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Human scribble, a novel tumor suppressor identified as a target of high-risk HPV E6 for ubiquitin-mediated degradation, interacts with adenomatous polyposis coli

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Recently, we have identified human scribble (hScrib), human homolog of the *Drosophila* tumor suppressor Scribble, as a substrate of human papillomavirus E6 oncoproteins for ubiquitin-mediated degradation dependent on ubiquitin-protein ligase E6AP. Human Scribble, classified as a LAP protein containing leucine-rich repeats and PDZ domains, interacts with E6 through its PDZ domains and C-terminal PDZ domain-binding motif of E6 protein. Interaction between human Discs Large (hDlg), which is a substrate of E6 for the ubiquitin-mediated degradation, and adenomatous polyposis coli (APC) has been shown. Here, we investigated whether hScrib and APC interact with each other *in vitro* and *in vivo*. Interaction between hScrib and APC is mediated by the PDZ domains 1 and 4 of hScrib and C-terminal PDZ domain-binding motif of APC. Human Scribble co-localized with APC at the synaptic sites of hippocampal neuron and at the tip of membrane protrusion in the epithelial cell line. Interference of the interaction between hScrib and APC caused disruption of adherens junction. Knockdown of hScrib expression by RNAi disrupts localization of APC at the adherens junction. These data suggest that hScrib may participate in the hDlg-APC complex through its PDZ domains and regulate cell cycle and neural function by associating with APC.

Introduction

Epithelial cells are characterized by a regular columnar or cuboidal shape with defined apical-basal polarity (Peifer & Tepass 2000; Bissell & Radisky 2001; Muller & Bossinger 2003). During the development of neoplastic tumors from their precursor lesions to invasive cancers, epithelia lose their regular cell shape, defined apical-basal polarity, and tissue architecture. Formation of the two integral junctions, the tight junction and the adherens junction, is required for vertebrate epithelial cells to establish the cell polarity (Bryant & Huwe 2000; Muller & Bossinger 2003). Recently, Bilder & Perrimon (2000) reported that *Drosophila* tumor suppressor Scribble which localizes at the septate junction, a structure equivalent to the vertebrate tight junction, serves as an apical-basal polarity determinant in epithelial cells. Loss of *scribble* mutation causes disruption of the cell polarity and leads to the overgrowth of epithelial cells in the imaginal discs, follicle, and brain in *Drosophila* (Bilder *et al.* 2000b; Greaves 2000; Peifer 2000; Wodarz 2000). Human Scribble (hScrib), a human homolog of *Drosophila* Scribble,

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was identified as a substrate of human papillomavirus (HPV) E6 oncoprotein for the ubiquitin-mediated degradation dependent on E6AP, ubiquitin-protein ligase (Nakagawa & Huibregtse 2000). Human Scribble has two typical protein-protein interaction domains: leucine-rich repeats (LRRs) and PDZ-domains (Nakagawa & Huibregtse 2000). Recently, the proteins with 16 canonical leucine-rich repeats (LRRs) and 1 or 4 PDZ-domains are grouped as LAP (leucine-rich repeats and PDZ-domains) proteins (Bilder *et al.* 2000a; Bilder & Perrimon 2000; Bryant & Huwe 2000). LAP proteins share common features such as the basolateral localization and apical-basolateral polarity determination in epithelial cells (Bryant & Huwe 2000). In addition to hScrib, Densin-180 (Apperson *et al.* 1996; Izawa *et al.* 2002; Ohtakara *et al.* 2002), Erbin (Borg *et al.* 2000; Huang *et al.* 2001; Jaulin-Bastard *et al.* 2002; Laura *et al.* 2002), and Lano (Saito *et al.* 2001) have joined the list of mammalian LAP proteins. Erbin, which has one PDZ domain, was shown to bind to EGF-receptor ErbB2/HER (Borg *et al.* 2000). Loss of binding domain of ErbB2/HER causes the mis-localization of Erbin, indicating the *in vivo* interaction between these two proteins (Borg *et al.* 2000).

The genetic study on *Drosophila* established an interaction among the three tumor suppressors, Scribble, Discs large (Dlg) and Lethal giant larvae (lgl) (Bilder *et al.* 2000b). Loss of *dLg* or *lgl* mutant resulted in loss of apical-basolateral polarity and massive overgrowth of epithelial cells, as was observed with *scribble* mutation (Bilder *et al.* 2000b). These data indicate that these tumor suppressors act cooperatively in a common pathway to regulate cell polarity and tissue growth (Bilder *et al.* 2000b; Bilder *et al.* 2003). Human Dlg (hDlg) was shown to be a substrate of high-risk HPV E6 for the ubiquitin-mediated degradation (Gardiol *et al.* 1999; Mantovani *et al.* 2001). The interaction between these two proteins needs the PDZ domains of hDlg and the C-terminal S/T-X-V/L motif conserved among the high-risk but not the low-risk E6 proteins (Kiyono *et al.* 1997). The PDZ domains of hDlg were reported to interact with the C-terminal region of the tumor suppressor adenomatous polyposis coli (APC) (Matsumine *et al.* 1996). The high-risk HPV E6 and APC share the class 1 PDZ-binding motif, Threonine/Serine-X-Leucine/Valine at their C-terminus (Morais Cabral *et al.* 1996; Kiyono *et al.* 1997). We recently reported that the interaction between hScrib and E6 depends on PDZ domains of hScrib and the conserved C-terminal motif (Threonine-X-Leucine/Valine) among the high-risk HPV E6 proteins (Nakagawa & Huibregtse 2000), as the interaction between hDlg and the high-risk HPV E6 does (Kiyono *et al.* 1997). These data suggest the possibility that hScrib binds to APC, a regulator of cell proliferation and cell cycle (Ishidate *et al.* 2000). If it is the case, it gives us a clue to solve an unanswered question how scribble determines cell polarity and exerts its tumor suppressive effect during the establishment of tissue architecture of epithelia. Here, we demonstrate that hScrib binds to APC *in vitro* and *in vivo* and thereby is possibly involved in the control of cell proliferation by interacting with APC.

Results

Specificity of the anti-hScrib PDZ domains 1–4 antibody

The specificity of the affinity-purified anti-hScrib PDZ domains 1–4 was examined by Western blotting. The anti-hScrib PDZ domains 1–4 polyclonal antibody detected a sharp band around 220 kDa, which corresponds to the *in vitro* translated hScrib in the rabbit reticulocyte lysate and the endogenous hScrib in 293-T cell lysate (Fig. 1B, lanes 2 and 3). In contrast, the anti-hScrib PDZ domain antibody did not react to the *in vitro* translated hDlg, another PDZ domain containing protein, in the rabbit reticulocyte lysate (Fig. 1B, lane 1). These data indicate that the anti-hScrib PDZ domain antibody can recognize hScrib specifically.

In vitro interaction between hScrib and APC

To examine which PDZ domain of hScrib binds to the high-risk HPV E6, we expressed each PDZ domain of hScrib as a GST-fusion protein. GST-fusion PDZ domains 1, 3 and 4 of hScrib bound to E6, but GST-fusion PDZ domain 2 did not bind to E6 (Fig. 1D). We compared the amino acid sequence of mucosotropic HPV E6 proteins found in anogenital lesions. The C-terminal of the high-risk HPV type 16, 18, 31, 33, 51, 52 and 58 E6 proteins, but not the low-risk HPV type 11 E6 conserved the PDZ domain-binding motif, Threonine-X-Leucine/Valine (Fig. 1C). The high-risk HPV E6 proteins and the tumor suppressor APC have the conserved C-terminal amino acid sequence Threonine-X-Leucine/Valine, which is essential for interaction with the PDZ domains of hDlg (Fig. 1C) (Matsumine *et al.* 1996; Kiyono *et al.* 1997). We next examined whether the *in vitro* translated APC binds to GST fusion hScrib PDZ domains. The *in vitro* translated APC bound to the PDZ domains 1 and 4 of hScrib (Fig. 1D).

