PhoSL APVPVTKLVCDGDTYKCTA YLDFGDGRWVAQWDTNVFHTG 1–40aa

RSL IDPVNVKKLQCDGDTYKCTA DLDFGDGR 1–28aa

FIGURE 2. **Multiple alignment of PhoSL and RSL.** The residues in the *first row* describe the amino acid sequence of PhoSL, and those in the *second row* describe the amino acid sequence of RSL. Shown in *gray shading* are the amino acid residues that are identical between PhoSL and RSL.

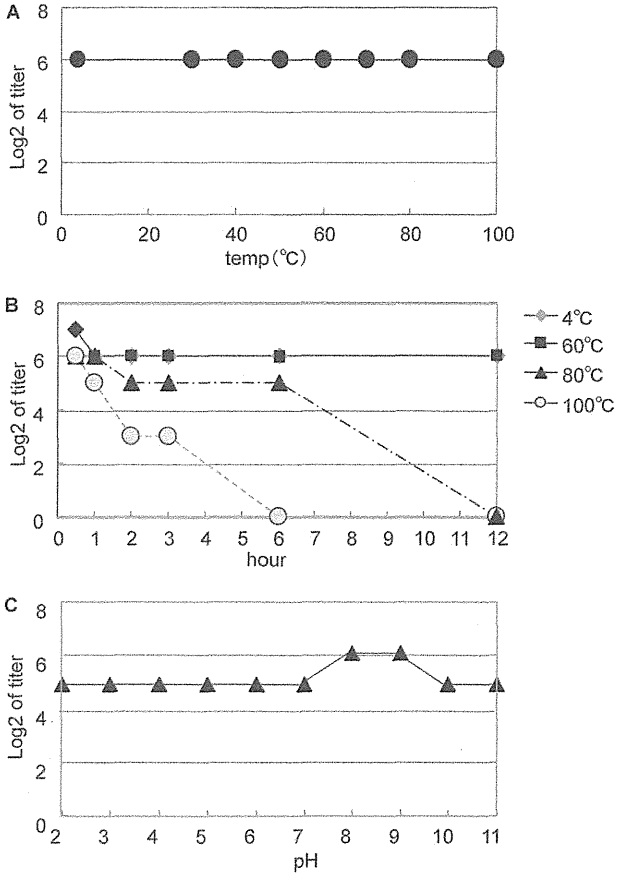

FIGURE 3. **The stability of PhoSL over a range of temperature and pHs.** The stability of PhoSL was investigated over a broad range of temperatures for 30 min (*A*), long incubation times for four different temperatures (*B*), and incubation in different pH buffers (*C*).

PBS, 10 mM potassium phosphate buffer (pH 7.4), or 10 mM sodium citrate buffer (pH 7.4) (data not shown).

Hemagglutination and Inhibition Assays—As shown in Table 1, PhoSL agglutinated intact erythrocytes from rabbit, horse, pig, goose, and guinea pig. As shown in Table 2, various monosaccharides, oligosaccharides, and glycopeptides were able to inhibit the hemagglutination activity of PhoSL. None of the mono- and oligosaccharides used bound to PhoSL. Among the tested glycoproteins, only IgG and thyroglobulin inhibited the hemagglutination activity of PhoSL. A peptide possessing the determined amino acid sequence was synthesized chemically. The synthetic peptide did not agglutinate intact rabbit erythrocytes (data not shown).

FAC Analysis of PhoSL—The detailed sugar binding specificity of PhoSL was also elucidated by FAC analysis. Among 132 kinds of PA-glycans used (supplemental Fig. S1), only the 21 glycans possessing the core α1–6 fucose bound to the lectin (Fig. 4*B*). The Bt and K_d values were determined to be 0.09 nmol

TABLE 1
Agglutination profiles of PhoSL(0.5 mg/ml)

Group of erythrocytes	Titer[a]
Rabbit	2^9
Sheep	NA[b]
Bovine	NA
Horse	2^8
Pig	2^8
Chicken	NA
Goose	2^{10}
Guinea pig	2^8
Human A	NA
Human B	NA
Human O	NA

[a] The hemagglutination titer was defined as the reciprocal of the highest dilution exhibiting hemagglutination.
[b] NA, not agglutinated.

TABLE 2
Inhibition of PhoSL-mediated hemagglutination by glycoproteins

Inhibitor[a]	MIC[b]
	μg/ml
Thyroglobulin	125
IgG	250

[a] Glucose, galactose, mannose, fucose, L-fucose, xylose, L-rhamnose, GlcNAc, GalNAc, ManNAc, LacNAc, lactose, maltose, fructose, saccharose, melibiose, and raffinose did not inhibit at concentrations up to 0.2 M. *N*-Acetylneuraminic acid and *N*-glycolylneuramic acid did not inhibit at concentrations up to 20 mM. Fetuin, asialo-fetuin, α_1-acid glycoprotein, transferrin, BSM, asialo-BSM, PSM, asialo-PSM, and albumin did not inhibit at concentrations up to 500 μg/ml.
[b] Minimum inhibitor concentration required for inhibition of four hemagglutination dose of the lectin.

and 3.3×10^{-6} M, respectively, for the immobilized PhoSL (1 mg/ml) using PA-402 (Fig. 4*A*). The strength of affinity of each PA-glycan for the immobilized lectin is shown as a K_a value (M^{-1}) in Figs. 4*B* and 5*B*. Manα1-3(Manα1-6 Manβ1-4GlcNAcβ(Fucα1-6)1-4GlcNAc-PA (PA-15, $K_a = 5.0 \times 10^{-5}$ M^{-1}) showed the strongest affinity to the immobilized lectin (Fig. 4*B*). The sialylated *N*-glycans ± Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-PA (PA-601, $K_a = 2.4 \times 10^{-5}$ M^{-1} and 602, $K_a = 1.2 \times 10^{-5}$ M^{-1}) also bound to the lectin. In contrast, *O*-glycans having L-Fuc (PA-718 to PA-723, PA-726 to PA-731, PA-739, PA-909) or Fucα1–3 linkages (PA-419 and -420) did not show any significant affinity to the immobilized PhoSL.

The detailed oligosaccharide binding specificity of PhoSL was compared with that of LCA, which has been reported previously (Fig. 5) (3). For an easier understanding of the structural elements required for the recognition of PhoSL and LCA, the K_a values for a series of core-fucosylated glycans have been arranged in the order of affinity strength, and the core-fucosylated glycans have been represented using the "GRYP" code proposed in a previous report (3, 32). In this system the branch positions of *N*-glycans (GlcNAc) are numbered from I to VI according to the corresponding mammalian GlcNAc-transferases, and the nonreducing end sugars are shown in different colors: GlcNAc (*blue*), Gal (*yellow*), and NeuAc (*purple*). The presence of the core fucose (α1–6Fuc) is emphasized with another box colored in *red*. Both PhoSL and LCA showed high specificity for mannose-type (PA-015), mono- (PA-201, -401, -402), and bi-antennary (PA-202, -203, -403, -404, -405, -406, -601, -602) *N*-glycans containing core fucose. PhoSL recognized not only mono- or biantennary oligosaccharides but also tri- or tetra-antennary oligosaccharides (PA-

Downloaded from www.jbc.org at USAKA UNIVERSITY, on February 3, 2013

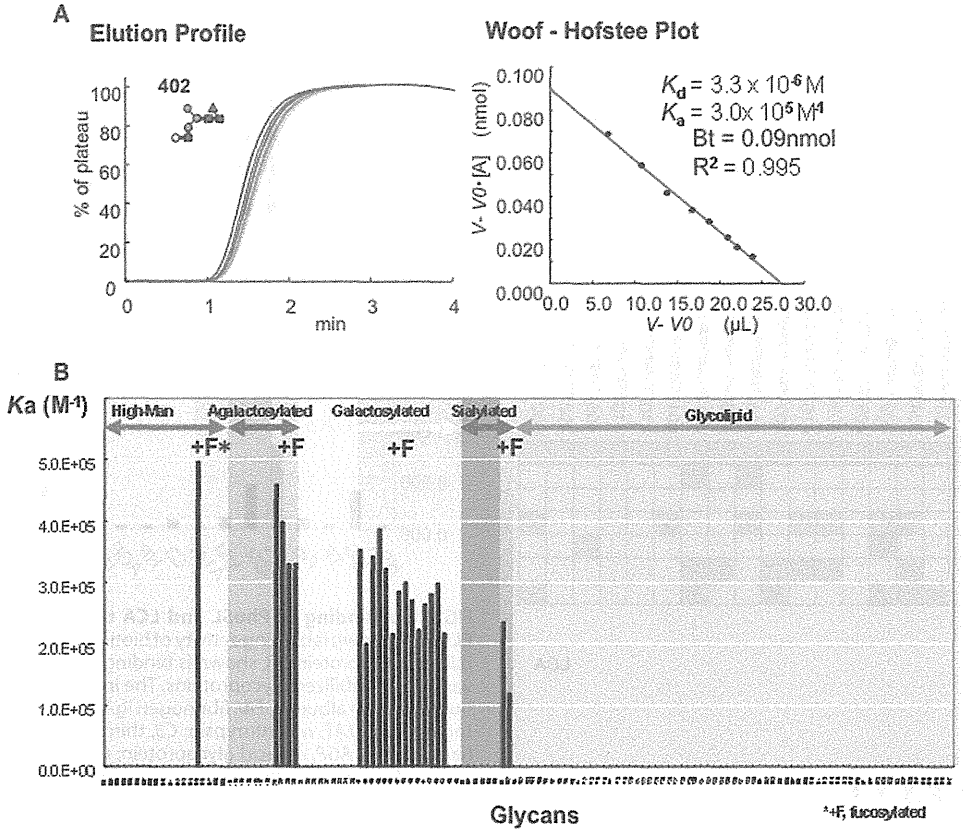

FIGURE 4. **FAC analysis of PhoSL.** *A*, shown is the elusion pattern of several types of PA-oligosaccharides on the PhoSL-immobilized column. *B*, shown are association constants (K_a) values of the purified PhoSL to various types of PA-glycans.

015, -201–204, -401–411, -413, -601, -602) (Fig. 5*B*). However, LCA bound to only mono- and biantennary oligosaccharides (PA-015, -202, -203, -205, -401, -402, -403, -406, -601, -602). No binding of LCA to core-fucosylated tri- (PA-407, -408, -409, -410) and tetra-antennary (PA-411, -413, -415, -418) *N*-glycans was observed (Fig. 5*B*). The sugar binding specificity of the synthetic peptide was almost the same as that of PhoSL (supplemental Fig. S3).

ELISA of PhoSL—To compare the detailed carbohydrate binding specificity of PhoSL with that of LCA, we examined the binding of biotinylated lectins to immobilized proteins or glycoproteins using ELISA (Fig. 6). Each protein or glycoprotein was immobilized on the plates, and biotinylated PhoSL or LCA was used as the analyte. Among the major human serum glycoproteins (HSA, IgG, transferrin, fibrinogen, IgA, α2-macroglobulin, IgM, α1-antitrypsin, C3 (third components of complement), haptoglobin, and α1-acid glycoprotein) tested, the most potent binding glycoproteins for PhoSL were IgA and IgG (Fig. 6*A*). Among the serum tumor markers (prostate specific antigen (PSA), AFP, AFP-L3) tested, PhoSL bound to fucosylated AFP (AFP-L3). Thyroglobulin (bovine) and lactoferrin (human milk) were also bound to PhoSL. All the glycoproteins that bound to PhoSL in the assay possessed the core fucose. Although the profile of LCA was similar to that of PhoSL, the binding to the glycoproteins was much weaker than that of PhoSL (Fig. 6*B*).

Antibody-Lectin Sandwich ELISA—The sugar binding specificity of PhoSL to fucosylated AFP (AFP-L3) obtained from the sera of three patients with HCC (HCC-1–3) was further investigated by antibody-lectin sandwich ELISA (Fig. 7). Before the ELISA, the existence of AFP-L3 in the serum samples was confirmed by the conventional method, lectin affinity electrophoresis, using LCA. All the samples contained AFP-L3 (supplemental Fig. S4). In the antibody-lectin sandwich ELISA, *N*-glycosidase F-treated anti-AFP antibody was immobilized on the plate, as IgG has fucosylated oligosaccharides in its Fc site (fragment, crystallizable site of antibody). AFP from the three patients (HCC-1 to HCC-3) and 3 volunteers (NV-1 to NV-3) was detected by anti-AFP (Fig. 7*A*). Both PhoSL and LCA bound to the AFP in a dose-dependent manner (Fig. 7, *B* and *C*).

Application of PhoSL to Histochemistry—To demonstrate the utility of PhoSL in the immunohistochemical analysis of human cancer tissues, 124 colon cancer tissues, including primary and metastatic colon cancers, on tissue arrays were stained with biotinylated PhoSL and AAL. AAL is a lectin from *A. aurantia* that binds to all types of fucosyl linkage. Staining intensities were classified into 4 groups: negative staining (0), low staining intensity (1), medial staining intensity (2), and high staining intensity (3). A representative tissue from each group is shown in supplemental Fig. S5. Representative images of normal colon, primary cancer, and metastatic cancer are shown in Fig. 8*A*. Normal colon mucosa was stained with AAL, but not PhoSL, because of abundant mucin, which carries α1–3/4 fucosylated glycans on *O*-glycans, suggesting that PhoSL does not bind to α1–3/4 fucosylated glycans (Fig. 8, *A* and *B*). As shown in Fig. 8, *C* and *D*, both AAL and PhoSL bound to primary colon cancer tissue at a similar intensity. In contrast, only AAL, but

Downloaded from www.jbc.org at OSAKA UNIVERSITY, on February 3, 2013

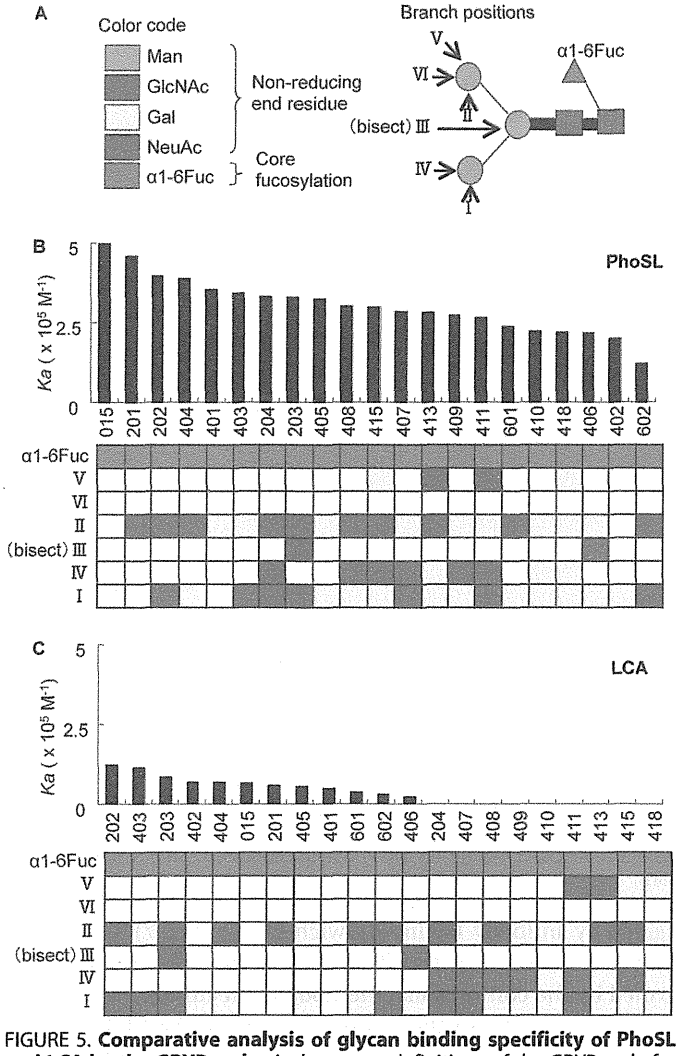

FIGURE 5. **Comparative analysis of glycan binding specificity of PhoSL and LCA by the GRYP code.** *A*, shown are definitions of the GRYP code for representing the branch positions and non-reducing end residues. The non-reducing end sugars and the core fucose are shown in different colors in the *left panel*. Each branch is numbered from I to VI corresponding to GlcNAc transferases, as shown in the *middle panel*. *B*, shown are *bar graph* representations of the association constants (K_a) of PhoSL toward core-fucosylated *N*-glycans. *Numbers at the bottom of the bar graphs* correspond to the sugar numbers indicated in supplemental Fig. S2. *C*, shown are *bar graph* representations of the association constants (K_a) of LCA.

