Protein kinase CK2 phosphorylation regulates the interaction of Kaposi’s sarcoma-associated herpesvirus regulatory protein ORF57 with its multifunctional partner hnRNP K

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ABSTRACT

ORF57 protein of Kaposi’s sarcoma-associated herpesvirus has a counterpart in all herpesvirus of mammals and birds and regulates gene expression at transcriptional and post-transcriptional levels. ORF57 was capable of self-interaction and bound a rapidly migrating form of heterogeneous nuclear ribonucleoprotein K (hnRNP K), a multifunctional cellular protein involved in gene expression. In virus infected cell extracts, ORF57 was present in a complex with hnRNP K that had protein kinase CK2 activity, and was phosphorylated by CK2. Different regions of ORF57 bound both catalytic α/α′ and regulatory β subunits of CK2. CK2 modification enhanced the ORF57–hnRNP K interaction, and may regulate the presence and activities of components in the complex. We suggest that ORF57 and hnRNP K interaction may modulate ORF57-mediated regulation of viral gene expression. Herpesviral ORF57 (Rhadinovirus) and ICP27 (Simplexvirus) proteins both interact with hnRNP K and CK2 implying that adaptation of the ancestral hnRNP K and CK2 to associate with viral regulatory ancestor protein likely pre-dates divergence of these Herpesviridae genera that occurred 200 million years ago.

INTRODUCTION

Herpesviruses infect a wide range of fish, amphibians, reptiles, birds, marsupials and other mammals including humans. A distinguishing feature is their ability to establish lifelong latent infections that can cause recurrent rounds of disease (1). These viruses utilize cellular RNA polymerase II to transcribe genes in a temporal cascade (2). Kaposi’s sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus 8) (3), a member of the γ-2 herpesvirus sub-family that includes Epstein–Barr virus (EBV) and herpesvirus saimiri (HVS), is associated with Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman’s disease [reviewed in (4,5)]. Treatment of latently infected B-cell lines such as BCBL-1, generated by culture of PEL material (6) with the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) efficiently induces the KSHV lytic cycle and produces virions (6).

ORF57 (also known as MTA) and ORF50 (also known as RTA), two of the earliest KSHV regulatory proteins to be expressed (7,8), are required for lytic replication (9). A counterpart of ORF57 protein is present in every herpesvirus of mammals and birds sequenced so far, reflecting its importance. These proteins include herpes simplex virus type 1 (HSV-1) ICP27 (10), EBV MTA (11) and HVS ORF57 (12). ICP27 protein is a multifunctional protein that affects transcription (13), pre-mRNA 3 processing (14), RNA splicing (15) and promotes export of viral RNAs (16). KSHV ORF57 protein also regulates gene expression at transcriptional and post-transcriptional levels. Expression of ORF50 protein alone is necessary and sufficient for the switch from latent to lytic KSHV replication (17,18). We have shown that ORF57 associates with the ORF50 promoter and with ORF50 protein cooperatively activates expression from this promoter (19), and others have shown that ORF57 and ORF50 proteins synergistically activate expression from different ORF50-responsive KSHV promoters (20). ORF57 protein localizes to the nucleus (20–22) and can shuttle between the nucleus and cytoplasm (22). Expression of ORF57 increases the accumulation of cytoplasmic reporter mRNA, suggesting a post-transcriptional action (20,21), and promotes nuclear export of a reporter RNA (23). Herpesvirus ORF57 counterparts like human immunodeficiency virus type 1 (HIV-1) Rev protein promote nuclear export of viral mRNAs [reviewed in (24)].

In HSV-1-infected cells, ICP27 protein was found in a complex with heterogeneous nuclear ribonucleoprotein K (hnRNP K) and the cellular protein kinase CK2 (25), and phosphopeptide mapping identified serine residues in ICP27 as targets for CK2 phosphorylation in vivo (26). An evolutionarily conserved protein, hnRNP K [reviewed in (27,28)] can bind RNA and single- or double-stranded DNA via a GRGG box and three repeats of a motif termed the K homology domain (29). The protein contains classical nuclear localization signals and a KNS domain (30) that confers the ability to shuttle...
bi-directionally between the nucleus and cytoplasm. HnRNP K is involved in a variety of processes that affect gene expression such as chromatin re-modelling, translational silencing of mRNAs and transcriptional activation, where its binding to a CT-element within the c-myc promoter has been shown to activate or repress gene expression [see (27) and references therein]. The involvement of hnRNP K in these various processes reflects the interactions of its several domains with nucleic acids, protein kinases and other proteins involved in different aspects of gene regulation (31,32).

CK2, a pleiotropic and ubiquitous protein kinase [reviewed in (33,34)] consisting of two catalytic subunits (α or α′) and two regulatory β subunits can be found as αβ2, ααβ2, or α′β2 combinations in the tetrameric holoenzyme. The sub-strate specificity of CK2 α is dramatically changed by its association with CK2 β (35). DNA sequence analysis of CK2 subunits from yeast to humans has revealed strong con-servation of both subunits, making it one of the most evolu-tionarily conserved protein kinases. The CK2 β subunit amino acid sequence is identical between mammals and birds with Xenopus laevis differing by a single conservative substitution [reviewed in (34,36)]. Deletion of either catalytic subunit is lethal in Saccharomyces cerevisiae (37) and functional studies have shown that CK2 α from the human or Caenorhabditis elegans can substitute for the S.cerevisiae catalytic subunits (38). CK2 is known to phosphorylate more than 300 proteins and is involved in signal transduction, transcriptional control, apoptosis, cell cycle regulation and cancer [reviewed in (33)]. Many viral proteins are substrates for CK2 [reviewed in (33,39)] including the HSV-1 structural proteins VP22 and VP16. HSV-1 infection stimulated CK2 activity at early times, an effect that required expression of ICP27 protein and was not shown by viral mutants with defects in ICP27 nucleocytoplasmic shuttling (40). Although CK2 has been considered to be constitutively active, stimulation of its activity has been reported by stress signalling agents and heat shock while other agents inhibit its activity [reviewed in (34,41)].

Modern herpesviruses replicate in a variety of different cell types and tissues, and their genomes exhibit wide variation in organization, which could be of functional significance in gene expression. In contrast to the majority of unspliced HSV-1 genes, although transcript mapping is not yet complete, the KSHV genome contains several spliced genes [reviewed in (4,42)], including the ORF57 gene itself (22). The ORF57 herpesvirus counterparts may have shared and separate fea-tures, e.g. EBV MTA protein only partially complemented ICP27 when inserted into an ICP27-null virus (43). Comparing ORF57 with its different herpesvirus counterparts will identify common functions, and may reveal differences related to virus specialization and speciation. Thus, it was important to determine if the γ-herpesvirus KSHV ORF57 interacted with hnRNP K and CK2, earlier shown to bind the α-1 herpesvirus HSV-1 ICP27, and, if so, any functional consequences of these interactions.