To explore the mechanism of the interaction between hScrib and APC, we investigated *in vitro* binding between these two proteins by the immunoprecipitation. First, we

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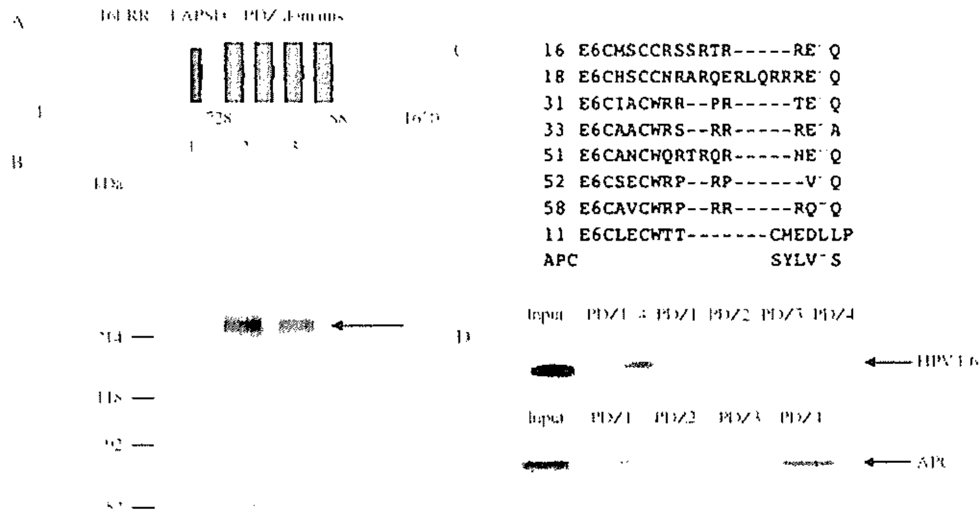

Figure 1 The scheme of the protein structure of hScrib and Western blotting of the *in vitro* translated hScrib and the endogenous hScrib. The high-risk HPV E6 proteins and the tumor suppressor APC share the class 1 PDZ-binding motif, which is essential for the interaction with PDZ domain of hScrib. (A) Human scribble has 16 canonical leucine-rich repeats (LRRs) at its N-terminal region, 4 PDZ domains at its central region, and LAP specific domain (LAPSD) between LRRs and PDZ domains. (B) The Western blotting of hScrib by using the polyclonal antibodies raised against PDZ domains of hScrib. Lane 1, negative control (*in vitro* translated hDlg); lane 2, *in vitro* translated hScrib; lane 3, the endogenous hScrib in 293T cells. (C) Comparison of the C-terminal sequences of the mucosotropic HPV E6 proteins found in the anogenical lesions and the tumor suppressor APC. (D) The identification of the binding domain for the interaction between HPV 16 E6 and hScrib by the GST-pull down assay (upper). The identification of the binding domain for the interaction between APC and hScrib (lower).

examined whether our anti-hScrib PDZ domain and C-terminus antibodies recognize the *in vitro* translated hScrib by immunoprecipitation. The anti-hScrib PDZ domain antibody (Fig. 2A, lane 2) and anti-hScrib C-terminus antibody (data not shown) immunoprecipitated the *in vitro* translated hScrib successfully. The anti-APC C-terminus antibody also immunoprecipitated the *in vitro* translated APC C-terminal 369 amino acids (Fig. 2A, lane 1). We next examined whether *in vitro* translated hScrib and APC make complex *in vitro*. After incubation for 2 h at 4 °C, the mixture of the *in vitro* translated two proteins was analyzed by immunoprecipitation. The anti-hScrib C-terminus antibody immunoprecipitated hScrib and it also co-precipitated APC, confirming our data by GST-pull down assay (Fig. 2A, lane 5). In contrast, anti-APC C-terminus antibody co-precipitated smaller amount of hScrib compared with co-precipitation analysis of the same complex by anti-hScrib C-terminus antibody (Fig. 2A, lane 4), presumably because the epitope of anti-APC C-terminus antibody could overlap with the region interacting with hScrib PDZ domains. The anti-hScrib PDZ domain antibody failed to co-precipitate the APC probably for the same reason (Fig. 2A, lane 3). To test whather APC and E6 are competitive for binding to hScrib, we performed a GST-pull down assay of hScrib, with or without the presence of E6. The E6 interfered the interaction between hScrib and APC (Fig. 2B).

In vivo interaction between hScrib and APC

We examined whether hScrib and APC make complex *in vivo*. We subjected the embryonic mouse brain tissue extract to the immunoprecipitation with the anti-hScrib or the anti-APC antibody. The embryonic mouse brains extract immunoprecipitated with the anti-APC N-terminus antibody was followed by Western blotting with anti-APC, anti-hScrib and anti-β catenin antibodies. The anti-APC N-terminus antibody co-precipitated hScrib and β catenin (Fig. 3), indicating that hScrib is a member of a protein complex containing APC and β catenin. The anti-hScrib C-terminus antibody also co-precipitated APC and β catenin. In contrast, the anti-APC C-terminus antibody and the anti-hScrib PDZ domain antibody co-precipitated negligible amount of hScrib and APC, respectively (Fig. 3). We confirmed that the non-immune immunoglobulin does not immunoprecipitate hScrib or APC (data not shown).

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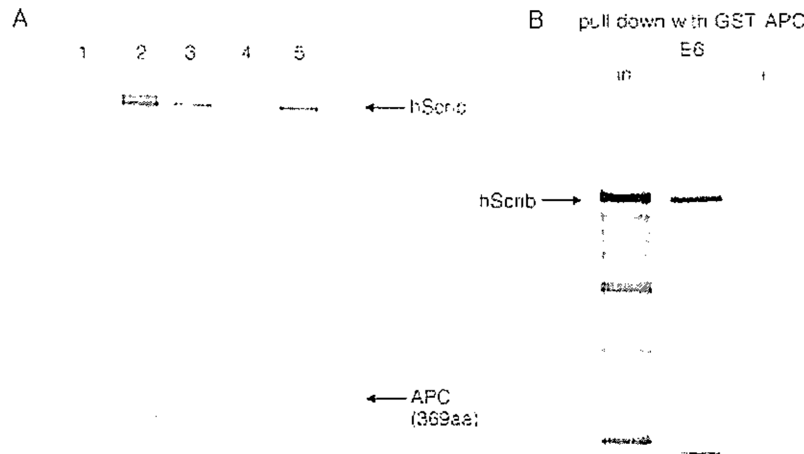

Figure 2 (A) *In vitro* interactions between hScrib and APC through the C-terminal region of APC and PDZ domains of hScrib. Lane 1, immunoprecipitation of the *in vitro* translated APC by anti-APC C-terminus antibody; lane 2, immunoprecipitation of the *in vitro* translated hScrib by anti-hScrib PDZ domain antibody; lane 3, co-precipitation assay of hScrib and APC by anti-hScrib PDZ domain; lane 4, co-precipitation assay of hScrib and APC by anti-APC C-terminus antibody; lane 5, co-precipitation assay of hScrib and APC by anti-hScrib C-terminus antibody. (B) The *in vitro* translated hScrib was analyzed by the GST fusion APC with or without presence of HPV E6.

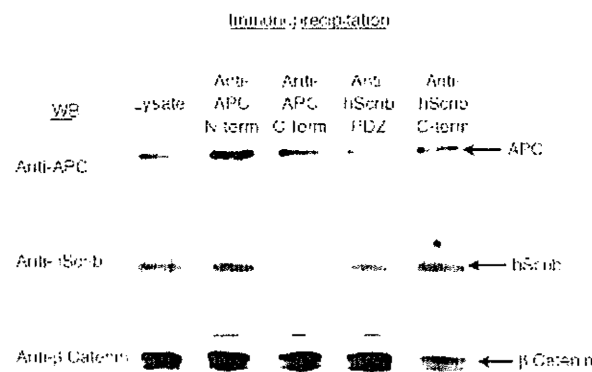

Figure 3 *In vivo* interaction between hScrib and APC. The *in vivo* interaction between hScrib and APC was analyzed using the immunoprecipitation assay of the mouse brain extract followed by Western blotting. Note that the anti-APC C-terminus antibody co-precipitated negligible amount of hScrib, which indicates that C-terminal epitope of APC is masked by the interaction with hScrib or hDlg. For the negative control, the anti-hScrib C-terminus antibody pre-absorbed against the antigen was used for the immunoprecipitation.