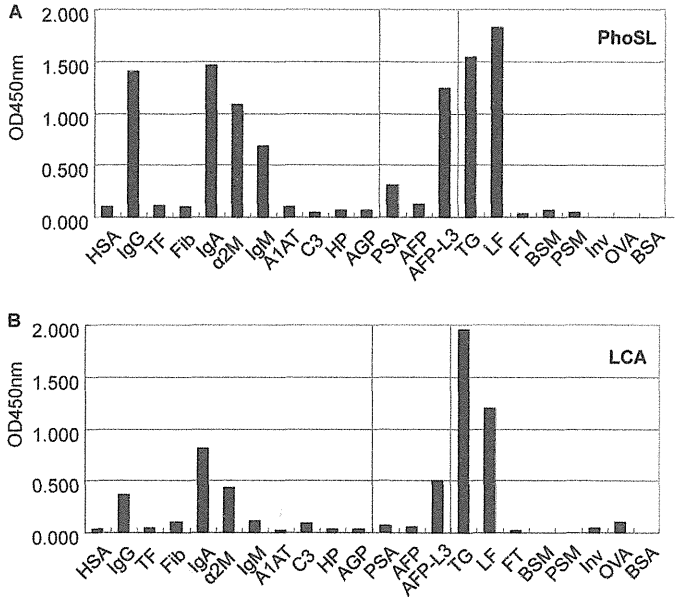

FIGURE 6. **Binding of PhoSL and LCA to immobilized glycoproteins by ELISA.** *A*, shown is binding activity of biotin-labeled PhoSL and various immobilized glycoproteins. *B*, shown is binding activity of biotin-labeled LCA and various immobilized glycoproteins. The immobilized glycoproteins are: *HAS*, human serum albumin; *Fib*, fibrinogen IgA Immunoglobulin A; *α2M*, α2-macroglobulin; *A1AT*, α1-antitrypsin; *C3*, third components of complement; *HP*, haptoglobin; *AGP*, α1-acid glycoprotein; *PSA*, prostate specific antigen; *AFP-L3*, 1–6-fucosylated fetoprotein; *LF*, lactoferrin; *FT*, fetuin; *BSM*, bovine submaxillary gland mucin; *PSM*, porcine stomach mucin; *Inv*, invertase; *OVA*, ovalbumin.

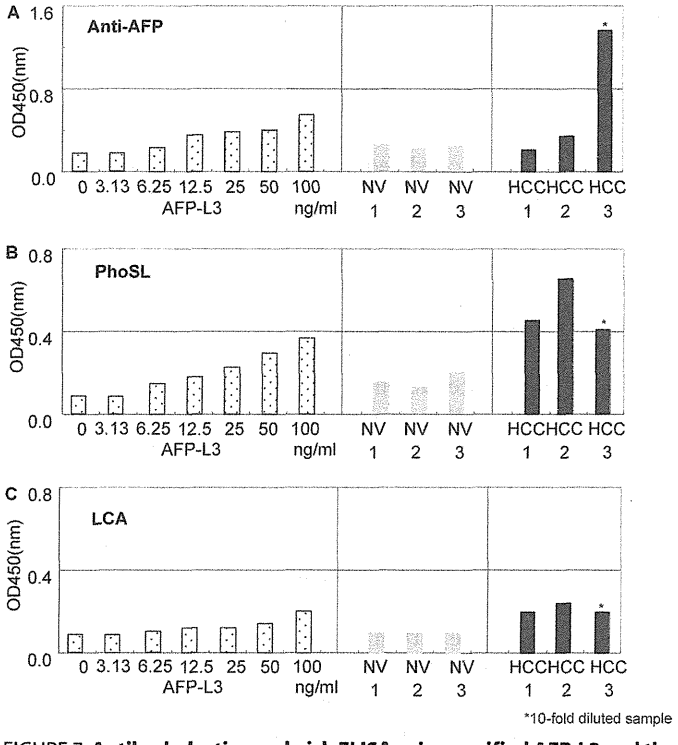

FIGURE 7. **Antibody-lectin sandwich ELISA using purified AFP-L3 and the sera of HCC patients and normal volunteers.** ELISA with Anti-AFP (*A*), PhoSL (*B*), and LCA (*C*).

not PhoSL, bound to metastatic colon cancer tissue (Fig. 8, *E* and *F*). The staining intensities of all tissues examined in this study are summarized in Table 3. Approximately 70% of the primary and metastatic cancer tissues were classified into a high intensity group after AAL staining (Fig. 8, *G* and *H*, and Table 3). No difference in AAL staining intensity was observed between primary and metastatic cancer tissues. In contrast, PhoSL exhibited a significantly lower binding capacity to the metastatic cancer tissues than the primary tissues (Fig. 8, *G* and *H*, and Table 3). Only 25% of the metastatic tissues showed medial and strong intensities (group 2 and 3) after PhoSL staining despite the fact that 84% of these tissues were stained with AAL.

DISCUSSION

PhoSL was purified from the edible mushroom *P. squarrosa*. The lectin gave a band with a mass of 4.5 kDa on SDS-PAGE in the presence (Fig. 1, *A*, *lane 1*, and *B*, *lane 1*) and two bands with masses of 4.5 and 14 kDa in the absence of 2-mercaptoethanol (Fig. 1*B*, *lane 2*). Its primary structure consisted of 40 amino acids, as determined by N-terminal amino acid sequence anal-

Downloaded from www.jbc.org at OSAKA UNIVERSITY, on February 3, 2013

ysis. The synthetic PhoSL peptide corresponding to the determined sequence exhibited identical binding specificity to native PhoSL in FAC analysis (supplemental Fig. S2) but did not exhibit hemagglutination activity. HPLC gel filtration of the intact lectin gave a peak at an elution volume corresponding to a molecular mass of 14 kDa. PhoSL possessed no sugar chains or phosphate groups. All the results mentioned above indicated that PhoSL is composed of three or four 4.5-kDa subunits with S-S linkage and exhibits true polyvalent binding during agglutination of erythrocytes and/or precipitation of appropriate cell surface polysaccharides, but the oligomeric form is not necessarily in direct binding assays (FAC analysis, ELISA, etc.).

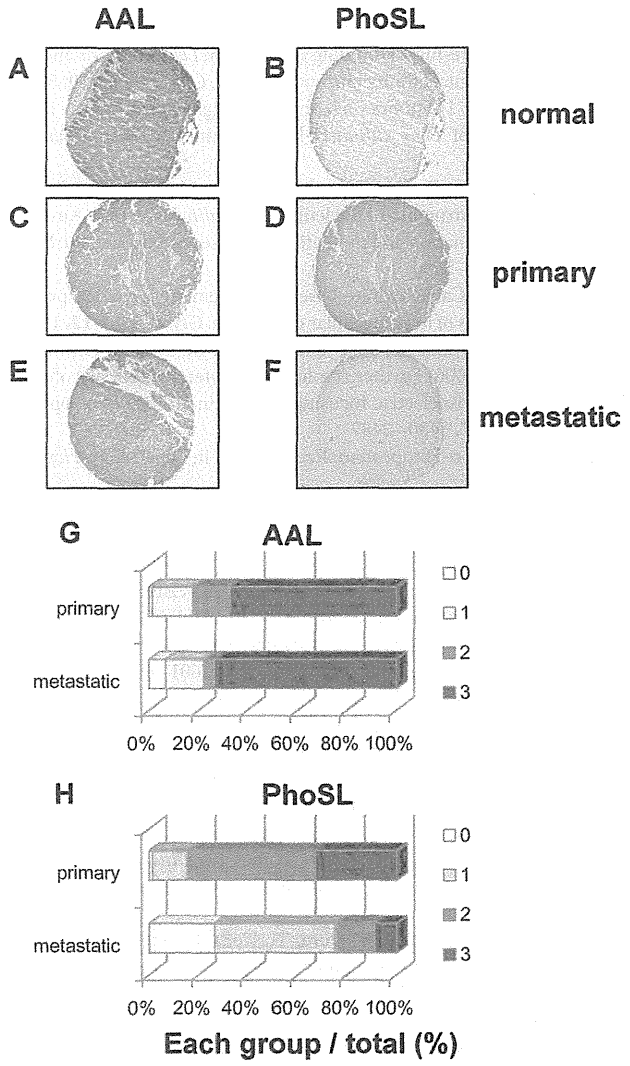

FIGURE 8. **Immunohistochemical analysis of human colon cancer tissues with PhoSL and AAL.** Human colon cancer tissue arrays comprising normal colon tissues (*A* and *B*), primary colon cancers (*C* and *D*), and metastatic colon cancers (*E* and *F*) were subjected to immunohistochemical analysis with PhoSL and AAL. *G* and *H* show the ratio of the numbers in each staining-intensity group to the total number is shown.

The BLAST search revealed that PhoSL has 85% sequence homology (22/26 amino acids) with the α1–6-linked fucose-specific lectin from *R. stolonifer*. RSL has also been isolated as a core fucose-specific lectin. RSL has high affinity toward saccharides with α1–6Fuc and weak affinity toward saccharides with α1–2Fuc, α1–3Fuc, and α1–4Fuc (33).

The unique property of PhoSL is its strict sugar binding specificity to α1–6Fuc (Fig. 4*B*). α1–6 fucosylation is one of the most important oligosaccharide modifications in carcinogenesis; however, although many studies related to fucosylation have been conducted, they have not completely clarified the difference between α1–2, α1–3, or α1–4 fucosylation and α1–6 fucosylation. A hindrance to this clarification has been the lack of a tool for the specific detection of α1–6 fucosyl linkage; AAL, which is used in many studies, recognizes all types of fucosyl linkages (13, 17).

The FAC results indicate that PhoSL recognizes α1–6 fucosyl linkages exclusively, that all the α1–6 oligosaccharides were bound to the lectin, and that LCA could not bind to some α1–6 oligosaccharides (Figs. 4 and 5). In addition, the K_a value of PhoSL was 3.2×10^5 M^{-1} for the fully galactosylated, biantennary *N*-glycan with a core fucose (PA-405), which is the major *N*-glycan in AFP-L3 from Huh7 cells and HCC patients (Fig. 4*B*) (34). On the other hand, the K_a value of the binding between LCA and PA-405 was 4.7×10^4 M^{-1} (3). The affinity of PhoSL toward the oligosaccharide was higher than that of LCA. Furthermore, LCA also bound to non-fucosylated, high mannose-type *N*-glycans. For example, LCA showed affinity for a larger high mannose-type *N*-glycan, Man8 (Manα1-2Manα1-2Manα1-3 (Manα1-2Manα1-6 (Manα1-3) Manα1-6) Manβ1–4GlcNAcβ1–4 (Fucα1–6) GlcNAc-PA, PA-012), with a K_a of 2.5×10^4 M^{-1} (3). LCA is now the only commercially available diagnostic agent that can detect α1–6 fucosyl-linked sugar chains specifically.

The superiority of PhoSL over LCA was confirmed by ELISA using biotin-labeled PhoSL and LCA and immobilized glycoproteins. Although the specificity of both lectins showed similar tendencies, the binding strength of PhoSL to the α1–6-fucosylated glycoproteins was greater than that of LCA (Fig. 6). The promising potential of PhoSL as a diagnostic agent was also shown by antibody-lectin sandwich ELISA using AFP-L3 and the partially purified AFP from the sera of HCC patients and normal volunteers (*NV*) (Fig. 7). The sensitivity and selectivity of PhoSL to AFP-L3 in the sera of HCCs and normal volunteers were much greater than those of LCA and even anti-AFP (Fig.

TABLE 3
Staining intensities of AAL and PhoSL in human colon cancer tissue analyses

	AAL[a]			PhoSL[b]		
Staining intensity	Normal	Primary	Metastatic	Normal	Primary	Metastatic
0	0	1 (1.3%)	3 (6.7%)	7	1 (1.3%)	12 (26.7%)
1	0	13 (16.5%)	7 (15.6%)	4	11 (13.9%)	22 (48.9%)
2	5	12 (15.2%)	2 (4.4%)	0	41 (51.9%)	7 (15.6%)
3	6	53 (67.1%)	33 (73.3%)	0	26 (32.9%)	4 (8.9%)
Total	11	79	45	11	79	45

[a] not significant.
[b] $p < 0.01$, compared between primary and metastatic cancers (χ^2 test).

Downloaded from www.jbc.org at OSAKA UNIVERSITY, on February 3, 2013

7). AFP is a biomarker that was discovered in 1963 by Abelev (35) and belongs to the albumin-like superfamily. This protein, whose molecular mass is 65 kDa, consists of 590 amino acids and has a biantennary sugar chain at Asn^{232} (36). A variety of sugar chains are on the protein, and AFP-L3 is one of them. The structure of AFP-L3 has been determined to be GlcNAcβ1-2Manα1-3 (Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-AFP by lentil lectin affinity electrophoresis. Because slightly increased serum concentrations of total AFP have been observed in patients with chronic hepatitis and liver cirrhosis, conditions that are known to be associated with pre-malignant HCC lesions, a wide overlap in total AFP has been observed between HCC and such benign liver diseases. The sera of patients with HCC are known to contain relatively large amounts of AFP-L3. Therefore, AFP-L3 has been recognized as a specific marker for HCC. Furthermore, analysis using this marker could be useful for monitoring treatment responses and disease recurrence and could also be a tool for recognition of HCC earlier than that possible by using imaging modalities (34, 37–42). In recent years, in addition to AFP, new biomarkers possessing fucosides have been discovered. For example, Golgi protein 73 (GP73) content in the blood of patients with HCC was found to be elevated, and the protein was α1–6-hyperfucosylated (43–46). In addition, patients with liver cirrhosis and liver cancer had increased levels of triantennary glycan containing outer arm (α1–3)-fucosylation in α-antitrypsin in the blood, but increases in core (α1–6)-fucosylation were observed only on α1-antitrypsin from patients with liver cancer (47). Physiological functions of the core fucose has been investigated recently. The lack of core fucosylation of transforming growth factor-β1 receptors induces severe growth retardation and death during postnatal development (48, 49). Mutations of the GDP-mannose-4,6-dehydratase gene that plays a pivotal role in fucosylation in human colon cancer resulted in resistance to TRAIL-induced apoptosis followed by escape from immune surveillance. This pathway by GDP-mannose-4,6-dehydratase mutation could be a novel type of cancer progression through cellular fucosylation and natural killer cell-mediated tumor surveillance. However the cellular fucosylation type has not been determined yet (50, 51).

Fig. 8 shows that AAL bound to both primary and metastatic colon cancer tissues with a similar intensity. However, PhoSL bound to the primary colon cancer tissues more strongly than it did to the metastatic tissues. These results suggest that in some cases α1–6 fucosylation is increased in the early phase of colon cancer development and subsequently decreased in the metastatic phase. The decreased expression of α1–6 fucosylation in metastatic cancer tissues may be responsible for the escape of cancer cells from natural killer cell-mediated tumor surveillance. The mechanism and meaning underlying the decreased expression of α1–6 fucosylation in metastatic cancer tissues should be revealed in a future study. PhoSL, the lectin characterized in this study, may be useful for the detection of AFP-L3 and other new biomarkers and for determining the physiological functions of oligosaccharides (19).

In summary, PhoSL very strongly and specifically binds to Fucα-oligosaccharides. Moreover, it is highly stable over a wide range of pHs and temperatures and is highly soluble in various buffers. These advantages indicate that PhoSL can become a powerful tool to analyze biological functions of core fucoses and serve as a diagnostic agent in the near future.

