The chromatin-tethering domain of human cytomegalovirus immediate-early (IE) 1 mediates associations of IE1, PML and STAT2 with mitotic chromosomes, but is not essential for viral replication

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Human cytomegalovirus (HCMV) immediate-early (IE) 1 protein associates with chromosomes in mitotic cells using its carboxyl-terminal 16 aa region. However, the role of this IE1 activity in viral growth has not been evaluated in the context of mutant virus infection. We produced a recombinant HCMV encoding mutant IE1 with the carboxyl-terminal chromosome-tethering domain (CTD) deleted. This IE1ΔCTD virus grew like the wild-type virus in fibroblasts, indicating that the CTD is not essential for viral replication in permissive cells. Unlike wild-type virus infections, PML and STAT2, which interact with IE1, did not accumulate at mitotic chromosomes in IE1ΔCTD virus-infected fibroblasts, demonstrating that their associations with chromosomes are IE1 CTD-dependent. IE1 SUMOylation did not affect IE1 association with chromosomes. Our results provide genetic evidence that the CTD is required for the associations of IE1, PML and STAT2 with mitotic chromosomes, but that these IE1-related activities are not essential for viral replication in fibroblasts.
primate CMV IE1 homologues, it was not necessary for complementation of CR208 replication in IE1-transfected cells (Reinhardt et al., 2005). However, the role of the IE1 association with mitotic chromosomes during productive infection has not been directly evaluated using a mutant virus.

To investigate the role played by the chromatin-tethering activity of IE1 during viral replication, we produced a recombinant HCMV encoding CTD-deleted IE1(ΔCTD) and its revertant virus. The Towne-BAC (T-BAC) clone (Marchini et al., 2001) was used as a template for mutagenesis (Fig. 1a). To create a transfer vector (pSHJ14) for IE1 mutagenesis, a 4.1 kb SalI–PvuII restriction fragment containing the mutant UL123 allele, which has a stop codon at its 476 aa position, was cloned into pGS284 (AmpR). This was conjugated into RecA+ Escherichia coli harbouring the T-BAC clone (CmR). Exoconjugates and the T-BAC clone encoding IE1(ΔCTD) (pSHJ15) were selected as described previously (Lee et al., 2004; Marchini et al., 2001). The revertant T-BAC clone (pSHJ16) was also generated by allelic exchange of the mutated allele in pSHJ15 with the wild-type SalI–PvuII DNA fragment cloned in pGS284 (pHR8). Mutagenesis was confirmed by direct sequencing (data not shown). In addition, the lack of any other alterations in the viral genomes was checked by comparing the restriction fragment patterns of viral genomes using pulsed-field gel electrophoresis (Fig. 1b). The recombinant viruses were produced in permissive human foreskin diploid fibroblast (HF) cells that were transfected via electroporation with the purified T-BAC DNAs as described previously (Lee et al., 2004; Marchini et al., 2001). In all cells that were transfected with wild-type, IE1(ΔCTD) mutant or revertant T-BAC DNAs, GFP signals (from the GFP cassette within the viral genome) appeared at 2 weeks after electroporation and spread into surrounding cells, indicating that the IE1 CTD is not essential for viral growth (Fig. 1c). Immunoblotting of total cell lysates prepared from cells at 4 weeks after electroporation confirmed that IE1(ΔCTD) was expressed as a slightly faster migrating form than wild-type IE1, and that IE2 (86 kDa) from T-BAC encoding IE1(ΔCTD) was normally expressed at levels comparable to those of wild-type and revertant T-BACs (Fig. 1d).

We investigated the need for the CTD of IE1 for the targeting to mitotic chromosomes during infection. Virus stocks were prepared as described previously (Ahn et al., 1998; Kim et al., 2008). HF cells were infected with wild-type, IE1(ΔCTD) mutant or revertant viruses and indirect immunofluorescence assays (IFA) were performed to determine the localization patterns of IE1. It was found that, unlike wild-type and revertant IE1, IE1(ΔCTD) did not associate with mitotic chromosomes (Fig. 2a). In the context of virus infection, this result confirms that the CTD is required for IE1 to associate with mitotic chromosomes.