Here, we show that ORF57 and hnRNP K proteins interact using pull down assays and both co-immunoprecipitate from extracts of KSHV positive TPA-treated BCBL-1 cells. In TPA-treated cells, ORF57 was present in a complex with hnRNP K containing CK2 activity and was phosphorylated by CK2. ORF57 protein directly interacted with catalytic α/α′ and regulatory β subunits of cellular protein kinase CK2. A CK2 inhibitor reduced in vitro and in vivo phosphorylation of ORF57 protein. Modification by CK2 enhanced ORF57 interaction with its hnRNP K partner, indicating that CK2 activity regulates the presence and activities of components within the ORF57 complex.

MATERIALS AND METHODS

Recombinant plasmids

The plasmid expressing an N-terminal glutathione S-transferase (GST) fusion protein of ORF57 amino acids 181–455 termed pGST-ORF57 small (gift from Dr L. Bello) was constructed by cloning ORF57 second exon DNA (22) into the EcoRI site of pGEX-2T(N+1). Plasmids pGST-ORF57 full-length (FL) expressing a GST fusion protein of ORF57 FL (19), pGBK7-ORF57 FL and small, expressing ORF57 amino acids 1–455 and 181–455 (23) for in vitro transcription/translation of ORF57 protein, pcDNA4-cORF57 FL (amino acids 1–455) and pcDNA4-ORF57 deletion mutants expressing ORF57 amino acids 17–455, 1–215, 181–328, 329–455 and 387–455 (19) were constructed as described. Plasmid pEGFP-gORF57 FL contains ORF57 genomic DNA cloned into pEGFP-C1 (22). Plasmids pGEX-27 expressing the GST–ICP27 fusion protein kindly provided by Dr S. Rice (44), and pCITE-ICP27 for in vitro transcription/translation of ICP27 protein (16), were con-structed as described. pGEX-5X-1-hnRNP K kindly provided by Dr D. Levens (45) expresses GST–hnRNP K FL. HnRNP K deletion mutants in pGEX-KT vector (31,45,46) were kindly provided by Dr K. Bomsztyk. Plasmids encoding GST–CK2 α FL, histidine (His)–CK2 β FL deletion mutants, maltose binding protein (MBP)–CK2 β FL fusion protein and its deletion mutants, GST–CK2 β deletion mutants and GST–CK2 α′ FL fusion protein (25,47,48) were kindly provided by Dr O. Filhol-Cochet. Plasmid pcDNA3-gORF50 (18) was kindly provided by Dr. D. Ganem.

Cell culture and antibodies

BCBL-1 cells from a KSHV-positive, EBV-negative primary effusion lymphoma (6) were grown as described in (49). Human embryonic kidney 293 epithelial cells were grown in DMEM (GibcoBRL) supplemented with 10% foetal calf serum (FCS), 1% l-glutamine, 1% non-essential amino acids and 1% penicillin–streptomycin. HeLa cells were grown in DMEM (GibcoBRL) supplemented with 2.5% FCS, 2.5% newborn calf serum and 100 units/ml penicillin and 0.01% streptomycin.

A rabbit antibody (Ab) against a peptide (DGESPFRFDD-SIIPR) corresponding to KSHV ORF57 amino acids 182–195, referred to as anti-ORF57 (GH) Ab (19) was kindly provided by Dr G. Hayward. Anti-ORF57 rabbit 718 and 721 Abs against synthetic peptides were raised as described in (19). Anti-hnRNP K Ab was a rabbit Ab, kindly provided by Dr K Bomsztyk (50), raised against a synthetic peptide corresponding to hnRNP K C-terminal amino acids 452–464, which are conserved in human and murine hnRNP K (51). The rabbit polyclonal β-c Ab against the CK2 β subunit and Rg403 Ab against the CK2 α and α′ subunits (52) were gifts from Dr O Filhol-Cochet, and pre-immune serum Abs (purified IgG) were from Santa Cruz Biotechnology.
TPA induction of KSHV lytic replication in BCBL-1 cells, radiolabelling and preparation of cell extracts

BCBL-1 cells (0.2 × 10⁶ cells/ml) were either treated with 20 ng/ml TPA for 72 h (6) or left untreated. Under these conditions, in TPA-treated cells, a readily detectable 50–52 kDa ORF57 protein band was visible 4 h after treatment, and, at later times, a faint faster migrating ORF57 processed product (~45 kDa) was present; whereas these bands were absent from untreated cells (19). As appropriate, TPA-treated or untreated cells (2 × 10⁶) were labelled with [³²P]-orthophosphate (150 μCi/ml) as described in (20) with medium containing 20% of normal phosphate and 2% newborn calf serum. As indicated, cells were treated with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of CK2, that acts in vivo and in vitro (25,53,54). Soluble protein cell extracts were prepared essentially as described in (19,23), and protein concentration was determined by Bradford assay (Bio-Rad). As appropriate, cell extracts were treated with 100 U RNase (ONE™ Ribonuclease, Promega) at 37°C for 15 min.

Recombinant protein expression, in vitro pull down assays and western blotting

Expression and preparation of GST–hnRNP K FL, GST–hnRNP K deletion mutants, GST- and MBP-fusions of FL and deletion mutants of CK2 β and CK2 α/α′ was as described in (25). GST–ORF57 FL was expressed as described in (19), and GST–ORF57 small was made following the same protocol. Fusion proteins immobilized on beads were pretreated with 0.2 U DNase I and 0.2 μg RNase A per μl for 30 min at 20°C in 50 mM Tris–HCl pH 8.0, 5 mM MgCl₂, 2.5 mM CaCl₂, 100 mM NaCl, 5% glycerol, 1 mM DTT. Beads were washed twice with 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT, and blocked in the same buffer containing 10 μg of BSA/ml for 30 min at 4°C. After blocking, the beads were resuspended in binding buffer containing 1 mg of BSA/ml, prior to use.

Pull down assays (16,25) were performed using BCBL-1 extracts (200 μg protein) or [³²S]-methionine-labelled ORF57 FL, small or deletion mutants synthesized in vitro using the TNT T7 Quick Coupled transcription/translation system (Promega), plus 5 μg of recombinant GST–hnRNP K FL or GST–CK2 α or α′, MBP–CK2 β, or GST, or MBP alone bound to glutathione- or amylose-agarose beads. As appropriate, pull down assays used 5 μg GST–ORF57 FL or small, GST–ICP27, various GST–hnRNP K deletion mutants, His–CK2 α FL, MBP–CK2 β FL, MBP–CK2 β deletion mutants, or His-thioredoxin with 10 μl of [³²S]-labelled His–CK2 α deletion mutants or luciferase proteins. Bound proteins were eluted, resolved by SDS–PAGE and visualized by autoradiography or western blotting with ECL detection (Amersham Biosciences) (25) using anti-ORF57 Ab (GH) (1:2500 dilution), anti-hnRNP K Ab (1:10000 dilution), anti-ORF57 FL (GibcoBRL), anti-GFP mouse mAb (1:1000 dilution, Clontech), anti-CK2 α and β Abs (1:2000 dilution), anti-His mouse mAb (1:1000 dilution, BD Biosciences) or anti-MBP Ab (1:5000, New England Biolab).

