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ORIGINAL ARTICLE

Regulation of CCAAT/enhancer-binding protein (C/EBP) α in human-cytomegalovirus-infected fibroblasts

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Abstract CCAAT/enhancer-binding protein (C/EBP) α, a member of the C/EBP family of transcription factors, is known to be involved in gene expression and DNA replication of human cytomegalovirus (HCMV). This study aimed to understand the regulation of endogenous C/EBPα during HCMV infection using an *in vitro* infection model. The expression and localization of C/EBPα were investigated in fibroblasts infected with HCMV. The overexpression of C/EBP homologous protein (CHOP), the endogenous inhibitor of C/EBP, was also employed to test the involvement of C/EBPα during HCMV infection. Our data showed that HCMV infection increases the expression of the full-length C/EBPα isoform (p42) especially during the late stage of infection at the transcriptional and post-translational levels. The increased p42 accumulated in the viral DNA replication compartment. p42 expression was not induced in cells treated with UV-irradiated virus or in cells infected with normal virus in the presence of ganciclovir. CHOP-mediated inhibition of C/EBP activity suppressed viral gene expression and DNA replication, which lowered the level of viral production. Together, our data suggest that HCMV-mediated C/EBPα regulation might play a beneficial role in the lytic cycle of HCMV.

Introduction

Human cytomegalovirus (HCMV/HHV-5), a member of the subfamily *Betaherpesvirinae*, is a widespread pathogen that causes serious diseases in congenitally infected infants and immunologically compromised individuals [1]. Upon primary infection, HCMV establishes lifelong latency with periodic reactivation. During latency, viral gene expression is limited to a small number of latency-associated transcripts [2]; however, during the lytic infection stage, most of the viral genes are expressed in three different temporal classes: immediate-early (IE), delayed-early (E), and late (L). Viral DNA replication occurs after E gene expression, and the onset of viral DNA synthesis is necessary for L gene expression [3].

The CCAAT/enhancer-binding proteins (C/EBPs), a family of basic leucine/zipper (bZIP) transcription factors, are host factors thought to be involved in the HCMV lytic cycle by regulating viral gene expression and DNA replication. There is a C/EBP-binding site in the major immediate early promoter (MIEP) of HCMV [4]. Because MIEP activity controls the expression of HCMV IE1 and IE2 genes, C/EBP is likely to regulate the expression of these genes and thus contribute to HCMV lytic replication. Two additional C/EBP-binding sites are also present in the lytic origin of replication (oriLyt) [5], at which HCMV DNA replication is initiated. Interestingly, one of these two sites has been found to be necessary for the replication of an oriLyt-containing plasmid in a transient replication assay, suggesting a crucial role of C/EBP in the viral DNA replication process [5].

The C/EBP family proteins form homodimers or heterodimers with other family members or with members of other transcription factor families [6–8]. Intra-family dimers bind to the consensus sequence RTTGCGYAAY

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(R = A or G and Y = C or T) and regulate the expression of target genes [9]. C/EBPα, the founding member of this family, plays important roles in adipocyte differentiation, hematopoiesis, and cell cycle regulation [10–16]. C/EBPα exists as two isoforms, full-length p42 and N-terminally truncated p30, which is generated by translation initiation at a downstream AUG codon within the same transcript [17]. C/EBPα-p30 is a dominant-negative inhibitor of p42 and plays additional roles that are not yet clearly understood [18, 19].

In this study, we investigated the effect of HCMV infection on endogenous C/EBPα using permissive fibroblasts. HCMV infection resulted in an increase in the level of C/EBPα-p42 and the translocation of C/EBPα-p42 to the viral DNA replication compartments. By overexpressing C/EBP homologous protein (CHOP), which inhibits the DNA-binding activity of C/EBP, it was found that C/EBP activity plays a beneficial role in the lytic cycle of HCMV.

