**Human papillomavirus type 1 (HPV1) E1^E4 protein is a potent inhibitor of the serine-arginine (SR) protein kinase SRPK1 and inhibits phosphorylation of host SR proteins and of the viral transcription and replication regulator E2**

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**ABSTRACT**

The serine-arginine-specific protein kinase SRPK1 is a common binding partner of the E1^E4 protein.of diverse human papillomavirus types. Here, we show for the first time, that the interaction between HPV1 E1^E4 and SRPK1 leads to potent inhibition of SRPK1 phosphorylation of host SR proteins that have critical roles in mRNA metabolism, including pre-mRNA processing, mRNA export and translation. Furthermore, we show that SRPK1 phosphorylates serine residues of SR/RS dipeptides in the hinge region of the HPV1 E2 protein in *in vitro* kinase assays and HPV1 E1^E4 inhibits this phosphorylation. Following mutation of the putative phosphoacceptor serine residues, the localization of the E2 protein was altered in primary human keratinocytes; with a significant increase in the cell population showing intense E2 staining of the nucleolus. A similar effect was observed following co-expression of E2 and E1^E4 that is competent for inhibition of SRPK1 activity, suggesting that the nuclear localization of E2 is sensitive to E1^E4-mediated SRPK1 inhibition. Collectively, these data suggest that E1^E4 mediated inhibition of SRPK1 could affect the functions of host SR proteins and those of the virus transcription/replication regulator E2. We speculate that the novel E4 function identified here is involved in the regulation of E2 and SR protein function in posttranscriptional processing of viral transcripts.

**IMPORTANCE**

The HPV life cycle is tightly linked to the epithelial terminal differentiation programme, with the virion-producing phase restricted to differentiating cells. While the most abundant HPV protein expressed in this phase is the E4 protein, we do not fully understand the role of this protein. Few E4 interaction partners have been identified, but we had previously shown that E4 proteins from diverse papillomaviruses interact with the serine-arginine-specific protein kinase SRPK1, a kinase important in the replication cycles of a diverse range of DNA and RNA viruses. Here, we show that HPV1 E4 is a potent inhibitor of this host cell kinase. We show that E4 inhibits SRPK1 phosphorylation, not only of cellular SR proteins involved in regulating alternative splicing of RNA, but also the viral transcription/replication regulator E2. Our findings reveal a potential E4 function in regulation of viral late gene expression through inhibition of a host cell kinase.

**INTRODUCTION**

Human papillomaviruses cause hyperproliferative warts and papillomas of the squamous epithelium at different body sites. Infection of the anogenital tract and oropharynx can lead to benign and malignant disease. There are thirteen HPV types defined as causative agents of cancers at these sites, the most common being HPV16 and HPV18. Of those viruses that infect cutaneous surfaces, some such as human papillomavirus type 1 (HPV1), cause only benign warts, whilst infection with others, such as HPV5 and HPV8 can, in immune-compromised individuals, cause the formation of lesions that are at risk of malignant conversion.

Despite the heterogeneity in pathogenesis, HPVs show a high degree of conservation in their infectious cycles ([1](#_ENREF_1)). The virion-producing phase of the infectious cycle – viral DNA amplification, capsid protein expression and the assembly of new progeny - occurs in suprabasal keratinocytes. While high levels of the viral protein E4 are expressed during this stage of the life cycle, E4’s role is an enigmatic one. Genetic knockdown of E4 expression in papillomavirus life cycle models can result in aberrations in the productive cycle, including reduced viral DNA amplification and decreased viral late gene expression ([2-5](#_ENREF_2)). These studies indicated that E4 function(s) are important at multiple stages of virion production, although there may be differences in the role of E4 between genotypes ([6](#_ENREF_6)). Overexpression of E4 proteins leads to a G2/M arrest of the cell cycle, a function common to HPV types with different epithelial tropisms ([7-9](#_ENREF_7)). However, abrogation of this function in an HPV18 replication model did not affect viral genome amplification or expression of viral late genes ([10](#_ENREF_10)). Reorganization of the keratin networks is another function that is conserved between virus types and while it is predicted to compromise the structural integrity of the superficial cells to aid release of newly synthesized virus, confirmation of this role has yet to obtained ([11-13](#_ENREF_11)).

During HPV infection, E4 is first synthesized as an E1^E4 fusion protein from spliced E1^E4 transcripts, such that the first few amino acids of the E4 protein are derived from the N-terminus of E1 ([14](#_ENREF_14)). The number of E4 species expressed in the virus life cycle however is expanded by phosphorylation and proteolysis ([15](#_ENREF_15), [16](#_ENREF_16)). Posttranslational modification of E4 likely serves to regulate E4 function during the different late stages of the infectious cycle ([9](#_ENREF_9), [17-20](#_ENREF_17)). As well as E4 being a substrate for a range of different protein kinases, the primarily cytoplasmic protein can also interfere with the cellular distribution of some kinases. Several studies have shown, in cells grown in monolayer cell culture, that the E4 proteins of HPV16 and HPV18 sequester the cyclin dependent kinases (CDK) 1 and 2 in complex with their cyclin partners, to cytoplasmic E4-cytokeratin structures ([10](#_ENREF_10), [21](#_ENREF_21), [22](#_ENREF_22)). The retention of the active CDK2-cyclin A and CDK1-cyclin B complexes in the cytoplasm has been linked to the G2-M cell cycle arrest function of E4 ([10](#_ENREF_10), [21](#_ENREF_21), [22](#_ENREF_22)). The serine-arginine specific kinase SRPK1 is a binding partner of the E4 proteins of HPV1, 16 and 18 ([23](#_ENREF_23)). Overexpression of HPV1 E4 in cultured keratinocytes induces the sequestration of SRPK1 to cytoplasmic E4 inclusion bodies. That the cellular kinase is also present in E4 inclusions formed in the upper layers of HPV1 induced palmar warts, is a strong indication that the association is of physiological relevance ([23](#_ENREF_23)).

SRPK1 is one of a family of serine-threonine kinases (SRPK1a, SRPK1-3), which specifically phosphorylate serine residues in serine-arginine/arginine-serine (SR/RS) dipeptide motifs ([24](#_ENREF_24), [25](#_ENREF_25)). The most studied substrates of these kinases have been the RNA-binding serine-arginine (SR)-rich proteins involved in multiple stages of mRNA maturation, including constitutive and alternative splicing, mRNA transport from the nucleus and mRNA translation. SRPKs have a predominantly cytoplasmic localisation, where they phosphorylate SR proteins and facilitate their nuclear import ([26](#_ENREF_26)). The kinases can translocate into the nucleus following stress, or other signals, and during the cell cycle ([25](#_ENREF_25), [27](#_ENREF_27), [28](#_ENREF_28)). Here they are known to target substrates found in nuclear speckles causing their release and redistribution to the cytoplasm ([29](#_ENREF_29)). They have also been shown to associate with small nuclear ribonucleoproteins involved in spliceosome assembly ([30](#_ENREF_30)). Furthermore, SRPKs have also been shown to phosphorylate the RS domains present in proteins that are not directly linked to pre-mRNA metabolism suggesting that the kinases have diverse functions in the cell ([25](#_ENREF_25)).