To investigate the mechanism by which hScrib interacts with APC, we constructed GST-fusion APC C terminal mutants (Fig. 4A,B) and examined whether these mutants associate with hScrib. These GST fusion APC C terminal mutants were incubated with 293T cell extract and those binding ability to hScrib was analyzed by Western blotting. While GST fusion APC C terminal 369 and 72 amino acids associated with hScrib, these GST fusion proteins lacking three C terminal amino acids, Threonine-Serine-Valine (GST APC C369DTSV and APC C72D TSV), did not associate with hScrib (Fig. 4C,D). To explore which of these three amino acids is critical for the interaction with hScrib, we constructed GST fusion APC mutants with a single amino acid substitution (Fig. 4A). GST fusion APC V2843Q, which has a Glycine substitution for Valine at amino acid 2843, lost the ability to associate with hScrib (Fig. 4C,D). GST fusion APC T2841L, which has a Leucine substitution for Threonine at amino acid 2841, and APC ΔV, which has a deletion of 2843, Valine, also lost the association with hScrib. In contrast, GST fusion APC S2842L, which has a Leucine substitution for Serine at amino acid 2842, still associated with hScrib. These data provided the evidence that APC interacts with hScrib through its C terminal class 1 PDZ domain-binding sequence Threonine-X-Valine, which is shared by the C terminal sequences of the high-risk HPV E6 proteins.

Co-localization of hScrib and APC in the epithelial cells and cultured rat hippocampal neurons

To further explore the *in vivo* interaction between hScrib and APC, we examined whether these two proteins show the co-localized expression in cultured MDCK cells. APC accumulates at their plus ends along the microtubules and associates with membrane protrusions (Mimori-Kiyosue *et al.* 2000). In the fully confluent and polarized culture condition, hScrib showed the membrane-associated localization, as we previously described (Nakagawa *et al.* 2004). In the growing MDCK cells, hScrib localized at the membrane protrusions, where the tumor suppressor APC localized (Fig. 5B-1). To confirm our results of the *in vivo* association of hScrib with APC

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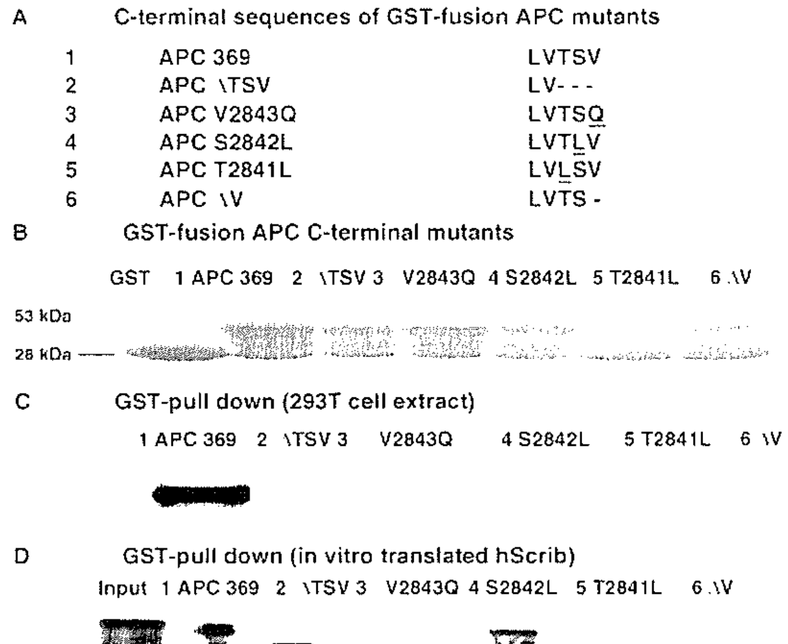

Figure 4 The class 1 PDZ- binding motif of APC is essential for the association with hScrib. (A) The alignment of amino acid sequences of the GST-fusion APC C-terminal mutants. (B) The amount of GST fusion APC C-terminal constructs is shown. (C) GST-pull down assay of the 293T cell extracts using the APC C-terminal region mutants followed by Western blotting with the anti-hScrib PDZ domain antibody. The C-terminal 72 amino acids of APC showed the binding to hScrib, but the same construct lacking the last three amino acids lost the ability to interact with hScrib. Threonine at amino acid 2841 and Valine at amino acid 2843 of APC are critical for the association with hScrib. (D) GST-pull down assay of the *in vitro* translated hScrib using the APC C-terminal region mutants. Threonine at amino acid 2841 and Valine at amino acid 2843 of APC are again critical for the association with the *in vitro* translated hScrib.

and β catenin, we analyzed the co-localization between hScrib and β catenin. The β catenin also localized at the membrane protrusions in the growing MDCK cells and showed the co-localization with hScrib (Fig. 5C-1).

Because APC is highly expressed in the central nervous system and scribble is also related to the neural function (Senda *et al.* 1998; Li *et al.* 2001; Mathew *et al.* 2002; Roche *et al.* 2002; Albertson & Doe 2003; Murdoch *et al.* 2003), we analyzed the localization of both proteins in the rat hippocampal neurons. APC showed the highly condensed dot-like expression at the synaptic sites along the dendrites, where the synaptic membrane protein synaptotagmin localizes beside the cell bodies of the neurons (Matsumine *et al.* 1996) (Fig. 5A-2,B-2). Human scribble showed the synapse-associated expression along the dendrites and co-localization with APC at the synaptic sites (Fig. 5C-2).

Over-expression of the C-terminal PDZ domain-binding motif of APC and high-risk HPV E6 disrupts the junctional integrity of the epithelial cells

To explore the importance of the association between hScrib and APC in the epithelial cells, we examined the effect of over-expression of the C-terminal region of APC on the formation of the cellular junctions by disrupting the endogenous interaction between hScrib and APC. We over-expressed the 369 amino acids C-terminal region of APC in addition to GFP vector at the ratio of 1000 : 1, as a marker of the transfection. In 85 of the GFP-labeled transfected cells with APC C-terminal 369 amino acids in 356 cells (24%), the formation of the adherens junctions were disrupted, as revealed by the loss of expression of hScrib (Fig. 6A-1) and the adherens junction marker, E-cadherin (data not shown). In contrast, the 450 cells transfected with the same construct lacking the last three amino acids still showed the normal expression of hScrib and the regular formation of the adherens junction as the untransfected cells (Fig. 6B-1).

As described above, APC and the high-risk HPV E6s share the class 1 PDZ domain-binding motif. Furthermore, APC and the high-risk HPV E6s share the PDZ domains 1 and 4 of hScrib as their binding sites. These data suggest the possibility that E6 exerts its oncogenic functions partly by competing with APC for binding to these PDZ domains of hScrib. To investigate this possibility, we over-expressed the high-risk type 16 E6 in MDCK cells and analyzed the junctional integrity in the E6 transfected cells. In the E6 transfected 265 cells, the expression of E-cadherin, the adherens junctional marker, as well as hScrib, was markedly reduced in the 78 cells transfected with E6 (29.4%), indicating that E6 disrupts the structure of adherens junction (Fig. 6A-2). In contrast, the structure of the adherens junction was less affected in the three cells transfected with E6 lacking the last three amino acids (287 cells, 0.01%), comparing with the cells tranfected with the wild-type E6 (Fig. 6B-2). These data strongly suggest the possibility that E6 is

