Biphasic translocation of a 70 kDa heat shock protein in human cytomegalovirus-infected cells

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Human cytomegalovirus (HCMV) reportedly induces the expression of a 70 kDa heat shock protein (hsp70) with no known function in the virus replication cycle. We report here a remarkably specific translocation pattern of hsp70 during HCMV infection of human diploid fibroblasts. Immunofluorescent observation and Western blotting of subcellular fractions revealed nuclear localization of hsp70 early in infection and predominantly cytoplasmic localization of hsp70 late in infection. Treatment of HCMV-infected cells with cycloheximide followed by treatment with actinomycin D allowed virus immediate-early gene expression but inhibited hsp70 nuclear localization. Phosphonoacetic acid and tunicamycin, both of which reportedly inhibit HCMV DNA replication, did not inhibit HCMV-induced nuclear localization of hsp70 but inhibited hsp70 translocation from the nucleus to the cytoplasm. These results indicate a correlation between HCMV multiplication and hsp70 localization, suggesting that hsp70 may play a role in HCMV multiplication.

The heat shock proteins (hsp) are a set of proteins that are synthesized in response to physical, chemical or biological stresses, including virus infection (Morimoto & Milarski, 1991). Induction of hsp by virus infection can be divided into two classes: induction of several species of hsp, possibly due to cellular stress responses (Collins & Hightower, 1982; Jindal & Young, 1992; Notarianni & Preston, 1982), and virus induction of the major nucleo-cytoplasmic 70 kDa hsp (hsp70). The second category includes responses to adenovirus (Nevins, 1982), herpes simplex virus (Kobayashi et al., 1994; Phillips et al., 1991) and human cytomegalovirus (HCMV) (Santomenna & Colberg, 1990). The major hsp70 is the type member of the hsp70 family, whose members are involved in, for example, thermotolerance, prevention of misfolding of nascent polypeptides, transmembrane protein transport and nuclear protein transport (Welch, 1993). However, little is known about the function of induced hsp70 in virus infections.

Of the viruses so far reported to induce hsp70 expression, HCMV is one of the best studied. HCMV immediate-early proteins 1 (IE1) and 2 (IE2) reportedly transactivate the hsp70 promoter in a TATA box independent- and a TATA box dependent-fashion, respectively (Caswell et al., 1993, 1996; Hagemeier et al., 1992a, b). HCMV US3 and UL37 gene products were also reported to transactivate the hsp70 promoter alone and in concert (Colberg et al., 1992; Tenney et al., 1993). Although the mechanism of hsp70 induction by HCMV has been elucidated in detail, the function of the induced hsp70 in HCMV-infected cells remains unknown.

To clarify the significance of hsp70 induction in HCMV infection, we studied the subcellular localization of hsp70 in HCMV-infected cells. Human diploid fibroblasts, TIG-3 (Matsuo et al., 1982), obtained from the Japan Cancer Research Resource Bank, were grown in Eagle’s minimum essential medium (MEM; Nissui) containing 5% foetal bovine serum (FBS; GEMINI Bio-products). TIG-3 cells (1–5 × 10⁴ cells per 15 mm diameter coverslip) were infected with HCMV strain AD169 (a kind gift of Y. Minamishima, Miyazaki Medical College, Japan) at an m.o.i. of about 4 p.f.u. per cell for 60 min or mock-inoculated, and cultured in MEM containing 2% FBS.

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In uninfected cells, hsp70 gave a diffuse staining pattern (Fig. 1a, c) and this pattern was unchanged 3 h after HCMV infection (Fig. 1a, c). Unlike in Fig. 1(k), hsp70 was barely visible in Fig. 1(a, c) because of differences in photographic conditions (see legend for Fig. 1). Expression of HCMV IE1 was not observed at 1 h post-infection (p.i.) (Fig. 1b) and was first observed at 3 h p.i. (Fig. 1d), when hsp70...

