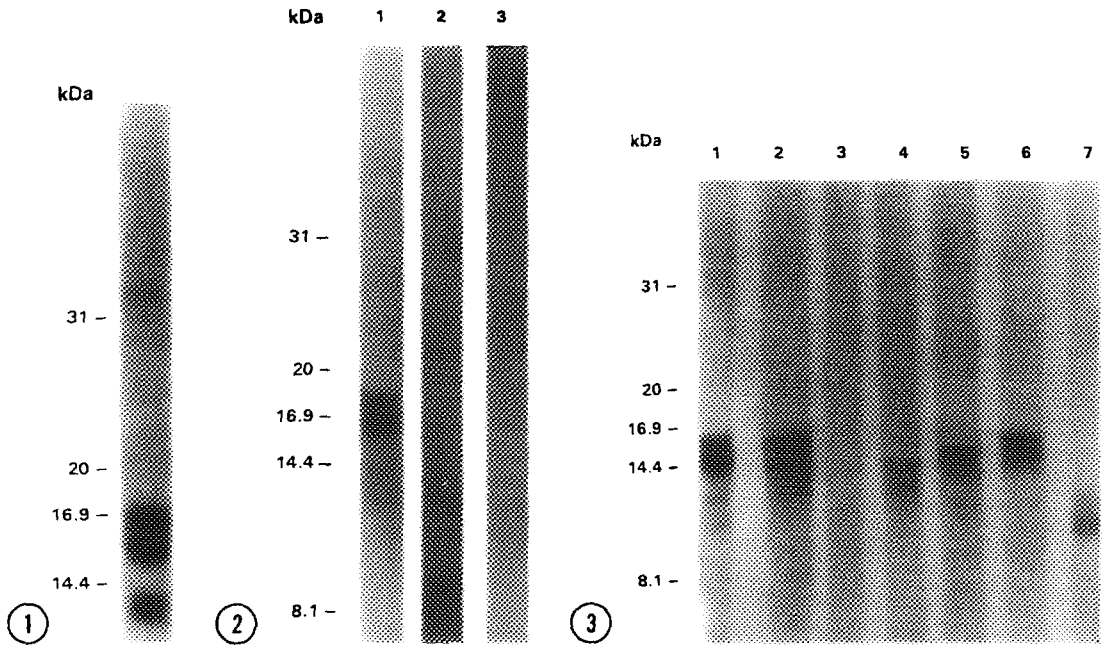

Figure 1. Ligand blotting of [^{125}I]*M.tuberculosis* cpn10 on a rat thymocyte lysate. A lysate derived from 2x10^6 thymocytes was separated by SDS-PAGE, transferred onto nitrocellulose filters and tested for binding of [^{125}I]*M.tuberculosis* cpn10.

Figure 2. Ligand blotting of [^{125}I]*M.tuberculosis* cpn10 on sera. A lysate from 2x10^6 thymocytes (lane 1), human serum (lane 2) or mouse serum (lane 3; for each 150 μg proteins/lane, corresponding to the protein content of 2x10^6 thymocytes) was separated by SDS-PAGE, transferred onto nitrocellulose filters and tested for binding of [^{125}I]*M.tuberculosis* cpn10.

Figure 3. Ligand blotting of [^{125}I]*M.tuberculosis* cpn10 on histones. A lysate from 2x10^6 thymocytes (lane 1), a pool of purified histones (lane 2; H1, H2A, H2B, H3 and H4, 10 μg of each) and single purified histones (lane 3, H1; lane 4, H2A; lane 5, H2B; lane 6, H3; lane 7, H4; 10 μg of each/lane) were separated by SDS-PAGE, transferred onto nitrocellulose filters and tested for binding of [^{125}I]*M.tuberculosis* cpn10.

hepatocytes, murine and human cell lines, results not shown). On the other hand, no binding was observed to mouse or human serum that were tested under similar conditions (Fig. 2).