Materials and methods

Cell culture and reagents

HFFs (passages 12 to 20) and HEK 293T cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin/streptomycin solution (Invitrogen-Gibco) at 37°C in a humidified atmosphere containing 5 % CO_2. GCV and cycloheximide were obtained from Sigma.

HCMV and infections

For the preparation of HCMV (Towne strain), HFFs were infected at a multiplicity of infection (m.o.i.) of 0.01. The culture supernatants were collected at 10-14 days postinfection (d p.i.), filtered through a 0.45-μm membrane, and concentrated by ultracentrifugation at 25,000 r.p.m. (92,600 × *g*) using a Beckman SW32 rotor for 1.5 h at 4 °C. The viral stocks were stored in aliquots at -70 °C. The titers of the viral stocks were determined using an infectious center assay [20]. To prepare UV-irradiated HCMV, viral stocks were placed in 1.5-ml tubes and exposed to 254-nm UV light (40 W) for 1 h. For HCMV infection, the cells were incubated with the viral inocula for 2 h at 37 °C in a humidified atmosphere of 5 % CO_2. All infections were performed at an m.o.i. of 3.

Plasmids

The CHOP gene was amplified from the cDNA of uninfected HFFs using the following PCR primers: forward, 5'-ACGCGTATGGAGCTTGTTCCAGCC-3'; reverse, 5'-GGATCCTCATGCTTGGTGCAGATT-3'. The PCR products were cloned into pGEM-T Easy Vector (Promega) to generate the plasmids pGTZ-hCHOP, which was then sequenced. pGTZ-hCHOP was digested with *Mlu*I and *Bam*HI. The digested products were purified and cloned into the retroviral vector MSIG [21], which had been digested with the same pairs of enzymes, generating the plasmid MSIG-hCHOP.

Cell transduction

The retroviral vectors were prepared by the three-plasmid transfection method using Lipofectamine® LTX and PLUS™ Reagent (Invitrogen), according to the manufacturer's protocols. The packaging constructs included pVM-gp for the Gag-pol genes from MLV and pCA-VSVG for the Env gene [22]. Two days later, the supernatants were collected and concentrated as described for HCMV. The retroviral vector titers were measured by counting the GFP-positive cells using fluorescence-activated cell sorting (FACS) as described previously [21]. To obtain the transduced HFFs, 10^5 cells were transduced at an m.o.i. of 3; polybrene (8 μg/ml) was included to increase the transduction efficiency (over 80 %). The cultures were expanded and sorted by FACS.

Immunoblotting

Cell lysates were made using Cytobuster (Novagen) supplemented with protease- and phosphatase-inhibitor cocktails (cOmplete and PhosSTOP, respectively, Roche Diagnostics Ltd.) according to the manufacturer's protocol. The protein concentration was determined using a Bradford assay (Bio-Rad). Equal amounts of protein (5–10 μg) were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked for 1 h at room temperature (RT) with TBST (150 mM NaCl, 10 mM Tris-HCl, 0.1 % (v/v) Tween 20, pH 8.0) containing 1 % (w/v) bovine serum albumin (Invitrogen-Gibco) and incubated overnight at 4 °C with primary antibodies that were diluted in the blocking buffer. After washing with TBST, the membranes were incubated with horseradish-peroxidase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (A0168 or A0545; 1:100000; Sigma) for 1 h at RT. The protein bands were detected using an enhanced chemiluminescence system (WBKL S0500; Millipore). The immunoblotting analyses were performed using the following primary antibodies: rabbit polyclonal anti-C/EBPα (SC-61; 1:500; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-IE (Mab810; 1:2000; Millipore), mouse monoclonal anti-UL44 (CA006; 1:1000; Virusys), mouse monoclonal anti-pp28 (CA004;

1:2000; Virusys), mouse monoclonal anti-CHOP (2895; 1:500; Cell Signaling Technology), and mouse monoclonal anti-β-actin (A5441; 1:10000; Sigma).