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Fig. 1. Biphasic translocation of hsp70 in HCMV-infected cells. (a)–(j) Cells were infected with HCMV at 4 p.f.u. per cell and fixed at 1 (a, b), 3 (c, d), 6 (e, f), 12 (g, h) and 24 (i, j) h.p.i. Pairs of micrographs represent the same fields. Cells were double-stained with anti-hsp70 MAb (a, c, e, g and i) and anti-HCMV IE1 MAb (b, d, f, h and j). (k)–(p) Cells were infected with HCMV at 0.5 p.f.u. per cell and fixed at 12 h p.i. Cells were first treated with a mixture of anti-hsp70 MAb (IgG1) and anti-HCMV IE1 MAb (IgG2a) (k, l), anti-HCMV IE1 MAb alone (m, n) or anti-hsp70 MAb alone (o, p) and then with a mixture of FITC-labelled anti-mouse IgG1 and TRITC-labelled anti-mouse IgG2a goat antibodies. Photographs were taken with either colour reversal film (a–j) or negative colour film (k–p) (both from Fuji) under very similar conditions. These films differed in sensitivity and therefore gave different image brightness. Upward arrows indicate cells positive for HCMV IE1 but negative for hsp70 induction. Downward arrows indicate cells with nuclear hsp70 staining 24 h p.i. Leftward arrows indicate uninfected cells. Bars represent 60 µm (a–j) or 50 µm (k–p). (q)–(r) Western blot analysis of hsp70 in nuclear (q) and cytoplasmic (r) fractions from mock-inoculated or HCMV-infected cells. A total protein extract from heat-shocked TIG-3 cells was included as a standard (r, lane 7). Mock-inoculated (q, lanes 1–3; r, 8–10) and HCMV-infected (q, lanes 4–6; r, lanes 11–13) cells were harvested and fractionated at 1 (q, lanes 1, 4, r, 8, 11), 6 (q, lanes 2, 5, r, 9, 12) or 24 (q, lanes 3, 6, r, 10, 13) h.p.i. Positions of hsp70 are indicated to the right. Positions of molecular mass standards are shown to the left.
induction was not detected. At 6 h p.i., nuclear localization of hsp70 and continuous accumulation of HCMV IE1 were observed in some HCMV-infected cells (Fig. 1e, f), although some HCMV IE1-positive cells had not yet accumulated hsp70 (Fig. 1e, upward arrows). The most intense nuclear staining for hsp70 was observed at 12 h p.i. (Fig. 1g). At 12 h p.i. and thereafter, the cytoplasm also stained for hsp70 (Fig. 1g, i). Although nuclear staining for hsp70 was still observed in some HCMV-infected cells at 24 h p.i. (Fig. 1i, downward arrow), nuclear hsp70 staining decreased in intensity and cytoplasmic staining was dominant at this time (Fig. 1i). At 24 h p.i., all HCMV IE1-positive cells were positive for hsp70 induction (Fig. 1i, j). HCMV IE1 remained in the nucleus throughout the experiment (Fig. 1h, j). Staining specificity for hsp70 and HCMV IE1 under our double-staining conditions was confirmed by omitting either anti-hsp70 MAb (Fig. 1m, n) or anti-HCMV IE1 MAb (Fig. 1o, p). Comparison with control staining including both MAbs (Fig. 1k, h) shows clearly that the MAbs detected the corresponding antigen specifically. These observations suggest that hsp70 accumulates in HCMV-infected cell nuclei early after infection and is then translocated to the cytoplasm in the late phase of infection.

To confirm the biphasic translocation of hsp70 in HCMV-infected cells, we fractionated HCMV-infected cells into a nuclei-enriched fraction and a cytoplasmic fraction and analysed the fractions for the presence of hsp70 by Western blotting. Mock-inoculated and HCMV-infected (m.o.i. = 1) cells were harvested at 1, 6 and 24 h p.i., lysed with buffer A (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP40) and centrifuged at 3000 r.p.m. for 5 min at 4 °C. These supernatants were retained as cytoplasmic fractions and the pellets were washed twice with buffer A. The final pellets were resuspended in 20 mM Tris–HCl, pH 7.5 containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS, sonicated and cleared by centrifugation. The supernatants from this final step were retained as protein extracts from nuclear fractions. Fractions were analysed by Western blotting as described previously (Kobayashi et al., 1994) with anti-hsp70 MAb 43 (generated in this laboratory, unpublished results) as the primary antibody (Fig. 1q, r).

In mock-infected cells, hsp70 accumulated gradually in the nucleus (Fig. 1q, lanes 1–3), which may be the result of either cell cycle-regulated hsp70 expression (Morimoto & Milarski, 1991) or of an unidentified stress factor in the culture. In contrast, hsp70 accumulated dramatically in HCMV-infected cell nuclei at 6 h p.i. and had decreased by 24 h p.i. (Fig. 1q, lanes 4–6). Although a small amount of mitochondrial hsp60 was found in the nuclear fraction under our cell fractionation conditions, hsp60 levels in the nuclear fractions were not affected by either HCMV-infection or differences in culture.