REFERENCES

1. Miyoshi, E., Moriwaki, K., and Nakagawa, T. (2008) Biological function of fucosylation in cancer biology. *J. Biochem.* **143,** 725–729
2. Uozumi N, Yanagidani S, Miyoshi E, Ihara Y, Sakuma T, Gao CX, Teshima T, Fujii S, Shiba T, and Taniguchi, N. (1996) Purification and cDNA cloning of porcine brain GDP-L-Fuc:*N*-acetyl-β-D-glucosaminide α1→6fucosyltransferase. *J. Biol. Chem.* **271,** 27810–27817
3. Tateno, H., Nakamura-Tsuruta, S., and Hirabayashi, J. (2009) Comparative analysis of core-fucose-binding lectins from *Lens culinaris* and *Pisum sativum* using frontal affinity chromatography. *Glycobiology* **19,** 527–536
4. Howard, I. K. (1971) Studies on a phytohemagglutinin from the lentil. II. Multiple forms of *Lens culinaris* hemagglutinin. *J. Biol. Chem.* **246,** 1590–1595
5. Foriers, A., Lebrun, E., Van Rapenbusch, R., de Neve, R., and Strosberg, A. D. (1981) The structure of the lentil (*Lens culinaris*) lectin. Amino acid sequence determination and prediction of the secondary structure. *J. Biol. Chem.* **256,** 5550–5560
6. Aoyagi, Y. (1995) Carbohydrate-based measurements on α-fetoprotein in the early diagnosis of hepatocellular carcinoma. *Glycoconj. J.* **12,** 194–199
7. Ichikawa, E., Kuriyama, S., Yuji, J., Masaki, T., Uchida, N., Nishioka, M., and Taketa, K. (2006) Further resolution of α-fetoprotein glycoforms by two-dimensional isoelectric focusing and lectin affinity electrophoresis. *Electrophoresis* **27,** 3480–3487
8. Taketa, K. (1990) α-Fetoprotein. Reevaluation in hepatology. *Hepatology* **12,** 1420–1432
9. Debray, H., Decout, D., Strecker, G., Spik, G., and Montreuil, J. (1981) Specificity of twelve lectins toward oligosaccharides and glycopeptides related to *N*-glycosylproteins. *Eur. J. Biochem.* **117,** 41–55
10. Amano, J., Osanai, M., Orita, T., Sugahara, D., and Osumi, K. (2009) Structural determination by negative-ion MALDI-QIT-TOF MSn after pyrene derivatization of variously fucosylated oligosaccharides with branched decaose cores from human milk. *Glycobiology* **19,** 601–614
11. Harada, H., Kamei, M., Tokumoto, Y., Yui, S., Koyama, F., Kochibe, N., Endo, T., and Kobata, A. (1987) Systematic fractionation of oligosaccharides of human immunoglobulin G by serial affinity chromatography on immobilized lectin columns. *Anal. Biochem.* **164,** 374–381
12. Matsumura, K., Higashida, K., Hata, Y., Kominami, J., Nakamura-Tsuruta, S., and Hirabayashi, J. (2009) Comparative analysis of oligosaccharide specificities of fucose-specific lectins from *Aspergillus oryzae* and *Aleuria aurantia* using frontal affinity chromatography. *Anal. Biochem.* **386,** 217–221
13. Yamashita, K., Kochibe, N., Ohkura, T., Ueda, I., and Kobata, A. (1985) Fractionation of L-fucose-containing oligosaccharides on immobilized *Aleuria aurantia* lectin. *J. Biol. Chem.* **260,** 4688–4693
14. Matsumoto, I., Uehara, Y., Jimbo, A., and Seno, N. (1983) Immunochemical and spectral studies on *Vicia faba* agglutinin. *J. Biochem.* **93,** 763–769
15. Jordinson, M., El-Hariry, I., Calnan, D., Calam, J., and Pignatelli, M. (1999) *Vicia faba* agglutinin, the lectin present in broad beans, stimulates differentiation of undifferentiated colon cancer cells. *Gut* **44,** 709–714
16. Allen, A. K., Desai, N. N., and Neuberger, A. (1976) Purification of the glycoprotein lectin from the broad bean (*Vicia faba*) and a comparison of its properties with lectins of similar specificity. *Biochem. J.* **155,** 127–135
17. Matsumura, K., Higashida, K., Ishida, H., Hata, Y., Yamamoto, K., Shigeta, M., Mizuno-Horikawa, Y., Wang, X., Miyoshi, E., Gu, J., and Taniguchi, N. (2007) Carbohydrate binding specificity of a fucose-specific lectin from *Aspergillus oryzae*. A novel probe for core fucose. *J. Biol. Chem.* **282,** 15700–15708
18. Mun, J. Y., Lee, K. J., Kim, Y. J., Kwon, O., Kim, S. J., Lee, S. G., Park, W. S., Heo, W. D., and Oh, D. B. (2012) Development of fluorescent probes for the detection of fucosylated *N*-glycans using an *Aspergillus oryzae* lectin. *Appl. Microbiol. Biotechnol.* **93,** 251–260
19. Matsumoto, H., Shinzaki, S., Narisada, M., Kawamoto, S., Kuwamoto, K., Moriwaki, K., Kanke, F., Satomura, S., Kumada, T., and Miyoshi, E. (2010)

Downloaded from www.jbc.org at OSAKA UNIVERSITY, on February 3, 2013

Clinical application of a lectin antibody ELISA to measure fucosylated haptoglobin in sera of patients with pancreatic cancer. *Clin. Chem. Lab. Med.* **48,** 505–512
20. Kawagishi, H. (1995) Mushroom lectins. *Food Rev. Int.* **11,** 63–68
21. Kobayashi, Y., Ishizaki, T., and Kawagishi, H. (2004) Screening for lectins in wild and cultivated mushrooms from Japan and their sugar binding specificities. *Int. J. Med. Mushr.* **6,** 113–125
22. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685
23. Kawagishi, H., Yamawaki, M., Isobe, S., Usui, T., Kimura, A., and Chiba, S. (1994) Two lectins from the marine sponge *Halichondria okadai*. An *N*-acetyl-sugar-specific lectin (HOL-I) and an *N*-acetyllactosamine-specific lectin (HOL-II). *J. Biol. Chem.* **269,** 1375–1379
24. Kawagishi, H., and Mizuno, T. (1988) Purification and properties of a β-galactosyl-specific lectin from the fruiting bodies of ischnoderma resinosus. *FEBS Lett.* **227,** 99–102
25. Kobayashi, Y., Kobayashi, K., Umehara, K., Dohra, H., Murata, T., Usui, T., and Kawagishi, H. (2004) Purification, characterization, and sugar binding specificity of an *N*-glycolylneuraminic acid-specific lectin from the mushroom *Chlorophyllum molybdites*. *J. Biol. Chem.* **279,** 53048–53055
26. Horibe M, Kobayashi Y, Dohra H, Morita T, Murata T, Usui T, Nakamura-Tsuruta S, Kamei M, Hirabayashi J, Matsuura M, Yamada M, Saikawa Y, Hashimoto K, Nakata M, and Kawagishi, H. (2010) Toxic isolectins from the mushroom *Boletus venenatus*. *Phytochemistry* **71,** 648–657
27. Hirabayashi, J., Arata, Y., and Kasai, K. (2000) Reinforcement of frontal affinity chromatography for effective analysis of lectin-oligosaccharide interactions. *J. Chromatogr. A.* **890,** 261–271
28. Tateno, H., Nakamura-Tsuruta, S., and Hirabayashi, J. (2007) Frontal affinity chromatography. Sugar-protein interactions. *Nat. Protoc.* **2,** 2529–2537
29. Nakamura-Tsuruta, S., Uchiyama, N., and Hirabayashi, J. (2006) High-throughput analysis of lectin-oligosaccharide interactions by automated frontal affinity chromatography. *Methods Enzymol.* **415,** 311–325
30. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254
31. Lundy, F. T., and Wisdom, G. B. (1999) An antibody-lectin sandwich assay for quantifying protein glycoforms. *Mol. Biotechnol.* **12,** 203–206
32. Nakamura-Tsuruta, S., Kominami, J., Kamei, M., Koyama, Y., Suzuki, T., Isemura, M., and Hirabayashi, J. (2006) Comparative analysis by frontal affinity chromatography of oligosaccharide specificity of GlcNAc-binding lectins, Griffonia simplicifolia lectin-II (GSL-II) and *Boletopsis leucomelas* lectin (BLL) *J. Biochem.* **140,** 285–291
33. Oda, Y., Senaha, T., Matsuno, Y., Nakajima, K., Naka, R., Kinoshita, M., Honda, E., Furuta, I., and Kakehi, K. (2003) A new fungal lectin recognizing α(1–6)-linked fucose in the *N*-glycan. *J. Biol. Chem.* **278,** 32439–32447
34. Nakagawa, T., Miyoshi, E., Yakushijin, T., Hiramatsu, N., Igura, T., Hayashi, N., Taniguchi, N., and Kondo, A. (2008) Glycomic analysis of α-fetoprotein L3 in hepatoma cell lines and hepatocellular carcinoma patients. *J. Proteome Res.* **7,** 2222–2233
35. Abelev, G. I., Perova, S. D., Khramkova, N. I., Postnikova, Z. A., and Irlin, I. S. (1963) Production of embryonal α-globulin by transplantable mouse hepatomas. *Transplantation* **1,** 174–180
36. Morinaga, T., Sakai, M., Wegmann, T. G., and Tamaoki, T. (1983) Primary structures of human α-fetoprotein and its mRNA. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 4604–4608
37. Taketa, K., and Hirai, H. (1989) Lectin affinity electrophoresis of α-fetoprotein in cancer diagnosis. *Electrophoresis* **10,** 562–567
38. Li, D., Mallory, T., and Satomura, S. (2001) AFP-L3. A new generation of tumor marker for hepatocellular carcinoma. *Clin. Chim. Acta* **313,** 15–19
39. Yamagata, Y., Shimizu, K., Nakamura, K., Henmi, F., Satomura, S., Matsuura, S., and Tanaka, M. (2003) Simultaneous determination of percentage of *Lens culinaris* agglutinin-reactive α-fetoprotein and α-fetoprotein concentration using the LiBASys clinical auto-analyzer. *Clin. Chim. Acta* **327,** 59–67
40. Sterling, R. K., Jeffers, L., Gordon, F., Sherman, M., Venook, A. P., Reddy, K. R., Satomura, S., and Schwartz, M. E. (2007) Clinical utility of AFP-L3% measurement in North American patients with HCV-related cirrhosis. *Am. J. Gastroenterol.* **102,** 2196–2205
41. Kagebayashi, C., Yamaguchi, I., Akinaga, A., Kitano, H., Yokoyama, K., Satomura, M., Kurosawa, T., Watanabe, M., Kawabata, T., Chang, W., Li, C., Bousse, L., Wada, H. G., and Satomura, S. (2009) Automated immunoassay system for AFP-L3% using on-chip electrokinetic reaction and separation by affinity electrophoresis. *Anal. Biochem.* **388,** 306–311
42. Tamura, Y., Igarashi, M., Kawai, H., Suda, T., Satomura, S., and Aoyagi, Y. (2010) Clinical advantage of highly sensitive on-chip immunoassay for fucosylated fraction of α-fetoprotein in patients with hepatocellular carcinoma. *Dig. Dis. Sci.* **55,** 3576–3583
43. Morota, K., Nakagawa, M., Sekiya, R., Hemken, P. M., Sokoll, L. J., Elliott, D., Chan, D. W., and Dowell, B. L. (2011) A comparative evaluation of Golgi protein-73, fucosylated hemopexin, α-fetoprotein, and PIVKA-II in the serum of patients with chronic hepatitis, cirrhosis, and hepatocellular carcinoma. *Clin. Chem. Lab. Med.* **49,** 711–718
44. Norton, P. A., Comunale, M. A., Krakover, J., Rodemich, L., Pirog, N., D'Amelio, A., Philip, R., Mehta, A. S., and Block, T. M. (2008) *N*-Linked glycosylation of the liver cancer biomarker GP73. *J. Cell. Biochem.* **104,** 136–149
45. Wang, M., Long, R. E., Comunale, M. A., Junaidi, O., Marrero, J., Di Bisceglie, A. M., Block, T. M., and Mehta, A. S. (2009) Novel fucosylated biomarkers for the early detection of hepatocellular carcinoma. *Cancer Epidemiol. Biomarkers Prev.* **18,** 1914–1921
46. Yamamoto, K., Imamura, H., Matsuyama, Y., Kume, Y., Ikeda, H., Norman, G. L., Shums, Z., Aoki, T., Hasegawa, K., Beck, Y., Sugawara, Y., and Kokudo, N. (2010) AFP, AFP-L3, DCP, and GP73 as markers for monitoring treatment response and recurrence and as surrogate markers of clinicopathological variables of HCC. *J. Gastroenterol.* **45,** 1272–1282
47. Comunale, M. A., Rodemich-Betesh, L., Hafner, J., Wang, M., Norton, P., Di Bisceglie, A. M., Block, T., and Mehta, A. (2010) Linkage specific fucosylation of alpha-1-antitrypsin in liver cirrhosis and cancer patients. Implications for a biomarker of hepatocellular carcinoma. *PLoS One* **5,** 12419–12422
48. Fukuda, T., Hashimoto, H., Okayasu, N., Kameyama, A., Onogi, H., Nakagawasai, O., Nakazawa, T., Kurosawa, T., Hao, Y., Isaji, T., Tadano, T., Narimatsu, H., Taniguchi, N., and Gu, J. (2011) α1,6-Fucosyltransferase-deficient mice exhibit multiple behavioral abnormalities associated with a schizophrenia-like phenotype. Importance of the balance between the dopamine and serotonin systems. *J. Biol. Chem.* **286,** 18434–18443
49. Wang, X., Inoue, S., Gu, J., Miyoshi, E., Noda, K., Li, W., Mizuno-Horikawa, Y., Nakano, M., Asahi, M., Takahashi, M., Uozumi, N., Ihara, S., Lee, S. H., Ikeda, Y., Yamaguchi, Y., Aze, Y., Tomiyama, Y., Fujii, J., Suzuki, K., Kondo, A., Shapiro, S. D., Lopez-Otin, C., Kuwaki, T., Okabe, M., Honke, K., and Taniguchi, N. (2005) Dysregulation of TGF-β1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 15791–15796
50. Moriwaki, K., Noda, K., Furukawa, Y., Ohshima, K., Uchiyama, A., Nakagawa, T., Taniguchi, N., Daigo, Y., Nakamura, Y., Hayashi, N., and Miyoshi, E. (2009) Deficiency of GMDS leads to escape from NK cell-mediated tumor surveillance through modulation of TRAIL signaling. *Gastroenterology* **137,** 188–198
51. Moriwaki, K., Shinzaki, S., and Miyoshi, E. (2011) GDP-mannose-4,6-dehydratase (GMDS) deficiency renders colon cancer cells resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor- and CD95-mediated apoptosis by inhibiting complex II formation. *J. Biol. Chem.* **286,** 43123–43133

Downloaded from www.jbc.org at OSAKA UNIVERSITY, on February 3, 2013

BMB
Reports

Invited Mini Review

Physiological roles of *N*-acetylglucosaminyltransferase V (GnT-V) in mice

Eiji Miyoshi, Mika Terao & Yoshihiro Kamada*

Department of Molecular Biochemistry and Clinical Investigation, Osaka University Graduate School of Medicine, Suita 565-0871, Japan

Oligosaccharide modification by *N*-acetylglucosaminyltransferase-V (GnT-V), a glycosyltransferase encoded by the *Mgat5* gene that catalyzes the formation of β1,6GlcNAc (*N*-acetylglucosamine) branches on *N*-glycans, is thought to be associated with cancer growth and metastasis. Overexpression of GnT-V in cancer cells enhances the signaling of growth factors such as epidermal growth factor by increasing galectin-3 binding to polylactosamine structures on receptor *N*-glycans. In contrast, GnT-V deficient mice are born healthy and lack β1,6GlcNAc branches on *N*-glycans, but develop immunological disorders due to T-cell dysfunction at 12-20 months of age. We have developed *Mgat5* transgenic (Tg) mice (GnT-V Tg mice) using a β-actin promoter and found characteristic phenotypes in skin, liver, and T cells in the mice. Although the GnT-V Tg mice do not develop spontaneous cancers in any organs, there are differences in the response to external stimuli between wild-type and GnT-V Tg mice. These changes are similar to those seen in cancer progression but are unexpected in some aspects. In this review, we summarize what is known about GnT-V functions in skin and liver cells as a means to understand the physiological roles of GnT-V in mice. [BMB Reports 2012; 45(10): 554-559]

INTRODUCTION

Changes in glycosylation are observed during cellular differentiation and carcinogenesis and are regulated by proteins known as glycosyltransferases. Every oligosaccharide is synthesized by a characteristic glycosyltransferase encoded by a specific glyco-gene. These genes make up approximately 1% of the human genome. Recent advances in glycotechnology and molecular biology have clarified the biological functions of oligosaccharides (1) and shown that these functions are often the result of complicated molecular interactions. Even if a single glyco-gene is knocked out or over-expressed in mice, a variety of different glycoproteins will undergo changes to their oligosaccharide structures. It is impossible to only change the oligosaccharide structure on one single glycoprotein with current molecular biology techniques. Therefore, comprehensive and focused analyses are required to understand the specific biological functions of each oligosaccharide, and researchers have focused on the important target glycoproteins that play pivotal roles in a specific phenomenon or organ. For example, epidermal growth factor (EGF) is studied for its role in skin proliferation, and transforming growth factor-β (TGF-β) receptor and its signaling pathways are the focus of research into liver fibrosis.

