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Nuclear translocation of stress protein Hsc70 during S phase in rat C6 glioma cells

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Abstract The expression and the nuclear translocation of the constitutive heat shock protein 70 (Hsc70) were determined during the cell cycle in synchronized rat astrocytomic C6 glioma cells. Cells were first shifted to the G0 by serum starvation. Twelve hours after a subsequent growth stimulation by transfer to 20% newborn calf serum, about 50% of the cells entered S phase. Western blot analysis with different monoclonal antibodies showed that only the constitutively expressed and moderately stress-activated Hsc70 is induced during serum stimulation. Maximal cellular Hsc70 content (170% of the control) was observed in early to mid S phase followed by a drastic decline while cells pass through G2/M (20% of the control). Hsp70, the major heat-inducible heat shock protein in C6 cells, is not detected in either asynchronously proliferating, serum-starved or in serum-stimulated C6 cells. Analysis of the nuclear and cytoplasmic protein fractions showed a significant increase of Hsc70 translocation into the nucleus during early S phase. These results indicate a role for Hsc70 but not for Hsp70 in the process of S phase entry and/or progression in C6 cells under physiological conditions.

INTRODUCTION

Heat shock proteins (Hsp) represent a set of proteins that show increased synthesis during or after stress (e.g. hyperthermia, UV-radiation or heavy metals). The Hsp70 family comprises proteins which are constitutively synthesized and which are only moderately stress-activated (Hsc70, heat shock cognate) as well as inducible proteins which are expressed only after stress (Hsp70) (Morimoto 1991; Feige and Polla 1994; Minowada and Welch 1995).

The remarkable feature of all Hsc/Hsp70 isoforms is their ability to act as 'molecular chaperones' which protect nascent or denaturated proteins from aggregation and support their folding or refolding into the correct conformation (Elis et al 1989; Agard 1993; Craig 1993; Becker and Craig 1994; Hartl et al 1994; Wynn et al 1994; Buchner 1996). Furthermore, Hsc70 supports the translocation of proteins into their target compartments (Shi and Thomas 1992; Okuno et al 1993).

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It was hypothesized, in addition, that members of the Hsp70 family were actively involved in the regulation of the cell cycle (Milarski and Morimoto 1986; Milarski et al 1989). Subsequent studies demonstrated different or even contradictory cell cycle-dependent expression patterns for Hsc and/or Hsp70 under physiological or under stressful conditions (Kao et al 1985; Milarski and Morimoto 1986; Wu et al 1987; Hang and Fox 1995; Hang et al 1995; Hang and Fox 1996; He and Fox 1996). However, there is agreement that Hsc/Hsp70 expression is stimulated after infection with different tumor viruses, e.g. adenovirus, SV40, polyoma virus, cytomegalo virus or HTLV-1 (Simon et al 1987; Sainis et al 1994; D'Onofrio et al 1995). This stimulation does not seem to constitute a cellular 'stress response' induced by the infection, because the E1A 13S and SV40-T transcription factors directly act on the Hsp70 promoter (Milarski and Morimoto 1986; Wu et al 1986). The most convincing evidence for a fundamental role of at least Hsp70 in the process of cell proliferation was obtained from *hsp70*-antisense application, which abolished progress through G1 and S phase in human tumor cells (Wei et al 1995).

It remains unclear, however, whether the constitutive or the inducible Hsp70 isoform plays the main role in cell cycle control of mammalian cells. This problem is additionally complicated by the fact that many investigators use tumor cell lines, which constitutively express the inducible Hsp70 (e.g. HeLa cells). Therefore we addressed the following question: Which member of the Hsp70 family is differentially expressed during the cell cycle under physiological conditions?

Since Hsc/Hsp70 isoforms were shown to be involved in the transport of proteins into the nucleus, we also determined the intracellular distribution of Hsc/Hsp70 in the course of the cell cycle. We chose the C6 glioma cell line because these cells do not express the inducible Hsp70 constitutively and because the expression of different stress proteins in C6 cells is well characterized (Neuhaus-Steinmetz et al 1994, 1996). Our results clearly demonstrate a cell cycle-dependent synthesis and nuclear translocation of the constitutive Hsc70.

MATERIAL AND METHODS

Cell culture

C6-glioma cells were maintained in Dulbecco's modified Eagle's Medium (DMEM, containing 100 U/ml penicillin and 100 µg/ml streptomycin) supplemented with 10% newborn calf serum (NCS). In order to arrest the cells they were seeded at low density. After 24 h, cells were washed twice and DMEM supplemented with 0.5% NCS was added. Within six days the cells were arrested in G0/G1. Cells were stimulated to reenter the cell cycle by adding DMEM containing 20% NCS.