Immunoprecipitation

BCBL-1 cell extracts (200 μg protein) were pre-cleared with 5 μl of rabbit pre-immune serum Ab (purified IgG) and 100 μl of a 50% slurry of protein A Sepharose beads for 1 h at 4°C. Then anti-ORF57 Abs (3 μl each of GH + 718 + 721 Ab) or control rabbit pre-immune serum Ab (purified IgG) were added and immunoprecipitations were performed as described in (16,23). Immunoprecipitated proteins were resolved by SDS–PAGE and visualized by western blotting (25). Rabbit polyclonal Ab 54 (50) was used for immunoprecipitation of hnRNP K as described in (40). Anti-GFP mAb and anti-hnRNP K Ab were used for western blotting of immunoprecipitates.

Transient transfections of plasmid DNAs

For immunoprecipitations, HeLa cells (3 × 10⁵ cells/well) were transfected with 600 ng of pEGFP-gORF57 FL or 500 ng of pEGFP-C1 empty vector DNAs using Polyfect (Qiagen) according to the manufacturers’ protocol. For CK2 activity assays, 293 cells (6 × 10⁵ cells/well) were transfected with 600 ng of pEGFP-ORF57 small, or pEGFP-gORF57 FL and/or 500 ng of pcDNA3-gORF50 DNAs keeping the final DNA amount at 2 μg by adding empty vector pcDNA3 or pEGFP-C1 DNAs. For electroporation, BCBL-1 cells were washed once in PBS-A and aliquoted at 2 × 10⁶ cells in 0.5 ml RPMI 1640 medium without antibiotics and FCS. Cells were transferred to a cuvette (0.4 cm) containing 10 μg of pEGFP-gORF57 FL or pEGFP-C1 and pCH110 encoding β-Gal (Amersham Biosciences) DNAs, kept on ice for 10 min and electroporated at 250 V, 950 μF (BioRad Gene Pulser). Following incubation on ice for 10 min, cells were resuspended in 3 ml complete RPMI 1640 medium (no antibiotics). As appropriate, TPA (20 ng/ml) and DRB (5 μm) were added 5 h post-transfection and harvested 24 h later.

CK2 activity assays

The CK2 activity assays (25) were performed on immunoprecipitates either with or without 0.1 mM of the specific peptide substrate (55,56). As appropriate, in vitro CK2 activity reactions were carried out in the presence of 50 μM DRB, a CK2 inhibitor, that acts in vitro (25,57) and in vivo (40,53,54).

In vitro phosphorylation

10 μg of glutathione beads containing GST–ORF57 FL were mixed with 3 μl CK2 holoenzyme from Escherichia coli (Boehringer Mannheim) in 30 μl kinase buffer (20 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM MgCl₂, 1 mM DTT, 1 mM EGTA and 100 μM γ-[³²P]ATP), and in vitro phosphorylation reactions were carried out for 30 min at 25°C as described in (26) in the presence or absence of 50 μM DRB. Bound proteins were resolved by SDS–PAGE and analysed either by phosphorimaging or transferred to nitrocellulose membranes for western blot analysis with anti-ORF57 Ab.

RESULTS

ORF57 protein is capable of self-interaction and interacts with hnRNP K from KSHV-infected cell extracts

ICP27 protein is capable of self-interaction, thus the possibility of ORF57 self-interaction was examined as was the ability
of ORF57 and ICP27 proteins to interact. GST–ORF57 FL protein (19) and GST–ORF57 small (containing ORF57 amino acids 181–455) or GST alone were expressed (Figure 1A) and equivalent amounts were used in pull down assays with [35S]-labelled ORF57 FL, ORF57 small or ICP27 proteins. On phosphorimager analysis, a band corresponding to labelled ORF57 FL was bound by GST–ORF57 FL (Figure 1B, lane 6) and by GST–ORF57 small (Figure 1B, lane 8). Neither GST–ORF57 FL nor small bound to labelled ICP27 protein (Figure 1B, lanes 3 and 4). GST–ICP27 pulled down labelled ICP27 (Figure 1B, lane 5) but not labelled ORF57 FL or small (Figure 1B, lanes 10 and 11). No labelled protein was bound by GST alone (Figure 1B, lanes 2, 7 and 9). Therefore, ORF57 protein is capable of interacting with itself but not with its HSV-1 ICP27 homologue. GST–ORF57 small bound to labelled ORF57 small (Figure 1C, lane 3), thus the ORF57 region involved in self-interaction lies outside the N-terminal 180 amino acids.

To examine for an ORF57–hnRNP K interaction, equivalent amounts of GST–ORF57 FL or GST alone were used in the pull down assays. (Figure 1B, lane 5) but not labelled ORF57 FL or small (Figure 1B, lanes 10 and 11). No labelled protein was bound by GST alone (Figure 1B, lanes 2, 7 and 9). Therefore, ORF57 protein is capable of interacting with itself but not with its HSV-1 ICP27 homologue. GST–ORF57 small bound to labelled ORF57 small (Figure 1C, lane 3), thus the ORF57 region involved in self-interaction lies outside the N-terminal 180 amino acids.

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pull down assays with cell extracts (200 μg protein) from TPA-treated or untreated BCBL-1 cells. Bound proteins were eluted, fractionated by SDS–PAGE, the gels were transferred to nitrocellulose membranes and western blots were performed using anti-hnRNP K Ab. GST–ORF57 FL bound hnRNP K present in both untreated and TPA-treated cell extracts whereas GST alone did not (Figure 1D, compare lanes 3 and 4 with lanes 1 and 2). When samples were separated for a longer duration, the hnRNP K fraction pulled down by GST–ORF57 FL from both untreated and TPA-treated cells migrated more rapidly than the majority fraction of hnRNP K (Figure 1D, compare lanes 3 and 4 with lanes 5 and 6). RNase treatment did not affect the ORF57–hnRNP K interaction (Figure 1D, lane 4) indicating that this was not mediated via RNA. In the reciprocal pull down assay, GST–hnRNP K FL or GST alone were used with extracts from BCBL-1 cells. Following SDS–PAGE, bound proteins were transferred to nitrocellulose membranes and western blotted with a mixture of anti-hnRNP K and anti-ORF57 Abs. HnRNP K was present in both TPA-treated and untreated extracts (Figure 1E, lanes 5 and 6) while ORF57 was present only in TPA-treated extracts (Figure 1E, compare lane 5 with 6). GST–hnRNP K FL pulled down a ~52 kDa ORF57 band from TPA-treated cell extracts (Figure 1E, lower band in lanes 1 and 5), and an hnRNP K band from both TPA-treated and untreated cells (Figure 1E, upper band in lanes 1, 2, 5 and 6) due to hnRNP K self-interaction (27). GST–hnRNP K bound approximately equal amounts of ORF57 and hnRNP K from TPA-treated cells (Figure 1E, compare lane 1 with 5) suggesting that they bind to each other stoichiometrically. GST alone did not pull down either ORF57 or hnRNP K (Figure 1E, compare lanes 3 and 4 with 5 and 6).