N-Acetylglucosaminyltransferase-V (GnT-V), a glycosyltransferase encoded by the *Mgat5* gene that catalyzes the formation of β1,6GlcNAc (*N*-acetylglucosamine) branches on *N*-glycans (Fig. 1A), is believed to be associated with cancer growth and metastasis (2, 3). It was first reported that GnT-V was directly associated with cancer metastasis in 1987 (4), and the *Mgat5* gene was subsequently cloned from rats (5) and humans (6) in 1993. Three distinct pathways are believed to be involved in the promotion of cancer metastasis by GnT-V. The first pathway involves the upregulation of EGF receptor (EGF-R) signaling through lattice formation between polylactosamine on β1,6GlcNAc branches and galectin-3, resulting in the inhibition of receptor endocytosis (7). In the second pathway, GnT-V promotes metastasis by inhibiting protease degradation through the addition of β1,6GlcNAc branches (8). The third pathway involves the inhibition of E-cadherin/β-catenin complex function, which leads to the release of cancer cells from the original tissue (9). Induction of mammary tumor growth and metastasis by the polyomavirus middle T oncogene is considerably reduced in GnT-V knockout mice (*Mgat5*$^{-/-}$ mice) compared to littermate controls, suggesting that GnT-V is essential for cancer growth as well as metastasis (10). However, clinical studies of GnT-V immunohistochemistry have demonstrated that GnT-V is not always a marker for poor prognosis. Although the prognosis is poor in colon, mammary, and esophageal cancers that exhibit high expression of GnT-V, the opposite has been observed in lung, thyroid, and liver cancers. This discrepancy might depend on the different target proteins of GnT-V in these various cancers. In contrast to its varying role as a prognosis marker, GnT-V expression is upre-

*Corresponding author. Tel: +81-6-6879-2590; Fax: +81-6879-2590;
E-mail: emiyoshi@sahs.med.osaka-u.ac.jp
http://dx.doi.org/10.5483/BMBRep.2012.45.10.190

Received 15 September 2012

Keywords: EGF-R, EMT, GnT-V, NASH, Oligosaccharide

ISSN: 1976-6696 (print edition) / 1976-670X (electronic edition)
Copyright © 2012 by the The Korean Society for Biochemistry and Molecular Biology
This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

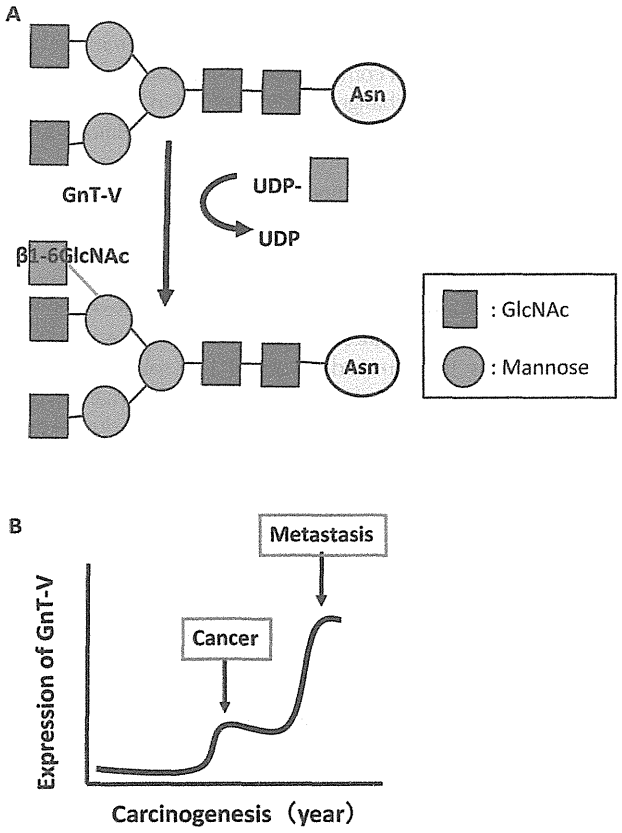

Fig. 1. GnT-V reaction and biological significance of GnT-V in cancer. (A) Reaction pathway catalyzed by GnT-V. (B) The expression of GnT-V is enhanced in the early stages of carcinogenesis as well as at the metastatic end stage.

gulated in the early stages of almost all cancers (Fig. 1B). Thus, the upregulation of EGF-R signaling and the activation of proteases could represent early events in carcinogenesis. Therefore, we have established GnT-V transgenic mice (GnT-V Tg mice) using a β-actin promoter to determine which organs would develop cancer in these mice. Unexpectedly, however, no spontaneous cancers were observed in any of the organs in the GnT-V Tg mice. We did, however, uncover physiological roles for GnT-V in skin and liver cells in response to certain kinds of stimuli in these tissues.

AN EPITHELIAL-MESENCHYMAL TRANSITION (EMT)-LIKE PHENOTYPE WAS OBSERVED IN GnT-V Tg MICE

EMT, which is characterized by the loss of epithelial adhesion and gain of mesenchymal features, plays a role in fundamental biological processes such as wound healing and embryonic development as well as in cancer invasion and metastasis (11). In adults, EMT, which is driven by the release of cytokines in response to tissue injury, mediates the production of fibroblasts during inflammation and wound healing (12-14). Re-epithelialization in wound healing involves the motility or migration of epithelial cells, and the migrating epithelial cells at the wound margins acquire mesenchymal features and go through the early stages of EMT (12). Previous *in vitro* studies by Demetriou *et al.* demonstrated that overexpression of GnT-V in Mv1Lu cells enhanced the migration of these cells to scratch wounds suggesting an effect of GnT-V on EMT induction in cells (15). Recently, we have reported the enhancement of an EMT-like phenotype in the skin of GnT-V Tg mice (Fig. 2A) (16). The skin of the GnT-V Tg mice was more easily damaged by repeated removal of the corneum with cellotape, suggesting that cell-cell adhesion was extremely impaired in these mice. As expected, the expression of E-cadherin, one of the most important cell-adhesion molecules, was dramatically decreased in cultured keratinocytes derived from the GnT-V Tg mice (Fig. 2B). In contrast, the expression of mesenchymal proteins such as N-cadherin and α-smooth muscle actin in these cells was increased (Fig. 2C). Additionally, the migration of keratinocytes derived from GnT-V Tg mice was significantly enhanced. These changes were dependent on the increased expression of EMT-regulatory transcription factors such as twist and snail (Fig. 2D) that are generally induced through the activation of the EGF or TGFβ receptor-mediated signaling pathways. It has been reported that the addition of β1-6GlcNAc branches onto these receptors inhibits endocytosis and prolongs the signaling of these molecules in cancer cells (7). EGF-R signaling was enhanced in GnT-V Tg mouse keratinocytes, suggesting that similar glyco-signaling occurs in normal keratinocytes. To evaluate the EMT-like phenotype and the enhanced migration of GnT-V Tg mouse keratinocytes *in vivo*, we performed a cutaneous wound-healing assay by creating 8-mm round wounds on the backs of GnT-V Tg and control mice. The wound areas in GnT-V Tg mice were significantly smaller than controls from 4 days onward (Fig. 2E). Notably, re-epithelialization was faster in the wounds on the GnT-V Tg mice, suggesting enhanced keratinocyte motility at the wound edge. Taken together, the EMT-like features observed in the skin of the GnT-V Tg mice contributed to keratinocyte motility and cutaneous wound healing, which was mediated in part by upregulated EGF receptor signaling.

GnT-V MAINTAINS SKIN HOMEOSTASIS BY REGULATING THE PROLIFERATION OF KERATINOCYTES THROUGH EGF-R SIGNALING

We previously reported that the expression of GnT-V was enhanced in inflammation and during cell proliferation (17, 18). Upregulation of GnT-V during these processes can enhance the signaling of growth factors and/or cytokines via the addition of β1-6GlcNAc branches onto their receptors. EGF-R-mediated signaling also plays a crucial role in maintaining homeostasis in the skin. The proliferation of mature, healthy epidermal cells is maintained by balancing signals from various

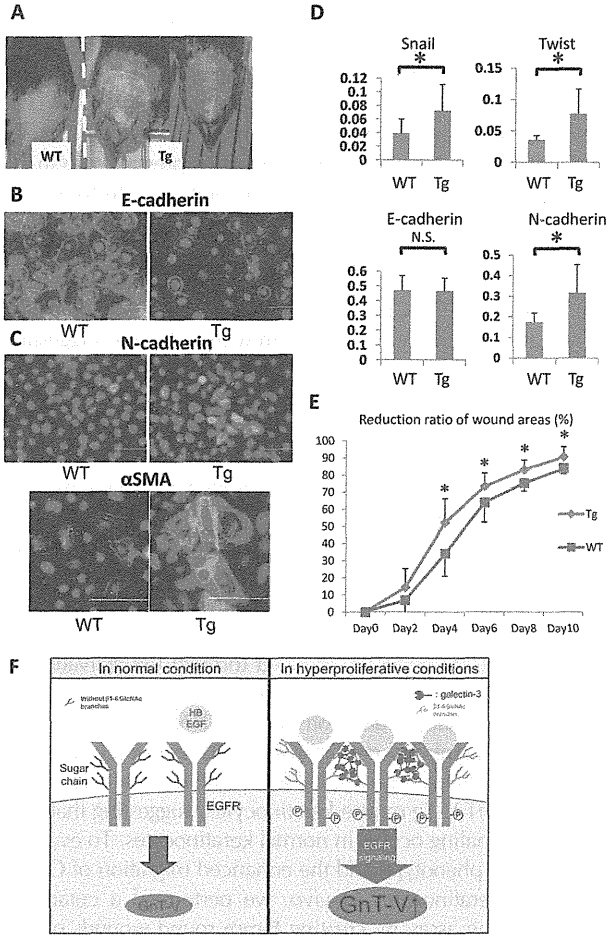

Fig. 2. EMT-like phenotypes are observed in GnT-V Tg mice. (A) Macro view of the skin on the backs of wild-type and GnT-V Tg mice 30 minutes after tape stripping. (B) Primary cultured keratinocytes derived from wild-type and GnT-V Tg mice were stained with anti-E-cadherin (green) and Hoechst 33342 (blue). Scale bar: 100 μm. (C) Primary cultured keratinocytes derived from wild-type and GnT-V Tg mice were stained with anti-N-cadherin (green), anti-αSMA (green), and Hoechst 33342 (blue). Scale bar: 100 μm. (D) The EMT-associated transcriptional factors (snail and twist), E-cadherin, and N-cadherin were evaluated by quantitative RT-PCR, and target gene expression was normalized to GAPDH. Results are expressed as means ± S.D. (n = 6). $*P < 0.05$. (E) Reduction of the wound area on days 2, 4, 6, 8, and 10. $*P < 0.05$. Photographs of re-epithelialization in wild-type and GnT-V Tg mice on day 6 are shown. Dotted yellow lines show the re-epithelialized edge of the epidermis. Bars indicate means ± S.D. (F) GnT-V cycle in the skin. GnT-V and HB-EGF co-operate in the proliferation of keratinocytes through the enhancement of each other's expression and function. All of the data in this figure are derived from references 22 and 26.

EGF family members such as heparin-binding epidermal growth factor-like growth factor (HB-EGF), transforming growth factor-α, and amphiregulin, all of which bind to EGF-R in the epidermis (19-21).

In spite of the fact that expression of GnT-V protein was extremely high in the skin compared to other organs in GnT-V Tg mice, no histological changes were observed in the GnT-V Tg mice or in the GnT-V skin under normal conditions (16). However, GnT-V deficient mice showed a decrease in epidermal hyperproliferation after treatment with phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) (22). TPA induces the production of HB-EGF, which plays important roles in keratinocyte proliferation. HB-EGF is produced in a membrane-anchored form that is then cleaved by proteases on the cell surface. TPA stimulates the activation of these proteases and this leads to increased levels of the soluble form of HB-EGF. The results of TPA-induced epidermal hyperproliferation in GnT-V-deficient mice suggested down-regulation of EGF-R signaling via HB-EGF, and EGF-R signaling in response to HB-EGF was shown to be downregulated in cultured GnT-V-deficient mouse keratinocytes. Furthermore, the addition of HB-EGF or TPA to conditioned medium induced up-regulation of GnT-V expression in cultured keratinocytes (22). Increased GnT-V activity contributes to the addition of β1-6GlcNAc branches onto the *N*-glycans of EGF, which leads to the lattice formation. This lattice formation inhibits the endocytosis of EGF-R and enhances EGF-R signaling. Thus, the induction of GnT-V by HB-EGF plays an important role in maintaining skin homeostasis during hyperproliferation. The effect of GnT-V in the skin under normal and hyperproliferative conditions is outlined in Fig. 2F and could be referred to as the "GnT-V cycle in the skin". GnT-V and HB-EGF co-operate in the proliferation of keratinocytes through the enhancement of each other's expression and function.

THE ROLE OF GnT-V IN THE DEVELOPMENT OF STEATO-HEPATITIS

Although the expression of GnT-V is quite low in normal liver tissue, it is increased under conditions of chronic hepatitis and liver regeneration (17, 18). Previous studies have shown that glycosyltransferase transgenic mice develop fatty liver due to the accumulation of lipoproteins in their hepatocytes (23, 24). However, GnT-V Tg mice did not show fatty liver phenotypes under normal conditions. One of the reasons for the lack of fatty changes in the GnT-V Tg mouse liver might be that the expression of GnT-V is lower in the liver than in the other organs in GnT-V Tg mice. Nonalcoholic fatty liver disease (NAFLD) is among the most common chronic liver diseases in the world and is a growing medical problem in industrialized countries (25). A wide spectrum of histological changes have been observed in NAFLD, ranging from simple steatosis, which is generally non-progressive, to nonalcoholic steatohepatitis (NASH), and a proportion of patients with NASH go on to develop cirrhosis and hepatocellular carcinoma (HCC) (26). Recent studies indicate that dietary cholesterol is an important risk factor for the progression of NASH in both humans and rodents.

When we treated GnT-V Tg and wild-type mice with a high fat and high cholesterol (HFHC) diet, lymphocyte infiltration was dramatically suppressed in the GnT-V Tg mice compared to wild-type mice (Fig. 3A). HFHC treatment is one of the best mouse models for NASH because it induces both inflammation and fibrosis in the mouse liver (27). Because the expression of GnT-V was shown to be elevated in chronic hepatitis (17), we gave the GnT-V Tg mice an HFHC diet as a means of understanding the biological effect of up-regulation of GnT-V in the liver (28). When mice were fed a normal chow (NC) diet, the total body weight, liver weight, and liver to body weight ratio were all significantly higher in GnT-V Tg mice compared to wild-type mice. In contrast, the liver weight and liver to body weight ratio were significantly lower in GnT-V Tg mice compared to wild-type mice when fed the HFHC diet. There were no differences in the serum triglyceride, liver triglyceride, or cholesterol levels between GnT-V Tg mice compared to wild-type mice with either the NC diet or the HFHC diet. There were no differences in the liver cholesterol contents between GnT-V Tg mice and wild-type mice fed the NC diet, but liver cholesterol levels were significantly lower in GnT-V Tg mice compared to wild-type mice when they were fed the HFHC diet. The suppression of lymphocyte infiltration in the GnT-V Tg mouse liver is thought to be due to a shift towards the Th2 response in their T cells. Accordingly, the levels of Th1-cytokines such as interleukin-6, interferon-γ, and tumor necrosis factor α were dramatically suppressed in GnT-V Tg mouse livers (28). Serum alanine aminotransferase levels are also lower in GnT-V Tg mice compared to wild-type mice.