Notably, SRPKs have been shown to be essential factors in the replication cycle of several DNA and RNA viruses. For example, the ICP27 protein of herpes simplex virus type 1 associates with SRPK1 to induce hypophosphorylation of SR proteins, leading to impairment in spliceosome assembly and inhibition of splicing ([31](#_ENREF_31)). SRPK1 and SRPK2 have been shown to phosphorylate the core protein of hepatitis B virus (HBV) ([32](#_ENREF_32)) and this phosphorylation is required for multiple steps in HBV DNA synthesis ([33](#_ENREF_33)). Interestingly, HBV hijacks and repurposes SRPKs to perform as chaperones to facilitate HBV genome packaging ([34](#_ENREF_34), [35](#_ENREF_35)), demonstrating that SRPKs have multiple roles in HBV replication. In fact, inhibition of SRPK activity using the selective isonicotinamide compounds (e.g. SRPIN340) leads to the suppression of replication of various RNA viruses, including hepatitis C and human immunodeficiency virus ([36](#_ENREF_36), [37](#_ENREF_37)).

In this study, we have investigated the effect of E4 on SRPK1 kinase activity and shown for the first time that HPV1 E4 is a potent inhibitor of SRPK1 phosphorylation of host SR proteins. Furthermore, we show that SRPK1 phosphorylates SR/RS dipeptides in the hinge region of the transcription/replication regulator E2 and that E4 can inhibit this phosphorylation. We provide evidence to suggest that E4 regulates the sub-nuclear distribution of E2 by inhibiting SRPK1 phosphorylation of the hinge region.

**MATERIALS AND METHODS**

**Cell culture**

The human cervical carcinoma cell line C33a and the non-small lung carcinoma cell line H1299 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) with HEPES modification (Sigma-Aldrich Co.), supplemented with 4 mM glutamine and 10% (v/v) foetal calf serum. Primary human foreskin keratinocytes (HFK) were isolated from neonate foreskins collected with informed written parental consent (ethical approval no. 06/Q1702/45), and maintained in SFM keratinocyte growth media (Invitrogen, Paisley, Scotland, United Kingdom) as previously described ([3](#_ENREF_3)).

**Bacterial expression of recombinant proteins**

The expression of His-SRPK1 and GST tagged proteins of HPV1 E1^E4, Δ44-48, Δ49-53, HPV16 E1^E4 and HPV18 E1^E4 have been described in our previous study ([23](#_ENREF_23)). HPV5 E1^E4 was cloned into the *Eco*RI site of pGEX-2T (GE Healthcare, Little Chalfont, United Kingdom) following amplification of the HPV5b total genome (a gift from Ethel de Villiers, Heidelberg, Germany) with the primer pairs of 5’-GCGCGAATTCATACGGATCCTAATCCTAAAGCTCCACGCCTCCAGGGTC-3’ and 5’-GCGCGAATTCTTACTGGGGGGTCGCGAGCTTCTTCC-3’.

HPV1 E2 and E2 hinge sequences (residues 198 to 323) were PCR amplified from pSG1aE2 (a gift from Saleem Khan, University of Pittsburgh, US) using the following primer pairs: E2 full-length: 5’-GCGCGAATTCATATGGAAAACCTCAGCAGTCGC-3’ and 5’-GCGCGAATTCTTAAGACCCATTAAACTGTCC-3’; E2 hinge region: 5’-GCGCGAATTCATGTTATGTCTTCCACTAGCTCC-3’ and 5’-GCGCGAATTCTTATACACAGACCACGGGTGG-3’. The PCR fragments were cloned into the *Eco*RI site of pGex-2T. The generation of GST-tagged mutant E2 proteins was performed using QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide primer used to generate the S265AS267A mutation was 5’-CCACCTCTGGGAGCTCTGGCTCTTCGCCGTCC-3’, and for S281A, 5’-TCCGGGTGTCCTAGCGGGCGTTGATTCTC-3’ (the nucleotide changes are underlined). The triple mutation, S265AS267AS281A, was generated using the S265AS267A template with the S281A mutagenic primer. Mutations were confirmed by bidirectional DNA sequencing of the complete insert.

SRSF1, SRSF3, SRSF4 and SRSF7 were PCR amplified using the following primer pairs: SRSF1: 5’-GCGCGGATCCATGTCGGGAGGTGGTGTGATTCGTG-3’ and 5’-GCGCGGATCCTTATGTACGAGAGCGAGATCTGCT-3’ (template pEGFP-ASF/SF2, a gift from Bettina Heinrich, University of Erlangen); SRSF3: 5’-GCGCGGATCCATGCATCGTGATTCCTGTCC-3’ and 5’-GCGCGAATTCCTATTTCCTTTCATTTGACCTAG-3’, (template IMAGE clone no. 3049167), SRSF4: 5’ GCGCGGATCCATGCCGCGGGTGTACATCGGC-3’ and 5’-GCGCGAATTCTTAGGACCTTGAGTGGGACC-3’, (template IMAGE clone no. 3619538, SRSF7); 5’-GCGCGGATCCATGTCGCGTTACGGGCGGTAC-3’ and 5’-GCGCGGATCCTCAGTCCATTCTTTCAGGACT-3’, (template IMAGE clone no. 2967417). IMAGE clones were obtained from MRC Geneservice, Cambridge, United Kingdom).

The amplified sequences were cloned into appropriately prepared pGEX-2T and transformed into *E. coli* BL21 CodonPlus® cells (Agilent Technologies, Wokingham, United Kingdom). Overnight cultures were used to inoculate 200 ml of LB and grown at 37°C with shaking. After 2 h, isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) was added to a final concentration of 1 mM and the cultures grown for 4 h at 30 °C. Proteins were purified as described previously ([23](#_ENREF_23)).