Flow cytometry

Cells were harvested by trypsination, fixed in 70% ethanol and stored overnight at – 20°C. For DNA measurements, cells were centrifuged for 10 min at 300 × *g*. The pellet was resuspended in phosphate-buffered saline (PBS). Cell doublets were excluded by filtering the solution through a 30 µm mesh nylon net. After a second centrifugation step, the cells were resuspended in PBS containing 50 µg/ml propidium iodide (PI) and 10 mg/ml RNase and stained for at least 45 min. DNA analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) using the Lysis II software.

Cell fractionation

Stimulated cells were washed twice with PBS, scraped from the culture dishes and suspended in ice-cold buffer (10 mM Tris, 3 mM $MgCl_2$, 10 mM NaCl, 0.5% NP-40, 0.1 mM phenylmethylsulfonylfluoride (PMSF), pH 7.5). After 30 min on ice, the cell membrane was disrupted by repeated trituration. Nuclear and cytoplasmic fractions were separated by centrifugation at 600 × *g* and 4°C for 10 min. To remove the NP-40, the pellet was washed in buffer without NP-40 four times. The purity was tested by staining in nuclei with methylene blue. The cytoplasmic fraction was centrifuged once at 35 000 × *g* and 4°C for 30 min. Both fractions were frozen in fluid nitrogen, freeze-dried and resuspended in sample buffer (62.5 mM Tris-HCl, 2% SDS, 16% glycerol, 5% β-mercapto-ethanol, pH 6.8). After boiling for 5 min samples were stored at –80°C. Whole cell extracts were prepared by scraping the cells from the culture dish into double distilled water, freezing them in fluid nitrogen and freeze-drying. Further steps were the same as described for the cell fractionation above.

Western blot analysis

For all samples the protein concentration (µg/µl) was determined and 10% SDS-PAGE gels were loaded with equal amounts of protein per lane (25 µg). Proteins were separated by electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with PBS containing 0.2% Tween20 for 30 min. Antibodies were added for 60 min at RT. The antibodies used were: SPA-820 (clone N27F3-4, monoclonal, 1:1000, Biomol, Hamburg, Germany) which recognizes Hsc as well as Hsp70; SPA-810AP (clone C92F3A-5, monoclonal, 1:1000, Biomol, Hamburg, Germany) detecting only Hsp70; anti-β-tubulin (monoclonal, 1:1000, Sigma, Deisenhofen, Germany) and goat anti-mouse conjugated to alkaline phosphatase (1:1000, Sigma, Deisenhofen, Germany). The relative intensities of the bands were determined using a video scanner and CREAM™ software.

RESULTS

Cell cycle analysis

Flow cytometric measurements of single-cell DNA content in serum-starved cells (Fig. 1A) revealed that at least 85% of the cells were arrested in the G0/G1 phase at the time just before serum re-addition. Within the first hours of cell cycle progression, the morphological differentiation of the cells observed during cultivation at low serum was reversed without detectable induction of DNA-synthesis for up to 8 h (Fig. 1B). The G1/S transition was derived from the right-handed shift of the G1-peak in DNA histograms and occurred synchronously after 12 h. A maximum of cells in S phase (about 50%) was observed after 14–16 h (Fig. 1C). It was followed by a significant increase of the G2/M fraction after 16 h (Fig.1D). The maximum of G2/M cells was detected after

