be very helpful to avoid unnecessary treatment and its associated complications.

Persistent infections by oncogenic human papillomaviruses (HPVs) are strongly associated with cervical carcinogenesis [3]. Data from immunocompromised patients indicate that a defective immune response against HPV infections may be the most important determinant of progression from HPV infections to cervical cancer. Although cell-mediated immunity is believed to be more important to induce viral clearance, immunological mechanisms associated with CIN regression are not well understood [4]. In clinical trials, therapeutic vaccines induce specific T cells without necessarily causing CIN regression.

Serum IgG antibodies to HPV L1-capsids (virus-like particles, VLPs) are known to be a type-specific biomarker of past and present HPV infections [5]. A recent study has shown that antibody responses to L1-capsids are induced together with E6/E7-specific CTL (cytotoxic T lymphocytes) responses in the majority of women with viral clearance [6]. Also, it is likely that cross-reactive immunity arising from previous viral clearance may contribute to spontaneous regression of CIN lesions induced by other HPV types [7,8]. Thus, antibody responses to previous or current HPV infections may serve as a predictive marker of CIN regression.

In the present study, we examined whether baseline IgG antibodies to various L1-capsids of HPV16, 52, 58 and 6 act as a predictor for natural regression of CIN lesions. In Japan, HPV16, 52 and 58 are most commonly associated with CIN [9], while HPV6 is primarily associated with genital warts.

2. Patients and methods

The present study was conducted on women who previously participated in a Japanese cohort study of CIN I/II. The details of this cohort study have been provided elsewhere [10]. Eligible subjects with biopsy-proven CIN I/II were followed at 3–6 months intervals and received cytology and colposcopic examinations at each visit. In this study, we defined regression as normal colposcopy and at least two consecutive negative smears. Among a total of 185 cases, serum samples from 69 cases were not available for HPV capsid serology. Therefore, the present analysis was restricted to 116 cases (80 CIN I and 36 CIN II) that were tested for both cervical HPV DNA and serum HPV antibodies at enrolment. We examined HPV DNA in cervical samples by polymerase chain reaction (PCR) using consensus primers for the HPV L1 region [11]. HPV types were identified by restriction fragment length polymorphism (RFLP) that has been shown to identify at least 26 types of genital HPVs [12]. Detection of IgG antibodies to HPV16, 52, 58 and 6 was performed by enzyme-linked immunosorbent assay (ELISA) using purified L1-capsids as antigens. The type specificity and sensitivity of our HPV capsid serology have been described elsewhere [13,14]. Since serum samples included in the present study were tested for HPV capsid serology together with those from our case-control study of CIN [14,15], the same cut-off values for determining seropositivity were employed (0.319 for anti-HPV16, 0.272 for anti-HPV52, 0.304 for anti-HPV58, and 0.273 for anti-HPV6). These cut-off points were determined statistically based on the data from HPV DNA-negative controls [16]. Cumulative regression curves of CIN lesions were constructed using the Kaplan–Meier method and compared with a log-rank test. The Cox regression model was used for statistical adjustments. All analyses were carried out using the JMP 5.01J statistics package (SAS Institute, Cary, NC). The P-values obtained in all tests were considered to be significant at $P<0.05$.

3. Results

In the present study, the clinical outcomes of 116 CIN lesions (80 CIN I and 36 CIN II) were monitored by cytologic and colposcopic testing at intervals of 3–6 months. The cumulative regression rate in the 2-year follow up was 44%, including 54% of CIN I and 29% of CIN II lesions. At enrollment, HPV DNA was detected in 86 patients (74% of all patients) with HPV16 (12%), 52 (10%) and 58 (14%) being the most prevalent HPV strains. IgG antibodies to any of HPV16/52/58/6 L1-capsids were detected in 77 patients (66%) at baseline. Of these, 40 patients were seropositive for more than one HPV type (21 for two types, 16 for three types, and three for all four types). As shown in our previous study [14], women with HPV16/52/58 DNA positive CIN had a significant

antibody response to homologous L1-capsids (data not shown).

We analyzed the regression rates of CIN lesions with respect to histological CIN grades, HPV DNA status and serological status (Fig. 1). CIN I lesions were significantly more likely to regress compared with CIN II lesions (Fig. 1A, $P=0.007$, hazard ratio (HR) for regression 2.5, 95% confidence interval (CI) 1.2–4.1). Also, the cumulative regression rate was significantly higher in HPV DNA negative CIN lesions than in HPV DNA positive CIN lesions (Fig. 1B, $P=0.01$, HR for regression 2.0, 95% CI 1.2–4.7). These findings were consistent with previous studies [2,10,17,18]. By contrast, the CIN regression curve in seropositive patients was very similar to that in seronegative patients (Fig. 1C, $P=0.47$, HR for regression 1.1,

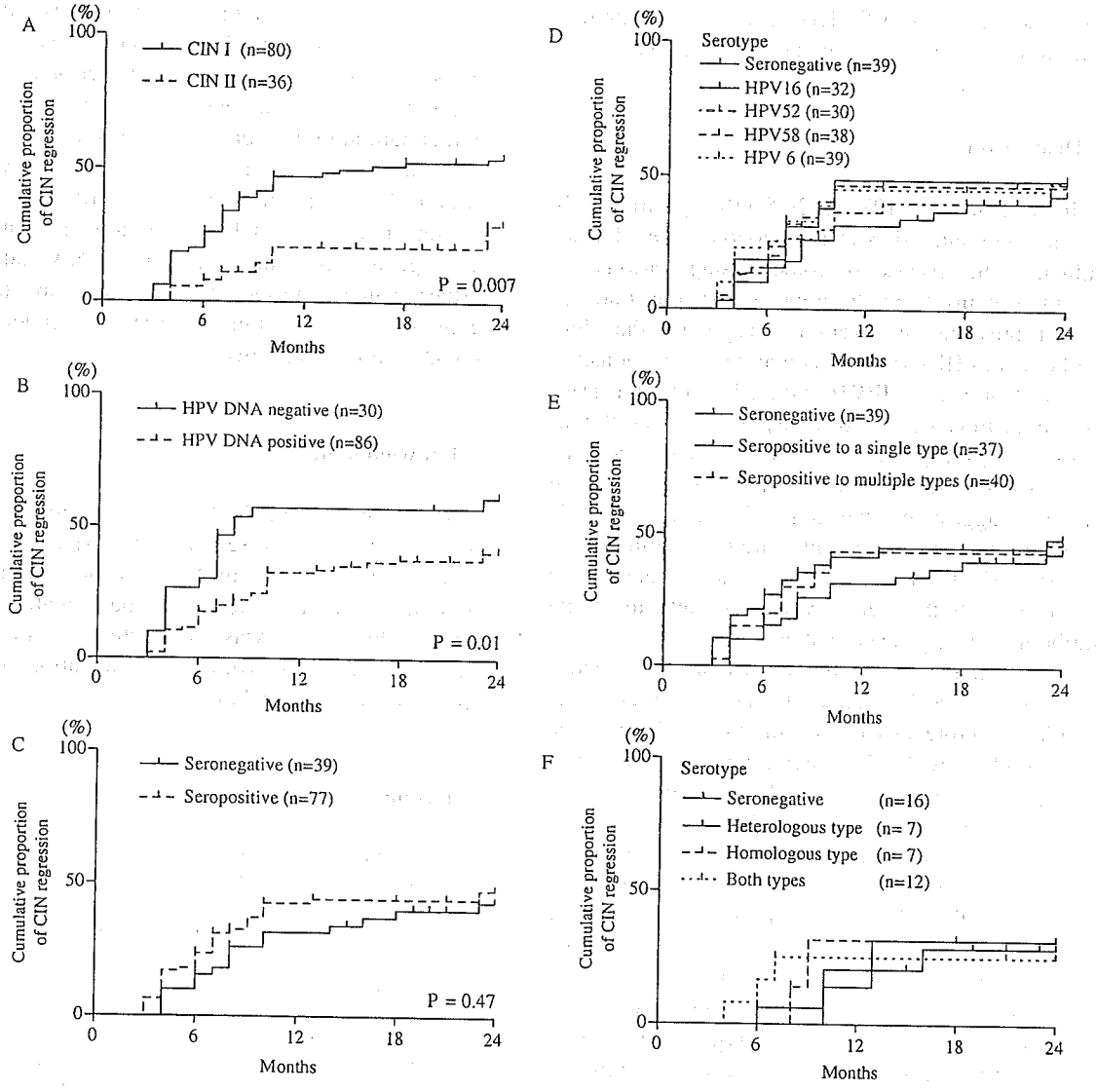

Fig. 1. Univariate analyses of possible predictors for CIN regression. In the analyses of all patients, CIN grades (A) and HPV DNA positivity (B) were significantly associated with CIN regression, while baseline seropositivity (C) did not correlate with CIN regression. Neither HPV serotypes (D) nor seropositivity to multiple types (E) influenced CIN regression. In the analysis restricted to HPV16/52/58 DNA positive patients, IgG reactivities to homologous or heterologous strains did not correlate with CIN regression (F).

95% CI 0.4–1.4). Neither HPV serotypes nor seropositivity to multiple types influenced the CIN regression (Fig. 1D and E). Furthermore, in 42 patients with HPV16/52/58 DNA positive CIN, IgG antibodies to homologous or heterologous L1-capsids failed to correlate with CIN regression in the 2-year follow-up (Fig. 1F). The statistical adjustments for age, CIN grades and HPV DNA types did not change the results (data not shown). These findings suggest that baseline IgG reactivities to HPV L1-capsids do not predict natural regression of untreated CIN I/II lesions.

4. Discussion

In women with HPV16/52/58 DNA positive CIN lesions, IgG antibodies to homologous types did not influence the disease outcome. Using various L1 capsid proteins from different strains, our findings extend previous observations suggesting that IgG antibodies to HPV16 capsids may not be responsible for the clearance of HPV16-induced CIN lesions [19]. In animal models, antibody responses to papillomavirus L1-capsids prevent animals from new infections, but do not induce tumor regression [20]. These findings suggest that the humoral response to HPV capsid proteins may not contribute to the elimination of established HPV infections, and appear to be consistent with the high prevalence of anticapsid IgG antibodies among cancer patients [13,21].

Interestingly, IgG antibody responses to heterologous L1-capsids did not influence CIN regression. Similarly, seroreactivity to multiple HPV types did not correlate with CIN regression. Although two seroepidemiological studies have suggested that immune responses against HPV6 may reduce the risk of cervical cancer induced by oncogenic HPVs [7,8], HPV6 seropositivity also failed to correlate with CIN regression. Since HPV capsid serology is largely type specific [14,22], these findings suggest that host adaptive immunity arising from previous viral clearance may not favor the clearance of CIN lesions induced by other types. This may be supported by recent studies suggesting that epithelial cells expressing HPV antigens may not reactivate memory CTL due to impaired antigen presentation [23,24]. Alternatively, immune responses inducing CIN regression may be HPV type specific with implications for the design of therapeutic vaccines against CIN [25].

To date, several serological studies have shown that the IgG1 to IgG2 ratio of antibodies detected against HPV16 antigens is higher in cancer patients compared with CIN patients, suggesting that Th1 immune responses may be crucial in the immunological control of HPV infections [26–28]. In addition, recent studies have demonstrated directly that cell-mediated immunity to HPV16 E7 oncoproteins correlates with CIN regression [29,30]. Thus, IgG subclass analysis among seropositive patients or the direct detection of cellular immunity to viral antigens may be more useful to predict natural regression of CIN lesions.

In summary, the present study has shown that baseline IgG antibodies to various HPV L1-capsids are not predictive of CIN regression, in contrast to histological CIN grades and HPV DNA status. To further evaluate the factors that predict spontaneous regression of CIN lesions, a large-scale cohort study of CIN is now in progress.

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References

[1] D.M. Parkin, Global cancer statistics in the year 2000, Lancet Oncol. 2 (2001) 533–543.
[2] A.G. Ostor, Natural history of cervical intraepithelial neoplasia: a critical review, Int. J. Gynecol. Pathol. 12 (1993) 186–192.
[3] H. zur Hausen, Papillomaviruses and cancer: from basic studies to clinical application, Nat. Rev. Cancer 2 (2002) 342–350.
[4] I.H. Frazer, Prevention of cervical cancer through papillomavirus vaccination, Nat. Rev. Immunol. 4 (2004) 46–54.
[5] J. Dillner, The serological response to papillomaviruses, Semin. Cancer Biol. 9 (1999) 423–430.
[6] M. Nakagawa, R. Viscidi, I. Deshmukh, M.D. Costa, J.M. Palefsky, S. Farhat, A.B. Moscicki, Time course of

