

## Human cytomegalovirus infection results in altered Cdk2 subcellular localization

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**Human cytomegalovirus (HCMV) stimulates numerous cellular pathways upon infection. One of these pathways involves activation of cyclin E/Cdk2. Recent reports have demonstrated that Cdk2 is retained in the cytoplasm of cells arrested in G0 by serum deprivation, sequestered from its regulatory subunit cyclin E which is located within the nucleus. Cdk2 rapidly enters the nucleus and becomes active upon stimulation of these cells with serum growth factors. The ability of HCMV to activate cyclin E/Cdk2 in both serum-arrested cells and contact-inhibited cells suggests that HCMV infection may also result in the translocation of Cdk2 into the nucleus. In this report, we demonstrate that Cdk2 is sequestered in the cytoplasm of cells arrested in G0 by contact inhibition, as well as those arrested by serum deprivation. HCMV infection results in translocation of Cdk2 from the cytoplasm into the nucleus within 24 h of infection, both in serum-arrested and contact-inhibited cells.**

Human cytomegalovirus (HCMV) is a ubiquitous pathogenic herpesvirus that infects over 80% of the population, and causes a variety of pathological manifestations, especially in patients with suppressed immune functions (e.g. following organ transplantation or HIV infection) (Schooley, 1990; Rubin, 1990; Alford *et al.*, 1990). Increasing use of therapeutic immunosuppression and organ transplantation and the incidence of AIDS have focused attention upon the HCMV life-cycle, with a view towards achieving a thorough understanding of virus–cell interactions and thereby identifying approaches to attenuate primary and/or reactivated HCMV infection. HCMV can mitogenically stimulate growth-arrested cells (reviewed in Albrecht *et al.*, 1989). The mitogenic stimulation that one observes upon HCMV infection shares many aspects in common with the cellular immediate early response that is

provoked by addition of serum growth factors to serum-arrested cells. Such virally induced mitogenic responses include increases in second messengers (e.g. cAMP,  $[Ca^{2+}]_i$ ,  $IP_3$ ) (Albrecht *et al.*, 1990) and activation of proto-oncogenes (e.g. *c-myc*, *c-fos*, *c-jun*) (Boldogh *et al.*, 1990; Monick *et al.*, 1992; Hagemeyer *et al.*, 1992). However, HCMV activation of mitotic responses in productively infected cells is incomplete in that cellular DNA synthesis is inhibited (Bresnahan *et al.*, 1996a; Dittmer & Mocarski, 1997; Lu & Shenk, 1996; Albrecht *et al.*, 1989; DeMarchi, 1983). Recently, we proposed a mechanism by which HCMV arrests cells in late G1 but still allows for activation of biosynthetic machinery necessary for virus replication (Bresnahan *et al.*, 1996a). We and others have shown that HCMV infection of G0 cells results in activation of specific G1 cyclin-dependent kinases (Cdks) (Bresnahan *et al.*, 1996a; Jault *et al.*, 1995). These enzymes are crucial regulatory molecules involved in controlling cell-cycle progression and particularly entry into S phase (see Draetta, 1994; Sherr & Roberts, 1995; and references therein). In particular, HCMV causes a dramatic increase in cyclin E/Cdk2 activity following infection (Bresnahan *et al.*, 1996a; Jault *et al.*, 1995). We have proposed that cyclin E/Cdk2 activity is essential for activating cellular genes such as dihydrofolate reductase and thymidine kinase. Both of these enzymes are induced following HCMV infection (Wade *et al.*, 1992; Estes & Huang, 1977) and are involved in nucleotide biosynthesis. However, HCMV does not induce cyclin D1 or cyclin A (Bresnahan *et al.*, 1996a), which may preclude cellular DNA synthesis in productively infected cells (Resnitzky *et al.*, 1995; Girard *et al.*, 1991).

