Effect of Growth State and Heat Shock on Nucleolar Localization of the 110,000-Da Heat Shock Protein in Mouse Embryo Fibroblasts

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ABSTRACT
We have shown previously that the mammalian 110,000-Da heat shock protein (hsp110) associates with nucleoli in several cell types and that in 2-day postconfluent mouse 10T½ cells, a segregation of the antigen from the nucleolar phase-dense body is seen (J. R. Subjeck, T. Shyy, J. Shen, and R. J. Johnson. J. Cell Biol. 97: 1389-1395, 1983). Here we further characterize the nucleolar segmentation of hsp110 in mouse 10T½ and 3T3 cells with respect to the formation of this structure in dense cultures and investigate the behavior of this protein following conditions (serum deprivation, actinomycin D, and heat shock) known to affect the functional and morphological integrity of the nucleolus. It is shown that in addition to its nucleolar locale, an affinity of hsp110 for the nonnucleolar, nuclear compartment in actively proliferating cells is also observed. When proliferating cells are treated with actinomycin D (1 μg/ml) for 8 h, hsp110 separates from the nucleolar phase-dense body to form a fluorescent nucleolar cap which resembles that seen in confluent cultures. This drug also results in a disappearance of hsp110 from the nucleoplasm. Incubation of cells for 24 h in media without serum also results in the nucleolar segmentation of hsp110 and a reduction in nucleoplasmic staining. A moderate nonlethal heat treatment does not lead to segmentation of hsp110 in proliferating cells but conversely results in a transient reversal of segmentation in confluent cultures. Examination of segmented nucleoli of postconfluent cells by immunoelectron microscopy reveals that hsp110 is associated with the fibrillar component of these nucleoli, the site of ribosomal DNA.

INTRODUCTION
Although many studies have focused on the molecular mechanisms responsible for the induction of heat shock proteins as well as their function and intracellular localization, the role of these proteins and of the heat shock response is still unclear (reviewed in Refs. 1-3). Earlier studies using cell fractionation, autoradiography, and microdissection have shown that some of the heat shock proteins exhibit a nuclear localization (4-7), suggesting a nuclear role for these proteins. We have previously described the preparation of a polyclonal antiserum against the major mammalian heat shock protein at 110,000 Da and have demonstrated that this antigen is associated with nucleoli in several cell types (8). In addition, antisera against another mammalian heat shock protein approximately 70 kDa (9, 10) indicates that this heat shock protein exhibits an affinity for nucleoli. The nucleolar localization of two different mammalian heat shock proteins suggests that aspects of nucleolar function may be associated with the role(s) which these proteins play in the cell.

It is also recognized that the nucleolus is highly sensitive to changes in environmental conditions, i.e., temperature (11-14), growth state (15-17), and certain drugs (18), all of which can affect the staining pattern of anti-hspl10 as a nucleolar marker. This study presents an analysis of the effects of heat shock, growth state, serum deprivation, and actinomycin D on the nucleolar localization of the hsp110 antigen in mouse embryo fibroblasts. It is demonstrated that these conditions and treatments significantly affect both the abundance of the antigen in the nucleus and its nuclear/nucleolar distribution.

MATERIALS AND METHODS
Cell Culture. Mouse C3H 10T½ fibroblasts (19) originally obtained from Dr. John Bertram (University of Hawaii) were cultured as monolayers on glass coverslips (18 x 18 mm) in plastic Petri dishes (Corning) and incubated at 37°C in a humidified 5% CO2 atmosphere. The medium used was Eagle's basal medium with Earle's salts [Gibco Laboratories, Grand Island, NY] supplemented with 10% heat-inactivated fetal bovine serum [Gibco] and antibiotics [penicillin G (50 μg/ml), streptomycin (50 μg/ml), and neomycin (100 μg/ml) (Gibco)]. Cultures were seeded at 2 x 10³ cells/100-mm plate. Swiss mouse embryo 3T3 cells (20) were plated at 2 x 10³ cells on glass coverslips in a 100-mm Petri dish with Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% calf serum (Gibco). Cultures were maintained at 37°C in a 10% CO2 environment.