Fig. 2. Comparison of hsp70 and hsc73 localization in HCMV-infected cells. (a)–(c) Uninfected, unstressed TIG-3 cells; (d)–(f) heat-shocked TIG-3 cells; (g)–(i) HCMV-infected (m.o.i. = 1) TIG-3 cells at 9 h p.i. Cells were stained with anti-hsc73 MAb (a, d, g) or with anti-hsp70 (b, e, h) or photographed by phase-contrast (c, f, i). Bar represents 30 µm.
Fig. 3. Effect of compounds that halt HCMV multiplication on HCMV-induced hsp70 relocalization. (A) Effect of sequential treatment with CHX and Act D. Cells were treated as indicated at the top of the figure. These compounds inhibit the entire HCMV replicative cycle, including hsp70-induction, except immediate-early gene expression. Mock-inoculated (a) and HCMV-infected (b,c) cells were double-stained for hsp70 (a, b) and HCMV IE1 (c). Bar represents 50 µm. (B)–(C) Effect of PAA and TM on hsp70 translocation. HCMV-infected (m.o.i. = 1) cells were cultured in normal (control), PAA-containing or TM-containing medium for 12, 24 and 48 h and stained for hsp70. (B) Representative micrographs show nuclear staining of PAA-treated HCMV-infected cells at 12 h p.i. (a, also observed in control and TM-treated HCMV-infected cells); nucleo-cytoplasmic staining of PAA-treated HCMV-infected cells at 48 h p.i. (b, also observed in control and TM-treated HCMV-infected cells); predominantly cytoplasmic staining of control HCMV-infected cells (c, barely observed in PAA- or TM-treated HCMV-infected cells); and perinuclear staining of TM-treated HCMV-infected cells (d, indicated by arrows; also observed in PAA-treated HCMV-infected cells but not in control HCMV-infected cells). Bar represents 50 µm. (C) Cell numbers were counted for each staining pattern at each time-point and for each treatment. Shading in (C) corresponds to that indicated in (B); the open bars in (C) indicate cells not showing hsp70 induction.
duration up to 24 h (data not shown). Since no hsp70 induction was apparent at 6 h p.i. in HCMV-infected cells (Fig. 1r, lane 12), incomplete cell fractionation could not have caused false nuclear accumulation of hsp70 in HCMV-infected cells. Similarly, the decrease in nuclear hsp70 at 24 h p.i. could not be an artefact of incomplete cell fractionation, since at this time-point the cytoplasm contained large amounts of hsp70 (Fig. 1r, lane 13). Biochemical testing therefore confirmed the biphasic translocation of hsp70 in HCMV-infected cells.

Hsc73, a constitutively expressed molecular species of the hsp70 family, is reported to interact physically with and be functionally related to heat-inducible hsp70 in both normal and stressed cells (Brown et al., 1993). HCMV-infected cells were double-stained with anti-hsc73 (IgM, Wako Pure Chemicals) and anti-hsp70 MAb 43 (IgG2b) followed by TRITC-conjugated goat anti-mouse IgG2b antibody and FITC-conjugated goat anti-mouse IgM antibody. Phase-contrast micrographs show the position of the nucleus in each field (Fig. 2, c, f, i). In uninfected, unstressed cells, both hsc73 and hsp70 gave homogeneously diffuse staining patterns (Fig. 2, a, b). After heat shock (42 °C for 2 h), hsp70 was apparently induced and concentrated in the nucleus and nucleolus, together with hsc73 (Fig. 2, d, e). The subcellular localization of hsc73 apparently had not changed at 9 h p.i., although hsp70 was relocated to the nuclei of infected cells (Fig. 2, g, h). These results suggest that hsp70 functions in HCMV-infected cell nuclei in the presence of low levels of hsc73 and therefore the function of hsp70 in HCMV-infected cells is different to that in normal or heat-stressed cells.

