Comparison of the biological and biochemical activities of several members of the alphaherpesvirus ICP0 family of proteins

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Short title: Comparative analysis of ICP0 related proteins

Key words: HSV-1, ICP0, PML, RING finger domain, E3 ubiquitin ligase

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ABSTRACT

Immediate-Early protein ICP0 of herpes simplex virus type 1 (HSV-1) is an E3 ubiquitin ligase of the RING finger class that is required for efficient lytic infection and reactivation from latency. Other alphaherpesviruses also express ICP0-related RING finger proteins, but these have limited homology outside of the core RING domain. Existing evidence indicates that ICP0 family members have similar properties, but there has been no systematic comparison of the biochemical activities and biological functions of these proteins. Here we describe an inducible cell line system that allows expression of the ICP0 related proteins of bovine herpes virus type 1 (BHV-1), equine herpesvirus type 1 (EHV-1), pseudorabies virus (PRV) and varicella zoster virus (VZV) and their subsequent functional analysis. We report that the RING domains of all the proteins have E3 ubiquitin ligase activity in vitro. The BHV-1, EHV-1 and PRV proteins complement ICP0-null mutant HSV-1 plaque formation and induce de-repression of quiescent HSV-1 genomes to extents similar to those achieved by ICP0 itself. VICP0 was found to be extremely unstable, which limited its analysis in this system. We compared the abilities of the ICP0 related proteins to disrupt ND10, induce degradation of PML and Sp100, to affect key components of the interferon signalling pathway and to interfere with induction of interferon stimulated genes. We found that the property that correlated most closely with their biological activities was the ability to preclude the recruitment of cellular ND10 proteins to sites closely associated with incoming HSV-1 genomes and early replication compartments.
INTRODUCTION

The members of the alphaherpesvirus subfamily are characterized by their ability to establish life long latent infections in neuronal tissues after the primary infection. Although certain core genes are conserved in all herpesviruses of all subfamilies, there are also genes that are characteristic of particular subfamilies. Amongst these are the genes that encode the ICP0 related proteins of the alphaherpesviruses, of which the most widely studied is ICP0 of herpes simplex virus type 1 (HSV-1). The interest in ICP0 stems from its biological roles in stimulating lytic infection and reactivation from latency (for reviews, see 17, 18, 33).

Members of the ICP0 family of proteins are characterized by the presence of a RING finger domain near their N-termini, a zinc stabilized fold that in many proteins confers E3 ubiquitin ligase activity (43). This has proved to be true of ICP0 (3), and the available evidence indicates that other members of the ICP0 family have similar biochemical functions (13, 61).

Although a number of ICP0 related alphaherpesvirus proteins have been studied in a variety of contexts, notably those expressed by Bovine herpesvirus 1 (BHV-1), Equine herpes virus 1 (EHV-1), Pseudorabies virus (PRV) and Varicella Zoster virus (VZV), there has been no systematic comparison of their abilities to complement ICP0 null mutant HSV-1 or to induce de-repression of quiescent HSV-1 genomes.

This paper describes a comparative study of the ICP0 related proteins expressed by the viruses listed above. In terms of nomenclature, the proteins expressed by BHV-1 and EHV-1 have been named BICP0 and EICP0, so although other names have been used for the PRV and VZV proteins (such as EP0 and orf61, respectively), we have adopted the names PICP0 and VICP0 for this study. Previous work found that, like ICP0 itself, all four proteins activate gene expression in reporter assays in a RING finger dependent manner (4, 5, 8, 29, 38, 41, 45, 51, 54, 59, 64, 75, 76, 78). VICP0 and EICP0 also complement, at least partially, ICP0 null mutant HSV-1 (15, 48, 53, 54). BHV-1, EHV-1, PRV and VZV virus mutants from which their ICP0 related genes have been deleted have been isolated and found to have reduced replication efficiencies, as expected by analogy with ICP0 null mutant HSV-1 (2, 7, 11, 12, 30, 46, 74, 77).

A prominent property of ICP0 is its localization to and disruption of cellular nuclear sub-structures known as ND10 or promyelocytic leukaemia (PML) nuclear domains. Interactions between ND10 and BICP0, EICP0, PICP0 and VICP0 have also been observed, with varied consequences to ND10 integrity (47, 60, 63). Whereas ICP0 achieves ND10 disruption through induction of the degradation of PML and SUMO-modified forms of Sp100 (21, 60), EICP0 appears less efficient than ICP0 in inducing PML degradation (60), while
VICP0 is inactive (47). While it is likely that all the ICP0 family members discussed here have RING finger mediated E3 ubiquitin ligase activity (61), the only other protein for which this has been confirmed is BICP0 (13).

The similarities between these members of the ICP0 family of proteins, and their apparent differences, prompted us to investigate in more detail the properties of these proteins in order to determine which of their properties correlate most closely with biological functions in complementing ICP0 null mutant HSV-1. In addition, there was no existing evidence on whether the related proteins could, like ICP0, induce de-repression of gene expression from quiescent HSV-1 genomes. We have taken two approaches to these issues. The first is the use of an inducible cell line system that has been used to study ICP0 itself (24, 26). Although inducible cell line systems have been described for VICP0 and BICP0 (53, 69), much of the work described in the current study is novel. The second approach is in vitro analysis of the E3 ubiquitin ligase activities of the isolated RING finger domains of the proteins. The major findings of the study are that (i) all the proteins studied are active in E3 ubiquitin ligase assays; (ii) VICP0 is extremely unstable, compromising comparative functional analysis in this system; (iii) BICP0, EICP0 and PICP0 complement to varying degrees the plaque forming defect of ICP0 null mutant HSV-1; (iv) these three proteins also stimulate efficiently de-repression of gene expression from quiescent HSV-1 genomes; (v) none of the ICP0 family members impede interferon (IFN) induced expression of IFN stimulated genes (ISGs), or affect the stability of important components of the IFN signalling system (namely STAT1, STAT2 and IRF3); (vi) BICP0, EICP0 and PICP0 cause some disruption of ND10 integrity and have varying effects on PML and Sp100 abundance, and (vii) the property of the proteins that correlated most closely with their stimulation of ICP0 null mutant HSV-1 infection and de-repression of quiescent genomes is their ability to inhibit the recruitment of PML and other ND10 proteins to sites associated with parental HSV-1 genomes and early replication compartments.