For heat shock experiments, Petri dishes containing coverslips were heated by placing the dishes 7-8 mm deep in a water bath (Haake FH-2) on the surface of an aluminum frame which allowed contact between the dish bottom and the bath. In this way dishes were exposed to a temperature of 45°C ± 0.1°C (SD) for 15 min and then postincubated at 37°C as required. For serum deprivation experiments, cells were grown with complete medium for 3 days followed by incubation in serum-depleted medium for 24 h. Actinomycin D (Sigma) was used at a concentration of 1.0 μg/ml. Following serum deprivation cells resumed normal growth after addition of serum and exhibited normal plating efficiency. Actinomycin D exposure did not significantly alter cell morphology during the exposure but led to a significant reduction in attached cells by 72 h posttreatment.

Antibody. Rabbit antiserum raised against hsp110 was prepared and characterized as described previously (8).

Indirect Immunofluorescence. Coverslips containing the cells were washed in Hanks' balanced salt solution (Gibco) and fixed in 2% formaldehyde in PBS for 20 min at room temperature. They were then rinsed in PBS followed by extraction in PBS containing 0.5% Triton X-100 for 30 min at room temperature. Anti-hsp110 antiserum at a 1:40 dilution was then added to the coverslips and they were incubated in a moist chamber for 60 min at room temperature. The coverslips were then washed again in PBS, following which a 1:150 dilution of fluorescein-conjugated goat anti-rabbit IgG (Miles-Yeda, Rehovot, Israel) was added and incubated again at room temperature for 45 min. Coverslips were then rinsed well and mounted in Elvanol (21). Cells were examined using a Zeiss photomicroscope II (Carl Zeiss, Inc., Thornwood, NJ) and photographed using Kodak Tri-X Pan film (Eastman Kodak Co., Rochester, NY) with the film speed automatic control set at ASA 400.

Immunoelectron Microscopy. Postconfluent cells were used in this study. Cells were fixed for 20 min in 1% glutaraldehyde-Puck's solution (0.11 M CaCl2, 5.4 M KCl-1.1 M KH2PO4-0.6 M MgSO4-0.14 M NaCl-1.1 M Na2HPO4-0.1 M D-glucose). The cells were then washed in Puck's solution and dehydrated in a graded ethanol series. After dehydration the cells were transferred to 100% 2-propanol and embedded in Epon. Silver thin sections were cut and mounted on nickel grids and processed for colloidal gold labeling. Etching was not performed on these sections. For labeling the nickel grids containing thin...
sections of cells were incubated for 30 min on a drop of 0.02 M TBS containing 0.5% bovine serum albumin. The grids were then transferred to 1 drop of 1:40 diluted primary antiserum and incubated for 2 h at room temperature. After a washing in TBS the grids were labeled with 1:10 diluted 5-nm goat anti-rabbit colloidal gold (Janssen Pharmaceutica, Life Sciences Products Division) for 60 min at room temperature. They were then thoroughly washed in TBS with 0.5% bovine serum albumin, rinsed in distilled water, and dried. Staining with 5% aqueous uranyl acetate and Reynold's lead citrate was performed before examination under a Siemens Elmiskop 1A electron microscope. In order to demonstrate the specificity of the labeling, control studies using preimmune serum were performed.

Fig. 1. Rearrangement of hspl10 as 10T½ cells reach a confluent state. Sequential changes of phase contrast (a, c, e) and corresponding hspl10 fluorescence (b, d, f) in growing and confluent 10T½ cells. In cells in exponential growth state (a, b), hspl10 fluorescence is uniformly distributed in the phase-dense body. As the cells become initially confluent (c, d), hspl10 localizes with a phase-dense ring structure. After 48 h of confluency (e, f), hspl10 migrates into a phase-lucent cap attached to a phase-dense structure to form the segmented nucleolus. Note general nucleoplasmic staining. Arrows, some sites where anti-hspl10 appears to react. Bar, 7.5 μm.
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RESULTS