humoral and cell-mediated immune responses to human papillomavirus type 16 in infected women, Clin. Diagn. Lab. Immunol. 9 (2002) 877–882.
[7] T. Luostarinen, V.A.F. Geijersstam, T. Bjørge, C. Eklund, M. Hakama, T. Hakulinen, et al., No excess risk of cervical carcinoma among women seropositive for both HPV16 and HPV6/11, Int. J. Cancer 80 (1999) 818–822.
[8] I. Silins, Z. Wang, E. Åvall-Lundqvist, B. Frankendal, U. Vikmanis, M. Sapp, et al., Serological evidence for protection by human papillomavirus (HPV) type 6 infection against HPV type 16 cervical carcinogenesis, J. Gen. Virol. 80 (1999) 2931–2936.
[9] H. Yoshikawa, C. Nagata, K. Noda, S. Nozawa, A. Yajima, S. Sekiya, et al., Human papillomavirus infection and other risk factors for cervical intraepithelial neoplasia in Japan, Br. J. Cancer 80 (1999) 621–624.
[10] M. Yokoyama, T. Iwasaka, C. Nagata, S. Nozawa, S. Sekiya, Y. Hirai, et al., Prognostic factors associated with the clinical outcome of cervical intraepithelial neoplasia: a cohort study in Japan, Cancer Lett. 192 (2003) 171–179.
[11] H. Yoshikawa, T. Kawana, M. Kitagawa, M. Mizuno, H. Yoshikura, A. Iwamoto, Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers, Jpn. J. Cancer Res. 82 (1991) 524–531.
[12] H. Nagano, H. Yoshikawa, T. Kawana, H. Yokota, Y. Taketani, H. Igarashi, et al., Association of multiple human papillomavirus types with vulvar neoplasias, J. Obstet. Gynecol. Res. 22 (1996) 1–8.
[13] K. Matsumoto, H. Yoshikawa, Y. Taketani, K. Yoshiike, T. Kanda, Antibodies to human papillomavirus 16, 18, 58, and 6b major capsid proteins among Japanese females, Jpn. J. Cancer Res. 88 (1997) 369–375.
[14] K. Matsumoto, H. Yoshikawa, T. Yasugi, S. Nakagawa, K. Kawana, A. Takeoka, et al., IgG antibodies to human papillomavirus 16, 52, 58 and 6 L1 capsids: case-control study of cervical intraepithelial neoplasia in Japan, J. Med. Virol. 69 (2003) 441–446.
[15] K. Matsumoto, T. Yasugi, A. Oki, H. Hoshiai, Y. Taketani, T. Kawana, H. Yoshikawa, Are smoking and chlamydial infection risk factors for CIN? Different results after adjustment for HPV DNA and antibodies, Br. J. Cancer 89 (2003) 831–833.
[16] R.G. Hoffmann, Statistics in the practice of medicine, J. Am. Med. Assoc. 185 (1963) 864–873.
[17] J.K. Chan, B.J. Monk, C. Brewer, K.A. Keefe, K. Osann, S. McMeekin, et al., HPV infection number of lifetime sexual partners are strong predictors for 'natural' regression of CIN 2 and 3, Br. J. Cancer 89 (2003) 1062–1066.
[18] N.F. Schlecht, R.W. Platt, E. Duarte-Franco, M.C. Costa, J.P. Sobrinho, J.C.M. Prado, et al., Human papillomavirus infection and time to progression and regression of cervical intraepithelial neoplasia, J. Natl Cancer Inst. 95 (2003) 1336–1343.
[19] T.D. de Gruijl, H.J. Bontkes, J.M.M. Walboomers, J.T. Schiller, M.J. Stukart, B.S. Groot, et al., Immunoglobulin G responses against human papillomavirus type 16 virus-like particles in a prospective nonintervention cohort study of women with cervical intraepithelial neoplasia, J. Natl Cancer Inst. 89 (1997) 630–638.
[20] R. Kirnbauer, L.M. Chandrachud, B.W. O'Neil, E.R. Wagner, G.J. Grindlay, A. Armstrong, et al., Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization, Virology 219 (1996) 37–44.
[21] B. Nonnenmacher, N.L. Hubbert, R. Kirnbauer, K.V. Shah, N. Munoz, F.X. Bosch, et al., Serologic response to human papillomavirus type 16 (HPV-16) virus-like particles in HPV-16 DNA-positive invasive cervical cancer and cervical intraepithelial neoplasia grade III patients and controls from Colombia and Spain, J. Infect. Dis. 172 (1995) 19–24.
[22] L. Wideroff, M. Schiffman, P. Hinderer, A. Armstrong, C.E. Greer, M.M. Manos, et al., Seroreactivity to human papillomavirus types 16, 18, 31, and 45 virus-like particles in a case-control study of cervical squamous intraepithelial lesions, J. Infect. Dis. 180 (1999) 1424–1428.
[23] M.C. Wolkers, N. Brouwenstijn, A.H. Bakker, M. Toebes, T.N. Schumacher, Antigen bias in T cell cross-priming, Science 304 (2004) 1314–1317.
[24] K. Matsumoto, G.R. Leggatt, J. Zhong, X. Liu, R.L. de Kluyver, T. Peters, et al., Impaired antigen presentation and effectiveness of combined active/passive immunotherapy for epithelial tumors, J. Natl Cancer Inst. 96 (2004) 1611–1619.
[25] A.S. Kadish, G.Y. Ho, R.D. Burk, Y. Wang, S.L. Romney, R. Ledwidge, R.H. Angeletti, Lymphoproliferative responses to human papillomavirus (HPV) type 16 proteins E6 and E7: outcome of HPV infection and associated neoplasia, J Natl Cancer Inst. 89 (1997) 1285–1293.
[26] T.D. de Gruijl, H.J. Bontkes, J.M.M. Walboomers, M.J. Stukart, A.A.J.P. Robbesom, B.M.E. von Blomberg-van der Filer, et al., Analysis of IgG reactivity against human papillomavirus type-16 E7 in patients with cervical intraepithelial, Int. J. Cancer 68 (1996) 731–738.
[27] K. Matsumoto, H. Yoshikawa, T. Yasugi, S. Nakagawa, K. Kawana, S. Nozawa, et al., Balance of IgG subclasses toward human papillomavirus type 16 (HPV16) L1-capsids is a possible predictor for the regression of HPV16-positive cervical intraepithelial neoplasia, Biochem. Biophys. Res. Commun. 258 (1999) 128–131.
[28] Z.H. Wang, L. Kjellberg, H. Abdalla, F. Wiklund, C. Eklund, P. Knekt, et al., Type specificity and significance of different isotypes of serum antibodies to human papillomavirus capsids, J. Infect. Dis. 181 (2000) 456–462.
[29] R. Höpfl, K. Heim, N. Christensen, K. Zumbach, U. Wieland, B. Volgger, et al., Spontaneous regression of CIN and delayed-type hypersensitivity to HPV-16 oncoprotein E7, Lancet 356 (2000) 1985–1986.
[30] A.S. Kadish, P. Timmins, Y. Wang, G.Y.F. Ho, R.D. Burk, J. Ketz, et al., Albert Einstein cervix dysplasia clinical consortium regression of cervical intraepithelial neoplasia and loss of human papillomavirus (HPV) infection is associated with cell-mediated immune responses to an HPV type 16 E7 peptide, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 483–488.

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CCAAT/enhancer binding protein β binds to and activates the P_{670} promoter of human papillomavirus type 16

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Abstract

The P_{670} promoter of HPV16 directs transcription of the virus late genes in the differentiating epithelium. We found that CCAAT/enhancer binding protein β (C/EBPβ), a key transcription factor that induces the terminal differentiation of keratinocytes, enhanced the P_{670}-driven transcription in transient reporter assays in HeLa cells and human primary keratinocytes, whereas it inhibited, as reported previously, the transcription from the early P_{97} promoter. An electrophoretic mobility shift analysis identified two binding sites in the upstream region of P_{670} for a bacterially expressed C/EBPβ. A chromatin immunoprecipitation analysis demonstrated that C/EBPβ bound to these sites of the P_{670} reporter plasmid in HeLa cells. Nucleotide substitutions in these sites in the reporter plasmid abrogated the enhancement by C/EBPβ in the transient HeLa and keratinocyte assays, indicating that the C/EBPβ-binding to these sites is required for the enhancement of transcription from P_{670}. These results suggest that C/EBPβ is involved in enhancing transcription from the P_{670} during keratinocyte differentiation.

Keywords: HPV16; P_{670}; C/EBPβ; Keratinocyte differentiation

Introduction

Human papillomaviruses (HPVs), small icosahedral viruses with circular double-stranded DNA genomes of 8 k base pairs (bp), cause proliferative lesions of the skin or mucosa (zur Hausen, 1996). To date more than 100 genotypes of HPVs have been identified and classified based on the homology of genomic DNA. HPVs that infect the genital epithelia are divided into two groups: low-risk types such as HPV type 6 (HPV6) and HPV11 found mainly in benign condyloma and high-risk types such as HPV16, HPV18, HPV31, and HPV33 found in cervical cancer (Longworth and Laimins, 2004; zur Hausen, 2000).

All HPVs have an overall similarity in the genomic organization: the early genes encoding viral nonstructural proteins (E1, E2, E4, E5, E6, and E7 proteins), the late genes encoding two capsid proteins (L1 and L2 proteins), and the non-coding long control region (LCR) (between the L1 and E6 genes) carrying the replication origin (Fehrmann and Laimins, 2003). Most of the early genes are transcribed from the promoter in LCR (such as HPV16 P_{97} and HPV31 P_{97}), and the E1 and late genes are transcribed from the promoter that is located within E7 gene (such as HPV16 P_{670} and HPV31 P_{742}) (Grassmann et al., 1996; Hummel et al., 1992).

The life cycle of HPVs is closely associated with epithelial differentiation (Longworth and Laimins, 2004). HPVs reach and infect the basal cells of the stratified epithelia through small epithelial lesions. In the basal cells, the viral DNA is maintained as episomes, and the early genes are transcribed at a very low level. When the host cells initiate terminal differentiation, the HPV DNA starts to replicate and to be transcribed efficiently. Then, HPV virions are generated in the upper layers of the epidermis or mucosa and released from them.

Transcriptional activities of the HPV promoters are drastically changed during the terminal differentiation of the host keratinocytes. Studies using immortalized human keratinocytes that harbor HPV16 or HPV31 DNA have demonstrated that HPV16 P_{97} or HPV31 P_{97} is active in undifferentiated cells, while HPV16 P_{670} or HPV31 P_{742} is suppressed (Grassmann et al., 1996; Hummel et al., 1992). Differentiation of the host cells induces a massive increase in transcripts from P_{670} or P_{742}, leading to expression of the L1 and L2 capsid proteins and E1 replication protein (Klumpp and Laimins, 1999; Ozbun and

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Meyers, 1997; Ruesch et al., 1998). Although the viral DNA replication coincides with the activation of P_{670} or P_{742}, it is not a prerequisite for the activation (Bodily and Meyers, 2005; Spink and Laimins, 2005). Cellular proteins in the differentiating epithelium are expected to directly regulate the transcriptional activities of the promoters, yet the molecular mechanisms of the activation and suppression are largely unknown.

Epidermal differentiation is regulated through the action of cellular transcription factors, which include AP-1 family proteins (Eckert et al., 1997), POU-domain proteins (Ryan and Rosenfeld, 1997), NF-kB family proteins (Seitz et al., 1998), and CCAAT/enhancer binding proteins (C/EBPs) (Maytin et al., 1999; Zhu et al., 1999). The C/EBP family of transcription factors are known to play a role in a wide range of biological processes, such as inflammation, the control of energy metabolism, and cellular proliferation and differentiation (Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002).

The C/EBP family is composed of six members: C/EBPα, -β, -δ, -γ, -ε, and -ζ (Ramji and Foka, 2002). In keratinocytes, C/EBPα, -β, -δ, and -ζ are expressed, and their expression levels fluctuate during the differentiation (Maytin and Habener, 1998; Smith et al., 2004). Of particular interest is that overexpression of C/EBPβ in keratinocytes causes growth arrest and the induction of early differentiation markers (Zhu et al., 1999), suggesting a crucial role of C/EBPβ in keratinocyte differentiation.

The C/EBP members contain a basic leucine zipper (bZIP) domain, which mediates the formation of homodimers or heterodimers with other bZIP family members, at their C-termini (Ramji and Foka, 2002). The dimers bind to the consensus DNA sequences near the core promoters (Akira et al., 1990). Translation of endogenous C/EBPβ mRNA produces three different products: two transactivator isoforms of 46 and 42 kDa (called as the liver-enriched transcriptional activator proteins, LAPs) and one inhibitory isoform of 20 kDa (called as the liver-enriched transcriptional inhibitory protein, LIP) by the use of internal translation initiation sites (Descombes and Schibler, 1991). The LIP consists only of the bZIP domain and acts as a dominant negative form of C/EBPβ (Descombes and Schibler, 1991).

C/EBPβ regulates cellular differentiation and proliferation through the generation of its multiple isoforms. Changes of the relative levels of the isoforms were observed in many cellular processes such as liver development/regeneration (Diehl et al., 1994), mammary grand development (Robinson et al., 1998; Seagroves et al., 1998), and tumorigenic conversion (Raught et al., 1996). The ectopic expression of the LIP isoform in 3T3-L1 cells disrupts terminal differentiation and induces a transformation phenotype (Calkhoven et al., 2000). The LIP isoform expression is also upregulated in mammary epithelial cells by the epidermal growth factor and is associated with cellular proliferation (Baldwin et al., 2004). These findings suggest an important role of the full-length C/EBPβ in inducing cellular differentiation.

A number of studies have demonstrated that C/EBPβ affects the transcription from the HPV promoters in LCR. C/EBPβ binds to a region of the HPV16 LCR and negatively regulates transcription from HPV16 P_{97} (Kyo et al., 1993). The complex of C/EBPβ and YY1 binds to the "switch region" in the HPV18 LCR and activates transcription from the promoter in the LCR (Bauknecht et al., 1996). The downregulation of endogenous C/EBPβ results in enhancement of transcription from the promoter in the HPV11 LCR (Wang et al., 1996).

In this study, we focused on the effect of C/EBPβ on HPV16 P_{97} and P_{670}. HeLa cells and primary human foreskin keratinocytes were transfected with plasmids expressing a reporter (luciferase) gene driven by HPV16 P_{97} or P_{670}, along with a C/EBPβ-expressing plasmid. The overexpression of C/EBPβ in the cells resulted in the activation of P_{670} and repression of P_{97}. The activation of P_{670} was mediated through the direct binding of C/EBPβ to two sites near the promoter.

Results

C/EBPβ was expressed from the plasmid introduced in HeLa cells and human primary foreskin keratinocytes (HFKs)

A newly constructed plasmid for FLAG-tagged C/EBPβ (FLAG-C/EBPβ), pFLAG-C/EBPβ, was introduced into HeLa cells and HFKs to test its expression. The transfection caused no significant morphological changes of the cells within 48 h. Immunoblot analyses of the cell lysates by using anti-C/EBPβ antibody showed that full-length FLAG-C/EBPβ was expressed in the two types of cells, and revealed the presence of two C/EBPβ isoforms, LAP and LIP (Fig. 1A). The endogenous LAP was more abundant than the endogenous LIP in HeLa cells and LIP was not detected in HFKs. In HeLa cells, the expression of FLAG-C/EBPβ induced efficient transcription of the keratin 10 (one of differentiation marker

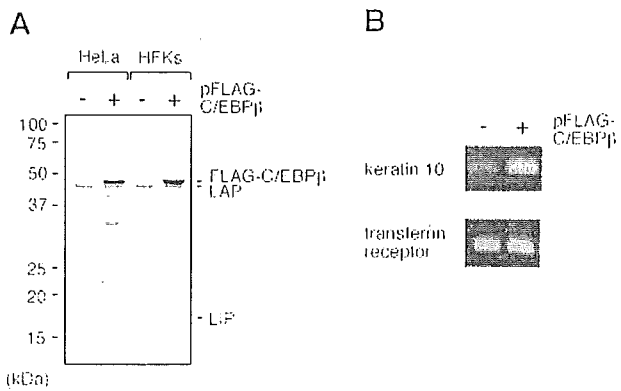

Fig. 1. Expression of FLAG-C/EBPβ in HeLa cells and human foreskin keratinocytes (HFKs). (A) Immunoblot analysis detecting endogenous and exogenous C/EBPβ. HeLa cells or HFKs were transfected with 0.4 ng of pFLAG-C/EBPβ or pFLAG-CMV2. At 48 h after the transfection, total cell extracts were prepared and subjected to immunoblotting with antibodies against the C-terminus of C/EBPβ. FLAG-C/EBPβ: FLAG-tagged full-length C/EBPβ; LAP: endogenous full-length C/EBPβ; LIP: endogenous shorter isoform of C/EBPβ. (B) RT-PCR analysis of mRNA for keratin 10 and transferrin receptor. HeLa cells were transfected with pFLAG-C/EBPβ or pFLAG-CMV2. cDNA libraries were constructed from the mRNAs extracted from the cells at 48 h after the transfection. cDNAs for keratin 10 and transferrin receptor were amplified by PCR with specific primers. The cDNA fragments were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide.

proteins) gene, as reported previously (Zhu et al., 1999), but did not affect transcription of the transferrin receptor gene (Fig. 1B). The results show that exogenous C/EBPβ can be introduced in HeLa cells and HFKs, and at least the one in the former is functional.

