Comparison of the Biological and Biochemical Activities of Several Members of the Alphaherpesvirus ICP0 Family of Proteins

Roger D. Everett,* Chris Boutell, Carol McNair, Louise Grant, and Anne Orr
MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, Scotland, United Kingdom

Received 4 December 2009/Accepted 15 January 2010

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 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 herpesvirus 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 derepression of quiescent HSV-1 genomes to levels similar to those achieved by ICP0 itself. VICP0, the ICP0 expressed by VZV, 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, to induce degradation of PML and Sp100, to affect key components of the interferon signaling 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.

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. Among 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 references 17, 18 and 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 is characterized by the presence of a RING finger domain near their N termini, a zinc-stabilized fold that in many other 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 type 1 (BHV-1), equine herpesvirus type 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 derepression 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 mutants in which the 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 substructures known as ND10 or promyelocytic leukemia (PML) nuclear domains. Interactions between ND10 and BICP0, EICP0, PICP0, and VICP0 have also been observed, with various consequences for 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 derepression 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 the following: (i) that all the proteins studied are active in E3 ubiquitin ligase assays; (ii) that VICP0 is extremely unstable, compromising comparative functional analysis in this system; (iii) that BICP0, EICP0, and PICP0 complement to various degrees the plaque-forming defect of ICP0 null mutant HSV-1; (iv) that these three proteins also efficiently stimulate derepression of gene expression from quiescent HSV-1 genomes; (v) that none of the ICP0 family members impedes interferon (IFN)-induced expression of IFN-stimulated genes (ISGs) or affects the stability of important components of the IFN signaling system (namely STAT1, STAT2, and IRF3); (vi) that BICP0, EICP0, and PICP0 cause some disruption of ND10 integrity and have various effects on PML and Sp100 abundance; and (vii) that the property of the proteins that correlated most closely with their stimulation of ICP0 null mutant HSV-1 infection and derepression 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 d11403 (71). HSV-1 mutants viruses in1374 contains the tk temperature-sensitive lesion in ICp4, a deletion of the ICP0 gene, and a mutation within VP16 that inactivates its ability to stimulate immediate-early (IE) gene expression (62). All viruses were grown in baby hamster kidney (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 was grown in the presence of 2.5 mM hexamethylene bisacetamide (HMBA) (62). U2OS, HEK-293T, and human fibroblast (HF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). BHK cells were grown in Glasgow modified Eagle's medium (GMEM) supplemented with 10% newborn 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 glutathione S-transferase (GST) fusion protein containing 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, respectively, 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 ICP0 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 cotransfection into HEK-293T cells of a pLKO series plasmid with pVSV-G (expressing the vesicular stomatitis virus [VSV] envelope protein) and pCMV.DR.DR.8.91 (expressing all necessary lentivirus helper functions), as described previously (23). HA-TetR (where HA is HepaRG) 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 and 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 × 105 cells per well and then infected the following day with appropriate sequential 3-fold dilutions of m13863 or d11403/ CMVlacZ. After virus adsorption, medium containing 1% human serum was added, and then the cells were stained for β-galactosidase-positive plaques 24 h later, as described previously (42). For reactivation (deerepression) assays, cells in 24-well dishes were infected with in1374 at a multiplicity of infection (MOI) of 5 PFU per cell and at nonpermissive temperature (NPT; 38.5°C) and then incubated at the NPT for 24 h. Derepression of the lacZ marker gene in the in1374 genome was induced by treatment with tetracycline (0.1 μg/ml) for 24 h to enhance 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 × 105 cells per well. After the relevant experimental manipulations, the cell monolayers were washed twice with phosphate-buffered saline before harvesting in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins were resolved on 7.5% SDS-PAGE gels and then transferred to nitrocellulose membranes by Western blotting. The following antibodies (MAbs) were used: anti-ICP0 MAb MAB 11060 (19), anti-actin MAb AC-40 (Sigma-Aldrich), anti-pML MAB SE10 (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-Sp10 SpGH (70), anti-enhanced green fluorescent protein (EGFP) ab290 (Abcam), anti-IGS15 H-150 (sc-50366; Santa Cruz Biotechnology), anti-STAT1 pAb (item 610119; BD Biosciences), and anti-enhanced green fluorescent protein (EGFP) (BD Biosciences) anti-STAT2 (sc-476; Santa Cruz Biotechnology), 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 overexpressed from baculoviral plasmids in insect cells and purified from crude extracts by nickel affinity chromatography. Ubiquitin was purchased from Sigma-Aldrich, and methylated ubiquitin was from Boston Biochem. E3 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 mixture included 20 ng of E1, 30 ng of UbcH5a, 5 μg of ubiquitin (or methylated ubiquitin), and 30 ng of purified GST fusion protein. After incubation at 37°C for 1 h, reactions were stopped by the addition of SDS-PAGE gel loading buffer supplemented with 8 M urea and 100 mM dithiothreitol (DTT). The products were analyzed on 4 to 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 cover-slips were fixed and prepared for immunofluorescence as described previously (22). PMD was detected with rabbit serum r8 and MAB SE10, Sp100 was detected with rabbit serum SpGh, ICP4 was detected with MAB 58S, myc-tagged proteins were detected with MAB 9E10, and human Daxx (hDaxx) was detected 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, and each channel was scanned separately under image capture conditions that eliminated channel crosstalk. These images were exported as TIFF files and then processed using Photoshop.