Immunofluorescence

Cells grown on coverslips were extracted using CSK buffer before fixation and permeabilization as described previously [23]. The CSK-extracted cells were fixed in PBS containing 4 % (w/v) paraformaldehyde, permeabilized with 0.5 % (v/v) Triton X-100 in PBS, and blocked for 1 h with 10 % (v/v) human serum (S1; Millipore) and 5 % (w/v) glycine in PBS. The primary antibodies diluted in blocking buffer were incubated with the cell monolayers overnight at 4 °C. After washing with PBS, the samples were incubated with Alexa Fluor® 488- or Alexa Fluor® 555-conjugated secondary antibodies (Invitrogen) diluted in PBS for 1 h at RT. Hoechst 33258 (0.1 mM in PBS) was used to stain the nuclear DNA. Immunofluorescence analysis was performed using antibodies to C/EBPα (SC-61), UL44 (CA006), and GFP (A11122; 1:1000; Invitrogen). Immunoglobulin (IgG) from normal rabbit serum was used as a negative control (I5006; 1:1000; Sigma).

Genomic DNA extraction and real-time quantitative PCR

The genomic DNA was isolated from 2×10^6 cells using a Gentra® Puregene® Kit (QIAGEN), according to the manufacturer's protocol. The genomic DNA (1–2 ng) was subjected to real-time quantitative PCR using the LightCycler SYBR® Green I technology (Takara Bio Inc.) according to the manufacturer's protocol. PCR was performed using the following primers:

HCMV genome: forward, 5'-AAAGATCCGAACTTTAAAATTGTGTGTTTTT-3'; reverse, 5'-TGCTCACCGCCTCGCCGGCCACGGGGTTGA-3'. Cellular DNA: forward, 5'-GCTGCCCAAACCACTTCTGT-3'; reverse, 5'-GCCCTTTCACCTCCCACCTA-3'.

Quantitative reverse transcription PCR (quantitative RT-PCR)

Total RNA (0.5 μg) extracted using TRIzol Reagent was used for cDNA synthesis with AMV reverse transcriptase (Life Science) and oligo(dT) primers (QIAGEN). Real-time quantitative PCR was performed in triplicate with LightCycler SYBR® Green I technology (Takara Bio Inc.) according to the manufacturer's protocol using the following primers to detect human C/EBPα and GADPH as an internal control:

C/EBPα: forward, 5'-TGGACAAGAACAGCAACGAGTA-3'; reverse, 5'-ATTGTCACTGGTCAGCTCCAG-3' [24]. GAPDH: forward, 5'-GTCGGAGTCAACGGATTTGGTCGT-3'; reverse, 5'-GACGGTGCCATGGAATTTGCCATG-3' [25]. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method [26].

Statistical analysis

The data are presented as the mean ± SD of triplicate samples. The significance of the difference between values was determined using an unpaired *t*-test or a one-way ANOVA, followed by Bonferroni multiple comparisons tests, using GraphPad Prism software (Version 5). *P*-values less than 0.05 were considered to represent significant differences (ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

Effect of HCMV infection on the expression and localization of C/EBPα

To test the effect of HCMV infection on C/EBPα expression, human foreskin fibroblasts (HFFs) were infected with HCMV (Towne strain). Total proteins were prepared at the indicated time points and subjected to immunoblotting analysis using an antibody to C/EBPα. In uninfected HFFs, the expression levels of C/EBPα were very low (Fig. 1a, lanes 1, 3, and 5). However, HCMV infection greatly increased the expression levels of C/EBPα, especially the p42 isoform of C/EBPα, during the late stage of infection (Fig. 1a, compare lanes 3 and 4 or 5 and 6). These data showed that HCMV infection regulated the expression of the p42 isoform of C/EBPα in infected cells. Because p42 is the full-length C/EBPα containing the transcriptional activity, these data also suggested that HCMV infection might induce C/EBPα activity during the late stage of infection.