We next investigated the growth kinetics of mutant virus in HF cells at high and low m.o.i. values. To measure virus titres, the infectious centre assays were performed as

![Fig. 1. Production of IE1(ΔCTD) mutant and its revertant viruses. (a) Schematic diagram for the generation of T-BAC clones. Positions of the stop codon introduced at aa 476 are indicated by arrowheads. (b) Restriction fragment DNA patterns obtained by BamHI/EcoRV digestion of BAC DNAs were analysed by pulsed-field gel electrophoresis. Marker, λ–HindIII fragments. (c and d) HF cells were transfected by electroporation with wild-type, IE1(ΔCTD) mutant or revertant T-BAC DNA. GFP and phase-contrast images taken at 15 days after electroporation are shown (c). Total cell lysates were prepared and subjected to SDS-8% PAGE and then immunoblotted with anti-IE1/IE2 Ab (d).](http://vir.sgmjournals.org)
**Fig. 2.** Lack of an association between IE1(ΔCTD) and mitotic chromosomes and the growth curves of recombinant viruses.

(a) HF cells were infected with wild-type, IE1(ΔCTD) mutant or revertant (R) virus at an m.o.i. of 1. At 72 h post-infection, cells were fixed with methanol and subjected to IFA with anti-IE1 Ab. A mounting solution containing DAPI was used to stain cell nuclei. Mitotic chromosomes are indicated by arrowheads. Images were obtained by confocal microscopy. 

(b) (Top) HF cells in 12-well plates were infected with recombinant viruses at m.o.i. of 3 or 0.1. The growth curves shown present the total numbers of infectious centre-forming units (IFU) (averages of triplicate) in 1 ml culture supernatant at the indicated sampling times. Total numbers of IFUs were measured as described previously (Huh et al., 2008). (Bottom) HF cells in six-well plates were infected at m.o.i. of 3 or 0.1. Total cell lysates were prepared at the indicated time points after infection, and then immunoblotted using antibodies for IE1/IE2, p52 (encoded by UL44) and pp28 (encoded by UL99). β-Actin was used as a loading control.
described previously (Huh et al., 2008). These assays showed that IE1(ΔCTD) virus grew as well as the wild-type and revertant viruses at m.o.i. of 3 and 0.1 (Fig. 2b, top). Furthermore, immunoblot analysis consistently showed that the expression levels of immediate-early (IE1 and IE2), early (p52) and late (pp28) proteins at different time points after infection were comparable in wild-type, mutant and revertant virus-infected cells at both m.o.i. values (Fig. 2b, bottom). These results indicate that the absence of the CTD of IE1 does not affect the normal growth kinetics of virus in permissive HF cells.

We also investigated whether the associations between PML and STAT2 and mitotic chromosomes in HCMV-infected cells occur in an IE1-dependent manner. HF cells were infected with wild-type, IE1(ΔCTD) mutant or revertant viruses, and double-label IFAs were performed. The results obtained demonstrated that, in contrast to what was observed in wild-type and revertant virus infections, both PML and STAT2 failed to accumulate at mitotic chromosomes in IE1(ΔCTD) virus-infected cells (Fig. 3a and b). In a control experiment, it was found that histone deacetylase 1 (HDAC1) associated with mitotic chromosomes in normal HF cells and that, however, this association was not affected by wild-type or IE1(ΔCTD) mutant virus infections (Fig. 3c). It has previously been reported that for PML binding, IE1 requires its large central hydrophobic region but not its CTD (Lee et al., 2007, 2004; Wilkinson et al., 1998), and in-line with these observations, IE1(ΔCTD) was found to disrupt PML NBs in infected cells (data not shown).