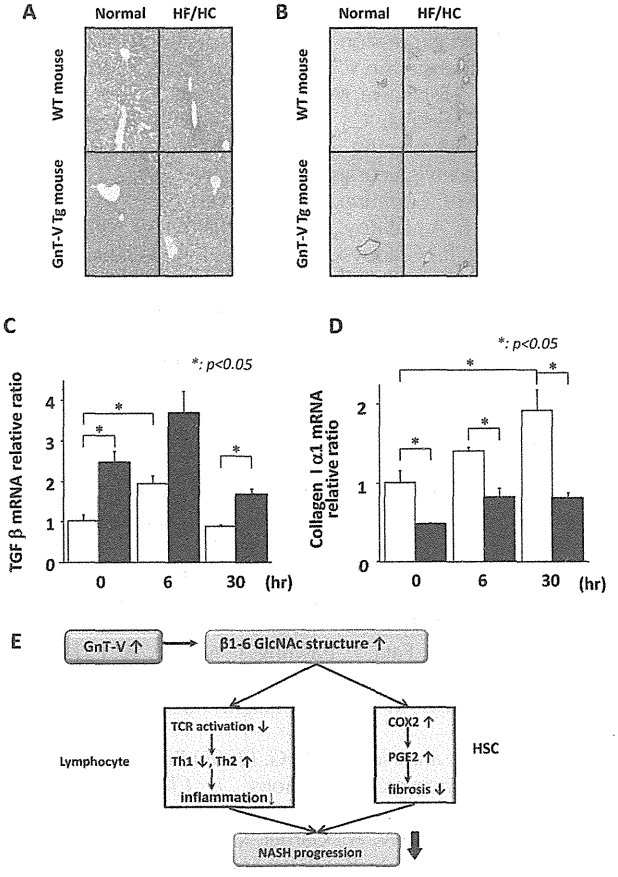

Fig. 3. Role of GnT-V in the development of steato-hepatitis in mouse models. (A) Representative photomicrographs of livers from mice fed a normal chow (NC) diet or high-fat and high-cholesterol (HFHC) diet. (B) Representative photomicrographs of livers from mice fed a NC diet or HFHC diet, stained with picrosirius red. (C) Expression levels of TGF-β mRNA in HSCs before and after TGF-β stimulation were measured with RT-PCR. HSCs derived from GnT-V Tg mice and wild-type mice were stimulated with 1 ng/ml of TGF-β for the indicated times. (D) Expression levels of collagen I α1 mRNA in HSCs before and after TGF-β stimulation were measured by RT-PCR. (E) Summary of the mechanism by which GnT-V suppresses NASH progression in mice.

MOLECULAR MECHANISMS OF INHIBITION OF LIVER FIBROSIS IN GnT-V Tg MOUSE LIVERS

Feeding GnT-V Tg mice an HFHC diet for 4 weeks inhibited fibrosis in their livers compared to wild-type mice (Fig. 3B). Although liver fibrosis is related to hepatic inflammation, the accumulation of extracellular matrix proteins is dependent on the functions of non-parenchymal cells in the liver. Hepatic stellate cells (HSCs) play the most important roles among non-parenchymal cells during liver fibrosis (29). HSCs produce TGF-β, which is a very important cytokine in fibrosis, and TGF-β stimulates HSCs to produce more TGF-β in a positive feedback cycle. When HSCs in primary culture were treated with exogenous TGF-β, the HSCs from GnT-V Tg mice showed higher TGF-β production than HSCs derived from wild-type mice (Fig. 3C). Even in the absence of exogenous TGF-β, RT-PCR showed that TGF-β mRNA levels are increased in GnT-V Tg HSCs. These results suggest that GnT-V enhances TGF-β signaling, which is dependent on similar mechanisms as EGF-R signaling (7). Determination of Smad3 localization is a useful indicator of whether or not TGF-β signaling is enhanced in a cell. Smad3 protein expression in cytoplasmic extracts of HSCs was decreased after TGF-β stimulation, and TGF-β stimulation significantly increased nuclear Smad3 protein levels in both wild-type and GnT-V Tg HSCs. Interestingly, nuclear Smad3 protein levels were significantly higher in the GnT-V Tg HSCs than in wild-type HSCs, both before and after TGF-β stimulation. These results indicated that TGF-β signaling in HSCs was enhanced in GnT-V Tg mice. However, the expression of collagen I was dramatically decreased in GnT-V Tg HSCs (Fig. 3D). To determine why collagen expression was decreased in GnT-V Tg HSCs in spite of the enhanced TGF-β signaling, we performed microarray analysis comparing GnT-V Tg HSCs and wild-type HSCs. One of the genes found to be elevated in GnT-V Tg HSCs compared to wild-type HSCs was

cycloogenase-2 (COX-2), the rate limiting enzyme in prostaglandin E2 (PGE2) production. It has been reported that TGF-β stimulates COX-2 expression and that COX-2-derived PGE2 inhibited TGF-β1-induced collagen production in HSCs via negative feedback (30). Our hypothesis for how GnT-V is related to this process is shown in Fig. 3E. Celecoxib, a selective COX-2 inhibitor, decreased PGE2 production and upregulated collagen I gene expression in GnT-V Tg HSCs. Continuous induction of TGF-β stimulation by GnT-V should enhance COX2 expression, and this negative feedback signal could reduce collagen expression in the livers and HSCs of GnT-V Tg mice. The precise mechanism of how this might occur is under investigation.

CLOSING

Knockout mice of glyco-genes are sometimes lethal if they are involved in the initial step of oligosaccharide synthesis (31). In the cases of other glyco-genes, however, the knockout mice often show no phenotypes or only small differences in a limited number of organs compared to wild-type mice. In such cases, stimulation experiments should be performed. In human diseases, the complete deficiency of a particular gene is relatively rare. On the other hand, partial deficiencies in multiple genes and abnormal inductions of some genes involved in inflammation and regeneration are common. The phenotypes that we observed in the skin and livers of GnT-V Tg mice could not be detected under normal conditions. It is believed that certain changes in the micro- and macro-environment can lead to the onset of disease, and we and other groups have performed these kinds of experiments using transgenic glyco-gene knockout mice (10, 22, 28, 32, 33). In next era of glycobiology research, such experiments will produce mouse models of diseases that involve oligosaccharide remodeling, and tumor induction in GnT-V Tg mice is one of the most promising studies. It is also important to take into consideration which kinds of carcinogens and carcinogenic mice are used in these experiments.

Acknowledgements

We would like to thank Drs. Naoyuki Taniguchi (Rikken, Wako) and Hitoshi Matsumoto, Susumu Nakahara, Ichiro Katayama, Tetsuo Takehara, Akiko Ishikawa, Akihiro Kimura, Arisa Kato, and Kanako Mori of the Osaka University Graduate School of Medicine for performing the experiments discussed in this review. These works were supported in part by the Global COE Program of Osaka University funded by the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a Grant-in-Aid for Scientific Research (A), No. 21249038, from the Japan Society for the Promotion of Science.

REFERENCES

1. Hart, G. W. and Copeland, R. J. (2010) Glycomics hits the big time. *Cell* **143**, 672-676.
2. Taniguchi, N., Miyoshi, E., Ko, J. H., Ikeda, Y. and Ihara, Y. (1999) Implication of N-acetylglucosaminyltransferases III and V in cancer: gene regulation and signaling mechanism. *Biochim. Biophys. Acta.* **1455**, 287-300.
3. Lau, K. S. and Dennis, J. W. (2008) N-Glycans in cancer progression. *Glycobiology* **18**, 750-760.
4. Dennis, J. W., Laferte, S., Waghorne, C., Breitman, M. L. and Kerbel, R. S. (1987) Beta 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science* **236**, 582-585.
5. Shoreibah, M., Perng, G. S., Adler, B., Weinstein, J., Basu, R., Cupples, R., Wen, D., Browne, J. K., Buckhaults, P., Fregien, N. and Pierce, M. (1993) Isolation, characterization, and expression of a cDNA encoding N-acetylglucosaminyltransferase V. *J. Biol. Chem.* **268**, 15381-15385.
6. Saito, H., Nishikawa, A., Gu, J., Ihara, Y., Soejima, H., Wada, Y., Sekiya, C., Niikawa, N. and Taniguchi, N. (1994) cDNA cloning and chromosomal mapping of human N-acetylglucosaminyltransferase V+. *Biochem. Biophys. Res. Commun.* **198**, 318-327.
7. Partridge, E. A., Le Roy, C., Di Guglielmo, G. M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I. R., Wrana, J. L. and Dennis, J. W. (2004) Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis. *Science* **306**, 120-124.
8. Guo, H. B., Lee, I., Kamar, M. and Pierce, M. (2003) N-acetylglucosaminyltransferase V expression levels regulate cadherin-associated homotypic cell-cell adhesion and intracellular signaling pathways. *J. Biol. Chem.* **278**, 52412-52424.
9. Ihara, S., Miyoshi, E., Ko, J. H., Murata, K., Nakahara, S., Honke, K., Dickson, R. B., Lin, C. Y. and Taniguchi, N. (2002) Prometastatic effect of N-acetylglucosaminyltransferase V is due to modification and stabilization of active matriptase by adding beta 1-6 GlcNAc branching. *J. Biol. Chem.* **277**, 16960-16967.
10. Granovsky, M., Fata, J., Pawling, J., Muller, W. J., Khokha, R. and Dennis, J. W. (2000) Suppression of tumor growth and metastasis in Mgat5-deficient mice. *Nat. Med.* **6**, 306-312.
11. Zeisberg, M. and Neilson, E. G. (2009) Biomarkers for epithelial-mesenchymal transitions. *J. Clin. Invest.* **119**, 1429-1437.
12. Yan, C., Grimm, W. A., Garner, W. L., Qin, L., Travis, T., Tan, N. and Han, Y. P. (2010) Epithelial to mesenchymal transition in human skin wound healing is induced by tumor necrosis factor-alpha through bone morphogenic protein-2. *Am. J. Pathol.* **176**, 2247-2258.
13. Kalluri, R. and Neilson, E. G. (2003) Epithelial-mesenchymal transition and its implications for fibrosis. *J. Clin. Invest.* **112**, 1776-1784.
14. Desmouliere, A. (1995) Factors influencing myofibroblast differentiation during wound healing and fibrosis. *Cell Biol. Int.* **19**, 471-476.
15. Demetriou, M., Nabi, I. R., Coppolino, M., Dedhar, S. and Dennis, J. W. (1995) Reduced contact-inhibition and

substratum adhesion in epithelial cells expressing GlcNAc-transferase V. *J. Cell Biol.* **130**, 383-392.
16. Terao, M., Ishikawa, A., Nakahara, S., Kimura, A., Kato, A., Moriwaki, K., Kamada, Y., Murota, H., Taniguchi, N., Katayama, I. and Miyoshi, E. (2011) Enhanced epithelial-mesenchymal transition-like phenotype in N-acetylglucosaminyltransferase V transgenic mouse skin promotes wound healing. *J. Biol. Chem.* **286**, 28303-28311.
17. Miyoshi, E., Nishikawa, A., Ihara, Y., Gu, J., Sugiyama, T., Hayashi, N., Fusamoto, H., Kamada, T. and Taniguchi, N. (1993) N-acetylglucosaminyltransferase III and V messenger RNA levels in LEC rats during hepatocarcinogenesis. *Cancer Res.* **53**, 3899-3902.
18. Miyoshi, E., Ihara, Y., Nishikawa, A., Saito, H., Uozumi, N., Hayashi, N., Fusamoto, H., Kamada, T. and Taniguchi, N. (1995) Gene expression of N-acetylglucosaminyltransferases III and V: a possible implication for liver regeneration. *Hepatology* **22**, 1847-1855.
19. Pastore, S., Mascia, F., Mariani, V. and Girolomoni, G. (2008) The epidermal growth factor receptor system in skin repair and inflammation. *J. Invest. Dermatol.* **128**, 1365-1374.
20. Schneider, M. R., Werner, S., Paus, R. and Wolf, E. (2008) Beyond wavy hairs: the epidermal growth factor receptor and its ligands in skin biology and pathology. *Am. J. Pathol.* **173**, 14-24.
21. Yoshida, A., Kanno, H., Watabe, D., Akasaka, T. and Sawai, T. (2008) The role of heparin-binding EGF-like growth factor and amphiregulin in the epidermal proliferation of psoriasis in cooperation with TNFalpha. *Arch. Dermatol. Res.* **300**, 37-45.
22. Kimura, A., Terao, M., Kato, A., Hanafusa, T., Murota, H., Katayama, I. and Miyoshi, E. (2012) Upregulation of N-acetylglucosaminyltransferase-V by heparin-binding EGF-like growth factor induces keratinocyte proliferation and epidermal hyperplasia. *Exp. Dermatol.* **21**, 515-519.
23. Ihara, Y., Yoshimura, M., Miyoshi, E., Nishikawa, A., Sultan, A. S., Toyosawa, S., Ohnishi, A., Suzuki, M., Yamamura, K., Ijuhin, N. and Taniguchi, N. (1998) Ectopic expression of N-acetylglucosaminyltransferase III in transgenic hepatocytes disrupts apolipoprotein B secretion and induces aberrant cellular morphology with lipid storage. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2526-2530.
24. Wang, W., Li, W., Ikeda, Y., Miyagawa, J. I., Taniguchi, M., Miyoshi, E., Sheng, Y., Ekuni, A., Ko, J. H., Yamamoto, Y., Sugimoto, T., Yamashita, S., Matsuzawa, Y., Grabowski, G. A., Honke, K. and Taniguchi, N. (2001) Ectopic expression of alpha1,6 fucosyltransferase in mice causes steatosis in the liver and kidney accompanied by a modification of lysosomal acid lipase. *Glycobiology* **11**, 165-174.
25. Ford, E. S., Giles, W. H. and Dietz, W. H. (2002) Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *JAMA* **287**, 356-359.
26. Bugianesi, E., Leone, N., Vanni, E., Marchesini, G., Brunello, F., Carucci, P., Musso, A., De Paolis, P., Capussotti, L., Salizzoni, M. and Rizzetto, M. (2002) Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* **123**, 134-140.
27. Matsuzawa, N., Takamura, T., Kurita, S., Misu, H., Ota, T., Ando, H., Yokoyama, M., Honda, M., Zen, Y., Nakanuma, Y., Miyamoto, K. and Kaneko, S. (2007) Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. *Hepatology* **46**, 1392-1403.
28. Kamada, Y., Mori, K., Matsumoto, H., Kiso, S., Yoshida, Y., Shinzaki, S., Hiramatsu, N., Ishii, M., Moriwaki, K., Kawada, N., Takehara, T. and Miyoshi, E. (2012) N-Acetylglucosaminyltransferase V regulates TGF-beta response in hepatic stellate cells and the progression of steatohepatitis. *Glycobiology* **22**, 778-787.
29. Friedman, S. L. (2008) Mechanisms of hepatic fibrogenesis. *Gastroenterology* **134**, 1655-1669.
30. Hui, A. Y., Dannenberg, A. J., Sung, J. J., Subbaramaiah, K., Du, B., Olinga, P. and Friedman, S. L. (2004) Prostaglandin E2 inhibits transforming growth factor beta 1-mediated induction of collagen alpha 1(I) in hepatic stellate cells. *J. Hepatol.* **41**, 251-258.
31. Ioffe, E., Liu, Y. and Stanley, P. (1996) Essential role for complex N-glycans in forming an organized layer of bronchial epithelium. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11041-11046.
32. Shinzaki, S., Iijima, H., Fujii, H., Kuroki, E., Tatsunaka, N., Inoue, T., Nakajima, S., Egawa, S., Kanto, T., Tsujii, M., Morii, E., Takeishi, S., Asano, M., Takehara, T., Hayashi, N. and Miyoshi, E. (2012) Altered oligosaccharide structures reduce colitis induction in mice defective in beta-1,4-galactosyltransferase. *Gastroenterology* **142**, 1172-1182.
33. Gao, C., Maeno, T., Ota, F., Ueno, M., Korekane, H., Takamatsu, S., Shirato, K., Matsumoto, A., Kobayashi, S., Yoshida, K., Kitazume, S., Ohtsubo, K., Betsuyaku, T. and Taniguchi, N. (2012) Sensitivity of heterozygous alpha1,6-fucosyltransferase knock-out mice to cigarette smoke-induced emphysema: implication of aberrant transforming growth factor-beta signaling and matrix metalloproteinase gene expression. *J. Biol. Chem.* **287**, 16699-16708.

Virology 432 (2012) 452–459

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Interferon-α suppresses hepatitis B virus enhancer II activity via the protein kinase C pathway

Takatoshi Nawa [a,1], Hisashi Ishida [a,1], Tomohide Tatsumi [a], Wei Li [a], Satoshi Shimizu [a], Takahiro Kodama [a], Hayato Hikita [a], Atsushi Hosui [a], Takuya Miyagi [a], Tatsuya Kanto [a], Naoki Hiramatsu [a], Norio Hayashi [b], Tetsuo Takehara [a,*]

[a] Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Kansai-Rosai Hospital, Amagasaki, Hyogo 660-8511, Japan

ARTICLE INFO

Article history:
Received 12 April 2012
Returned to author for revisions
3 May 2012
Accepted 1 July 2012
Available online 24 July 2012

Keywords:
HBV
Enhancer II
Interferon-α
Protein kinase C

ABSTRACT

HBV has two enhancer (En) regions each of which promotes its own transcription. En II regulates production of pregenomic RNA, a key product of HBV replication, more strongly than En I. Although IFN-α has been found to suppress En I activity, its effect on En II activity has not been examined. Here we used luciferase assay to demonstrate that IFN-α suppresses En II activity. Analysis with several deletion/mutation constructs identified two major segments, nt 1703–1727 and nt 1746–1770, within the En II sequence as being responsible for the suppressive effects of IFN-α. Pre-treatment with protein kinase C (PKC) inhibitors blocked this effect regardless of the expression levels of phospho-STAT1 and Mx upon IFN-α stimulation. These results indicate that IFN-α suppresses En II activity via the PKC pathway, which may be an alternative suppressive pathway for HBV replication. (136 words).