The intracellular localization of the C/EBPα protein at 48 hours post-infection (h p.i.) was also investigated using immunofluorescence. The HCMV infection increased the fluorescence intensity of C/EBPα (red), which was low in uninfected control cells (Fig. 1b, compare panels 2 and 10). No change was observed when purified IgG from normal serum was used instead of the C/EBPα-specific antibody (Fig. 1b, compare panels 6, and 10). C/EBPα was present within the nuclei of the infected cells in areas with a globular shape that resembled the viral DNA replication compartment (Fig. 1b, panels 10 and 11). The fluorescence corresponding to C/EBPα also co-localized with that of the viral protein UL44 (green fluorescence), which is known to be localized to this sub-nuclear structure (Fig. 1b, panel 12) [27]. These results indicated that C/EBPα accumulated

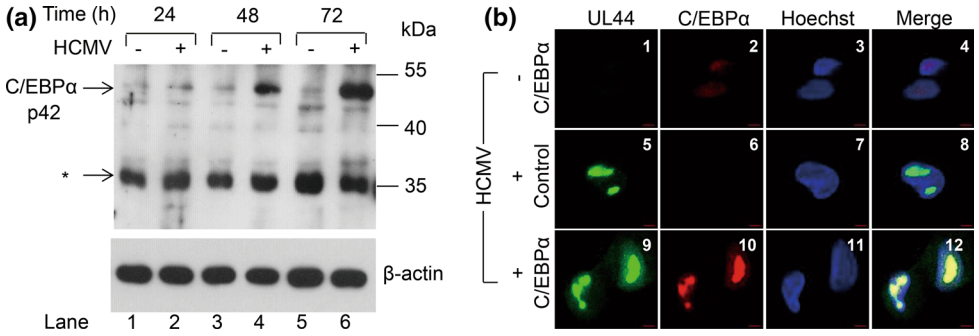

Fig. 1 Effects of HCMV infection on C/EBPα. (a) HFFs were infected with HCMV at an m.o.i. of 3 and harvested at the indicated time points. The expression level of C/EBPα was measured by immunoblotting analysis using a C/EBPα-specific antibody. β-actin protein was detected as a loading control. An asterisk (*) indicates a band corresponding to an unidentified protein of ~35 kDa that is attributed to nonspecific binding of the antibody [35]. (b) HFFs were infected with HCMV and examined by immunofluorescence at 48 h p.i. using UL44- (green) and C/EBPα- (red) specific antibodies. IgG purified from preimmune serum served as a negative control for the C/EBPα-specific antibody. Nuclei were stained with Hoechst stain (blue). Scale bar: 5 μm (color figure online)

in the viral DNA replication compartment in infected cells during the late stage of infection.

Involvement of the expression of viral genes and the initiation of viral DNA replication in the HCMV-mediated induction of C/EBPα-p42 expression

To examine the possible involvement of viral gene products in HCMV-mediated induction of p42 expression, the effect of UV-irradiated HCMV on the p42 level was examined. Cells were infected with either HCMV or UV-irradiated HCMV, and total proteins were harvested at 48 h p.i. Immunoblot analysis was then performed using antibodies specific for IE1/IE2 and C/EBPα. UV irradiation of the virus stock prior to infection abolished the expression of IE1 and IE2, as expected (Fig. 2a, compare lanes 2 and 3). Unlike non-irradiated virus infection, UV-irradiated HCMV infection had no effect on p42 expression (Fig. 2a). These data showed that viral gene expression was required for the induction of p42 expression by HCMV infection.

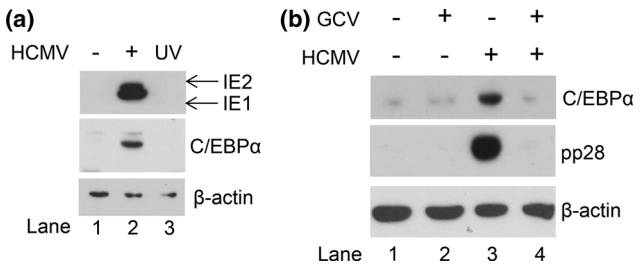

Fig. 2 Involvement of viral gene expression and the onset of viral DNA replication in HCMV-mediated regulation of C/EBPα expression. (a) HFFs were treated with UV-inactivated HCMV, followed by immunoblotting analysis at 48 h p.i. (b) HFFs were infected with HCMV in the absence or presence of 30 μM GCV. Protein lysates were prepared at 72 h p.i., followed by immunoblotting analysis using specific antibodies to C/EBPα, pp28, and β-actin

