RESEARCH COMMUNICATION

Heat-inducible proteins that react with antibodies to chaperonin60 are localized in the nucleus of a fish cell line

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We report in the present paper that proteins which react with a polyclonal antibody (pAb) raised against the heat-shock protein chaperonin60 (cpn60) were revealed by indirect immunofluorescence in the nucleus of a fish (fathead minnow, *Pimephales promelas*) cell line after heat-shock. This immunoreactive cpn60 associated with the nucleolus and with discrete foci. An increased abundance of two nuclear proteins of approx. 57 and 42 kDa, present in approximately equal amounts, was detected by Western blotting using an anti-cpn60 pAb as a probe during the same time period that cpn60 was revealed in the nucleus. These

proteins also reacted with a monoclonal antibody (mAb) against human cpn60 but did not react with an mAb against the cytoplasmic chaperonin, TCP1. The kinetics of translocation and pattern of nuclear localization of this immunoreactive cpn60 differed from that of stress70, another major family of heat-shock proteins. We suggest that these nuclear immunoreactive cpn60 proteins are members of the cpn60 family and that they play a chaperone role in folding and assembly of proteins in the nucleus which is distinct from that of stress70.

INTRODUCTION

Two families of chaperonins have been shown to form ringed structures that facilitate protein folding and assembly in different compartments of eukaryotic cells (Horwich and Willison, 1993; Saibil et al., 1993). The chaperonin60 (GroEL/cpn60) family, found in bacteria as well as in the mitochondria and chloroplast of eukaryotes, are major heat-shock proteins (McMullin and Hallberg, 1988; Ellis, 1990). In contrast, members of the TF55-TCP1 family appear to be part of a hetero-oligomeric folding complex which carries out folding functions in the cytoplasm (Trent et al., 1991; Gao et al., 1992; Lewis et al., 1992; Yaffe, 1992). No functionally analogous chaperonin has yet been identified in the nucleus.

In the present study we demonstrate that proteins which react with a polyclonal antibody raised against moth (*Heliothis*) cpn60 (Miller, 1987) were revealed by indirect immunofluorescence in the nucleolus, and as discrete foci in the nucleus of a fish cell line after heat-shock. Additional studies are described which (i) further characterize this immunoreactive cpn60, (ii) independently confirm this nuclear localization and (iii) compare the kinetics of translocation and nuclear localization of cpn60 with that of stress70, another major heat-shock protein involved in the folding, protection and repair of proteins which also migrates into the nucleus after heat-shock (Lindquist, 1986; Welch and Feramisco, 1984).

MATERIALS AND METHODS

Indirect Immunofluorescence

Fathead-minnow (*Pimephales promelas*, A.T.C.C. CCL 42) epithelial cells were grown on coverslips in CRCM medium (Sigma) supplemented with 10% (v/v) calf serum. Cells were subjected to a 38 °C/30 min heat-shock treatment and returned

to 24 °C for recovery. Non-heat-shocked cells and cells collected at 0, 2, 4, 8, 12, 16 and 24 h after heat-shock were fixed in ice-cold acetone/methanol (1:1, v/v), rinsed in PBS and then probed with either anti-stress70 or anti-cpn60 antibody. The anti-(moth cpn60) polyclonal antibody (pAb), which had been shown previously to cross-react with this species (Dyer et al., 1993), was used at a 1:100 dilution, and a 1:100 dilution of goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) was used to detect the antibody probe. The anti-(human stress70) pAb (Ehrhart et al., 1988) was used at a 1:500 dilution, and a 1:40 dilution of a goat anti-rabbit antibody conjugated with FITC was used to detect the antibody probe. As a cytoplasmic control, an actin mAb (Sigma) was used at a 1:500 dilution and a 1:500 dilution of goat anti-rabbit antibody conjugated with FITC was used to detect the antibody probe. The coverslips were then mounted using Citifluor glycerol from Ted Pella Inc. (Redding, CA, U.S.A.). Cells were examined and photographed in colour using a Nikon UFX-II optishot microscope. The colour photographs were scanned on a Kodak Coloredge 1550 scanner and imported via an EFI Postscript Interpreter. Black-and-white thermal-dye-transfer prints were printed on a Kodak XL 7700 digital continuous tone printer.