We and others have recently reported that Cdk2 is predominantly located in the cytoplasm of G0, serum-arrested cells, and therefore sequestered from its regulatory subunit, cyclin E, which is located within the nucleus (Bresnahan *et al.*, 1996b; Baptist *et al.*, 1996). Serum stimulation of subconfluent, serum-arrested cells results in increased Cdk2 in the nucleus, which is essential for activation of cyclin E/Cdk2. Since cyclin E/Cdk2 activity is induced when G0 cells are infected with HCMV, we wanted to test whether the virus could promote nuclear uptake of Cdk2 in a fashion similar to that observed for serum. We undertook to test this prediction in HCMV-infected cells that had been arrested in G0 by either serum deprivation or contact inhibition.

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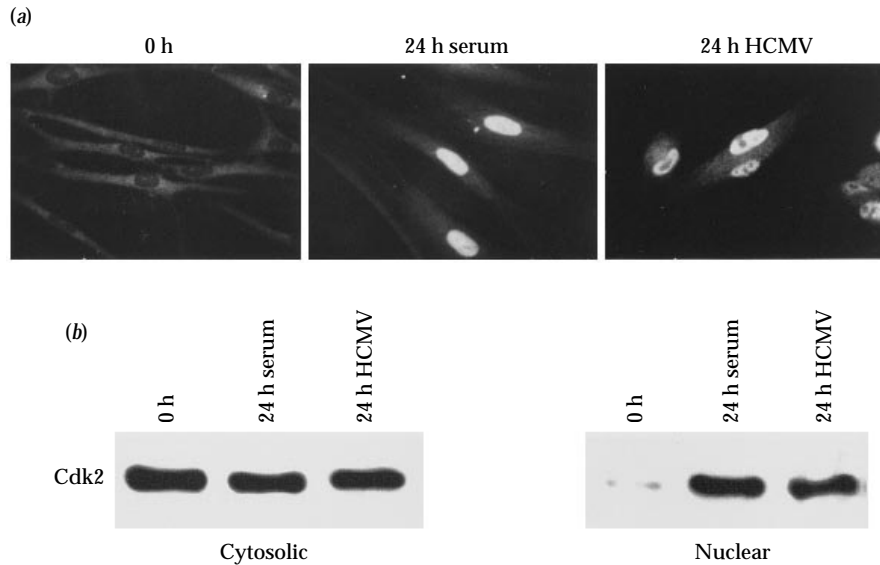


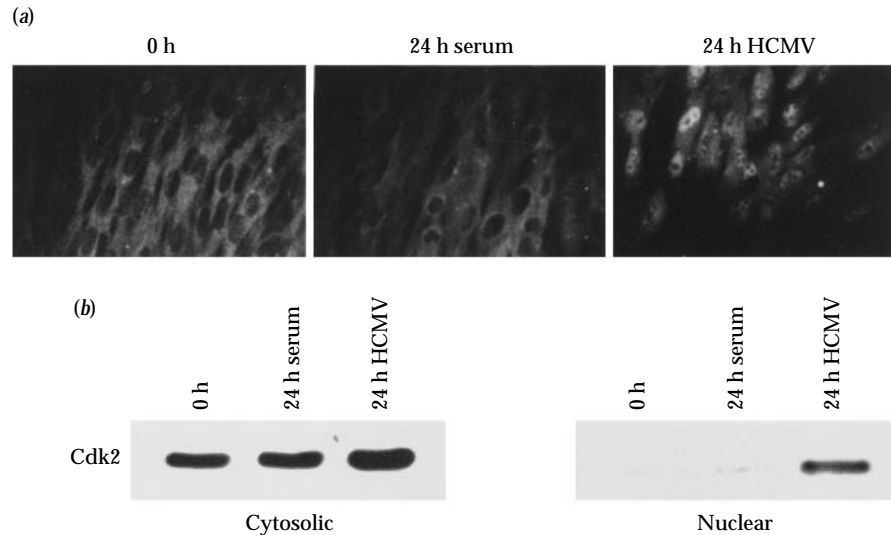
Fig. 1. Subcellular localization of Cdk2 in serum-arrested cells following serum stimulation or HCMV infection. LU cells were growth arrested by serum deprivation at subconfluent densities for 48 h. Cells were then stimulated with 20% FBS or infected with HCMV for 24 h as described. (a) Cells were fixed for immunofluorescence and stained with Cdk2 antibody (Santa Cruz Biotechnology M2). (b) Cytosolic and nuclear fractions were prepared. Aliquots of each fraction were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with Cdk2 antibody (Santa Cruz Biotechnology M2). The abundance of Cdk2 in both nuclear and cytosolic fractions in quiescent cells (0 h) and 24 h after serum stimulation or HCMV infection are shown.

