**The Multifunctional Herpes Simplex Virus IE63 Protein Interacts with Heterogeneous Ribonucleoprotein K and with Casein Kinase 2**

(Received for publication, March 15, 1999, and in revised form, June 10, 1999)

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Herpes simplex virus type 1 (HSV-1), the prototype α-herpesvirus, causes several prominent diseases. The HSV-1 immediate early (IE) protein IE63 (ICP27) is the only regulatory gene with a homologue in every mammalian and avian herpesvirus sequenced so far. IE63 is a multifunctional protein affecting transcriptional and post-transcriptional processes, and it can shuttle from the nucleus to the cytoplasm. To identify interacting cellular proteins, a HeLa cDNA library was screened in the yeast two-hybrid system using IE63 as bait. Several interacting proteins were identified including heterogeneous nuclear ribonucleoprotein K (hnRNP K), a multifunctional protein like IE63, and the β subunit of casein kinase 2 (CK2), a protein kinase, and interacting regions were mapped. Confirmation of interactions was provided by fusion protein binding assays, co-immunoprecipitation from infected cells, and CK2 activity assays. hnRNP K co-immunoprecipitated from infected cells with anti-IE63 serum was a more rapidly migrating subfraction than hnRNP K immunoprecipitated by anti-hnRNP K serum. Using anti-IE63 serum, both IE63 and hnRNP K were phosphorylated in vitro by CK2, while in immunoprecipitates using anti-hnRNP K serum, IE63 but not hnRNP K was phosphorylated by CK2. These data provide important new insights into how this key viral regulatory protein exerts its functions.

The involvement of herpesviruses in a range of prominent medical or veterinary diseases makes them one of the most important virus families. Herpes simplex virus type 1 (HSV-1), a common and effective human pathogen, is capable of establishing both lytic and latent infectious life cycles, and up to 80% of adults in the developed world are seropositive for this virus. HSV-1 is a nuclear replicating virus with a large double-stranded DNA genome that encodes some 80 gene products (1). During lytic infection, virus genes are expressed in a temporal cascade and are categorized as immediate early (IE), early (β), or late (γ) based on the time postinfection of their expression (2). The five IE gene products, which do not require prior viral protein synthesis for their expression, regulate early and late gene expression and subvert the host cytotoxic T-lymphocyte response (3, 4). A key IE protein is the 63-kDa IE phosphoprotein IE63 also called ICP27 (5). IE63 is one of two HSV IE proteins essential for lytic virus replication (6) and is the only regulatory gene with a homologue in every herpesvirus of mammals and birds sequenced so far (7), suggesting that aspects of its regulatory role are maintained throughout the herpesvirus family.

Studies of IE63 have shown that its expression is required for the switch from early to late virus gene expression (8) and have highlighted the multifunctional nature of this protein that acts both at the transcriptional and post-transcriptional levels (reviewed in Ref. 9). Acting post-transcriptionally, IE63 binds RNA in vivo with a reported specificity for intronless viral transcripts (10), enhances pre-mRNA 3′ processing (11), inhibits splicing of viral and cellular transcripts (12), causes the nuclear retention of intron-containing viral transcripts (13), and co-localizes with nuclear antigens such as small nuclear ribonucleoproteins (14). IE63 is capable of shuttling from the nucleus to the cytoplasm (15, 16) and may facilitate the nuclear export of intronless RNAs, which form the vast majority of viral transcripts (10). IE63 and the other essential HSV-1 IE protein, IE175, a transcriptional transactivator of early and late virus genes, colocalize within HSV-1 replication compartments (17), and IE63 affects the post-translational modification (18, 19) and in vivo function of IE175 (20), supporting an involvement in transcription.

Domains within IE63 (Fig. 1) include an N-terminal acidic region essential for lytic replication (21), an N-terminal nuclear/nucleolar localization signal (22), a methylated internal RGG box required for RNA binding (23), C-terminal transactivator and transrepressor regions required for IE63 co-immunoprecipitation with anti-5m serum (24), and a zinc-finger-like region located toward the extreme C terminus (25). IE63 has an N-terminal leucine-rich region, homologous to regions of other proteins known to shuttle from the nucleus such as Rev (26), which promotes its export to the cytoplasm (10). Infection with a virus containing a mutation at the IE63 C terminus also prevents the protein from shuttling (27).