**MATERIALS AND METHODS**

**Viruses and cells**

HSV-1 strain 17+ was the wild type (wt) strain used, from which was derived the ICP0 null mutant dl1403 (71). Viruses in1863 and dl1403/CMV lacZ are derivatives of the above that contain the lacZ gene under the control of the HCMV promoter/enhancer inserted into the tk gene (kindly provided by Chris Preston). HSV-1 mutant virus in1374 contains the tsK temperature sensitive lesion in ICP4, a deletion of the ICP0 gene, and a mutation within VP16.
that inactivates its ability to stimulate IE gene expression (62). All viruses were grown in BHK cells and titrated in U2OS cells, in which ICP0 is not required for efficient replication of HSV-1. Virus in1374 was propagated at the permissive temperature of 31°C, and the latter was grown in the presence of 2.5 mM HMBA (62). U2OS, HEK-293T and HF cells were grown in Dulbecco’s Modified Eagles’ Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Baby hamster kidney (BHK) cells were grown in Glasgow Modified Eagles’ Medium (GMEM) supplemented with 10% new born calf serum and 10% tryptose phosphate broth. HepaRG hepatocyte cells (32) and their derivatives were grown in William’s Medium E supplemented with 10% fetal bovine serum Gold (PAA Laboratories Ltd), 2 mM glutamine, 5 µg/ml insulin and 0.5 µM hydrocortisone. All cell growth media were supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. Lentivirus transduced cells were maintained with continuous antibiotic selection, as appropriate.

**Plasmids**

The backbones of the lentivirus vector plasmids used in the inducible cell line system have been described previously (26). The coding regions of the ICP0 related proteins expressed by BHV-1, EHV-1, PRV and VZV (strain Dumas) were excised from a series of pET-based plasmids described previously (60) and inserted downstream of the TetO sites in the vector plasmid used in the construction of pLKO.DCMV.TetO.cICP0 (26) in place of the ICP0 cDNA. In each case, an oligonucleotide encoding an initiation codon and the myc tag was inserted in frame into the NcoI site located at the N-terminal end of each open reading frame. A plasmid for bacterial expression of a GST fusion protein including the first 241 residues of ICP0 (pGEX-241) has been described previously (3). Analogous vectors for expression of GST fusion proteins including the first 108, 113 and 165 residues of BICP0, PICP0 and VICP0 were constructed by insertion of NcoI-StuI, NcoI/SalI and NcoI/XhoI fragments from the respective pET-based plasmids in place of the ICP0 fragment in pGEX-241. A vector for expressing the first 63 residues of EICP0 as a GST fusion protein was constructed by transfer of the NcoI/XbaI fragment of pT7-ENX (1) into the GST expression vector.

**Lentivirus transduction**

Lentivirus supernatants were prepared after co-transfection into HEK-293T cells of a pLKO series plasmid with pVSV-G (expressing the VSV envelope protein) and pCMV.DR.8.91 (expressing all necessary lentivirus helper functions), as described previously (23). HA-TetR cells (26) were transduced with lentiviruses derived from the pLKO.DCMV.TetO series of plasmids to create cells named HA-BICP0, HA-EICP0, HA-PICP0 and HA-VICP0 according to the protein that could be expressed. These cells were selected with G418 and puromycin.
(initially 1 µg/ml, then reduced to 0.5 µg/ml during subsequent passage). All cells were maintained with continuous antibiotic selection.

**Induction of ICP0 and related protein expression**

Cells were treated with medium containing tetracycline (Sigma-Aldrich) at 0.1 µg/ml for various times as indicated in the text. Tetracycline was maintained in the medium throughout the duration of an experiment after the initial induction in order to maintain inducible protein expression.

**Virus plaque, yield and reactivation assays**

For plaque assays, cells were seeded into 24-well dishes at 1 x 10⁵ cells per well, then infected the following day with appropriate sequential 3-fold dilutions of *in1863* or *dl1403/CMVlacZ*. After virus adsorption, medium containing 1% human serum was added then the cells were stained for β-galactosidase positive plaques 24 h later, as described (42).

For reactivation (de-repression) assays, cells in 24-well dishes were infected with *in1374* at a multiplicity of infection (MOI) of 5 plaque forming units (pfu) per cell and at non-permissive temperature (npt; 38.5°C), then incubated at npt for 24 h. De-repression of the *lacZ* marker gene in the *in1374* genome was induced by treatment with tetracycline (0.1 µg/ml) for 24 h to induce ICP0 expression. Cells were stained for β-galactosidase activity the following day.

**Infections and western blot analysis.**

Cells were seeded into 24-well dishes at 1 x 10⁵ cells per well. After the relevant experimental manipulations, the cell monolayers were washed twice with phosphate buffered saline before harvesting in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins were resolved on 7.5% SDS-PAGE gels, then transferred to nitrocellulose membranes by western blotting. The following monoclonal antibodies (mAb) were used: anti-ICP0 mAb 11060 (19), anti-actin mAb AC-40 (Sigma-Aldrich), anti-PML mAb 5E10 (72), anti-myc tag Mab 9E10 (Santa Cruz Biotechnology), anti-ICP4 mAb 58S (68) and anti-ubiquitin mAb P4D1 (Santa Cruz Biotechnology). Rabbit polyclonal sera were as follows: anti-Sp100 SpGH (70), anti-EGFP ab290 (Abcam), anti-ISG15 H-150 (sc-50366; Santa Cruz Biotechnology), anti-STAT1 pAb (BD Biosciences, Cat. No. 610119), anti-STAT2 (Santa Cruz Biotechnology, sc-476), anti-IRF3 (sc-9082; Santa Cruz Biotechnology). Goat anti-GST polyclonal antibody was obtained from GE Healthcare.