Transcription from HPV16 P_{670} was enhanced by C/EBPβ

Two reporter plasmids, pGL3-P_{670} and pGL3-P_{97}, in which a firefly luciferase gene replaced the E1 and E6 genes, were constructed to monitor the transcription from P_{670} and P_{97}, respectively (Fig. 2A). HeLa cells or HFKs were transfected with these reporter plasmids with increasing amounts of pFLAG-C/EBPβ followed by measurement of the luciferase activity. In HeLa cells, increasing FLAG-C/EBPβ enhanced the luciferase activity from pGL3-P_{670} up to 8-fold, but repressed the activity from pGL3-P_{97} down to half (Fig. 2B, left panel). In HFKs, C/EBPβ similarly enhanced and repressed the luciferase activities from pGL3-P_{670} and from pGL3-P_{97}, respectively (Fig. 2B, right panel). The similar effects of C/EBPβ on pGL3-P_{670} and pGL3-P_{97} were observed in HaCat (immortalized human skin keratinocytes) and 293 (a human fibroblast cell line) cells (data not shown). The results show that C/EBPβ enhances and represses transcription from P_{670} and P_{97}, respectively, in these human cells.

The full-length FLAG-C/EBPβ enhanced transcription from P_{670}. C/EBPβ contains a transactivation domain (TAD) at its N-terminus and a basic leucine-zipper (bZIP) type DNA-binding domain at its C-terminus (Fig. 2C, upper panel). To examine the effect of the LIP isoform on transcription from P_{670}, an expression plasmid for the FLAG-C/EBPβ lacking bZIP domain (FLAG-dbZIP) and an expression plasmid for LIP were constructed and used to test for their abilities to enhance transcription from P_{670} in HeLa cells by the transient

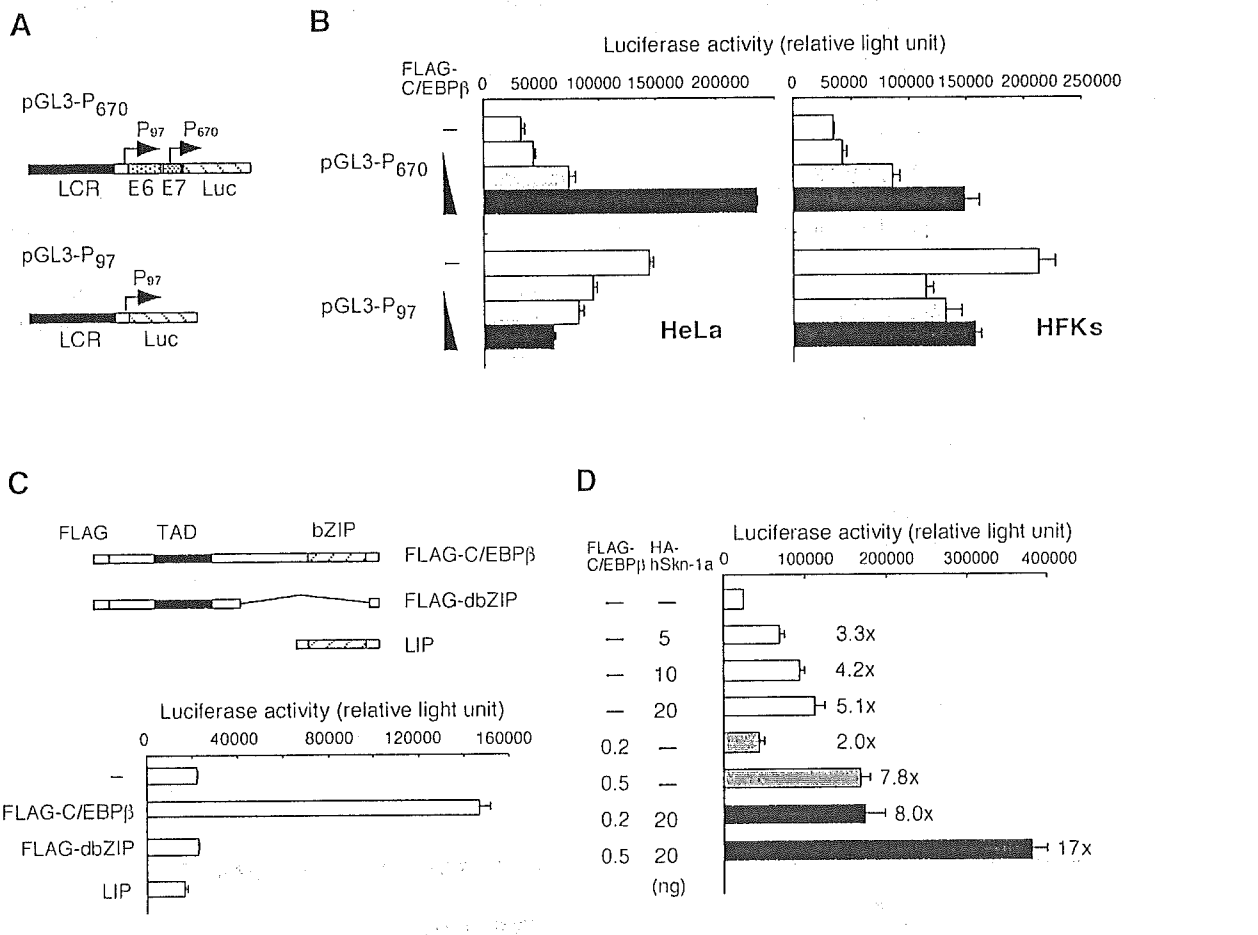

Fig. 2. Activation of HPV16 P_{670} and repression of P_{97} by C/EBPβ. (A) Schematic representation of the HPV16 reporter plasmids, pGL3-P_{670} and pGL3-P_{97}. A firefly luciferase gene was placed in the position of E1 gene (pGL3-P_{670}) or E6 gene (pGL3-P_{97}) to monitor transcription from P_{670} and P_{97}, respectively. (B) Effects of C/EBPβ on expression of luciferase from pGL3-P_{670} and pGL3-P_{97} in HeLa cells and HFKs. Cells were co-transfected with 200 ng of pGL3-P_{670} or pGL3-P_{97} together with increasing amounts of pFLAG-C/EBPβ (0, 0.1, 0.2, and 0.5 ng). At 48 h after the transfection, luciferase activities of cell lysates were measured. Results are presented as means ± standards deviations of three experiments. (C) Effects of domain-deleted C/EBPβs on expression of luciferase from pGL3-P_{670}. HeLa cells were transfected with 200 ng of pGL3-P_{670} and 0.5 ng of pFLAG-CMV2 (backbone plasmid), pFLAG-C/EBPβ or the expression plasmids for C/EBPβs with deletions (FLAG-dbZIP or LIP). The structures of the C/EBPβs with deletions are schematically illustrated at the top. Luciferase activities of the cell lysates were measured at 48 h after the transfection. (D) Effects of C/EBPβ and hSkn-1a on expression of luciferase from pGL3-P_{670}. HeLa cells were transfected with 200 ng of pGL3-P_{670} and indicated amounts of pFLAG-C/EBPβ and/or pHM-hSkn-1a. The total amounts of the expression plasmids were adjusted to 20.5 ng using pFLAG-CMV2 and pHM6 (backbone plasmid for pHM-hSkn-1a). Activation expressed in multiples was calculated from comparison of the luciferase activities with and without the plasmid expressing hSkn-1a and/or the plasmid expressing C/EBPβ.

reporter assay. FLAG-dbZIP and LIP did not affect transcription from pGL3-P_{670} and only full-length FLAG-C/EBPβ enhanced transcription from pGL3-P_{670} (Fig. 2C, lower panel), indicating that combined function of TAD and bZIP enhanced the transcription from P_{670}.

Enhancing effect by C/EBPβ on the P_{670} transcription was additive to that by the keratinocytes-specific transcription factor hSkn-1a, which also enhances the transcription from P_{670} as shown by our previous study (Kukimoto and Kanda, 2001). HeLa cells were transfected with pFLAG-C/EBPβ, pHM/hSkn-1a (an expression plasmid for HA-tagged hSkn-1a) (Kukimoto and Kanda, 2001), and a mixture of pFLAG-C/EBPβ and pHM/hSkn-1a in the pGL3-P_{670} transient expression assay. Expressions of FLAG-C/EBPβ and HA-hSkn-1a each enhanced luciferase activity, and the combined expression of FLAG-C/EBPβ and HA-hSkn-1a resulted in further enhancement (Fig. 2D). Although the enhancement by HA-hSkn-1a alone reached nearly maximum with 20 ng of pHM/hSkn-1a, co-expression of C/EBPβ induced further enhancement.

RT-PCR detecting hSkn-1a mRNA and Western blotting using anti-hSkn-1a antibody did not show expression of hSkn-1a in HeLa cells transfected with pFLAG-C/EBPβ (data not shown). HA-hSkn-1a also did not increase level of an endogenous C/EBPβ in HeLa cells (data not shown). These results suggest that C/EBPβ and hSkn-1a probably enhance the transcription from P_{670} by independent molecular mechanisms.

The major cis-element(s) required for the C/EBPβ-mediated transcriptional enhancement is located in the E7 region from nt 567 to nt 684

A series of 5′ deletions were introduced into pGL3-P_{670} by digestion with *Nde*I (pGL3-P_{670}/d*Nde*I), *Eco*T22I (pGL3-P_{670}/d*Eco*T22I), or *Pvu*II (pGL3-P_{670}/d*Pvu*II) and expressions of the reporter gene from the deletion mutants were examined in HeLa cells with or without FLAG-C/EBPβ (Fig. 3). Being consistent with the previous data shown by others (Bodily and Meyers, 2005), deletion of total LCR (pGL3-P_{670}/d*Nde*I) resulted in significant reduction of luciferase activity, suggesting that LCR has the enhancer function on P_{670}. However, C/EBPβ-mediated transcriptional enhancement occurred much clearly with the reporters lacking LCR. FLAG-C/EBPβ significantly enhanced luciferase activity from pGL3-P_{670}/d*Nde*I (deletion from the 5′ of LCR to nt 280) up to 16-fold and from pGL3-P_{670}/d*Eco*T22I (deletion from the 5′ of LCR to nt 566) up to 6-fold. The enhancement of transcription did not occur from pGL3-P_{670}/d*Pvu*II (deletion from the 5′ of LCR to nt 684). By a yet unidentified mechanism, luciferase expression from promoter-less plasmids, pGL3-P_{670}/d*Pvu*II and pGL3-Basic, was enhanced by less than 2-fold. The data indicate that the region between nt 567 and nt 684 contains the major element responsible for the enhancement, although there may be minor elements between nt 281 to nt 566.

C/EBPβ bound to the upstream region of P_{670} in vitro

An electrophoretic mobility shift assay (EMSA) demonstrated that a recombinant C/EBPβ protein bound to at least two sites in the HPV16 sequences from nt 501 to nt 670. An LIP isoform fused with glutathione *S*-transferase (GST-LIP) was bacterially expressed and used for the assay, since LIP contains a minimum DNA-binding domain and shows a higher DNA-binding affinity to its target sequence than the full-length C/EBPβ does (Descombes and Schibler, 1991). GST-LIP was incubated with the radiolabeled DNA probes (30 bp) having nucleotide sequences of the HPV16 genome designated A to J (Fig. 4A), and the complex formation of GST-LIP with each probe was detected by mobility shift. GST-LIP clearly bound to probes E and G (Fig. 4B). Faint bindings to the other probes seemed to be non-specific because the bindings were not sequence-specific. A similar binding profile was observed with a recombinant full-length C/EBPβ fused with GST (data not shown).

The probes E and G contain sequences similar to a consensus binding motif for the C/EBP family proteins, $(N)_3$TTGCNNAA$(N)_3$ (Osada et al., 1996) (Fig. 4C). The regions from nt 580 to nt 593 (within probe E) and from nt 601 to nt 614 (within probe G) were designated as CEB#1 and CEB#2, respectively (Fig. 4A). The C/EBP consensus motif partially resembles the binding motif for Skn-1a, WTGCAWNN (W is A or T), and thus the previously described hSkn-1a-binding sites Skn#2 and Skn#3 (Kuki-

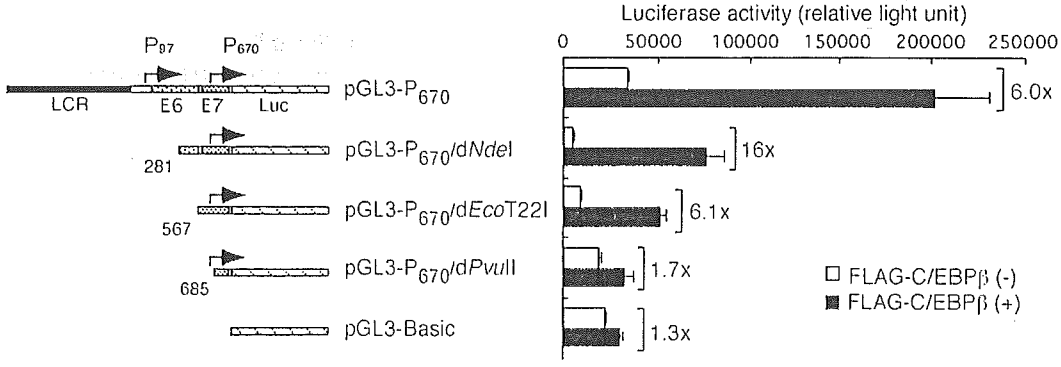

Fig. 3. Effects of C/EBPβ on expression of luciferase from pGL3-P_{670} having 5′-deletion. HeLa cells were transfected with 200 ng of pGL3-P_{670} having 5′-deletion and 0.5 ng of pFLAG-CMV2 or pFLAG-C/EBPβ. The luciferase activities of cell lysates were measured at 48 h after the transfection. Activation expressed in multiples was calculated from comparison of the luciferase activities with and without pFLAG-C/EBPβ for each deleted reporter.