© 2012 Elsevier Inc. All rights reserved.

Introduction

Hepatitis B virus (HBV) causes acute and chronic hepatitis in humans, and chronic infection is closely associated with the development of liver cirrhosis and hepatocellular carcinoma (Lok and McMahon, 2009). HBV has a partially double-stranded 3.2-kb DNA genome (relaxed circular (RC) DNA) in its nucleocapsid. When HBV invades host cells, RC-DNA is converted into a plasmid-like covalently closed circular DNA (cccDNA) inside the nucleus. From the cccDNA, the 3.5-, 2.4-, 2.1-, and 0.8-kb mRNAs are transcribed by cellular RNA polymerase II (Beck and Nassal, 2007). Among these RNAs, 3.5-kb pregenomic RNA (pgRNA) serves as the template of reverse transcription for synthesis of negative-strand DNA. Thus, transcription of pgRNA from cccDNA is one of the key steps in HBV replication.

In the HBV genome, there are four promoters (CP, SPI, SPII, and XP) and two transcriptional enhancer regions. Both enhancers stimulate transcription from the promoters (Antonucci and Rutter, 1989; Moolla et al., 2002; Su and Yee, 1992; Vannice and Levinson, 1988; Yee, 1989). Enhancer I (En I), which is located upstream of the X gene, activates transcription in a relatively cell-independent manner (Vannice and Levinson, 1988). In contrast, enhancer II (En II) (Fig. 1), located just upstream of CP, specifically activates transcription in hepatocytes (Wang et al., 1990; Yee, 1989; Yuh and Ting, 1990). Hepatocytes selectively express transcription factors which activate En II activity, such as HNF1 (Wang et al., 1998), HNF3 (Johnson et al., 1995; Li et al., 1995), HNF4 (Guo et al., 1993; Raney et al., 1997), CCAAT/enhancer binding protein (C/EBP) (López-Cabrera et al., 1990, 1991; Yuh and Ting, 1991) and FTF (Ishida et al., 2000; Li et al., 1998). This characterizes En II as a hepatocyte-specific *cis*-acting element. A previous report showed that, upon transfection with HBV genome, human hepatic cells, but not non-hepatic cells, were able to express pgRNA (Sureau et al., 1986). For this reason, En II is considered to regulate the production of pgRNA more strongly than En I (Yee, 1989).

Interferon-α (IFN-α) has been used as an anti-viral agent against HBV. It suppresses HBV viral load and ameliorates hepatic inflammation (Jonas et al., 2010; Liaw, 2009). Type I IFN activates the Janus kinase (JAK) bound to the cytoplasmic domain of its receptor. JAK phosphorylates transcription factors such as signal transducers and activators of transcription (STAT) 1 and STAT2. Phosphorylated STAT1 and STAT2 bind to IFN regulatory factor 9 (IRF9). These transcription factors form a complex, IFN-stimulated gene factor 3 (ISGF3). This complex binds to IFN stimulation response element (ISRE) in the promoter region of various genes, and activates interferon-stimulated genes (ISGs) (Der et al., 1998). Some of the ISGs including RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetases (OAS), and Mx have been shown to possess antiviral activity. ISG induction by type I IFN is considered to be the main pathway to suppressing viral replication.

* Corresponding author. Fax: +81 6 6879 3629.
E-mail address: takehara@gh.med.osaka-u.ac.jp (T. Takehara).
[1] T.N. and H.I. contributed equally to this work and share first authorship.

0042-6822/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2012.07.002

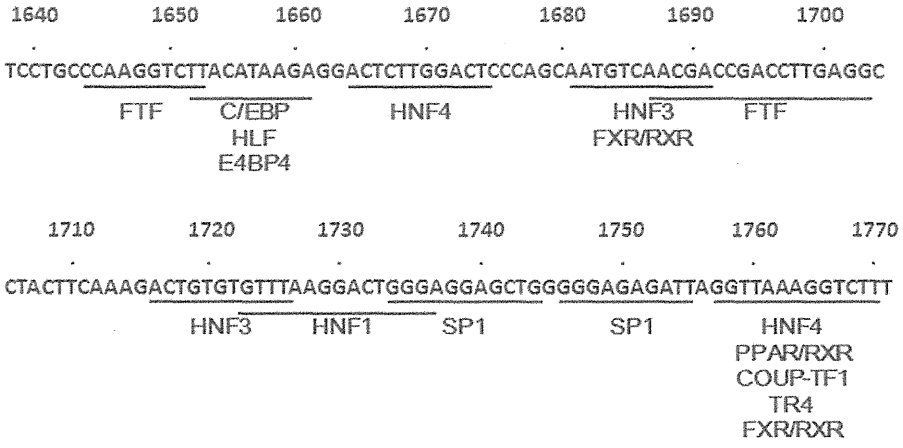

Fig. 1. Nucleotide sequences of the HBV En II region. The HBV sequence used in this study was of the *adw2* subtype (GenBank accession no. X02763). Numbering of the HBV sequence started at the unique *Eco*RI site. The underlined sequences represent the transcription factor binding sites mentioned in previous reports.

Type I IFN has been reported to inhibit HBV En I and core promoter activities (Nakao et al., 1999; Romero and Lavine, 1996; Schulte-Frohlinde et al., 2002; Tur-Kaspa et al., 1990). Nakao et al. demonstrated that IFN-α suppressed En I transcriptional activity by the binding of ISGF3 to the ISRE-like sequence in En I region (Nakao et al., 1999). However, there has been no study on the effect of IFN-α on HBV En II activity. In this study, we demonstrated that IFN-α suppressed En II activity via activation of PKC. Notably, STAT1 activation and ISG induction may be dispensable for IFN-α-mediated suppression of En II activity. This might shed light on understanding the inhibition of HBV replication by IFN-α.

Results

En II activity is down-regulated by IFN-α

We constructed a luciferase gene expression vector by inserting the En II sequence (nt 1640 to 1771) into pGL4LUC (pGL4LUC-En II). Huh-7 cells were transfected with pGL4LUC or pGL4LUC-En II, treated with or without IFN-α, and luciferase activities were evaluated. Insertion of En II increased the luciferase activity (about 228-fold) (Fig. 2A). IFN-α down-regulated the luciferase activity of pGL4LUC-En II, but did not affect that of pGL4LUC (Fig. 2B). This result suggested that IFN-α inhibited the activity of En II, and we examined the time course of IFN-α-induced suppression of En II activity. The suppressive effect of IFN-α on En II activity appeared at 3 h after administration of IFN-α, peaked at 6–12 h, and was gradually attenuated (Fig. 2C). Next, dose-response analysis showed that the En II activity was down-regulated by IFN-α in a dose-dependent manner, with the maximal suppressive effect at 300–1000 IU/m (Fig. 2D). We also examined the IFN-α-mediated suppression of En II activity in other hepatoma cell lines, PLC/PRF/5 and Hep3B. IFN-α significantly suppressed En II activities in both these cell lines (Fig. 2E). We next assessed whether or not IFN-α regulated HBV transcription in the HBV genome transfected cells by RT-PCR. HBV-RNA levels were significantly reduced by IFN-α (Fig. 2F). These results indicate that IFN-α suppresses HBV En II activity as well as its expression at a transcriptional level.

Both nt 1703–1727 and nt 1746–1770 within the En II region are required for suppression of En II activity by IFN-α

To determine the region responsible for the inhibitory effect of IFN-α on En II activity, we divided the En II sequence into six segments (Fig. 3A), and constructed plasmids containing En II sequences with deletion of each segment (pGL4LUC-En II-D1∼6). Huh-7 cells were transfected with these deleted constructs, treated with IFN-α, and then assayed for luciferase activity. None of the deletions could restore the suppressive activity by IFN-α (Fig. 3B), suggesting that there are several responsible regions for the IFN-α-induced suppression of En II activity. Next, we constructed plasmids containing four iterations of each segment within the En II sequence in tandem (pGL4LUC-En II-T1∼6) to examine the contribution of individual short fragments. IFN-α significantly suppressed the activities of pGL4LUC-En II-T2, T3, T4, T5 and -T6 in luciferase assay. Among them, the activities of pGL4LUC-En II-T4 and -T6 showed the largest suppression by IFN-α (Fig. 3C). On the basis of this result, we constructed a luciferase reporter vector with deletions of both segment 4 (nt 1703–1727) and segment 6 (nt 1746–1770) (pGL4LUC-En II-D4+6). The activity of this dual-deleted construct did not show a significant change due to IFN-α (Fig. 3D). These results suggest that both nt 1703–1727 and nt 1746–1770 within the En II region are required for the suppression of En II activity by IFN-α.

IFN-α-mediated suppression of En II activity is dependent on JAK activation

IFN-induced signal transduction occurs through the sequential activation of JAKs and STATs (Darnell et al., 1994). We examined the role of JAK in the inhibition of En II activity. JAK inhibitor alone did not affect En II activity. But the pre-treatment of the cells with JAK inhibitor completely blocked the suppressive effect of IFN-α on En II activity (Fig. 4A). The effect of JAK inhibitor was confirmed by the reducion of Mx induction in Western blot analysis (Fig. 4B). This result demonstrates that JAK activation is necessary for the IFN-α-induced suppression of En II activity.

The PKC pathway is involved in IFN-α-mediated suppression of En II activity

Previous reports demonstrated that type I IFN activated various kinases such as MAPK family members (MEK/ERK and p38 MAPK) (David et al., 1995; Goh et al., 1999), PI3K/Akt (Uddin et al., 1995), JNK (Caraglia et al., 1999) and protein kinase C (PKC) (Uddin et al., 2002). Here we examined the involvement of alternative pathways by pre-treatment with inhibitors for various kinases, including MEK, p38 MAPK, PI3K/Akt, JNK and PKC. The name of each inhibitors and its target kinase is commented in Table 1. As shown in Fig. 5A, only staurosporine, a PKC inhibitor, blocked the inhibitory effect of IFN-α, and other inhibitors did

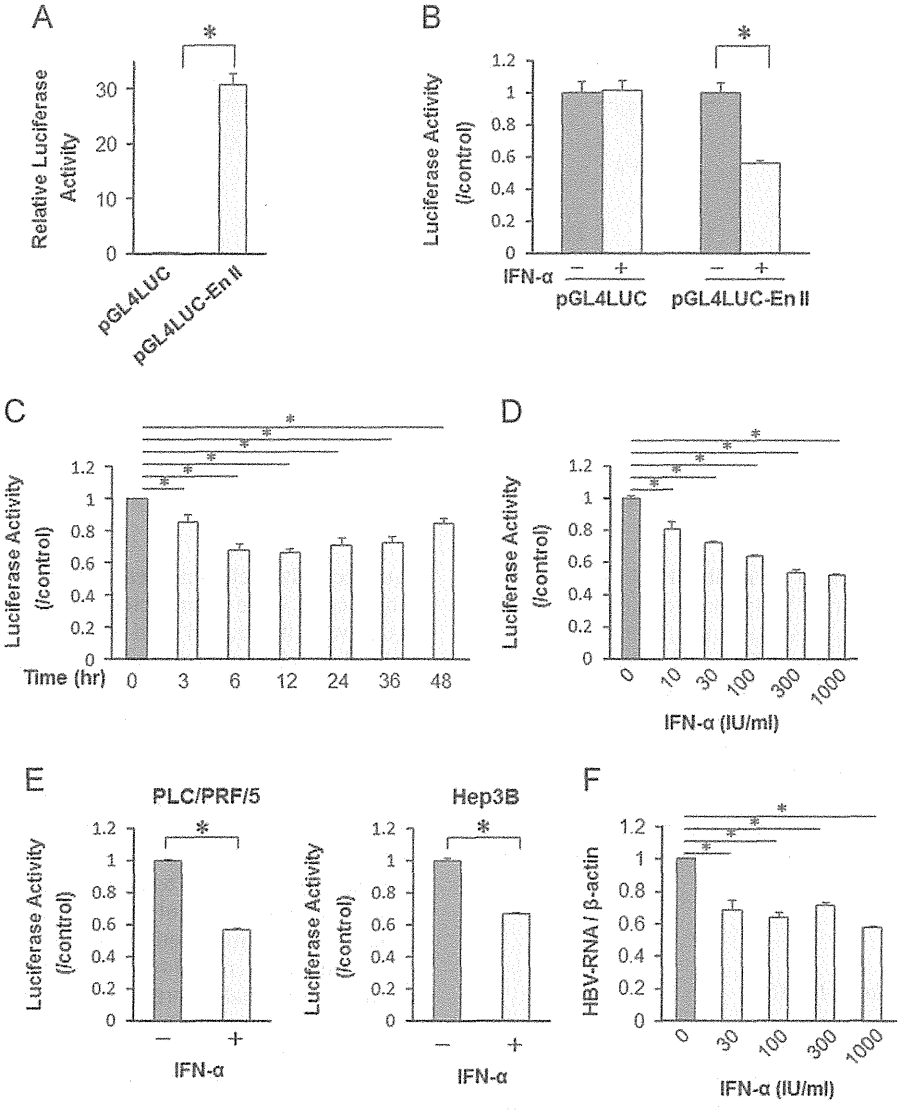

Fig. 2. Suppression of HBV En II transcriptional activity and reduction of HBV-RNA by IFN-α. A, B. Huh-7 cells were transfected with pGL4LUC or pGL4LUC-En II or incubated with or without IFN-α (100 IU/ml). After 24 h, the activity of firefly luciferase was evaluated. C. Huh-7 cells were transfected with pGL4LUC-En II, and incubated with IFN-α (100 IU/ml). Luciferase activities were evaluated at the indicated times. D. Huh-7 cells were transfected with various concentrations (0–1000 IU/ml) of IFN-α for 12 h and luciferase activities were evaluated. E. PLC/PRF/5 cells (left panel) and Hep3B (right panel) cells were transfected with pGL4LUC-En II, and incubated with or without IFN-α (300 IU/ml). Luciferase activities were evaluated. F. Huh-7 cells were transfected with pHBV1.5, and treated with IFN-α at various concentrations (0–1000 IU/ml). At 72 h after IFN-α treatment, cells were harvested, and the abundances of HBV-RNA were evaluated by quantitative RT-PCR. The HBV-RNA level of the IFN-α treated cells was normalized with that of non-treated cells. $^*p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

not. Since staurosporine is a PKC inhibitor showing broad-spectrum activity (Marte et al., 1994), we also examined other inhibitors specific for PKC isoforms. Previous reports demonstrated that IFN-α activated PKC-α/β and PKC-δ (Pfeffer et al., 1990; Uddin et al., 2002). Indeed, activation of PKC-α/β and PKC-δ by IFN-α was confirmed by immunoblot analysis (Fig. 5B). Thus, we examined the PKC inhibitors rottlerin and Gö6976 (Gschwendt et al., 1994; Martiny-Baron et al., 1993). All PKC inhibitors blocked the suppression of En II activity by IFN-α (Fig. 5C). These results suggest that several isoforms of PKC are involved in the IFN-α-mediated suppression of En II activity. We also examined STAT1 activation and ISGs induction by IFN-α in cells pre-treated with these PKC inhibitors using immunoblot analysis (Fig. 5D). Expression levels of phospho-STAT1 and Mx differed among these PKC inhibitors. Staurosporine and Gö6976 slightly diminished the activation of STAT1, but rottlerin did not. This result suggests that PKC isoforms might not strongly regulate activation of STAT1. Rottlerin, a specific inhibitor for PKC-δ, inhibited the induction of Mx, which agreed with previous findings (Kaur et al., 2005). Staurosporine and Gö6976 did not suppress Mx expression. Taken together, all these PKC inhibitors blocked the suppression of En II activity by IFN-α regardless of the expression levels of phospho-STAT1 and Mx. These results suggest that STAT1 activation and ISG induction may be dispensable for the IFN-α-mediated suppression of En II activity. Next, we examined the effect of phorbol 12-myristate 13-acetate (PMA), a PKC activator (Castagna et al., 1982; Griner and Kazanietz, 2007). PMA suppressed En II activity (Fig. 5E), and PMA stimulation did not result in STAT1 phosphorylation and Mx induction (Fig. 5F), suggesting that suppression of En II by PMA is independent of STAT1 activation and ISG induction. On the basis of these findings, we conclude that IFN-α suppresses En II activity via the PKC pathway, which may not involve STAT1 activation and ISG induction.