The possible involvement of the viral DNA replication process in the regulation of C/EBPα expression was also investigated. HFFs were infected with HCMV in the absence or presence of 30 μM ganciclovir (GCV), an inhibitor of viral DNA synthesis. Cells were harvested at 72 h p.i., followed by Western blot analysis to monitor the C/EBPα expression level. In control cells, HCMV infection increased the level of p42 expression (Fig. 2b, compare lanes 1 and 3), which was strongly inhibited by GCV (Fig. 2b, compare lanes 3 and 4). As a control, the level of the late protein, UL99 (pp28), was measured and found to be undetectable in the presence of GCV (Fig. 2b, compare lanes 3 and 4). These data showed that initiation of viral DNA replication was necessary for the HCMV-mediated induction of C/EBPα-p42 expression.

HCMV-mediated increase in the level of C/EBPα mRNA and the stability of p42 protein

To identify the step at which the HCMV infection regulated C/EBPα expression, the level of C/EBPα mRNA was measured. HFFs were infected with HCMV, and the total RNA was extracted and analyzed by quantitative reverse transcription-PCR using the primer specific for C/EBPα. The steady-state level of C/EBPα mRNA began to increase at 12 h p.i. and reached its highest level at 24 h p.i., followed by a decrease (Fig. 3a). These results suggested that transcriptional activation might be involved in the HCMV-mediated induction of C/EBPα expression.

Next, the effect of HCMV infection on the stability of p42 protein was tested using cycloheximide (CHX) to inhibit protein synthesis. Prior to CHX treatment, HFFs were cultured in a confluent state for 4 days (Fig. 1a, compare lanes 1 and 5) or infected with HCMV and cultured for 2 days to induce p42 expression. Then, cells were treated with 100 μM CHX. Total proteins were harvested at

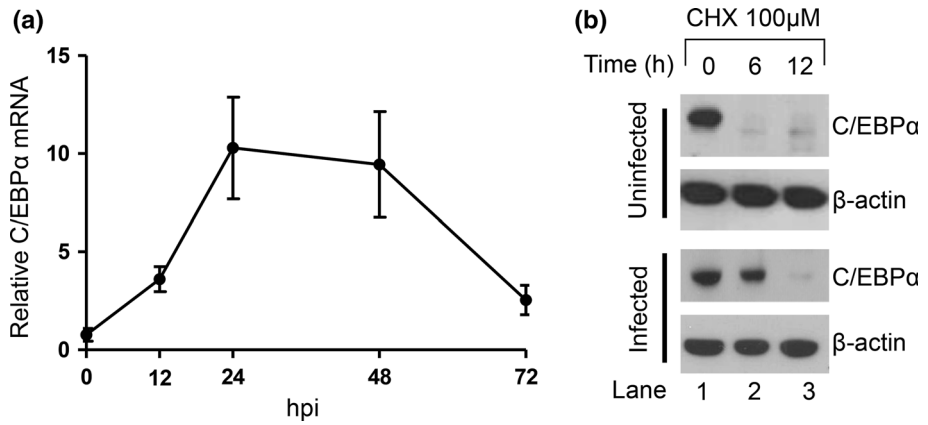

Fig. 3 Involvement of transcriptional and post-translational mechanisms in HCMV-mediated induction of C/EBPα expression. (a) C/EBPα mRNA levels were determined by quantitative reverse transcription PCR using the primer specific for C/EBPα. GAPDH RNA levels served as a loading control. (b) Confluent HFFs were cultured for 4 days (Uninfected) or infected with HCMV and cultured for 2 days (Infected). Cells were then treated with 100 μM cycloheximide (CHX). Total proteins were harvested at the indicated time points after CHX treatment, followed by immunoblotting analysis using specific antibodies to C/EBPα and β-actin

