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Molecular cloning and characterization of a novel human papillomavirus, HPV 126, isolated from a flat wart-like lesion with intracytoplasmic inclusion bodies and a peculiar distribution of Ki-67 and p53

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ABSTRACT

Infection with certain human papillomavirus types induces warts with specific macroscopic and microscopic features. We observed multiple flat wart-like lesions on the chest, neck and extremities of an adult T-cell leukemia patient. Histologically, atypical intracytoplasmic inclusion bodies currently known to be pathognomonic for genus gamma or mu papillomaviruses were disclosed in some cells of the epidermis showing histological features compatible with flat warts. In the present study, a novel human papillomavirus was identified and its whole genome, 7326 bp in length, was cloned and characterized. Phylogenetic analysis showed the virus designated as HPV126 to be a novel type of genus gamma papillomavirus. Strikingly, Ki-67 and p53 expression was found to be increased in all layers of the epidermis except for horny layer, contrasting to expression restricted to the basal and lower spinous layers in ordinary flat warts.

Introduction

So far, more than one hundred twenty human papillomaviruses (HPVs) have been characterized based on nucleotide sequence diversity (Bernard et al., 2010). Infections of distinct types of HPVs are characterized by type-specific cytopathic/cytopathogenic effects (CPEs), i.e., macro- and microscopic features, pathological properties, and tissue tropisms. Hence, unusual CPEs which had not previously been described may suggest that lesions could be induced by a novel type of HPV (Egawa, 2005). We recently observed intracytoplasmic inclusion bodies (ICBs) resembling the HPV 4/60/65-associated homogenous ICB (Hg-ICB) (Egawa, 1994, 2005; Egawa et al., 1993) in flat wart-like lesions of a patient with adult T-cell leukemia (ATL). However, the clinical features of the lesions proved quite different from those of HPV 4/60/65-associated skin lesions, i.e., pigmented warts (Egawa, 1988; Egawa et al., 1993) or ridged warts (Honda et al., 1994), suggesting the presence of a previously unidentified papillomavirus. While the HPV type-specific CPEs are important in understanding the biological nature of the viruses, many of the novel HPV genotypes recently isolated lacked specific cell biological aspects.

The present report describes not only isolation and molecular biological characterization of a novel HPV genotype, HPV126, but also a clinical, histopathological and immunohistochemical characterization of HPV 126-associated skin lesions, revealing this novel human genus gamma papillomavirus to induce flat wart-like lesions with Hg-ICBs. Strikingly, Ki-67 and p53, well-known cell cycle proteins, were established to be expressed in all layers of the epidermis except for horny layer in the lesions, quite different from the expression pattern restricted to basal and lower spinous layers seen in ordinary flat warts.

Results

Histopathological features of wart lesions

Disseminated hypopigmented macules clinically resembling flat warts or epidermodysplasia verruciformis-related tinea versicolor-like lesions (Jablonska and Orth, 1985) were seen on the chest, neck, and extremities of a 56-year-old Japanese patient (Fig. 1A) (Kawai et al., 2009). A biopsy was taken from the disseminated fused lesion and adjacent normal-looking skin. Microscopically, at least two independent wart-

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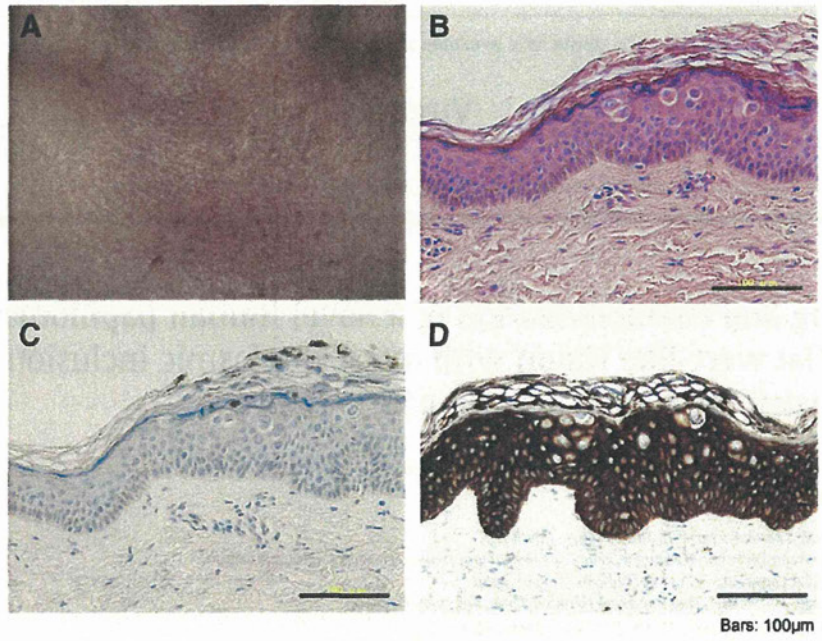

Fig. 1. Clinical (A), histopathological (B) and immunohistochemical (C and D) findings for the HPV 126-associated flat wart-like lesion. Macroscopic appearance of the flat wart-like lesions (A). In contrast to the normal skin adjacent to the flat wart-like lesion, Hg-ICBs are evident in balloon cells in the upper epidermal cell layers (B); strong positive signals for papillomavirus common antigen (L1 protein) in the nuclei of the cells in horny layer and cells with Hg-ICBs in granular layer (C); and positive signals for pan-cytokeratin are lacking in the cells with ICB (D). Scale bars; 100 μm.

like lesions separated by normal epidermis were included in the specimen. The epidermis showed mild acanthosis with basket-weave-like hyperkeratosis, partial hypergranulosis and mild papillomatosis, basic histological features compatible with those of flat warts (Fig. 1B) (Jablonska et al., 1985). However, additional unique histopathological features were also seen, i.e., keratinocytes with an enlarged nucleus, abundant blue-gray cytoplasm, occasional perinuclear haloes, and prominent keratohyalin granules observed in the granular and spinous layers, which are histopathological features consistent with EV (Jablonska and Orth, 1985). In addition, large clear cells contained homogeneous eosinophilic ICBs (Fig. 1B) resembling the homogeneous ICBs (Hg-ICBs) previously described in HPV 4/60/65-associated cutaneous warts (Egawa, 1994, 2005; Egawa et al., 1993).

Cloning and characterization of the HPV 126 genome

Although highly sensitive PCR failed to detect the DNA of either genus beta or mu papillomaviruses from the frozen biopsy specimen, a segment of a putative novel type genus gamma papillomavirus was amplified with a gamma papillomavirus-specific degenerate primers (Kawai et al., 2009) (Supplementary Fig. 1). Based on the nucleotide sequence, the full genome was cloned as described in Materials and methods. Sequencing of two clones from independent PCR reactions revealed the full genome consists of 7326 bp in length with a GC content of 50.5%. With a cutaneotropic papillomavirus primer set FAP59/FAP64 (Forslund et al., 1999), only the corresponding region of the cloned genome was amplified, further indicating the HPV is a single type in the lesions of this patient. The cloned HPV was found to be closely related to genus gamma papillomavirus types with an L1 ORF nucleotide similarity ranging from 60.1% to 68.7% (Table 1). According to the established criteria for a new type of papillomavirus that a new type should have 10% divergence of the L1 ORF nucleotide sequence from that of any other papillomavirus type {de Villiers, 2004 #16}, the cloned HPV qualified as a new type of papillomavirus designated as HPV126. According to the proposed criteria for species that should share between 60% and 70% nucleotide identity within a genus, we propose that HPV126, which has less than 70% nucleotide identity with any other papillomaviruses, constitutes a new species of genus gamma papillomavirus. Generation of a phylogenetic tree based on complete L1 nucleotide sequences of representative HPV types indicated that HPV 126 is most closely related to HPV 129 (Fig. 3), with similarity of 68.7% (Table 1). HPV 126 has a typical genomic organization for a genus gamma papillomavirus, and it has seven ORFs, E6, E7, E1, E2, E4, L2 and L1, but no E5 (Supplementary Fig. 2).

Immunohistochemical features of the wart lesions

Strong signals of L1 capsid proteins were seen in the nuclei of the cells in horny layer and cells with the ICBs in granular layer (Fig. 1C), suggesting active production of virions. In the cells with ICBs, little cytokeratin staining was observed while strong staining was observed in all epidermal cell layers of the lesions as well as its adjacent normal skin (Fig. 1D).

Table 1
Nucleotide sequence pairwise comparison of HPV 126 ORFs with those of representative genus gamma papillomaviruses.

ORF / HPV type	E6	E7	E1	E2	E4	L1	L2
HPV 4	52.2	53.2	66.2	57.3	57.3	63.5	51.6
HPV 48	54.0	60.3	61.3	55.6	52.7	61.4	52.7
HPV 50	49.1	55.9	61.6	58.5	55.2	61.5	52.7
HPV 60	54.3	54.0	65.5	59.8	59.5	62.9	53.6
HPV 65	53.4	50.5	67.0	54.7	55.0	61.4	51.9
HPV 88	51.5	55.4	64.5	57.1	55.6	62.8	52.6
HPV 95	52.6	51.5	66.9	57.2	57.5	62.2	52.6
HPV 112	55.0	53.0	61.0	58.4	53.7	62.4	50.4
HPV 116	60.3	53.7	66.4	60.9	58.6	67.6	57.7
HPV 119	52.5	53.4	62.0	58.3	52.9	61.5	50.3
HPV 121	55.0	55.1	63.8	55.8	53.4	63.1	52.3
HPV 123	47.1	48.0	60.5	55.6	51.4	60.1	51.6
HPV 129	58.3	57.4	67.3	60.2	61.1	68.7	58.7

Similarities (%). Sequence for the genus gamma papillomaviruses were obtained from GenBank.

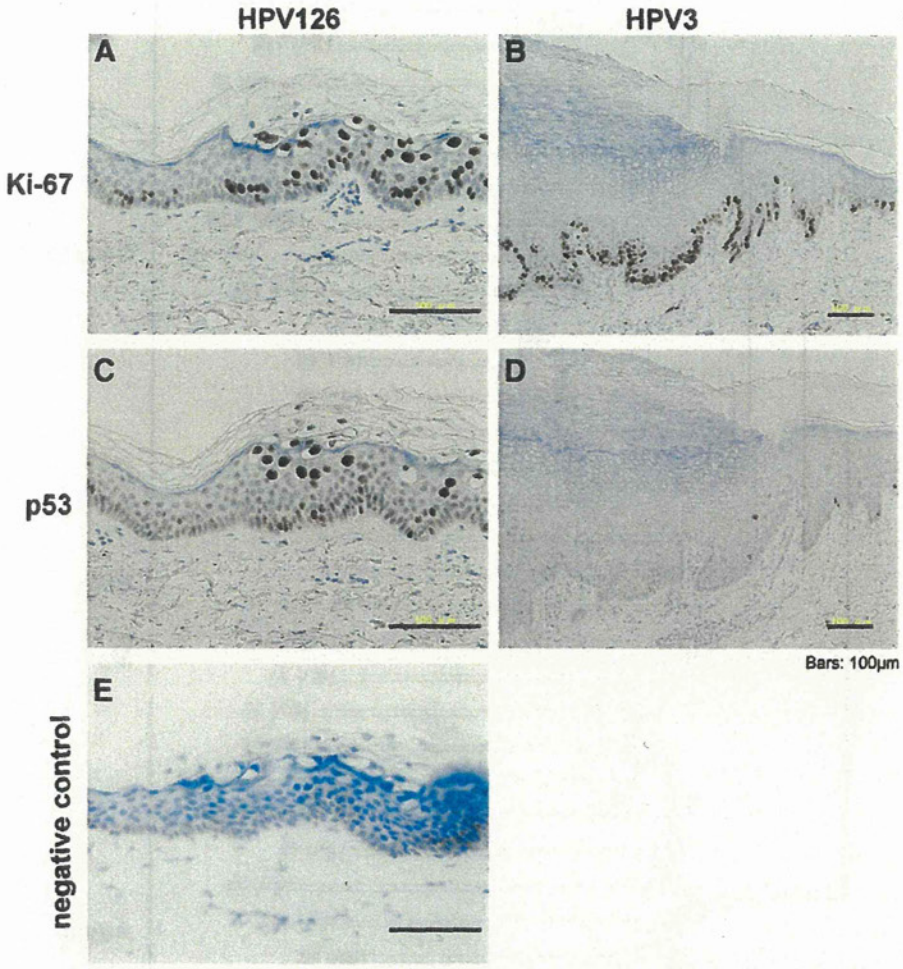

Fig. 2. Immunohistochemical features of the HPV 126-associated flat wart-like lesion. In contrast to the normal skin adjacent to the flat wart-like lesion (left side), positive signals for both Ki-67 (A) and p53 (C) are apparent in all compartments of the epithelium except for horny layer of the lesion (right side). However, a divergence is seen between Ki-67 and p53 staining. In contrast to that strong staining for Ki-67 is seen parabasal cells as well as cells of upper epidermal layers, the strong signal is seen predominantly in cells of upper epidermal cell layers. In contrast, weak (B) and faint (D) signals are restricted to the basal and parabasal (B) or lower spinous (D) layers of typical HPV 3-associated flat warts. Scale bars; 100 μm. As a negative control, no signals are observed in the staining of normal non-immune serum from the same source as the primary antibody (E).