Sequential changes in phase density and nuclear patterns of hsp110 fluorescence in 10T½ cells as they reach confluency are shown in Fig. 1. During exponential growth the nucleoli appear large and irregular in shape, containing small light vacuoles (Fig. 1a). At this stage fluorescent localization of the hsp110 antigen coincides exactly with the phase-dense body (Fig. 1b). However, as cells reach a confluent, contact-inhibited state, the nucleoli concurrently undergo major structural alterations and a rearrangement of hsp110 is seen to occur (Fig. 1, c and d). During this phase, nucleoli become more spherical and smaller. Occasionally phase-dense patches appear to migrate to the periphery of the nucleolus and seem to exclude the fluorescent stain and in some instances an affinity of hsp110 for the periphery of the nucleolus is observed (a ring-like structure). In many cells a total nucleolar segregation of hsp110 fluorescence from the phase-dense body is evident, even at these early stages of confluency. As a period of time of postconfluency increases (24 to 48 h) the segregation of the hsp110 fluorescent caps becomes more obvious and in most instances the phase-dense bodies generally exclude this antigen (Fig. 1, e and f). However, not all nuclear phase-dense structures interact with anti-hsp110. In addition to the studies on 10T½ cells, Swiss mouse embryo 3T3 cells also exhibit an identical pattern of nucleolar segregation (data not shown) of hsp110 fluorescence which occurs concomitantly with the onset of confluency in this contact-inhibited cell line.

In addition to the specific fluorescent staining of the nucleolus, the extranucleolar nucleoplasm of both cell lines also clearly binds the antisera, although to a reduced degree. The nucleoplasmic staining observed in growing 10T½ and 3T3 cells is brighter than that found in confluent cells, indicating an enhanced accumulation of hsp110 in this compartment during growth.

The nucleolus is thought to be a particularly sensitive nuclear site to supranormal temperatures (13, 14, 22). Cells in exponential phase of growth or in a postconfluent state were heated and the nucleolar/nuclear localization of hsp110 was examined. In the case of actively proliferating cells the coincident pattern of hsp110 fluorescence with the phase-dense body is unaltered (Fig. 2). However, the nucleoli appear to be round and of a reduced size after heat. When postconfluent cells are subjected to a heat treatment, the segmented nucleoli observed in this situation are seen to undergo a marked change (Fig. 3). The fluorescent cap exhibited by confluent cells (Fig. 3, a and b) is eliminated by heat shock and the phase and fluorescent bodies are again observed to coincide. This disappearance of segregation is observed as early as 1 h after heat shock and continues up to 8 h postshock (Fig. 3, c and d). However, at later times (8 h+, postshock) nucleoli once again begin to segregate and the fluorescent cap reappears (Fig. 3, e and f). This disappearance and reappearance of segregation of hsp110 in response to heat shock correlates with the induction and repression phases of the heat shock response as determined using a pulse label analysis of protein synthesis (data not shown). In addition, shortly after heat treatment, a distinct increase in nuclear fluorescent staining, indicative of a rapid accumulation of hsp110 in the nucleus, is observed (Fig. 3d). However, at later times when the response has subsided, the nucleoplasm is again observed to exhibit a diminished fluorescence (Fig. 3f), suggesting a reduction of hsp110 levels in the nucleus. Thus the presence of coincident nucleoli appears to predict enhanced nucleoplasmic hsp110 levels.

Both confluent and serum-depleted cells are considered to be arrested in the G1 phase of the cell cycle (16, 23), in a state of
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Fig. 3. Effect of heat shock on the localization of hsp110 in postconfluent 10T½ cells. a, c, e, phase contrast; b, d, f, hsp110 fluorescence. Nucleolar segmentation (a, b) disappears at 1 to 8 h after heat shock (c, d) and then reappears at times exceeding 8 h postshock (e, f). Note general nucleoplasmic staining. Arrows, some sites where anti-hsp110 reacts. Bar, 7.5 μm.