Identification of the binding sites as histones. On the basis of the above results that suggested an intracellular location of the binding sites and on the Mr of the bands that had been identified, we argued that the bands could be histones. This hypothesis was confirmed by ligand blotting experiments performed with [^{125}I]*M.tuberculosis* cpn10 on purified histones. Fig. 3 shows that the proteins recognized by [^{125}I]*M.tuberculosis* cpn10 in the thymocyte lysate migrate at a Mr corresponding to that of core histones (H2A, H2B, H3 and H4), while no binding to the linker histone H1 was observed. In other experiments, weak binding was observed to this histone (apparent Mr ~ 33kDa) as well (see Fig. 1).

The observed interaction between acidic *M.tuberculosis* cpn10 (pI 4.5) and basic histones suggested the possibility that the binding was solely the result of electrostatic interactions between oppositely charged proteins. In order to test this possibility we evaluated the binding of [^{125}I]*M.tuberculosis* cpn10 to two highly basic proteins (lysozyme, pI 11 and ribonuclease A, pI 9) under the same experimental conditions as above, but no binding to these two proteins could be detected (data not shown). In addition, we tested if *M.tuberculosis* cpn10 could bind to native histones, i.e. histones that had not been subjected to denaturing SDS-PAGE. For this purpose we studied the binding of [^{125}I]*M.tuberculosis* cpn10 to native core histones that had been immobilized onto solid phase. Fig. 4 shows that also under these conditions binding to all core histones was detected and that it could be displaced by an excess of free histones in solution but not, or only marginally, by an unrelated, highly basic protein (lysozyme).

To investigate if the observed interaction was specific for *M.tuberculosis* cpn10 or could be extended to other cpn10 as well, we tested, in ligand blotting, two other cpn10, *E.coli* GroES and human cpn10. Fig. 5 shows a pattern of binding for [^{125}I]GroES that is similar to that observed with [^{125}I]*M.tuberculosis* On the other hand, nothing can be said for human cpn10 because it proved to be a sticky protein and to bind nonspecifically to the nitrocellulose membranes (result not shown).

DISCUSSION

In the present report we show that cpn10 from two different prokaryotic species (*M.tuberculosis* cpn10 and *E.coli* GroES) are histone-binding proteins. They were found to bind to all 4 core histones (H2A, H2B, H3 and H4). Binding to the linker histone (H1) was generally weaker and varied from experiment to experiment.

With regard to the specificity of the observed interaction we performed experiments in order to exclude that it was solely due to electrostatic interactions between acidic proteins (*M.tuberculosis* cpn10 and GroES) and basic proteins (histones). In fact, no binding of *M.tuberculosis* cpn10 to two other highly basic proteins (lysozyme and ribonuclease A) was observed. Moreover, the same cpn10 was found to bind both to histones that had undergone treatments in denaturing conditions as well as to histones in native conditions. Altogether, these results suggest that cpn10 recognition of histones is based, at least in part, on discrete