the indicated time points after CHX treatment, followed by immunoblotting analysis. Unlike uninfected control cells, where the level of p42 decreased to an undetectable level at 6 h after CHX treatment (Fig. 3b, uninfected panel, compare lanes 1 and 2), the level of p42 in HCMV-infected cells was comparable to that of the untreated control cells at the same time point after CHX treatment (Fig. 3b, "infected", compare lanes 1 and 2). These data indicated that the p42 protein had a longer half-life in HCMV-infected cells than in uninfected cells and suggested that this stabilization of p42 might contribute to the high level of expression of C/EBPα-p42 observed during HCMV infection.

Generation of HFFs overexpressing a dominant negative inhibitor of C/EBP

To investigate whether C/EBPα is involved in the lytic cycle of HCMV, HFFs were engineered to stably express CHOP, a protein that inhibits the DNA binding activity of C/EBP in a dominant-negative fashion by directly associating with C/EBPs such as C/EBPα and C/EBPβ [28]. HFFs were transduced with retroviral vectors expressing CHOP and GFP from a bicistronic construct. The transduced cells were enriched for GFP-expressing cells, and the remaining GFP-negative cells served as a negative control. GFP expression was confirmed by immunofluorescence assay after the sorting procedure (Fig. 4a, upper panel). CHOP expression was also confirmed by immunoblotting analysis with a specific antibody. CHOP protein was virtually undetectable in the GFP-negative cells, whereas it was present at high levels in the GFP-positive cells (Fig. 4a, lower panel).

The kinetics of endogenous CHOP expression in HCMV-infected cells was investigated by Western blot analysis using a CHOP-specific antibody. The CHOP protein was undetectable in uninfected control cells (Fig. 4b, lanes 1). When HFFs were infected with HCMV, the CHOP protein was not detected at 2 d p.i., and its level was increased at 3 d p.i. (Fig. 4b, lanes 2, 3, and 4). The HCMV-induced level of CHOP expression was lower than the level of CHOP expression in retrovirally transduced cells. These data indirectly suggest that CHOP overexpression may inhibit the DNA-binding activity of C/EBP in HCMV-infected cells, at least during the first 48 h p.i.

Suppression of viral gene expression and DNA replication in CHOP-overexpressing cells

To test the involvement of C/EBP during HCMV infection, control and CHOP-overexpressing cells were infected with HCMV and the expression of selected viral genes was compared using immunoblotting analysis. The elevated CHOP expression reduced the levels of IE1 and UL44 at 1 d p.i. (Fig. 5a, compare lanes 3 and 4). These differences were less prominent at 2 d p.i. (Fig. 5a, compare lanes 5 and 6). However, at this time point, the expression levels of IE2 and pp28, which are both known to be dependent on viral DNA synthesis, were greatly decreased in the CHOP-overexpressing cells (Fig. 5a, compare lanes 5 and 6). These data showed that C/EBP activity was required for the expression of at least some viral genes.

The effect of CHOP overexpression on the replication of the viral genome was also investigated. Control and CHOP-overexpressing cells were infected with HCMV. The genomic DNAs were extracted at the indicated time points and subjected to real-time quantitative PCR analysis. At 1 d p.i., the amount of the viral genome was low in both the CHOP-overexpressing and control cells (Fig. 5b,