Using the yeast two-hybrid system and in vitro binding assays, we have shown that IE63 interacts with heterogeneous nuclear ribonucleoprotein K (hnRNP K) and with the casein...
kinase 2 (CK2) β subunit and has mapped regions of these proteins responsible for their interaction. Confirmation of the interaction between IE63, hnRNP K, and CK2 in infected cell extracts was obtained by immune precipitation using either anti-IE63 serum or anti-hnRNP K serum and by detection of CK2 activity in these immunoprecipitates. Interestingly, the formation of hnRNP K immunoprecipitated with anti-IE63 serum was a fraction of cellular hnRNP K, and, unlike the hnRNP K precipitated with anti-hnRNP K serum, this fraction and the co-immunoprecipitated IE63 were phosphorylated in vitro by CK2 activity present in the immunoprecipitate.

Like IE63, hnRNP K is a multifunctional protein (reviewed in Refs. 28 and 29) capable of shuttling from the nucleus to the cytoplasm with a possible role in the processing and transport of pre-mRNA (30). hnRNP K has both RNA-binding and DNA-binding properties, interacts with proteins of cellular and viral origin, acts as a transcription regulator, and affects translation. CK2 is a ubiquitous serine/threonine eukaryotic kinase known to phosphorylate, and in some cases to interact with, several proteins to regulate their activity (reviewed in Ref. 31).

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids—**Mouse monoclonal anti-IE63 H1113 (5) was supplied by the Goodwin Institute for Cancer Research. Anti-hnRNP K serum was a rabbit antibody (32) generously supplied by Dr. Karol Bomsztyk (University of Washington) raised against a synthetic peptide representing the C-terminal aa 452–464, conserved in the murine and human hnRNP K (33). GST-IE63 (23) was generously supplied by Dr. Steve Rice. The plasmid pGST-RNPK, encoding GST fused to full-length hnRNP K (34), was a kind gift from Dr. David Levens (National Institutes of Health). GST-CK2α and maltose-binding protein (MBP)-CK2β constructs have been described previously (35, 36). GST-CK2β fusion constructs were made from CK2β DNA (aa 1–150) obtained by polymerase chain reaction amplification of pGEX provided by Dr. Eric Nigg (Swiss Institute for Experimental Cancer Research), and the truncations (aa 1–55; 55–150) were generated using appropriate primers and sequenced to confirm their structures. The CK2 peptide substrate was supplied by Calbiochem. Unless otherwise stated, all chemicals were from Sigma.

**Cells and Viruses—**Baby hamster kidney (BHK C13) cells were grown in BHK 21 medium supplemented with 100 units/ml penicillin, 0.01% streptomycin, 10% newborn calf serum, and 10% tryptose phosphate broth. Stocks of wild type (WT) HSV-1 strain 17* and of the IE63 insertion mutant HSV-1 1-2LacZ (37) were grown as described previously (11).

**Cell Infection, Labeling, and Preparation of Extracts—**90% confluent BHK C13 monolayers (4 × 106 cells) were then infected with HSV-1 ( multiplicities of infection [m.o.i.] of 10 or 27-LacZ virus (multiplicity of 10) or no virus (mock-infected). After 1 h of absorption at 37 °C, cells were labeled with [35S]methionine (30 Ci/ml) for 16 h in Eagle’s medium containing 20% of the normal methionine level and 2% newborn calf serum. Unlabeled cell extracts were similarly prepared. For preparation of extracts, monolayers were washed with PBS, and cells were lysed by suspension in 1 ml of cell extract buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 0.1% Nonidet P-40) containing a protease inhibitor mixture (Roche Molecular Biochemicals); equal amounts of fusion protein were used as judged by Coomassie Blue staining. Beads were washed in binding buffer, and bound proteins eluted with 50 mM HEPES, 1 mM NaCl, 0.1% Nonidet P-40 with protease inhibitor at 4 °C overnight were separated by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS, transferred to nitrocellulose membranes, and analyzed by Western blotting. Appropriate radioactivity was visualized using a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA).