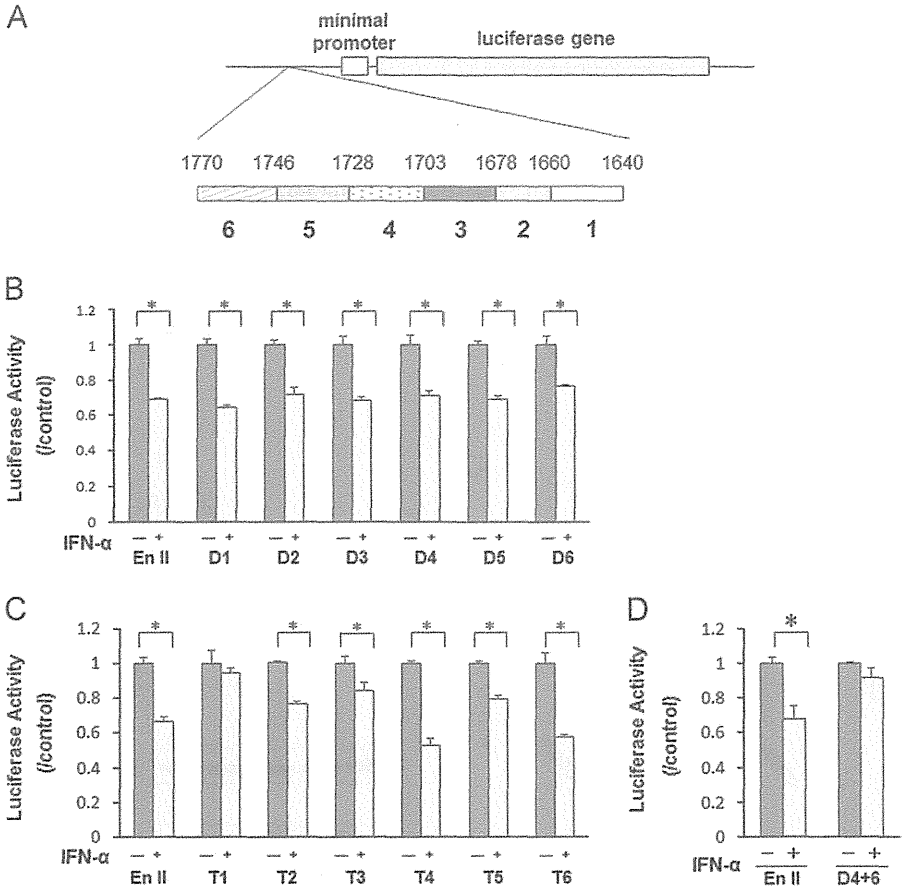

Fig. 3. Deletion/mutational analysis to identify the responsive sequence for the suppressive effect of IFN-α on En II. A. Scheme of pGL4LUC-En II and six segments defined within the En II sequence. The En II sequence was integrated just upstream of the minimal promoter of pGL4LUC. B. Huh-7 cells were transfected with the reporter vectors with deletion of each segment (pGL4LUC-En II-D1~6), incubated with 300 IU/ml IFN-α for 12 h, and luciferase activities were evaluated. C. Plasmids containing four iterations of each segment within En II sequence in tandem (pGL4LUC-En II-T1~6) were generated and luciferase activities were evaluated similarly. D. Plasmid with deletion of both nt 1703–1727 and nt 1746–1770 (pGL4LUC-En II-D4+6) was constructed and luciferase activities were evaluated similarly. $^*p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

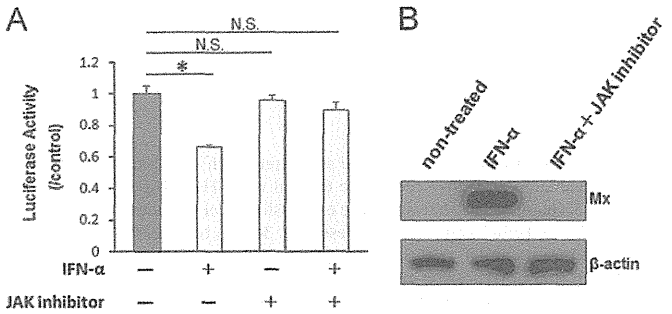

Fig. 4. Involvement of JAK activation in the IFN-α-induced suppression of En II activity. A. Huh-7 cells were transfected with pGL4LUC-En II and treated with JAK inhibitor (1 μM) for 1 h. The cells were then incubated with IFN-α(150 IU/ml) for 12 h, followed by luciferase assay. B. Huh-7 cells were pre-treated with JAK inhibitor for 1 h, and then incubated with IFN-α(150 IU/ml) for 12 h, followed by immunoblot analyses to detect Mx protein. $^*p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized by that of non-treated cells.

Table 1
A comment of the inhibitors and its target kinase.

PD98059	MEK inhibitor
SB203580	P38MAPK inhibitor
LY294002	PI3K inhibitor
Akt-1-1/2	Akt inhibitor
SP600125	JNK inhibitor
Staurosporine	PKC inhibitor with broad spectrum
Rottlerin	Inhibitor specific for PKC-δ
Gö6976	Inhibitor specific for Ca^{2+}-dependent PKC isoforms

Knockdown of a single transcription factor does not influence IFN-α-induced suppression of En II activity

We anticipated that IFN-α suppressed En II activity by functional down-regulation of some transcription factor(s) phosphorylated in a PKC-dependent manner. Among transcription factors which bind the En II region, previous reports showed that Specificity Protein 1 (Sp1) (Mahoney et al., 1992; Pal et al., 1998; Rafty and Khachigian, 2001), Retinoid X Receptor α (RXRA) (Delmotte et al., 1999) and C/EBP (Mahoney et al., 1992) were inactivated by PKC. Thus, we examined the En II response to IFN-α after knockdown of these transcription factors. C/EBP, RXR and Sp1 expression was efficiently reduced by siRNA (Fig. 6A). We observed no significant change in the suppression of En II activity compared with control siRNA (Fig. 6B). This result suggests that several transcription factors (including unknown proteins) might be involved in the IFN-α-mediated suppression of En II activity.

Discussion

In the present study, we demonstrated that IFN-α suppressed HBV En II activity. The inhibition by IFN-α of En II activity could be blocked by pre-treatment with PKC inhibitors, and this

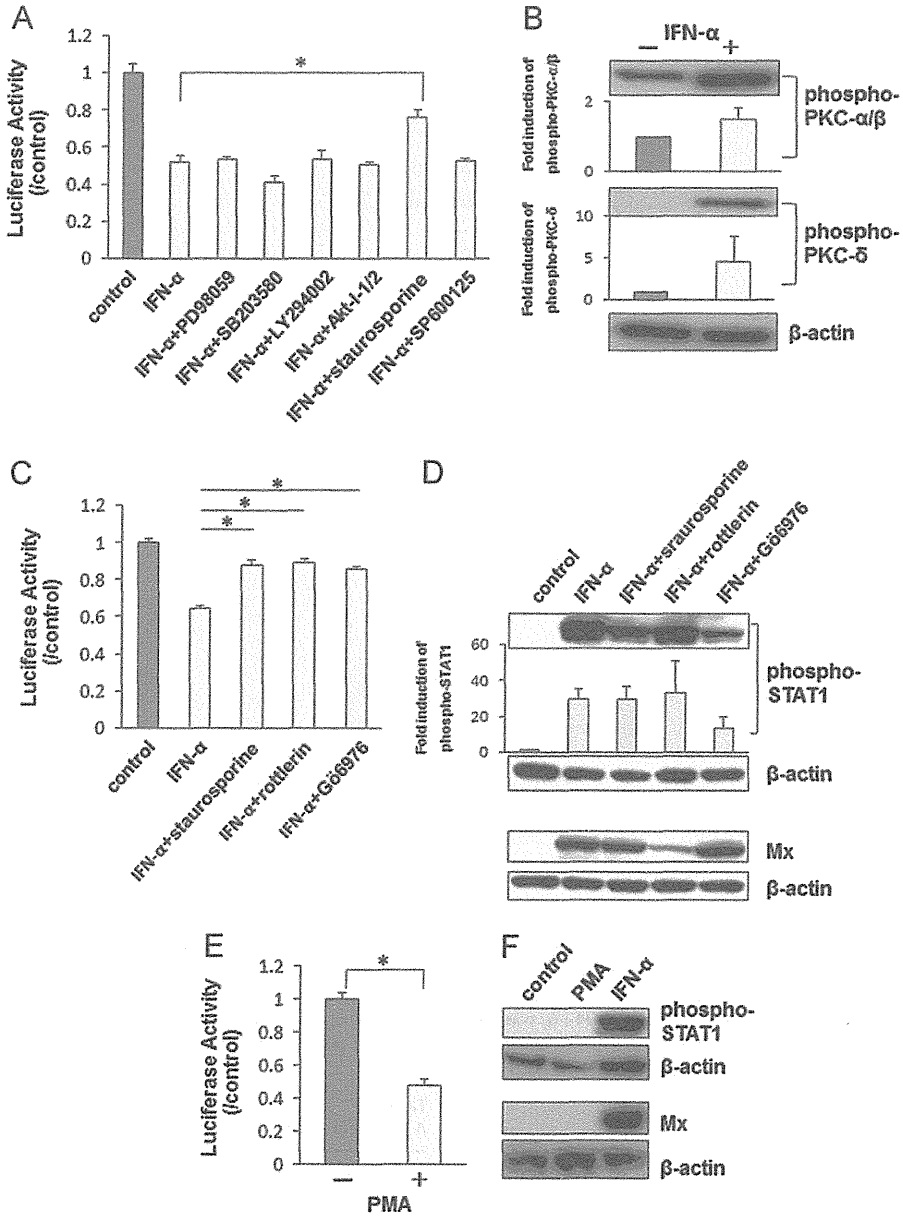

Fig. 5. PKC-dependent suppression of En II activity by IFN-α. A and C. Huh-7 cells were transfected with pGL4LUC-En II, treated separately with each kinase inhibitor for 1 h. The cells were then treated with IFN-α(1000 IU/ml) for 12 h, and luciferase activities were evaluated. B. Huh-7 cells were treated with IFN-α(1000 IU/m) for 12 h. Immunoblot analyses were performed to detect phosphorylated PKC-α/β and phosphorylated PKC-δ. Quantitative analysis of the expression level of phopho- PKC-α/β and -δ was performed by using ImageJ. Each level was normalized with that of IFN-α-non-treated cells. D. Huh-7 cells were harvested at 30 min to detect phosphorylated STAT1 and at 12 h to detect the expression of Mx after administration of IFN-α (1000 IU/m), and immunoblot analyses were performed. Quantitative analysis of the expression level of phopho-STAT1 was performed by using ImageJ. Each level was normalized with that of IFN-α-non-treated cells. E. Huh-7 cells were transfected with pGL4LUC-En II, treated with PMA (100 nM) for 12 h, and luciferase activities were evaluated. F. Huh-7 cells were treated with PMA (100 nM) or IFN-α(1000 IU/m). The cells were harvested at 30 min to detect phosphorylated STAT1 and at 12 h to detect the expression of Mx, and immunoblot analyses were performed. $^{*}p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α/ PMA treated cells normalized with that of non-treated cells.

blocking effect may not involve STAT1 activation and ISG induction. The latter, ISG induction via the JAK-STAT pathway, has been considered to be the main mechanism suppressing viral replication. Our findings suggest a pathway for IFN-α repression of HBV transcription other than ISG induction.

PKCs are involved in a wide variety of cell functions and signal transduction pathways regulating cell migration and polarity, proliferation, differentiation and cell death (Nishizuka, 1988). In the PKC family, there are at least ten isoforms which can be divided into three sub-groups based on their structural characteristics and cofactor requirements. These include the classical PKC (cPKC: α, βI, βII, and γ), the novel PKC (nPKC: δ, ε, η and θ), and the atypical PKC (aPKC: ζ and ι/λ) (Azzi et al., 1992; Breitkreutz et al., 2007; Kikkawa et al., 1989). IFN-α can activate multiple PKC isoforms: not only PKC-δ, but also PKC-α/β (Pfeffer et al., 1990), PKC-ε (Pfeffer et al., 1991), and PKC-θ (Srivastava et al., 2004). Despite the variety of PKC isoforms, most phosphorylate similar sequences (Breitkreutz et al., 2007). Both the PKC-α/β inhibitor (Gö6976) and PKC-δ inhibitor (rottlerin) blocked the inhibitory effect of IFN-α on En II activity. Thus, it was speculated that each PKC isoform might be similarly involved in suppressing of En II activity.

Other studies have examined the role of the PKC pathway in HBV replication. Kang et al. (2008) reported that PKC-mediated phosphorylation increased capsid assembly and stability (von Hahn et al., 2011), and von Hahn et al. (2011) reported that the

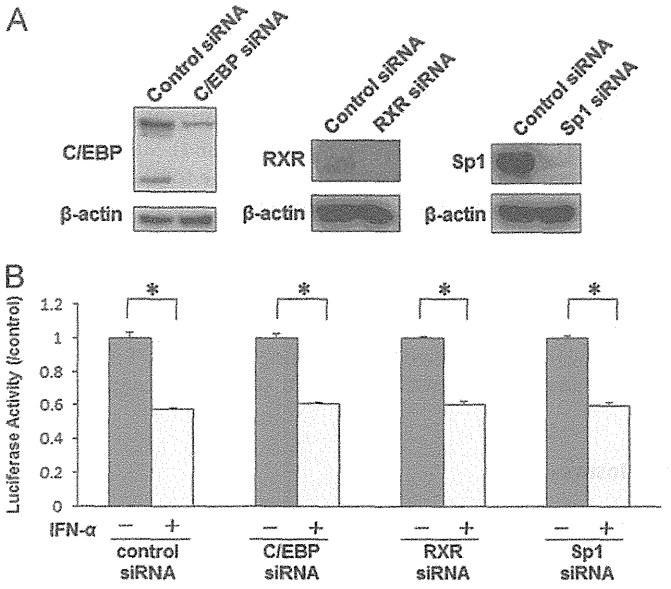

Fig. 6. IFN-α-mediated suppression on En II activity with knockdown of C/EBP, RXR and Sp1. A. Huh-7 cells were transfected with 10 nM siRNA (negative control or specific for C/EBP, RXR and Sp1). Immunoblot analyses for expressions of C/EBP, RXR, Sp1 and β-actin were performed at 48 h post siRNA transfection. B. Huh-7 cells were transfected with 10 nM siRNA (negative control or specific for C/EBP, RXR and Sp1). On the next day, si-RNA treated cells were transfected again with pGL4LUC-En II. On the following day, these transfected cells were incubated with IFN-α(1000 IU/ml) for 12 h, and luciferase activities were evaluated. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

pan-PKC inhibitor sotrastaurin did not affect HBV replication. While the role of PKC in the HBV life cycle is still controversial, our findings suggest that PKC isoforms activated by IFN-α play inhibitory roles in HBV transcription by down-regulation of En II activity. As von Hahn et al. reported, sotrastaurin alone did not affect HBV replication. But, based on our present data about another pan-PKC inhibitor, staurosporine, we speculate that sotrastaurin may also block the inhibitory effect of IFN-α on En II activity.

We showed that knockdown of a single transcription factor did not influence the IFN-α-mediated suppression of En II activity, suggesting that several transcription factors might be involved in this suppression. We also showed that both segment 4 (nt 1703–1727) and segment 6 (nt 1746–1770) within the En II region are required for the IFN-α-induced suppression of En II activity. Although these two regions seem to be more important than the others, all the deleted version of reporter constructs showed almost completely similar suppression activities (Fig. 3B). We speculate that there may be some transcription factors which affect both the segment 4 and 6. Even if one of these regions is deleted, some factors may affect the other region, and result in the suppression of En II activity. Further study will be needed to clarify the mechanism.