Increased expression of Ki-67, an indicative marker of cycling cells, was observed in all compartments of the epithelium except for horny layer of the lesions, whereas its expression was restricted to the basal proliferative compartment of the adjacent normal epidermis (Fig. 2A) and to basal to parabasal cells in the typical HPV 3-positive flat warts (Fig. 2B).

Increased p53 staining was also observed in all compartments of the epithelium except for horny layer of the lesions in the HPV 126-associated lesions. However, unlike Ki-67, strong signals were not seen in parabasal cells for p53. In the adjacent normal epidermis, weak staining for p53 was restricted to the basal proliferative compartment (Fig. 2C), and faint staining was in the basal and lower spinous layers in HPV 3-positive typical flat warts (Fig. 2D). Five cases of typical HPV 3-associated flat warts were examined for comparison to confirm the unusual distribution of Ki-67 and p53 expression in the present flat wart-like lesion though the present case is the only patient with HPV 126-asssociated inclusion warts studied thus far. These observations are reminiscence of high grade cervical intraepithelial neoplasia. However, neither the HPV 126-associated flat wart-like lesions nor ordinary flat warts showed positive staining for $p16^{INK4a}$, while cervical cancer biopsy examined as a positive control exhibited strong positive signals (data not shown).

Discussion

In the present study, the full-length genome of a novel papillomavirus, HPV 126, was cloned from flat wart-like lesions arising in a Japanese ATL patient and characterized. The DNA genome of HPV 126 consists of 7326 base pairs and shows the gene arrangement characteristic for a cutaneous HPV. The nucleotide sequence of the L1 ORF of HPV 126 shares the highest homology of 68.7% to that of HPV 129, a genus gamma papillomavirus, thereby defining HPV 126 a novel type possibly constituting a novel species of the genus gamma papillomavirus (de Villiers et al., 2004). The HPV 126-associated cutaneous lesions on the chest, neck and extremities of our Japanese ATL patient were disseminated hypopigmented macules clinically resembling flat warts or tinea versicolor-like lesions seen in epidermodysplasia verruciformis (EV) and acquired EV patients (Jablonska and Orth, 1985; Lutzner et al., 1983).

On microscopy balloon cells with pale blue cytoplasm like those seen in flat wart-like lesions of EV or acquired EV patients (Jablonska and Orth, 1985; Lutzner et al., 1983). Additional features characteristic for the present case were ICBs most resemble those associated with HPV 4/60/65, which are members of species 1 (HPV 4/65) and species 4 (HPV 60) of genus gamma papillomaviruses. (Egawa,

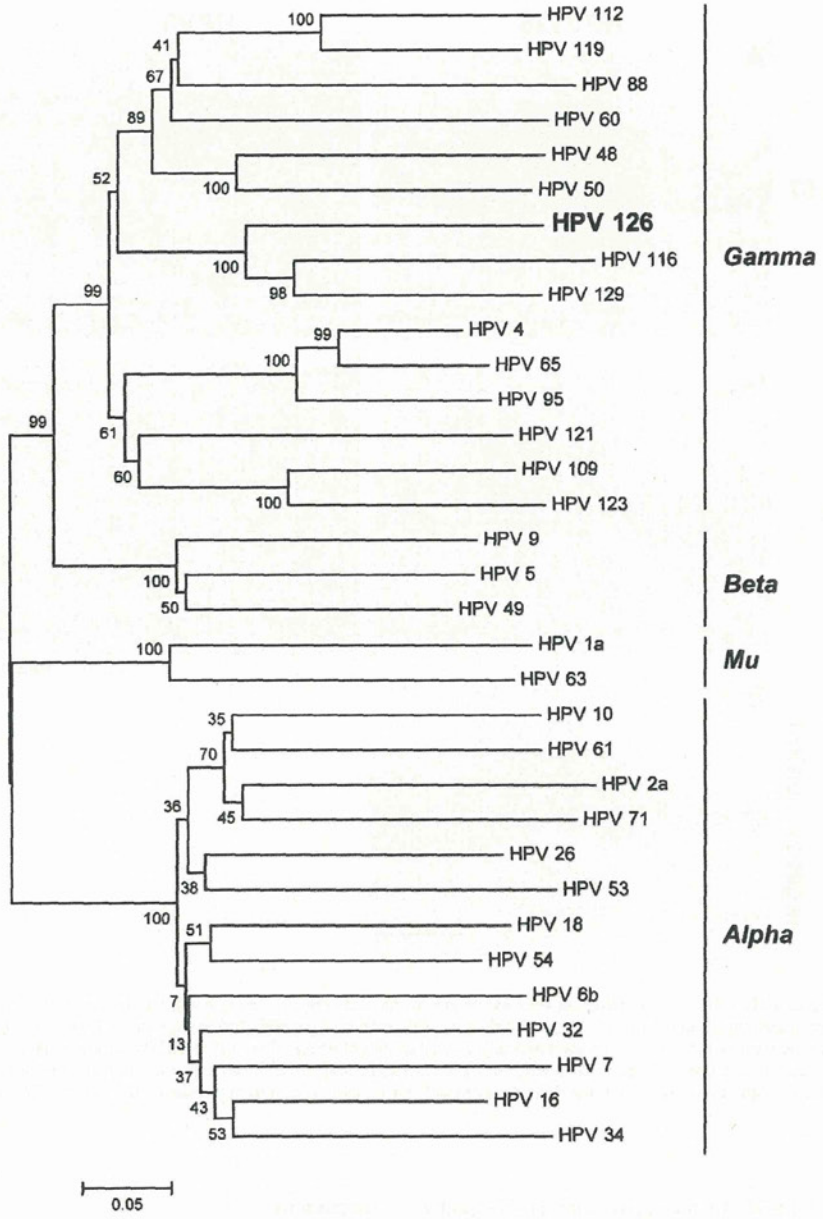

Fig. 3. Phylogenic relationships among HPV 126 and representative HPV types. A phylogenetic tree was constructed based on L1 ORF sequences using the neighbor-joining (NJ) method with 1000 bootstrap replicates. Numbers near branches indicate support index from NJ bootstrap percentage. Nucleotide sequences of representative HPVs were obtained from GenBank.

1994, 2005, 2007; Egawa et al., 1993). It is well known that distinct ICBs are pathognomonic for genus gamma and mu papillomaviruses (Egawa, 2007), although the histological features of recently isolated genus gamma papillomaviruses, including HPV 129 and HPV 116, have yet to be described (Bernard et al., 2010; Li et al., 2009). Cytokeratins were absent from cells containing Hg-ICBs (Fig. 1D), in which E4 proteins are thought to be a major component, though E4 protein expression was not examined in the present case. Thus like HPV16 E4 (Doorbar et al., 1989), HPV 126 E4 might be involved in interference with keratin filament assembly.

Another striking feature of the present case was its peculiar immunohistochemical localization of Ki-67 and p53, namely, they were expressed strongly and distributed in all compartments of the epithelium except for horny layer of the HPV 126-associated lesions (Fig. 2). Antigen Ki-67 is expressed during all phases of the cellular cycle, G1, S, G2, and M, of proliferating cells, but is absent in quiescent cells (G0). It is, therefore, a marker of cellular proliferation, which can be detected with monoclonal antibodies. Interaction of human papillomavirus oncoproteins E6 and E7 with cell cycle proteins leads to disturbance of the cell cycle and subsequent alteration in expression of some cell cycle proteins, such as $p16^{INK4a}$, cyclin D1, p53 and Ki-67. Abrupt inactivation of pRB can induce p53 accumulation though activation $p14^{ARF}$ (Bates et al., 1998). Like other HPVs, E7 protein of HPV 126 conserves the pRB binding motif and potentially inactivates pRB. Indeed some of the E7 proteins of cutaneous HPVs, such as HPV1 E7, can strongly bind and inactivate pRB (Hiraiwa et al., 1996; Schmitt et al., 1994). Thus it will be interesting to examine the activity of HPV 126 E7 protein and relationship among expression levels of HPV 126 E7, Ki-67 and the p53 accumulation. Unlike high grade CIN lesions where a positive correlation between the expression of the $p16^{INK4a}$

and Ki-67 has been reported (Nam et al., 2008; Queiroz et al., 2006), accumulation of $p16^{INK4a}$ was not detected in the Ki-67 positive cells (data not shown) in the present case whose clinical behavior and histopathological findings were benign (Kawai et al., 2009).

In conclusion, HPV 126, isolated and characterized in the present study, is a novel type of genus gamma papillomavirus and associated with flat wart- or EV-related tinea versicolor-like clinical features: histological ICBs as with other genus gamma papillomaviruses; and immunohistochemical expression of Ki-67 and p53 in characteristic manner not typical for benign cutaneous warts. It is probable that conditions accompanying immunosuppression in this ATL patient may have contributed to stimulate viral production of HPV 126, thus leading to wart formation, as known for acquired EV (Lutzner et al., 1983). To ascertain the true nature of HPV 126 and its associated warts, we need to perform epidemiological as well as further clinicopathological and virological studies on a larger number of lesions and patients, including immunocompromised individuals.

Materials and methods

Patient

A 56-year-old Japanese man was referred to us in August 2008 for evaluation of a 5-year history of disseminated hypopigmented macules clinically resembling flat warts or epidermodysplasia verruciformis-related tinea versicolor-like lesions (Jablonska and Orth, 1985) on the chest, neck, and extremities (Fig. 1A) (Kawai et al., 2009). The patient might have been suffering from immunodeficiency, because he was diagnosed as having chronic type of adult T-cell leukemia at the age of 52 years and manifested recurrent fungal pneumonia, rapidly progressing oral squamous cell carcinoma and multiple brain abscesses. A biopsy specimen was taken from the flat wart-like lesions with adjacent normal skin under suspicion of acquired EV (Lutzner et al., 1983). The biopsy specimen was cut into two pieces, one of which was fixed in 20% buffered formalin and embedded in paraffin for conventional histopathological and immunohistochemical analyses, and the other was frozen and stored in −70 °C for further analyses including DNA extraction.

Microscopical examination

Four-micrometer thick sections were obtained from the formalin-fixed and paraffin-embedded biopsy specimen, stained with hematoxylin and eosin (H&E), and examined microscopically.