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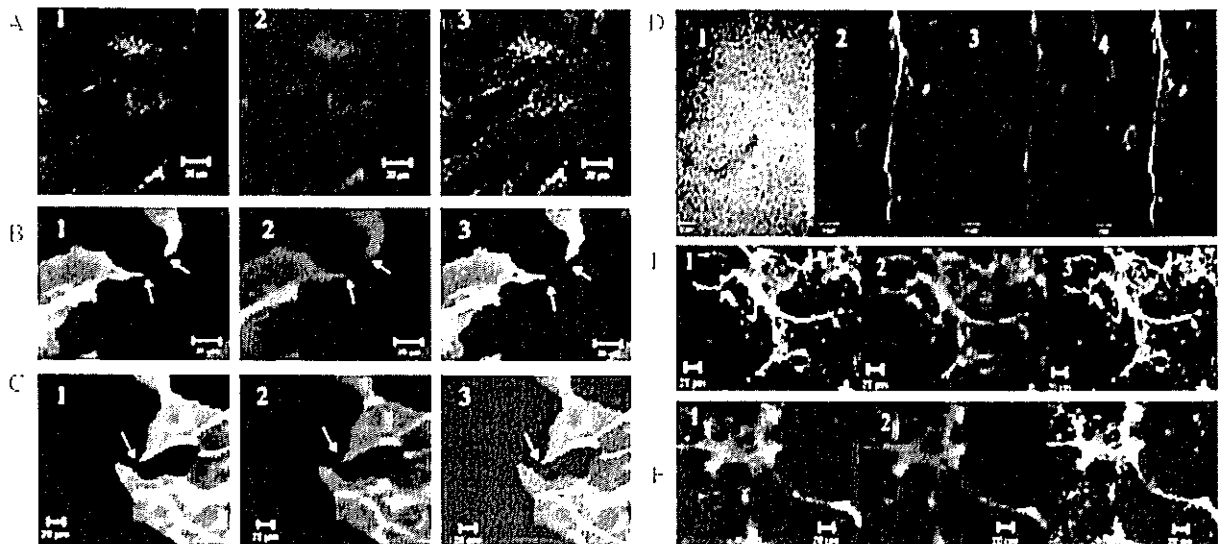

Figure 5 Human scribble co-localized with APC at the membrane protrusions in the growing epithelial cells. (A) The negative immunofluorescence staining of hScrib and APC in the epithelial cell line, MDCK using the antibodies pre-absorbed against the antigens. 1, immunofluorescence staining with the anti-hScrib antibody pre-absorbed against the antigen; 2, immunofluorescence staining with the anti-APC antibody pre-absorbed against the antigen; 3, the merge. (B) Immunofluorescence staining of hScrib and APC in the epithelial cell line, MDCK. 1, hScrib; 2, APC; 3, the merge. The arrows indicate that hScrib and APC co-localize at the membrane protrusions of MDCK cells. (C) Immunofluorescence staining of hScrib and β catenin in the epithelial cell line, MDCK. 1, hScrib; 2, β catenin; 3, the merge. The arrows indicate that hScrib and β catenin co-localize at the membrane protrusions of MDCK cells. Co-localization of hScrib with APC in the synaptic sites of cultured rat hippocampal neurons. (D) The immunofluorescence staining of APC (2) in the cultured neurons showed the dot-like staining along the dendrites, where the synaptic membrane protein synaptotagmin (3) localized beside the cell bodies of the neurons. 4. The merged image of Fig. APC (2) and synaptotagmin (3). The phase contrast image is shown in Fig 5D-1. (E) The immunofluorescence staining of hScrib (1) and synaptotagmin (2) revealed the co-localization of these proteins at the synaptic sites. 3. The merged image of hScrib (1) and synaptotagmin (2). (F) The immunofluorescence staining of hScrib (1) and APC (2) revealed the co-localization of these proteins at the synaptic sites. 3. The merged image of hScrib (1) and APC (2).

involved in the process of carcinogenesis of the cervical epithelia partly through the interference with the association between the tumor suppressor APC and the cellular apical-basal polarity determinant hScrib.

Knockdown of hScrib disrupts proper localization of APC at the adherens junction

To reveal whether the complex formation between hScrib and APC is required for the normal expression and localization of APC, we investigated the expression of APC in Caco-2 cells transfected with the siRNA against human scribble. In Caco-2 cells transfected with three RNAis against human scribble, the expression of hScrib was almost lost, while its expression was not affected in cells transfected with the control RNAi (Fig. 7A). The expression level of tubulin was not affected with treatments of RNAis against human scribble (Fig. 7A). Human Scribble and APC co-localize at the basolateral membranes of Caco-2 cells transfected with control RNAi (Fig. 7B). The expression of hScrib was lost in cells transfected with siRNA against human scribble, confirming the Western blot analysis (Fig. 7A,B). The membrane bound expression of APC was almost negative in cells transfected with siRNA against human scribble (Fig. 7B). These data underscore that the complex formation between hScrib and APC is necessary to the proper localization of APC at the adherens junction and may be required for the signal transduction through this protein complex (Fig. 7B).

Discussion

Scribble was first identified as an apical-basolateral cell polarity determinant in the *Drosophila* epithelia (Bilder & Perrimon 2000). To date, about 50 *Drosophila* tumor suppressor genes in which mutation gives rise to the over-proliferation of the larvae imaginal discs and brain tissues have been reported and they are classified as hyperplastic tumor suppressors (Bilder *et al.* 2000b; Humbert *et al.*

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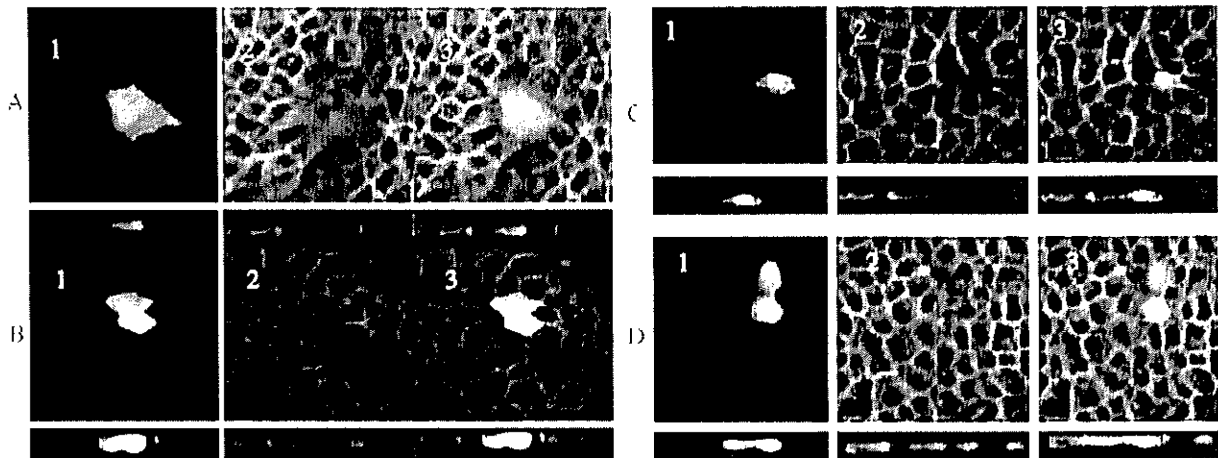

Figure 6 Over-expression of the C-terminal PDZ domain-binding motif of APC disrupts the adherens junctional architecture. (A) Over-expression of the C-terminal 369 amino acids of APC with GFP vector as a marker for the transfection disrupted the localization of hScrib at the adherens junction. 1. GFP signal was used as a marker of the expression of the C-terminal 369 amino acids of APC. 2. The immunofluorescence staining of anti-hScrib antibody revealed the loss of the expression of the endogenous hScrib in the cells transfected with the 369 C-terminal amino acids of APC. 3. The merged image of Fig 6A-1 and Fig 6A-2. Z-section images showed that the cells transfected with the C-terminal 369 amino acids of APC lost the basolateral expression of hScrib. (B) Over-expression of the C-terminal region of APC lacking the PDZ domain-binding motif lost the ability to disrupt the junctional integrity, as revealed by the normal expression of hScrib and regular shape of the adherens junction in the transfected cells. 1. GFP signal was used as a marker of the expression of the 369 C-terminal amino acids of APC lacking the PDZ domain-binding motif. 2. The immunofluorescence staining of anti-hScrib antibody revealed the normal basolateral expression of hScrib and conserved regular structure of the adherens junction in the cells transfected with the 369 C-terminal amino acids of APC lacking the PDZ domain-binding motif. 3. The merged image of Fig. 6B-1 and Fig. 6B-2. Z-section images showed that the cells transfected with the C-terminal 369 amino acids of APC lacking the PDZ domain-binding motif show the normal basolateral expression of hScrib. (C) Over-expression of the high-risk HPV E6 elicits its oncogenic effect on the epithelial cells partly through the interference with the association between hScrib and APC. Over-expression of the HPV 16 wild-type E6 in addition to the GFP vector as a marker for the transfection disrupted the adherens junction. 1. GFP signal was used as a marker of the expression of the HPV 16 wild-type E6. 2. The immunofluorescence staining of the adherens junction marker, E-cadherin, revealed the disruption of the adherens junction in the cells transfected with the HPV 16 wild-type E6. 3. The merged image of Fig. 6C-1 and Fig. 6C-2. Z-section images showed that the cells transfected with the HPV 16 wild-type E6 lost the integrity of the adherens junction. (D) Over-expression of the HPV 16 E6 construct lacking the last three amino acids showed the weaker ability to disrupt the adherens junction. The GFP vector was also used as a marker for the transfected cells. 1. GFP signal was used as a marker of the expression of the E6 construct lacking the last three amino acids. 2. The immunofluorescence staining of the adherens junction marker, E-cadherin, revealed the conserved regular structure of the adherens junction in the cells transfected with the E6 construct lacking the last three amino acids. 3. The merged image of Fig. 6D-1 and Fig. 6D-2. Z-section images showed that the cells transfected with the HPV 16 wild-type E6 lacking the last three amino acids preserved the integrity of the adherens junction.