ORF57 and hnRNP K proteins co-immunoprecipitate from KSHV-infected cell extracts and from HeLa cells transfected with an ORF57 expression vector

Co-immunoprecipitation experiments confirmed an ORF57–hnRNP K interaction. Cell extracts were incubated with anti-ORF57 Ab and immunoprecipitates were separated by SDS–PAGE, then following transfer to nitrocellulose membranes, anti-hnRNP K Ab was used for western blotting. HnRNP K co-immunoprecipitated with ORF57 from extracts of TPA-treated but not from untreated cells (Figure 2A, compare lanes 1 and 2) and not using pre-immune Ab with TPA-treated extracts (Figure 2A, lane 3). In the reciprocal immunoprecipitation using anti-hnRNP K Ab, ORF57 protein was not apparent as the IgG heavy chain of the rabbit anti-hnRNP K Ab is of similar size and masked the signal in western blots (data not shown).

To see if other viral proteins were required for the ORF57–hnRNP K interaction, immunoprecipitations were performed using anti-hnRNP K Ab or pre-immune Ab with extracts of HeLa cells transfected with plasmids pEGFP-gORF57 FL (expressing ORF57 protein) or empty vector pEGFP-C1. Immunoprecipitates were fractionated by SDS–PAGE, proteins were transferred to nitrocellulose membranes and then western blotting was performed with anti-GFP mAb (Figure 2B). GFP–ORF57 fusion protein (82–86 kDa) was detected in cells transfected with pEGFP-gORF57 FL but not with cells transfected with pEGFP-C1 (Figure 2B, compare lanes 2 and 3) or with cells transfected with pEGFP-gORF57 FL and treated with pre-immune Ab (Figure 2B, lane 5). The band co-immunoprecipitated with anti-hnRNP K Ab in lane 2 was GFP–ORF57 fusion protein as confirmed by western blotting the membranes with anti-ORF57 (GH) Ab (data not shown). Western blotting with anti-hnRNP K Ab using the samples shown in Figure 2B showed that hnRNP K was immunoprecipitated from extracts treated with anti-hnRNP K Ab (Figure 2C, lanes 2 and 3) and not with extracts treated with pre-immune Ab (Figure 2C, lanes 5 and 6).
Mapping the regions of hnRNP K and ORF57 proteins required for their interaction

Recombinant GST–hnRNP K FL (27), its deletion mutants containing hnRNP K amino acids 1–209, 171–337, 240–337 and 318–464 expressed as described previously in (31,45) or GST alone (Figure 3A) were used in the pull down assay with [35S]-labelled ORF57 FL. Despite the larger predicted size for GST-K3 (166 amino acids) in comparison to GST-K7 (146 amino acids), it migrated more rapidly as described earlier (31,45), perhaps due to differential processing. Bound proteins were separated by SDS–PAGE, gels were dried and analysed by autoradiography. GST–hnRNP K FL bound labelled ORF57 deletion mutants

Figure 3. Mapping the regions of hnRNP K and ORF57 proteins required for their interaction. (A) Left panel: GST–hnRNP K FL (amino acids 1–464) or four deletion mutants were used in the pull down assay with [35S]-labelled ORF57 FL. GST–hnRNP K FL and deletion mutant proteins were separated by SDS–PAGE and visualized by Coomassie blue staining as follows: GST–hnRNP K FL (lane 1); GST–hnRNP K 3 amino acids 171–337 (lane 2); GST–hnRNP K 7 amino acids 318–464 (lane 3); GST–hnRNP K 12 amino acids 1–209 (lane 4); GST–hnRNP K 31 amino acids 240–337 (lane 5); GST alone (lane 6). Right panel: cartoon showing GST–hnRNP K FL and the various deletion mutants used. (B) Mapping regions of hnRNP K that interacted with ORF57. Bound proteins were resolved by SDS–PAGE, gels were analysed by autoradiography using the following: input labelled ORF57 FL (lane 1); labelled ORF57 + GST (lane 2); labelled ORF57 + GST–hnRNP K FL (lane 3); labelled ORF57 + GST–hnRNP K 7 amino acids 318–464 (lane 4); labelled ORF57 + GST–hnRNP K 12 amino acids 1–209 (lane 5); labelled ORF57 + GST–hnRNP K 31 amino acids 240–337 (lane 6). (C) Expression of [35S]-labelled ORF57 deletion mutant proteins used in mapping as follows: ORF57 FL amino acids 1–455 (lane 1); ORF57 amino acids 17–455 (lane 2); ORF57 amino acids 1–215 (lane 3); ORF57 amino acids 181–328 (lane 4); ORF57 amino acids 329–455 (lane 5); ORF57 amino acids 387–455 (lane 6). (D) Cartoon showing ORF57 FL and the various ORF57 deletion mutants used. (E) Mapping regions of ORF57 that interacted with hnRNP K. GST–hnRNP K FL was used in pull down assays with labelled ORF57 proteins, bound proteins were separated by SDS–PAGE, gels were analysed by phosphorimaging using the following: labelled ORF57 amino acids 1–455 (lane 1); labelled ORF57 amino acids 17–455 (lane 2); labelled ORF57 amino acids 1–215 (lane 3); labelled ORF57 amino acids 181–328 (lane 4); labelled ORF57 amino acids 329–455 (lane 5); labelled ORF57 amino acids 387–455 (lane 6); GST alone + labelled ORF57 FL (lane 7). (F) Use of similar amounts of GST–hnRNP K FL in the pull down assays shown in Figure 3E was demonstrated by western blotting similar amounts of samples eluted from the pull downs using anti-GST Ab.
ORF57 protein (Figure 3B, lane 3) as did GST–hnRNP K3 and 31 deletion mutants, containing hnRNP K amino acids 171–337 and 240–337, respectively (Figure 3B, lanes 5 and 7) whereas GST–hnRNP K7 and 12 deletion mutants, containing hnRNP K amino acids 318–464 and 1–209, respectively, did not (Figure 3B, lanes 4 and 6) and GST alone did not bind (Figure 3B, lane 2). Thus, the hnRNP K region containing amino acids 240–337 was capable of interaction with ORF57 protein.

In the reciprocal mapping experiment, GST–hnRNP K FL or GST alone were used in pull down assays with labelled ORF57 FL (amino acids 1–455) or radiolabelled fragments containing ORF57 amino acids 17–455, 1–215, 181–328, 329–455 and 387–455 (Figure 3C and D). GST–hnRNP K FL and ORF57 amino acids 17–455, 1–215 and 329–455 bound ORF57 FL (Figure 3E, lanes 1, 2, 3 and 5 respectively) but no detectable binding was observed with ORF57 amino acids 181–328, 387–455 or with GST alone (Figure 3E, lanes 4, 6 and 7). Use of equivalent amounts of GST–hnRNP K FL or GST alone in the pull down assay was demonstrated by western blotting similar amounts of samples eluted from the pull downs shown in Figure 3E using anti-GST Ab (Figure 3F). These data indicated that two separate ORF57 regions were capable of interaction with hnRNP K, involving amino acids 17–181 and 329–387.