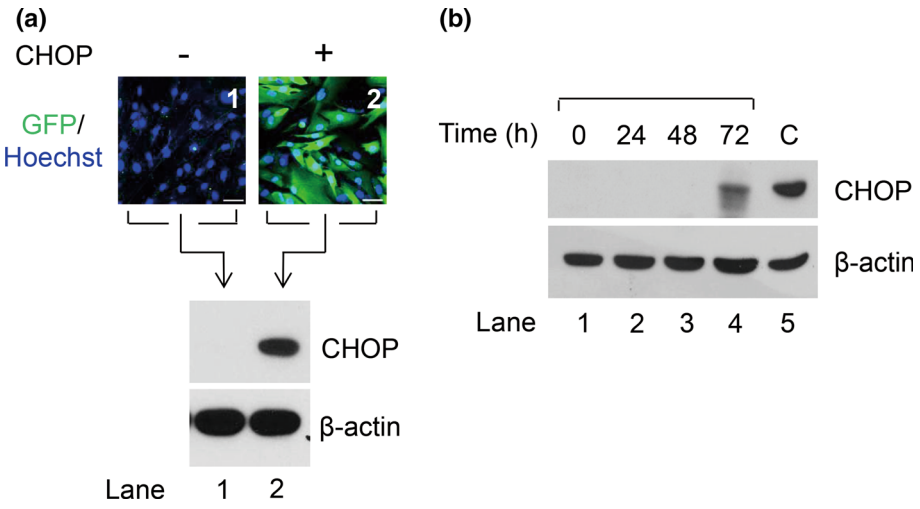

Fig. 4 Generation of CHOP-overexpressing cells. (a) GFP-positive cells acquired by FACS were immunostained with an anti-GFP antibody along with the control GFP-negative cells (upper panel). Nuclei were stained with Hoechst stain (blue). Scale bar: 50 μm. Protein lysates were prepared from each type of cell, and the expression level of CHOP was determined by immunoblotting analysis using CHOP- and β-actin-specific antibodies (lower panel). (b) HFFs were infected with HCMV, and protein lysates were obtained at the indicated time points. The level of endogenous CHOP protein was determined by immunoblotting analysis using antibodies specific for CHOP and β-actin (color figure online)