***In vitro* and *in vivo* kinase assays**

*In vitro* kinase assays were performed essentially as described previously ([23](#_ENREF_23)), 1 µg of His-SRPK1 added to 1-5 µg of substrate protein (GST-SR proteins). For the inhibition studies, 0.3 - 10 µg of the inhibitory protein (GST and GST-E1^E4 proteins) was added to the reaction. The reaction was incubated at 30°C for 30 min and either stopped by the addition of Laemmli loading buffer (2X) and resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or by the addition of 20% (w/v) trichloroacetic acid (TCA) and 0.2 mg bovine serum albumin. Following centrifugation at 16100 *x g* for 5 min the pellet was washed with 10% (w/v) TCA three times and then re-suspended in acetone and centrifuged at 16100 *x g* for 5 min. The pellet was allowed to air dry and re-suspended in scintillation cocktail (OptiPhase ‘HiSafe’ 3, Perkin Elmer). The amount of phosphate incorporated was determined using a scintillation counter (Packard Tri-Carb liquid scintillation counter) and calculated using the following equations: pmol incorporated = scintillation count/count 1 pmol gives (count 1 pmol gives =1 μCi count/([32P]/specific activity), where 1 μCi count was determined by measuring the scintillation count of 1 μCi of radioisotope. The concentration of radioisotope [32P] and the specific activity of the batch of radioisotope were both determined by using the Perkin Elmer decay calculator (<http://www.perkinelmer.co.uk/tools/RadCalculator>).

For E2-containing *in vitro* kinase assays, 1 g of His-SRPK1 added to 1-5 µg of GST-tagged wild type or mutant E2 proteins (triple, E2-FLS265-267-281A and E2-HS256-267-281A, double, E2-FLS265-267A and E2-HS265-267A, and single, E2-FLS281A and E2-HS281A substitutions). For inhibition analysis, increasing amounts of GST or GST-HPV1 E1^E4 proteins were added. After a 30 min incubation at 30°C, the reaction was stopped and resolved by SDS-PAGE. Quantification of the level of E2 phosphorylation was obtained from the dried gels using a Storm860 PhosphorImager® combined with Image-Quant 5.0 software (GE Healthcare, Chalfont St Giles, UK).

For the *in vivo* kinase assays, H1299 cells were transfected with plasmids encoding Flag-SRPK1, or Flag-MCM7, along with plasmids expressing the wild type HPV1 E1^E4 protein or the SRPK1 binding defective E1^E4 mutant Δ44-48 ([23](#_ENREF_23)) using LipofectamineTM LTX (Invitrogen) according to the manufacturer’s instructions.

Cells were washed with ice-cold saline and lysed in kinase lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton® X-100, 1 mM phenylmethylsuphonyl fluoride, 5 mM dithiothreitol). Following incubation on ice for 30 min the lysate was cleared by centrifugation at 16100 x *g* for 30 min. One milligram of protein lysate was mixed with 1 µg of mouse anti-Flag antibody (M2, Sigma-Aldrich) for 30 min at 4°C, then added to protein G Sepharose and rotated at 4°C for 1 h. Beads were washed twice with kinase lysis buffer, then twice with 50 mM Tris-HCl pH 7.5, 150 mM NaCl. Beads were resuspended in 50 µl of 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 µg GST-SRSF1 added, followed by 5 μCi [γ-32P] ATP (Perkin Elmer) in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl2 and 20 µM ATP. Following 30 min at 30°C, the reaction was stopped by the addition of 50 µl Laemmli loading buffer (2X) and resolved by SDS PAGE.

***In vitro* co-precipitations**

To remove the GST-tag from GST-HPV1 E1^E4, the fusion protein was expressed in 200 ml of LB and isolated on glutathione agarose beads ([23](#_ENREF_23)). The beads were incubated with 5 units of thrombin protease (GE Healthcare) in phosphate–buffered saline (PBS) per 100 μl bed volume of beads, overnight at 30°C. The cleaved protein was loaded onto a pre-equilibrated Benzamidine FF Hitrap column (GE Healthcare) and washed with 50 mM Tris-HCl pH 7.5, 0.5 M NaCl followed by 50 mM Tris-HCl pH 7.5, 1 M NaCl. The protein was collected in the high salt wash and the salt reduced to 200 mM by dialysis. To “pull-downs” containing GST-SRSF1, 10 µg of the cleaved HPV1 E1^E4 was added. Following washing the beads were re-suspended in Laemmli loading buffer (2X), resolved by SDS-PAGE and analysed by Western blotting.

**Generation of HA-tagged wild type and mutant HPV1 E2 proteins**

A cassette containing a multiple cloning region and the sequence of the HA tag was created using the following primers: 5’-AGCTTATGTACCCATACGATGTTCCAGATTACGCTGGATCCCCGGGAATTCC-3’ and 5’-TCGAGGAATTCCCGGGGATCCAGCGTAATCTGGAACATCGTATGGGTACA

TA-3’. The primers were resuspended to 100 μM and 10 μl of each primer was mixed with 2 μl of restriction buffer M (Roche) and heated to 95 °C for 2 min, then cooled slowly to room temperature. The cassette mixture was diluted to 50 nM and ligated into the plasmid pcDNA3 digested with *Hind*III and *Xho*I to form plasmid pcDNA3-HA.

HPV1 E2 sequences were isolated from the pGEX2T-HPV1E2FL plasmid by *Eco*RI digestion and cloned into *Eco*RI-digested pcDNA3-HA. Correct orientation of insert was identified by bidirectional DNA sequencing. Mutant HA-tagged HPV1 E2 proteins were generated by site directed mutagenesis using the mutagenic primers as described for the generation of mutant GST-HPV1 E2 proteins.

**Western blotting and immunofluorescence microscopy**

Resolved proteins were transferred onto Biotrace® NT nitrocellulose membrane (VWR International, Lutterworth, United Kingdom) and blocked in 2% (w/v) dried skimmed milk in PBS. HPV1 E4 was detected using a mouse antibody 4.37 ([38](#_ENREF_38)) was used at a dilution of 1/150 and mouse anti-SRPK1 (Clone 12, BD Transduction Labs) used at a dilution of 1/1000. Epitope tags were detected using a rabbit anti-Flag antibody (Sigma-Aldrich), a mouse anti-His antibody (Sigma-Aldrich), a goat anti-GST antibody (GE Healthcare) and a mouse anti-HA antibody (Covance), all used at a dilution of 1/1000. Secondary antibodies conjugated to horseradish peroxidise were used 1/3000 and were from Sigma-Aldrich (mouse and goat) and DAKO (rabbit). Membranes were developed by enhanced chemiluminescence (AmershamTM ECLTM Western blotting detection reagents, GE Healthcare).

HFK were grown on collagen-coated glass coverslips and transfected with plasmid DNA by using Lipofectamine™ 2000 as described by the manufacturer. After 24 h, cells were fixed in 3.6% formaldehyde (EM-grade, TAAB Laboratories, Aldermaston, Berks, UK) for 20 min and permeabilized using 0.2% Triton X-100 for 5 min.