Cellular fractionation

Fathead-minnow cells were subjected to a 38 °C/30 min heat-shock treatment and collected at 0, 1, 2, 3, 4, 6, 8, 10, 12, 13, 16, 18, 20, 22 and 24 h after heat-shock. Controls were maintained at 24 °C. Cells were homogenized in Nonidet P40 lysis buffer [10 mM Tris (pH 7.4)/10 mM NaCl/3 mM MgCl₂/0.4% (v/v) Nonidet P40] and the nuclear fraction was separated from postnuclear supernatant (PNS) by centrifugation at 300 g for 5 min at 4 °C. Microscopy confirmed the integrity of the nuclei. The total protein concentration in each fractionated sample was

Abbreviations used: pAb, polyclonal antibody; mAb, monoclonal antibody; cpn60, chaperonin60; FITC, fluorescein isothiocyanate; PNS, postnuclear supernatant; snRNP, small nuclear ribonucleoprotein.

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Figure 1 Indirect immunofluorescence micrographs of fish epithelial cells probed with moth anti-cpn60 pAb and detected by antibody conjugated with FITC

a, Cells cultured at 24 °C; b, 2 h; c, 4 h; d, 6 h; e, 8 h; f, 12 h; g, 16 h and h, 24 h after heat-shock. The scale bar equals 10 μ m.

determined by the method of Bradford (1976), using Coomassie Brilliant Blue. One- and two-dimensional SDS/PAGE were used to separate the samples, after which they were subjected to Western-blot analysis.

One-dimensional electrophoresis

The homogenate was boiled for 3 min with SDS sample buffer (Laemmli, 1970). Samples containing equal amounts of total protein (25 μ g) were loaded on a 12.5%-polyacrylamide gel with a 5% stacking gel as described by Blattler et al. (1972), using the buffer system described by Laemmli (1970). After electrophoresis the gels were analysed by Western-blotting techniques.

Two-dimensional electrophoresis

Fathead-minnow proteins were analysed by two-dimensional SDS/PAGE as described by Garrels (1979), with the modifications described by Sanders et al. (1992). After electrophoresis the gels were analysed by Western-blotting techniques.

Western blotting

Samples were separated by one- and two-dimensional electrophoresis and then electroblotted on to nitrocellulose membranes (Hybond C-extra; Amersham) as described by Towbin et al.

(1979), with the modifications described by Sanders et al. (1992). Polyclonal antibodies directed against cpn60 from the moth *Heliothis* (Miller, 1987) and against the highly conserved C-terminus of stress-70 were used as probes (Ehrhart et al., 1988), as were monoclonal antibodies (mAbs) directed against human cpn60 (Boog et al., 1992) and mouse TCP1 (Willison et al., 1989). To verify that differences in protein band density were not due to differences in transfer efficiency, an internal protein standard was loaded on to each gel. Densitometry was used to verify that the density of the standard on each gel was within 10% of the expected range for that protein standard.

RESULTS

Subcellular compartmentalization of cpn60 was revealed by indirect immunofluorescence microscopy using a pAb raised against moth cpn60 (Miller, 1987) in an epithelial-cell line of fathead minnow after a 30 min heat-shock of 38 °C (Figure 1). While immunoreactive cpn60 was present in control cells cultured at the physiologically relevant temperature of 24 °C (Figure 1a), its abundance increased within the mitochondria 2 h after heat-shock (Figures 1b–1d). By 8 h, cpn60 was less abundant in this region (Figure 1e). From 12 to 24 h after heat-shock, cpn60 was revealed in the nucleus, where it associated with the nucleolus (Figure 1g) and as discrete foci (Figures 1f and 1h). The percentage of cells with multiple foci was greatest at 12 h, where

Figure 2 Western blots of equal protein (25 μ g) from the nuclear fraction (a) and the PNS (b) which were collected over time after a heat-shock and probed with a pAb to cpn60

C indicates non-heat-shocked cells; numbers designate time (h) after a 38 °C/30 min heat-shock treatment. Arrows at the left designate prestained molecular-mass markers of 84 and 47 kDa.

it was observed in approx. 60 % of the cells, while 30 % had one foci and 10 % had no foci. At 16 h, 20 % of the cells had multiple foci, 40 % had a single discrete foci and 40 % had no foci. At 24 h only 20 % had discrete foci and the remainder were comparable with controls. These results were in contrast with the subcellular compartmentalization of actin, which was also revealed by using an anti-actin mAb and indirect immunofluorescence microscopy after a 30 min heat-shock of 38 °C. Although the abundance of this cytoplasmic protein did not increase, it became localized in the perinuclear region after heat-shock and remained absent from the nucleus for the 24 h period examined (results not shown).