We and others have previously demonstrated that IE1 interacts with STAT2 via its near carboxyl-terminal region, which includes its acidic domains, but not via the CTD (Huh et al., 2008; Krauss et al., 2009). To confirm that the lack of a
mitotic chromosome association by IE1(ΔCTD) is not due to the abrogation of its ability to bind STAT2, co-immunoprecipitation (co-IP) assays were carried out by using virus-infected cells. HF cells were infected with wild-type virus or mutant virus expressing IE1(ΔCTD) or IE1(Δ421−475), which contains a deletion within its acidic region (Huh et al., 2008; Krauss et al., 2009). Total cell lysates prepared at 12 h after infection were immunoprecipitated with anti-IE1 Ab followed by immunoblotting with anti-STAT2 Ab. The results of co-IP assays showed that IE1(ΔCTD) interacted with STAT2 as efficiently as wild-type IE1 in infected cells, whereas IE1(Δ421−475) showed markedly reduced or almost no STAT2 binding after thorough washing steps (Fig. 3d). These results show that STAT2 interacted with IE1 independently of the CTD. Collectively, these results demonstrate that both PML and STAT2 are recruited to mitotic chromosomes through interaction with IE1, and that the CTD is required for this process.

Since the lysine at the 450 aa position of IE1, which is located near the CTD, is conjugated by SUMO (Spengler et al., 2002; Xu et al., 2001), we investigated the effect of CTD deletion on the SUMOylation level of IE1 during mutant virus infection. When SUMOylation levels were compared in wild-type, IE1(ΔCTD) and revertant viruses by immunoblotting with IE1-specific Ab, IE1(ΔCTD) was still modified by SUMO, although its SUMOylation level was slightly lower than those in wild-type and revertant virus infections (Fig. 3e). We also investigated whether IE1 SUMOylation inhibits its association with mitotic chromosomes. For this, the IE1−SUMO−1 fusion protein, which is used as a mimic of SUMO-modified IE1 (Huh et al., 2008), was expressed in HF cells via retroviral transduction, and its ability to associate with mitotic chromosomes was investigated by IFA. The results obtained showed that IE1−SUMO−1 protein co-localized with chromosomes in mitotic cells, demonstrating that IE1 SUMOylation does not interfere with the association between IE1 and mitotic chromosomes (Fig. 3f).

This recombinant virus study confirms the findings of earlier transient transfection assays that the CTD of IE1 is required for its association with mitotic chromosomes (Reinhardt et al., 2005; Wilkinson et al., 1998). In transfection assays, IE1 CTD was found to be sufficient to deliver fused GFP to mitotic chromosomes (Reinhardt et al., 2005). Structural analyses of IE1 suggest the presence of highly disordered unstructured domains at the carboxyl-terminal region of IE1 (Krauss et al., 2009; E. T. Kim & J. H. Ahn, unpublished data), but it remains unclear which chromosome components are targeted by the CTD. The normal growth properties of IE1(ΔCTD) virus in HF cells in the present study are fully consistent with the previous observation that IE1(ΔCTD) protein complemented the growth defect of CR208 at a low m.o.i. value in HF cells at levels comparable with wild-type IE1 (Reinhardt et al., 2005). Despite no obvious deleterious effect of CTD deletion on viral replication in permissive HF cells, it is still possible that the association between IE1 and mitotic chromosomes benefits the virus in different cell types or in vivo.

The expressions of PML and Sp100 proteins have inhibitory effects on efficient HCMV replication (Adler et al., 2011; Ahn & Hayward, 2000; Kim et al., 2011; Tavalaï et al., 2006) and STAT2 is an integral player in the anti-HCMV responses mediated by IFNs (Huh et al., 2008; Krauss et al., 2009; Le et al., 2008; Paulus et al., 2006). Therefore, the IE1-mediated segregation of these host factors to mitotic chromosomes could provide a favourable cellular environment for viral replication in mitotic cells. In gammaherpesviruses, Epstein–Barr virus nuclear antigen 1 and latency-associated nuclear antigen of Kaposi’s sarcoma-associated herpesvirus target metaphase chromosomes and these activities are required for genome maintenance during latency and the control of viral genome replication (Marechal et al., 1999; Pirolot et al., 2001). Although no role has been assigned to IE1 during HCMV latency, it would be intriguing to determine whether the chromatin-tethering activity of IE1 promotes persistence of the viral genome in dividing cells.

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References

Association of HCMV IE1 with mitotic chromosomes