**E3 ubiquitin ligase assays**

E3 ubiquitin ligase assays were carried out in a similar manner to that described previously (3, 9). Polyhistidine tagged E1 ubiquitin activating enzyme and E2 ubiquitin conjugation enzyme
UbcH5a were expressed from baculovirus and bacterial plasmid expression vectors and purified from crude extracts by nickel affinity chromatography. Ubiquitin was purchased from Sigma-Aldrich and methylated ubiquitin from Boston Biochem. Ubiquitin conjugation assays were carried out in a buffer containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl and 5 mM ATP. Each reaction included 20 ng E1, 30 ng UbcH5a, 5 µg ubiquitin (or methylated ubiquitin) and 30 ng of purified GST fusion protein. After incubation at 37°C for 1 h, reactions were stopped by addition of SDS-PAGE gel loading buffer supplemented with 8 M urea and 100 mM DTT. The products were analyzed on 4-12% Bis-Tris Novex gels followed by Western blotting for detection of ubiquitin or the GST fusion proteins.

**Immunofluorescence and confocal microscopy**

Cells on 13 mm glass coverslips were fixed and prepared for immunofluorescence as described (22). PML was detected with rabbit serum r8 or mAb 5E10, Sp100 with rabbit serum SpGH, ICP4 with mAb 58S, myc-tagged proteins with mAb 9E10, hDaxx with rabbit polyclonal antibody 07-471 (Upstate). The secondary antibodies used were Cy3 conjugated goat anti-mouse IgG and Cy5 conjugated goat anti-rabbit IgG (GE Healthcare). The samples were examined using a Zeiss LSM 510 confocal microscope, with 488 nm, 543 nm and 633 nm laser lines, scanning each channel separately under image capture conditions that eliminated channel overlap. The images were exported as tif files then processed using Photoshop.

**Interferon methods**

For IFN treatment experiments, medium containing human β-IFN (Calbiochem, Catalogue Number 407318) at the stated concentrations was added to the cells one day after seeding, then the cells were incubated for a further 24 h before the next stage of experimentation (as detailed in the text). The cells were maintained with medium containing β-IFN at the same concentration throughout the course of the subsequent experiment. For induction of ISG expression by double stranded RNA, cells were seeded into 24-well plates, then the following day they were washed twice with serum-free medium before addition of serum-free medium containing poly(I):poly(C) (Sigma-Aldrich cat. no. P1530) at 100 µg/ml. After incubation for 2 h, the cells were washed 4 times with serum-free medium then normal medium was replaced with or without tetracycline as relevant. The cells were then incubated overnight before being harvested for western blot analysis.
RESULTS

Expression of ICP0 family members in an inducible cell line system

The initial steps of this study involved insertion of the complete coding regions of the four ICP0 family members, taken from previously characterised plasmids (60), into the lentivirus vector used for inducible expression of ICP0 (26). Figure 1 shows a map of the ICP0 expression vector and the myc tagged coding region fragments that were inserted in place of that of ICP0. Lentiviruses prepared from these plasmids were used to transduce HA-TetR cells (that express the tetracycline repressor linked to EGFP; (26) and cell lines, named HA-BICP0, HA-EICP0, HA-PICP0 and HA-VICP0 were isolated, as described for the ICP0 inducible HA-cICP0 cells (26). A time course of treatment with tetracycline showed that BICP0, EICP0 and PICP0 were expressed efficiently and were detectable as early as 2 h after induction (Figure 2A). In contrast, VICP0 was weakly expressed (as observed previously; 60).

To assess the efficiency of expression of these proteins compared to that of ICP0 in the same system as studied previously, a myc tagged ICP0 lentiviral vector was constructed and used to isolate an analogous cell line (HA-mycICP0). Tetracycline induction of HA-cICP0, HA-mycICP0 and HA-BICP0 followed by western blotting for ICP0 itself and the myc tag showed that BICP0 and the ICP0 proteins were expressed at similar levels (Figure 2B), which in the case of ICP0 corresponds to expression levels at around 2 h after infection of HepaRG cells at MOI 1 (24). These findings opened an avenue towards comparative functional analyses of ICP0, BICP0, EICP0 and PICP0.

A recent publication put forward the idea that VICP0 might be unstable (47). To test whether this might explain the low expression of VICP0 in this system, HA-cICP0 and HA-VICP0 cells were treated with tetracycline, then at 24 h MG132 was added. After a further 2 h, the cells were washed and medium containing cycloheximide without MG132 was added, and wells were harvested at various time points thereafter. Inhibition of proteasome mediated degradation caused a substantial increase in the levels of VICP0, which appeared as a ladder of bands (Figure 2C). This complex band pattern is consistent with previous studies (47, 60). Treatment with cycloheximide resulted in the rapid loss of VICP0, indicating extreme instability, certainly much greater than in the case of the parallel ICP0 samples (Figure 2C). The rapid degradation of VICP0 is consistent with the hypothesis that its RING finger domain induces autoubiquitination and subsequent proteasome mediated degradation. At least some of the components of the VICP0 band ladder might be ubiquitinated forms. This scenario mirrors that of the instability of ICP0 through RING finger mediated autoubiquitination (9).
but to a much more pronounced degree (at least in HepaRG cells and in the absence of other viral gene products). The low level of VICP0 expression complicates any interpretation of activities in comparison with the other members of the family in this study.