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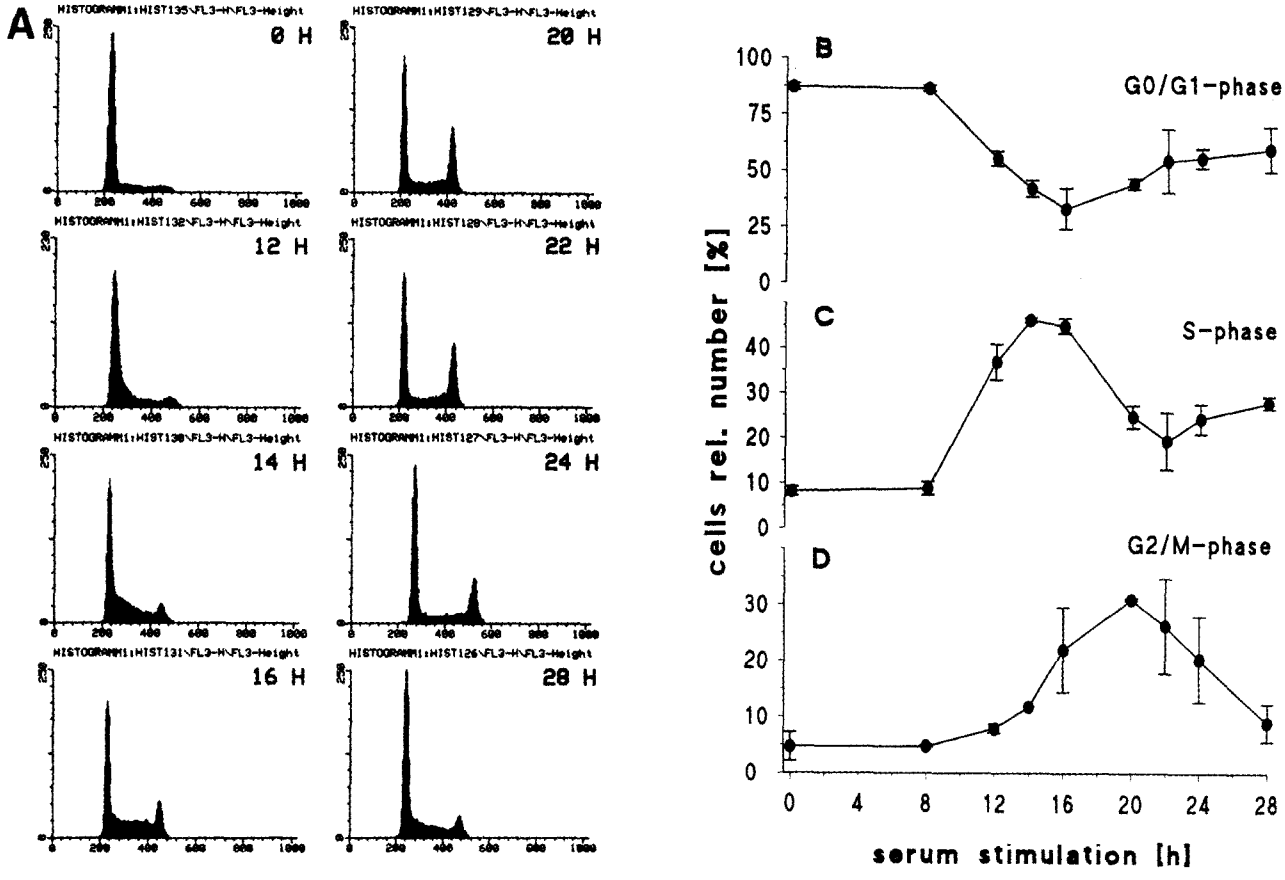

Fig. 1 Cell cycle phase distribution of serum-stimulated rat C6 cells analysed by flow cytometry. (A) DNA-histograms of C6 cells harvested at the indicated times after serum stimulation corresponding to the abscissa of B–D. Abcissa: DNA-content based on PI-fluorescence, ordinate: relative number of cells (10 000 cells were analysed). (B–D) Quantitative analysis of DNA-histograms using the LYSIS II research program (version 1.1; Becton Dickinson, San Jose, CA, USA). The proportion of cells in different phases of the cell cycle (ordinate; b) G0/G1 fraction; (C) S phase fraction; (D) G2/M fraction) is plotted against the time of serum stimulation (abcissa). Values are means (± SE) of three independent experiments.

20 h, thereafter this fraction gradually declined to a minimum 28 h after serum readdition. The initial synchrony decreased after the first cycle as shown by the smaller increase of the S phase fraction which began after 24 h.

In order to distinguish G2 and M phases, we additionally counted the number of cells (data not shown). The counts confirmed the flow cytometric results and revealed that the onset of cell division occurs about 24 h after serum stimulation. All cells finished the first division cycle within 28 h.

Time course of Hsc70 expression and intracellular distribution

Figure 2B shows that the amount of Hsc70 in whole cell extracts and in the cytoplasmic fraction slightly decreases during the first hours of serum-stimulation, when cells are progressing through G1 phase. A distinct increase of Hsc70 can be observed in the nuclear fraction within the first 12 h after the addition of serum. The initial decrease of Hsc70 may be due to an increase in protein synthesis initiated by the addition of serum that causes the relative amount of Hsc70 to decrease with respect to total cellular protein.