Cells were stained for E4 using antibody 4.37 ([38](#_ENREF_38)), at a dilution of 1/200. HA-tagged E2 proteins were detected using a rabbit anti-HA antibody used at 1/200 dilution (Abcam, Cambridge, UK). Nucleoli were visualised by using an antibody specific to C23 (Santa Cruz, sc-8031) at 1/100 dilution. Immune complexes were then detected using species - specific Alexa-fluor® (Molecular Probes, Invitrogen) conjugates and nuclei were counterstained with 4’,6-diamino-2-phenylindole. Imaging was performed on a Nikon E600 microscope fitted with epifluorescence detection and images acquired using a DXM1200F digital camera and assembled in Adobe Photoshop® CS5.

Quantification of the different immunofluorescent staining patterns of HPV1 E2 in primary keratinocytes was performed blind by two investigators (CLB and SR). The data was taken from a minimum of three independent experimental repeats.

**RESULTS**

**HPV1 E1^E4 inhibits SRPK1 phosphorylation of cellular SR proteins**

An *in vitro* kinase assay system was used to establish if SRPK1 binding to HPV1 E1^E4 alters the activity of the kinase. The assay contained purified forms of bacterially expressed His-tagged SRPK1 and a GST fusion of the SRPK1 cellular substrate SRSF1. GST or GST-HPV1 E1^E4 proteins were titrated into the assay and after 30 min half of the reaction was resolved by SDS polyacrylamide gel electrophoresis and the dried gel exposed to X-ray film. The level of phosphate incorporated into GST-SRSF1 was quantitated by scintillation counting of trichloroacetic acid (TCA) precipitated proteins isolated from the remaining half of the reaction. GST-SRSF1 was phosphorylated by His-tagged SRPK1 (Fig. 1A, lane a) and the amount of phosphate incorporation into the SR protein did not vary upon addition of increasing amounts of GST protein to the *in vitro* reactions (Fig. 1A). In contrast, in the presence of the GST-HPV1 E1^E4 protein, phosphorylation of GST-SRSF1 decreased in a dose-responsive manner. At the maximum amount of GST-HPV1 E1^E4 added to the assay, the phosphate incorporated into GST-SRSF1 was reduced by 65% compared to an equivalent amount of GST protein (p <0.001) (Fig. 1A).

Previously, we have shown that SRPK1 was also an *in vitro* binding partner of the E1^E4 proteins of *Alphapapillomaviruses* HPV types 16 and 18, ([23](#_ENREF_23)) and of the *Betapapillomavirus* HPV5 ([39](#_ENREF_39)). To determine if these E1^E4 proteins affected SRPK1 activity, GST proteins encoding E1^E4 of HPV5, 16 and 18 were also added to the SRPK1 kinase assay. Here, the level of phosphate incorporation into GST-SRSF1 was not altered significantly by the presence of these E1^E4 proteins when compared to the control reactions (Fig. 1B). Therefore, we conclude that only the type 1 E1^E4 protein is an inhibitor of SRPK1 *in vitro*.

To establish that HPV1 E1^E4 induced inhibition of SRPK1 activity is valid for more than one SRPK1 substrate, GST-HPV1 E1^E4 was added to *in vitro* kinase assays containing GST-tagged fusion proteins of SRSF3, SRSF4 and SRSF7. HPV1 E1^E4 inhibited SRPK1 phosphorylation of all the SR proteins tested (Fig. 2A). While the level of inhibition by GST-HPV1 E1^E4 in comparison to the GST protein alone was significant in all cases, there was a degree of variation in the level of inhibition between substrates; SRSF4 showed the greatest reduction in phosphorylation (86% ±2.3) and SRSF3 the lowest (58% ±0.02) (Fig. 2B).

**An interaction between HPV1 E1^E4 and SRPK1 is necessary for the inhibition of kinase activity**

To confirm that an interaction between HPV1 E1^E4 and SRPK1 is required for inhibition of kinase activity, two E1^E4 deletion mutants were tested in the kinase assay. Deletion of residues 44 to 48 from the HPV1 E1^E4 protein (∆44-48) has been shown previously to disrupt the binding of HPV1 E1^E4 to SRPK1, whereas deletion of the amino acids 49 to 53 (∆49-53) does not abrogate this interaction ([23](#_ENREF_23)). These mutants were bacterially-expressed as GST-fusion proteins and included in the assay with His-SRPK1 and GST-SRSF1. The E1^E4 mutant Δ44-48 did not inhibit the phosphorylation of GST-SRSF1, whereas the mutant competent for SRPK1 binding, Δ49-53, reduced GST-SRSF1 phosphorylation to a level comparable to that of the wild type protein (Fig. 3A).

Whether the interaction between E1^E4 and SRPK1 blocks binding between the kinase and substrate was tested in an *in vitro* binding assay. The association between His-SRPK1 and GST-SRSF1 was reduced in the presence of increasing amounts of recombinant HPV1 E1^E4 protein that had been cleaved from the GST tag (Fig. 3B). Together these findings suggest that inhibition of SRPK kinase activity requires binding between the kinase and the E1^E4 protein and that this interaction is sufficient to block binding of SRPK1 to the substrate. There was no evidence of an association between HPV1 E1^E4 and the SR proteins themselves following pull-down experiments using the different GST-SR proteins and purified HPV1 E1^E4 protein (data not shown).

**HPV1 E1^E4 inhibits SRPK1 activity in cells**

To establish whether HPV1 E1^E4 inhibits the activity of SRPK1 in cells, the Flag-SRPK1 expression plasmid was co-transfected into H1299 cells with plasmids expressing E1^E4 or the deletion mutant Δ44-48 which is unable to bind or inhibit SRPK1. A plasmid expressing Flag-tagged MCM7 (minichromosome maintenance complex component 7), which is known to interact with HPV1 E1^E4 ([40](#_ENREF_40)), was also added to control for non-specific effects. Following transfection, the cells were harvested after 48 h and immunoprecipitations performed on the cell lysates with an anti-Flag antibody. The immunoprecipitated proteins were then added to an *in vitro* kinase assay containing GST-SRSF1 and 32P labelled γ-ATP. Following resolution by SDS-PAGE, 32P-incorporation was quantified by phosphorimaging. A low level of phosphorylation of GST-SRSF1 was observed with Flag-MCM7 immunoprecipitates that was most likely due to non-specific sticking of the kinase to the beads and was therefore considered to be the background level of phosphorylation (Fig. 4). In the presence of the HPV1 E1^E4 protein, the immunoprecipitated Flag-SRPK1 showed a significant (p < 0.05) reduction in phosphorylation of GST-SRSF1 compared to immunoprecipitates from cells transfected with the deletion mutant Δ44-48 (Fig. 4).