Next we examined the relationship between the HCMV replication cycle and HCMV-induced relocalization of hsp70 by halting virus replication with inhibitors. First, the HCMV replication cycle was held in the immediate-early phase by infecting cells with HCMV (m.o.i. = 1) in the presence of 100 μg/ml cycloheximide (CHX), culturing for 6 h in CHX-containing medium and then for 12 h in medium containing 10 μg/ml actinomycin D (Act D). Since this treatment inhibits hsp70 induction by HCMV, cells received a mild heat shock (40 °C for 30 min) 12 h before HCMV infection in order to elevate cellular hsp70 levels. Under these treatment conditions, mock-inoculated cells exhibited diffuse hsp70 staining (Fig. 3, A, a) and HCMV-infection did not recruit hsp70 to the nucleus (Fig. 3, A, b and c). This suggests that viral immediate-early proteins are not sufficient to induce hsp70 relocalization, although some of them induce hsp70 gene expression (Colberg et al., 1992; Hagemeier et al., 1992b; Tenney et al., 1993). In the next experiment, phosphonoacetic acid (PAA, 400 μg/ml) and tunicamycin (TM, 1 μg/ml) were added to the cells just after HCMV infection (m.o.i. = 1) and cells were examined by immunofluorescence at 12, 24 and 48 h p.i. for localization of hsp70. Neither PAA nor TM inhibited the HCMV-induced nuclear translocation of hsp70 observed at 12 h p.i. (Fig. 3, B, a). At 24 h p.i., hsp70 was distributed in the nucleus and cytoplasm in both untreated and treated cells (Fig. 3, B, b). At 48 h p.i., most of the untreated cells gave predominantly cytoplasmic hsp70 staining (Fig. 3, B, c), while treated cells mostly gave nuclear, perinuclear and nucleo-cytoplasmic staining (Fig. 3, B, a, d and b, respectively). The number of cells with each staining pattern was counted (about 200 cells in total for each time-point and treatment) and the ratio of each staining pattern for each treatment is shown (Fig. 3C). The results suggest that PAA and TM inhibit hsp70 translocation from the nucleus to the cytoplasm in HCMV-infected cells. Both PAA and TM had little effect on hsp70 induction by HCMV and on hsp70 localization in uninfected TIG-3 cells (data not shown). The modes of action of PAA and TM are different; PAA inhibits viral DNA polymerase activity itself and TM inhibits HCMV replication by down-regulating glycosylated viral gene products (Radsak & Weder, 1981). The effects of both compounds on hsp70 translocation were similar, which suggests that the inhibition of nucleo-cytoplasmic translocation of hsp70 is the result of an interruption of viral DNA replication. Therefore, hsp70 is associated in HCMV-infected cells with a molecular event(s) involved in viral DNA synthesis or subsequent processes.

Our results indicate that hsp70 localizes transiently in the infected cell nucleus and then moves to the cytoplasm, suggesting that hsp70 may play a significant role in both the nucleus and cytoplasm of HCMV-infected cells. Hsps, including hsp70, have been characterized as molecular chaperones. Hsp70 has been reported to interact physically with several viral and cellular proteins (for reviews see Cripe et al., 1995; Kitay & Rowe, 1996, and references therein). In HCMV-infected cells, nuclear hsp70 may be involved in viral or host transcription, viral DNA replication and/or nucleocapsid assembly by interacting with a protein(s) which directly drives these molecular events.

Following the transient nuclear localization of hsp70, we observed large amounts of hsp70 in the cytoplasm of HCMV-infected cells. Cytoplasmic hsp70 probably acts as a molecular chaperone and may be involved in maturation or transport of virions. Two additional functions can be suggested for cytoplasmic hsp70. One is that cytoplasmic hsp70 protects HCMV-infected cells from host defence mechanisms. Over-expression of hsp70 reportedly protects cells from the cytotoxic activity of monocytes or tumour necrosis factor (Jaattela & Wissing, 1993; Jaattela et al., 1992). Recently, hsp70 has been shown to protect cells from stress-induced apoptosis (Mosser et al., 1997). HCMV IE1 and IE2 proteins, which induce hsp70 expression, reportedly block apoptosis (Zhu et al., 1995). Hsp70 may therefore function in the blocking of apoptosis by viral immediate-early proteins. The other possible function of cytoplasmic hsp70 is its involvement in the HCMV-induced transport of major histocompatibility complex class I molecules from the endoplasmic reticulum to the cytosol (Wiertz et al., 1996). This suggestion is an extrapolation of the proposal that hsp70 functions as a force-generating motor in transmembrane protein transport (Glick, 1995).
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References


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