To determine whether HCMV is capable of causing Cdk2 translocation into the nucleus of serum-arrested cells, human diploid embryonic lung fibroblasts (LU cells) in passage 12–20 (Albrecht *et al.*, 1980) were grown on glass coverslips in Eagle's minimum essential medium with Earle's salts (EMEM) containing 10% foetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml) in a 37 °C incubator with a 5% CO<sub>2</sub> atmosphere until they reached 70–80% confluence. The medium was removed and the cells were washed with warm, serum-free EMEM. Fresh serum-free EMEM was placed on the cells, and the incubation was continued for another 48 h. The medium was removed after 48 h and reserved. The cells were either fixed for immunofluorescence, stimulated by addition of 20% FBS in EMEM, or infected with strain AD169 of HCMV at an m.o.i. of 5–10 p.f.u. per cell, as described previously (Bresnahan *et al.*, 1996a). Infected cells were maintained in the reserved 'spent' serum-free medium to ensure that no stimulation would result from the presence of serum growth factors. The cells were fixed 24 h after HCMV infection, mock infection or serum stimulation in acetone-methanol (1:1) for 10 min at –20 °C. Cdk2 antigen was detected by immunofluorescence using an anti-Cdk2 antibody and an FITC-conjugated secondary antibody as previously described (Bresnahan *et al.*, 1996b).

After serum deprivation for 48 h, greater than 90% of the cells are arrested in G<sub>0</sub>/G<sub>1</sub> of the cell cycle (Bresnahan *et al.*, 1996b). Cells arrested in G<sub>0</sub> (0 h) by serum deprivation exhibited a diffuse cytoplasmic immunofluorescence, with little or no nuclear staining (Fig. 1a). HCMV-infected or serum-

stimulated cells exhibited a diffuse cytoplasmic staining pattern and intense nuclear immunofluorescence (Fig. 1a). Mock-infected cells exhibited a staining pattern identical to that observed for G<sub>0</sub> cells (see Fig. 3a). These results suggest that HCMV, like serum, is capable of dramatically increasing the abundance of nuclear Cdk2 within 24 h of infection, at which time cyclin E/Cdk2 activity is maximal (Bresnahan *et al.*, 1996a). Subcellular fractionation was carried out to confirm our immunocytochemical data. Nuclear and cytosolic fractions were prepared, as previously described (Bresnahan *et al.*, 1996b), from subconfluent, serum-arrested cells and from cells that had been HCMV-infected or serum-stimulated for 24 h. The abundance of Cdk2 present in these fractions was measured by Western blotting as shown in Fig. 1(b). Very little Cdk2 was detected in the nuclear fraction of serum-arrested cells (0 h), whereas the amount of nuclear Cdk2 increased dramatically in both HCMV-infected and serum-stimulated cells (Fig. 1b). These results confirm the immunocytochemical data and demonstrate that HCMV (like serum) is capable of altering the subcellular localization of Cdk2 in serum-arrested, subconfluent cells.

HCMV, but not serum, is also capable of activating cyclin E/Cdk2 kinase in contact-inhibited cells (Bresnahan *et al.*, 1996a). Accordingly, we determined if Cdk2 was sequestered in the cytoplasm of cells arrested in G<sub>0</sub> by contact inhibition, and, if it was, if HCMV infection would cause translocation of Cdk2 into the nucleus. LU cells were cultured on glass coverslips and allowed to proliferate until the cells became confluent. The medium was removed and replaced with fresh



**Fig. 2.** Subcellular localization of Cdk2 in contact-inhibited cells following serum stimulation or HCMV infection. LU cells were growth arrested by contact inhibition and then stimulated with 10% FBS or infected with HCMV for 24 h as described. (a) Cells were fixed for immunofluorescence and stained with Cdk2 antibody. (b) Cytosolic and nuclear fractions were prepared. Aliquots of each fraction were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with Cdk2 antibody. The abundance of Cdk2 in both nuclear and cytosolic fractions in arrested cells (0 h) and 24 h after serum stimulation or HCMV infection is shown.