The Hsc70 level increases in all cellular fractions when cells pass the G1/S boundary (Fig. 2B; 12 h) and reaches a maximum during S phase (Fig. 2B, 12–16 h). The most pronounced increase of Hsc70 can be detected in the nuclear fraction (approximately 400% of the control at 0 hours). The considerable nuclear accumulation of Hsc70 indicates a S phase-specific translocation. A drastic decrease of the Hsc70 level occurs while cells progress through the G2/M phase (Fig. 2B; 20–22 h). In this phase the most significant decline is observed in whole cell extracts while the Hsc70 level of the nuclear fraction does not decrease below the level of serum-starved cells. When the cells enter the second G1 and S phase, the Hsc70 level of all cellular fractions rises again. To rule out the possibility that the

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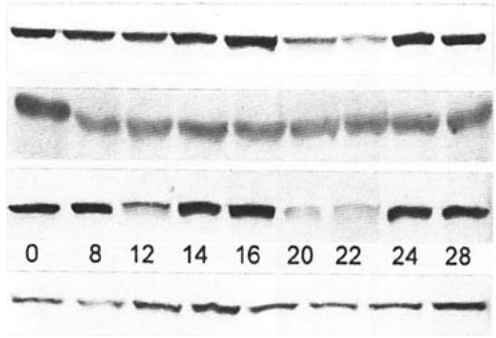
2A



2B

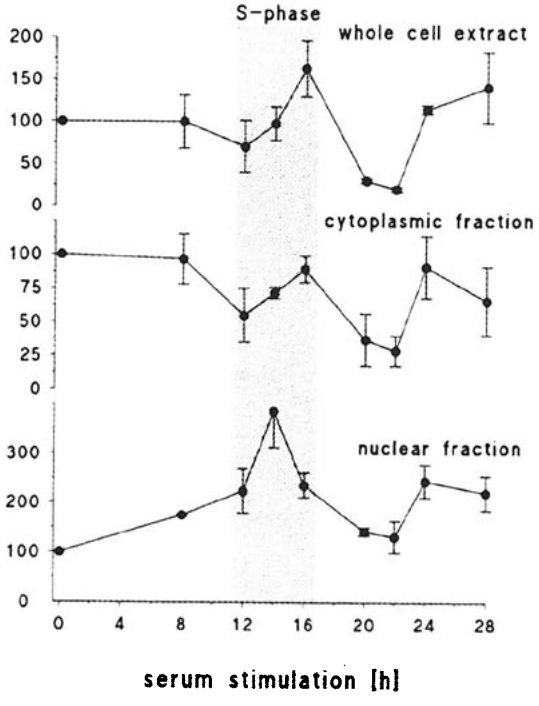


Fig. 2 Hsc70-amount in different cellular fractions of serum-stimulated C6 cells analysed by Western blot. (A) The Hsc70 content of different cellular fractions prepared at the indicated times after serum stimulation was determined by SDS-PAGE and a subsequent immunoblot using the SPA-820 antibody (Biomol, Hamburg, Germany). Every lane was loaded with 25 µg of protein. Due to the different protein contents of the fractions the amounts of Hsc70 in the nuclear and cytoplasmic fractions are not directly comparable. β-tubulin is shown in the second lane as an internal control; **T**: total cell extract, **C**: cytoplasmic fraction, **N**: nuclear fraction. (B) Western blots were quantitatively evaluated by video scanning and measurement with CREAM™ research software (Ver 4.1). Values are relative amounts of pixel density (ordinate; Hsc70 content of serum-deprived cells = 100%) plotted against the time of serum stimulation (abcissa). Shaded box: time interval during which maximal numbers of cells (30–50%, compare Fig. 1) are in S phase. Values are means (± SE) of three independent experiments.

differential expression of the Hsc70 reflects a general expression pattern of serum-induced protein synthesis, we also analysed a housekeeping protein (β-tubulin). As shown in Figure 2A, the amount of β-tubulin does not exhibit significant cell cycle-dependent variations.

In order to ensure that the Western blots actually represent the amount of Hsc70 when the antibody SPA-820 (clone N27F3–4) which recognizes both Hsc70 and Hsp70 isoforms was applied, identical samples were also incubated with the SPA-810AP antibody (clone C92F3A-5). This antibody specifically binds to the inducible isoform (Hsp70). The SPA-810AP antibody failed to produce any signals with the exception of a heat stressed sample as positive control. The Western blot data thus unequivocally reflect the expression of the constitutively expressed Hsc70.