Cloning and characterization of HPV DNA

Degenerate primers to detect genus gamma papillomaviruses were as described previously (Kawai et al., 2009). The amplified sequence turned out to correspond to nt 4641 to 5632 of the cloned HPV126 (Supplementary Fig. 1). Then abutting primers were designed juxtaposing the *HpaI* site present in the L1 region (forward primer: 5′-GTTAACAGTAGGCCATCCCTATTTTGATATTGTTG-3′, reverse primer: 5′-GTTAACAGTCTTTCAGTATTTGCATGAAAATAAATATCG-3′). The genome was amplified by 30 cycles of PCR using KOD plus DNA polymerase (Toyobo, Japan) according to the supplier's instruction; annealing at 60 °C, elongation at 68 °C for 8 min. About 8 kbp PCR products were purified and then cloned into pBluescriptIISK(−) (Stratagene, La Jolla, CA), in which 15-bp overlapping sequence of HPV 126 was added to the *NotI* site by PCR (forward primer: 5′-TGAAAGACTGTTAACGCGG-CCGCTCTAGAACTAGTGGATC-3′, reverse primer: 5′-TGGCCTACTGT-TAACGCGGCCGCCACCGCGGTGGAGCTCC-3′), by In-Fusion reaction (Clontech, Mountain View, CA). The complete genomic sequence of a clone was initially determined using primer walking by Nihon Gene Research Laboratories Inc. With the same set of primers (Supplementary Table 1), we confirmed the sequence of another clone from an independent PCR reaction to be identical (Supplementary Fig. 1). The DNA clone was submitted to the Human Papillomavirus Reference Laboratory (Heidelberg, Germany) for official designation, HPV 126, and the sequence was reconfirmed. HPV 126 sequence was submitted to DNA Data Bank of Japan (DDBJ) under accession number AB646346. Nucleotide sequence pairwise comparison of HPV 126 ORFs with types representing genus gamma papillomaviruses and L1 nucleotide global multiple sequence alignments were analyzed using ClustalW program (Thompson et al., 1994). Each gap was included and counted as one position. Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007).

Immunohistochemical examination

Formalin-fixed and paraffin-embedded tissue sections (4 μm-thick) were deparaffinized in xylene and rehydrated through a series of graded ethanols (100–70%). For antigen retrieval, slides were immersed in citrate buffer (pH6.0) and were heated for 20 min in a microwave. The slides were then incubated in methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity. After washing, primary antibodies (Anti-papillomavirus common antigen, DAKO, clone K1H8, 1:200; Ki-67, DAKO, Clone MIB-1, 1:50; p53 protein, DAKO, Clone DO-7, 1:50; $p16^{INK4a}$, Santa Cruz, Clone JC8, 1:200; cytokeratin, Nichirei, polyclonal,1:2) were applied for 1 h and binding was detected using an Envision Kit (Dako Cytomation; K4006). Color development was achieved with 3, 3-diaminobenzine (DAB) as the chromogen and hematoxylin counterstaining was performed to aid in orientation. As a negative control, normal non-immune serum from the same source as the primary antibody was applied. Formalin-fixed, paraffin-embedded sections from an invasive uterine cervix squamous cell carcinoma biopsy served as a positive control for $p16^{INK4a}$.

Supplementary materials related to this article can be found online at doi: 10.1016/j.virol.2011.10.011.

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A Novel Interaction between hScrib and PP1γ Downregulates ERK Signaling and Suppresses Oncogene-Induced Cell Transformation

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Abstract

Previous studies have shown that the cell polarity regulator hScrib interacts with, and consequently controls, the ERK signaling pathway. This interaction occurs through two well-conserved Kinase Interacting Motifs, which allow hScrib to bind ERK1 directly, resulting in a reduction in the levels of phospho-ERK. This suggests that hScrib might recruit a phosphatase to regulate this signaling pathway. Using a proteomic approach we now show that Protein Phosphatase 1γ (PP1γ) is a major interacting partner of hScrib. This interaction is direct and occurs through a conserved PP1γ interaction motif on the hScrib protein, and this interaction appears to be required for hScrib's ability to downregulate ERK phosphorylation. In addition, hScrib also controls the pattern of PP1γ localization, where loss of hScrib enhances the nuclear translocation of PP1γ. Furthermore, we also show that the ability of hScrib to interact with PP1γ is important for the ability of hScrib to suppress oncogene-induced transformation of primary rodent cells. Taken together, these results demonstrate that hScrib acts as a scaffold to integrate the control of the PP1γ and ERK signaling pathways and explains how disruption of hScrib localisation can contribute towards the development of human malignancy.

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Introduction

The control of cell polarity and the maintenance of tissue architecture are intimately related and are, in part, controlled by a tri-partite macromolecular signaling complex consisting of the Scrib complex, the Par complex and the Crumbs complex [1,2]. Through a series of antagonistic interactions the components of these three complexes control a variety of downstream signaling pathways that, in turn, directly contribute to the regulation of cell polarity and cell proliferation [3]. It is now clear that the loss of control of these pathways is a common event during the development of diverse human malignancies [1,4–7]. These defects are particularly evident at the later stages of malignant progression, and a variety of studies in both Drosophila and transgenic mice have provided additional supporting evidence of tumour suppressor activity for the various components of these signaling complexes [8–11].

The hScrib complex consists of three proteins, hScrib, hDlg1 and Hugl-1/2. In *Drosophila*, loss of either Scrib or Dlg produces imaginal disc overgrowth with invasive characteristics [8] [12], phenotypes that can be functionally complemented by the mammalian equivalents [13–15]. More recently Scrib has been implicated in the control of the JNK and ERK signaling cascades, and loss of hScrib appears to enhance the effects of the Ras and Myc oncogenes, and can contribute to mammary tumour development [16–21]. Recent studies have also demonstrated that hScrib can interact directly with ERK, and control both ERK activation and its nuclear translocation [19]. However, the physical interaction between ERK and hScrib is not sufficient to explain the inactivation of ERK, since high levels of hScrib appear capable of directly reducing the levels of ERK phosphorylation [19]. Since hScrib has no known phosphatase activity itself, it therefore seemed possible that a protein phosphatase might be recruited by hScrib to fully inactivate the ERK signaling pathway.

Control of ERK activation reflects an exquisite balance between the activities of the activating kinases and the de-activating protein phosphatases. Activated ERK can translocate to the nucleus, where it activates several transcription factors and also phosphorylates cytoplasmic and nuclear kinases [22–24]. Since phosphorylation of both the threonine and tyrosine residues of ERK is required for its activation, dephosphorylation of either is sufficient for its inactivation [25]. There are several reports demonstrating that dephosphorylation of active ERK can be achieved by tyrosine-specific phosphatases, by serine/threonine-specific phos-

phatases or by dual specificity (threonine/tyrosine) protein phosphatases [26–29]. One of the important negative regulators of the ERK signaling pathway is PP2A, a member of the PPP family of protein serine/threonine phosphatases which also includes PP1 [30,31]. However, PP2A is thought to exert its activity mainly upon other activating kinases within the cascade, rather than upon ERK itself [32–34]. In addition, recent studies have also shown that hScrib can directly regulate the Akt signaling cascade by recruitment of the protein phosphatase PHLPP1 to the plasma membrane, thereby resulting in de-phosphorylation of Akt [35]. Here, we have used a proteomic approach to extend our investigations into the regulation of the ERK signaling cascade by hScrib. We now show that hScrib interacts with PP1γ, and that this association correlates with the ability of hScrib to downregulate ERK activation. We also provide compelling evidence that hScrib directly contributes to the regulation of PP1γ function by controlling its translocation between the cytoplasm and the nucleus. Thus, loss of hScrib expression results in both ERK activation and aberrant nuclear translocation of PP1γ.

Materials and Methods

Cells and treatments

HEK293 (human embryonic kidney cells) and HaCaT (Human keratinocytes) were obtained from ATCC [36,37]. HEK293, HaCaT and Baby Rat Kidney (BRK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/mL) and glutamine (300 μg/mL) in a humidified 5%CO_2 incubator. Transfection was carried out using calcium phosphate precipitation as described previously [37] or using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The depleted Scribble cell lines were generated as described previously [19]. Cell transformation assays were done using BRK cells obtained from 9 day old Wistar rats with a combination of HPV-16 E7 and EJ-ras, plus the appropriate hScrib and PP1γ expression plasmids. Cells were placed under G418 selection for three weeks, and then fixed and stained.

Plasmids

The wild type pCDNA3-HA-PP1γ was the kind gift of Dr. Wilhelm Krek (Swiss Federal Institute of Technology (ETH) Zurich). The wild type HA-tagged pcDNA hScrib expression plasmid and the truncated mutant pGEX hScrib PDZ1-C, PDZ1-4, S1445A, S1445D, and CT expression plasmids have been described previously [19]. The L1266Y1268→AA mutation (KADA) to doubly change the Leucine (L) and Tyrosine (Y) residues to Alanine (A) in hScrib was done using the QuikChange site-directed mutagenesis kit from Stratagene Cloning Systems (Celbio) according to the manufacturer's instruction. The mutants were confirmed by DNA sequencing. See Figure S1 for a detailed description of the location of the different hScrib mutations.

Antibodies

The following commercial antibodies were used at the dilution indicated: anti-hScrib goat polyclonal antibody (Santa Cruz, WB 1:1000), anti-PP1γ goat polyclonal antibody (Santa Cruz, WB 1:1000), anti-PP1γ sheep polyclonal antibody (Abcam, WB 1:1000), anti-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology, WB 1:1000), anti-phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology, WB 1:1000), anti-HA monoclonal antibody 12CA5 (Roche, WB 1:500), anti-γ-tubulin monoclonal antibody (Sigma, WB 1:5000), anti-p84 mouse monoclonal antibody (Abcam, WB 1:1000), anti-E-Cadherin rabbit polyclonal antibody (Santa Cruz, WB 1:500), anti-α-tubulin mouse monoclonal antibody (Abcam, WB 1:1000).

Immunofluorescence and Microscopy

For immunofluorescence cells were grown on glass coverslips and fixed in 3.7% paraformaldehyde in PBS for 20 mins at room temperature. After washing in PBS the cells were permeabilised in PBS/0.1% Triton for 5 mins, washed extensively in PBS and then incubated with primary antibody diluted in PBS for 1 hour followed by the appropriately conjugated secondary antibodies. Secondary antibodies conjugated to Alexa Fluor 488 or 548 were obtained from Invitrogen. The cells were then washed several times in water and mounted on glass slides. Cells were visualized by using a Zeiss Axiovert 100 M microscope attached to a LSM 510 confocal unit.

siRNA transfection

HEK293 cells were seeded on 6 cm dishes and transfected using Lipofectamine 2000 (Invitrogen) with control siRNA against Luciferase (siLuc), or siRNA against hScrib and PP1γ sequences (Dharmancon). 48 hours post-transfection cells were harvested and total cells extracts or cell fractionated extracts were then analysed by western blotting.

Fusion protein purification and in vitro binding assays

GST-tagged fusion proteins were expressed and purified as described previously [19]. Proteins were translated in vitro using the Promega TNT kit and radiolabelled with (^{35}S) cysteine or (^{35}S) methionine (Perkin Elmer). Equal amounts of in vitro-translated proteins were added to GST fusion proteins bound to glutathione agarose (Sigma) and incubated for 1 hour at 4°C. After extensive washing with PBS containing 0.25% NP-40, or as otherwise indicated, the bound proteins were analysed by SDS-PAGE and autoradiography.

In vitro phosphorylation

Purified GST fusion proteins were incubated with commercially purified ERK1 (Cell Signaling Technology) or PKA (Promega) for 20 mins at 30°C in phosphorylation buffer (0.25 M Tris pH7.5, 1 M $MgCl_2$, 3 M NaCl, 0.3 mM aprotinin, 1 mM Pepstatin) or using the kinase buffer supplied by New England Biolabs supplemented with 56 nM (^{32}P) γ-ATP (Perkin Elmer) and 10 mM ATP following the manufacturer's instruction. After extensive washing, the phosphorylated proteins were monitored by SDS-PAGE and autoradiography.

Mass spectrometry analysis

HEK293 cells were transfected with HA-tagged Scrib and after 24 hours the cells were extracted in mass spectrometry lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.25% NP40) and extracts incubated with anti-HA beads (Sigma) for 2–3 hours on a rotating wheel at 4°C. The beads were then extensively washed with PBS, dried and the immunoprecipitated proteins were subjected to proteomic analysis as described previously [38].

