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Nuclear translocation of constitutive heat shock protein 70 during S phase in synchronous macroplasmodia of *Physarum polycephalum*

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Abstract

The level of constitutive heat shock protein 70 (HSC70) in *Physarum polycephalum* was analyzed by means of Western blots during the synchronous cell cycle of macroplasmodia. Total amounts as well as nuclear and cytoplasmic contents were determined separately and evaluated densitometrically. A drastic increase of nuclear HSC70 was observed 10–40 min after the initiation of S phase (600% of the M phase value) and thereafter a slow decline toward the next M phase. Total HSC levels showed a slight (30%) increase during S phase whereas cytoplasmic HSC70 was about 30% lower during S phase compared to mitosis.

Keywords: *Physarum polycephalum*; Macroplasmodium; Heat shock protein 70; Cell cycle; Nuclear localization

1. Introduction

Cell cycle-dependent changes in the expression of heat shock proteins of the 70-kDa family have suggested an active role for constitutive and induced isoforms (HSC and HSP70 respectively) in the control of the cell cycle [1,2]. These observations concerning an increase of HSC in the early S phase have been corroborated (for example [3,4]) but a maximum in late S phase has also been described [5]. Evidence in support of a role for HSC70 during S phase was obtained from experiments in which hsp70 antisense mRNA was applied, which stopped the progress of human tumor cells through the G1 and S phases [6]. We analyzed the macroplasmodia of *Physarum polycephalum*, in which millions of nuclei undergo precisely synchronized mitosis within 4 ± 2.1 min [7] because evidence for cell cycle-dependent changes of HSC 70 levels, particularly for a phase-specific nuclear localization of HSC70 [1], is hampered in mammalian cell cultures by incomplete synchronization of the cells.

Physarum polycephalum – like almost all organisms – increases HSP synthesis after exposure to heat shock [8]. This property is useful to identify HSP70 on Western blots. We applied several antibodies raised against HSP70, the most specific was a polyclonal antibody against a fragment of yeast SSA1 (HSC70) protein [9]. A drastic increase in nuclear HSC70 was found during the beginning of

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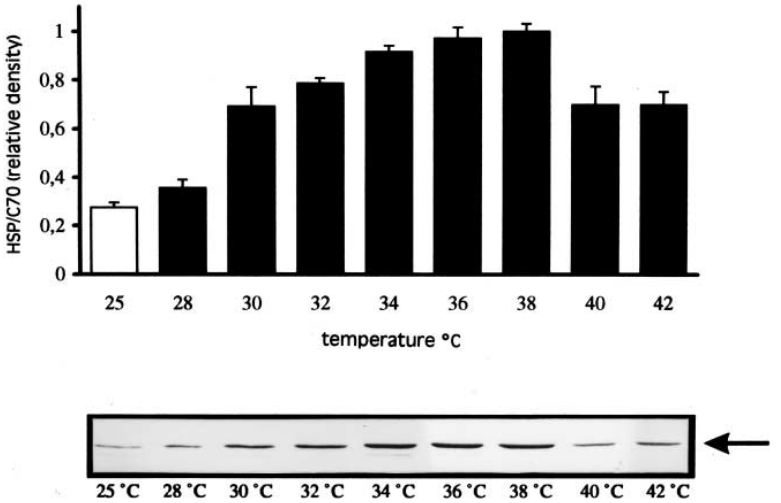

Fig. 1. Heat shock induction of 69 kDa HSC/HSP isoforms. Above: mean values of 3 independent experiments (±S.D.) after video densitometric evaluation. Light column: control (25°C). Ordinate: relative density in relation to the highest value (1.0). Abscissa: heat shock temperature. Below: Western blot analysis of HSP/HSC70 (arrow) after 45 min exposure of microplasmodia of *Physarum polycephalum* to different temperatures.

S phase while the total amount and the cytoplasmic portion of HSC70 changed only little.

2. Materials and methods

2.1. Culture conditions

Microplasmodia of *Physarum polycephalum* (CL-Mü, haploid strain, Colonia Leicester) were kept in 50 ml axenic growth medium plus 0.8 ml hemin solution [10] in 250 ml Erlenmeyer flasks. The flasks were shaken continuously and the microplasmodia inoculated in new medium every 2 days.