2003). Only *scrib*, *dLg* and *lgl* are grouped as a malignant neoplastic tumor suppressor in which mutation causes disruption of the tissue structure and marked overgrowth of the epithelial tissues (Bilder *et al.* 2000b; Humbert *et al.* 2003). Human Scribble was first identified as a substrate of the high-risk HPV E6 for ubiquitin-mediated degradation dependent on E6AP, a ubiquitin protein-ligase (Nakagawa & Huibregtse 2000). Recently, we (Nakagawa *et al.* 2004) and Legouis *et al.* (2003) revealed its proper localization along the basolateral membrane, very similar to the site of the adherens junction, by using the GFP-tagged full-length hScrib clone and the immunofluorescence staining of the endogenous hScrib protein with the antibody raised against hScrib. Audebert *et al.* (2004) demonstrated that hScrib forms complex with the βPIX exchange factor and these complex may have a role in neural transmission. However, the function of hScrib as a tumor suppressor is barely understood.

Here we demonstrate that the tumor suppressor APC is a binding partner of hScrib. The germ line mutation of the APC gene is found in over 95% of patients with familial adenomatous polyposis (FAP), which is an autosomal-dominant intestinal disorder characterized by the development of hundreds to thousands colorectal polyposis

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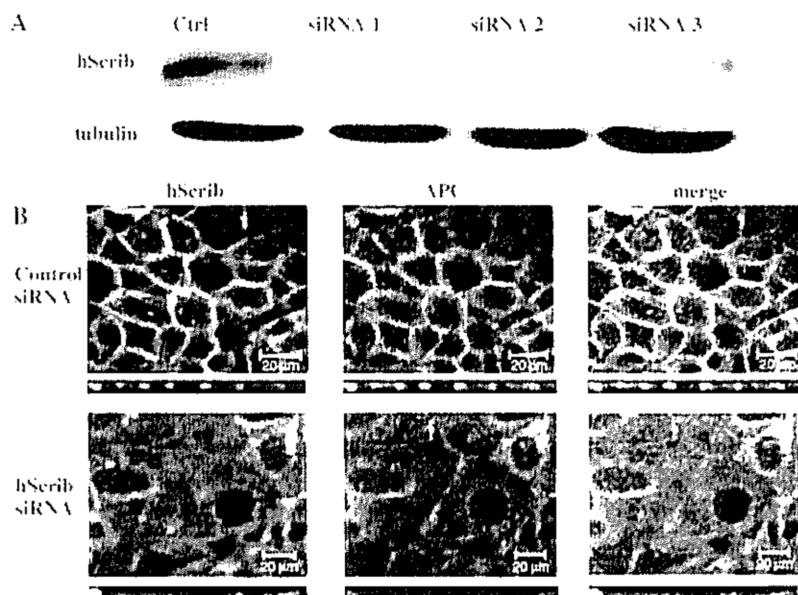

Figure 7 Knockdown of hScrib by siRNAs disrupts proper localization of APC at the adherens junction. (A) Western blot analysis of hScrib and α-tubulin expression in Caco-2 cells transfected with the hScrib siRNA. (B) Immunofluorescence analysis of hScrib and APC expression in Caco2 cells transfected with the hScrib siRNA.

(Fodde *et al.* 2001). Moreover, the somatic mutations of APC are found in the majority of sporadic colorectal cancers (Fodde *et al.* 2001). APC is known to have a broad range of functions from the control of the Wnt signal transduction to cell migration, cell-cell adhesion, and cell cycle control (Henderson 2000; Ishidate *et al.* 2000; Fodde *et al.* 2001). Most of APC mutations found in cancers lose its C-terminal coding sequence, leading to the loss of the interaction with partner proteins, such as β catenin (Fodde *et al.* 2001). The intracellular level of β catenin is controlled by the degradation in the proteosomes depending on the 'destruction complex' containing APC, axin/conductin, and glycogen synthase kinase 3β (GSK3β) (Nakamura *et al.* 1998). APC gene mutations allow β catenin to escape from this destruction complex and abnormally high level of β catenin in the nucleus leads to the transactivation of the transforming genes (Henderson 2000).

Our data on the analysis of the embryonic mouse brain extract confirmed the *in vivo* interaction among hScrib, APC and β catenin. Because hScrib does not bind to β catenin directly, it is thought to interact with β catenin by binding to the C-terminal region of APC. While the anti-hScrib C-terminus antibody co-precipitated APC and β catenin, the anti-APC C-terminus antibody co-precipitated negligible amount of hScrib, suggesting the possibility that the C-terminal epitope region of APC could be masked by binding to hScrib or hDlg PDZ domains and it could be important for the association with these proteins. Moreover, knockdown of hScrib expression by RNAi against human scribble disrupted proper localization of APC at the adherens junction. These data also underline the evidence of interaction between these proteins. It is possible that the PDZ domains of hScrib and hDlg are competitive for interaction with the C-terminal PDZ domain-binding motif of APC and that these two human homologs of Drosophila tumor suppressor are involved in formation of protein complex at the adherens junction through their interaction with APC.

Our finding that hScrib associates with APC and β catenin *in vitro* and *in vivo* and it co-localizes with them at the membrane protrusion in the MDCK cell line strongly suggests that hScrib elicits its inhibitory effect on the cell proliferation through the transduction of cell cycle regulatory signal of APC. Actually, hDlg, which is another target of HPV E6 for the ubiquitin-mediated degradation (Gardiol *et al.* 1999; Mantovani *et al.* 2001), makes a complex with APC and thereby negatively controls cell cycle from G0/G1 to S phase (Ishidate *et al.* 2000). Our unpublished observations on the inhibitory effect of cell-cycle progression of hScrib indicate that hScrib is involved in suppression of cellular proliferation of epithelia by controlling cell-cycle. Loss of PDZ domains of hScrib, which are critical to interact with APC, lost its negative regulatory ability of cell-cycle progression from G1 to S phase (unpublished observations, K.N. & S.N.). These data underscore that hScrib negatively controls cell-cycle by associating with APC. APC moves along microtubules through its interaction with

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kinesin superfamily (KIF) 3 A-KIF3B proteins and accumulates at their plus ends in migrating epithelial cells (Kawasaki *et al.* 2000). Based on the report that C-terminal region of APC including the class 1 PDZ domain-binding motif is important for the association with microtubule cytoskeleton, it is intriguing to speculate that hScrib, as well as hDlg, could represent potential export cargo for APC (Matsumine *et al.* 1996; Mimori-Kiyosue *et al.* 2000; Kawasaki *et al.* 2000, 2003). If it is the case, hScrib might be involved in cell–cell contact and adhesion and thus the early formation of the adherens junction through its association with APC. This is conceivable considering that *Drosophila* scribble is essential for the formation of apical-basal cell polarity and tissue architecture in the epithelia and hScrib can rescue the polarity loss and overgrowth of epithelia in the *Drosophila scrib* mutant (Bilder & Perrimon 2000; Bilder *et al.* 2000b, 2003; Dow *et al.* 2003). Furthermore, the mouse homolog of *Drosophila scrib* has been identified as a candidate gene of the most severe form of neural tube defect, termed craniorhachischisis (Murdoch *et al.* 2003). In *Circletail*, which is one of the two mouse mutants exhibiting this phenotype, the entire brain and spinal code remain open. In *Circletail*, a single base insertion in Scrib1 causes a frameshift that leads to a truncation of the protein with loss of two PDZ domains (Murdoch *et al.* 2003). These data also underscore the importance of hScrib as a determinant of tissue architecture.