EMEM containing 10% FBS, and the cells were cultured for a further 48 h to obtain strict growth arrest. Under these conditions greater than 90% of the cells were arrested in G<sub>0</sub>/G<sub>1</sub> of the cell cycle (data not shown). The arrested cells were then fixed for immunofluorescence, infected with HCMV, mock-infected or stimulated with fresh EMEM containing 10% FBS, as described previously (Bresnahan *et al.*, 1996a). The cells were washed 24 h later, and fixed for immunofluorescence as described above. Cells arrested in G<sub>0</sub> by contact inhibition demonstrated a diffuse cytoplasmic staining pattern with Cdk2 antibodies. Little or no Cdk2 was detected in the nuclei of these cells (Fig. 2a). Contact-inhibited cells that were HCMV-infected, mock-infected or treated with serum were also stained for Cdk2. Cells treated with 10% FBS or mock-infected showed diffuse cytoplasmic staining with little or no nuclear staining, similar to that seen in untreated cells (Figs 2a and 3a). This result is not surprising, considering that contact-inhibited cells are refractory to serum stimulation and do not enter the cell cycle following serum stimulation (data not shown). However, cells infected with HCMV demonstrated an intense nuclear staining (Fig. 2a). Subcellular fractionation was done to confirm our immunocytochemical data. Contact-arrested LU cells were infected with HCMV or treated with 10% FBS for 24 h as described above. Nuclear and cytosolic fractions were prepared, and Cdk2 abundance was determined by Western blotting. In contact-arrested LU cells, Cdk2 was predominantly located in the cytosolic fraction; and very little Cdk2 was contained within the nuclear fraction (Fig. 2b). Similar results were obtained for contact-arrested cells that had been treated with 10% FBS for 24 h (Fig. 2b). HCMV infection resulted in

a large increase in the abundance of Cdk2 in the nuclear fraction (Fig. 2b). These results confirmed the immunocytochemical data, showing that Cdk2 is predominantly located within the cytoplasm of contact-arrested cells. However, HCMV infection, but not serum growth factors, caused a dramatic increase in the abundance of Cdk2 in the nuclei.

We also investigated if HCMV gene expression is necessary for Cdk2 translocation into the nucleus. To inhibit HCMV gene expression, virus stocks were UV-irradiated on an ice-bed at 254 nm at a dose rate of  $8 \times 10^{-6}$  J/s/mm<sup>2</sup> for 30 min as described previously (Boldogh *et al.*, 1990). Under these conditions, HCMV gene expression is abolished (Boldogh *et al.*, 1990). To ensure that our UV-irradiation protocol inhibited HCMV gene expression, subconfluent, serum-arrested or confluent, contact-inhibited LU cells were infected with HCMV or UV-irradiated HCMV and stained for the expression of HCMV immediate early (IE) proteins. Our UV-irradiation protocol inhibited HCMV IE gene expression by greater than 99% (data not shown). Subconfluent, serum-arrested and confluent, contact-inhibited LU cells were also stained for Cdk2 following infection with UV-irradiated HCMV. As Fig. 3(a) shows, cells stained for Cdk2 following infection with UV-irradiated HCMV demonstrated diffuse cytoplasmic staining with little or no nuclear staining. These results demonstrate that HCMV gene expression is necessary for HCMV-induced translocation of Cdk2 from the cytoplasm into the nucleus of infected cells. To further demonstrate that HCMV gene expression is required for Cdk2 translocation, serum-arrested, subconfluent cells were infected at an m.o.i. of 0.5 p.f.u. per cell and co-stained with antibodies against Cdk2 and HCMV IE