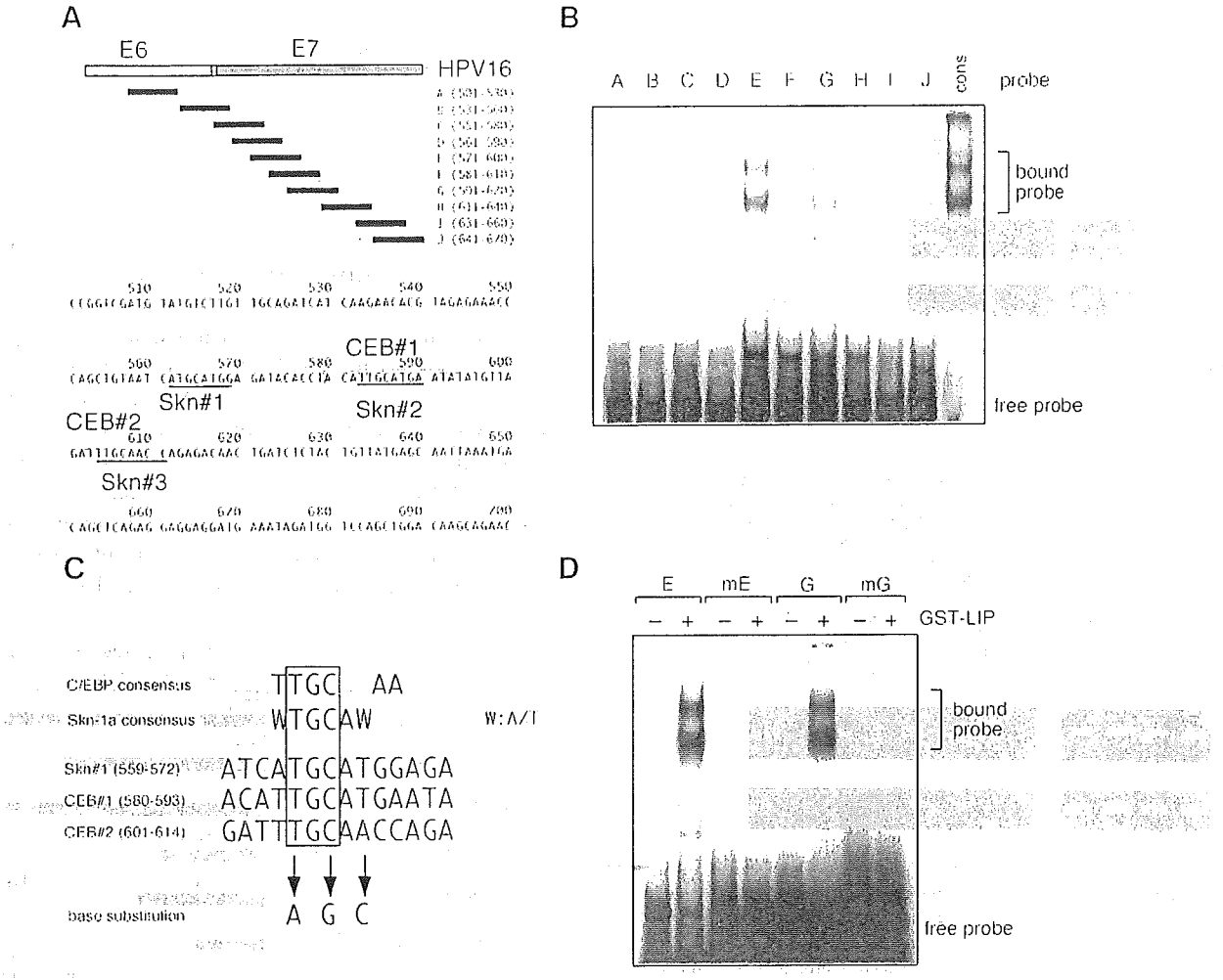

Fig. 4. In vitro binding of C/EBPβ with HPV16 E7 sequences. (A) The DNA probes (A to J) used in electrophoretic mobility shift assay (EMSA). Numbers in parentheses indicate nucleotide numbers of the HPV16 DNA (Los Alamos National Library database). Nucleotide sequence from nt 501 to nt 700 in the HPV16 genome is presented. The previously identified hSkn-1a binding sites are underlined and designated as Skn#1, Skn#2, and Skn#3. Binding sequences for the C/EBP family protein are boxed in gray and designated as CEB#1 and CEB#2. (B) EMSA detecting the complex of GST-LIP with the [^{32}P]-labeled HPV16 probe. The DNA–protein complex was electrophoresed on a 5% polyacrylamide gel and visualized by autoradiography. DNA probe having a consensus C/EBP binding sequence was used as positive control for EMSA (cons). (C) Base substitutions introduced in the probes E and G. (D) Inability of GST-LIP to bind to mutated E and G probes in EMSA. The probes E and G were mutated as shown in panel C to generate probes mE and mG, respectively, and used for EMSA with GST-LIP.

moto and Kanda, 2001) apparently overlap CEB#1 and CEB#2, respectively, on HPV16 DNA (Fig. 4A).

Nucleotide substitutions of TAGG for TTGC (Fig. 4C) were introduced into CEB#1 and CEB#2 in the probes E and G to produce mutated probes mE and mG, respectively. Probes mE and mG totally lost their capabilities of complexing with GST-LIP (Fig. 4D), indicating that the sequences of CEB#1 and CEB#2 are essential for probes E and G, respectively, to bind C/EBPβ.

C/EBPβ bound to the upstream region of P_{670} in vivo

Chromatin immunoprecipitation (ChIP) showed that C/EBPβ bound to the upstream region of P_{670} in HeLa cells. HeLa cells transfected with pGL3-P_{670} together with or without pFLAG-C/EBPβ were cross-linked with formaldehyde and lysed. The lysates were subjected to immunoprecipitation for a DNA–protein complex using either rabbit anti-C/EBPβ antibody or control rabbit IgG. After reversal of cross-links and the proteinase K digestion of the precipitates, a DNA fragment covering HPV16 nt 501 to nt 670 (170 bp) was amplified by PCR and electrophoresed on an agarose-gel (Fig. 5A). The DNA fragment associated with endogenous C/EBPβ was detectable in the precipitate with anti-C/EBPβ antibody and the DNA fragment became prominent in the lysate of HeLa cells transfected with pFLAG-C/EBPβ (Fig. 5A, upper panel). Amplification of Bcl-2 promoter sequence that does not bind to C/EBPβ (Heckman et al., 2003) from the precipitates with anti-C/EBPβ antibody was not successful (Fig. 5A, lower panel), indicating that the amplification is specific to HPV16 P_{670}. Anti-FLAG antibody also precipitated the 170 bp DNA from the lysate of HeLa cells transfected with pFLAG-C/EBPβ (data not shown).

The nucleotide substitutions in CEB#1 and CEB#2 reduced level of DNA precipitated with anti-C/EBPβ antibody. The nucleotide substitutions that abolished bindings of GST-LIP to

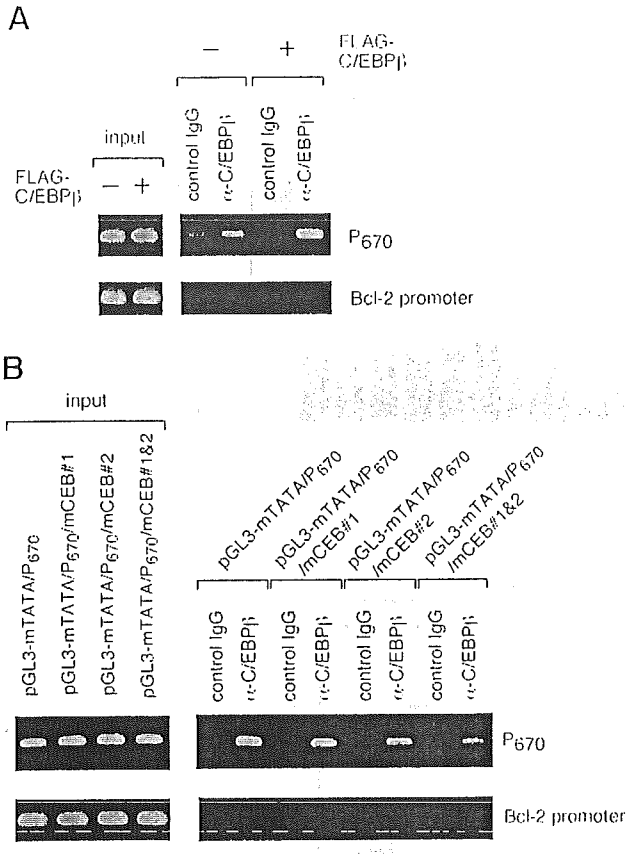

Fig. 5. In vivo binding of C/EBPβ to P_{670} in HeLa cells. (A) Chromatin immunoprecipitation analysis of complex of C/EBPβ and HPV DNA. HeLa cells transfected with pGL3-P_{670} together with pFLAG-CMV2 or pFLAG-C/EBPβ were cultured for 48 h and cross-linked with formaldehyde. The cells were lysed in SDS lysis buffer and sonicated. Chromatin–C/EBPβ complex was immunoprecipitated with anti-C/EBPβ antibody or normal rabbit IgG. DNA was extracted from the precipitate and used as a template for PCR amplification of DNA fragments of nt 501 to nt 670 (P_{670}) and of the Bcl-2 promoter. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Data are representative of three independent experiments. Part (0.2%) of the total input chromatin was used for PCR analyses (input). (B) Chromatin immunoprecipitation analysis of complex of C/EBPβ and HPV DNA with the mutations in C/EBP-binding sites. HeLa cells were transfected with pGL3-mTATA/P_{670} (a TATA box of P_{97} in pGL3-P_{670} was disrupted), pGL3-mTATA/P_{670}/mCEB#1, pGL3-mTATA/P_{670}/mCEB#2, or pGL3-mTATA/P_{670}/mCEB#1&2 together with pFLAG-C/EBPβ, then analyzed by chromatin immunoprecipitation as described in panel A. The nucleotide substitutions introduced into the upstream region of P_{670} in these plasmids are shown in Fig. 4C.

probes E and G (Fig. 4C) were introduced into corresponding regions of pGL3-mTATA/P_{670}, in which a TATA box of P_{97} in pGL3/P_{670} was disrupted to reduce transcription from P_{97}, to produce pGL3-mTATA/P_{670}/mCEB#1 having the mutations in CEB#1, pGL3-mTATA/P_{670}/mCEB#2 having the mutations in CEB#2, and pGL3-mTATA/P_{670}/mCEB#1&2 having the mutations in both CEB#1 and CEB#2. ChIP was conducted with lysates of HeLa cells transfected with pFLAG-C/EBPβ together with one of these plasmids (Fig. 5B). The levels of the 170bp DNA fragments obtained from pGL3-mTATA/P_{670}/mCEB#1 and pGL3-mTATA/P_{670}/mCEB#2 were lower than that from pGL3-mTATA/P_{670}. The level of the 170 bp DNA obtained from pGL3-mTATA/P_{670}/mCEB#1&2 was the lowest. These results strongly suggest that C/EBPβ appears to bind to the upstream region of P_{670} at CEB#1 and CEB#2 in a sequence-specific manner in vivo.

The nucleotide substitutions in CEB#1 and CEB#2 abolished the C/EBPβ-mediated enhancement of transcription from P_{670}

The transcription from the reporter lacking capability of binding with C/EBPβ was examined by introducing the nucleotide substitutions into CEB#1 and CEB#2. To know the possible contribution of P_{97} to the transcription from pGL3-P_{670}, luciferase activity from pGL3-P_{670} was compared with that from pGL3-mTATA/P_{670} in which TATA-box of P_{97} in pGL3-P_{670} was abolished by the nucleotide substitutions as previously described (Kukimoto and Kanda, 2001). The luciferase activity from pGL3-P_{670} was slightly higher than that from pGL3-mTATA/P_{670} (Fig. 6, upper panel), indicating that P_{97} contributes to the transcription from P_{670} in pGL3-P_{670}. The P_{97} contribution was clearer in HFKs than in HeLa cells (Fig. 6,

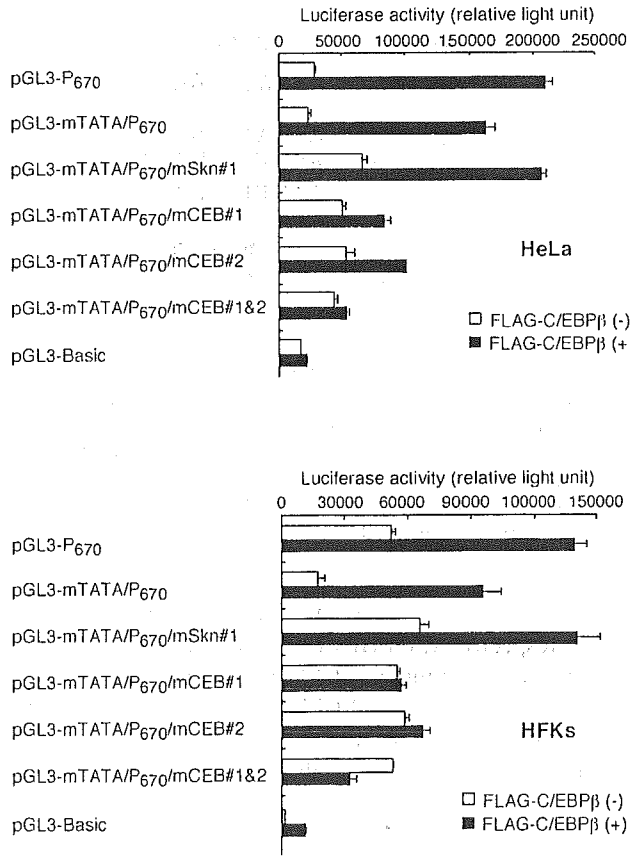

Fig. 6. Effects of C/EBPβ on expression of luciferase from pGL3-P_{670} with the mutations in C/EBP-binding sites in HeLa cells and HFKs. HeLa cells or HFKs were transfected with 200 ng of pGL3-mTATA/P_{670} (a TATA box of P_{97} in pGL3-P_{670} was disrupted), pGL3-mTATA P_{670}/mSkn#1, pGL3-mTATA/P_{670} mCEB#1, pGL3-mTATA/P_{670}/mCEB#2, or pGL3-mTATA/P_{670}/mCEB#1&2 together with 0.5 ng of pFLAG-CMV2 (bars in gray) or pFLAG-C/EBPβ (bars in black). The nucleotide substitutions introduced into the upstream region of P_{670} in these plasmids are shown in Fig. 4C. At 48 h after the transfection, luciferase activities of the cellular extracts were measured. Results are presented as means ± standards deviations of three experiments. Upper and lower panels show data obtained by using HeLa cells and HFKs, respectively.

lower panel). Therefore, for further mutational analyses of CEB#1 and CEB#2, pGL3-mTATA/P_{670} was used as a parental plasmid. Luciferase expressions in HeLa cells transfected with pGL3-mTATA/P_{670}/mCEB#1 and pGL3-mTATA/P_{670}/mCEB#2 were not efficiently enhanced by expression of FLAG-C/EBPβ (Fig. 6, upper panel). Luciferase expression from pGL3-mTATA/P_{670}/mCEB#1&2 was not enhanced. Nucleotide substitutions introduced into hSkn-1a-binding site #1 (pGL3-mTATA/P_{670}/mSkn#1) did not affect C/EBPβ-mediated enhancement. Similar results were obtained in HFKs (Fig. 6, lower panel). The results strongly support the idea that the direct binding of C/EBPβ to CEB#1 and CEB#2 is required for the enhancement of transcription from P_{670} by C/EBPβ.