10 ml medium with microplasmodia was centrifuged and the plasmodia distributed on filter paper, where they fuse to macroplasmodia during 90 min. Thereafter, filters with the macroplasmodium were transferred to stainless steel grids in Petri dishes. Growth medium was then added and cultures kept at 25°C [7,11]. 'Mitosis 0' indicates the time at initiation of macroplasmodia cultivation.

2.2. Cell cycle synchrony

The cell cycle of *Physarum polycephalum* consists of S, G2 and M phase, but lacks G1 phase [12]. Cell cycle synchrony within a plasmodium is high: in different parts of the plasmodium nuclei undergo mitosis within 4 min [7]. The S phase lasts approximately 3 h [13], and the G2 phase 4–9 h. Mitosis, particularly telophase, was determined by cutting small pieces from a plasmodium and analyzing ethanol-fixed smears of these pieces under a phase-contrast microscope. These tests were done every 15 min shortly before and continuously after the second mitosis (approximately 'mitosis 0'+13 h). Macroplasmodia which had their cell cycle phases thus determined were frozen in liquid nitrogen and stored at −80°C [7].

2.3. Cell fractionation

3.5–4 g material (4 macroplasmodia) was homogenized by means of a Waring blender (40 s) at 4°C in a 'nuclear buffer' (5×) containing mM Tris-HCl, 75 mM $MgCl_2$, 15 mM EGTA, 2.5 M hexylene glycol, 4% Surfynol, pH 7.5 [14].

The homogenate was then centrifuged for 5 min at 200×*g* and the pellet mixed with a gradient solution (1.0 vol. Percoll, 0.25 vol. nuclear buffer (5×), 2.8 vol. nuclear buffer (1×)) and centrifuged for 25 min at 48 000×*g*. The nuclear pellet was suspended in nuclear buffer (1×), again centrifuged for 5 min at

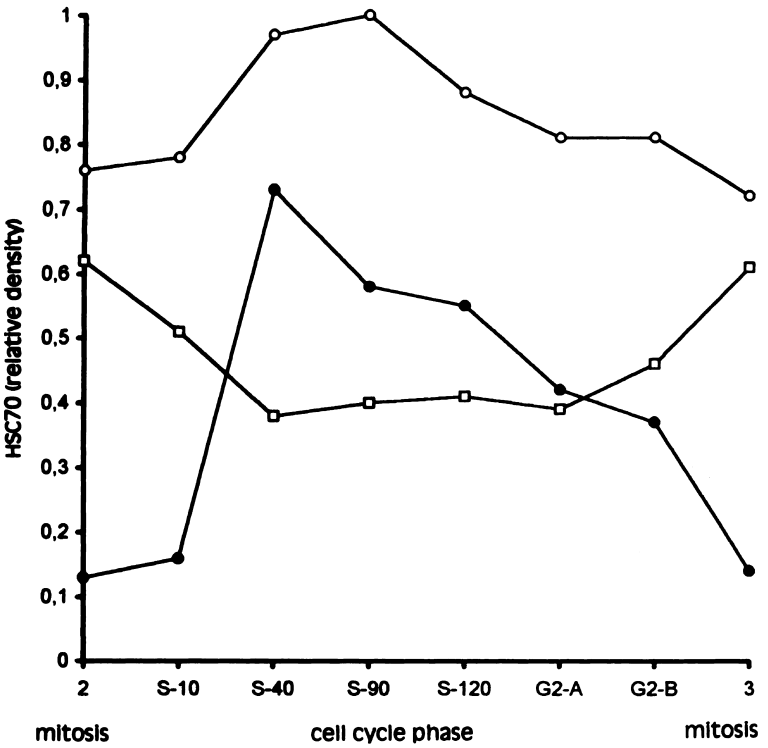

Fig. 2. Cell cycle-dependent changes in HSC70 levels between mitosis 2 and 3. Densitometric evaluation; ○, total amount; □, cytoplasmic; ●, nuclear amount of HSC70. Ordinate: relative density with respect to an internal standard (microplasmodial HSC70) present on all blots. Abscissa: cell cycle phases.