IFN methods. For interferon (IFN) treatment experiments, medium containing human IFN-β (catalogue number 407518; Calbiochem) at 100 U/ml was added to the cells 1 day after seeding, and then the cells were incubated for a
The cells were maintained with medium containing IFN-α 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, and then the following day they were washed twice with serum-free medium and then normal medium was added by 2 h after induction (Fig. 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 (Fig. 2B), which in the case of ICP0 corresponds to expression levels at around 2 h after infection of HepaRG cells at an MOI of 1 (24). These findings opened an avenue toward comparative functional analyses of ICP0, BICP0, EICP0, PICP0, and VICP0.

A recent publication put forward the idea that VICP0 might be unstable (47). To test whether this might explain the low expression level of VICP0 in this system, HA-cICP0 and HA-VICP0 cells were treated with tetracycline, and then at 24 h MG132 was added. After a further 2 h, the cells were washed, 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 (Fig. 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 (Fig. 2C). The rapid degradation of VICP0 is consistent with the hypothesis that its RING finger domain induces auto-ubiquitination 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 auto-ubiquitination (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 and 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 Fig. 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 (Fig. 3). This was true even for VICP0 (albeit at a lower fluorescence intensity), indicating that VICP0 was detectable on Western blots only at low levels not because of inefficient transduction but because each cell was expressing low levels of...
the protein. As might be expected from the Western blot analysis (Fig. 2A), fluorescence intensities for BICP0, EICP0, and PICP0 increased with longer periods of induction, and 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) and 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 were treated with tetracycline for 24 h. Samples were treated with MG132 (5 μM) for 2 h, and then replicate samples were maintained in tetracycline medium with cycloheximide (Cx) but without MG132 for 1, 2, and 4 h (all as indicated below the panels).

**FIG. 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, and 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) and 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 were treated with tetracycline for 24 h. Samples were treated with MG132 (5 μM) for 2 h, and then replicate samples were maintained in tetracycline medium with cycloheximide (Cx) but without MG132 for 1, 2, and 4 h (all as indicated below the panels).

**FIG. 3.** Expression of ICP0 family members in induced cell lines. Untreated HA-BICP0 cells (top row) 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 panel). The left-hand panels show the EGFP staining from expression of the EGFP-linked tetracycline repressor protein in all cells.

**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 pre-
pared by tetracycline induction for 24 h, and 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 titers 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 (Fig. 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; although this value is greater than the complementation observed here, this implies that VICP0 is not a complete functional homologue of ICP0 (48).

**Derepression 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 shown in Fig. 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 derepression assay (Fig. 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 relative to ICP0, whereas the VICP0 result was scarcely above background (Fig. 4C). Note that the process by which ICP0 proteins stimulate lytic infection is not necessarily the same as that of derepression since the viral genomes are nucleosome free when released into the nucleus, while in the latter situation they are likely to have been assembled into a repressed chromatin state.