To test the proportion of cells that were positive for expression after induction, the BICP0, EICP0, PICP0 and VICP0 cell lines were treated with tetracycline for various times then analyzed by immunofluorescence. In all cases, an extremely low proportion of cells had any detectable myc tagged signal before induction (a BICP0 example is shown in Figure 3, top row). Expression was detectable by 2 h after induction (data not shown) and by 4 h almost all the cells in the populations were positive for myc-tagged protein expression (Figure 3). This was true even for VICP0 (albeit at a lower fluorescence intensity), indicating that the low levels of VICP0 detectable on western blots was not due to inefficient transduction, but because each cell was expressing low levels of the protein. As might be expected from the Western blot analysis (Figure 2A), fluorescence intensities for BICP0, EICP0 and PICP0 increased with longer periods of induction, but the 4 h samples are shown in Figure 3 to illustrate that induction is rapid and quite synchronous in the cell population. Repeat analysis of HA-cICP0 cells indicated that induction of ICP0 expression was also detectable at early times of induction (data not shown). This aspect was not analysed in detail in the previous study of HA-cICP0 cells (26).

**Complementation of ICP0 null mutant HSV-1 by ICP0 family members**

Having constructed cell lines in which high proportions of cells express the ICP0 family members at similar levels, it was possible to compare their abilities to complement the plaque forming defect of ICP0 null mutant HSV-1. Cells expressing ICP0 and the other four family members were prepared by tetracycline induction for 24 h, then plaque assays using ICP0 null mutant HSV-1 were conducted. The negative control parental HA-TetR cells were analyzed in parallel. Plaque numbers were counted and titres were compared in the various cell lines. In these experiments, the average increase in plaque numbers in ICP0 expressing cells compared to the control was of the order of 350-fold. Plaque formation efficiencies in the other cell lines were expressed as a percentage of this value (Figure 4A). By this method of analysis, EICP0 complemented ICP0 null mutant HSV-1 plaque formation at close to 100% efficiency, whereas BICP0 and PICP0 complemented at around 50% and 30% respectively. These reflect very substantial increases in plaque numbers over background. There was a slight increase in plaque numbers in VICP0 expressing cells (around 5-fold), but we were unable to determine whether this was due to the low levels of expression of VICP0 or because the protein is inherently less active. A recent study estimated that plaque formation by an HSV-1
recombinant in which VICP0 is expressed in place of ICP0 is about 10% as efficient as the wt virus, which, although greater than the complementation observed here, implies that VICP0 is not a complete functional homologue of ICP0 (48).

**De-repression of quiescent HSV-1 genomes by ICP0 family members**

We next compared the abilities of the ICP0 family members to enable renewed expression of a β-galactosidase marker gene in quiescently infected cells. The cell lines used in the experiment of Figure 4A were infected with in1374 and incubated at 38.5°C for 24 h. This virus includes a deletion of the ICP0 gene, a temperature sensitive lesion in ICP4, and a mutation that inactivates the transactivation function of VP16, ensuring that expression even from the HCMV promoter driven marker gene is efficiently and rapidly repressed (62). We previously demonstrated that induction of ICP0 expression in HA-cICP0 cells causes renewed transcription of the repressed marker gene in cells quiescently infected with in1374, which can be detected by simple assay for β-galactosidase activity (26). We found that all the ICP0 family members, except VICP0, were active in this de-repression assay (Figure 4B). Quantification of the number of positive cells in random fields of view at higher magnification indicated that BICP0, EICP0 and PICP0 achieved 70% or greater frequencies of reactivation compared to ICP0, whereas the VICP0 result was scarcely above background (Figure 4C). Note that the process by which ICP0 proteins stimulate lytic infection from viral genomes soon after they enter the nucleus is not necessarily the same as that of de-repression, since the viral genome at the start of the former process is not chromatinized, while in the latter situation the viral genomes are likely to have been assembled into a repressed state.

Figure 4 indicates that BICP0, EICP0 and PICP0 are able to reproduce the core functions of ICP0 in the basic assays of stimulating plaque formation and de-repression of quiescent genomes. It was therefore possible to comparing the biochemical and other functions of these proteins in order to investigate which properties correlate with their biological functions at the level of virus replication.

**E3 ubiquitin ligase activities of ICP0 family members**

The RING finger mediated E3 ubiquitin ligase activity of ICP0 (3) is essential for its functions in stimulating HSV-1 plaque formation, de-repression from quiescence and reactivation from latency in terms of production of infectious virus (16, 26, 36, 73). Since the ICP0 family members are defined on the basis of the presence of a related RING finger domain, we investigated whether these domains also confer E3 ubiquitin ligase activity in *vitro*. GST fusion proteins including the first 241, 108, 63, 113 and 165 residues of ICP0, BICP0, EICP0, PICP0 and VICP0 were purified from bacteria harbouring the cognate GST
expression plasmids (Figure 5B), then incubated with purified E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzyme UbcH5a and ubiquitin. These segments of the ICP0 related proteins were chosen to include their core RING finger domains, plus amounts of downstream sequence that were similar to that in the analogous ICP0 fragment used as the positive control (Figure 5A). Analysis of the reaction products showed that all members of the family were active E3 ubiquitin ligases, producing large amounts of high molecular weight polyubiquitin chains (Figure 5C). Substitution of normal ubiquitin with methylated ubiquitin in the reactions, which limits conjugation to a single ubiquitin moiety at each substrate lysine residue, followed by western blotting to detect the GST fusion proteins indicated that all undergo efficient autoubiquitination (Figure 5D). Therefore all four additional members of the ICP0 family exhibit \textit{in vitro} E3 ubiquitin ligase activity similar to that of ICP0.