2800×*g* and suspended in 10 μl nuclear buffer (1×). The nuclei were observed using a fluorescence microscope after staining with 4-diamidino-2-phenylindole dihydrochloride (DAPI).

In order to isolate the cytoplasmic fraction, 3 g macroplasmodia were homogenized for 20 s in 5 ml nuclear buffer (1×) by means of a Waring blender. The homogenate was then centrifuged for 10 min at 1500×*g*. The supernatant was subsequently centrifuged for 15 min at 15 000×*g* and this supernatant again centrifuged for 2 h at 48 000×*g*. The supernatant was tested for nuclear contamination by DAPI staining, frozen in liquid nitrogen and stored at −80°C.

2.4. Heat shock

Heat shocks were applied to microplasmodia by transfer to a preheated water bath. 20 min were necessary to reach the heat shock temperature in the culture flask ('warm up'). The actual exposure lasted 45 min.

2.5. Sample preparation

Sample buffer (3×) [15] was added to the total cell homogenate as well as to the nuclear and cytoplasmic fractions (1:2), and the samples were then sonicated (20–25 pulses of 1 s) and boiled for 5 min. After another centrifugation (5 min, 5000×*g*), the samples were analyzed by 1-dimensional SDS-polyacrylamide (10%) gel electrophoresis [15]. The protein content of the samples was determined by the method of Neuhoff et al. [16] and equal amounts of protein were loaded on each lane of the gel.

2.6. Western blot analysis

Gels were transferred to a nitrocellulose mem-

brane using a Trans Blot Cell (Bio-Rad [17]). The blots were blocked with phosphate-buffered saline plus 0.2% Tween 20 (PBST) for 30 min at 25°C. After removal of the blocking solution, primary antibody diluted in PBST (1:1000) and 0.02% NaN_3 were added and left for 1 h at 25°C. Subsequently, the blots were washed with PBST. Secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate, SIGMA Deisenhofen), diluted in PBST (1:500), was added and after 1 h at 25°C the blots were washed with PBST plus substrate buffer. The immune complex was detected by 4-nitroblue tetrazolium chloride in 70% dimethyl formamide and 5-bromo-4-chloro-3-indolylphosphate (Boehringer, Mannheim).

We tested various anti-HSP/HSC70 antibodies. The only antibody reacting specifically with *Physarum* HSC70 was a polyclonal rabbit antibody directed against SSA1 in *Saccharomyces cerevisiae* [9]. The antibody was raised against the 90 C-terminal amino acids of this HSC70 (gift of Dr. J. Becker, Dortmund). It cross-reacts slightly with the other members of the SSA group.

2.7. Densitometry

Digital evaluation of Western blots were performed by using a video scanner and computer software (CREAM™) which allowed subtraction of background staining. We either evaluated the Western blots of a single gel and normalized all pixel values of individual bands to the highest value (1.0), or when comparing different gels, we ran a standard sample of a microplasmodium preparation on each gel. Differences in the staining intensity of this reference HSC70 were used as a correction factor. It is also important to standardize the light intensity and direction when illuminating the blots during the video scanning.

3. Results

We first tested whether the main signal at 69 kDa in the Western blots of total cell extracts of microplasmodia represented HSC70. The signals of heat shock induced HSP isoforms (as well as the enhanced constitutive isoforms) should increase after heat shock. When we exposed microplasmodia for 45 min (in addition to 20 min warm up time) to different temperatures, the intensity of the staining increased from 28°C to a maximum at 38°C and declined at higher temperatures (Fig. 1). This result showed that the 69-kDa band determined on 1-dimensional gels contains the HSC/HSP70 isoforms.