Protein kinase CK2 activity is present in complexes containing ORF57 and hnRNP K proteins

HSV-1 ICP27 protein interacts with and is phosphorylated by protein kinase CK2. To determine if the ORF57 complex contained CK2 activity, GST pull down assays using TPA-treated and untreated BCBL-1 cell extracts were followed by an assay using a synthetic peptide substrate specific for CK2 activity (25,55). The peptide substrate binds the CK2 subunit where it is held for phosphorylation by the CK2 α subunit. GST–ORF57 FL pulled down considerable and similar amounts of CK2 activity from TPA-treated and untreated cell extracts in comparison to GST alone (Figure 4A, compare bars 1 and 2 with bars 9 and 10) and all activities were much reduced in the absence of peptide substrate (Figure 4A, bars 3, 4, 11 and 12). When the GST–ORF57 pull downs were assayed in the presence DRB, a CK2 specific inhibitor (25,53,54), the activities were considerably reduced (Figure 4A, bars 7 and 8).

CK2 activity associated with ORF57 protein was further examined using extracts of KSHV-infected BCBL-1 cells treated with TPA, or BCBL-1 cells transfected with pEGFP-gORF57 FL following immunoprecipitations with anti-ORF57 Ab (Figure 4B, bars 1, 2, 4, 5 and 7), anti-GFP mAb (Figure 4B, bars 3 and 6) or pre-immune serum Ab (Figure 4B, bars 8–10). High CK2 levels were obtained with anti-ORF57 immunoprecipitates from TPA-treated cell extracts and anti-GFP immunoprecipitates from pEGFP-gORF57 FL transfected cells but not with anti-ORF57 immunoprecipitates from untreated cells (Figure 4B, compare bars 4 and 6 with bar 5). Little CK2 activity was detected in the absence of peptide substrate (Figure 4B, bars 1, 2 and 3) or in immunoprecipitates obtained with pre-immune Ab (Figure 4B, bars 8–10), and the assay activity was almost completely inhibited by DRB (Figure 4B, bar 7).

Using immunoprecipitates obtained with anti-hnRNP K Ab, CK2 activity was detected in extracts of BCBL-1 cells treated with TPA (Figure 4C, bar 3) but not in the presence of DRB (Figure 4C, bar 5) or with immunoprecipitates from untreated cell extracts (Figure 4C, compare bars 3 and 4) or with immunoprecipitates obtained with pre-immune Ab (Figure 4C, bar 7). CK2 activity was not detected in the absence of peptide substrate (Figure 4C, bars 1, 2 and 6). CK2 activity assays were performed on extracts of 293 cells transfected with pGFP-gORF57 FL and small, and pcDNA3-gORF50 that expresses the KSHV ORF50 lytic switch regulatory protein (18). Using immunoprecipitates obtained with anti-hnRNP K Ab, high levels of CK2 activity were detected in cells transfected with pGFP-gORF57 FL, with less activity in cells transfected with pGFP-gORF57 small (Figure 4D, compare bars 5 and 2). Much lower CK2 activity was detected in the absence of substrate (Figure 4D, bars 4 and 1), in the presence of DRB (Figure 4D, bars 6 and 3), using immunoprecipitates obtained with pre-immune Ab in the presence or absence of substrate, and with DRB (Figure 4D, bars 12, 13 and 11). With cells transfected with pcDNA3-gORF50, little CK2 activity was detected in the presence or absence of peptide substrate (Figure 4D, bars 8 and 7), while co-expression of ORF57 and ORF50 proteins gave CK2 activities similar to those obtained with ORF57 alone (Figure 4D, compare bars 9 and 5). Hence, expression of ORF57 protein alone is sufficient for CK2 activity to be present in a complex that also includes hnRNP K protein.

ORF57 protein interacts directly with CK2 α, α′ and β subunits

Pull down assays were performed using recombinant GST–CK2 α or α′, MBP–CK2 β fusion proteins, GST or MBP alone and [35S]-labelled ORF57 FL, ORF57 small or luciferase protein. The fusion proteins were visualized by Coomassie blue staining (Figure 5A) and adjusted equivalent amounts were used in the pull down assays. ORF57 was pulled down by both α and α′ catalytic subunits (Figure 5B, lanes 3, 4, 5 and 6), by the regulatory β subunit (Figure 5B, lanes 7 and 8), but not by GST or MBP alone (Figure 5B, lanes 9 and 10) and GST–CK2 α did not pull luciferase control (Figure 5B, lane 11). As both ORF57 FL and small proteins bound the three CK2 subunits, ORF57 amino acids 1–180 were not required for these interactions.

These pull down assays were repeated using BCBL-1 cell extracts. Following separation of samples by SDS–PAGE, gels were transferred to a nitrocellulose membrane, and then western blotted with anti-ORF57 Ab. ORF57 protein was absent from untreated cells (Figure 5C, lane 1) and present in TPA-treated cells (Figure 5C, lane 2) where a faint faster migrating product was present. CK2 α/α′ and β subunits interacted with ORF57 protein from TPA-treated cells whereas GST and MBP alone did not (Figure 5C, compare lanes 3, 5 and 10 with lanes 7 and 8), and no interactions were observed using untreated cell extracts (Figure 5C, lanes 1, 4, 6 and 9). The band just above ORF57 protein (Figure 5C, lanes 9 and 10 marked with asterisk) is an MBP–CK2 β species that cross-reacts with anti-ORF57 Ab and was present in both TPA-treated and untreated cell extracts (Figure 5C, lanes 9 and 10) whereas in the input cell extracts
ORF57 protein was present only in the TPA-treated cells (Figure 5C, lane 2) and not in the untreated cells (Figure 5C, lane 1).