Discussion

Activation of the HPV late promoter occurs in host cells that have started terminal differentiation (Longworth and Laimins, 2004). Therefore, it is reasonable to speculate that the late promoter is activated by some of the cellular transcription factors regulating cell differentiation. In this study, we examined the involvement of C/EBPβ, a key transcription factor that induces the terminal differentiation of keratinocytes (Zhu et al., 1999), in the regulation of the HPV16 promoters. It was found that C/EBPβ bound to two sites in the upstream region of the P_{670} late promoter in a sequence-specific manner and enhanced the transcription in HeLa cells and HFKs. As reported previously (Kyo et al., 1993), C/EBPβ repressed the transcription from the P_{97} early promoter.

C/EBPβ is known to interact with SWI/SNF chromatin remodeling complexes and induces transcription of several cellular genes in a chromatin environment (Kowenz-Leutz and Leutz, 1999). A recent study of an episomal HPV31 genome maintained in a human cervical cell line showed that a nucleosome-free region appears in the upstream region of the late promoter upon host cell differentiation (del Mar Pena and Laimins, 2001). The appearance of the nucleosome-free region strongly coincides with induction of the transcription from the late promoter. It is possible that C/EBPβ-mediated recruitment of the SWI/SNF chromatin remodeling complex to the upstream region of the HPV late promoter induces rearrangement of the nucleosome structure around the promoter, resulting in the appearance of a nucleosome-free region where transcription takes place.

The sequences of CEB#1 and CEB#2 are present in the corresponding positions in the E7 sequences of HPV31 and HPV18, strongly suggesting that the late promoters of HPV31 and HPV18 are activated by C/EBPβ. The identical sequences are not present in the E7 sequences of HPV6, HPV11, HPV33, and HPV58. However, substantial variations are tolerated for C/EBP-binding sequences (Osada et al., 1996), and several sequences containing TTGC are present in the E7 region of these HPVs. It remains to be examined experimentally whether C/EBPβ enhances transcription from the late promoter of these HPVs through its direct binding to the upstream region.

We have previously shown that hSkn-1a, a POU-transcription factor inducing keratinocyte differentiation, binds to Skn#1 and Skn#2 (Fig. 4A) and activates HPV16 P_{670} by displacement of YY1 repressor (Kukimoto and Kanda, 2001). hSkn-1a does not bind to the region previously designated as Skn#3. When both C/EBPβ and hSkn-1a were expressed in HeLa cells, the enhancement of transcription from P_{670} seemed to be additive (Fig. 2D). Probably hSkn-1a binds to Skn#1 and C/EBPβ binds to CEB#2 (Fig. 4A). Since CEB#1 and Skn#2 partially overlap (Figs. 4A and C), it is not clear which of C/EBPβ and hSkn-1a binds to the site dominantly. Our real-time RT-PCR analysis of differentiating primary keratinocytes showed that induction of hSkn-1a preceded induction of C/EBPβ during differentiation (T. Takeuchi et al., unpublished data). It is therefore possible that hSkn-1a first binds to Skn#2 and relieves the YY1-mediated repression, and then hSkn-1a is displaced by C/EBPβ.

C/EBPβ repressed transcription from HPV16 P_{97} as described previously (Kyo et al., 1993). C/EBPβ disrupts the binding of TATA-binding protein to the TATA box in the early promoter of HPV18 (Bauknecht and Shi, 1998). It is possible that the reduction of E6 and E7, which are produced from transcripts from P_{97} and have function to delay the terminal differentiation, is required for the later stage of the differentiation-associated viral propagation.

This study has shown that C/EBPβ enhances the transcription from the HPV16 late promoter. In the epidermis, C/EBPβ is expressed at a low level in the basal cell layers where the HPV late genes are not transcribed and at a high level in the middle and upper stratum spinosum where the HPV late genes are actively transcribed. It is very likely that C/EBPβ is involved in the differentiation-associated life cycle of HPV16.

Materials and methods

Construction of plasmids

The cDNA of C/EBPβ was provided by DNA Bank, BioRecource Center, RIKEN (Ibaraki, Japan), and was inserted in flame between *Eco*RI and *Sal*I sites of pFLAG-CMV2 (Sigma-Aldrich, St. Louis, MO) to generate an expression plasmid for an N-terminal FLAG-tagged C/EBPβ, pFLAG-C/EBPβ. The expression plasmid for C/EBPβ with deletion of the DNA-binding domain was constructed by digestion of pFLAG-C/EBPβ with *Sac*I and self-ligation. The DNA fragment encoding an LIP isoform of C/EBPβ was synthesized by PCR using pFLAG-C/EBPβ as a template with primers (forward, 5′-CGA ATT CGC CAT GGC GGC GGG CTT CCC GTA-3′; reverse, 5′-GCG TCG ACT CTA GCA GTG GCC GGA-3′; restriction digestion sites were underlined), and was inserted into pCMV5 (a kind gift from Dr. Michael Megner) as a *Eco*RI–*Sal*I fragment to generate an expression plasmid for LIP. The bacterial expression plasmid for an LIP fused with glutathione *S*-transferase (GST-LIP) was made by insertion of the *Eco*RI–*Sal*I fragment of LIP into pGEX-6P-1 (Amersham Biosciences, Piscataway, NJ).

To construct the P_{670} reporter plasmid, a *Pst*I–*Nco*I fragment of HPV16 (from nt 7003 to 7904 and from nt 1 to 868) was inserted between *Sma*I and *Nco*I sites of pGL3-Basic

(Promega, Madison, WI). In the resultant plasmid pGL3-P_{670}, the HPV16 DNA was fused with the luciferase DNA at the first ATG codon of the HPV16 E1 gene. To construct the P_{97} reporter plasmid pGL3-P_{97}, a DNA fragment of HPV16 (from nt 7003 to 7904 and from nt 1 to 101) was synthesized by PCR using primers (forward, 5′-CCC AAG CTT CTG CAG ACC TAG ATC AGT-3′; reverse, 5′-CAT GCC ATG GCA GTT CTC TTT TGG TGC ATA A-3′; restriction digestion sites were underlined), and was inserted between *Hin*dIII and *Nco*I sites of pGL3-Basic. Deleted P_{670} reporter plasmids, pGL3-P_{670}/d*Nde*I, pGL3-P_{670}/d*Eco*T22I, and pGL3-P_{670}/d*Pvu*II, were made by digestion of pGL3-P_{670} with *Sac*I and *Nde*I, with *Sac*I and *Eco*T22I, and with *Sac*I and *Pvu*II, respectively, followed by blunting and self-ligation. All nucleotide substitutions in the reporter constructs were introduced using a Mutan-Super Express Km site-directed mutagenesis kit (Takara, Osaka, Japan) or standard PCR techniques based on KOD-plus polymerase (TOYOBO, Osaka, Japan). All mutations were verified by sequencing.

Cell culture and luciferase assay

HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. Primary human foreskin keratinocytes (HFKs) were purchased from Kurabo (Osaka, Japan), and grown in serum-free keratinocyte growth medium (Kurabo) in 5% CO_2 at 37 °C. For luciferase assays, 3×10^4 HeLa cells or 4×10^4 HFKs were seeded onto 24-well plates. HeLa cells and HFKs were cultured for 4 h and overnight, respectively, before transfection. The cells were transfected with 200 ng of luciferase reporter plasmids and 0.1–0.5 ng of pFLAG-C/EBPβ by using FuGENE-6 reagent (Roche Applied Science, Indianapolis, IN). To monitor transfection efficiency, the cells were co-transfected with 5 ng of the cytomegalovirus promoter-driven *Renilla*-luciferase plasmid. Firefly and *Renilla* luciferase activities were measured at 48 h after transfection by using the Dual-Glo luciferase assay kit (Promega) and a TopCount microplate luminometer (Perkin-Elmer Sciences Inc, Boston, MA). Firefly luciferase activities were normalized using *Renilla*-luciferase activities. Each experiment was done in triplicate and repeated at least three times.

Western blot analysis

Total extracts of 1.2×10^5 HeLa cells or 1.6×10^5 HFKs transfected with 0.4 ng of pFLAG-C/EBPβ or pFLAG-CMV2 were electrophoresed on a 12% SDS-Polyacrylamide gel. After transfer of proteins to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), the membrane was blocked with 5% skim milk in PBS-0.1% Tween 20 at room temperature and then incubated with rabbit anti-C/EBPβ antibody (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The membranes were washed and incubated with peroxidase-conjugated goat anti-rabbit antibody for 1 h. Peroxidase activity was detected with the enhanced chemiluminescence detection method (ECL plus kit, Amersham Biosciences).

Electrophoretic mobility shift assay

The GST-LIP fusion protein was expressed in *Escherichia coli* strain JM109 and purified by GSTrap affinity-column chromatography (Amersham Biosciences) with AKTA*prime* (Amersham Biosciences). A mixture of double-stranded [^{32}P]-labeled oligonucleotides (0.4 pmol), 1 μg of GST-LIP, and 1 μg of poly (dI/dC) in a final volume of 10 μl of binding buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 40 μg/ml BSA) was incubated at room temperature for 30 min. Then, the samples were loaded on a 5% polyacrylamide gel and electrophoresed in 0.5× Tris–borate/EDTA buffer at room temperature. The gels were dried and visualized by autoradiography on X-ray films. The sense sequences of double-stranded oligonucleotides are as follows: A (501–530), 5′-CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3′; B (531–560), 5′-CAA GAA CAC GTA GAG AAA CCC AGC TGT AAT-3′; C (551–580), 5′-CAG CTG TAA TCA TGC ATG GAG ATA CAC CTA-3′; D (561–590), 5′-CAT GCA TGG AGA TAC ACC TAC ATT GCA TGA-3′; E (571–600), 5′-GAT ACA CCT ACA TTG CAT GAA TAT ATG TTA-3′; F (581–610), 5′-CAT TGC ATG AAT ATA TGT TAG ATT TGC AAC-3′; G (591–620), 5′-ATA TAT GTT AGA TTT GCA ACC AGA GAC AAC-3′; H (611–640), 5′-CAG AGA CAA CTG ATC TCT ACT GTT ATG AGC-3′; I (631–660), 5′-TGT TAT GAG CAA TTA AAT GAC AGC TCA GAG; J (641–670), 5′-AAT TAA ATG ACA GCT CAG AGG AGG AGG ATG-3′; mE, 5′-GAT ACA CCT ACA TAG GAC GAA TAT ATG TTA-3′; mG, 5′-ATA TAT GTT AGA TTA GGA CCC AGA GAC AAC-3′; C/EBP-cons, 5′-AAG CTG CAG ATT GCG CAA TCT GCA GCT T-3′. Numbers in parentheses indicate nucleotide numbers of the HPV16 genome (the HPV Sequence Database of Los Alamos National Laboratory) and nucleotides used for substitution mutations are underlined.

Chromatin immunoprecipitation

HeLa cells (8×10^5 cells) were grown on a 100-mm dish for 4 h and then transfected with 6 μg of the reporter construct and 6 ng of pFLAG-C/EBPβ or pFLAG-CMV2 using FuGENE-6. At 48 h after the transfection, the cells were cross-linked with 1% formaldehyde for 5 min at 37 °C followed by a 5-min treatment with 125 mM glycine to quench cross-linking. The cross-linked cells were lysed in 200 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0) supplemented with protease inhibitor mixture (Roche Applied Science), incubated on ice for 10 min, and sonicated using a Bioruptor (Cosmobio, Tokyo, Japan). The sonication resulted in an average DNA fragment size of 500 bp. One hundred microliters of the sonicated materials was diluted with 900 μl of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris–HCl, pH 8.0) and precleared with salmon sperm DNA/protein G-agarose beads. Part (2%) of the precleared lysate was taken as input chromatin for PCR analyses. The supernatant was incubated overnight at 4 °C with anti-C/EBPβ (Santa Cruz) or normal rabbit IgG. The chromatin–antibody complex was collected through incuba-

tion with the agarose beads for 2 h at 4 °C and centrifugation. The beads were washed sequentially for 5 min at 4 °C in wash buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, Tris–HCl, pH 8.0), wash buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, Tris–HCl, pH 8.0), wash buffer III (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0), and TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) twice, then extracted with 200 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$, 10 mM DTT). The eluted chromatin and the input chromatin were heated at 65 °C for 4 h to reverse the cross-links, followed by proteinase K digestion overnight at 37 °C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The purified DNA fragments were used as templates for PCR amplification to detect the protein occupancy of the HPV P_{670}. PCR primers were as follows: forward, 5′-CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3′; reverse, 5′-CAT CCT CCT CCT CTG AGC TGT CAT TTA ATT-3′. These primers amplify 170 bp DNA from nt 501 to nt 670 of the HPV16 sequence. PCR consisted of one cycle of initial denaturation at 94 °C for 1 min and 21 cycles of the following conditions: denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. Human Bcl-2 promoter sequence (from −748 to −587) (Heckman et al., 2003) was amplified as a negative control for ChIP analysis. PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide.

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References

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., Kishimoto, T., 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. 9 (6), 1897–1906.

Baldwin, B.R., Timchenko, N.A., Zahnow, C.A., 2004. Epidermal growth factor receptor stimulation activates the RNA binding protein CUG-BP1 and increases expression of C/EBPbeta-LIP in mammary epithelial cells. Mol. Cell. Biol. 24 (9), 3682–3691.

Bauknecht, T., Shi, Y., 1998. Overexpression of C/EBPbeta represses human papillomavirus type 18 upstream regulatory region activity in HeLa cells by interfering with the binding of TATA-binding protein. J. Virol. 72 (3), 2113–2124.

Bauknecht, T., See, R.H., Shi, Y., 1996. A novel C/EBP beta·YY1 complex controls the cell-type-specific activity of the human papillomavirus type 18 upstream regulatory region. J. Virol. 70 (11), 7695–7705.

Bodily, J.M., Meyers, C., 2005. Genetic analysis of the human papillomavirus type 31 differentiation-dependent late promoter. J. Virol. 79 (6), 3309–3321.