**Immunoprecipitation—**Beads for immunoprecipitation were prepared by mixing protein A-Sepharose beads with anti-hnRNP K serum or preimmune serum in binding buffer (5 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, and 0.05% Nonidet P-40) at 4 °C for 1 h (1 μl of serum/10 μl of beads) as described (38). Beads then were mixed with Western Blotting—Nitrocellulose membranes were blocked using PBS with 5% (w/v) dried milk and then washed in PBST. Primary antibody was added at dilutions of 1:2000 (anti-IE63) or 1:10000 (anti-hnRNP K) in PBST for 1 h at 20 °C. After washing, secondary antibody (either anti-mouse-horseradish peroxidase conjugate or protein A-horseradish peroxidase conjugate) was added (diluted 1:1000) in PBST for 1 h at 20 °C. Membranes were washed and developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

**CK2 Activity Assays—**Immunoprecipitates were suspended in 30 μl of CK2 reaction buffer (50 mM Tris, pH 7.2, 20 mM MgCl2) containing 10 μCi of [γ-32P]ATP per reaction, either with or without 0.1 mM peptide substrate (Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu) (39). Reactions were for 30 min at 25 °C. After brief centrifugation, the supernatant was applied to a SpinZyme column (Pierce), which was washed three times with 75 μl phosphoric acid, and phosphorylated peptide bound to the column was detected by direct scintillation counting.

**Phosphorylation Assays—**Immunocomplexes were washed with 50 μl of PBS, pH 7.4, and then suspended in 20 μl of 25 mM Tris, pH 7.4, and to this, 5 μl radioactive solution (50 mM Tris, pH 7.4, 20 mM MgCl2, 10 μM ATP, 2.5 μCi of [γ-32P]ATP) was added. The reactions were for 15 min at 25 °C either in the presence or absence of 50 μM 5,6-dichloro-1-β-D-ribofuranoxylosylbenzimidazolide (DRB), an inhibitor of CK2 activity that acts in vitro and in vivo (40, 41). Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis or PhosphorImager analysis.

**Yeast Two-hybrid Strains and Scaffolds—**The IE63 bait constructs 502CBD (aa 10–512), 440CBD (aa 72–512), 270CBD (aa 242–512), and N397BD (aa 10–397) were made by polymerase chain reaction amplification and fused in frame to the GAL4 DNA-binding domain (DNA-BD) in the cloning vector pAS2−1, which encodes the TRP1 gene and the LEU2 gene (CLONTECH); structures of the truncations were confirmed by DNA sequencing. The target constructs consisted of a human HeLa cDNA library (CLONTECH) fused in frame to the GAL4 activation domain (AD) sequences of the cloning vector pGADGH, which encodes the LEU2 gene (CLONTECH). The yeast (Saccharomyces cerevisiae) strains used were Y187 and CG1945 (CLONTECH).

**Yeast Two-hybrid Screen—**The yeast strain CG1945, which has a trp-, leu-, his- phenotype and contains the two reporter genes his3 and lacZ was simultaneously transformed with the IE63 bait plasmid 502CBD and the target plasmids encoding the HeLa cDNA library (CLONTECH) fused to the AD using a basic lithium acetate protocol (42). Colonies that had a trp-, leu-, his- phenotype were selected and assayed for β-galactosidase activity as described in the Matchmaker System 2 manual (CLONTECH). Candidate colonies with the his- and lacZ- phenotype were treated with cycloheximide (1.0 μg/ml) to eliminate the IE63 bait plasmid while retaining the target library AD plasmid and then mated with strain Y187 transformed with either the bait or a series of control plasmids as described for Matchmaker System 2. The resulting diploids were tested for histidine expression and β-galactosidase activity to determine the specificity of the interactions. The candidate library plasmids were isolated from yeast and transformed by calcium chloride/heat shock into E. coli DH5α cells. Plasmid DNA was isolated using alkaline lysis/PEG precipitation, and DNA inserts of around 500 bp were sequenced using a 17-mer primer (CLONTECH) by cycle sequencing using the ABI Prism BigDye Terminator Sequencing Ready Reaction Kit (Applied Biosystems) with an ABI Prism automated
**RESULTS**