The results shown in Fig. 4 indicate that BICP0, EICP0, and PICP0 are able to reproduce the core functions of ICP0 in the basic assays of stimulating plaque formation and derepression of quiescent genomes. It was therefore possible to compare 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, derepression from quiescence, and reactivation from latency in terms of production of infectious virus (16, 26, 36, 39).
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 \textit{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 harboring the cognate GST expression plasmids (Fig. 5B) and 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 the amounts in the analogous ICP0 fragment used as the positive control (Fig. 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 (Fig. 5C). Replacing normal ubiquitin with methylated ubiquitin in the reaction mixtures, 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 auto-ubiquitination (Fig. 5D). Therefore, all four additional members of the ICP0 family exhibit \textit{in vitro} E3 ubiquitin ligase activity similar to that of ICP0.

**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 (Fig. 6A). VICP0 had no effect, BICP0 had at best a marginal effect on 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 (Fig. 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 both EICP0 and PICP0 induced relative reductions in the higher-molecular-weight Sp100 isoforms with a concomitant increase in unmodified Sp100-A (Fig. 6B). Even VICP0 caused a slight change in Sp100 isoform abundance (Fig. 6B), despite its very low levels of expression (Fig. 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). Whereas it is not possible to conclude that ICP0 has a direct effect on Sp100 because depletion of PML causes the same changes in Sp100 isoform abundance (25, 27), the effect of BICP0 on Sp100 cannot occur through this indirect mechanism because the BMV-1 protein does not affect PML.

**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 showing punctate foci within a diffuse background, with the foci frequently colocalizing 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

**FIG. 5.** The RING domains of the ICP0 family members exhibit E3 ubiquitin ligase activity \textit{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 to 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 (Ub) 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. For the blot analysis in panel C, 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 (Me Ub), which reveals multiple monoubiquitin-conjugated species of the GST fusion proteins produced by auto-ubiquitination.
PML in the Western blot assay, BICP0 had little or no measurable effect on punctate PML staining (Fig. 7, two left 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 (Fig. 7, two left 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 (Fig. 7, two middle columns). The effect of BICP0 on Sp100 was very rapid, with the proteins strongly colocalizing 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 (Fig. 7, two right columns). This probably represents a redistribution of hDaxx as there was no significant change in intensity (Fig. 7, two right columns). This probably represents a redistribution of hDaxx as there was no significant change in intensity (Fig. 7, two right columns).

These data are similar to those from a previous study based on high-level expression in transfected cells, particularly in terms of VICP0 having the least marked effects on ND10 and 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 a low multiplicity of infection. 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 (Fig. 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 derepression 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 were treated with tetracycline continuously over a period of 6 days, and 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 (Fig. 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 trypsinized and reseeded twice weekly or when the flasks became confluent, generally at a split factor of about 1 in 4 (as used for the uninduced cells), and 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

FIG. 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 analyzed by Western blotting for PML (A), Sp100 (B), and myc-tagged proteins or ICP0 (C). Uninduced samples (no Tet) of each cell line are shown for comparison. The panels show analysis of the same samples on replicate gels.
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 pretreated 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). 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 (Fig. 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 (Fig. 2) (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 the possibility 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. This activity 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 the proteins are expressed in cultured cells, however, their stabilities will depend on the balance between any auto-ubiquitination activity leading to instability and protection from this self-destructive activity. In the case of
ICP0 itself, the protein is stabilized by binding to the ubiquitin-specific protease USP7 (which removes ubiquitin adducts [9]) and other uncharacterized 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 considerably less than 2 h (Fig. 2). Although VICP0 expression could be detected by fluorescence microscopy in a high proportion of induced cells and although 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 either 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...
derepression 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 sequences (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 does so to the degree of ICP0 itself, and BICP0 has no effect on PML in these assays (Fig. 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 indirect 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 (49a). Clearly, the effects of ICP0 family members on ND10 and their constituent proteins are 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 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 pretreatment (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 pretreatment (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) (Fig. 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 derepression 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 (Fig. 8). This is far more pronounced, at least in terms of PML distribution, than their effects on ND10 in uninfected cells (compare Fig. 7 and 8). We have observed previously that, of all the effects that have been attributed to ICP0, 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 lie 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, 49a). Further comparison of the properties and activities of the ICP0 family members is likely to be a valuable approach.

ACKNOWLEDGMENTS

The work in our laboratory is mainly funded by the Medical Research Council. C.M. and L.G. are supported by an MRC Technology Drug Discovery Group Development Gap Fund grant.

REFERENCES