Subcellular Fractionation assays

Differential extraction of HEK 293 cells to obtain cytoplasmic, membrane, cytoskeleton, and nuclear fractions was performed using the Calbiochem ProteoExtract Fractionation Kit according to the manufacturer's instructions. To inhibit phosphatase activity during the preparation of cell lysates, phosphatase inhibitors

A

log (e)+	#	total	HA-Scribble
-1189.3	91	200	SCRIB, scribble
-327.5	29	37	VIM, vimentin
-73.2	6	10	NPM1P21, nucleophosmin
-60.5	6	6	PPP1CC, protein phosphatase 1, catalytic subunit, gamma isoform
-59.6	6	6	NCL, nucleolin

ADLDKLNIDS IIQR [15]llev
LFEYGGFPPE SNYLFLGDYV DR [96]gkqs
IKYPENFFLL R [122]gnhe
YPENFFLLR [122]gnhe
AHQVVEDGYE FFAK [260]rqlv
KKPNATRPVT PPR [314]gmit

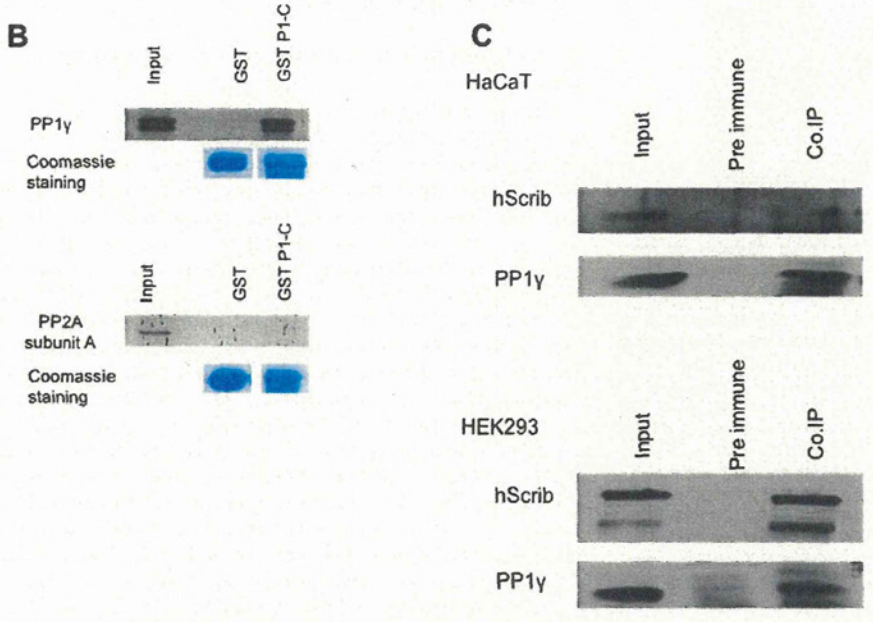

Figure 1. Interaction between hScrib and PP1γ in vivo. A) Results from the mass spectroscopy analysis of hScrib containing immunoprecipitates identified 6 peptides (indicated) corresponding to PP1γ. B) In vitro translated PP1γ (upper panels) and PP2A subunit A (lower panels) were incubated for 1 hour at 4°C with purified GST-hScribP1-C or GST alone immobilized on Glutathione agarose. After extensive washing, the bound proteins were analysed by SDS–PAGE and autoradiography which are shown in each of the upper panels. The gels were rehydrated and stained with Coomassie to show equal levels of GST loading in the respective lower panels. C) Endogenous PP1γ was immunoprecipitated from HaCaT (upper panels) and HEK 293 cells (lower panels), with pre-immune antibody used as control. The immunoprecipitated proteins were then analysed by western blotting using anti-hScrib and anti-PP1γ antibodies.
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(1 mM Na_3VO_4, 1 mM β-Glycerophosphate, 2.5 mM Sodium Pyrophosphate, 1 mM Sodium Fluoride) were also included.

Immunoprecipitation and Western blotting

Total cellular extracts were prepared by directly lysing cells from dishes in SDS lysis buffer. Alternatively cells were lysed in either E1A buffer (25 mM HEPES pH 7.0, 0.1% NP-40, 150 mM NaCl, plus protease inhibitor cocktail; Calbiochem) or RIPA buffer (50 mM Tris HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, plus protease inhibitor cocktail; Calbiochem) and the cell extracts were analysed by SDS-PAGE and western blotting. For immunoprecipitations, total cell lysates were transferred into a tube of equilibrated EZview Red Anti-HA Affinity Gel beads (Sigma), and incubated for 2 hours at 4°C. Immunoprecipitates were extensively washed four times in lysis buffer and solubilised in SDS-PAGE sample buffer. For western blotting, 0.45 μm nitrocellulose membrane (Schleicher and Schuell) was used and membranes were blocked for 1 hour at 37°C in 10% milk/PBS followed by incubation with the appropriate primary antibody diluted in 10% milk/0.5% Tween 20 for 1 hour. After

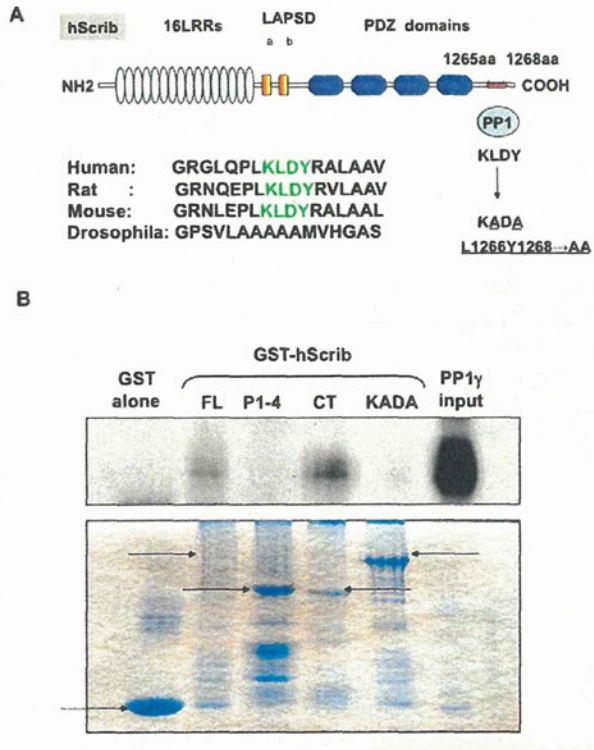

Figure 2. hScrib contains a consensus PP1-binding motif. A) The schematic shows the arrangement of the functional domains on the hScrib protein, highlighting the LRR, LAPSD and PDZ domains. The putative PP1-binding site, the RVXF (the consensus sequence is K/R/H/N/S V/I/L X F/W/Y) motif is also shown, where X is any amino acid. The hScrib mutant in which the PP1-binding site KLDY was mutated to KADA in order to disrupt the interaction with PP1 is shown. A comparison sequence alignment of the region of hScrib containing the PP1-binding motif indicating its absence in Drosophila also shown. B) In vitro translated and radiolabeled PP1γ was incubated with purified full length GST-hScrib fusion protein (FL), GST-hScrib PDZ1-4 (P1-4), GST-hScrib CT (CT), GST-hScrib L1266Y1268→AA (KADA) and GST alone as a control. After extensive washing the bound PP1γ was ascertained by SDS PAGE and autoradiography. The upper panel shows the autoradiograph, with the input of PP1γ also shown for comparison. The lower panel shows the Coomassie stain of the gel showing the levels of GST fusion protein loading, with the arrows indicating the relevant full length fusion proteins.
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several washings with PBS 0.5% Tween 20, secondary antibodies conjugated with HRP (DAKO) in 10% milk/0.5% Tween 20 were incubated for 1 hour. Blots were developed using Amersham ECL reagents according to the manufacturer's instructions.

Results

PP1γ is a direct binding partner of hScrib

Based on our previous studies we reasoned that down-regulation of ERK phosphorylation by hScrib might involve the recruitment of a protein phosphatase [19]. To investigate this possibility we performed proteomic analyses to identify additional interacting partners of hScrib. HEK293 cells were transfected with an HA-tagged hScrib expression plasmid and after 24 hours the cells were extracted, and hScrib–bound protein complexes were immunoprecipitated with anti-HA agarose beads and then subjected to mass spectroscopy analysis. Several previously reported interacting partners were identified, including vimentin. However, of the novel interacting partners, the most prominent phosphatase identified was the catalytic subunit of PP1γ (Figure 1A), a major eukaryotic serine/threonine protein phosphatase. To investigate whether hScrib can interact with PP1γ, an in vitro pull-down assay was performed using purified GST-hScrib P1-C fusion protein and in vitro translated radiolabeled PP1γ. For comparison a similar assay was also done using in vitro translated radiolabeled protein phosphatase 2A (PP2A). After extensive washing the bound PP1γ and PP2A were detected by SDS PAGE and autoradiography, and the results in Figure 1B demonstrate strong interaction between hScrib and PP1γ. In contrast, no interaction was observed between hScrib and PP2A, confirming the specificity of the association between hScrib and PP1γ. To determine whether endogenous hScrib and PP1γ could exist in a complex in vivo, immunoprecipitations were performed on cell extracts from HEK293 and HaCaT epithelial cells using anti-PP1γ antibody. Co-immunoprecipitated hScrib was then detected by western blotting, and the results in Figure 1C show a significant degree of co-immunoprecipitation of hScrib with PP1γ in both cell lines. Taken together, these results demonstrate that hScrib and PP1γ can exist as a complex in vivo.

hScrib interacts with PP1γ through a conserved RVxF motif

The PP1 holoenzyme is composed of a catalytic subunit and several regulatory subunits, which target the catalytic subunit to specific subcellular locations. The RVxF motif is a short conserved PP1-binding motif initially identified in previous studies showing that these residues can block the interaction of regulatory subunits with the PP1 catalytic subunit [39]. As shown in Figure 2A, analysis of the hScrib sequence reveals the presence of a putative PP1 binding motif, KLDY (the consensus sequence is: {K/R/H/N}.{S/V/I/L}.X.{ F/W/Y}) [40,41] spanning residues 1265–1268. This sequence is also highly conserved in mammalian Scrib proteins, but is absent in Drosphila. Based on previous studies, mutation of the L and Y residues would be expected to severely perturb the interaction with PP1 [39–42]. To investigate whether this KLDY motif is responsible for the capacity of hScrib to bind to PP1γ, a panel of GST-hScrib fusion proteins consisting of the full length (FL), two truncated proteins encompassing PDZ domains 1–4 (P1-4) and the carboxy terminal third of hScrib (CT), plus a full length hScrib with the KLDY/KADA mutation, were used in pull-down assays with in vitro translated radiolabeled PP1γ. The levels of bound PP1γ were then assessed by SDS PAGE and autoradiography and, as can be seen from Figure 2B, PP1γ binds to the carboxy terminal region of hScrib which contains the predicted PP1 binding motif. Furthermore the KLDY/KADA mutation significantly decreases the capacity of PP1γ to interact with hScrib, confirming that the major site of interaction is through the KLDY consensus motif.

hScrib and ERK are substrates of PP1γ

We have previously shown that hScrib is a substrate for both PKA and ERK. Furthermore, hScrib can downregulate ERK activation through a direct protein-protein interaction [19], although the precise mechanism by which hScrib can achieve this is still unknown. We therefore wanted to determine whether phosphorylation of hScrib by either PKA or ERK1 could influence the ability of hScrib to interact with PP1γ and, furthermore, whether hScrib itself was a substrate of PP1γ. To do this, purified GST-hScrib fusion protein was subject to phosphorylation by either PKA or ERK1 in the presence of non-radiolabeled ATP, and after extensive washing binding assays