**IE63 Is Capable of Self-interaction**—The domain structure of IE63 is represented in Fig. 1 together with details of the truncations used to identify the regions required for interaction of IE63 with itself and other proteins. Initially, we tested the ability of IE63 itself to activate gene expression in the yeast two-hybrid assay. When full-length IE63 fused to the GAL4 DNA-BD was used as bait, the inclusion of amino acids (aa) 1–9 caused IE63 alone to transactivate the his3 and lacZ reporter genes. Thereafter, IE63 502CBD (aa 10–512) or its truncations were used as bait. None of these derivatives alone when linked to GAL4 DNA-BD activated gene expression in yeast. To examine the ability of IE63 to interact with itself, IE63 502CBD was screened against the IE63 truncations fused to the GAL4 AD. The activation of his3 and lacZ reporter genes relative to the respective positive (pVA3–1/pTD-1) and negative (pTD-1/pLAM59) controls are shown in Fig. 1A. The results show that sequences within aa 397–512, which contains the C-terminal putative zinc finger, were required for IE63 to interact with itself and that sequences within aa 10–72 aided this interaction; there is a report showing that the zinc finger like region is required for IE63 self-interaction (45). The ability of IE63 to interact with itself was further studied using a GST-IE63 fusion protein bound to glutathione beads. A GST pull-down experiment followed by Western blotting (Fig. 1B) showed that IE63 from infected cells interacted with GST-IE63 but not with GST alone.

**hnRNPK Interacts with IE63 Using the Yeast Two-hybrid Screen: Mapping the Regions of IE63 and K Involved**—To identify cellular proteins capable of interacting with IE63 in the yeast two-hybrid assay, we screened a HeLa cDNA library fused to the GAL4 AD expressed in pGADGH, and 82 clones were used as bait. None of these derivatives alone when linked to GAL4 DNA-BD activated gene expression in yeast. To examine the ability of IE63 to interact with itself, IE63 502CBD was screened against the IE63 truncations fused to the GAL4 AD. The activation of his3 and lacZ reporter genes relative to the respective positive (pVA3–1/pTD-1) and negative (pTD-1/pLAM59) controls are shown in Fig. 1A. The results show that sequences within aa 397–512, which contains the C-terminal putative zinc finger, were required for IE63 to interact with itself and that sequences within aa 10–72 aided this interaction; there is a report showing that the zinc finger like region is required for IE63 self-interaction (45). The ability of IE63 to interact with itself was further studied using a GST-IE63 fusion protein bound to glutathione beads. A GST pull-down experiment followed by Western blotting (Fig. 1B) showed that IE63 from infected cells interacted with GST-IE63 but not with GST alone.

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vollving IE63 with these cellular proteins. Interactions involving other cellular proteins identified in the yeast two-hybrid screen will be described elsewhere. To map the regions of IE63 required for interaction with hnRNP K, the series of IE63 truncations, expressed as hybrids with the GAL4 DNA-BD, was mated into cells transformed with the hnRNP K clone identified from the library screen. The results (Fig. 2A) show that sequences within aa 242–397 were sufficient for the interaction and indicate that sequences within aa 166–242 also contribute to the interaction.

To map the regions of hnRNP K required for interaction with IE63, a series of hnRNP K truncations (44) was expressed as hybrids with the GAL4 AD. These were mated into cells transformed with IE63 aa 10–512 fused to the GAL4 DNA-BD. The results (Fig. 2B) indicate that sequences within aa 250–276 and aa 327–463 are required for the interaction. The interaction of IE63 and hnRNP K was confirmed by in vitro binding assays using a GST-hnRNP K fusion protein bound to glutathione beads. A GST pull-down experiment followed by Western blotting (Fig. 3A, lane 1), showed that IE63 interacted with GST-K (lane 4) but not with GST alone (lane 3). Fig. 3C, lane 2, shows the 35S-labeling pattern of proteins from WT-infected BHK cells that bound to GST-hnRNP K and reveals labeled bands of appropriate size for IE63 and hnRNP K, present due to its ability to interact with itself, together with other labeled bands that are under investigation.