When analyzing cell cycle dependent differences in HSC70 levels it is important to exactly determine the cell cycle phase of the macroplasmodia. This was done by testing small parts of the plasmodium for the appearance of typical features of mitosis: disintegration of the nucleolus, metaphase chromosomes or adjacently located newly formed nuclei in telophase. At 'mitosis 0' (see Section 2) plus 13 h the tests were begun and the second mitosis determined. When the second mitosis had been determined in a macroplasmodium, this macroplasmodium was cultivated for a further defined time interval of 0, 10, 90 and 120 min and then frozen in liquid nitrogen. In order to determine the later cell cycle phase (G2) before the third mitosis, macroplasmodia were divided and the larger part frozen, whereas the smaller part was tested for the time of the third mitosis. The time interval determined in the small part was used to estimate the cell cycle phase of the larger part. This method produced reliable data compared to uncut plasmodia, despite cutting the macroplasmodium into two parts [7].

A total of 57 macroplasmodia were thus examined in 8 cell cycle phases, 37 for the determination of mitosis 2 and four S phase stages. Thus, an average of 7 macroplasmodia (3–4 for the nuclear fraction and 3 for the cytoplasmic fraction) was pooled for each early data point, whereas 20 macroplasmodia were analyzed for G2A = 200–175 min before mitosis 3, G2B = 100–55 min before mitosis 3 and mitosis 3 itself.

The results show that the main HSC changes occur in the nuclear fraction: HSC amounts are lowest during M-2, but increase drastically at S40 (by a factor of 6) and then slowly decrease to M-3 (Fig. 2). The HSC70 level in total cell extracts is slightly higher during S40 and S90 than during other phases, whereas opposite changes of the HSC70 level occur in the cytoplasmic fraction.

4. Discussion

We consider the HSC70 changes, particularly the nuclear localization differences, significant for the following reasons: (a) each data point of the nuclear and cytoplasmic HSC content is the result of at least three independently maintained plasmodia and (b) a mean value for the nuclear content at mitosis 2, S10 and mitosis 3 is significantly different from a mean value of S40, S90 and S120. Furthermore, the sum of cytoplasmic and nuclear values at each phase is in good agreement with the independently determined total amount, which shows the reliability of the measurements.

Our results show a strong cell cycle dependence of HSC translocation into the nucleus and thus support the hypothesis of a role of HSC (or HSP) 70 in the S phase of the cell cycle [1]. In spite of their nuclear localization signal (NLS) heat shock 70 proteins are localized primarily in the cytoplasm, possibly due to a masking of the NLS, which may be controlled by the carboxy-terminal end of the molecule. Truncated HSC70 lacking this region were greatly reduced in their nuclear accumulation rate [18]. Masking seems to be altered upon heat shock or during S phase, the mechanism of which, however, is not clear. Possibly, different proteins bind to HSC/HSP70 during different cell cycle phases [19].

The role of HSC/HSP70 in the nucleus is equally unclear. It might assist the nuclear import of transcription factors and histones, because it colocalizes with karyophilic proteins [20]. It has been shown that the kinetics of NLS-directed nuclear transport in yeast was stimulated by an elevated expression of SSA1, which encodes a cytoplasmic HSC70. Increased levels of this HSP70 were able to overcome defects of the NLS receptor complex in the nuclear pores [21]; in fact, injection of anti-HSC70 antibodies abolished the nuclear translocation of nucleoplasmin [22] and SV40 large T antigen while the import of the glucocorticoid hormone receptor was not affected [23].

Nuclear HSC/HSP70 might directly bind to proteins and enzymes involved in DNA synthesis, as reported for the bacterial Dnak in the replication of bacteriophage λ [24]. In mammalian cells, there is some evidence that HSC/HSP cotranslocates with c-Myc and forms distinct globules in the nucleus. This was particularly observed when c-*myc* was overexpressed [25]. Furthermore, the binding of HSC/HSP70 to tumor suppressor proteins such as p53 has been reported [26] as well as a stimulation of HSC/HSP synthesis by c-Myc and E1A [27,28].

We conclude that HSC70 is involved in replication-associated processes particularly at the beginning of S phase. The large increase of nuclear HSC70 concentration is mainly achieved by increased nuclear translocation rather than higher expression. This suggests conformational changes and/or differential binding of other proteins to HSC70 in order to unmask the NLS during this cell cycle phase.

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