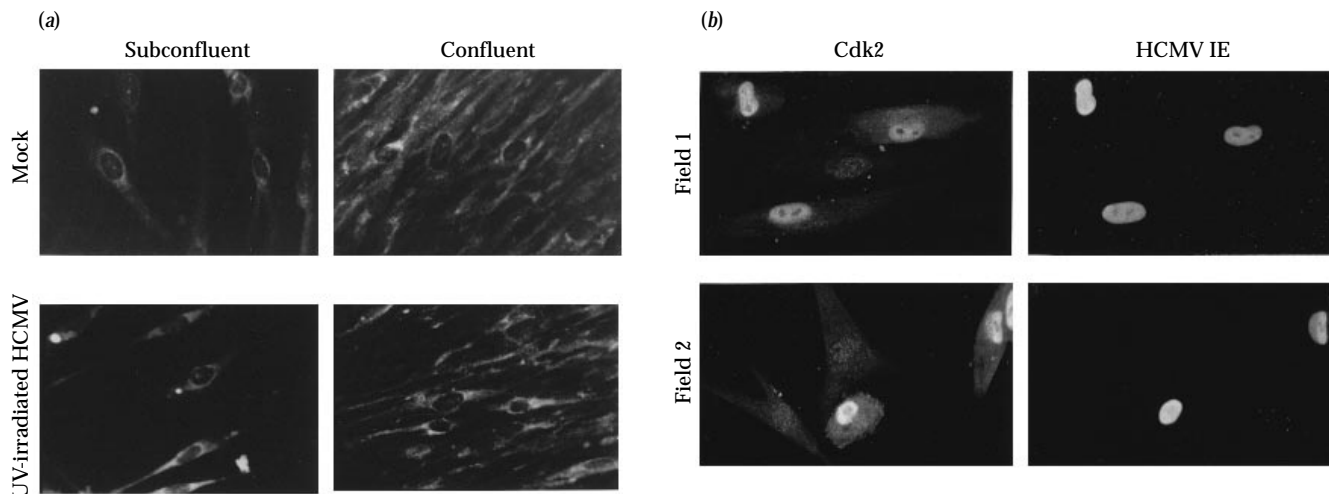


Fig. 3. HCMV gene expression is required for Cdk2 translocation into the nucleus. LU cells were growth arrested either by serum deprivation (subconfluent) or contact inhibition (confluent) and then either infected with stock HCMV, UV-irradiated HCMV or mock-infected. (a) Cells were mock-infected or infected with UV-irradiated HCMV for 24 h and then fixed for immunofluorescence and stained with Cdk2 antibody. (b) Subconfluent, serum-arrested cells were infected with HCMV at an m.o.i. of 0.5 p.f.u. per cell for 24 h, fixed for immunofluorescence and co-stained with HCMV immediate early antibody or Cdk2 antibody.

antigens. Fig. 3 (b) shows two fields of cells which demonstrate that only cells infected and expressing IE antigens expressed abundant Cdk2 in the nucleus.

HCMV is a DNA virus that replicates in the nucleus of post-mitotic cells *in vivo* (Weller, 1971). Therefore, the virus must be able to overcome the normal constraints upon mitotic activation of arrested cells, and thereby activate the biosynthetic machinery that is required for HCMV replication. We propose that the ability to induce cyclin E and activate Cdk2 is a necessary step in generating the biosynthetic precursors necessary for HCMV replication (Bresnahan *et al.*, 1996 a). The results presented here demonstrate that HCMV is capable of eliciting nuclear translocation of Cdk2, which is essential for Cdk2 activation. When contact-inhibited cells are treated with serum growth factors, this translocation and activation of Cdk2 is not observed (Bresnahan *et al.*, 1996 a). This suggests that the sequestration of Cdk2 in the cytoplasm of contact-inhibited cells may be a key constraint in blocking cell proliferation during contact-inhibition. However, HCMV is capable of overcoming these constraints and results in translocation and activation of Cdk2. These results are entirely consistent with our hypothesis that cyclin E/Cdk2 activity is required for HCMV replication. This is also the first demonstration that Cdk2 is sequestered in the cytoplasm of contact-inhibited cells. The present findings provide further insights into cell-cycle regulation, and the ability of HCMV to regulate key cell-cycle regulatory molecules that help prepare the cell for efficient virus replication.

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