\textbf{Effects of ICP0 family members on PML and Sp100}

The abilities of the ICP0 family members to induce degradation of PML and Sp100 were compared by Western blot analysis of cell extracts made before and 24 h after tetracycline treatment. Whereas expression of ICP0 itself resulted in the disappearance of all PML isoforms and SUMO modified forms, the effects of the ICP0 related proteins were less clear-cut (Figure 6A). VICP0 had no effect, BICP0 had a best a marginal effect of the SUMO modified forms of PML, and both EICP0 and PICP0 induced reductions in the SUMO modified forms but not the other isoforms of PML (Figure 6A). Therefore the ability to target PML varies greatly between the members of the group of ICP0 proteins. Analysis of Sp100 revealed a surprisingly different picture. ICP0 induced the loss of SUMO modified Sp100-A and the higher molecular weight Sp100 isoforms, as observed before (26, 60). BICP0 was at least as active on Sp100 as ICP0, and EICP0 and PICP0 both induced relative reductions in the higher molecular weight Sp100 isoforms with a concomitant increase in unmodified Sp100-A (Figure 6B). Even VICP0 caused a slight change in Sp100 isoforms abundance (Figure 6B), despite its very low levels of expression (Figure 6C). The effect of BICP0 on Sp100 was remarkably rapid, as the change in Sp100 profile was almost complete as early as 4 h after induction (data not shown). The BICP0 results are particularly interesting, because in the case of ICP0 it is not possible to conclude that it has a direct effect on Sp100 since depletion of PML (in the absence of ICP0) causes the same changes in Sp100 isoform abundance (25, 27). Because BICP0 does not have a marked effect on PML, its effect on Sp100 cannot be through this indirect mechanism.
Effects of ICP0 family members on ND10 integrity

We next compared the effects of the ICP0 family members on the integrity of ND10. At 4 h after induction, all the family proteins exhibited nuclear staining with punctate foci within a diffuse background, with the foci frequently co-localising with PML (data not shown). At later times of induction the staining of all proteins became more diffuse within the nucleus. At 24 h after induction, as might be expected from its lack of affect on PML in the Western blot assay, BICP0 had little or no measurable effect on punctate PML staining (Figure 7, left-hand set of columns). Expression of both EICP0 and PICP0 resulted in the appearance of enlarged PML foci that were fewer in number than the average complement of ND10, while VICP0 had little noticeable effect on PML (Figure 7, left-hand set of columns). BICP0 caused almost complete loss of Sp100 staining, while both EICP0 and PICP0 resulted in abnormal Sp100 distributions, with VICP0 again having a lesser effect (Figure 7, middle set of columns). The effect of BICP0 on Sp100 was very rapid, with the proteins strongly co-localising at 2 h after induction and loss of Sp100 staining from most cells at 4 h (data not shown). BICP0, EICP0 and PICP0 all caused a reduction in the numbers of hDaxx foci and also in their apparent fluorescence intensity (Figure 7, right-hand set of columns). This probably represents a redistribution of hDaxx as there was no significant change in hDaxx levels in these cells as detected by Western blotting (data not shown). Once again, VICP0 had a lesser effect than the other proteins.

These data are similar to a previous study based on high level expression in transfected cells, particularly in terms of VICP0 having the least marked effects on ND10, and of the very clear effects of the other proteins on Sp100 (60). One difference, however, is the comparative lack of an apparent effect of BICP0 on PML distribution in this study. This is most likely due to differences in expression level.

Inhibition of the formation of ND10-like structures associated with HSV-1 genomes by ICP0 family members

At the earliest stages of HSV-1 infection, several ND10 component proteins are recruited to novel sites that are closely associated with incoming viral genomes and early replication compartments (22). This occurs only weakly and transiently during wt virus infection because ICP0 disrupts these virus-induced ND10-like foci very rapidly. This property of ICP0 correlates closely with its ability to stimulate lytic infection and subsequent plaque formation (26). Therefore we tested whether the other ICP0 family members could replicate this function by infecting cells expressing these proteins, and control HA-TetR cells, with ICP0 null mutant HSV-1 at low multiplicity. Recruitment of PML to the novel virus-induced foci is
readily visualized in cells at the edges of developing plaques at 24 h after infection (22). Induced expression of ICP0 itself virtually eliminates recruitment of PML in this assay (26). While recruitment of PML was readily observed in control HA-TetR cells, expression of BICP0, EICP0 and PICP0 substantially reduced or eliminated such recruitment, whereas VICP0 had little or no effect (Figure 8). Analogous results were obtained after staining for hDaxx (data not shown). Therefore, despite variations in their abilities to disrupt ND10 and affect PML and Sp100 abundance and/or modification, all the ICP0 related proteins that stimulate HSV-1 plaque formation and de-repression impede the cellular response that leads to assembly of viral genome associated novel ND10-like foci.

**Viability of cells expressing ICP0 family members**

It is known that prolonged expression of ICP0 is incompatible with cell survival (20, 39, 44, 67). To test whether this also applies to the other members of the family, cultures of cells were left uninduced or treated with tetracycline continuously over a period of 6 days, then the cells were stained with Giemsa to facilitate photography. Whereas the toxic effect of ICP0 was clearly evident, at this level of analysis expression of the other family members did not lead to cell loss (Figure 9). Therefore we extended the analysis to investigate whether these cells could be passaged in culture in the continuous presence of tetracycline over a period of 3 weeks. The cells were trypsinised and re-seeded twice weekly or when the flasks became confluent, generally at a split factor of about 1 in 4 (as used for the uninduced cells), then the cumulative split factor was calculated. Expression of VICP0 did not appear to impede cell growth (cumulative split factor of 1 in 240, the same as for uninduced cells), whereas growth of the other cells was retarded, with cumulative split factors of 1 in 36, 1 in 8 and 1 in 48 for cells expressing BICP0, EICP0 and PICP0, respectively. This equates to a 5- to 30-fold reduction in growth rate of the various cell lines. Although it was possible that any cell growth could reflect preferential multiplication of cells that did not express the ICP0 proteins, immunofluorescence analysis showed that nearly all the cells in each population remained positive for the ICP0 related proteins at this time point. Therefore the ICP0 family members that can complement ICP0 null mutant HSV-1 efficiently are detrimental to cell growth, but to a much lesser extent than ICP0 itself. It is possible that further analysis of these proteins and variants thereof might allow the development of an ICP0 related protein that retains many of its functions without major long-term detriment to the cell.