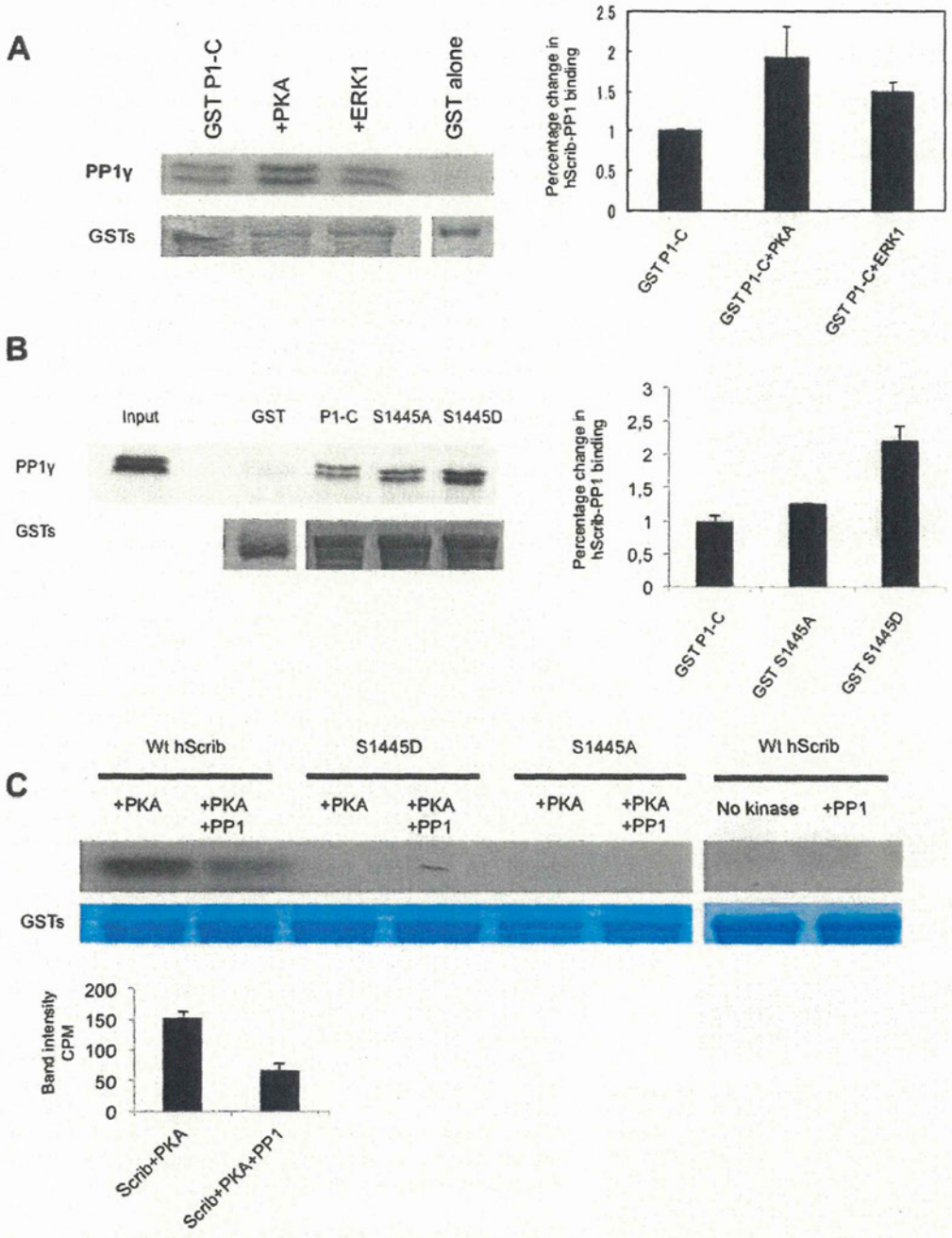

Figure 3. hScrib is a substrate of PP1γ. A) Purified GST-hScrib fusion protein was in vitro phosphorylated with purified PKA or ERK1 as described previously (19) and then incubated with PP1γ for 20 mins at 30°C. Bound PP1γwas detected by western blotting with anti PP1γ antibody. The lower panel shows the ponceau stain of the nitrocellulose, and the upper right panel shows the quantitations from three independent experiments. Note that hScrib phosphorylated by PKA exhibits increased association with PP1γ. B) Purified PP1γ was incubated with purified full length wild type GST-hScrib fusion protein (P1-C), the mutants S1445A, S1445D or GST alone as a control. After extensive washing the bound PP1γ was ascertained by western blotting. The upper panel shows the result of the western blot, with the 20% input of PP1γ also shown for comparison. The lower panel shows the ponceau stain of the nitrocellulose. The histogram shows the quantitation from three independent experiments. C) Purified GST-hScrib wild type and PKA phospho-site mutants of hScrib were in vitro phosphorylated with purified PKA in the presence of radiolabeled ATP as described previously (19) and incubated with PP1γ for 20 mins at 30°C. The remaining level of phosphorylated hScrib was then determined following SDS PAGE and autoradiography. The two right-hand lanes show lack of phosphorylation of hScrib in the absence of PKA, whilst the lower panels show the Coomassie stain of the gel demonstrating equal levels of the GST-hScrib fusion protein throughout. The quantitation of hScrib phosphorylation from three independent experiments is also shown.
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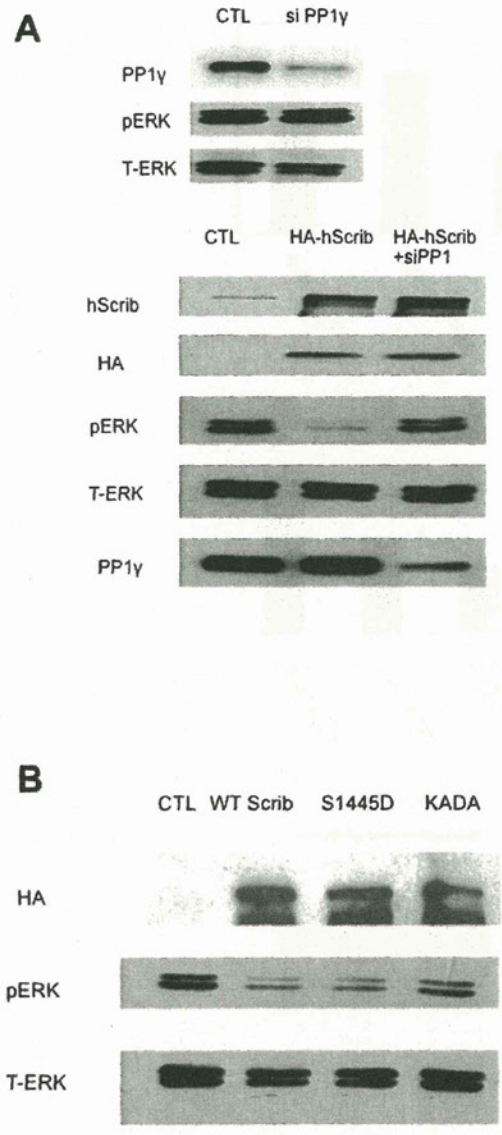

Figure 4. PP1γ is required for hScrib-induced de-phosphorylation of ERK. A) HEK 293 cells were transfected with PP1γ si RNA or si Luc RNA as control (CTL) and after 24 hours were then transfected with a plasmid expressing HA-tagged hScrib. After a further 24 hours the cells were extracted and levels of phospho and total ERK determined by western blot analysis. The upper three panels shows the changes in the ERK profiles when cells were transfected with siRNA PP1γ alone, whilst the lower set of five panels show the effects in the presence of ectopically expressed hScrib. B) HEK 293 cells were transfected with HA-tagged wild type hScrib, or the S1445D and KADA mutants. Total cell extracts were then made after 48 hours and the hScrib, phospho-ERK and total ERK were detected by western blotting.
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were performed using commercially purified PP1γ. The bound protein was then detected by western blotting using anti-PP1γ antibodies. The results in Figure 3A demonstrate a number of important features. In the absence of phosphorylation there is a strong interaction between hScrib and the purified PP1γ, demonstrating that the interaction between hScrib and PP1γ is indeed direct. However, there is also a clear increase in the association between hScrib and PP1γ when hScrib is phosphorylated by PKA, but not when it is phosphorylated by ERK1. We had previously shown that the major PKA phosphorylation site on hScrib was S1445 [19]. Therefore, to further confirm that phosphorylation of hScrib by PKA at S1445 can influence its capacity to interact with PP1γ, we repeated the pull down assays using the phospho-mimic mutation of hScrib, S1445D. As can be seen from Figure 3B, the S1445D mutant exhibits a significantly increased capacity to interact with PP1γ, which is similar to that seen following phosphorylation by PKA. These results demonstrate that phosphorylation of hScrib by PKA at S1445 can indeed increase the ability of hScrib to directly interact with PP1γ.

We then analysed whether hScrib was a potential substrate of PP1γ. Purified GST-hScrib fusion protein was subjected to in vitro phosphorylation with purified PKA and radiolabeled ATP. After extensive washing the radiolabeled hScrib fusion protein was incubated with purified PP1γ, and the amount of phosphorylated protein determined by SDS PAGE and autoradiography. The results obtained in Figure 3C demonstrate that the level of phosphorylated hScrib is decreased following incubation with PP1γ, demonstrating that hScrib is a potential substrate of the phosphatase and, furthermore, that hScrib can directly recruit active PP1γ. Also shown are the non-phosphorylatable mutants of hScrib, confirming the specificity of the phosphorylation reaction.

We then proceeded to determine whether the interaction of hScrib with PP1γ might be involved in the capacity of hScrib to downregulate ERK activation. Cells were transfected with control siRNA against luciferase or against PP1γ, and after 24 hours the cells were then transfected with an hScrib expression plasmid. After a further 24 hours the cells were extracted and the levels of activated phospho-ERK analysed by western blotting. The results obtained are shown in Figure 4A. As can be seen, in the absence of hScrib, siRNA PP1γ has minimal effect on the levels of phospho-ERK (Figure 4A upper three panels). In contrast, overexpression of hScrib significantly reduces the levels of phospho-ERK (Figure 4A lower five panels), and this is in agreement with previous studies [19]. However, the ability of hScrib to down-regulate the levels of phospho-ERK is largely abolished following treatment with siRNA PP1γ, suggesting that this activity of hScrib is in part PP1γ-dependent. To further investigate this, we repeated the assay using the PKA phospho-mimic mutant (S1445D) and the non-PP1γ binding mutant (KADA) of hScrib. After 24 hours the levels of phospho-ERK were analysed by western blotting and the results obtained are shown in Figure 4B. As can be seen the wild type and S1445D mutant of hScrib both strongly inhibit the levels of phospho-ERK, whilst the non-PP1γ binding mutant of hScrib is decreased in this activity. Taken together these results demonstrate that the ability of hScrib to interact with PP1γ correlates with its ability to down-regulate the levels of phospho-ERK.