DISCUSSION

After serum stimulation of resting C6 cells, the subsequent S phase coincides with increased expression and significant nuclear translocation of Hsc70. Whereas a drastic decrease of Hsc70 expression is observed during G2/M phase. In contrast to the cell cycle-dependent expression of the constitutive form, the inducible Hsp70 was not detected under physiological conditions in cells of any cell cycle phase. The inducible form is thus cell cycle-independently restricted to the stress response in C6 cells. The finding that Hsp70 is expressed only under pathological conditions in rodents is confirmed by in vitro and in vivo studies using other rodent cells and tissues (Subjek and Shyy 1986).

Members of the Hsp70 family were proposed to be cell cycle-regulated based upon cell cycle-dependent changes in the amount of *hsp70*-mRNA in several human cell lines (Kao et al 1985; Milarski and Morimoto 1986; Milarski et al 1989). Recent investigations indicated that the cell cycle-dependent expression might be restricted to the inducible Hsp70 isoforms (Hang and Fox 1995; Hang et al 1995; Hang and Fox 1996; He and Fox 1996).

In contrast to these results, cell cycle-dependent expression of both the *hsc70* and *hsp70* genes was observed in monkey kidney CV1 cells: *hsp70* expression was restricted to late S/G2, whereas *hsc70* expression was S phase-specific after serum stimulation as well as after virus infection (Sainis et al 1994). In agreement with our results, a drastic decline of *hsc70* mRNA was observed while cells passed through G2/M (Sainis et al 1994).

The controversial results concerning cell cycle-specific expression of different Hsp70 species may be due to the type and degree of transformation among various mammalian cell lines, because different cellular and viral protooncogene products (c-Myc, p53, E1A and SV40 T-antigen) were shown to regulate the expression of Hsp70

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isoforms (Kingston et al 1984; Milarski and Morimoto 1986; Agoff et al 1993; Protti et al 1994; Tsutsumi-Ishii et al 1995). Some protooncogenes exhibit a cell cycle-dependent expression pattern themselves (Kao et al 1985; Rosenwald et al 1995) which may activate or interfere with Hsp70 isoform expression.

Our observation that Hsc70 molecules enter the nucleus during the S phase of the cell cycle in C6 cells may be of particular interest: even though Hsc/Hsp70 are predominantly cytoplasmic, they contain a NLS sequence and can selectively be transported into the nucleus (Dang and Lee 1989; Mandell and Feldherr 1992). When cells are subjected to heat shock, both Hsp70 isoforms translocate into the nucleus where they facilitate the recovery of nucleolar function (Pelham 1984; Yamane et al 1995). Under physiological conditions Hsc70 is apparently involved in the active import of karyophilic proteins into the nucleus, where the chaperone activity of Hsc/Hsp70 probably supports the disassembly of the nuclear translocation complex (Mandell and Feldherr 1992; Shi and Thomas 1992; Okuno et al 1993; Goldfarb 1994; Yang and DeFranco 1994; Yamane et al 1995; Shulga et al 1996). Microinjection of anti-Hsc70 antibodies abolished the nuclear translocation of various NLS bearing proteins (Imamoto et al 1992). Nuclear cotranslocation of Hsc70 and c-Myc has been observed in cells transiently overexpressing *c-myc* even though c-Myc also bears a NLS itself (Koskinen et al 1991; Henriksson et al 1992). In virus-infected cells, the adenovirus E1A protein and members of the Hsp70 family similarly cotranslocate into the nucleus (White et al 1988). The capacity of Hsp70 to associate with other celullar proteins and to modify their destiny and function may be one way to regulate the activity of essential proteins which are involved in cell cycle regulation. In addition, specific interactions of Hsc/Hsp70 with various cell cycle-regulating proteins have been observed, e.g. with p53 and the retinoblastoma protein pRb (Hainaut and Milner 1992; Nihei et al 1993; Matsumoto et al 1994; Inoue et al 1995; Ohnishi et al 1995).

Shi and Thomas (1992) suggested that an increased constitutive expression of Hsp/Hsc70 might be correlated with an enhanced requirement of nuclear transport capacity. Thus enhanced expression of Hsc70 combined with nuclear accumulation observed in the S phase of C6 cells may serve an enhanced transport demand of replicating cells.

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