Western blots of the nuclear fraction and PNS which were collected over time after a 30 min heat-shock of 38 °C and probed with anti-cpn60 (Miller, 1987) pAb corroborated the nuclear localization observed in the micrographs (Figures 2a and 2b). A protein of approx. 57 kDa was detectable in the nuclear fraction of non-heat-shocked cells. Within 2 h after heat-shock a protein of approx. 42 kDa was also detectable. The abundance of both proteins increased by 12 h after heat-shock and was abundant through the 24 h period. In contrast with these data, a protein of approx. 57 kDa was detected by the pAb in the PNS in approximately equal abundance in heat-shocked and non-

Figure 3 Western blots of equal-protein (25 μ g) subcellular fractions from cells collected 16 or 20 h after a heat-shock and probed with antibodies to cpn60

(a) A pAb to moth cpn60 was used to probe cells collected 20 h after a 38 °C/30 min heat-shock treatment. Numbers at the bottom of the blot indicate: 1, total homogenate; 2, PNS; 3, nuclear fraction; 4, yeast cpn60. Arrows at the left designate molecular-mass markers of 66 and 45 kDa. (b) A pAb to moth cpn60 was used to probe cells collected 16 h after a 38 °C/30 min heat-shock treatment. Numbers at the bottom of the blot indicate: 1, PNS; 2, nuclear fraction; 3, PNS and nuclear fraction combined. (c) A mAb to human cpn60 was used to probe cells collected 16 h after a 38 °C/30 min heat-shock treatment. Numbers at the bottom of the blot indicate: 1, PNS; 2, nuclear fraction; 3, PNS and nuclear fraction combined. Unless stated otherwise, arrows at the left designate prestained molecular-mass markers of 84 and 47 kDa. (d) Two-dimensional Western blots of the nuclear fraction (N) and PNS. Cells were either maintained at 24 °C (C) or exposed to 38 °C for 30 min and returned to 24 °C for 12 h [heat-shock (HS)]. The arrow indicates the 57 kDa protein, ◀ indicates the 63 kDa protein and ▲ indicates the 42 kDa protein.

Figure 4 Indirect immunofluorescence micrographs of fish epithelial cells probed with anti-(human stress70) pAb and detected by antibody conjugated with FITC

a, Cells cultured at 24 °C; b, 2 h; c, 4 h; d, 6 h; e, 8 h; f, 12 h; g, 16 h and h, 24 h after heat-shock. The scale bar equals 10 μ m.

heat-shocked cells. A larger protein of approx. 63 kDa was also detected in lower abundance in non-heat-shocked cells. This protein increased 2 h, and decreased 12 h, after heat-shock.

To characterize further the relative size of these proteins, the total cellular homogenate, nuclear fraction and PNS of cells collected 20 h after a heat-shock, and yeast cpn60, were run on the same gel and blotted with the moth anti-cpn60 (Figure 3a). A 57 kDa protein was detected in the total homogenate, 57 and 63 kDa proteins were detected in the PNS, and 57 and 42 kDa proteins were detected in the nuclear fraction. Yeast cpn60 co-migrated with the 63 kDa protein in the PNS. Further, when 25 μ g of protein from the homogenate of both the nuclear fraction and PNS were incubated together for 30 min before loading on to a gel, the larger immunoreactive cpn60 of 57 kDa in the nucleus co-migrated with the 57 kDa protein in the PNS, and the 42 kDa protein could still be detected (Figure 3b). A monoclonal antibody to human cpn60 (Boog et al., 1992) also used to probe these samples demonstrated cross-reactivity that was similar to that of the pAb to cpn60. A 57 kDa protein was detected in the PNS, 57 and 42 kDa proteins were detected in the nuclear fraction, and in the combined fractions the 57 kDa proteins co-migrated and the 42 kDa protein was also detected. (Figure 3c). In addition, each of these proteins resolved as an individual spot by two-dimensional Western-blot analysis (Figure 3d).