Loss of hScrib enhances PP1γ nuclear localization

Having found that PP1γ plays a role in hScrib regulation of ERK signaling, we were next interested in determining whether hScrib could also potentially affect PP1γ localisation. Therefore, we first analysed the pattern of PP1γ expression in human keratinocytes after stably silencing hScrib expression in these cells. The distribution of PP1γ in control and shScrib HaCaT cells were analysed by immunofluorescence. The results in Figure 5A and Figure 5B, show that most of the PP1γ localises in the nucleus, although some also co-localises with hScrib at the plasma membrane and within the cytoplasm. More importantly, however, upon loss of hScrib expression there is a significant increase in the amount of nuclear PP1γ, with a corresponding decrease in the cytoplasmic pool. In order to verify these results we also performed

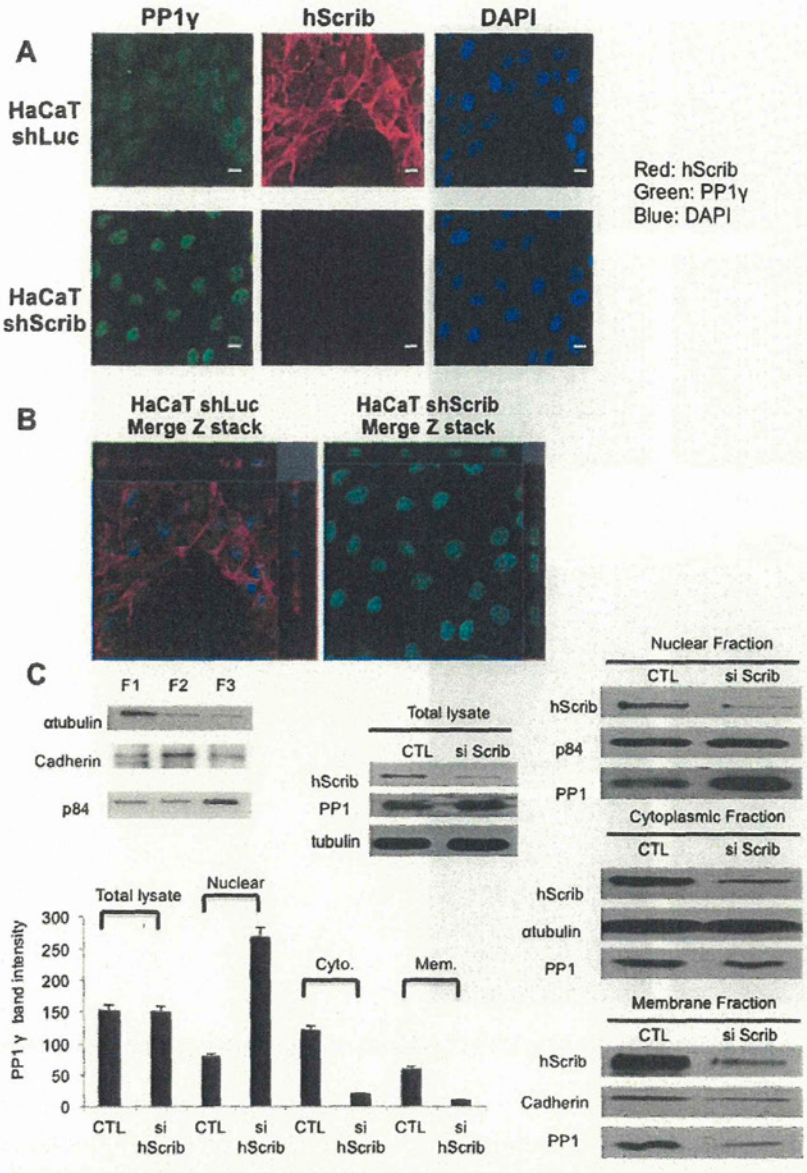

Figure 5. hScrib regulates PP1γ nuclear localization. A) Immunofluorescence analysis of hScrib and PP1γ expression in sh-Luc control HaCaT cells and sh-hScrib knockdown cells. The cells were grown on coverslips and then fixed and double-stained with the anti-hScrib antibody and the anti-PP1γ antibody. Note the significant increase in the levels of nuclear PP1γ in the absence of hScrib expression. B) Z-reconstruction (x-z direction) of a z-stack (15 planes, z-distance 0.2 μm), showing sh-hScrib knockdown cells have enhanced PP1γ localisation into the nucleus. C) HEK 293 cells were transfected with hScrib siRNA and si Luc RNA as control. Cells were either extracted in SDS PAGE sample buffer (Total lysate) or were fractionated into cytoplasmic (F1), membrane (F2) and nuclear (F3) pools (the example shows the integrity of a typical extraction procedure) and then PP1γ was detected by western blotting. p84 was used as a loading control for the nuclear fraction, cadherin was used as a loading control for the membrane fraction and α-tubulin was used as the loading control for the cytoplasmic fraction and total cell extracts. Note the relative increase in nuclear PP1γ following hScrib knockdown but no overall change in total PP1γ levels.
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a series of transient siRNA experiments, where hScrib levels were ablated in 293 cells, and the levels of PP1γ, both in total cell extracts or in the respective cellular fractions (Fig. 5C), were analysed by western blotting. As can be seen, loss of hScrib resulted in decreases in the cytoplasmic and membrane pools of PP1γ, but a corresponding increase in the amounts of the nuclear form of the protein.

To investigate the pattern of pERK expression following hScrib depletion we repeated the immunofluorescence assays staining for hScrib, PP1γ and pERK. The results obtained are shown in Figure 6. As can be seen, under conditions of hScrib depletion there is a marked increase in the levels of both nuclear and cytoplasmic pERK, consistent with previous observations [19]. This is also accompanied by an increase in the levels of nuclear PP1γ.

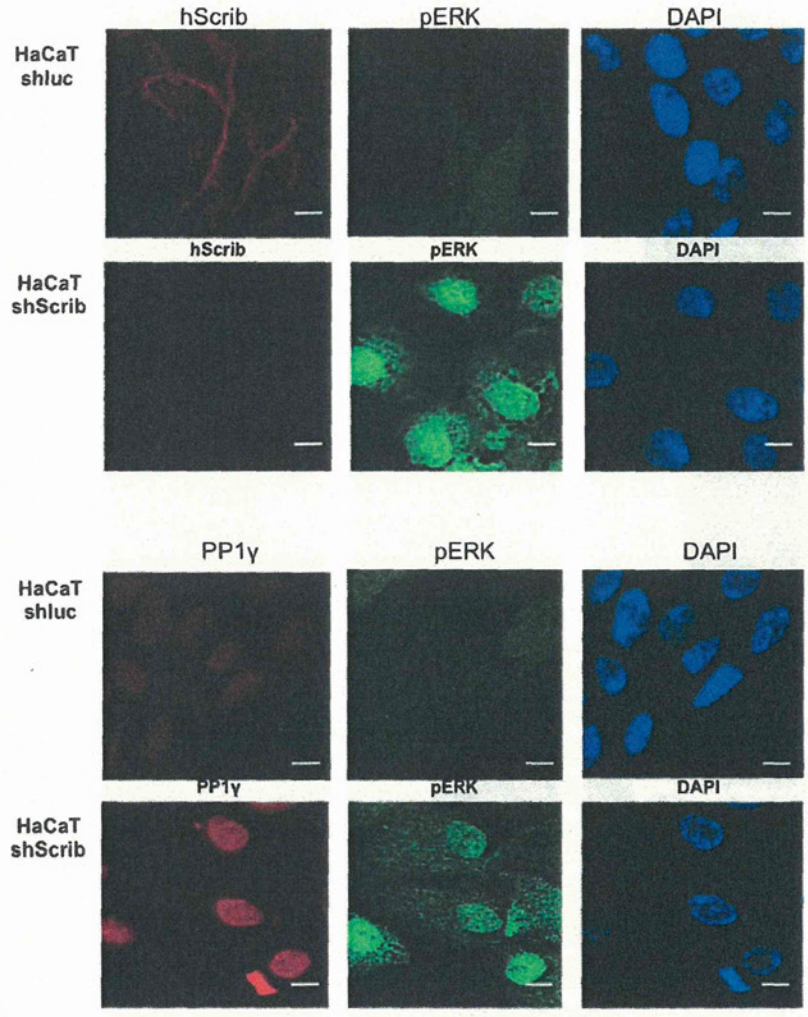

Figure 6. Loss of hScrib results in enhanced nuclear accumulation of both PP1γ and pERK. Control and shScrib HaCaT cells were stained for hScrib, phospho-ERK and PP1γ as indicated.
doi:10.1371/journal.pone.0053752.g006

hScrib tumour suppressor activity requires an intact PP1γ binding motif

We have previously shown that hScrib can suppress cell transformation induced by EJ-ras and Human Papillomavirus (HPV)-16 E7 [19]. To determine whether the interaction between hScrib and PP1γ was physiologically relevant in this context, primary BRK cells were transfected with HPV-16 E7 plus EJ-ras in the presence or absence of the hScrib wild type and KLDY/KADA mutant hScrib expressing plasmids, with or without the PP1γ expression plasmid. After 3 weeks the cells were fixed and stained and the numbers of colonies counted. As can be seen from Figure 7, co-expression of wild type hScrib and PP1γ strongly inhibits the oncogene cooperation between E7 and EJ-ras, whilst the KADA mutant of hScrib is compromised in this activity. These results demonstrate that the hScrib-PP1γ interaction is functionally relevant in an assay of oncogene cooperation.

Discussion

We have shown previously that hScrib can regulate ERK signaling in two ways. The first involves a direct protein interaction, which is mediated via two KIM binding sites located within hScrib. The second appears to involve the recruitment of a protein phosphatase [19]. In this study we provide evidence that a candidate phosphatase is PP1γ. We have also found that hScrib can control PP1γ sub-cellular localisation, with a loss of hScrib promoting PP1γ nuclear translocation.

Regulation of the ERK signaling cascade can occur at multiple levels and can involve Raf dephosphorylation, MEK1,2 phosphorylation, and also MEK1,2 dephosphorylation [24,43–44]. Furthermore, it has been reported that whilst the kinases in the pathway control signal amplitude, the phosphatase PP2A mediates both signal amplitude and signal duration [32–33]. Previous studies have also implicated PP1 in regulating ERK signaling through its ability to dephosphorylate Raf-1 at Ser 259 [45]. Since we have consistently observed that overexpressed hScrib results in a decrease in ERK phosphorylation, we initiated a series of studies to identify the potential phosphatases with which hScrib might interact. Using a proteomic approach we identified PP1γ as a direct interacting partner of hScrib, an interaction that we could confirm both in vitro and in vivo. Analysis of the hScrib amino

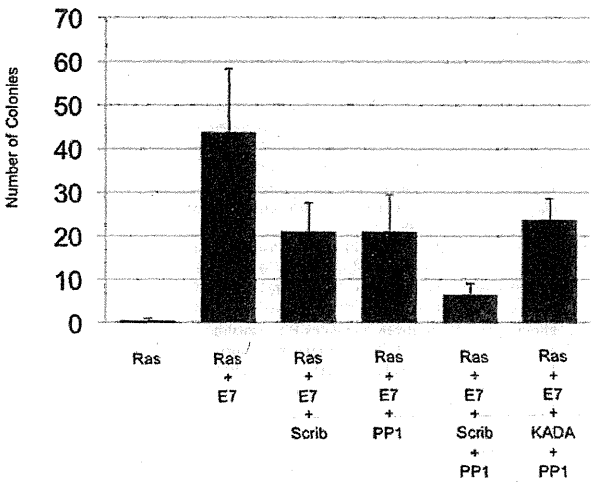

Figure 7. hScrib suppresses HPV-16 E7 and EJ-ras induced transformation in cooperation with PP1γ in a RVxF motif-dependent manner. BRK cells were transfected with EJ-ras alone, HPV-16 E7 plus EJ-ras, HPV-16 E7 plus EJ-ras and wild type hScrib, HPV-16 E7 plus EJ-ras and PP1γ, and HPV-16 E7 plus EJ-ras and wild type hScrib with PP1γ, and HPV-16 E7 plus EJ-ras and PP1γ plus the KADA non-PP1γ binding mutant of hScrib. After three weeks the dishes were fixed and stained and the colonies counted. Results represent the mean number of colonies from 3 independent assays and standard deviations are shown.
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acid sequence identified a potential site of interaction, KLDY, mutation of which abolished the ability of hScrib to bind PP1γ. Furthermore, this consensus PP1 recognition motif is conserved in mammalian forms of Scrib, but is absent in Drosophila.

We also analysed the effects of PP1γ ablation upon hScrib control of ERK phosphorylation, and found that loss of PP1γ greatly diminished the ability of hScrib to downregulate the levels of phospho-ERK in vivo. Furthermore, we also found that this activity of hScrib was in part dependent upon an intact PP1γ binding site motif. Interestingly, we also noted that the interaction between PP1γ and hScrib was increased following PKA phosphorylation of hScrib, one potential consequence of which is PP1γ-mediated de-phosphorylation of hScrib. Whether this has an important role with respect to other functions of hScrib remains to be determined and is worthy of further study. Taken together these studies demonstrate that hScrib can interact with PP1γ, an activity which appears to play a role in the ability of hScrib to downregulate the ERK signaling pathway. Interestingly, this regulation of ERK by hScrib has many parallels with a recent study showing that hScrib could also regulate Akt signaling [35]. This required hScrib interaction with the phosphatase, PHLPP1, resulting in the de-phosphorylation of Akt. In this case the interaction between hScrib and PHLPP1 requires sequences in the LRR region of hScrib. Thus hScrib could potentially interact simultaneously with multiple protein phosphatases to control diverse signaling pathways. It should also be emphasized that hScrib is a multifunctional protein, and loss of hScrib also results in increased levels of MEK activity, suggesting multiple mechanisms by which hScrib can control ERK signaling [46].