In Vivo Co-immunoprecipitation of IE63 and hnRNP K: IE63 Interacts with a Subfraction of hnRNP K—Extracts of BHK cells infected with HSV-1 WT, the IE63 mutant 27-LacZ, or mock-infected were subjected to immunoprecipitation by anti-IE63 or anti-hnRNP K sera. The immunoprecipitated proteins, separated by SDS-PAGE, were transferred to nitrocellulose membranes and analyzed by Western blotting using antisera directed against hnRNP K or IE63. Anti-hnRNP K antibodies precipitated hnRNP K from all three extracts (Fig. 4A, lanes 1–3) and IE63 from the WT-infected extract only (Fig. 4B, lane 1); the preimmune serum did not immunoprecipitate hnRNP K or IE63 (Fig. 4A, lane 4, and B, lane 4). The broad (50-kDa) contaminating bands are due to the heavy chain of the antibody used to immunoprecipitate. The addition of RNase A to immu-
noprecipitates had no effect on co-immunoprecipitation of IE63 and hnRNP K (data not shown).

The predominant form of hnRNP K that was co-immunopre-

FIG. 4. In vivo co-immunoprecipitation of IE63 and hnRNP K using antibodies directed against IE63 or hnRNK K identifies a faster migrating form of hnRNP K with anti-IE63 serum. HSV-1-infected, 27-LacZ-infected, or mock-infected BHK cell extracts were immunoprecipitated with anti-IE63 serum or anti-hnRNP K serum. The precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by Western blotting using hnRNP K or IE63 antisera. A, immunoprecipitates by anti-hnRNP K serum blotted with hnRNP K antibody. B, immunoprecipitates by anti-hnRNP K serum blotted with IE63 antibody. C, immunoprecipitation of WT-infected extracts by anti-IE63 serum followed by Western blotting with anti-hnRNP serum detected faster migrating forms of hnRNP K (lane 1) than the hnRNP K band detected following immunoprecipitation with anti-hnRNP K serum (lane 4). D, [35S]methionine-labeled profile of the Western blot shown in Fig. 4C revealed labeled bands corresponding to IE63 and hnRNP K. A faster moving band (lane 1) immunoprecipitated by anti-IE63 serum reacted with anti-hnRNP K serum as compared with a more slowly migrating band (lane 4), which also reacted with anti-hnRNP K serum following immunoprecipitation by anti-hnRNP K serum. 

FIG. 5. The IE63 regions involved in interaction with the CK2β subunit. A, interaction of various IE63 truncations with CK2β in the yeast two-hybrid assay. B, regions of the CK2β subunit required for interaction with IE63. GST and MBP fusion proteins of CK2β and different truncations were expressed in E. coli BL21. The proteins were coupled to glutathione-Sepharose 4B or amylose beads and mixed with infected cell extracts. Bound proteins were eluted, separated by SDS-PAGE, and Western blotted using anti-IE63 serum. C, Coomassie Blue staining of the GST-CK2α and β- and MBP-CK2β proteins used. D, the CK2β structure is shown together with the regions involved in the interaction with IE63.
versions of the CK2β subunit. The expressed fusion proteins bound to beads were mixed with a [35S]methionine-labeled WT-infected cell extract, and bound proteins were analyzed by Western blotting using anti-IE63 serum. The results (Fig. 5B) demonstrate that sequences within aa 150–182 of CK2β are required for the interaction with IE63. An interaction between IE63 and a GST-CK2α fusion protein was also detected (Fig. 5B, lane 2), although this is likely to be via CK2β present in the extract, since no interacting CK2α clones were identified in the library screen using IE63 as bait.

**CK2 Activity Present following Co-immunoprecipitation of IE63 and hnRNP K Can Phosphorylate IE63 and hnRNP K in Vitro**—CK2 activity assays were performed on the immunoprecipitates generated by anti-IE63 or anti-hnRNP K sera. Results show that high levels of CK2 activity were specific to precipitates generated using anti-IE63 or anti-hnRNP K sera. Treatment with DRB made little difference to the phosphorylation of IE63 by at least 70% (Fig. 7A, compare lanes 1 and 5). By contrast, using immunoprecipitates of WT-infected cells obtained with anti-hnRNP K serum, the addition of DRB made little difference to the phosphorylation of hnRNP K but consistently reduced the phosphorylation of IE63 by at least 70% (Fig. 7A, compare lanes 1 and 5). Western blotting showed that similar amounts of IE63 and hnRNP K were present in DRB-treated and -untreated samples. These in vitro phosphorylation data further demonstrate a difference between the fraction of hnRNP K immunoprecipitated with anti-hnRNP K serum compared with the hnRNP K fraction immunoprecipitated with anti-IE63 serum.