reduced rRNA synthesis (16, 17). The effect of serum deprivation on nucleolar localization of hsp110 in exponential growing 10T½ cells is shown in Fig. 4. Cells are grown with complete medium for 3 days and then switched into a serum-depleted medium for 24 h. Under normal growth conditions the hsp110 fluorescence is observed to precisely coincide with the phase-dense structure (Fig. 4, a and b) as described above. However, in serum-starved cells which are subconfluent, a segregation of fluorescence from the phase-dense body is again seen (Fig. 4, c and d), consistent with the above study of postconfluent cells (Fig. 1, e and f). Therefore, arrest of the cell cycle by serum deprivation leads to a similar morphology with respect to
Fig. 4. Effect of serum deprivation on the nucleolar localization of hsp110 in growing 10T½ cells. a, c, phase contrast; b, d, hsp110 fluorescence. Exposing growing cells to serum deprivation for 24 h, the coincident hsp110 fluorescent pattern with the phase-dense body (a, b) undergoes a segregation phenomenon (c, d) similar to that observed in postconfluent cells. Arrows, some sites where anti-hsp110 reacts. Bar, 7.5 μm.

hsp110 localization as is seen in postconfluent, contact-inhibited cells. Low doses of actinomycin D have been shown to inhibit rRNA synthesis (18). When actinomycin D at a concentration of 1 μg/ml is added to a subconfluent, proliferating population of cells for 8 h, a segregation of hsp110 fluorescence and the phase-dense body is once again observed (data not shown), identical to the data obtained for serum deprivation and postconfluency. In addition, intermediate degrees of segmentation, analogous to that seen in Fig. 1, occur at shorter time intervals.

Segmentation has been shown previously to separate nucleoli into two ultrastructural elements, the fibrillar and granular segments, as presented by others (18). To ultrastructurally examine the localization of hsp110 in segmented nucleoli, postconfluency was chosen as the method of inducing nucleolar segmentation. Fig. 5a presents a typical electron micrograph of a segregated nucleolus from 48-h postconfluent 10T½ cells, which has been reacted with the antibody and labeled with colloidal gold. The gold staining is found exclusively on the densely uranyl acetate–citrate-stained nucleolar segment which strongly resembles the fibrillar component recognized by others (18), while no binding is observed in the less electron-dense granular segment. Control specimens utilizing preimmune serum showed no specific or significant labeling of either nuclear or cytoplasmic structures (Fig. 5b). Therefore the segregated fluorescent hsp110 cap structure described above appears to represent the nucleolar fibrillar element. In addition to this association, gold may also associate with other nuclear electron-dense structures.

DISCUSSION

Several laboratories have recently used an immunological approach to study the localization and behavior of specific heat shock proteins. Valazquez and Lindquist (24) prepared a monoclonal antibody against the Drosophila 70-kDa hsp and have demonstrated that it moves into the nucleus during periods of stress. Welch and Feramisco (10) have shown that the mammalian nonconstitutive hsp near 70 kDa is found transiently in the nucleolus after heat shock and Pelham (9) has recently demonstrated a similar effect at the mammalian level using a Drosophila monoclonal antibody. In addition to these studies we have reported (8) that mammalian hsp110 occupies a nucleolar site in proliferating cells in the absence of heat shock and that this hsp separates from the phase-dense component of the nucleolus in 2-day postconfluent 10T½ cells. We demonstrate in this study that the initiation of this segregation occurs as cells begin to reach confluency and proceed through certain
transition forms which precede total segregation. It is also shown that other conditions inhibitory to RNA synthesis also result in a similar change in nucleolar morphology. Interestingly, this segmented nucleolus is not a terminal structure but readily reverts to a coincident state by a mild (nonlethal) heat shock, following which the segmented form reappears. These rapid changes, which appear to result from changes in nucleolar activity, suggest a highly labile or reversible association between the hsp110 and an active nucleolar structure. Immunoelectron microscopy additionally indicates that during the quiescent

Fig. 5. Immunoelectron micrography of a segregated nucleolus from a 48-h postconfluent 10T½ cell. In a, staining with colloidal gold is found exclusively on the fibrillar component (F) and no staining is observed in the granular component (G). Gold is also bound to other nuclear structures (arrows). b, corresponding preimmune control.
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Phase hsp110 is associated with the component of the nucleolus which is the locale of ribosomal DNA.