Our data that the over-expression of the C-terminal region of APC in the epithelial MDCK cell line, but not the same construct without the PDZ domain-binding motif, disrupts the configuration of the adherens junction underscore the importance of the association between hScrib and APC for the formation of proper adherens junction and tissue architecture in epithelial cells. We previously showed that the high-risk HPV E6, but not the low-risk HPV E6, target hScrib for ubiquitin-mediated degradation depending on E6AP ubiquitin protein-ligase (Nakagawa & Huibregtse 2000). Moreover, destruction of the adherens junction structure by the over-expression of the high-risk HPV E6, but not that lacking the last three amino acids, strongly support the evidence that the E6 elicits its oncogenic effect partly through the interference with the association between the hScrib and APC with its class 1 PDZ domain binding motif, in addition to that between hDlg and APC. The E6 of HPV type 18, which is known to its more aggressive character, has the same C-terminal conserved amino acids, T-x-V, as the C-terminal amino acids of APC. The E6 of HPV type 16, which is more frequent type of HPV found in cervical cancer, has different amino acids in its C-teriminal PDZ domain-binding motif from that of APC (T-x-L, Fig. 1). Loss of hScrib expression led to disruption of the adherens junction. Our data are in line with the recent study reporting the potential link between E-cadherin and hScrib (Navarro *et al.* 2005). Taken together, the interaction between the hScrib and APC through the C-terminal motif of APC and the PDZ domains of hScrib have a critical role in the regulation of the formation of regular shape and control of the cell cycle in the epithelia. The high-risk HPV E6 might be involved in cancer generation and development through both the ubiquitin-mediated degradation of hScrib and the interference with the association of hScrib with APC through its C-terminal PDZ domain-binding motif. It is possible that HPV 18 E6 has a stronger effect on the interference in the interaction between hScrib and APC, which is involved in its more aggressive character in the cervical tumor genesis.

In our study, hScrib co-localizes with APC at the synaptic sites in the cultured neurons, indicating that hScrib is involved in the synapse formation and control of neural growth by cooperating with APC. The previous report by Matsumine *et al.* (1996) describing that hDlg co-localizes with APC at the presynaptic nerve terminals and our data showing that hScrib co-localizes with APC and synaptotagmin in the cultured primary neurons support the idea that hScrib makes a complex with APC and hDlg at the presynaptic sites and thereby controls the neural growth and signal transduction. The shared phenotype of the *Drosophila scrib* and *dLg* mutants and the genetic interaction between *Drosophila scrib* and *dLg* also support the possibility that hScrib interacts with hDlg and these two tumor suppressors corporate and negatively regulate the growth of the neurons and epithelial cells (Bilder *et al.* 2000b; Bilder 2003). The investigation of the mechanisms underlying the association of these two human homologs of the *Drosophila* malignant neoplastic tumor suppressors is under the way in our laboratory.

In summary, we identified that hScrib associates *in vitro* and *in vivo* with the tumor suppressor APC through its PDZ domains 1 and 4 and the class 1 PDZ domain-binding motif of APC. The association between hScrib and APC is essential for the structure of the adherens junction in the epithelia and also might be important for the proper formation of the synapse in the neuron. Dissociation of the interaction between hScrib and APC, as well as that between hDlg and APC could be a part of the oncogenic potential of the high-risk HPV E6 besides the ubiquitin-mediated degradation of these two tumor suppressors. The identification of the association between hScrib and APC shed light on the mechanism of how the LAP protein hScrib is involved in the control of epithelial cell growth through the proper formation of adherens junction.

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Experimental procedures

Preparation of the anti-hScrib antibodies and the anti-APC antibodies

The hScrib has 16 canonical leucine-rich repeats (LRRs) at its N-terminus and 4 PDZ domains at its central region (Fig. 1A). To further explore the interaction between hScrib and APC, we generated the polyclonal antibody against the PDZ-domains 1–4 (amino acids 728–1188) of hScrib in rabbits. The DNA sequence, which encodes PDZ-domains 1–4 (amino acids 728–1188), was subcloned into the pGEX-6P-1 vector (Amersham Biosciences, Little Chalfont, UK). Glutathione S-transferase (GST) fusion protein, GST-hScrib PDZ-domain was made in the bacteria and purified according to the manufacturer's recommendation. The amino acids encoding hScrib PDZ-domain were cleaved from the GST-fusion proteins with the PreScission Protease (Amersham Biosciences) and purified. This hScrib PDZ-domain was injected to rabbits as an antigen. The hScrib PDZ-domain antibody (anti-hScrib PDZ-domain) was purified from the serum of the immunized rabbits by affinity chromatography. The construction of the polyclonal antibody against the C-terminal region (amino acids 1189–1630) of hScrib was previously described (Nakagawa *et al.* 2004). The anti-β catenin and E-cadherin antibodies were purchased from BD Transduction Laboratories, Inc. (Lexington, KY, USA). The construction of the anti-APC antibodies was previously described (Matsumine *et al.* 1996).

Western blotting

293T cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Protein extracts of 293T cells were made in the NP-40 lysis buffer containing 100 mM Tris (pH 8.0), 100 mM NaCl, and 1% NP-40. Protein concentration was determined by standard Bradford assay. Equal amount of extracts were fractionated by SDS-PAGE and electrophoretically transferred on to the polyvinylidene difluoride membranes (Millipore Co., Bedford, MA, USA). The anti-hScrib PDZ-domain and C-terminus antibodies were used at the dilution of 1 : 1000 to detect the expression of hScrib as indicated. The *in vitro*-translated hScrib and hDlg with the reticulocyte lysate system (Promega Corp., Madison, WI, USA) were used for the immunoblotting as the positive and negative control, respectively. The level of protein expression was analyzed by the STORM 860 according to the manufacturer's recommendation (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

In vitro and *in vivo* binding assay

In vitro translations were performed in the reticulocyte lysate system (Promega Corp.) in the presence of ^{35}S-labeled methionine. The plasmids for *in vitro* translation of HPV 16 E6 and hScrib were previously described (Nakagawa & Huibregtse 2000). The construction of the plasmid for *in vitro* translation of APC was previously described (Matsumine *et al.* 1996). Five to 10 μL of *in vitro* translation reaction mixture was incubated with 100 ng of GST-fusion protein immobilized on to glutathione-Sepharose. The GST-fusion hScrib and APC constructs were made by subcloning PCR fragments into the pGEX-6P-1 vector. The GST-fusion APC C-terminal mutants were made by subcloning the amplified PCR products with primers containing mutations into the pGEX-6P-1 vector. Binding reactions were performed in 250 μL total volume of buffer containing 25 mM Tris (pH 8.0), 125 mM NaCl and the cell lysis buffer (100 mM Tris (pH 8.0), 100 mM NaCl and 1% NP-40) at the ratio of 9-1. Reaction mixtures were rotated at 4 °C for 2 h and glutathione-Sepharose beads were washed three times with the cell lysis buffer. Then, the proteins were released in SDS-PAGE loading buffer and analyzed by SDS-PAGE followed by autoradiography. For the analysis of *in vitro* binding between hScrib and APC, both *in vitro* translated proteins were incubated at 4 °C for 2 h under the conditions described above and mixed with the anti-hScrib or anti-APC antibody and protein A sepharose 4B for 1 h. Then, the immunoprecipitated proteins were analyzed by SDS-PAGE. Recombinant baculovirus for HPV39 E6 was produced using the BaculoGold system (Pharmingen) in High5 insect cells (Invitrogen). Protein was isolated from infected cells 48 h postinfection and partially purified by cation exchange chromatography on Bio-Rad MacroPrep S. To analyze whether HPV E6 interfere the association between hScrib and APC, immunoprecipitation was done with or without of the presence of 100 nanograms of HPV39 E6 protein.

The cell extracts prepared from embryonic mouse brain were subjected to the immunoprecipitation with anti-APC N-terminus, anti-APC C-terminus, anti-hScrib PDZ domain, or anti-hScrib C-terminus antibodies. The immunoprecipitated proteins were fractionated by the 6–8% SDS-PAGE, transferred to the PVDF membrane, and then immunoblotted with the indicated antibodies.