**DISCUSSION**

Like IE63, hnRNP K affects transcriptional and post-transcriptional processes, is capable of self-interaction, shuttling from the nucleus to the cytoplasm, and is present in multiprotein complexes. Other multifunctional proteins include the Y-box proteins, the Wilms’ tumor gene product, TF II A, and La protein (46). A proposal is that hnRNP K could create a docking platform, regulated by nucleic acid to facilitate communication among molecules involved in gene expression and signal transduction (28). There are structural similarities between IE63 and hnRNP K; both have acidic N termini required for function, possess methylated RGG boxes, and have C-terminal regions that facilitate protein/protein interactions. A feature of hnRNP K is the presence of repeated KH regions that are required for

**FIG. 6.** CK2 activity present in immunoprecipitates of WT-infected cells generated by anti-IE63 or anti-hnRNP K sera. A, CK2 activities present in immunoprecipitates generated by anti-IE63 serum of HSV-1 infected, 27-LacZ-infected or mock-infected cell extracts. Assays were performed in the absence (lanes 1–3) or presence (lanes 4–7) of the CK2 peptide substrate and in the presence of the CK2 inhibitor DRB (lane 7). B, CK2 activities present in immunoprecipitates generated by anti-hnRNP K serum or preimmune serum (lanes 2 and 8) in the absence (lanes 1–4) or presence (lanes 5–8) of the CK2 peptide substrate.

**FIG. 7.** The CK2 inhibitor DRB has different effects on phosphorylation activities of immunoprecipitates obtained with anti-IE63 or anti-hnRNP K antisera. A, effects of DRB on in vitro phosphorylation using immunoprecipitates generated by anti-hnRNP K serum. Treatment had little effect on the phosphorylation of hnRNP K but decreased the phosphorylation of IE63 by more than 70% (compare lanes 1 and 5). B, Western blotting to show the relative amounts of hnRNP K present in the DRB-treated samples above. The relative amounts of hnRNP K in untreated samples are as shown in Fig. 4A. C, effects of DRB treatment on in vitro phosphorylation using immunoprecipitates generated by anti-IE63 serum. Treatment reduced the phosphorylation of hnRNP K by some 60%, while phosphorylation of IE63 showed a greater reduction (compare lanes 1 and 2). D, Western blotting to determine the relative amounts of IE63 present in samples assayed in C.

IE63 and hnRNP K to phosphorylate IE63 or hnRNP K in vitro, a series of CK2 phosphorylation activity assays was performed on immunoprecipitates in the presence or absence of DRB. Proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes, and the radiolabeled bands were visualized. Using immunoprecipitates from infected or mock-infected cells, obtained with anti-hnRNP K serum, the addition of DRB made little difference to the phosphorylation of hnRNP K but consistently reduced the phosphorylation of IE63 by at least 70% (Fig. 7A, compare lanes 1 and 5). By contrast, using immunoprecipitates of WT-infected cells obtained with anti-IE63 serum, in vitro phosphorylation of the co-immunoprecipitating hnRNP K band consistently was reduced at least 60% by DRB treatment, with phosphorylation of IE63 showing an even greater reduction (Fig. 7C, compare lanes 1 and 2). Western blotting the extracts showed that similar amounts of IE63 and hnRNP K were present in DRB-treated and -untreated samples (Figs. 4A and 7, B and D). These in vitro phosphorylation data further demonstrate a difference between the fraction of hnRNP K immunoprecipitated with anti-hnRNP K serum compared with the hnRNP K fraction immunoprecipitated with anti-IE63 serum.

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RNA binding activity (47).