When taken together, the data given in Figures 1 to 4 indicate that HPV1 E1^E4 is a potent inhibitor of the protein kinase SRPK1, both *in vitro* and *in vivo*.

### HPV1 E1^E4 inhibits SRPK1 phosphorylation of the HPV1 E2 protein

Several unrelated viruses encode proteins with similar characteristics to cellular SR proteins ([41](#_ENREF_41)). Most notable are E2 proteins of HPV types within the *Betapapillomavirus* genus (<http://pave.niaid.nih.gov>); the hinge regions, separating the amino-terminal transactivation and carboxy-terminal DNA binding and dimerization domains, contain multiple RS/SR repeats. For example, the HPV5 E2 hinge region contains 27 RS/SR motifs. The HPV5 E2 protein has been shown to be a substrate for SRPK1 *in vitro* and evidence suggests that this phosphorylation is restricted to the hinge region ([42](#_ENREF_42), [43](#_ENREF_43)). Examination of the sequence of the HPV1 E2 hinge region identified four RS/SR dipeptide motifs, with the serines at positions 265, 267, 281 and 306 (Fig. 5A). Therefore, to determine whether HPV1 E2 is a substrate for SRPK1, the HPV1 E2 protein (E2-FL) and the hinge region only (E2-H, residues 198 to 323) were expressed as GST fusion proteins and included in an *in vitro* kinase assay containing His-SRPK1. Both E2-FL and E2-H were phosphorylated by His-SRPK1 (Fig. 5 B and C).

To establish whether the serine residues within the RS/SR dipeptides are SRPK1 phospho-acceptors, GST-tagged mutant full-length and hinge region E2 proteins containing alanine replacement of three out of the four serine residues (triple (T), S265-267-281A) were tested in the SRPK1 phosphorylation assay. SRPK1 mediated phosphorylation of the mutant full-length protein was markedly reduced in comparison to the wild type E2 protein (by > 80%, Fig. 5B). A similar level of reduction in phosphorylation occurred upon inclusion of a double mutant (D, S265-267A) in the *in vitro* assay, whilst substitution of serine 281 alone (S, S281A) had a less dramatic effect on SRPK1 phosphorylation (Fig. 5 B, > 50%). These data suggest that one or both of the serine residues at 265 and 267 are major SRPK1 phospho-acceptors and serine 281 is not a significant target site of the kinase acceptors. These mutations when expressed in the hinge region alone produced a similar profile of SRPK1 phosphorylation (Fig. 5C). Since mutation of serine 265 and 267 was sufficient to block SRPK1 phosphorylation, the serine in the fourth RS motif (306-307) was judged not to be a relevant SRPK1 target.

Having established that the HPV1 E2 protein is phosphorylated by SRPK1, we then examined if E1^E4 affects SRPK1 phosphorylation of the E2 protein. Increasing amounts of GST-HPV1 E1^E4 were added to *in vitro* kinase assays containing His-SRPK and GST-E2-FL proteins. The level of SRPK1 mediated phosphorylation of the full-length E2 protein was markedly reduced in the presence of HPV1 E1^E4 in a dose-dependent fashion whereas the addition of increasing amounts of the GST protein alone had no effect on E2 phosphorylation (Fig. 5D).

**Mutation of the SRPK phospho-acceptor residues within the E2 hinge region affects the sub-nuclear localization of the E2 protein**

We next wanted to identify a role for the SRPK1 phospho-acceptor residues in HPV1 E2 function. The hinge region of E2 is thought to be an unstructured flexible hinge between the transactivation and DNA-binding domains, though it is not required for the function of these two domains ([44](#_ENREF_44)). However, it has been shown that E2 hinge sequences influence the nuclear distribution of the protein ([45](#_ENREF_45), [46](#_ENREF_46)) and that phosphorylation of hinge residues can lead to changes in E2 protein turnover ([45](#_ENREF_45), [47](#_ENREF_47), [48](#_ENREF_48)). Therefore, the cellular localization of the HPV1 E2 protein, and the triple and double mutant proteins was examined in primary human foreskin keratinocytes (HFK) by immunofluorescence staining. The wild type HA-tagged E2 protein was primarily restricted to the nucleus, but showed variation in sub-nuclear distribution (Fig. 6A). The patterns of nuclear staining were classified as either diffuse throughout the nucleoplasm (Fig. 6A, i), excluded from the nucleolus (Fig. 6A, ii), or concentrated in the nucleolus (Fig. 6A, iii – vi), with nucleolar staining ranging from faint (Fig. 6A, iii) through to very intense staining (Fig. 6A, vi). Nucleolar localization was confirmed by co-staining with the nucleolar protein C23 (Fig. 6A, right-hand panel). Small E2-positive nuclear foci were also observed in some of the cells (Fig. 6A, v) and a very small subset of cells showed very bright cytoplasmic staining (data not shown). The staining patterns of both the triple and double mutants were similar to wild type protein (Fig. 6A, HA-E2-T and –D, respectively). However, quantification of the different phenotypes formed by the mutant E2 proteins showed that the population of both triple and double mutant E2-positive cells with intense nucleolar-staining significantly increased in comparison to cells expressing the wild-type protein and furthermore, there was a concomitant significant decrease in cells with less intense nucleolar-staining (Fig. 6B, i).

Because the transfection efficiency of the E2 expressing plasmids in HFK was very low (< 5%), it was not possible to determine by western blotting if the mutation of the serine residues in the hinge affected E2 protein turnover. However, we observed little variation in the steady-state expression levels of the mutants compared to the wild type E2 protein following transient expression in C33a keratinocytes, or of the wild type E2 protein following co-transfection with a Flag-tagged SRPK1 expression plasmid (Fig. 6C and data not shown).

The data described above indicated that the serine residues of the RS dipeptide motifs have a role in determining the sub-nuclear localization of E2 and suggest that SRPK1 phosphorylation may regulate the distribution of E2 in the nucleus. However, coexpression of Flag-tagged SRPK1 and full-length HPV1 E2 in HFK did not affect the distribution of the wild type E2 between the different staining phenotypes (data not shown). This observation may reflect an existing high level of endogenous SRPK1 activity in the primary cells and so silencing SRPK1 protein expression using siRNAs transfected into primary cells transiently expressing E2 was also examined. Western blot analysis of total cell lysates confirmed efficient knock-down of SRPK1 protein expression, however, immunofluorescence staining of these cells for E2 and SRPK1 showed that there was no silencing of SRPK1 expression in cells positive for E2 expression (data not shown). We have no evidence that E2 upregulates expression of SRPK1 in primary keratinocytes. All of which leads us to conclude that E2 is able to overcome siRNA-mediated silencing of SRPK1 expression by some unexplained mechanism.