Calkhoven, C.F., Muller, C., Leutz, A., 2000. Translational control of C/EBPalpha and C/EBPbeta isoform expression. Genes Dev. 14 (15), 1920–1932.

del Mar Pena, L.M., Laimins, L.A., 2001. Differentiation-dependent chromatin rearrangement coincides with activation of human papillomavirus type 31 late gene expression. J. Virol. 75 (20), 10005–10013.

Descombes, P., Schibler, U., 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell 67 (3), 569–579.

Diehl, A.M., Michaelson, P., Yang, S.Q., 1994. Selective induction of CCAAT/enhancer binding protein isoforms occurs during rat liver development. Gastroenterology 106 (6), 1625–1637.

Eckert, R.L., Crish, J.F., Robinson, N.A., 1997. The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. Physiol. Rev. 77 (2), 397–424.

Fehrmann, F., Laimins, L.A., 2003. Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. Oncogene 22 (33), 5201–5207.

Grassmann, K., Rapp, B., Maschek, H., Petry, K.U., Iftner, T., 1996. Identification of a differentiation-inducible promoter in the E7 open reading frame of human papillomavirus type 16 (HPV-16) in raft cultures of a new cell line containing high copy numbers of episomal HPV-16 DNA. J. Virol. 70 (4), 2339–2349.

Heckman, C.A., Wheeler, M.A., Boxer, L.M., 2003. Regulation of Bcl-2 expression by C/EBP in t(14;18) lymphoma cells. Oncogene 22 (39), 7891–7899.

Hummel, M., Hudson, J.B., Laimins, L.A., 1992. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J. Virol. 66 (10), 6070–6080.

Klumpp, D.J., Laimins, L.A., 1999. Differentiation-induced changes in promoter usage for transcripts encoding the human papillomavirus type 31 replication protein E1. Virology 257 (1), 239–246.

Kowenz-Leutz, E., Leutz, A., 1999. A C/EBP beta isoform recruits the SWI/SNF complex to activate myeloid genes. Mol. Cell 4 (5), 735–743.

Kukimoto, I., Kanda, T., 2001. Displacement of YY1 by differentiation-specific transcription factor hSkn-1a activates the P(670) promoter of human papillomavirus type 16. J. Virol. 75 (19), 9302–9311.

Kyo, S., Inoue, M., Nishio, Y., Nakanishi, K., Akira, S., Inoue, H., Yutsudo, M., Tanizawa, O., Hakura, A., 1993. NF-IL6 represses early gene expression of human papillomavirus type 16 through binding to the noncoding region. J. Virol. 67 (2), 1058–1066.

Lekstrom-Himes, J., Xanthopoulos, K.G., 1998. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J. Biol. Chem. 273 (44), 28545–28848.

Longworth, M.S., Laimins, L.A., 2004. Pathogenesis of human papillomaviruses in differentiating epithelia. Microbiol. Mol. Biol. Rev. 68 (2), 362–372.

Maytin, E.V., Habener, J.F., 1998. Transcription factors C/EBP alpha, C/EBP beta, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. J. Invest. Dermatol. 110 (3), 238–246.

Maytin, E.V., Lin, J.C., Krishnamurthy, R., Batchvarova, N., Ron, D., Mitchell, P.J., Habener, J.F., 1999. Keratin 10 gene expression during differentiation of mouse epidermis requires transcription factors C/EBP and AP-2. Dev. Biol. 216 (1), 164–181.

Osada, S., Yamamoto, H., Nishihara, T., Imagawa, M., 1996. DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. J. Biol. Chem. 271 (7), 3891–3896.

Ozbun, M.A., Meyers, C., 1997. Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b. J. Virol. 71 (7), 5161–5172.

Ramji, D.P., Foka, P., 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem. J. 365 (Pt. 3), 561–575.

Raught, B., Gingras, A.C., James, A., Medina, D., Sonenberg, N., Rosen, J.M., 1996. Expression of a translationally regulated, dominant-negative CCAAT/enhancer-binding protein beta isoform and up-regulation of the eukaryotic translation initiation factor 2alpha are correlated with neoplastic transformation of mammary epithelial cells. Cancer Res. 56 (19), 4382–4386.

Robinson, G.W., Johnson, P.F., Hennighausen, L., Sterneck, E., 1998. The

C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes Dev. 12 (12), 1907–1916.

Ruesch, M.N., Stubenrauch, F., Laimins, L.A., 1998. Activation of papillomavirus late gene transcription and genome amplification upon differentiation in semisolid medium is coincident with expression of involucrin and transglutaminase but not keratin-10. J. Virol. 72 (6), 5016–5024.

Ryan, A.K., Rosenfeld, M.G., 1997. POU domain family values: flexibility, partnerships, and developmental codes. Genes Dev. 11 (10), 1207–1225.

Seagroves, T.N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G.J., Rosen, J.M., 1998. C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes Dev. 12 (12), 1917–1928.

Seitz, C.S., Lin, Q., Deng, H., Khavari, P.A., 1998. Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. Proc. Natl. Acad. Sci. U.S.A. 95 (5), 2307–2312.

Smith, C., Zhu, K., Merritt, A., Picton, R., Youngs, D., Garrod, D., Chidgey, M., 2004. Regulation of desmocollin gene expression in the epidermis: CCAAT/enhancer-binding proteins modulate early and late events in keratinocyte differentiation. Biochem. J. 380 (Pt. 3), 757–765.

Spink, K.M., Laimins, L.A., 2005. Induction of the human papillomavirus type 31 late promoter requires differentiation but not DNA amplification. J. Virol. 79 (8), 4918–4926.

Wang, H., Liu, K., Yuan, F., Berdichevsky, L., Taichman, L.B., Auborn, K., 1996. C/EBPbeta is a negative regulator of human papillomavirus type 11 in keratinocytes. J. Virol. 70 (7), 4839–4844.

Zhu, S., Oh, H.S., Shim, M., Sterneck, E., Johnson, P.F., Smart, R.C., 1999. C/EBPbeta modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. Mol. Cell. Biol. 19 (10), 7181–7190.

zur Hausen, H., 1996. Papillomavirus infections—A major cause of human cancers. Biochim. Biophys. Acta 1288 (2), F55–F78.

zur Hausen, H., 2000. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J. Natl. Cancer Inst. 92 (9), 690–698.

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Human VAP-B Is Involved in Hepatitis C Virus Replication through Interaction with NS5A and NS5B

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The hepatitis C virus (HCV) nonstructural protein (NS) 5A is a phosphoprotein that associates with various cellular proteins and participates in the replication of the HCV genome. Human vesicle-associated membrane protein-associated protein (VAP) subtype A (VAP-A) is known to be a host factor essential for HCV replication by binding to both NS5A and NS5B. To obtain more information on the NS5A protein in HCV replication, we screened human brain and liver libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. Immunoprecipitation and mutation analyses revealed that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. VAP-A interacts with VAP-B through the transmembrane domain. NS5A interacts with the coiled-coil domain of VAP-B via 70 residues in the N-terminal and 341 to 344 amino acids in the C-terminal polyproline cluster region. NS5A was colocalized with VAP-B in the endoplasmic reticulum and Golgi apparatus. The specific antibody to VAP-B suppressed HCV RNA replication in a cell-free assay. Overexpression of VAP-B, but not of a mutant lacking its transmembrane domain, enhanced the expression of NS5A and NS5B and the replication of HCV RNA in Huh-7 cells harboring a subgenomic replicon. In the HCV replicon cells, the knockdown of endogenous VAP-B by small interfering RNA decreased expression of NS5B, but not of NS5A. These results suggest that VAP-B, in addition to VAP-A, plays an important role in the replication of the HCV genome.

Hepatitis C virus (HCV) infects 170 million people worldwide and frequently leads to cirrhosis or hepatocellular carcinoma (6, 29). HCV is classified in the family *Flaviviridae* and possesses a single-stranded positive-sense RNA with a length of 9.6 kb. The HCV genome encodes a single large precursor polyprotein composed of about 3,000 amino acids (aa) that is processed by cellular and viral proteases, resulting in at least 10 structural and nonstructural (NS) proteins (29). Details of HCV's replication cycle are unknown because of the low viral load in the sera of HCV-infected individuals and the lack of a reliable and robust cell culture system to support HCV infection and replication. The development of HCV RNA replicons in which a synthetic HCV genomic or subgenomic RNA replicates efficiently in the human hepatocarcinoma cell line Huh-7 has enabled the study of viral RNA replication in cell culture (4, 20, 24). The HCV RNA replication complex, composed of the viral NS proteins and host cellular proteins, replicates the viral RNA genome at the intracellular membrane. Thus far, the HCV replicon system has greatly contributed to the understanding of HCV replication and pathogenesis associated with the expression of viral NS proteins. Replication of positive-strand RNA viruses generally involves certain intracellular membrane structures, including the endoplasmic reticulum (ER), Golgi apparatus, endosome, and lysosome (39). Recently, several groups have succeeded in demonstrating cell-free replication activities of replication complexes in crude membrane fractions of HCV subgenomic replicon cells (2, 3, 14, 53). These cell-free systems provide semi-intact polymerase assays for biochemical dissection of HCV RNA replication and are a useful source for the isolation of HCV replication complexes. Replication complexes were detected in detergent-resistant membrane structures, most likely lipid raft structures (2, 14). Although HCV NS proteins presumably form a membrane-associated RNA replication complex with host proteins, the precise components and mechanisms for replication are poorly understood.

HCV NS5A is a phosphoprotein that appears to possess multiple and diverse functions in viral replication, interferon resistance, and pathogenesis (26, 35). Cell culture-adaptive mutations have been shown to cluster in the central portion of NS5A in subgenomic HCV replicons, indicating that NS5A is involved in the viral replication process either directly or by interacting with host cellular proteins (4, 55). This observation, together with the modulation of NS5A hyperphosphorylation by NS3, NS4A, and NS4B and physical interaction with other viral NS proteins, strongly supports the notion that NS5A is an essential component of the HCV replication complex (21, 30, 36). NS5A has been shown to be associated with a range of cellular proteins involved in cellular signaling pathways, such as interferon-induced kinase PKR (11), growth factor receptor-binding protein 2 (Grb2) (45), p53 (27, 37), phosphoinositide-3-kinase p85 subunit (15), and proteins in protein trafficking and membrane morphology, such as karyopherin β3 (8),

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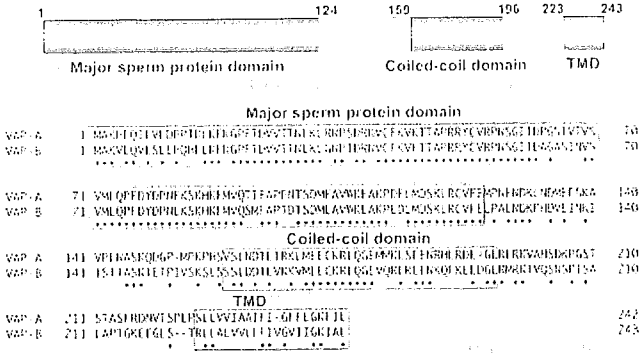

FIG. 1. Schematic representation of VAP-B and alignment of amino acid sequences of VAP-A and VAP-B. The major sperm protein domain, coiled-coil domain, and TMD are indicated. The asterisks indicate identical amino acid residues between VAP-A and VAP-B.

apolipoprotein A1 (40), amphiphysin II (56), and vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtype A (VAP-A), also called VAP-33 (48). Host fatty acids and geranylgeranylation appear to modulate the host and viral proteins involved in HCV RNA replication (19, 49, 54). Gao et al. showed that small interfering RNA (siRNA) or the dominant-negative mutant of VAP-A resulted in relocation of NS5B from detergent-resistant to detergent-sensitive membranes and reduced HCV RNA replication (12). In addition, Evans et al. suggested that NS5A hyperphosphorylation disrupts interaction with VAP-A and negatively regulates HCV RNA replication (9). Like many of the fusion proteins, VAP is a tail-anchored protein with a globular amino-terminal domain followed by a stalk region containing a coiled coil (Fig. 1), and it is ubiquitously expressed in human tissues (7). In humans, there are two isoforms of VAP, VAP-A and VAP-B, encoded by separate genes, and VAP-C is a splicing variant of VAP-B missing the C-terminal two-thirds (23, 32). VAP-B shows 63% amino acid identity to VAP-A (32, 51). The first proposed function for VAP arose from its initial identification as an interactor with the membrane fusion protein synaptobrevin/VAMP in *Aplysia* (43). Since then, it has been shown to be involved in vesicle transport, including the regulation of COP-I vesicle transport in the ER/Golgi pathway (13, 44), VAMP/synaptobrevin-mediated neurotransmitter release (38), and VAMP-2-mediated Glut-4 trafficking at the plasma membrane (10); it is also involved in the interaction between the microtubule network and tight junctions (22). Recently, VAP has been linked to the function of mammalian neurons, where VAP is enriched on microtubules (42), because a mutation in human VAP-B causes familial amyotrophic lateral sclerosis type 8 (32).

To gain a better understanding of the interactions between NS5A and host proteins involved in HCV replication, we screened human libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. In this study, we examined the biological significance of the interaction between VAP-B and NS proteins in HCV replication and found that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. Immunodepletion of VAP-B suppressed the replication of HCV RNA in a cell-free replication assay, and the knockdown of endogenous VAP-B by siRNA decreased the expression of NS5B but not that of NS5A. These results suggest that VAP-B plays an important role in HCV replication through interaction with NS5A and NS5B.

MATERIALS AND METHODS

Cells. Human embryo kidney 293T, human cervical carcinoma HeLa, and human hepatoma Huh-7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, Mo) containing 10% fetal calf serum (FCS), while the Huh-9-13 cell line, which possesses an HCV subgenomic replicon (4, 20, 23), was cultured in DMEM supplemented with 10% FCS and 1 mg/ml G418. All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Antibodies. Chicken anti-human VAP-B antibody was prepared by immunization using the synthetic peptides of residues from 188 to 203, KQFKEEDGL RMRKTVQ, of human VAP-B. A mouse monoclonal antibody to human VAP-A was purchased from BD Pharmingen (San Diego, CA). Mouse monoclonal antibodies to giantin, influenza virus hemagglutinin (HA), and GluGlu (EE) tag were from Covance (Richmond, CA). Mouse anti-FLAG antibody M2, horseradish peroxidase-conjugated antibody, and mouse monoclonal anti-beta-actin antibody were from Sigma. A mouse monoclonal antibody to protein disulfide isomerase (PDI) was from Affinity Bioreagents (Golden, CO). Rabbit polyclonal antibody to NS5A was prepared by immunization using peptides of residues from 409 to 422, DVESYSSMPPLEGE. Mouse monoclonal antibody to NS5B was described previously (41).