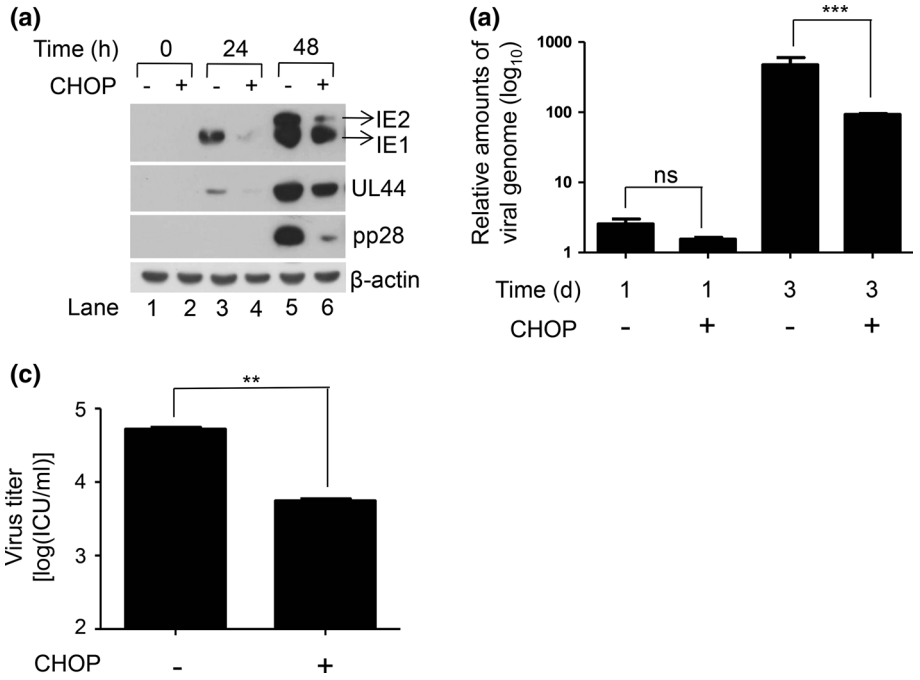

Fig. 5 Involvement of C/EBP activity in the lytic cycle of HCMV. (a-c) HFFs overexpressing CHOP and control cells were infected with HCMV. (a) Total proteins were harvested at the indicated time points, and the levels of viral IE1/IE2, UL44, and pp28 proteins were analyzed by immunoblotting using antibodies specific for each protein. (b) Genomic DNA was extracted at 1 and 3 d p.i. and subjected to real-time quantitative PCR using primers specific for the viral and cellular DNA. Cellular DNA served as an internal control. Data were analyzed by one-way ANOVA, followed by Bonferroni multiple comparisons tests (n = 3). (c) Culture supernatants were harvested at 72 h p.i., and the titers of progeny virus in the culture supernatant were measured using infectious center assays. Data were analyzed using an unpaired *t*-test (n = 3)

compare lanes 1 and 2), but at 3 d p.i., it was 5-fold lower in the CHOP-overexpressing cells than in the controls (Fig. 5b, compare lanes 3 and 4). Consistent with this result, the CHOP-overexpressing cells produced approximately 10-fold fewer viral progeny than the control cells at 3 d p.i., as measured by an infectious center assay (Fig. 5c).

These results showed that C/EBP transcriptional activity was required for the efficient replication of the viral genome and, ultimately, for the increased production of viral progeny.

Discussion

Our data showed that HCMV infection greatly increased the expression of C/EBPα-p42 at the transcriptional and post-translational levels and also induced the localization of C/EBPα-p42 to the viral DNA replication compartment. These effects seem to be the direct consequence of HCMV infection as evidenced by the comparable level of p42 expression when infected cells were treated with a viral DNA synthesis inhibitor.

Data from the experiment involving CHX indicated that the half-life of the C/EBPα-p42 protein might be increased by HCMV infection. It is not yet clear how the stability of p42 is enhanced by HCMV infection. One possibility is that a viral protein(s) directly associated with p42 inhibits a rapid degradation of p42 in the viral DNA replication compartment, similar to the case of p53 and IE2 [29]. Such a direct interaction between p42 and viral proteins is a possible scenario because we showed that p42 is localized to this compartment.

Because p42 is an unstable protein, with a half-life of approximately 30 min [30], it is a limiting factor for transcriptional activity of C/EBPα. Therefore, HCMV-mediated control of p42 is thought to produce a wide range of effects on the host cell. For example, C/EBPα has been shown to the control cell cycle, and thus, HCMV-mediated p42 activation may induce cell cycle arrest in HCMV-infected cells, similar to the case of EBV and KSHV infections [30] [31]. C/EBPα is also a key regulator of adipogenesis, and thus, the increased level of p42 might induce adipocyte-like differentiation of infected cells [32].

One can argue that CHOP overexpression could indirectly suppress HCMV lytic replication, for example, via the induction of apoptosis [33]. However, when cell rounding and DNA fragmentation were analyzed in HFFs overexpressing CHOP, CHOP overexpression did not seem to induce apoptosis in our experimental setting (data not shown). Therefore, the suppression of HCMV infection by elevated CHOP expression appears to be the consequence of its ability to inhibit the DNA-binding activity of C/EBP.

Because CHOP suppresses multiple members of the C/EBP family proteins such as C/EBPα, C/EBPβ, and possibly C/EBPγ and C/EBPδ, it is not clear that the suppressive effect of CHOP on HCMV infection can be attributed to the action of C/EBPα only. HCMV infection increased the level of C/EBPβ, C/EBPγ, and C/EBPδ according to a recent proteomic analysis [34], suggesting the possible roles of these factors during HCMV infection. However, based on its late-stage-specific induction and localization in the viral DNA replication compartments, it is tempting to speculate that the suppressive effect of CHOP on viral DNA replication and late gene expression has resulted from the inhibition of C/EBPα. The identification of a cis-acting element(s) that mediates the positive effect of C/EBPα on HCMV infection would be useful to demonstrate an exact role(s) of C/EBPα during HCMV infection and might provide novel insights into the regulation of the HCMV lytic cycle.

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