We have shown here that both serum deprivation and actinomycin D, two conditions known to affect the morphological and functional integrity of the nucleolus, also induce significant changes in the localization of anti-hsp110. In addition to these conditions, heat shock itself interferes with nucleolar function and would therefore be expected to result in a similar inhibitory effect (18, 22) in proliferating cells as described above using actinomycin D and serum deprivation. We show here that not only does heat have little effect on the nucleolar localization of hsp110 in proliferating cells, but during the recovery period it actively reverts the inactive nucleolar morphology observed in confluent cultures to a coincident form. In contrast to this, Welch and Suhan (25) have recently reported that heat shock results in the redistribution of hsp110 (utilizing the same antisera as described here) to form a "ring-like" structure in which antigen is concentrated at the nucleolar periphery. A similar ring pattern is also described in this report as a transition form leading to complete nucleolar segmentation in confluent cells. Our study utilizes a "slow transition" 15-min, 45°C shock (which is nonlethal) and examines the cells following various recovery times at 37°C, while the study of Welch and Suhan shows cells immediately following a 42°C, 3-h exposure. Thus, our study describes morphology associated with recovery and that of Welch and Suhan describes morphology during the heat exposure. It is likely that longer heat exposures than those used in this report would also result in one or more forms of segmentation morphology, when examined immediately following the exposure.

While hsp110 expresses a strong affinity for nucleoli, a significant affinity of the antisera for the nucleus as a whole was also observed. The level of this antigen in the nucleoplasm reflects the state of segmentation of the nucleolus. As cells reach confluence (or are subject to serum deprivation or actinomycin D), general nucleoplasmic staining is significantly diminished. The level of hsp110 in the nucleus is also briefly increased by a mild heat exposure of confluent cells. Welch and Feramisco (10) report that the nonconstitutive hsp at approximately 70 kDa is observed in the nucleolus after heat shock, from which it appears to migrate to a secondary site in the nucleus. A similar phenomenon was also observed in a study by Pelham (9). All of these studies are reminiscent of the earlier work of Roti Roti and Winward (26) who showed a significant increase in protein:DNA ratio after heat shock. Although hsp110 is observed in the nucleolus and nucleoplasm in proliferating cells in the absence of heat, perhaps a continuous migration of hsp110 through the nucleolus into the nucleoplasm is occurring.

In addition to the nucleolar locale of hsp110, a major nucleolar protein designated C23 by Orrick et al. (27) has a molecular size of 100,000 to 110,000 Da and contains several phosphorylated and highly acidic regions (28). Protein C23 is a major nucleolar silver-staining protein (29), which is localized in the fibrillar structure of segregated nucleoli (30), and possesses properties which suggest that it may have an organizational role (31, 32). In comparison, hsp110 is released from the nucleus following digestion with RNase (8). We have recently compared C23 with hsp110* and have shown by immunochemical and peptide mapping criteria that while C23 and hsp110 are clearly distinct proteins, they share a limited degree of homology. While it remains to be determined, it is possible that C23 represents a second component of a hsp110 gene family, analogous to the situation already known to occur in the case of hsp70 (reviewed in Ref. 2). Kistler et al. (33) have recently described a monoclonal antibody which cross-reacts with a 110-kDa nucleolar protein in HeLa cells. We have shown that this antibody reacts with hsp110 and stains nucleoli of mouse 10T½ cells in a manner which parallels that described for the polyclonal antisera studied here.*

It is well known that the major function of the nucleolus is the biogenesis of rRNA. Numerous proteins seem to participate in this synthesis and processing of pre-rRNA (34) or are involved in maintaining the structural integrity of nucleolus (35). Segregation of nucleoli into separate granular and fibrillar components following application of drugs, such as d-galactosamine (36, 37) or actinomycin D (18), has been well studied. In addition a similar phenomenon appears to occur during the differentiation of frog erythroblasts (38) and during maturation of human epithelial cells (39). This segregation phenomenon appears to reflect a state of relative nucleolar inactivity in rRNA accumulation (16, 40). Thus, the specific and reversible rearrangement of hsp110 during this segregation phenomenon suggests that an association between hsp110 and rRNA synthesis or processing may occur. Since the fibrillar component of the nucleolus is believed to be a transcription unit, hsp110 may be associated with actively transcribed rRNA genes.

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REFERENCES


* T-T. Shyy et al., unpublished data.

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