**ICP0 family members do not inhibit induction of IFN responsive genes**

ICP0 null mutant HSV-1 and PICP0 null mutant PRV replicate less efficiently in cells pre-treated with IFN (7, 35, 56). Therefore it has been debated whether ICP0 directly impedes the
IFN response (14, 50, 55, 57). Using the inducible cell line system we found that ICP0 does not inhibit the induction of ISG15, a typical ISG, after treatment with either IFN or poly I:C, which stimulate ISG expression through STAT1 and IRF3 dependent pathways respectively (24). We found that this was also true of the other ICP0 family members, in that induction of ISG expression by either IFN or poly I:C was not reduced in tetracycline treated cells compared to the untreated controls (Figure 10). As in the previous study (24), there is an important caveat to this conclusion in that the expression levels of BICP0, EICP0 and PICP0 are of a similar order to that of ICP0 in the first few hours of HSV-1 infection (see Figure 2 and 26). It is likely that, as in HSV-1 infection, expression of these proteins increases substantially at later times of infection so we cannot exclude that these increased amounts have an inhibitory effect on IFN related pathways. However, we can conclude that amounts sufficient to complement ICP0 null mutant HSV-1 are unable to impede these pathways directly. As with other experiments in this report, the very low levels of VICP0 expression preclude any conclusions on the properties of this protein in this assay.

**DISCUSSION**

This paper presents a comparative analysis of five members of the alphaherpesvirus ICP0 family of proteins. It has long been predicted that their RING finger domains would confer E3 ubiquitin ligase activity. That of ICP0 itself was confirmed in 2002 (3) and more recently BICP0 was also demonstrated to be a representative of this class of enzymes (13). Here we show that the RING finger domains of the other members of the family under study (EICP0, PICP0 and VICP0) have E3 ubiquitin ligase activity *in vitro*, and in the assays reported here there are no obvious differences in their relative activities. When expressed in cultured cells, however, the stabilities of the proteins will depend on the balance of any autoubiquitination activity leading to instability, and protection from this self-destructive activity. In the case of ICP0 itself, the protein is stabilised by binding to the ubiquitin specific protease USP7 (which removes ubiquitin adducts; 9), and other uncharacterised factors (31). In the particular experimental system adopted here, VICP0 was found to be extremely unstable, and although its accumulation could be massively enhanced by the proteasome inhibitor MG132, the half life of the protein after removal of the drug was of the order of only a few minutes (Figure 2). Although VICP0 expression could be detected by fluorescence microscopy in a high proportion of induced cells, and despite the fact that only small amounts of ICP0 itself are required for full activity (40), we cannot distinguish between the possibilities that the low biological activities of VICP0 in our assays are due to inefficient expression or inherently
reduced activity of the protein itself. Therefore the bulk of the following discussion will be restricted to ICP0, BICP0, EICP0 and PICP0.

As cited in the Introduction, previous work has demonstrated that certain members of the ICP0 family improve the replication efficiency of ICP0 null mutant HSV-1, and in a further example it was found that PICP0 can substitute for VICP0 (52). Here we extend these analyses in a quantitative manner and demonstrate their activities relative to ICP0 itself in stimulating plaque formation of ICP0 null mutant HSV-1. We demonstrate for the first time that all save VICP0 stimulate de-repression of marker gene expression from quiescent HSV-1 genomes, and we can therefore conclude that all share the core biological activities of ICP0 in stimulating lytic infection and reactivation from quiescence. The system that we have developed allows detailed analysis of several other functions of the proteins in a robustly comparative manner, and therefore we were able to determine which of their biochemical activities on selected cellular proteins correlate most closely with their shared biological functions.

The E3 ubiquitin ligase activities of the ICP0 family members imply that they target certain proteins for degradation. The RING finger provides their core catalytic domain, and in many cases the target specificity of a given E3 ligase resides in outlying motifs that confer interactions with target proteins or adaptors that themselves interact with the target proteins. In the case of the ICP0 family of proteins, however, there is little shared homology outside their RING fingers, and even within these domains there is considerable diversity in primary sequence (1). Given these differences, it is perhaps unsurprising that the activities of the family members on known ICP0 targets vary. For example, EICP0 and PICP0 cause changes in PML isoform abundance, particularly of the SUMO modified forms, but neither to the degree of ICP0 itself, while BICP0 has no effect on PML in these assays (Figure 6). Nonetheless, all disrupt ND10 to some extent but in distinguishable manners. The most notable of these differences is in the effect of BICP0 on Sp100, which is rapidly degraded in BICP0 expressing cells. It will be interesting to determine whether this is through a direct interaction, since the effect of ICP0 on Sp100 could be either direct or indirectly as a consequence of the loss of PML (27). While VICP0 did not induce changes in PML or Sp100 isoform abundance in the current studies, perhaps because of insufficient expression levels, a recent study in which VICP0 was expressed in place of ICP0 in the context of HSV-1 infection resulted in some loss of Sp100 without any apparent effect on PML (48). These findings are consistent with previous implications that Sp100 is involved in regulating the efficiency of HSV-1 infection (25, 58). We suspect, however, that this is only one of a
number of factors that are relevant to the mode of action of the ICP0 family of proteins. For example, like ICP0, the other members of the family that complement ICP0-null mutant HSV-1 in our assays also cause the dispersal of hDaxx from ND10. We have recently demonstrated that hDaxx and its interaction partner ATRX are involved in the cellular response that represses HSV-1 gene expression (V. Lukashchuk and R.D. Everett, submitted for publication). Clearly, the effects of ICP0 family members on ND10 and their constituent proteins is a complex issue involving perhaps several proteins and factors that impinge on the efficiency of virus infection.