Immunofluorescence of MDCK cells and the cultured hippocampal neurons

Subconfluent MDCK cells were grown on coverslips in the culture medium. Cells were washed three times with phosphate-buffered saline (PBS) and then fixed with 3.7% formaldehyde in PBS for 10 min. After washing with PBS three times, cells were rinsed with distilled water and permeabilized with acetone at −20 °C for 10 min. Then, cells were washed with PBS and incubated with the diluted anti-hScrib C-terminus, anti-APC, anti-β catenin or anti-E-cadherin antibodies (BD Transduction Laboratories) for 30 min at room temperature. After washing with PBS three times, cells were incubated with rhodamine or FITC-conjugated secondary antibodies (Sigma, St. Louis, MO, USA) and then washed three times with PBS. Finally, cells were mounted on a slide glass and analyzed under a confocal fluorescence microscopy (Zeiss LSM 410). Images were captured with a CCD camera. The subconfluent MDCK cells were transfected with the wild-type APC C-terminal or the APC C-terminal ΔTSV (the last three amino acids) expression plasmid along with pEGFP-C1 vector using Lipofectamine (Invitrogen Corp., Carlsbad, CA, USA). MDCK cells were also transfected with the HPV E6 *in vivo* expression plasmid, generously provided by Dr Thoru Kiyono (Virology Division,

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National Cancer Center Research Institute, Japan). The GFP signal was served as a marker for transfected cells. The effects of over-expression of the APC C-terminal sequences and HPV E6 were examined by analyzing the formation of adherens junction with immunofluoresence staining of E-cadherin in the transfected cells.

The primary neural cultures were obtained from the hippocampus of 15–18-day-old fetal rats, prepared in the dish culture, and then incubated for a week until the immunofluorescence analysis. The immunoflorescence staining was performed as described above.

siRNAs transfection

All siRNAs were obtained from Qiagen as purified. The target sequence of hScrib siRNA were designed using HiPerformance 2, for silencing siRNA duplexes, from Qiagen. The sequence was submitted to a BLAST seach against the human genome sequence to ensure that no gene of the human genome was targeted. An siRNA against hDlg was used as a positive control which has been previously described (Laprise *et al.* 2004). To minimize possible off-targeting effects, we detected three different target sequence against hScrib. The target sequence against hScrib were as follows hScrib 1: 5′-CAG GAT GAA GTC ATT GGA ACA; hScrib 2: 5′-CCG CAG GAG GAG GAT GGA GAA; hScrib 3: 5′-CTG GGA GGC AAC GAT CTG GAA.

Several cell-lines, Caco2, HaCaT and 293T cells were transfected by the use of 5μl of X-treme GENE siRNA transfection reagent (Roche) per ml and a final siRNA concentration of 10 nM according to the manufacturer's instructions. Alexa 568 labeled negative control siRNA (Qiagen) was used as a measure of transfection efficiency. The transfection efficiency was determined to be 80–90% for each cells. The cells were fixed at 72 h post-transfection for immunofluorescence or lysed in NP-40 buffer for Western blot analysis.

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Inhibitory cis-element-mediated decay of human papillomavirus type 16 L1-transcript in undifferentiated cells

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Abstract

Production of human papillomavirus type 16 major capsid protein L1 in undifferentiated cells is negatively regulated by several yet unidentified cis-acting inhibitory RNA elements, among which a major element is located within the first 514 nucleotides of the L1-mRNA. By Northern blotting we examined effect of the major element on the steady-state level of mRNA transiently transcribed in 293T cells from the firefly luciferase (Fluc) gene combined with the L1 DNA fragment encoding the major element. As reported previously, the element down-regulated steady-state level of the mRNA. The most efficient down-regulation was achieved by insertion of the element near the 5′ end of mRNA, resulting in an undetectable level of the mRNA. The longer the distance from the 5′ end of the mRNA to the element, the weaker the down-regulation. The half-life of the mRNA having the element was similar to that of normal Fluc-mRNA. When the element near the 5′ end was removed by splicing, the steady-state level of the resultant mRNA was raised to a readily detectable level. The steady-state level of RNA synthesized by RNA polymerase-I was not influenced by the presence of the element. Taken together, it is suggested that DNA region encoding the major inhibitory element does not disturb transcription and that the pre-mRNA is degraded by an RNA element-mediated mechanism after the splicing step in the course of mRNA maturation. (Mol Cell Biochem **288:** 47–57, 2006)

Key words: inhibitory RNA element, HPV16, L1 protein

Introduction

Human papillomavirus (HPV), a small icosahedral virus with a circular double-stranded DNA genome of 8k base pairs, has a strong epithelial tropism [1]. More than 100 genotypes of HPV have been identified so far [2]; HPV type 16 (HPV16) has been studied most extensively because of its association with 50% of cervical cancer, the second most prevalent cancer among women in the world. HPV infects the basal cells of the stratified epithelia through small epithelial legions. In the basal cells the viral DNA is maintained as episomes, and the genes encoding nonstructural proteins are transcribed at very low levels [3]. When the host cells initiate terminal differentiation, HPV DNA starts to replicate. The synthesis of

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the major (L1) and minor (L2) capsid proteins takes place only in the upper layers of the epithelium [4].

The production of L1 and L2 is primarily regulated at transcription. The HPV16 late promoter directing transcription of the L1 and L2 genes is negatively regulated by CCAAT displacement protein (CDP) [5] and YY1, a multifunctional protein acting as a transcriptional activator or repressor [6]. CDP and YY1 bind directly to the upstream region of the promoter in undifferentiated cells. Recently, it has been shown that YY1 in the YY1/DNA-complex is displaced by hSkn-1a, one of key factors to trigger epithelial differentiation [6]. The detailed regulatory mechanism for the late promoter has yet to be fully elucidated.

The production of L1 and L2 is further regulated at the posttranscriptional steps. The cis-acting negative regulatory RNA elements are found at the 3′ untranslated region (UTR) of HPV late mRNAs [7, 8]. hnRNPC1/C2 and HuR bind to an AU-rich element in 3′UTR of HPV1 L1-mRNA and reduces stability [9] and translation efficiency [10] of the L1-mRNA. HPV16 L1-mRNA contains a 79-nt negative regulatory element (NRE) in the 3′UTR [11]. The 5′ part of the NRE contains four weak consensus 5′ splice sites and the 3′ part is very GU rich [12]. Three cellular proteins, the U2 auxiliary splicing factor 65-kDa subunit, the cleavage stimulation factor 64-kDa subunit, and the HuR protein, are found to directly bind to NRE [13]. RNA having NRE becomes unstable *in vitro* degradation assay [14] and is not transported from nucleus to cytoplasm [13].

The additional cis-acting negative regulatory RNA elements, which are not yet precisely identified, are found in the HPV16 L1- and L2-coding regions of the mRNAs [15–17]. Transcripts encoding L1 are not detectable in HeLa and human 293T cells transfected with the expression plasmid directing the L1 gene by a heterologous strong promoter, such as the cytomegalovirus immediate early promoter [16, 18]. But certain changes in the DNA sequence that do not affect the protein sequence of HPV16 L1 destroy the inhibitory function and induce efficient production of the L1 [18]. Comparison of the levels of transcripts from series of fusion genes composed of a part of the HPV16 L1 gene and the p55gag gene of equine infectious anemia virus (EIAV) indicates that the coding region of L1-mRNA has multiple inhibitory elements [16]. The major inhibitory element is located within the first 129 nucleotides [16]. When the first 367 nucleotides sequences are fused to the 3′ end of the EIAV coding sequence, inhibition is substantially reduced [16].

The putative inhibitory RNA elements of the L1-mRNA-coding region act in the nucleus. HPV16 L1 is produced, when the human immunodeficiency virus type 1 (HIV1) Rev-responsive element is inserted just after the stop codon of the L1-DNA coding region and transcribed from HIV1 LTR under the presence of nuclear export protein Rev [19]. The L1 is also produced from a vaccinia virus [20], T7 RNA polymerase-based expression system [16] and from a recombinant Semliki forest virus directing the transcription in the cytoplasm [21]. The L1 is produced in HeLa cells transfected with in vitro synthesized L1-mRNA [16].