Plasmids. For expression in mammalian cells, a DNA fragment encoding NS5A was generated from HCV genotype 1b strain J1 (1) (GenBank database accession number D89815), and another was generated from genotype 1a strain H77 (52) (GenBank database accession number AF009606) by PCR using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). The fragments were then cloned into the appropriate sites in pEF-FLAG pGBK puro (18) and pEGFP-C3 (Clontech, Palo Alto, CA). The mutations of the NS5A gene were generated by a method known as "splicing by overlapping extension" (16, 17) and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding NS5B of the J1 strain was generated by PCR and cloned into pCAGGs-PUR (33). The DNA fragment encoding human VAP-A was amplified by PCR from a human fetal-brain library (Clontech) and was introduced into pEF-FLAG pGBK puro, pEF-EE hygro (34), pCHA3 (34), and pcDNA3.1-N-HA, in which an HA tag is inserted in the N terminus of the cloning site of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The cDNAs of human VAP-A and -B were amplified by PCR and cloned into pEF-FLAG pGBK puro, pEF-EE hygro, pcDNA3.1-N-HA, and pEGFP-C3. The genes encoding VAP lacking the transmembrane domain were amplified and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding the human VAP-B protein lacking a coiled-coil region was introduced into pEF-EE hygro. All PCR products were confirmed by sequencing them with an ABI PRSM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Yeast two-hybrid assay and library screening. The NS5A-binding protein was identified by a yeast two-hybrid assay according to the user manual of MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). The DNA fragment encoding amino acids 1973 to 2419 was amplified from HCV strain J1 by PCR and then was cloned into pGBKT7 (Clontech). The resulting plasmid was designated pGBK T7 HCV NS5A. A human brain library based on pACT2 was purchased from Clontech. The yeast *Saccharomyces cerevisiae* strain AH109, which secretes alpha-galactosidase under the control of MEL1 upstream activation sequence, was grown in yeast extract-peptone-dextrose medium and transformed with the bait and library plasmids. The transformed yeast cells were grown on 2.0% agar plates of dropout medium lacking tryptophan, leucine, histidine, and adenine. The resulting colonies were inoculated on the new dropout plate containing 20 μg/ml X-alpha-Gal (5-bromo-4-chloro-3-indolyl-alpha-*O*-galactopyranoside) and lacking leucine and tryptophan. The total DNA was prepared from all positive clones and then introduced into *Escherichia coli* strain JM109. The prey plasmids of isolated yeast cells were recovered from the clones grown on LB agar plates containing 10 μg/ml ampicillin and then purified. The insert DNA fragments of isolated clones were determined by sequencing. Finally, 48 alpha-galactosidase-positive clones were identified from 2 million clones screened in the fetal-brain library. One of the positive clones contained the complete cDNA of human VAP-B in frame.

Transfection, immunoblotting, and immunoprecipitation. Cells were seeded onto a six-well tissue culture plate 24 h before transfection. The plasmids were transfected into cells by liposome-mediated transfection using Lipofectamine 2000 (Invitrogen). Cells were harvested 36 h posttransfection, washed five times

with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.2 ml lysis buffer (20 mM Tris-HCl, pH 7.4, containing 135 mM NaCl and 1% Triton X-100) supplemented with 1 μg/ml leupeptin, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 5 mM $NaVO_4$. Cell lysates were sonicated at 4°C for 5 min, incubated for 30 min at 4°C, and centrifuged at 14,000 × *g* for 5 min at 4°C. The supernatant was immunoprecipitated with 1 μg of antibodies and 10 μl of Protein G-Sepharose 4B Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The immunocomplex was precipitated with the beads by centrifugation at 14,000 × *g* for 30 s and then was washed five times with lysis buffer by centrifugation. The proteins binding to the beads were boiled in 30 μl of loading buffer and then subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then reacted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

Immunofluorescence microscopy. Cells were seeded on an eight-well chamber slide at 2×10^4 per well 24 h before transfection. Transfected cells were washed twice with PBS, fixed with PBS containing 4% paraformaldehyde, and permeabilized with PBS containing 0.5% Triton X-100. The ER and Golgi apparatus of cells were stained with the mouse monoclonal antibody against luminal ER redox enzyme PDI and the rabbit polyclonal antibody against giantin, respectively, in PBS containing 5% bovine serum albumin. Bound primary antibody was revealed with Alexa Fluor 594-conjugated anti-mouse or anti-rabbit antibody. After additional washes with PBS, a coverslip was attached over PBS containing 50% glycerol and observed under an LSM 510 microscope (Carl Zeiss, Tokyo, Japan).

Gene silencing by siRNA. The siRNA target sequence against human VAP-B, 5′-GGUUAUGGAAGAAUGUAAGTT-3′, was synthesized and purified by Ambion (Austin, TX). Negative control siRNA, siCONTROL Non-Targeting siRNA-2, was purchased from Dharmacon (Lafayette, CO). The Huh-7 cells harboring a subgenomic HCV replicon on six-well plates were transfected with 80 nM or 160 nM of siRNA by using siFECTOR (B-Bridge International, Sunnyvale, CA) according to the manufacturer's protocol. Cells were incubated in DMEM supplemented with 10% FCS and harvested at 96 h posttransfection.

RNA replication assay. In vitro RNA replication was determined as previously described with some modification (3). Briefly, the Huh-7 cells harboring a subgenomic HCV-replicon grown in a 100-mm dish were treated with lysolecithin (Wako, Osaka, Japan) (250 μg/ml in wash buffer: 150 mM sucrose, 30 mM HEPES [pH 7.4], 33 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate), collected by scraping in 120 μl of incomplete replication buffer (100 mM HEPES [pH 7.4], 50 mM NH_4Cl, 7 mM KCl, and 1 mM spermidine), and centrifuged at 1,600 rpm for 5 min at 4°C. A total of 40 μl of cytoplasmic fraction (supernatant) was treated with 1% Nonidet P-40 (Boehringer Mannheim, Quebec, Canada) at 4°C for 1 h and incubated with antibody for 4 h at 4°C with rotation. Then, samples were incubated with 1 mM of ATP, GTP, and UTP; 10 μM CTP; [α-^{32}P]CTP (1 MBq; 15 TBq/mmol); 10 μg/ml actinomycin D; and 800 U/ml RNase inhibitor (Promega, Madison, WI) for 4 h at 30°C. RNA was extracted from the total mixture by TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The RNA was precipitated, eluted in 10 μl of RNase-free water, and analyzed by 1% formaldehyde agarose gel electrophoresis.

Real-time PCR. Total RNA was prepared from cell lines by using TRIzol LS (Invitrogen), and first-strand cDNA was synthesized by using a first-strand cDNA synthesis kit (Amersham) with random primers. Each cDNA was estimated by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed with an ABI PRISM 7000 (Applied Biosystems). The HCV NS5A gene was amplified using the primer pairs 5′-AGTCAGTTGTCTGCGCTTTC-3′ and 5′-CGGGGAATTTCCTGGTCTTC-3′. The human beta-actin gene was amplified with the primer pairs 5′-TGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTC-3′ and 5′-CGGACTCGTCATACTCCTGCTTGCTGATCCACATC-3′, which are located at different exons to prevent false-positive amplification from contaminated genomic DNA. The value of the HCV genome was normalized with that of actin mRNA. Each PCR product was found as a single band of the correct size on agarose gel electrophoresis (data not shown).

RESULTS

Isolation of VAP-B as a novel binding partner for HCV NS5A. To examine the protein(s) that interacts with NS5A in more detail, we screened a cDNA library of human fetal brain by a yeast two-hybrid system using a full-length NS5A of genotype 1b as bait. Among the 2 million transformants we screened, we obtained 48 positive clones containing cDNAs that encode proteins interactive with NS5A. A BLAST search against the GenBank database revealed each of two clones that have the cDNA encoding VAP-A and VAP-B in frame. Figure 1 shows the amino acid alignments of VAP-A and VAP-B and their predicted functional domains. VAP-A and VAP-B are composed of 242 and 243 amino acids, respectively. VAP-B shows 63% amino acid identity to VAP-A. VAP has three structural domains. The first 124 amino acids share high sequence similarity with the nematode major sperm protein and are conserved among all VAP family members (50). The central region on the protein contains an amphipathic helical structure and is predicted to form a coiled-coil protein-protein interaction motif (159 to 196 aa) and a hydrophobic carboxy-terminal transmembrane domain (TMD) (223 to 243 aa). The homology between their N-terminal regions is higher than that between their C-terminal regions (32, 48).

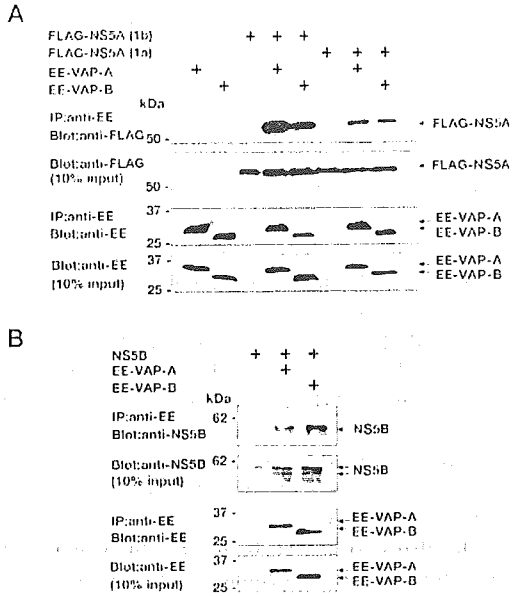

FIG. 2. VAP-A and VAP-B bind to both NS5A and NS5B in mammalian cells. N-terminally FLAG-tagged NS5A of genotype 1b, FLAG-NS5A (1b) of genotype 1a, FLAG-NS5A (1a), and N-terminally EE-tagged VAP (EE–VAP-A or EE–VAP-B) were coexpressed in HEK293T cells and immunoprecipitated with anti-EE antibody. The resulting precipitates were examined by immunoblotting using anti-FLAG antibody (A). NS5B was coexpressed with EE-tagged VAP-A or VAP-B and immunoprecipitated with anti-EE antibody, and NS5B in the precipitates was detected by anti-NS5B antibody (B). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

VAP-B interacts with NS5A and NS5B in mammalian cells. To confirm the specific interaction, FLAG-tagged NS5A was coexpressed with EE-tagged VAP-A or VAP-B in 293T cells, and cell lysates were immunoprecipitated by specific antibodies. NS5A was coprecipitated with VAP-A and VAP-B to similar extents (Fig. 2A). We also obtained the same results in the reverse experiments (data not shown). Recently, it was shown that hyperphosphorylation of NS5A disrupts interaction with VAP-A and negatively regulates HCV RNA replication, sug-

gesting that adaptive mutations detected in the HCV replicon prevent phosphorylation-dependent dissociation of the RNA replication complex (9). Amino acid residues at Tyr2185 and Lys2187 of NS5A genotype 1b were defined as key determinants for VAP-A binding, and the replacement of these residues with those of genotype 1a (Ala and Gly, respectively) reduced binding to VAP-A in yeast and enhanced hyperphosphorylation of NS5A (9). However, as shown in Fig. 2A, the NS5As of both the 1a and 1b genotypes were coimmunoprecipitated with VAP-A and -B in mammalian cells. Since a previous report indicated that VAP-A interacts with not only NS5A but also NS5B (12), we next examined the interaction of VAP-B with NS5B. EE-tagged VAP-A or VAP-B was coexpressed with NS5B in 293T cells and immunoprecipitated with anti-EE-tag antibody. NS5B was coprecipitated with VAP-B, as well as VAP-A (Fig. 2B). These results indicate that VAP-B participates in the complex of HCV NS proteins in a manner similar to that of VAP-A.

NS5A colocalizes with VAP-B in ER and Golgi compartments. To determine the subcellular localization of NS5A and VAP-B in mammalian cells, HeLa cells were cotransfected with plasmids encoding enhanced green fluorescent protein (EGFP)-tagged NS5A and FLAG-tagged VAP-B or FLAG-tagged VAP-A and examined by immunofluorescence analysis. EGFP-NS5A was colocalized exclusively with FLAG-VAP-B in the cytoplasm, as seen in FLAG-VAP-A (Fig. 3A). To further determine the precise subcellular localization of NS5A and VAP-B, the ER and Golgi apparatus were stained with specific antibodies against PDI and giantin, respectively. NS5A and VAP-B were colocalized with PDI and giantin in HeLa cells transfected with the plasmids (Fig. 3B), indicating that NS5A and VAP-B are colocalized in the membranes of the ER or ER-derived compartment. VAP-B was localized in a diffuse ER-like network, in small vesicles clustered around the nucleus, and predominantly in a perinuclear/Golgi region. Similar to the case with VAP-A, the colocalization of NS5A with VAP-B in the ER and Golgi apparatus suggests that NS5A specifically interacts with VAP-B under intracellular conditions.