A direct interaction between ORF57 protein and CK2 α and β subunits was seen when the pull down assay was performed with all proteins expressed from prokaryotic vectors. GST–ORF57 FL or GST alone were mixed with equivalent amounts of recombinant His–CK2 α, MBP–CK2 β or an unrelated His–thioredoxin fusion protein. Following separation of bound protein by SDS–PAGE, gels were western blotted using a mixture of anti-CK2 α and β Abs or anti-His mAb. Recombinant ORF57 protein bound both CK2 α and β sub-units (Figure 5D, lanes 1 and 3, respectively) whereas GST alone did not (Figure 5D, lanes 2 and 4), western blotting using anti-His Ab indicated that GST–ORF57 did not bind His–thioredoxin (Figure 5D, lane 5).
Figure 4. Protein kinase CK2 activity is present in complexes containing ORF57 and hnRNP K proteins. A sensitive and specific method to determine CK2 activity in immunoprecipitates uses an artificial peptide substrate (25). Error bars show the mean values (± SD) obtained using assays from three independent experiments performed in duplicate. (A) CK2 activity is present in complexes containing ORF57 protein. The pull down assay using GST–ORF57 FL (bars 1–8) or GST alone (bars 9–14) with extracts of BCBL-1 cells was followed by a CK2 activity assay. CK2 assays used TPA-treated (TPA) and untreated (Un) cell extracts, and were performed with (+P) or without (−P) peptide and in the presence (+DRB) or absence of 50 μM DRB, a specific CK2 inhibitor, that acts in vitro and in vivo. (B) Extracts of TPA-treated or untreated BCBL-1 cells and also transfected with pEGFP-gORF57 FL (Trans ORF57) were immunoprecipitated with anti-ORF57 Ab (bars 1, 2, 4, 5, and 7), anti-GFP Ab (bars 3 and 6) or pre-immune (PI) serum Ab (bars 8–10), and CK2 activity present in the immunoprecipitates was assayed. (C) CK2 activity is present in complexes containing hnRNP K and ORF57 proteins. CK2 activities present in immunoprecipitates generated by anti-hnRNP K Ab from extracts of TPA-treated or untreated BCBL-1 cells. Bars 1–5, immunoprecipitations with anti-hnRNP K Ab; bars 6 and 7, immunoprecipitations with pre-immune serum Ab. (D) CK2 activities present in immunoprecipitates generated from 293 cell extracts following transfection with expression plasmids, pEGFP-ORF57 small, pEGFP-gORF57 FL and pcDNA3-gORF50. Immunoprecipitations with anti-hnRNP K Ab (bars 1–10); immunoprecipitations with pre-immune serum Ab (bars 11–13); CK2 activities in the presence of DRB (bars 3, 6, and 13).
Regions of ORF57 protein involved in interaction with CK2 α/α' and β subunits and CK2 β subunit regions that interact with ORF57

ORF57 regions required for interaction with CK2 α/α' were mapped using the pull down assay with GST–CK2 α or GST alone and [35S]-labelled ORF57 FL (amino acids 1–455) or ORF57 small (amino acids 181–455) or ORF57 deletions containing amino acids, 1–215, 181–328, 329–455 and 387–455 (Figure 5B, lanes 1 and 2 and Figure 3C). ORF57 FL, small, amino acids 329–455 and 387–455 bound GST–CK2 α' (Figure 6A, lanes 1, 2, 5 and 6) whereas no binding was detected with amino acids 1–215 and 181–328 (Figure 6A, lanes 3 and 4) or with GST alone (Figure 6A, lane 7). Equal portions of the eluted samples were examined by western blotting using anti-GST Ab demonstrating that similar amounts of GST–CK2 α' or GST alone were used in the pull downs (Figure 6C). Thus, the minimum region of ORF57 protein sufficient for interaction with CK2 α' involves amino acids 387–455.

When the pull down assay was performed with MBP–CK2 β or MBP alone and labelled ORF57 FL or its deletion mutants, ORF57 FL and amino acids 17–455, 1–215 and 181–328 bound MBP–CK2 β (Figure 6B, lanes 1, 2, 3 and 4) while no interaction was observed with amino acids 329–455 and 387–455 (Figure 6B, lanes 5 and 6) or MBP (Figure 6B, lane 7). Equal portions of the eluted samples were examined by western blotting using anti-MBP Ab, demonstrating that...
similar amounts of MBP–CK2 β or MBP alone were used in the pull downs (Figure 6D). Therefore, the minimum ORF57 region sufficient for interaction with CK2 β involves amino acids 181–215.

CK2 β subunit regions interacting with labelled ORF57 FL and small were mapped using GST and MBP proteins fused with different regions of CK2 β (25). ORF57 FL protein- (Figure 6E, lane 1), which also showed a smaller truncation product of similar size to ORF57 small, bound MBP–CK2 β FL (amino acids 1–215) in the presence of RNase (Figure 6E, lane 2) and bound MBP–CK2 β amino acids 1–182 (Figure 6E, lane 3) whereas no ORF57 binding was detected with MBP–CK2 β amino acids 1–110, GST–CK2 β amino acids 1–150, GST–CK2 β amino acids 51–150 and GST–CK2 β amino acids 1–55 (Figure 6E, lanes 4, 7, 8 and 9). MBP–CK2 β FL (amino acids 1–215) also bound ORF57 small (amino acids 181–455) protein (Figure 6E, lane 6), but did not pull down the unrelated luciferase protein (Figure 6E, lane 11) and MBP or GST controls did not bind ORF57 FL (Figure 6E, lanes 5 and 10). Thus, CK2 β amino acids 150–182 are involved in interaction with ORF57 protein. The N- and C-terminal CK2 α subunit deletion mutants, containing amino acids 1–265 and 266–391 bound to GST–ORF57 FL while GST alone did not (data not shown).

**ORF57 is phosphorylated by CK2 in vitro and in KSHV-infected cells**

The ability of recombinant CK2 holoenzyme to *in vitro* phosphorylate GST–ORF57 FL was examined in the presence or absence of DRB. ORF57 FL was efficiently phosphorylated *in vitro* by CK2 holoenzyme as was a smaller truncation (Figure 7A, lane 1) and phosphorylation was inhibited by DRB (Figure 7A, lane 2). Western blot analysis with anti-ORF57 Ab showed comparable amounts of GST–ORF57 protein in the absence and presence of DRB (Figure 7B, lanes 1 and 2).

Next, phosphorylation of ORF57 protein by CK2 in KSHV-infected cells was examined using low, non-toxic doses of cell-permeable CK2 inhibitor DRB (40). BCBL-1 cells, TPA-treated or untreated, were labelled with either [35S]-methionine or [32P]-orthophosphate for 6 h in the presence or absence of DRB and immunoprecipitations were then performed on cell extracts using anti-ORF57 (GH) Ab.
In vivo phosphorylation of ORF57 protein (Figure 7C) in TPA-treated cells was reduced in the presence of 5 μM DRB (Figure 7C, compare lanes 4 and 5) and this inhibition was greater with 10 μM DRB (Figure 7C, compare lanes 4 and 5) but was not abolished completely, suggesting that ORF57 is phosphorylated by a kinase(s) other than CK2 that is sensitive to DRB at higher concentrations. ORF57 protein levels in the immunoprecipitates obtained with anti-ORF57 Ab could not be demonstrated by western blotting due to masking of the protein by the IgG heavy chain of the available rabbit ORF57 Ab. However, western blot analysis of same TPA-treated protein by the IgG heavy chain of the available rabbit ORF57 Ab showed that the accumulation of ORF57 protein was not affected by the presence of DRB (Figure 7D, compare lane 1 with lanes 2, 3, and 4), thus, excluding the possibility that in vivo the reduction of phosphorylation was due to DRB inhibition of ORF57 transcription. The anti-peptide ORF57 Ab used for detection was not directed against a phosphorylated form and thus may not recognize differentially phosphorylated forms of the protein from the band detected in presence of DRB (Figure 7D), and protein dephosphorylation does not necessarily result in retardation of migration (40,58). Immunoprecipitation of [35S]-labelled cell extracts with anti-ORF57 Ab showed the presence of a smaller band (Figure 7C, lane 2) that was not labelled with [32P]-orthophosphate.