If SRPK1 phosphorylation of the E2 hinge residues is important in the sub-nuclear distribution of the viral protein and E4 can inhibit this activity, then we would expect E4 to affect the distribution of E2. To investigate this, HFK were co-transfected with plasmids expressing E2 and E4 and the different E2 staining phenotypes quantified. Notably, in the presence of E4, the population of wild type E2 positive cells with intense nucleolar staining increased significantly when compared to cells expressing E2 alone (Fig. 6B, ii). However, there was no change in this level of this population in cells co-expressing E2 and the E4 deletion protein ∆44-48 that is unable to bind SRPK1 (Fig. 6B, ii). The distribution of the triple mutant E2 protein is unaffected by co-expression of E4 (Fig. 6B, iii). We have reported previously that HPV1 E4 is present in the nucleoli of SV40-immortalized keratinocytes ([49](#_ENREF_49)); however this was not the case in primary keratinocytes (Fig. 6D). In addition, unlike the effect of HPV16 E4 on E2 ([50](#_ENREF_50)), there was no relocalization of the E2 protein to the cytoplasm in primary cells, even in cells in which E4 had assembled into cytoplasmic inclusions (Fig. 6D). Collectively, these data show that HPV1 E1^E4 protein affects the sub-nuclear distribution of E2 and that this activity is linked to the SRPK1-binding function of E4.

**DISCUSSION**

While our data has shown that SRPK1 is a conserved host target of E1^E4 proteins of HPV types representing diverse genotypes (*Alpha*, *Beta* and *Mu*), only the E1^E4 protein of the *Mupapillomavirus* HPV1 inhibited SRPK1 activity. The interaction between HPV1 E1^E4 and SRPK1, which is mediated by direct binding ([23](#_ENREF_23)), was necessary for the inhibition of SRPK1 phosphorylation of the cellular SR protein SRSF1. Since this association was sufficient to prevent the binding of SRPK1 to SRSF1, it suggests that E1^E4 blocks access to the binding site on the kinase, either by steric hindrance and/or by provoking a change in the conformation of the SRPK1 protein. For those E1^E4 proteins that interact with the kinase, but do not inhibit its activity, at least in the *in vitro* assay used here, they may have a different mode of binding to that of the type 1 protein, or they may require specific post-translational modifications or additional cellular and/or viral factors in order to modulate SRPK1 activity. These differences may be a reflection of the different tropism and biology of the HPV types ([1](#_ENREF_1)).

In addition to SRSF1, HPV1 E1^E4 also inhibited SRPK1 phosphorylation of SRSF3, SRSF4 and SRSF7. The phosphorylation of SRSF4 and SRSF7 was more susceptible to inhibition than the phosphorylation of substrates SRSF1 or SRSF3. These data may reflect limitations of the *in vitro* assay since some of the GST-SR fusion proteins may not have been expressed as full-length proteins. However, it has recently been shown that SRPK1 uses different modes of phosphorylation of SR proteins that is dependent on the lengths and the topography of the RS dipeptide repeats in the RS domain ([51](#_ENREF_51)). Thus, SRPK1 activity towards those SR proteins such as SRSF4 and SRSF7 with shorter RS dipeptide repeats might be more prone to E1^E4 mediated inhibition than when acting upon SRSF1 and SRSF3 which have longer RS repeats. The variation in potency of E1^E4 inhibition of SRPK1 activity on different cellular SR protein substrates may indicate that there is differential regulation of SR protein function in HPV1 infections.

During the HPV life cycle, the expression of the late genes involves tight regulation of alternative splicing and polyadenylation. Cellular SR proteins seem to have a pivotal role in these events and in fact E2 from the high-risk virus HPV16 has been shown to regulate the transcription of some of these factors ([52-55](#_ENREF_52)). Selection of bovine and human papillomavirus late-specific splice sites has been shown to be controlled by SRSF3, with this cellular factor suppressing the activity of a key exonic splicing enhancer in the papillomavirus genomes ([56](#_ENREF_56)). In natural infections and organotypic raft systems of papillomavirus replication, the major capsid protein L1 only occurred in superficial cells containing low levels of the SRSF3 splicing factor ([56](#_ENREF_56)). Multiple exonic enhancers in the HPV16 genome that bind to SRSF1 are also linked to suppression of late gene expression ([55](#_ENREF_55)), and binding of SRSF1 to the negative regulatory element in the viral genome may be necessary to relieve repression of this element on late gene expression ([57](#_ENREF_57)). In contrast, SRSF9 stimulates splicing of the late HPV16 viral transcripts ([54](#_ENREF_54)). In HPV1 infections, the pattern of L1 and L2 expression is highly divergent; L1 is detected in the lower suprabasal cell layers, only a few cell layers after the initiation of E4 expression, while L2 protein appears in the higher, differentiated cell layers ([58](#_ENREF_58), [59](#_ENREF_59)). The analysis of capsid encoding transcripts confirmed that L1-specific transcripts were highly abundant in the lower and middle suprabasal cells but less abundant in the more differentiated cells, where L2-specific transcripts were preferentially expressed ([58](#_ENREF_58)). Our previous study showed that SRPK1 was sequestered to the E4 inclusions formed in the more differentiated cells and not to those present in the lower epithelial cells ([23](#_ENREF_23)). Therefore it is tempting to speculate that in the superficial cell layers, E4 mediated inhibition of SRPK1 activity towards cellular SR proteins leads to an alteration in the production of RNA splice isoforms to one(s) more suitable for L2 protein production.

Binding between SRPK1 and E1^E4 may influence E4 function itself in the upper layers of the infection. Indeed, in our previous study we had shown that the HPV1 E1^E4 protein was a substrate for SRPK1 in the *in vitro* assay ([23](#_ENREF_23)). However, in the experiments reported here, this phosphorylation was not observed. The most likely explanation of this anomaly is that the amounts of SRPK1 and HPV1 E1^E4 used in the assays described here were much lower than those used previously due to the cellular SR proteins being superior substrates for SRPK1 than E1^E4.