Dimerization of VAP-A and VAP-B and interaction with NS5A. Immunoprecipitation analyses revealed that NS5A and NS5B interact with VAP-A and VAP-B. Therefore, it might be reasonable to speculate that VAP-A and VAP-B interact with each other and are involved in RNA replication through the formation of a replication complex. It has been demonstrated that VAP-A interacts with VAP-A or VAP-B through their TMDs and forms a homodimer and a heterodimer in vitro (32). We constructed expression plasmids encoding mutant VAP-A and VAP-B lacking their TMDs and examined their dimer formation with authentic VAPs in vivo. Although coprecipitation of authentic VAP (FLAG–VAP-B or FLAG–VAP-A) with VAP-B–HA was clearly detected, no interaction between TMD deletion mutants (FLAG–VAP-AΔTMD or FLAG–VAP-BΔTMD) and VAP-B–HA was observed (Fig. 4A and B). Furthermore, a TMD deletion mutant, HA–VAP-BΔTMD, which lost the ability to form a dimer with VAP-B and VAP-A, retained the ability to bind to FLAG-NS5A (Fig. 4C), although the efficiency of interaction with NS5A was reduced. These results indicate that TMDs of VAP-A and VAP-B are required for hetero- and homodimerization, but

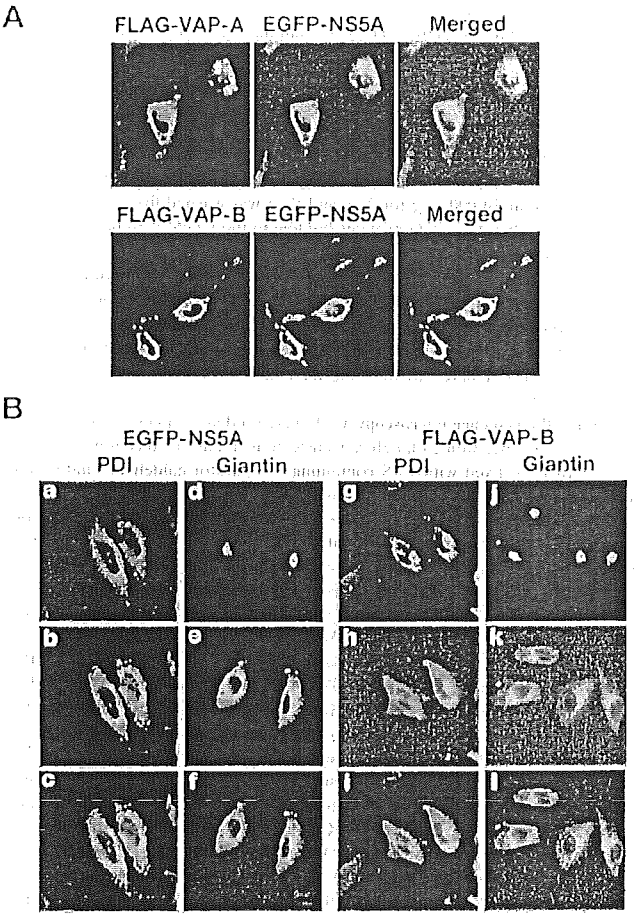

FIG. 3. Intracellular localization of VAPs and NS5A in mammalian cells. (A) N-terminally FLAG-tagged VAP (FLAG–VAP-A or FLAG–VAP-B) was coexpressed with N-terminally EGFP-fused NS5A of genotype 1b (EGFP-NS5A) in HeLa cells, fixed with 4% paraformaldehyde-PBS, permeabilized with 0.5% Triton X-100, and stained with anti-FLAG antibody and AlexaFluor 594-conjugated anti-mouse IgG antibody. (B) EGFP-NS5A of genotype 1b (b and e) or FLAG–VAP-B (h and k) was expressed and then stained with anti-PDI (a and g) or anti-giantin (d and j) antibodies and AlexaFluor 594-conjugated anti-mouse IgG antibody. FLAG–VAP-B was stained with biotinylated anti-FLAG antibody and fluorescein isothiocyanate-conjugated streptavidin. Overlapped images are shown in panels c, f, i, and l.

not for binding to NS5A. A region other than the TMD should be involved in the specific interaction between VAP-B and HCV NS5A. The coiled-coil domain of VAP-A was reported to be critical for binding to NS5A (48). Therefore, we examined whether the coiled-coil domain of VAP-B is also involved in interaction with NS5A. FLAG-NS5A was coimmunoprecipitated with EE–VAP-B but not with EE–VAP-BΔcoiled-coil, which lost the coiled-coil domain but retained the TMD (Fig. 4D), suggesting that the coiled-coil domain is also essential for interaction between NS5A and VAP-B.

Two separate domains in NS5A are critical for binding to VAP-B. Since NS5A specifically interacts with VAP-B, we tried to determine the region of NS5A responsible for interaction with VAP-B. Various deletion mutants of FLAG-tagged NS5A were prepared as shown in Fig. 5A. The mutants covering regions from amino acids 1 to 75, but not 1 to 50, and those

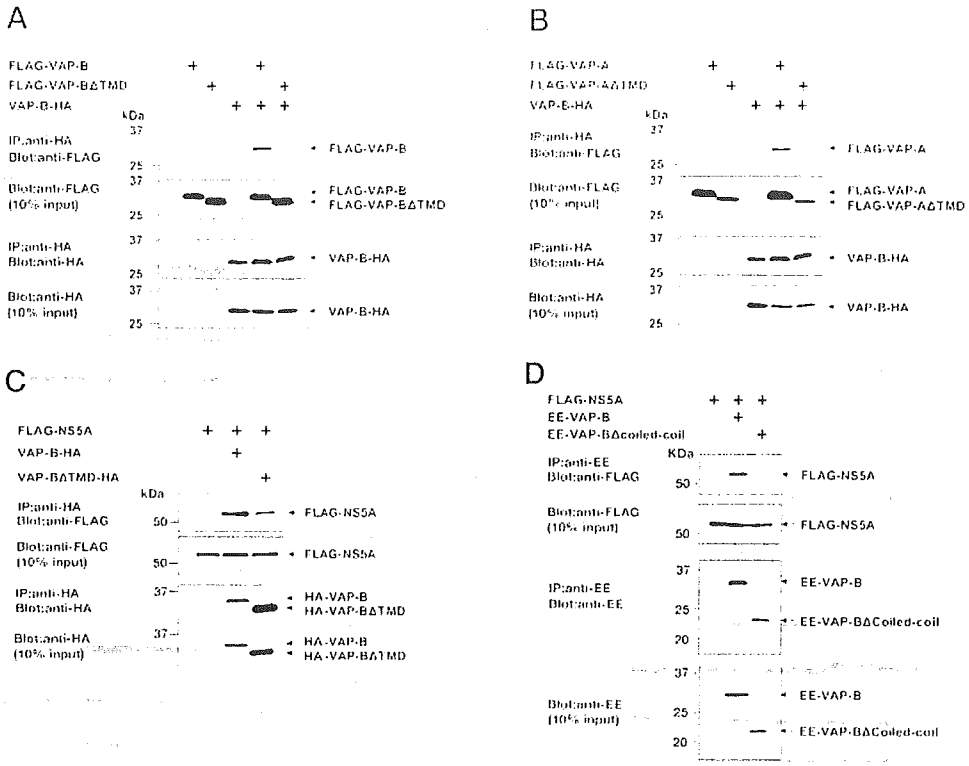

FIG. 4. VAP-B dimerizes with VAP-B and VAP-A through the TMD and interacts with NS5A via the coiled-coil domain. C-terminally HA-tagged VAP-B (VAP-B–HA) was coexpressed with FLAG–VAP-B or FLAG–VAP-B with TMD deleted (FLAG–VAP-BΔTMD). VAP-B–HA was immunoprecipitated with anti-HA antibody, and the immunoprecipitates were immunoblotted with anti-FLAG antibody (A). Interaction of VAP-B–HA with FLAG–VAP-A or FLAG–VAP-A with TMD deleted (FLAG–VAP-AΔTMD) was examined in a similar way (B). FLAG-NS5A was coexpressed with HA–VAP-B or HA–VAP-BΔTMD, and immunoprecipitates with anti-HA antibody and immunoprecipitates were immunoblotted with anti-FLAG antibody (C). FLAG-NS5A was coexpressed with EE–VAP-B or with EE–VAP-B in which the coiled-coil domain was deleted (EE–VAP-BΔcoiled-coil). EE-tagged VAP-B proteins were immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted with anti-FLAG antibody (D). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

from amino acids 325 to 447, but not 350 to 447, exhibited binding to VAP-B, suggesting that two separate regions of NS5A (amino acids 51 to 75 and 325 to 349) are involved in physical association with VAP-B. Further mutational analyses of NS5A revealed that regions from amino acids 1 to 70, but not 1 to 65, and those from amino acids 340 to 447, but not 345 to 447, interact with VAP-B (Fig. 5B and C), suggesting that amino acids 66 to 70 and 340 to 344 are required for interaction with VAP-B. According to Tellinghuisen et al., NS5A consists of three domains, domain I (amino acids 1 to 213), domain II (amino acids 250 to 342), and domain III (amino acids 356 to 477) (46, 47). In our results, the region from amino acids 340 to 344, which is essential for the physical interaction with VAP-B, belongs to the connecting segment between domains II and III of NS5A. Ala substitution analyses revealed that an NS5A construct covering amino acids 260 to 447 that replaced the five amino acid residues between 340 and 344 with Ala abrogated interaction with VAP-B (Fig. 5D), whereas that covering 75 N-terminal amino acids carrying an Ala substitution of between 66 and 70 residues retained binding activity to VAP-B (data not shown). Therefore, we focused on the region between 340 and 344 to determine the amino acid residues in NS5A responsible for specific binding to VAP-B. A FLAG-tagged full-length NS5A carrying an Ala substitution between amino acid residues 340 and 344 (FLAG-NS5A/340-344A) exhibited a clear reduction of binding to EE–VAP-B compared with the authentic NS5A (Fig. 5E). To further determine the critical amino acids of NS5A responsible for specific binding to VAP-B, each amino acid between 340 and 344 of the NS5A construct covering amino acids from 260 to 447 was replaced with Ala, and the effect of each substitution on the interaction with VAP-B was examined by immunoprecipitation. As summarized in Fig. 5F, the four amino acid residues 341 to 344 in the polyproline cluster region of NS5A, which are highly conserved among HCV genotypes, are suggested to be involved in the interaction with VAP-B.

VAP-B plays an important role in HCV RNA synthesis. To determine whether VAP-B is involved in HCV replication, cell lysates isolated from Huh-7 cells harboring a subgenomic HCV replicon were used for an in vitro RNA synthesis assay. Chicken anti-human VAP-B antibody raised against synthesized peptides specifically detected endogenous and overexpressed VAP-B (Fig. 6A). Cytoplasmic fraction from the HCV replicon was added to an assay mixture containing [α-^{32}P]CTP and incubated at 30°C for 4 h in the presence or absence of antibodies. Labeled RNA was analyzed by 1% formaldehyde agarose gel electrophoresis as described previously (2). Replication of the subgenomic HCV RNA was inhibited by the

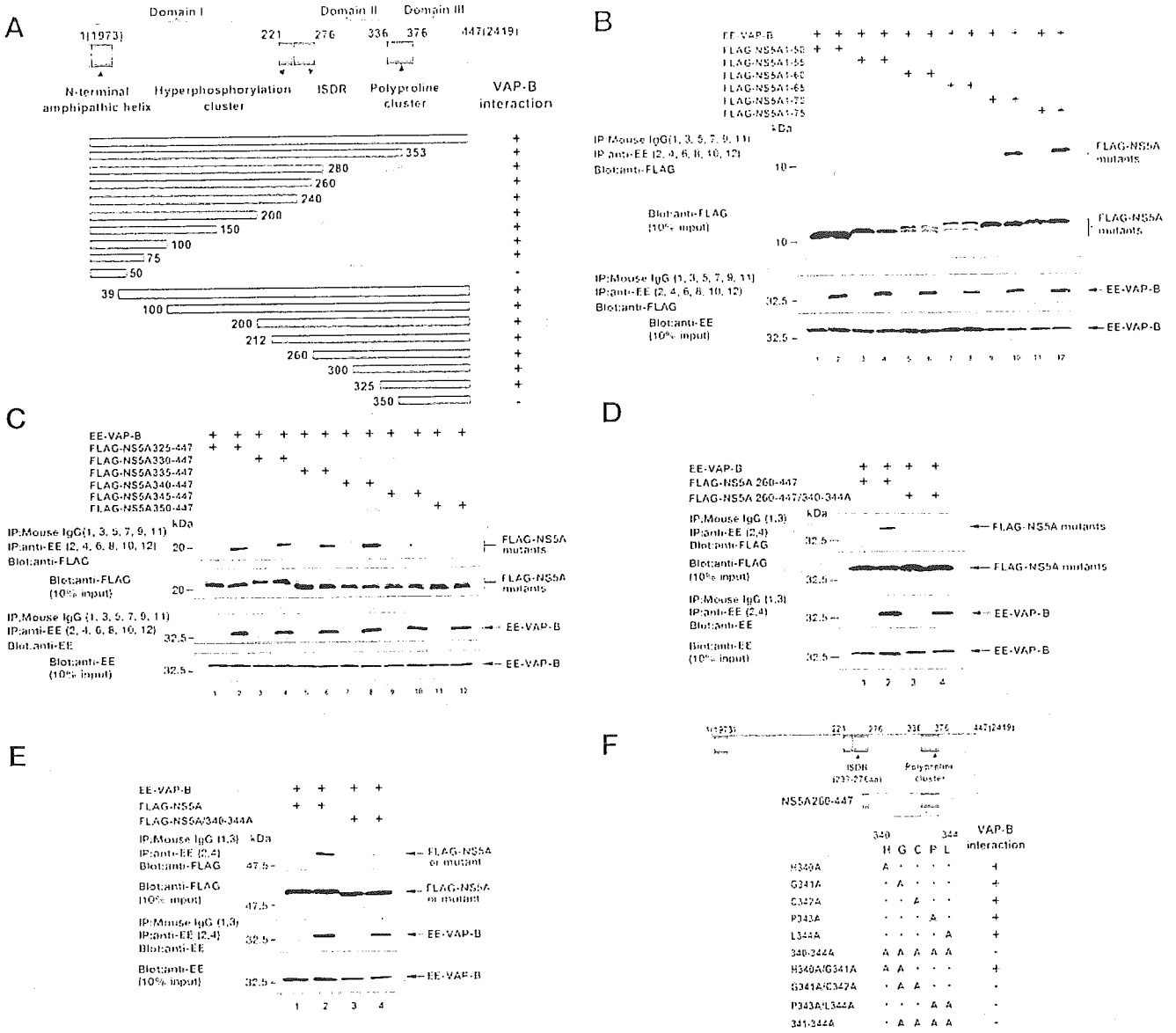

FIG. 5. Two regions of NS5A are required for VAP-B binding. N-terminal or C-terminal deletion mutants of NS5A were introduced into pEF-FLAG pGBK puro vector and coexpressed with EE–VAP-B. EE–VAP-B was immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted by anti-FLAG antibody. The reverse combination of immunoprecipitation was also examined. The results are summarized in panel A. Four functional domains in the NS5A protein and three domains based on the locations of the blocks of low-complexity sequence (46) are indicated. The numbers in parentheses indicate amino acid residues in the HCV polyprotein. To further determine the critical amino acids of NS5A for specific binding to VAP-B, deletion mutants of the N-terminal region from residues 1 to 75 (B) or those of the C-terminal region from residues 325 to 447 (C) were immunoprecipitated with EE–VAP-B. Replacement of the five residues 340 to 344 with Ala was introduced into a truncated NS5A possessing residues 260 to 447, FLAG-NS5A 260-447/340-344A (D), or full-length NS5A, FLAG-NS5A/340-(E), to examine the interaction with VAP-B. Further precise mutations were introduced into NS5A possessing residues 260 to 447. The resulting mutants were coexpressed with EE–VAP-B and immunoprecipitated as described above. The results are summarized in panel F. Four amino acids (Gly, Cys, Pro, and Leu) responsible for interaction with VAP-B are indicated by dotted squares. Plus and minus indicate binding and nonbinding, respectively (A and F). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

antibody to VAP-B but not by a control chicken immunoglobulin G (IgG) (Fig. 6B), suggesting that VAP-B plays a critical role in HCV replication. Aizaki et al. suggested that VAP-A sequesters NS5A at an appropriate site, such as the raft-like domain on the intracellular compartment, and that the TMD of VAP-A plays an important role in subcellular localization and dimerization (2). We demonstrated that the TMD of VAP is required for hetero- and homodimerization of VAP-A and VAP-B but not for interaction with NS5A (Fig. 4). Gao et al. indicated that a truncated VAP-A mutant lacking the TMD inhibited the association of HCV NS proteins with insoluble membrane fractions and reduced both the expression level of