There has been much interest in the concept that one role of ICP0 is in precluding or overcoming an IFN response to virus infection. Following the initial observations that ICP0 null mutant HSV-1 is more sensitive than the wt virus to IFN pre-treatment (35, 56), and evidence that ICP0 might interfere with the IFN response (14, 50, 57), a number of studies have investigated whether other ICP0 family members might have similar roles. For example, there is evidence that BICP0 can interact with components of the interferon response pathway and inhibit interferon induced gene expression (37, 65, 66), while a PICP0 deletion mutant of PRV is also more sensitive to interferon pre-treatment (6, 7). While it is incontrovertible that IFN pathways control HSV-1 and particularly ICP0 null mutant HSV-1 infections in mouse models (34, 49), it appears that IFN pathways do not contribute a dominant role to the phenotype of ICP0 null mutant HSV-1 in cultured cells. For example, ICP0 mutant virus replication is not enhanced in either STAT1 or IRF3 depleted human fibroblasts (28), and ICP0 itself does not preclude ISG induction through either STAT1 or IRF3 dependent pathways (24). Similarly, with the proviso that higher levels of expression may have additional effects, we report here that amounts of BICP0, EICP0 and PICP0 that are sufficient to complement ICP0 null mutant HSV-1 are also unable to inhibit ISG induction by either IFN itself or poly I:C (Figure 10). Consistent with these findings, we found that neither ICP0 nor the other family members had any effect on the abundance of STAT1, STAT2 or IRF3 in the inducible cell line system (data not shown). Therefore it is likely that the preferential loss of STAT2 observed in wt compared to ICP0 null mutant infections at late times (10) is due to indirect effects, and the effect of BICP0 on IRF3 (66) may require the very high levels of expression achieved in transfected cells.

A property common to all the ICP0 family members that are able to complement ICP0 null mutant HSV-1 and to induce de-repression of quiescent viral genomes is their ability to inhibit the recruitment of ND10 constituents to sites that are closely associated with incoming HSV-1 genomes and early replication compartments (Figure 8). This is far more
pronounced, at least in terms of PML distribution, than their effects on ND10 in uninfected cells (compare Figures 7 and 8). We have observed previously that of all the effects that have been attributed to ICP0, that of inhibition of this recruitment correlates most closely with biological activity in terms of efficiency of stimulation of lytic infection and derepression from quiescence (26). The current findings therefore remain consistent with the hypothesis that the recruitment to these sites of ND10 components, and in all likelihood other specific cellular proteins that are yet to be identified in this context, represents an intrinsic cellular response to virus infection that aims to inhibit gene expression from the introduced foreign DNA. Our current hypothesis is that the commonalities between the ICP0 family members lies not only in their targets in terms of individual specific proteins, but also in their ability to inhibit the pathways that are required for the recruitment of the varied cellular proteins to sites in close proximity to viral genomes soon after they enter the nucleus. A refinement of this model is that the total repression effect is a synthesis of several such recruited proteins, which would explain why several ND10 proteins appear to contribute to the ICP0 null mutant defect (25; V. Lukashchuk and R.D. Everett, submitted for publication). Further comparison of the properties and activities of the ICP0 family members is likely to be a valuable approach in testing this hypothesis and determining in more detail the most relevant cellular proteins and underlying biochemical mechanisms.

ACKNOWLEDGEMENTS
The work in the authors’ laboratory is mainly funded by the Medical Research Council. CMcN and LG are supported by a MRC Technology Drug Discovery Group Development Gap Fund grant.

FIGURE LEGENDS
Figure 1
A map of the lentiviral vector used for expression of ICP0 and its related proteins and a summary of the coding sequences utilized. The upper part shows lentivirus plasmid vector pLKO.DCMV.TetO.cICP0 that includes the ICP0 cDNA. The key features of the lentivirus vector are noted: pac – HIV packaging sequence; RRE – REV response element; RSV – RSV promoter; Amp r – ampicillin resistance; Puro-r, puromycin resistance; hGFPp, human phosphoglycerol kinase gene promoter; cppt, central polypyrimidine tract; DCMVp, truncated HCMV promoter/enhancer; TetO, tandem tetracycline operator sequences; other labels are self-explanatory. The lower section shows the fragments inserted in place of the ICP0 cDNA
in the above plasmid. Coding sequences are boxed, indicating the myc tag present in each and the length of the open reading frame; 3’ non-coding sequences are shown by lines.

**Figure 2**
Expression of ICP0 family member proteins in inducible cell lines. A: Cell lines transduced with lentivirus including the indicated open reading frames were treated with tetracycline then whole cell extracts were prepared at 2, 4, and 6 h after induction, as shown. Expressed proteins were detected by Western blotting with an anti-myc tag antibody. B: Comparative levels of ICP0 and BICP0 expression. Whole cell extracts of uninduced and induced cells expressing untagged ICP0 (HA-cICP0), myc tagged ICP0 and BICP0 were analyzed by Western blotting for ICP0 (upper panel) and the myc tag (middle panel). The lower panel shows the actin loading control. C: Instability of VICP0. HA-cICP0 and HA-VICP0 cells were left uninduced or treated with tetracycline for 24 h. Samples were treated with MG132 (5 µM) for 2 h, then replicate samples were maintained in tetracycline medium with cycloheximide but without MG132 for 1, 2 and 4 h (all as indicted below the panels).

**Figure 3.**
Expression of ICP0 family members in induced cell lines. Untreated HA-BICP0 cells (top row of panels) and HA-BICP0, HA-EICP0, HA-PICP0 and HA-VICP0 cells treated with tetracycline for 4 h were stained for expression of the myc tagged proteins. Images were captured on identical settings for the uninduced BICP0 cells and the induced BICP0, EICP0 and PICP0 samples. An enhanced setting was used for VICP0 cells because of the weaker signal (right-hand panels). The left-hand panels show the EGFP staining from expression of the EGFPnlsTetR protein in all cells.