We have also shown SRPK1 phosphorylates RS dipeptide motifs in the hinge region of the HPV1 E2 protein and E1^E4 inhibits this phosphorylation. The hinge regions of the *Beta-papillomavirus* E2 proteins are particularly rich in RS/SR dipeptides motifs and they have been shown to mediate interactions between E2 and cellular SR proteins ([60](#_ENREF_60)). Interestingly, the HPV16 E2 protein, whose hinge region does not contain RS dipeptide motifs, also associates with multiple SR proteins ([61](#_ENREF_61)). The interaction with cellular splicing factors may contribute to E2 mediated posttranscriptional regulation of cellular and/or viral gene expression during the virus life cycle ([60-62](#_ENREF_60)). However, in the case of HPV1 E2, we have been unable to identify interactions between SR proteins (HA-tagged forms of SRSF1 – 4, SRSF7) and un-phosphorylated or SRPK1 phosphorylated forms of the E2 protein (MH, ELP, SR, unpublished data).

In this study, mutation of the SRPK1 phospho-acceptor residues in the hinge region of E2 led to an alteration in the sub-nuclear localization of the protein in primary keratinocytes, specifically enhancing localization of E2 to the nucleolus. This suggests that SRPK1 phosphorylation may have a role in regulating the cellular localization of HPV1 E2. Indeed, SRPK1 activity is associated with the release of HPV5 E2 from nuclear speckles and transport to the cytoplasm ([42](#_ENREF_42), [43](#_ENREF_43)). Since E2 stability is sensitive to phosphorylation ([44](#_ENREF_44)), an alternative explanation is that SRPK1 phosphorylation of the E2 hinge regulates the stability of the fraction of E2 protein that is located within the nucleolus. Surprisingly, overexpression of SRPK1 did not affect the distribution of HPV1 E2, perhaps because of high endogenous levels of SRPK1. However, co-expression of HPV1 E4 enhanced E2’s nucleolar-association, but not so following co-expression of an E4 mutant that cannot inhibit SRPK1, suggesting that the cellular localization of E2 is sensitive to E4-mediated SRPK1 inhibition.

The biological significance of the nucleolar localization of HPV1 E2 is unclear. The HPV8 E2 protein locates to the nucleolus following removal of sequences required for localization of the protein to nuclear speckles and the hinge sequences alone are sufficient for localization to this sub-nuclear compartment ([45](#_ENREF_45)). This may indicate an association between HPV8 E2 and nucleolar proteins and/or RNA, or reflect attachment to ribosomal DNA loci, which are sequestered in the nucleolus during interphase; associations that are regulated by phosphorylation of hinge residues ([63](#_ENREF_63)). Interestingly, the chromatin binding sequence present in the hinge region of the *Betapapillomavirus* E2 proteins is highly conserved in the HPV1 E2 hinge region and partially conserved in the other *Mupapillomavirus* HPV63 (Fig. 7). Notably, the sequence most conserved between the *Mu-* and *Beta-papillomaviruses* covers the RS dipeptide repeat (HPV1, 264RSRS267). Phosphorylation of this HPV8 sequence by protein kinase A stabilizes E2 and promotes chromatin binding ([45](#_ENREF_45), [64](#_ENREF_64)). Within the HPV1 sequence a potential PKA recognition motif overlaps with the RS dipeptide repeat (262RRRS265) (Fig. 7). While we have been unable to show that HPV1 E2 is phosphorylated by PKA in cells treated with the activator forskolin and using antibodies that detect PKA phosphorylated substrates (CLB, unpublished data), it is possible that the function of this region in HPV1 is regulated by multiple host kinases, including SRPK1 and that there is differential regulation of this domain by these kinases during the HPV1 life cycle.

The E2 proteins of cutaneous and mucosal HPV types have been shown to induce late gene expression through inhibition of the early polyadenylation site in the papillomavirus genomes ([62](#_ENREF_62)). This function of E2 is unlikely to be relevant during viral genome amplification since the presence of E1 inhibited E2’s induction of late gene expression ([62](#_ENREF_62)). However, since several studies have suggested that there may be a functional relationship between the E4 and E2 proteins ([50](#_ENREF_50), [65](#_ENREF_65)), it is reasonable to speculate that if SRPK1 activity on E2 has a role to play in HPV1 genome replication then E4 inhibition of SRPK1 activity in the upper layers of an infection could switch E2 function to one that induces late gene expression. While the resolution of the biological function of HPV1 E1^E4 inhibition of SRPK1 activity is hampered by the lack of suitable cell-based models of the HPV1 infectious cycle, loss of E1^E4 expression in models of high-risk HPV replication repressed late gene expression ([2-4](#_ENREF_2)).

Collectively, our data suggest that inhibition of SRPK1 by E4 in the HPV1 life cycle could affect the functions of both host cell SR proteins and those of the virus transcription/replication regulator E2. While the work described here has primarily focussed on the type 1 virus that causes highly productive palmar and plantar warts, the interaction between E4 and SRPK1 is conserved between diverse genotypes including high-risk HPV types. Therefore, it will be important in future investigations to establish the role of SRPK1 in high-risk infections since selective inhibition of this kinase using chemical compounds can inhibit the replication of other clinically-relevant viruses ([36](#_ENREF_36), [37](#_ENREF_37), [66](#_ENREF_66)).

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**FIGURE LEGENDS**

**Figure 1 HPV1 E1^E4 inhibits SRPK1 kinase activity *in vitro.*** (**A**) GST-HPV1 E1^E4 was added in increasing amounts (0.15, 0.3, 0.6, 1.2, 2.5 or 5 µg) to an *in vitro* kinase assay containing 1 μg of GST-SRSF1 and 1 μg of His-SRPK1. Similar amounts of GST protein (0.3, 0.6, 1.2, 2.5, 5 or 10 µg) were added to separate reactions. Phosphorylation of GST-SRSF1 (P-GST-SRSF1) was shown by exposure of the Coomassie-stained gel to X-ray film (32P autoradiograph). Lane a, contains only GST-SRSF1 and His-SRPK1; lane b, contains only GST-SRSF1. The level of phosphate incorporation into GST-SRSF1 is shown in the graph as the percentage of phosphorylation normalised to the level of phosphorylation in the absence of GST proteins (lane a). (**B**) GST-tagged HPV1, 5, 16, and 18 E1^E4 proteins were added in increasing amounts (2.5, 5 and 10 µg) to an *in vitro* kinase assay containing 3 μg of GST-SRSF1 and 1 μg of His-SRPK1. Quantification of phosphate incorporation from four independent experiments is given in the bar graph as the mean percentage of phosphorylation (± standard deviation). Statistical significance was determined by using Student’s t-test.