**Phosphorylation by CK2 promotes interaction between ORF57 and hnRNP K proteins**

The effect of CK2 phosphorylation on the ORF57–hnRNP K interaction was examined. Endogenous viral ORF57 protein from TPA-treated cells could not be demonstrated by the western blotting of immunoprecipitates. Thus, to visualize co-immunoprecipitated ORF57, permissive BCBL-1 cells harbouring KSHV genome were transfected with pGFP-gORF57 FL or pEGFP-C1 empty vector, then cells were either TPA-treated to induce viral lytic cycle or left untreated in the presence or absence of 5 μM DRB. Immunoprecipitates obtained using anti-ORF57 K Ab were separated by SDS–PAGE, gels were transferred to nitrocellulose membranes and western blotting was performed using anti-GFP mAb. GFP-ORF57 was co-immunoprecipitated with hnRNP K from TPA-treated pGFP-gORF57 FL transfected but not from pEGFP-C1 transfected cells (Figure 8A, compare lanes 1 and 2 with lanes 3 and 4). Quantification of the amount of ORF57 protein immunoprecipitated with hnRNP K following DRB treatment showed a consistent 2- to 3-fold lowering based on three separate experiments (Figure 8A, compare lanes 2 and 1). Western blotting using anti-hnRNP K Ab on extracts with equivalent protein amounts demonstrated that hnRNP K protein was immunoprecipitated in similar amounts from the pGFP-gORF57 or pEGFP-C1 transfected cells (Figure 8C, lanes 1–4). GFP-ORF57 co-immunoprecipitated with hnRNP K in lesser amounts from untreated pGFP-gORF57 FL transfected cells (Figure 8A, lanes 5 and 6) in the absence of endogenous ORF57 expression, but did not co-immunoprecipitate from pEGFP-C1 transfected cells (Figure 8A, lanes 7 and 8). Quantification of the amount of ORF57 protein immunoprecipitated with hnRNP K following DRB treatment from untreated cells showed a similar 2- to 3-fold lowering (Figure 8A, compare lanes 6 and 5). Co-transfection of pCH110 encoding β-Gal served as an internal control for transfection efficiency and western blotting of the cell extracts with anti-β-Gal Ab (Invitrogen) indicated no difference in the amounts of β-Gal protein, and following DRB treatment (data not shown).

The effect of DRB on hnRNP K phosphorylation was examined using TPA-treated or untreated BCBL-1 cells labelled with [32P]-orthophosphate for 6 h in the presence or absence of 5 μM DRB. Immunoprecipitates obtained using anti-hnRNP K Ab were separated by SDS–PAGE, followed by autoradiography or western blot analysis using anti-hnRNP K Ab. The radiolabelled hnRNP K band showed greater labelling in TPA-treated in comparison to untreated cells (Figure 8B, compare lanes 2 and 4). And, phosphorylation of...
hnRNP K was inhibited by DRB in TPA-treated cells as compared to untreated cells (Figure 8B, compare lanes 1 and 2 with lanes 3 and 4). HnRNP K was immunoprecipitated in similar amounts from TPA-treated and untreated cells and in the presence of DRB (Figure 8C, lanes 5–8). Up to 72 h after TPA treatment, there was little change in the levels of CK2 α/α′ and β subunits as compared to untreated cells (Figure 8D, compare lanes 2–7 with lane 1), and the amounts of hnRNP K protein remained similar (Figure 8E, compare lanes 2–7 with lane 1).

**DISCUSSION**

HnRNP K shuttles from the nucleus to the cytoplasm, is capable of self-interaction, associates with multiprotein complexes, regulates transcriptional, post-transcriptional, and translational processes, and could act as a docking platform to facilitate communication among molecules involved in gene expression and signal transduction (27). ORF57 and hnRNP K proteins were shown to interact using pull down assays. They were co-immunoprecipitated from TPA-treated BCBL-1 cells and from cells transfected with an ORF57 expression construct, indicating that no other KSHV proteins were required for their association. ORF57 interacted with a rapidly migrating form of hnRNP K as does its ICP27 protein counterpart (25). HnRNP K primary transcripts are alternatively spliced to generate mRNAs that encode four protein isoforms (59). The more rapidly migrating hnRNP K form that interacted with ORF57 could represent a smaller or a less processed protein isoform. Our preliminary data suggest that the more rapidly migrating hnRNP K fraction is hypophosphorylated that confers a higher affinity for RNA than more phosphorylated forms (59).
The ORF57 domain organization has not yet been fully characterized, although on the basis of amino acid homology with other herpesvirus counterparts several putative domains have been predicted. The N-terminal region (amino acids 17–215), interacting with hnRNP K (Figure 3E) and RNA export factor (REF) protein (23), contains an Arg and Gly-rich putative RGG box (amino acids 122–152) characteristic of many RNA-binding proteins. And a putative nuclear localization domain is present between amino acids 1–180 as an N-terminal ORF57 deletion construct localized to the cytoplasm whereas full-length ORF57 showed a nuclear distribution (22). Unlike its homologues in other herpesviruses, KSHV ORF57 contains a putative leucine zipper motif between amino acids 343–364, generally found in DNA-binding proteins, with a possible role in self-interaction or DNA-binding and another hnRNP K interacting region (amino acids 329–387) was present in this domain. HnRNP K protein also is capable of self-interaction, a property that may enable simultaneous contact with several partners to form multi-component complexes.

Co-expression of hnRNP K with YB-1 (46) and C/EBP β transcription factor (60) caused repression of the gene activities, and activation of the agp gene by the interaction of Nopp140 with C/EBPβ was abolished by hnRNP K (60). A reduction of hnRNP K-mediated transcriptional activity was observed by co-expression of Sam68, a cellular partner protein that substitutes for and synergizes with HIV-1 Rev (61). HnRNP K suppression of reporter gene activity from the cellular TK promoter was relieved by expression of hepatitis C virus core protein (62), and hnRNP K suppression on cellular C/EBP β-mediated transcription activation could be reversed by Dengue virus core protein (63). HnRNP K and hnRNPs E1/ E2 mediate translational silencing of cellular LOX and human papillomavirus-16 mRNAs, an effect reversed by c-Src-mediated phosphorylation of hnRNP K which inhibits its RNA binding (64,65). Herpesvirus ORF57 counterparts, such as HSV-1 ICP27 and EBV MTA mediate viral transcription and gene regulation (11,13). We suggest that interaction with hnRNP K may modulate ORF57-mediated transactivation of viral promoters.