In this study, we further characterized the decay of the HPV16 L1-RNA mediated by the putative major inhibitory RNA element. It was found that pre-mRNA having the element was degraded after the splicing step in the course of mRNA maturation and that once matured, the mRNA having the element was stable.

Materials and methods

Production of the codon-modified HPV16 L1 gene

The codons of HPV16 L1 were changed to those used most frequently in human mRNAs; Ala: GCC; Cys: TGC; Asp: GAC; Glu: GAG; Phe: TTC; Gly: GGC; His: CAC; Ile: ATC; Lys: AAG; Leu: CTG; Asn: AAC; Pro: CCC; Gln: CAG; Arg; AGG; Ser: AGC; Thr: ACC; Val: GTG; Trp: TGG; Tyr: TAC. The entire 1518 nucleotide sequence of the codon-modified HPV16 L1 gene was produced by ligating 11 fragments (approximately 100 to 200 base pairs). Each DNA fragment was produced by PCR using sense and antisense synthetic oligonucleotides that had an annealing region (approximately 20 nucleotides) at its 3′ end. Each fragment was designed to have appropriate restriction enzyme sites at both ends available for ligation with fragments for adjacent segments. The codon-modified L1 DNA was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) at the *Not*I site to produce pGEM-L1Mut.

Construction of plasmids

(i) Expression plasmids for L1-chimeric genes

The HPV16 L1 gene was amplified from cloned HPV16 genome by PCR using primer pairs having *Not*I sites at their 5′ ends. The forward primer was designed to have Kozak sequence adjacent to the start codon. The PCR product was digested with *Not*I, and inserted into the *Not* I site of pGL3-Not that had been produced by replacement of the luciferase gene (from the *Hin*dIII site to *Xba*I site) of pGL3-control (Promega) with *Not*I linker to produce pSV-L1WT. The codon-modified L1 gene was excised from pGEM-L1Mut by digestion with *Not*I, and cloned into the *Not*I site of pGL3-Not to produce pSV-L1Mut.

Parts of the codon-modified L1 gene, nt1 to nt514, nt415 to nt914, nt915 to nt1518 (A at the first ATG of the codon-modified L1 gene was numbered as nt1) of pGEM-L1Mut were replaced with corresponding region of L1WT by procedures described previously [17] to produce pSV-L1Ch1, pSV-L1Ch2, and pSV-L1Ch3, respectively.

(ii) Expression plasmids for firefly luciferase genes having the L1-fragment encoding the major inhibitory element

The HPV16 L1 region of nt4 to nt513 (inhibitory region, IR) was amplified by PCR with sense-primer having *Hin*dIII site and Kozak sequence and antisense-primer having *Nco*I site. The amplified fragment was digested with *Hin*dIII and *Nco*I, and inserted between the *Hin*dIII and *Nco*I sites of pGL3-control (Promega) to produce p5′UTR-IRLuc. Similarly IR amplified by PCR with primers having *Xba*I sites at their 5′ ends was digested with *Xba*I, and inserted into the *Xba*I site of pGL3-control to produce p3′UTR-IRLuc.

IRs amplified by PCR with primers having *Bst*BI, *Bcl*I, and *Eco*O109I sites at their 5′ ends were inserted in frame into firefly luciferase gene (Fluc) of pGL3-control at the *Bst*BI (nt171, A of the first ATG of luciferase coding region was numbered as nt1), *Bcl*I (nt582), and *Eco*O109I (nt1181) sites, respectively, to produce p171-IRLuc, p582-IRLuc, and p1181-IRLuc. IR was inserted in frame at nt3, at nt780, at nt981, and at nt1650 by the standard PCR techniques [22] to produce p3-IRLuc, p780-IRLuc, p981-IRLuc, and p1650-IRLuc.

The DNA fragment containing SV40 splicing donor and acceptor signals (spl DNA) in pCMV-β (BD Bioscience Clontech, Palo Alto, CA, USA) (nucleotide 641 to 843 of pCMV-beta) was used to produce p5′UTR-IRspl and p3′UTR-IRspl. The *Ppu*MI site between the donor signal and the acceptor signal was converted to a *Nhe*I site by a linker insertion, and then used as the template for PCR to amplify the spl DNA. The spl DNA was inserted between the *Hin*dIII and *Nco*I sites and at the *Xba*I site of pGL3-control to produce pGL3-5′spl or pGL3-3′spl, respectively. Then, the IR amplified by PCR using primers having *Xba*I sites at their 5′ ends was digested with *Xba*I and inserted into the *Nhe*I site (nucleotide sequences of the cohesive ends of *Nhe*I and *Xba*I are identical) of pGL3-5′spl and pGL3-3′spl to produce p5′UTR-IRspl and p3′UTR-IRspl, respectively. The signal sequences for splicing donor (ACTTACCAG) and acceptor (TAGG) of p5′UTR-IRspl and p3′UTR-IRspl were converted to CTGGGAAGA and GTTC, respectively, by the standard PCR techniques [34] to produce p5′UTR-IRmspl and p3′UTR-IRmspl.

A DNA fragment containing the human ribosomal RNA promoter (HrD), which directs transcription by the RNA polymerase-I [23], was amplified from HeLa genomic DNA by PCR using a sense primer having *Kpn*I site (5′-ggtacccgcgatcctttctggagagtccc-3′) and an antisense primer having *Hin*dIII sites (5′-aagcttgacgagaacgcctgacacgcac-3′). The amplified DNA digested with *Kpn*I and *Hin*dIII was inserted between the *Kpn*I and *Hin*dIII sites of pGL3-control. A DNA fragment containing Sal box, which directs termination of transcription by the RNA polymerase-I [24], was produced by annealing of synthetic oligonucleotides of 5′-ctagatccgcacgggtcgaccaga-3′ and 5′-tcgatctggtcgacccgtgcggat-3′. The Sal box fragment was inserted between the *Xba*I and *Hpa*I sites of the pGL3-control having HrD to produce pHrD-Luc. Then, IR was inserted between the *Hin*dIII and *Nco*I site and at the *Xba* I site of the pHrD-Luc to produce pHrD-5′IRLuc and pHrD-3′IRLuc, respectively.

(iii) Expression plasmids for EGFP genes having the L1-fragment encoding the major inhibitory element

IR was inserted into the enhanced green fluorescence protein (EGFP) gene (pEGFP-Tub, BD Bioscience Clontech) at nt3, nt240, nt478, and nt717 (A at the first ATG of EGFP coding region was numbered as nt1) by the standard PCR techniques [22]. The fusion genes were inserted between the *Nco*I and *Xba*I sites of pGL3-control to produce p3-IREGFP, p240-IREGFP, p478-IREGFP and p717-IREGFP.

(iv) Expression plasmids for β-galactosidase genes having the L1-fragment encoding the major inhibitory element

The Fluc gene in pGL3-control was replaced with the β-galactosidase (β-gal) gene of pCMV-β (BD Bioscience Clontech) to produce pSV-β-gal. IR amplified by PCR with primers having the *Cla*I and *Aor*51HI sites at their 5′ ends were inserted in frame into β-gal gene in pSV-β-gal at the *Cla*I (nt909, A of the first ATG of β-gal coding region was numbered as nt1) and the *Aor*51HI (nt1920) sites, respectively, to produce p909-IRβ-gal and p1920-IRβ-gal. IR was inserted at nt3 and nt3141 of β-gal by the standard PCR techniques [22] to produce p3-IRβ-gal and p3141-IRβ-gal, respectively.

Cell culture and transfection

Human 293T cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and grown in 5% CO_2 at 37 °C. 6×10^5 cells in a 6 cm plate were transfected with 2 μg of plasmid DNA using FuGene 6 (Roche Diagnostics, Indianapolis, IN, USA). Each transfection mixture contained 0.05 μg of pEF1a-renilla, an expression plasmid for renilla luciferase gene driven by the human EF1 alpha promoter, as an internal control for transfection efficiency. RNA was extracted at 2 days after the transfection.

Western blotting

293 T cells were transfected with the expression plasmid. At 48 h after the transfection the cells were suspended in an appropriate volume of SDS-sample buffer (153 mM Tris-HCl, pH 7.5, 4.9% SDS, 6.13% 2-mercapt ethanol, 24.5% glycerol,