The hnRNP K region containing the RGG box and the C-terminal portion, known to bind the transcriptional repressor Zik 1 and the hnRNP K protein kinase, was involved in the interaction with IE63. The Xenopus hnRNP K RGG box was not required for poly(rC) binding (46), although a contribution to RNA binding was considered possible; RNA binding of the fragile X mental retardation gene product required the RGG box and KH domains (48). The IE63 region required for interaction with hnRNP K did not include the RGG box (R1) domain necessary for RNA binding, although adjacent arginine-rich (R2) sequences contributed to the interaction. Point mutations within this relatively large region have been shown to abolish the ability of WT virus to grow (49). It is possible that G + C-rich viral RNA (HSV-1 DNA is 68% G + C overall) could contribute to the interaction of IE63 and hnRNP K, this effect could apply in yeast two-hybrid assays and in co-immunoprecipitations, where RNA:protein binding may confer protection from RNase treatment.

Immune precipitation confirmed the interaction of IE63 and hnRNP K in extracts of infected cells and showed that the hnRNP K that interacted with IE63 was a less processed form or possibly a smaller form. Our preliminary data suggest that the hnRNP K fraction immunoprecipitated with anti-IE63 serum is a hypophosphorylated RNA binding form. IE63 was co-immunoprecipitated by anti-hnRNP K serum perhaps due to interaction with the more rapidly migrating hnRNP K, although less rapidly migrating hnRNP K forms may also have some affinity. Three forms of hnRNP K from human keratinocytes have been characterized (50); a rapidly migrating, non-phosphorylated form bound poly(rC) much more efficiently than two less rapidly migrating, more phosphorylated forms. Primary transcripts of hnRNP K are alternatively spliced to generate four variants that contain or lack two small coding exons, and changes in the relative proportions of variants are associated with alterations in cell proliferation (50); however, the antibody used in this study was directed against a peptide present in all four isoforms. The hnRNP K cDNA clone identified from our HeLa cell library screen contained both alternative exons, and protein expressed from this variant is a minor hnRNP K component of HeLa cells, although this may not be the form that interacts with IE63 in infected cells.

The similarities between IE63 and hnRNP K suggest that they may access common cellular pathways, and IE63 could prevent hnRNP K from accessing these pathways, thereby inhibiting competition. IE63-mediated phosphorylation of hnRNP K by CK2 could prevent the binding of hnRNP K to RNA, affecting transport of cellular RNAs or altering the subcellular localization of hnRNP K, perhaps sequestering it to inactive sites within the nucleus. Alternatively, many of the functions ascribed to IE63 may be due to its interaction with hnRNP K, which could play a key role in HSV-1 infection such as by targeting IE63 to transcriptionally active nuclear domains or facilitating its access to one of hnRNP K's molecular partners such as DNA or another cellular protein. Interestingly, DSEF-1, a member of the hnRNP H family of RNA-binding proteins, has been shown to increase the level of cross-linking of the 64-kDa protein of cleavage stimulation factor (CstF) to polyadenylation substrate RNAs (51). Cross-linking of 64-kDa CstF to poly(A) sites of all temporal classes of viral mRNA is increased during HSV-1 infection, and this binding requires the expression of IE63 (11). The region of IE63 that interacts with hnRNP K confers its ability to activate and repress gene expression, and the region of hnRNP K required for IE63 binding also binds the hepatitis C virus core protein, whose expression has been shown to relieve hnRNP K suppression of the cellular thymidine kinase promoter (44). hnRNP K interacts with TBP (34), and this interaction with IE63 could facilitate viral transcription. Tandem copies of a CT-rich DNA sequence similar to known hnRNP K DNA binding sites are present in an HSV-1 domain that has been proposed to act as a transcriptional regulator of virus immediate early genes (53).