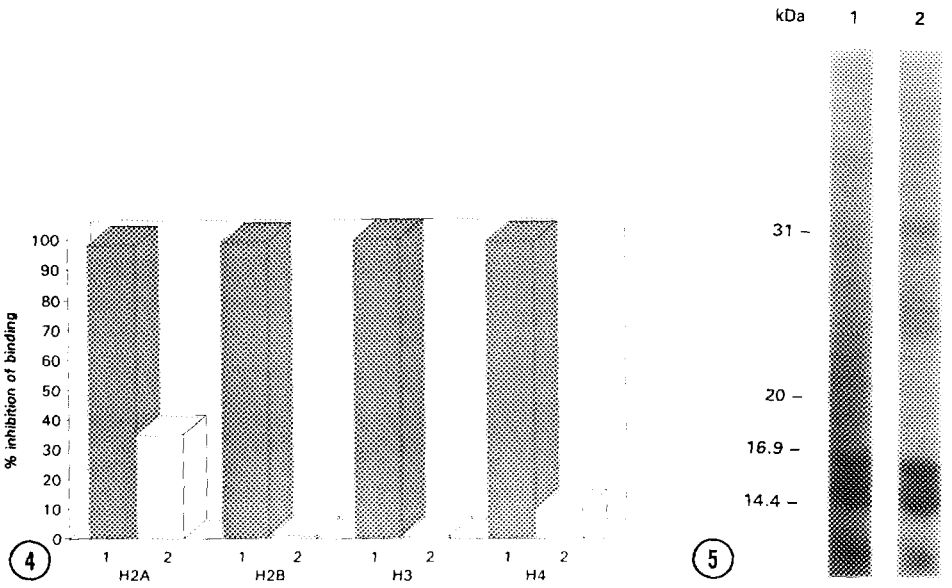

Figure 4. Binding of [^{125}I]*M.tuberculosis* cpn10 to native histones. Single, purified core histones were immobilized onto solid phase. [^{125}I]*M.tuberculosis* cpn10 (100.000 cpm/well, $8{,}7x10^{-9}$M) was added and binding was measured in the absence or in the presence, in solution, of a 100-fold molar excess of the same histone that been immobilized (1) or of lysozyme (2). Values are given as percent inhibition of specific binding. Specific binding is total binding minus binding in the absence of immobilized histone. Specific binding values were as follows: for H2A, 2592 cpm; for H2B, 2354 cpm; for H3, 2086 cpm; for H4, 3668 cpm.

Figure 5. Ligand blotting of [^{125}I]GroES on a rat thymocyte lysate. A lysate from $2x10^6$ rat thymocytes was separated by SDS-PAGE, transferred onto nitrocellulose filters and tested for binding of [^{125}I]*M.tuberculosis* cpn10 (lane 1) and [^{125}I]GroES (lane 2).

domains (either linear or conformational, the latter case supposing that the histones undergo renaturation upon transfer to nitrocellulose membranes) of the histone molecules. The identity of these domains is at present unclear because there is no significant overall sequence homology between the different histone molecules that are bound by the cpn10. The observation, however, is not unprecedented since other histone-binding proteins (e.g. serum amyloid P-component, ref. 13) have been found to possess a similarly broad specificity.

We have also tested human cpn10 for its capacity to bind to histones. This protein, however, proved to be sticky and to bind nonspecifically to the membranes used for the ligand blotting experiments (data not shown). Therefore, nothing can be said, at present, with regard to the possibility that eukaryotic cpn10 as well have the potential to interact with histones.

As to the possible consequences of the described interaction in living cells, one may anticipate possible effects on the regulation of gene expression. Thus, other histone-binding proteins and post-translational modifications of histones have been shown to tune the function of nucleosomes in repressing or facilitating access of the transciptional machinery to individual genes (14). These considerations could be of particular relevance for *M.tuberculosis* cpn10 for

several reasons. First, *M.tuberculosis* cpn10 is a major secretory product of this microorganism in early bacterial cell cultures (8-10). Second, *M.tuberculosis* infects cells of the monocyte-macrophage lineage (15) where mycobacterial cpn10 is probably produced and secreted in much larger amounts then in cell-free cultures because the microorganism is exposed to the action of host cell-derived oxygen radicals and other inducers of the stress response (16,17). Therefore, considering the intracellular location of this microorganism, it is conceivable that secreted cpn10 may interact with histones of the host cell thereby influencing its gene expression and, possibly, contributing to the pathogenesis of the disease. This interaction could take place either directly in the cytoplasm or, upon translocation of the cpn10 into the nucleus, with histones incorporated in nucleosomes. Consistent with this hypothesis and with the results presented in this report is a recent paper that describes an association between host-derived histones and *M.leprae* (18). On the basis of our findings, the possibility that this interaction could be mediated, at least in part, by *M. leprae* cpn10 deserves consideration.

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