The absence of the 63 kDa protein in the nuclear fractions,

and differences in the kinetics of induction of immunoreactive cpn60 in the two fractions, reduce the likelihood that leakage from mitochondria during cell lysis contaminated the nuclear fractions. Further, the absence of the 42 kDa protein in the PNS reduces the likelihood that this protein is a breakdown product of the 57 kDa protein. In addition, when immunoblotted and probed with mAb anti-mouse TCP1 (Willison et al., 1989), a 60 kDa protein was detected in the PNS, but not in the nuclear fraction (results not shown). Yeast cpn60 also did not react with the TCP1 mAb. These latter data lend further support that the nuclear fractions were not contaminated with cytosol.

To determine whether immunoreactive cpn60 and stress70 co-localized, we also revealed stress70 compartmentalization by indirect immunofluorescence using a pAb against mammalian stress70 (Ehrhart et al., 1988). Stress70 was present at low levels in cells cultured at 24 °C (Figure 4a), and it increased in the cytoplasm and in the nucleolus 2 h after heat-shock (Figure 4b). After 2 h, stress70 migrated from the nucleolus and dispersed throughout the nucleus (Figure 4c). Within 24 h after heat shock, stress70 abundance and distribution were similar to those of the non-heat-shocked cells (Figure 4h).

DISCUSSION

Several lines of evidence presented in this study indicate that members of the cpn60 family accumulated in the nucleus in a

heat-inducible manner in these cells. Immunoreactive proteins were revealed by indirect immunofluorescence in the nucleus with pAb to cpn60 from 12 to 24 h after heat-shock. Cellular fractionation and Western blotting confirmed these observations, detecting two major immunoreactive proteins of 57 and 42 kDa over the same time period. Both proteins also reacted with a mAb to human cpn60, and did not react with an mAb to mammalian TCP1.

Interestingly, cpn60 nuclear localization was revealed in a pattern of foci (Figure 1) which is strikingly similar to the *in vivo* distribution of the small nuclear ribonucleoprotein (snRNP)-rich organelles referred to as 'coiled bodies' (Carmo-Fonseca et al., 1991b, 1993). These organelles are suspected to be involved in pre-mRNA splicing, perhaps through the assembly and disassembly of multi-snRNP complexes (Matunis et al., 1993; Carmo-Fonseca et al., 1991a, 1991b, 1992; Zhang et al., 1992; Lamond and Carmo-Fonseca, 1993). They have also been shown to be disrupted by heat-shock (Carmo-Fonseca, et al., 1991a, 1991b, 1992; Zhang et al., 1992; Lamond and Carmo-Fonseca, 1993; Welch and Mizzen, 1988). It is tempting to speculate that cpn60 associates with these organelles and plays a role in the repair of RNP complexes damaged from the heat-shock treatment.

In addition, the data obtained in the present study indicate that the two major heat-shock proteins, cpn60 and stress70, interacted differently with complexes in the nucleus (Figures 1 and 4), suggesting that they have distinct roles in facilitating repair from heat-induced damage. In contrast with cpn60, nuclear localization of stress70 was much more rapid. Also, cpn60 localized as highly discrete foci, whereas, stress70 did not. Instead, nuclear staining of stress70 in this fish cell line appeared similar to the granular and speckled staining patterns observed for stress70 in mammalian cells (Welch and Feramisco, 1984). Further, although both proteins interacted with the nucleolus (Figures 1 and 4), the nucleolar association of cpn60 occurred well after stress70 had migrated from the nucleolus. This sequential interaction is similar to that observed during the folding of multimeric proteins in the mitochondria, where the mitochondrial member of the stress70 family (mhsp70) facilitates initial folding and passes the partly folded intermediate on to cpn60 for subsequent folding and assembly of higher-order complexes (Hartl et al., 1992; Manning-Krieg et al., 1991). Since stress70 and cpn60 perform different folding functions (Willison et al., 1989; Rothman, 1989; Gething and Sambrook, 1992; Landry et al., 1992) and the induced synthesis of heat-shock proteins is related to environmentally induced protein damage (Edington et al., 1989; Morimoto et al., 1992), we suggest that cpn60 plays a novel role in the folding and assembly of oligomeric structures in the nucleus as part of the repair process.

We are grateful to R. Hallberg, K. Willison and S. Ullrich for their gifts of yeast cpn60, antibody to TCP1 and antibody to stress70 respectively. We also thank L. Martin for assistance in preparing this manuscript, S. Nieto and D. Porter for the photography,

R. Pianalto for digitization and printing of Figures, and A. Tarnawski for the use of his microscope. This work was supported by an Exploratory Research Grant from the Environmental Protection Agency (R-817219-01-1).

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