Using a specific assay, CK2 activity was pulled down with GST–ORF57 fusion protein from extracts of KSHV containing TPA-induced BCBL-1 cells and activity was markedly reduced by the CK2 inhibitor DRB; CK2 activity was also present in immunoprecipitates obtained with anti-ORF57 and anti-hnRNP K Abs. ORF57 protein interacted directly with both CK2 catalytic α/α′ and regulatory β subunits, as demonstrated using purified recombinant proteins, and ORF57 amino acids 1–180 were not required for binding all three CK2 subunits. HSV-1 ICP27 showed an interaction with CK2 β in a yeast two-hybrid library screen, and was also pulled down with GST–CK2 α, an interaction regarded as bridged by CK2 β as no CK2 α clone was detected in the screen (25). Various CK2 substrates interact with both CK2 α and β subunits including HIV-1 Rev protein [reviewed in (66)] that like ORF57 and ICP27 herpesvirus proteins promotes nuclear export of unspliced viral RNAs and eukaryotic translation initiation factor elf2β [reviewed in (33,34)]. Intriguingly, recombinant HIV-1 Rev stimulates CK2 activity in vitro (67) while ICP27 stimulates CK2 activity in HSV-1-infected cells (40). We are examining for effects of KSHV lytic replication on cellular CK2 activation.

CK2 α and α′ subunits though structurally similar are not functionally identical in mammals and yeast [reviewed in (34)], and the holoenzyme may form transiently and dissociate in vivo (68). Free populations of α and β subunits exist alone or in association with different partners, and there is increasing evidence that they may have specific functions [reviewed in (34,36) and references therein]. Unlike CK2 β, CK2 α is capable of nuclear-cytoplasmic shuttling (69) and changes in its subcellular distribution could influence ORF57 localization and activity. In this regard, adenovirus infection causes redistribution of CK2 α and β subunits to morphologically distinct structures in the HeLa cell nucleus (70).

N- and C-terminal regions of CK2 α and amino acids 150–182 of CK2 β interacted with ORF57. From the human CK2 crystal structure of two α and two β subunits, the complex is shaped like a butterfly in which interacting β subunits make bridging contacts with C-termini of the α subunits (68). CK2 β amino acids 150–182 are involved in β subunit homodimerization and in α–β heterodimerization (71), and a similar region interacted with ICP27 (25). The minimum ORF57 region for interaction with CK2 β involved the central amino acids 181–215, in contrast, both central and N-terminal regions of ICP27 were involved (25). The ORF57 C-terminus was capable of interacting with CK2 α independently of other ORF57 regions; this does not exclude CK2 phosphorylation of the ORF57 N-terminus as kinase binding sites and sites of phosphorylation are not necessarily similar, as reported for cellular eIF2β (41). The ORF57 C-terminus region interacting with CK2 α (amino acids 387–455) contains a putative zinc finger like domain H(423)-X(427)-X4-C(432) with cysteine and histidine residues, some of which are conserved between the γ-herpesvirus and α-herpesvirus counterparts (72), and a hydrophobic GLFF domain (amino acids 448–451) highly conserved in the γ-herpesviruses. The region encompassing the putative zinc finger is predicted to form an α-helical secondary structure (data not shown) characteristic of the dimerization motifs in helix–loop–helix proteins and leucine zipper DNA binding proteins. ORF57 protein was capable of self-interaction, as shown for HSV-1 ICP27 protein (25,73), but did not interact with its ICP27 counterpart. In ICP27, this C-terminal region is involved in protein self-interaction (25,73) and point mutations in it were lethal in virus, suggesting that self-recognition is important for function (72).
HnRNP K interacts with inducible kinases (32,50) and via changes in phosphorylation regulates its interactions with proteins and RNA (75,76). Indeed, hnRNP K itself regulates the activity of inducible kinases (31,50,64). Although added CK2 is capable of phosphorylating hnRNP K in vitro (31,50), hnRNP K is not normally phosphorylated by this kinase in uninfected cells [reviewed in (77)], thus its association with CK2 and ORF57 is of considerable interest. In both TPA-treated and untreated BCBL-1 cell extracts, hnRNP K was phosphorylated but this was considerably reduced by DRB only in TPA-treated cells. Taken together with the observation that CK2 activity was associated with hnRNP K only in the presence of ORF57 in the peptide substrate assay, this indicates a requirement of ORF57 for CK2 phosphorylation of hnRNP K. In the presence of DRB in vivo at low concentrations specific for inhibiting CK2 phosphorylation, the amount of ORF57 present in a complex with hnRNP K showed a consistent 2- to 3-fold lowering. CK2 phosphorylation therefore serves to recruit ORF57 into a complex with hnRNP K. Phosphorylation by CK2 may regulate the presence and activities of ORF57 and/or hnRNP K, and perhaps other components in the ORF57 complex at various times during KSHV lytic gene expression. In different infected host cells, such as in B-cells or endothelial cells, and during the different phases of lytic KSHV replication, CK2 phosphorylation could alter the subcellular locations of hnRNP K isoforms, sequestering them to active or inactive sites. During the cell cycle, inhibiting CK2 phosphorylation of human chromatin protein DEK with 4,5,6,7-tetrabromobenzotriazole changed its DNA binding properties (58). ORF57 may act to subvert the multifunctional activities of hnRNP K, re-directing them for use in viral rather than cellular gene expression or, alternatively, prevent hnRNP K from accessing common pathways thereby inhibiting competition for cellular components used by both proteins.

ORF57 protein can regulate gene expression at the transcriptional level (20). We have shown that ORF57 associates with the ORF50 promoter and with ORF50 protein to co-operatively activate expression from this promoter (19). ORF50 protein expression is sufficient to induce lytic replication in latently infected cells (17,18), and this protein also is extensively phosphorylated in vivo and contains several serine/threonine-rich consensus phosphorylation sites for protein kinase CK2 and protein kinase C (8). CK2 phosphorylation of both ORF57 and ORF50 proteins could play an important role in the augmentation of ORF50 activity by ORF57 protein (19), facilitating the cascade of lytic KSHV gene expression and thereby breaking latency. ORF57 protein shuttles between the nucleus and cytoplasm (22), and also regulates gene expression post-transcriptionally (20,21), promoting the nuclear export of an intron-less reporter RNA (23). Nuclear import of Simian virus 40 large T antigen is enhanced by CK2 phosphorylation (78). Similarly, CK2 phosphorylation may regulate ORF57 protein transport and/or transport of hnRNP K with a viral RNA cargo. HnRNP K has a binding preference for poly (C) [reviewed in (28)], can shuttle from the nucleus to the cytoplasm and might export viral RNAs via an ORF57–RNA complex piggybacking on hnRNP K to the cytoplasm. Or, the ORF57 interaction might prevent hnRNP K from shuttling, permitting ORF57 to access a nuclear export or import route normally used by hnRNP K.

The γ-2 herpesvirus KSHV (genus, Rhadinovirus) ORF57 protein and the α-1 herpesvirus HSV-1 (genus, Simplexvirus) ICP27 protein both interact with hnRNP K and are phosphorylated by CK2. Thus, the functional adaptation of the ancestral herpesvirus ORF57 regulatory protein and its counterparts to interact with the hnRNP K and CK2 ancestral proteins is likely to have preceded divergence of these Herpesviridae genera. This divergence is estimated to have occurred 180–210 million years ago (79) and prior to the mammalian radiation.

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REFERENCES