**Figure 4**
A: Complementation of plaque formation efficiency of ICP0 null mutant HSV-1 by ICP0 family members. Cells expressing the indicated proteins were treated with tetracycline for 24 h, then used to titrate a stock of ICP0 null mutant HSV-1. The increase in titre in the ICP0 expressing cells was in these experiments an average of 330-fold greater than in the control HA-TetR cells. The data present average increases in titre over background in the other cell lines. The data show the mean of duplicate experiments, and the error bars show the range of the individual values. B: De-repression of repressed HSV-1 genomes by induction of ICP0 family members; analysis of HA-TetR control cells and cells expressing the indicated
proteins. Cells were infected (rows 2 and 3) or not (row 1) with virus in1374 at MOI 5 pfu per cell at npt, then 24 h later the cells in row 3 were treated with tetracycline (0.1 µg/ml). The cells were stained for β-galactosidase activity the following day to detect reactivated transcription from the lacZ marker gene in the in1374 genome. Cells positive for reactivated transcription turn blue, while negative cells give a clear background. C: Quantification of the extent of de-repression. Four random fields of view of the wells in row 3 of panel B were photographed, then positive and negative cells counted (approximately 250 cells per view). The proportions of positive (blue) cells in the induced wells are plotted with respect to that in the HA-cICP0 samples. The bars show mean results from the different fields of view counted, with standard deviations. The background level of blue cells in HA-TetR cells representing those in which expression of the marker gene was not initially repressed is 0.5%. This constitutes the limit of detection, meaning that the assay extends over a 200-fold range.

**Figure 5**
The RING domains of the ICP0 family members exhibit E3 ubiquitin ligase activity in vitro. A: A representation of the ICP0 family member amino acid sequences, with their RING fingers marked by boxes. The arrows indicate the C-terminal limits of the sequences fused to GST and expressed in bacteria. B: An image of a Coomassie stained 4-12% Bis-Tris Novex gel loaded with 200 ng of each purified GST fusion protein used in the analysis. The details of the amino acid coordinates of the RING domains used and their purification are given in Materials and Methods. C and D: Western blots showing the E3 ubiquitin ligase activities of the ICP0 family proteins. Each purified GST fusion protein was incubated with either ubiquitin or a ubiquitin mix containing ubiquitin, E1 ubiquitin activating enzyme and E2 ubiquitin conjugating enzyme UbcH5a, as indicated. The blot in panel C analyses samples containing wt ubiquitin, and high molecular weight unlinked polyubiquitin chains were detected with P4D1 anti-ubiquitin antibody. The blot in panel D shows similar reactions conducted with methylated ubiquitin, which reveals multiple monoubiquitin conjugated species of the GST fusion proteins, produced by autoubiquitination.

**Figure 6**
Degradation of PML and Sp100 induced by ICP0 family proteins. Control HA-TetR cells and cells transduced with lentiviruses encoding the ICP0 family members were treated with tetracycline for 24 h and analysed by western blotting for PML (A), Sp100 (B), and myc
tagged proteins or ICP0 (C). Uninduced samples of each cell line are shown for comparison (lanes labelled ‘no Tet’). The panels show analysis of the same samples on replicate gels.

**Figure 7**
Analysis of the effects of ICP0 family members on ND10 proteins PML (left-hand pairs of panels), Sp100 (central pairs of panels) and hDaxx (right-hand pairs of panels) by immunofluorescence. The top row shows uninduced HA-BICP0 cells as controls. These are identical to uninduced samples from the other cell lines and HA-TetR parental cells (not shown). Rows 2 to 5 show typical images of groups of cells induced to express BICP0, EICP0, PICP0 and VICP0, as indicated, at 24 h after induction.

**Figure 8**
Inhibition of recruitment of PML to the sites of viral genomes by ICP0 related proteins during the early stages of HSV-1 infection. Control HA-TetR cells and cells induced by tetracycline 24 h treatment to express BICP0 and the other family members were infected at low MOI with ICP0 null mutant HSV-1. At 24 h after infection, the cells were fixed and stained for PML (red) and ICP4 (green). Representative images of cells close to the edge of developing plaques were selected to show the sites of parental viral genomes and early replication compartments (detected by concentrations of ICP4 in distinct foci). The recruitment of PML to these foci, obvious in the HA-TetR and VICP0 expressing cells (top and bottom rows), is inhibited by expression of BICP0, EICP0 and PICP0 (rows 2 to 4, as indicated).

**Figure 9**
Viability of cells expressing ICP0 and related proteins. Control HA-TetR and cells expressing ICP0 and its related proteins were maintained in the absence (left-hand column of panels) or continuous presence (right-hand column of panels) of tetracycline for 6 days. The cells were then stained lightly with Giemsa and photographed.

**Figure 10**
ICP0 family members do not impede induction of ISG15 expression by either IFN or poly I:C treatment. Panels A and B: The indicated cells lines were seeded in triplicate wells of which two were treated with tetracycline for 24 h. The uninduced and one of the induced wells were then treated with β-interferon for a further 24 h, in the continuous presence of tetracycline. Whole cell extracts were analyzed by Western blotting for ICP0, myc tagged ICP0 family
members, ISG15 and actin. Panels C and D: A similar experiment was performed except that poly I:C was used instead of interferon. In this experiment the negative control lane for ISG15 induction had not been treated with tetracycline.

REFERENCES


repression of herpes simplex virus: implications for the regulation of viral latency. Virol J 3:44.


A. Complementation of ICP0 null mutant plaque formation

B. De-repression of quiescent HSV-1 by ICP0 family members

C. Number of de-repressed cells compared to ICP0, %

- **HA-TetR**
- **cICP0**
- **BICP0**
- **EICP0**
- **PICP0**
- **VICP0**
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**A**

**B**

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