CK2 is a multifunctional, second messenger-independent serine/threonine kinase present in the nucleus and cytoplasm of all eukaryotic cells, which exists as a heterotetramer composed of catalytic subunits (α, α′) and two regulatory (β) subunits. The α subunits are catalytically active, while the β subunit exerts a regulatory function by stimulating catalytic activity of the α subunits (31). Several cellular proteins interact with CK2 with interactions involving α or β subunits (54). Two regions of IE63 were involved in the interaction with CK2β, the C terminus containing the putative zinc-finger and a portion containing the arginine-rich R2 region also involved in interaction with hnRNP K. From interactions with truncated fusion proteins, the CK2β region required for interaction with IE63 mapped to part of the region involved in αβ subunit heterodimerization. As high levels of CK2 activity were detected, the interaction of CK2β with IE63 appeared insufficient to prevent its association with CK2α. Confirmation of the CK2 interaction came from immunoprecipitates generated by both anti-IE63 sera and anti-hnRNP K sera in which, specific to WT-infected cell extracts, CK2 activity was readily detectable in vitro. CK2α was present with CK2β in the complex involving IE63 and hnRNP K as the GST-CK2α fusion protein pulled down IE63 from infected cell extracts, most likely due to its interaction with the β subunit. The interaction of IE63 with CK2 is consistent with reports that show that IE63 affects the post-translational modification of IE175 and that more highly phosphorylated forms of the UL 70-kDa protein and an 80-kDa protein are present in extracts of WT-infected cells than in cells infected with 27-LacZ (24).

IE63 contains several consensus sites for phosphorylation by CK2 and other cellular kinases, and two serine residues located in the N-terminal portion have been shown to serve as targets for CK2 phosphorylation in vivo (55). We show here that IE63 is capable of being phosphorylated in vitro by co-immunoprecipitated CK2 activity; thus, CK2 could modify IE63 activity or modify partner proteins in the complex. CK2 activity has been reported in preparations of purified HSV-1 virions (56), a CK2 phosphorylation site in the VP16 structural protein facilitates assembly of a multicomponent transcription complex to induce IE gene expression (57), and several other HSV-1 proteins are phosphorylated by CK2 (58). Added CK2 was able to nucleotidylate ICP22 (59), one of four HSV-1 IE proteins (including IE63) shown to be guanylylated and adenylylated (60). By contrast, phosphorylation of the hnRNP K fraction immunoprecipitated with anti-hnRNP K serum was not inhibited by the CK2 inhibitor DRB, which is consistent with reports that show that, while added CK2 is capable of phosphorylating hnRNP K in vitro, the hnRNP K protein kinase activity that normally co-immunoprecipitates with hnRNP K is not CK2 (32). hnRNP K protein kinase may therefore be present in the immunoprecipitates obtained using anti-hnRNP K serum. hnRNP K protein is phosphorylated in vivo by inducible kinases, one of which may be protein kinase Cα, and it is suggested that the ability of this kinase to bind and phosphorylate hnRNP K may alter its activities and those of its interacting partners (61). Strikingly, phosphorylation of the hnRNP K fraction immunoprecipitated by anti-IE63 serum from WT-infected cells was capable of being partially inhibited by DRB, further distinguishing between the fractions of hnRNP K immunoprecipitated with anti-IE63 serum or anti-hnRNP K se-
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rum, and the presence of IE63 was required for CK2 activity to phosphorylate co-precipitating hnRNP K. Interactions involving hnRNP K and CK2 have been described previously; CK2β interacts with the nuclear protein Nopp 140 (52), which in synergy with CCAAT/enhancer-binding protein β causes transcriptional activation with hnRNP K (interacting with CCAAT/enhancer-binding protein β) repressing this activation (62).

These data provide firm evidence for the interaction of IE63 with a cellular nuclear shutting protein and a cellular protein kinase and allow important new insights into how this key herpesvirus protein exerts its various activities. Future studies will be directed at determining the functional significance for the virus and cell of these interactions and the role played by CK2 in modulating the activities of these proteins.

Acknowledgments—We thank Dr Karol Bomsztynk for providing anti-hnRNP K serum, Dr. David Levens for supplying the GST-hnRNP K fusion protein plasmid, and Dr. Steve Rice for the GST-IE63 plasmid. We are grateful to Dr. Alasdair MacLean and Dr. John McLauchlan for comments on the manuscript.

REFERENCES
23. O'Reilly, D., Hanesombe, O., and O'Hare, P. (1997) EMBO J. 16, 2420–2430
The Multifunctional Herpes Simplex Virus IE63 Protein Interacts with Heterogeneous Ribonucleoprotein K and with Casein Kinase 2

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doi: 10.1074/jbc.274.41.28991

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