To investigate whether the capacity of hScrib to interact with PP1γ had any physiological relevance, we made use of an oncogene cooperation assay in primary rodent cells. Previous studies had shown that hScrib could suppress cell transformation induced by HPV-16 E7 and EJ-ras in these cells, and that this activity was dependent in part upon the ability of hScrib to interact with ERK [19]. We reasoned that this activity of hScrib might also be influenced by the ability of hScrib to interact with PP1γ. Indeed, both hScrib and PP1γ, either alone or in combination, could dramatically decrease the levels of HPV-16 E7 and EJ-ras induced cell transformation. However, the additive effects upon the levels of cell transformation, seen with the combination of hScrib and PP1γ, was abolished if a mutant hScrib defective in its ability to interact with PP1γ was included in the assay. This demonstrates that, in the context of an oncogene cooperation assay, the ability of hScrib to interact with PP1γ does play a role in the ability of hScrib to suppress cell transformation.

PP1γ has been linked to the regulation of a variety of different cellular processes, including the DNA damage response, nuclear function and diverse aspects of the cell cycle [47–52]. One of the important aspects of PP1γ regulation is believed to be related to the control of its nuclear expression, which can be mediated by proteins possessing the consensus RVxF PP1 binding motifs, and which can thereby control the correct cellular localization of PP1 [42,52]. We therefore investigated whether hScrib might have a similar potential regulatory function with respect to the pattern of PP1γ localization within the cell. This was indeed found to be the case; in two different assay systems we observed that loss of hScrib resulted in an increased nuclear accumulation of PP1γ, with a concomitant decrease in the levels found in membrane and cytoplasmic fractions. Thus hScrib would appear to contribute directly to the regulation of PP1γ expression patterns. Whether this is related to some of hScrib's previously reported pleiotropic effects upon cell proliferation and cell survival remains to be determined. Taken together, these studies have defined PP1γ as a novel interacting partner of hScrib, an interaction which correlates with hScrib downregulation of ERK signaling and suppression of oncogene-induced cell transformation.

Supporting Information

Figure S1 Schematic diagram showing the different hScrib expression constructs. The schematic shows the arrangement of the functional domains on the hScrib protein, highlighting the LRR, and PDZ domains. The putative PP1-binding site, KLDY is also shown in the carboxy terminal third of hScrib. Also summarized are the results on the interaction assays with PP1γ.
(TIF)

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Author Contributions

Conceived and designed the experiments: KN LB. Performed the experiments: KN TS AY VKS CK PM MT. Analyzed the data: KN LB. Contributed reagents/materials/analysis tools: KN KK SN TY YT TF SK. Wrote the paper: KN LB.

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Therapeutic Human Papillomavirus (HPV) Vaccines: A Novel Approach

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Abstract: Cervical cancer is the second largest cause of cancer-related death in women worldwide, and it occurs following persistent infection, sometimes for decades, with a specific subset of human papillomavirus (HPV) types; the approximately 13 oncogenic subtypes. Prophylactic vaccines against HPV infections hold promise for cost-effective reductions in the incidence of cervical cancer, but this may not be enough. Two prophylactic HPV vaccines are presently available and both contain L1 virus-like particles (VLPs) derived from the HPV subtypes most frequently associated with cervical cancer, HPV-16 and -18. Since the L1-VLP vaccines can only effectively prevent infection by the specific HPV subtype against which the vaccine was developed, cervical cancers caused by high-risk HPV subtypes other than HPV-16 and -18 may still occur in recipients of the current HPV vaccines. Furthermore, HPV vaccination coverage for adolescents is insufficient in most countries and therefore even HPV-16 and -18 infections are unlikely to be fully eradicated using the existing strategies. The development of HPV therapeutic vaccines remains essential. Many therapeutic vaccines aimed at clearing HPV-related cervical lesions have been developed and tested in patients with HPV16-positive cervical intraepithelial lesions (CIN) or cervical cancers. To date, definitive clinical efficacy and appropriate immunological responses have never been demonstrated for cervical neoplasia although promising results have been reported in patients with vulvar intraepithelial neoplasia. Here we discuss shortcomings of previous HPV therapeutic vaccine candidates and propose a novel vaccination strategy that leverages newly gained knowledge about mucosal immunity and the induction of mucosal immune responses.

Keywords: HPV therapeutic vaccine, mucosal vaccination, cervical mucosal immune system, E7-expressing lactobacillus-bases vaccine.

EPIDEMIOLOGY OF HPV INFECTION

At present, there are about 100 identified genotypes (types) of human papillomavirus (HPV) of which about 40 are genital HPV types that invade genital organs such as the uterine cervix, vaginal wall, vulva, and penis. Genital HPV types are classified into high-risk types commonly associated with cervical cancer and low-risk types known to cause condyloma acuminatum. This classification varies among researchers, but, in general, types 16/18/31/33/35/39/45/51/52/56/58/66/68 are classified as high-risk and 6/11/40/42/43/44/54/61/72 as low-risk [1]. Interestingly, the HPV type distribution varies depending on the stage of cervical neoplasia (Fig. **1**).

The HPV DNA detection rate in the genital organs of healthy adult females varies between advanced and developing countries but is approximately 20-40% collectively [2, 3]. In Japan, the HPV-positive rate in pregnant females aged 20-29 years has been reported to be 20-30%, which is similar to or higher than that among similarly aged females in the U.S [4]. The World Health Organization (WHO) has estimated an annual increase of 3 hundred million in the number of HPV carriers in the world [5, 6]. Overall HPV prevalence with normal cervical cytology was estimated to be 10.4 % [6]. Epidemiological data show HPV infection at least once during their lifespan in approximately 75 % of U.S. women [3]. Thus, HPV infection is common and can affect any female. Frequent sexual activity has been reported to increase the risk of HPV infection but this is not always the case [7].

NATURAL HISTORY OF CERVICAL INTRAEPITHELIAL NEOPLASIA

Natural history studies of CIN show that most infections and CIN lesions resolve spontaneously but some persist and progress to cervical cancer. The incidence of cervical intraepithelial neoplasia (corresponding to squamous intraepithelial lesion: SIL) is about 1 per 10 females with HPV infection [8]. The incidence of high grade SIL (corresponding to cervical intraepithelial neoplasia 2 and 3: CIN2-3) is about 3 per 10 females with low grade SIL, and that of CIN3 is about 1-2 per 10 females with low grade SIL [9]. Without treatment, the incidence of the progression of CIN3 to cervical cancer is about 30% [10]. Therefore, the incidence of the spontaneous development of cervical cancer is about 1 per 200-300 females with HPV infection. Factors associated with progression to cervical cancer in females with HPV infection have been extensively studied [1]. Many prospective studies have identified persistent HPV infection as the most important risk factor. They have also shown that persistent infection tends to occur in women with high risk HPV subtypes.

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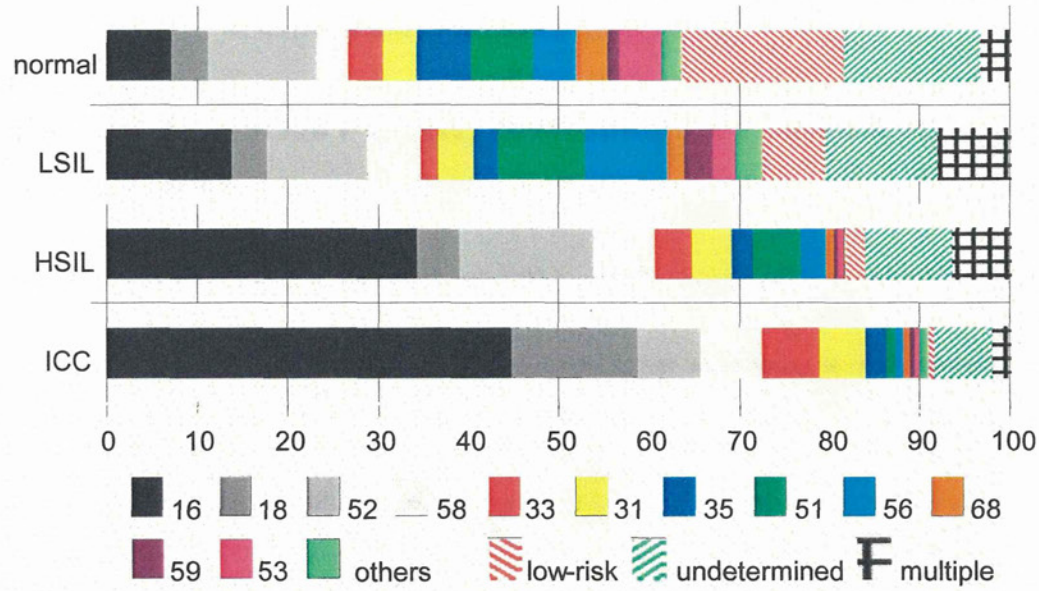

Fig. (1). HPV subtype distribution in cervical neoplastic lesions in Japan [18]. HPV16 and 18 are the most common subtypes found in invasive cervical cancer (ICC) but more than 40% of invasive lesions are associated with other oncogenic subtypes in Japan. HPV52 is the most common HPV subtype present among Japanese women with with normal cervical cytology [19].

Chronic virus proliferation induces the active proliferation/differentiation of infected epithelial cells, and some infected cells incidentally immortalize, which is the first step of carcinogenesis [1]. In contrast, transient infection involves short-term virus proliferation followed by the long-term latent presence of low copies of the viral genome in the basal cells of the genital epithelium [11]. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions [12, 13] suggest that cell-mediated immune response against HPV antigens is important in the control of HPV infection and progression to CIN. More controversial are the relative roles of systemic and local mucosal immune responses in HPV pathogenesis [14]. Trimble *et al.* reported that naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN 2/3 lesions [15] but Nakagawa *et al.* demonstrated a positive association between systemic cell-mediated immune responses to HPV E6 and the regression of HPV/CIN [16].

SHORTCOMINGS OF THE CURRENT L1-VLP VACCINES

Theoretically, if HPV infection could be completely eradicated, HPV-associated cancers could be prevented. With this in mind, HPV vaccines began to be studied nearly 10 years ago. In 2002, Koutsky *et al.* were the first to show the clinical prophylactic effects of an HPV vaccine [17]. Soon thereafter, Merck in the United States and Glaxo Smith Kline (GSK) in Europe launched full-scale development of prophylactic vaccines against HPV. These products were approved and became commercially available just a few years ago. The vaccine antigens used by the two companies are virus-like particles (VLP) produced by overexpressing HPV16 L1 protein in yeast or insect cells. These particles have a 3-dimensional external structure similar to that of virus particles, but having no internal contents, they are not infective. The vaccine first reported by Koutsky *et al.* also used HPV16L1-VLP as an antigen.

One integral drawback of L1-VLP based vaccines is their negligible prophylactic effect on many HPV subtypes not specifically targeted by the vaccine [18]. For this reason, GSK and Merck developed cocktail vaccines composed of L1-VLPs corresponding to several HPV subtypes. The vaccine developed by Merck is a quadrivalent vaccine against HPV types 6, 11, 16, and 18 (Gardasil®) [19] and that developed by GSK is a bivalent vaccine against types 16 and 18 (Cervarix®) [20]. Unfortunately these L1-VLP vaccines are very specific and may not protect for long time against HPV types that exhibit very close genetic similarities to HPV-16 or -18, such as HPV-58 or -45 respectively. Ultimately, the most effective L1-VLP-based vaccines vaccines would be multivalent for the 13 described oncogenic HPV types. Such prophylactic vaccines would likely be much more expensive than their current counterparts.