**Figure 2 HPV1 E1^E4 inhibits SRPK1 phosphorylation of multiple SR proteins.** (**A**) Five micrograms of GST (lanes 1-2), GST-SRSF1 (lanes 3-5), GST-SRSF4 (lanes 6-8), GST-SRSF3 (lanes 9-11) and GST-SRSF7 (lanes 12-14) were added to an *in vitro* kinase assay containing His-SRPK1. Five micrograms of GST (lanes 1, 4, 7, 10 and 13) or 5 μg of GST-HPV1 E1^E4 (lanes 2, 5, 8, 11 and 14) were added to the individual kinase assays. Additional control reactions containing the GST-tagged SR proteins alone are shown in lanes 3, 6, 9 and 12. Left hand-side panels, autoradiographs; right hand-side panels, Coomassie-stained gels. (**B**) Quantification of the level of phosphorylation, as determined by phosphorimaging analysis, shown from three independent experiments as the mean percentage of phosphorylation normalised to the level of phosphorylation in the presence of GST protein (± standard deviation). Statistical analysis was determined by using paired Student’s t-test; P values: \* <0.05, \*\* < 0.01, \*\*\* < 0.0005.

**Figure 3** HPV1 E1^E4 inhibitory action is dependent on an interaction with SRPK1 and E1^E4 blocks binding between the kinase and the substrate SRSF1. (**A**) GST-HPV1 E1^E4 protein (WT, 2.5, 5, 10 g) and deletion mutants Δ49-53 (5 g, SRPK1-binding mutant) and Δ44-48 (5 g, non SRPK1-binding mutant), were added in an *in vitro* kinase assay containing GST-SRSF1 and His-SRPK1. Quantification of phosphate incorporation is given in the graph as the percentage of phosphorylation normalised to the level of phosphorylation in the presence of GST protein. Data shown is one of two experimental repeats. (**B**) GST-SRSF1 was bound to glutathione agarose beads and incubated with His-SRPK1 alone or in the presence of increasing amounts of bacterially-expressed HPV1 E1^E4 cleaved from the GST tag. Following resolution of the washed beads by SDS PAGE the gels were blotted with anti-SRPK1 and anti-GST antibodies. The amount of His-SRPK1 co-precipitated with GST-SRSF1 in the presence of HPV1 E1^E4 relative to that bound in the absence of E1^E4 is shown below the blot.

**Figure 4 HPV1 E1^E4 inhibits SRPK1 activity in cells.** A plasmid expressing HPV1 E1^E4 was transfected into cells along with Flag-tagged SRPK1, or Flag-tagged MCM7, or cells were co-transfected with Flag-SRPK1 and the HPV1 E1^E4 deletion mutant ∆44-48 that is unable to bind to SRPK1. The proteins were subject to immunoprecipitation with an anti-Flag antibody and added to an *in vitro* kinase assay with GST-SRSF1 and after SDS-PAGE electrophoresis exposed to X-ray film (top panel). One twentieth of the input lysate was western blotted with anti-Flag and E4 antibodies. The graph shows the normalised data for phosphorylation of GST-SRSF1 as the mean (± standard deviation) from three experimental replicates (\* = p < 0.05, Student’s t-test).

**Figure 5 HPV1 E1^E4 inhibits SRPK1 phosphorylation of RS dipeptides in the hinge region of HPV1 E2.** (**A**) Amino acid sequence of HPV1 E2 hinge region (residues 198 to 323), RS/SR dipeptides are underlined and those serine residues altered to alanines are shown boxed. The triple (T), double (D) and single (S) alanine substitutions are as shown. (**B** and **C**) GST-HPV1 E2 full-length (FL) or GST-HPV1-E2 hinge (H) wild type and mutant proteins were added to *in vitro* kinase reactions containing His-SRPK1. SRSF1 was added as a positive control. Bar graphs, quantification of phosphorylation levels as determined from phosphorimager analysis, shown as the mean (± standard deviation) from three experimental replicates and the Student’s t-test determined significance (\*\*, <0.01; \*\*\*, <0.001) in comparison to the wild type E2 protein (WT). (**D**) Increasing amounts of GST-HPV1 E1^E4 or GST alone were added to *in vitro* kinase assays containing GST-HPV1 E2 FL and His-SRPK1. Data shown is one of two experimental replicates.

**Figure 6 Serine residues of the RS dipeptide motifs have a role in the sub-nuclear localization of the HPV1 E2 protein in primary keratinocytes.** (**A**) Primary human foreskin keratinocytes were transfected with plasmid expressing HA-tagged wild type or mutant HPV1 E2 proteins for 24 h and the various immunofluorescent staining patterns shown (E2, green; nucleus, blue). Right hand-side panel, location to nucleolus was confirmed by co-staining with anti-C23 antibody (red). Scale bar, 10 m. (**B**) Quantification of the patterns of E2 staining as “nuclear with faint to moderate nucleolar staining (Fig. 6B, iii and iv)”, “nuclear with intense nucleolar staining (Fig. 6B, v and vi))” and “other” (includes “diffuse throughout the nucleus”, “nuclear but excluded from the nucleolus” and “cytoplasmic”). The data is shown as the mean (± standard deviation) in three bar graphs and the Student’s T test determined significance (\*, <0.05; \*\*, <0.01) in comparison to the wild type E2 protein (E2WT). The data is taken from a minimum of three experimental repeats and the profile of staining patterns was the same in all of the experiments. The number of cells counted for each transfection falls in the range of 60 – 425. (i) The effect of mutations (double, D; triple, T) within the hinge region of E2 on localization in comparison to E2WT, (ii) the effect of co-expression of E4 on E2WT that is competent for SRPK1 inhibition (E4WT) or that lacks this function (E4∆44-48), (iii) the effect of coexpression of E4WT on the distribution of the mutant E2-T. (**C**)Western blot of equivalent amounts of cell lysate harvested from C33a keratinocytes transfected for 24 h with plasmids expressing HA-tagged wild type or triple mutant E2 proteins. Equivalent amounts of lysate from three independent transfections were blotted with anti-HA antibody. Levels of GAPDH were used as loading control. (**D**) Distribution of HA-tagged E2 (green) and E4 (red) in HFK (nucleus, blue). Scale bar, 10 m.

**Figure 7** **Homology between the hinge regions of *Betapapillomaviruses* HPV5 and 8 and the *Mupapillomaviruses* HPV1 and HPV63.**

Amino acids sequences of the E2 hinge regions showing conservation of the chromosome binding regions of HPV 5 and 8 in the HPV1 E2 hinge domain and partial homology with HPV63. Regions of homology are shaded. The serine residue 253 in HPV8 that is phosphorylated by PKA and the serines 265, 267 in the RS dipeptide repeat in HPV1 that is targeted by SRPK1, are underlined. Sequences were obtained from http://pave.niaid.nih.gov.