HPV-16 or -18-related cervical cancers, which constitute less than 60% of all invasive cervical cancer cases in Japan [21], could be prevented if the appropriate subtype cocktail vaccine were available (Fig. **1**). However, the HPV subtype distribution in cervical cancer varies (60-70%) by worldwide location [22] and current vaccines are unable to address all oncogenic subtypes in even a single population. While current HPV vaccines are distributed without cost to the patient due to government subsidies or full coverage by insurance [23] these facile approaches will ultimately fail to eradicate the disease. Further, even with broad vaccination coverage, deficiencies in vaccine design mandate that even vaccinated females must continue cervical cancer screening.

The commercially available GSK and Merck HPV vaccines are indicated for uninfected females to prevent

HPV infection/spread. Due to the high prevalence of HPV infection, effective mass prophylactic vaccination strategies for uninfected females should include girls age 10 and above to predate the onset of sexual activity. Ph-III clinical studies in which females approximately 20 years of age were randomly inoculated with Gardasil® or Cervarix® revealed protective efficacy on the development of CIN2-3 associated with HPV-16 or -18 in 93-98% of vaccine-type naïve females who completed the vaccination protocol [24, 25]. However, intention-to-treat analysis revealed protective efficacy was only 19-30% for non-vaccine HPV subtypes [24, 25].

DEVELOPMENT OF HPV THERAPEUTIC VACCINES

The limitations of current prophylactic HPV vaccines demonstrate a pressing need for novel approaches to the eradication of HPV-related neoplasia and suggest that the development of therapeutic vaccines for the treatment of HPV-associated lesions will remain an important goal even if worldwide prophylactic vaccine programs are successfully implemented [26]. The past two decades has seen several inroads into the development of therapeutic HPV vaccines. The combined actions of the high-risk E6 and E7 oncoproteins are essential for the maintenance of the neoplastic phenotype and the evasion of apoptosis. Several functions have been described for E6 and E7. Initial observations revealed that E6 interacts with p53 and E7 interacts with Rb to block the activity of these tumour suppressors [1]. There are only two possible antigenic targets, E6 and E7, since these are the only viral proteins that will be expressed in all cancers and precursor lesions [1]. The approach of deliberate immunization with E6 and/or E7 of HPV 16 and 18 predominantly, and the generation of antigen-specific CTL as an immunotherapy for HPVassociated cancer has been tested with a wide array of potential vaccine delivery systems. Here we will summarize the results of the therapeutic vaccine clinical trials reported to (Table **1**) [14].

1. SGN-00101 (s.c.) is a fusion protein consisting of a heat shock protein (Hsp) from *Mycobacterium bovis* and HPV16 E7. The Ph-II study looking at the effects of SGN-00101 in women with CIN3 revealed histological regression to CIN1or less (complete remission: CR) in 13 (22.5%) of 58 cases, although immunological responses were not studied [27]. Another Ph-II study of the same agent administered to women with CIN showed the induction of cytotoxic T lymphocyte (CTL) against HPV16E7 in peripheral monocytes in 5 of 7 patients which obtained CR [28].
2. L1VLP-E7 (s.c.) is a vaccine using chimeric particles composed of HPV16 L1-VLP and E7. In the Ph-I/II study of women with CIN2-3, histological regression to CIN2 (partial remission; PR) was shown in 39% of vaccine recipients compared with 25 % of placebo recipients. This was not significant significant [29]. Clinical response was coupled with detectable cellular immune responses in some cases.
3. TA-HPV (i.m.) is a recombinant vaccinia virus expressing E6 and E7 of HPV-16 and -18. The Ph-II study of TA-HPV in women with vulvar intraepithelial neoplasia (VIN) revealed PR of lesions in 8 of 13 cases and responders also had an increase in lesion-infiltrating CD4 and CD8 positive cells [30].
4. TA-CIN (i.m.) is a fusion protein consisting of E6, E7 and L2 from HPV-16 and -18. The Ph-II study in women with VIN revealed CR or PR in only 6 of 29 cases. CTL against E6/E7 were induced in 4 of 29 cases [31]. Correlations between clinical efficacy and cellular immune responses to the vaccine remain unclear.
5. MVA-E2 (TGA4001) (intrauterine) is also a recombinant vaccinia virus expressing bovine papilloma virus (BPV) E2. A Ph-II study in subjects with CIN2-3 confirmed the down grade of CIN in some cases (19/34 cases) [32].
6. ZYC-101a (i.m.) is a DNA vaccine synthesized from proteins containing CTL epitopes against E6 and E7 of HPV-16 and -18. A Ph-III study was performed in subjects with CIN2-3. CR or PR was observed in 41% of vaccinated women and 27% of those receiving placebo. This was not a significant difference. Subset-analysis limited to those subjects aged 25 years or less revealed a statistically significant increase in the percentage of women with CR or PR in the vaccination group (72%) when compared to placebo controls (23%). However, no correlation was shown between CTL induction against E6/E7 and clinical effect [33].

Table 1. Clinical Trials of Therapeutic Vaccine for HPV-Associated Cervical Lesion

Trial Phase	Target Proteins	Vaccine Vectors	Inoculation	Target Types
Ph-I/II [27]	L1, E7	Chimera-VLP	S.C.	16
Ph-II [26]	E7	Hsp (SGN-00101]	S.C.	16
Ph-II [28]	E6, E7	Vaccinia virus (TA-HPV)	I.M.	16, 18
Ph-II [29]	L2, E6, E7	Fusion protein L2E6E7 (TA-CIN)	I.M.	16, 18
Ph-II [30]	BPV E2	Vaccinia virus (MVA-E2]	intrauteral	all
Ph-III [31]	E6, E7	plasmid vaccine (ZYC101a)	I.M.	16, 18
Ph-II [32]	E6, E7	Cocktailed Synthetic peptide	S.C.	16

S.C.: subcutaneous injection, I.M.: intramuscular injection, BPV: bovine papillomavirus.

7. Synthetic long-peptide vaccine (s.c.) is a peptide vaccine comprised of nine HPV16 E6 peptides and four HPV16 E7 peptides solubilized in incomplete Freund's adjuvant. A Ph-II study was performed in patients with VIN3. 5 of 20 patients demonstrated complete regression of their lesions [34].

In summary, no therapeutic HPV vaccines are presently available that exert significant clinical efficacy against CIN. Some of the tested therapeutic vaccines elicited systemic cellular immunity after intramuscular or subcutaneous injection, but none of the trials have assessed local cellular immune responses to vaccine antigen in the cervix.. The outcomes of vaccination strategies involving intramuscular or subcutaneous injection of E6/E7-based antigens for the treatment of VIN have been more promising [30, 31, 34]. We hypothesize that these findings are the direct result of the predicted poor response of cervical mucosal lesions to systemic cellular immune responses when compared to the effects of systemic immunity on epidermal lesions including those of VIN.

THE CERVICAL MUCOSAL IMMUNE SYSTEM AND HPV THERAPEUTIC VACCINES

Induction of adaptive cellular immune responses to HPV in the cervical mucosa is indispensable for treating cervical mucosal lesions such as CIN. Since precancerous lesion of the cervix develops essentially exclusively in the mucosal epithelium it would be predicted that intraepithelial lymphocytes (IELs) should be central to the elimination of CIN. To this poin, there are substantial differences between cellular and humoral immune responses in the female reproductive tract mucosa. It is well-known that intramuscular injection of L1-VLP based vaccines leads to systemic humoral immune responses characterized by the induction of anti-L1 IgG neutralizing antibody which leaks from the serum to protect the reproductive tract mucosa from HPV infection. However, the requirements for induction of mucosal cellular immune responses against microbial infected lesions differ from and are independent of those for systemic cellular immunity. Therefore, systemic intramuscular or subcutaneous vaccination strategies may be unsuitable for the induction of mucosal cellular immunity, at least in the reproductive tract mucosa.

In the uninduced state, the specific lymphocytes involved in mucosal immunity reside in the inductive sites of organized mucosa-associated lymphoid tissues (MALT); these are present in a variety of effector sites, including the mucosa of the intestine, respiratory tract and genital tract [35]. Efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin $\alpha4\beta7$ and C-C chemokine receptor type 9 (CCR9). Lymphocyte-expressed integrin $\alpha4\beta7$ and CCR9 bind to their natural ligands, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCL25 (TECK), respectively, which are expressed on the cell surface of endothelial cells in submucosal post-capillary venules. In the intestine, mucosal dendritic cells (DCs) in gut-associated lymphoid tissues (GALT) regulate the expression of integrin $\alpha4\beta7$ on activated effector and regulatory lymphocytes in a retinoic acid-dependent manner [36]. Integrin $\alpha4\beta7$+ T cells reside the lamina propria in submucosa as lamina propria lymphocytes (LPL) and can differentiate into integrin $\alpha E\beta7$+ T cells upon exposure to TGF-β and expression of integrin $\alpha E\beta7$ facilitates retention of lymphocytes in the epithelium *via* interactions with E-cadherin [37] (Fig. **2**). Integrin $\alpha E\beta7$ is a specific marker of IELs residing in mucosal epithelia and those cells expressing this antigen on their surface were initially educated in the gut.

Several studies have demonstrated that human genital tract mucosa expresses MAdCAM-1 endogenously [38] and that GALT-derived integrin $\alpha4/E\beta7$+ T cells home to the genital mucosa [39-41]. This T cell homing and the expression of integrin αE increase in the presence of cervicitis and vaginitis [39, 40]. Although integrin $\beta7$+ mucosal T cells have been found in the cervical mucosa, a local inductive site (i.e., MALT) has never been demonstrated histologically [39, 40]. Taken together, GALT is thought to act as the inductive site for cervical IELs. GALT and the cervical mucosal connect through mucosa-specific T cells which express the homing receptors, integrin $\beta7$ and/or CCR9. Using flow cytometry, we have demonstrated that 25-30% of CD3-positive mucosal cervical lymphocytes are positive for the homing receptors integrin $\beta7$ and CCR9 and are thereby educated in GALT [41]. Approximately half of the integrin $\beta7$-positive T cells are CD45RO memory T cells while the other half are CD45RA effector T cells. Accumulation of integrin $\alpha E\beta7$+ IEL in CIN lesions varies markedly among patients and higher IEL numbers are associated with spontaneous regression of CIN [41]. These and related investigations have dramatically improved our understanding of cervical mucosal immunity which should hasten the development of a therapeutic HPV vaccine.

ORAL ADMINISTRATION OF HPV THERAPEUTIC VACCINES: A NOVEL APPROACH

Mucosal vaccination *via* oral administration of vaccine antigen is an effective method for the induction of mucosal immunity. Bermudez-Humaran *et al.* have evaluated the induction of CTL activity and the prevention/reduction of tumor formation following nasal or oral administration of live lactobacillus engineered to produce lactic acid-expressing HPV16E7 and IL-12, in tumor challenged murine models [42]. They found more marked induction of mucosal responses after nasal *vs* oral administration and a more effective induction of immunity when using *Lactobacillus plantarum vs Lactococcus lactis* [43]. Poo *et al.* have shown that oral immunization of C57BL/6 mice with *Lactobacillus casei* expressing HPV16 E7 reduces tumor formation induced by TC-1 cell administration. Immunization in these experiments elicited type 1 T cell immune responses to E7 in lymphocytes isolated from the spleen and from anogenital regional lymph nodes [44]. Although both studies used transmucosal immunization with Lactobacillus-based vaccines, they examined E7-specific systemic cellular immune response and regression of subcutaneous TC-1-induced tumors. These investigations provide no insight into mucosal cellular immune responses after immunization nor into the antigen specificity of mucosal lymphocytes. We have observed a marked induction of mucosal T cells possessing HPV16 E7-specific cellular immune recognition (E7-CMI) within intestinal mucosa after oral administration of *Lactobaccilus casei* expressing HPV16 E7 in mice [45].