Indeed, there are no identified transcription factors which could bind both segment 4 and 6. Only two transcription factors (HNF1 and 3) were reported to bind segment 4 (Johnson et al., 1995; Wang et al., 1998), and there have been no reports indicating that IFN-α or PKC inactivates HNF1 or 3. We also examined the expression levels of HNF1 and 3 of the IFN-α treated and the non-treated cells by RT-PCR. There was no significant difference in the expression of these transcription factors between the IFN-α treated and the non-treated cells (Nawa et al., unpublished data). Thus, we speculate that HNF1 or 3 might not be involved in the IFN-α mediated suppression of En II activity. There may be unknown transcription factors in the PKC pathway.

Previous reports showed that IFN-α suppressed En I activity (Nakao et al., 1999; Tur-Kaspa et al., 1990). Nakao et al. (1999) indicated that this occurred due to the binding of ISGF3 to an ISRE-like motif within the En I region. However, Rang et al. (2001) demonstrated that IFN-α reduced HBV-RNA levels derived from both HBV genome wild type and mutated ISRE-like motifs. This result contradicted the Nakao's result that the activity of the En I mutated ISRE-like motif was not suppressed by IFN-α. Schulte-Frohlinde et al. (2002) reported that IFN-α suppressed HBV core promoter regulated transcriptional activity, even when the ISRE-like motif of En I was deleted. The results of Rang et al. and Schulte-Frohlinde et al. suggest that IFN-α might suppress the activity of regions other than En I. In the present study, we demonstrated that IFN-α suppressed En II activity via the PKC pathway. En II might be one of the candidate regions down-regulated by IFN-α within the HBV genome.

Since En II activates viral transcription only in hepatocytes, it is responsible for the hepatocyte-specific gene expression of HBV. There had been no study on the effect of IFN-α on En II activity. Our study clarified that the PKC pathway is involved in the IFN-α-mediated suppression of En II activity, but may not involve ISG induction. Our result should aid in establishing better treatment with IFN-α against HBV infection. As we could not determine the molecule which inhibits En II activity by IFN-α, further study is needed to clarify this molecule and to control hepatitis B by IFN-α treatment.

Materials and methods

Plasmids

The HBV sequence used in this study was of the *adw2* subtype (GenBank accession no. X02763). Numbering of the HBV sequence started at the unique *Eco*RI site. The En II region in this study was defined as nt 1640–1771 of HBV sequence (Fig. 1) (Ishida et al., 2000). To construct pGL4LUC-En II, a plasmid containing the HBV En II region, the DNA fragment was amplified with PCR and inserted between *Hind* III and *Nhe* I site of pGL4 Luciferase Reporter Vector (pGL4LUC) (Promega, Madison, WI). The PCR primers were as follows: 5′-CCAAGCTTCTGCCCAAGGTC-3′ and 5′-CCCGCTAGCAAAGACCTTTAACCTAATCTCCTCC-3′. The constructs of the En II sequence with various deletions were generated by modifying pGL4LUC-En II using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The constructs containing four tandem repeats of short fragment in En II sequence were generated by inserting duplexes of synthesized oligonucleotides into the multi-cloning site of pGL4LUC. All of the En II sequences were inserted in the antisense orientation to evaluate their enhancer activity.

Plasmid pHBV1.5 containing a 1.5-fold-overlength genome of HBV-DNA (GenBank accession no. AF305422) has been described previously (Bruss and Ganem, 1991).

Cell lines and reagents

The human hepatocellular carcinoma cell lines Huh-7, PLC/PRF/5, and Hep3B were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 5% CO_2 and 37 °C. Human natural IFN-α was kindly provided by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan).

The inhibitors/activators and the final concentrations used were: JAK inhibitor I (1 μM), PD98059 (10 μM), SB203580 (10 μM), LY294002 (10 μM), Akt-I-1/2 (5 μM), staurosporine (10 or 20 nM), rottlerin (5 μM), Gö6976 (1 μM), SP600125 (10 μM)

(Calbiochem, San Diego, CA), phorbol 12-myristate 13-acetate (PMA) (100 nM) (Sigma-Aldrich, St. Louis, MO).

Plasmid transfection and luciferase assay

Huh-7 cells were co-transfected with the firefly luciferase plasmid and pGL4-RL-tk, an expression vector of renilla luciferase, which was used as an internal control, using FuGENE HD reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Activities of firefly luciferase and renilla luciferase were measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI), and then relative luciferase activity was calculated by normalizing firefly luciferase activity to renilla luciferase activity.

RNA extraction

Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The isolated RNA was treated with DNase I (Promega, Madison, WI) to avoid contamination with transfected plasmid, and then purified with a mixture of phenol, chloroform, and isoamylalcohol (pH 7.9), followed by ethanol precipitation.

Western blot analysis

Cultured cells were lysed with a lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protein inhibitor cocktail (Nacalai Tesque), in PBS, pH 7.4). Equal amounts of protein were electrophoretically separated by polyacrylamide gel and transferred onto PVDF membrane. For immunodetection, the following antibodies were used: anti-STAT1 antibody, anti-phospho-STAT1 antibody, anti-phospho-PKC-α/β II (Thr 638/641) antibody, anti-phospho-PKC-δ (Thr 505) antibody, anti-C/EBP antibody, anti-RXR antibody, anti-Sp1 antibody, anti-β-actin antibody from Cell Signaling Technology (Beverly, MA), and anti-Mx antibody from Abcam (Cambridge, UK). The signals of phosphorylated proteins such as phospho-PKC-α/β, -δ and phospho-STAT1 were analyzed quantitatively using image analyzing software (ImageJ; version 1.45).

Small RNA interference

Stealth Select RNAi specific for STAT1 (HSS 10273) was purchased from Invitrogen (Carlsbad, CA). Silencer Select siRNA specific for C/EBP (ID: S2890), RXR (ID: S12386) and Sp1 (ID: S13319) were purchased from Ambion (Austin, TX). Stealth RNAi Negative Control Low GC Duplex (Invitrogen, Carlsbad, CA) was used as a control for the off-target effect following Stealth Select RNAi delivery. The transfections were carried out using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the reverse transfection protocol.

Real-time reverse-transcription PCR

For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA). cDNA, equivalent to 20 ng RNA, was used as a template for real-time reverse-transcription PCR (RT-PCR) using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). mRNA expressions of C/EBP, FTF, HNF1, HNF3, and HNF4 were measured using TaqMan Gene Expression Assays and were corrected with the quantified expressions level of β-actin mRNA. Assay IDs for the genes were as follows: C/EBP (Hs00269972_s1), FTF (Hs00187067_m1), HNF1 (Hs00167041_m1), HNF3 (Hs00232754_m1), and HNF4 (Hs01023298_m1).

For the detection of pgRNA and pre-C mRNA, the primers and the probes were designed as follows according to a previous study (Laras et al., 2002): the sense primer was 5′-TCTTGTACATGTCCCACTGTTCAA-3′ (nt 1843–1866); the anti-sense primer was 5′-AATGCCATGCCCCAAAGC-3′ (nt 1890–1909); the probe was 5′-FAM-CTCCAAGCTGTGCCTT -3′ (nt 1869–1884). Since they were within precore/core coding sequence, only the total abundance of pgRNA and pre-C RNA could be detected.

Statistical analysis

Data were presented as mean ± SD. Differences between two groups were determined using Student's t-test for unpaired observations. $p < 0.05$ was considered statistically significant.

Disclosures

All authors have nothing to disclose.

References

Antonucci, T.K., Rutter, W.J., 1989. Hepatitis B virus (HBV) promoters are regulated by the HBV enhancer in a tissue-specific manner. J. Virol. 63, 579–583.
Azzi, A., Boscoboinik, D., Hensey, C., 1992. The protein kinase C family. Eur. J. Biochem. 208, 547–557.
Beck, J., Nassal, M., 2007. Hepatitis B virus replication. World J. Gastroenterol. 13, 48–64.
Breitkreutz, D., Braiman-Wiksman, L., Daum, N., Denning, M.F., Tennenbaum, T., 2007. Protein kinase C family: on the crossroads of cell signaling in skin and tumor epithelium. J. Cancer Res. Clin. Oncol. 133, 793–808.
Bruss, V., Ganem, D., 1991. The role of envelope proteins in hepatitis B virus assembly. Proc. Nat. Acad. Sci. U.S.A. 88, 1059–1063.
Caraglia, M., Abbruzzese, A., Leardi, A., Pepe, S., Budillon, A., Baldassare, G., Selleri, C., Lorenzo, S.D., Fabbrocini, A., Giuberti, G., Vitale, G., Lupoli, G., Bianco, A.R., Tagliaferri, P., 1999. Interferon-alpha induces apoptosis in human KB cells through a stress-dependent mitogen activated protein kinase pathway that is antagonized by epidermal growth factor. Cell Death Differ. 6, 773–780.
Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., Nishizuka, Y., 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257, 7847–7851.
Darnell, J.E., Kerr, I.M., Stark, G.R., 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415–1421.
David, M., Petricoin, E., Benjamin, C., Pine, R., Weber, M.J., Larner, A.C., 1995. Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. Science 269, 1721–1723.
Delmotte, M.H., Tahayato, A., Formstecher, P., Lefebvre, P., 1999. Serine 157, a retinoic acid receptor alpha residue phosphorylated by protein kinase C in vitro, is involved in RXR.RARalpha heterodimerization and transcriptional activity. J. Biol. Chem. 274, 38225–38231.
Der, S.D., Zhou, A., Williams, B.R., Silverman, R.H., 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc. Nat. Acad. Sci. U.S.A. 95, 15623–15628.
Goh, K.C., Haque, S.J., Williams, B.R., 1999. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. EMBO J. 18, 5601–5608.
Griner, E.M., Kazanietz, M.G., 2007. Protein kinase C and other diacylglycerol effectors in cancer. Nat. Rev. Cancer 7, 281–294.
Gschwendt, M., Müller, H.J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G., Marks, F., 1994. Rottlerin, a novel protein kinase inhibitor. Biochem. Biophys. Res. Commun. 199, 93–98.
Guo, W., Chen, M., Yen, T.S., Ou, J.H., 1993. Hepatocyte-specific expression of the hepatitis B virus core promoter depends on both positive and negative regulation. Mol. Cell. Biol. 13, 443–448.
Ishida, H., Ueda, K., Ohkawa, K., Kanazawa, Y., Hosui, A., Nakanishi, F., Mita, E., Kasahara, A., Sasaki, Y., Hori, M., Hayashi, N., 2000. Identification of multiple transcription factors, HLF, FTF, and E4BP4, controlling hepatitis B virus enhancer II. J. Virol. 74, 1241–1251.
Johnson, J.L., Raney, A.K., McLachlan, A., 1995. Characterization of a functional hepatocyte nuclear factor 3 binding site in the hepatitis B virus nucleocapsid promoter. Virology 208, 147–158.
Jonas, M.M., Block, J.M., Haber, B.A., Karpen, S.J., London, W.T., Murray, K.F., Narkewicz, M.R., Rosenthal, P., Schwarz, K.B., McMahon, B.J., Foundation, H.B., 2010. Treatment of children with chronic hepatitis B virus infection in the United States: patient selection and therapeutic options. Hepatology 52, 2192–2205.

Kang, H., Yu, J., Jung, G., 2008. Phosphorylation of hepatitis B virus core C-terminally truncated protein (Cp149) by PKC increases capsid assembly and stability. Biochem. J. 416, 47–54.
Kaur, S., Parmar, S., Smith, J., Katsoulidis, E., Li, Y., Sassano, A., Majchrzak, B., Uddin, S., Tallman, M.S., Fish, E.N., Platanias, L.C., 2005. Role of protein kinase C-delta (PKC-delta) in the generation of the effects of IFN-alpha in chronic myelogenous leukemia cells. Exp. Hematol. 33, 550–557.
Kikkawa, U., Kishimoto, A., Nishizuka, Y., 1989. The protein kinase C family: heterogeneity and its implications. Annu. Rev. Biochem. 58, 31–44.
Laras, A., Koskinas, J., Hadziyannis, S.J., 2002. In vivo suppression of precore mRNA synthesis is associated with mutations in the hepatitis B virus core promoter. Virology 295, 86–96.
Li, M., Xie, Y., Wu, X., Kong, Y., Wang, Y., 1995. HNF3 binds and activates the second enhancer, ENII, of hepatitis B virus. Virology 214, 371–378.
Li, M., Xie, Y.H., Kong, Y.Y., Wu, X., Zhu, L., Wang, Y., 1998. Cloning and characterization of a novel human hepatocyte transcription factor, hB1F, which binds and activates enhancer II of hepatitis B virus. J. Biol. Chem. 273, 29022–29031.
Liaw, Y.F., 2009. HBeAg seroconversion as an important end point in the treatment of chronic hepatitis B. Hepatol. Int.
Lok, A.S., McMahon, B.J., 2009. Chronic hepatitis B: update 2009. Hepatology 50, 661–662.
López-Cabrera, M., Letovsky, J., Hu, K.Q., Siddiqui, A., 1990. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: trans-activation and repression by CCAAT/enhancer binding protein. Proc. Nat. Acad. Sci. U.S.A. 87, 5069–5073.
López-Cabrera, M., Letovsky, J., Hu, K.Q., Siddiqui, A., 1991. Transcriptional factor C/EBP binds to and transactivates the enhancer element II of the hepatitis B virus. Virology 183, 825–829.
Mahoney, C.W., Shuman, J., McKnight, S.L., Chen, H.C., Huang, K.P., 1992. Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. J. Biol. Chem. 267, 19396–19403.
Marte, B.M., Meyer, T., Stabel, S., Standke, G.J., Jaken, S., Fabbro, D., Hynes, N.E., 1994. Protein kinase C and mammary cell differentiation: involvement of protein kinase C alpha in the induction of beta-casein expression. Cell Growth Differ. 5, 239–247.
Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marmé, D., Schächtele, C., 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J. Biol. Chem. 268, 9194–9197.
Moolla, N., Kew, M., Arbuthnot, P., 2002. Regulatory elements of hepatitis B virus transcription. J. Viral. Hepat. 9, 323–331.
Nakao, K., Nakata, K., Yamashita, M., Tamada, Y., Hamasaki, K., Ishikawa, H., Kato, Y., Eguchi, K., Ishii, N., 1999. p48 (ISGF-3gamma) is involved in interferon-alpha-induced suppression of hepatitis B virus enhancer-1 activity. J. Biol. Chem. 274, 28075–28078.
Nishizuka, Y., 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661–665.
Pal, S., Claffey, K.P., Cohen, H.T., Mukhopadhyay, D., 1998. Activation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription requires specific interaction with protein kinase C zeta. J. Biol. Chem. 273, 26277–26280.
Pfeffer, L.M., Eisenkraft, B.L., Reich, N.C., Improta, T., Baxter, G., Daniel-Issakani, S., Strulovici, B., 1991. Transmembrane signaling by interferon alpha involves diacylglycerol production and activation of the epsilon isoform of protein kinase C in Daudi cells. Proc. Nat. Acad. Sci. U.S.A. 88, 7988–7992.
Pfeffer, L.M., Strulovici, B., Saltiel, A.R., 1990. Interferon-alpha selectively activates the beta isoform of protein kinase C through phosphatidylcholine hydrolysis. Proc. Nat. Acad. Sci. U.S.A. 87, 6537–6541.
Rafty, L.A., Khachigian, L.M., 2001. Sp1 phosphorylation regulates inducible expression of platelet-derived growth factor B-chain gene via atypical protein kinase C-zeta. Nucleic Acids Res. 29, 1027–1033.
Raney, A.K., Johnson, J.L., Palmer, C.N., McLachlan, A., 1997. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. J. Virol. 71, 1058–1071.
Rang, A., Heise, T., Will, H., 2001. Lack of a role of the interferon-stimulated response element-like region in interferon alpha -induced suppression of Hepatitis B virus in vitro. J. Biol. Chem. 276, 3531–3535.
Romero, R., Lavine, J.E., 1996. Cytokine inhibition of the hepatitis B virus core promoter. Hepatology 23, 17–23.
Schulte-Frohlinde, E., Seidler, B., Burkard, I., Freilinger, T., Lersch, C., Erfle, V., Foster, G.R., Classen, M., 2002. Different activities of type I interferons on hepatitis B virus core promoter regulated transcription. Cytokine 17, 214–220.
Srivastava, K.K., Batra, S., Sassano, A., Li, Y., Majchrzak, B., Kiyokawa, H., Altman, A., Fish, E.N., Platanias, L.C., 2004. Engagement of protein kinase C-theta in interferon signaling in T-cells. J. Biol. Chem. 279, 29911–29920.
Su, H., Yee, J.K., 1992. Regulation of hepatitis B virus gene expression by its two enhancers. Proc. Nat. Acad. Sci. U.S.A. 89, 2708–2712.
Sureau, C., Romet-Lemonne, J.L., Mullins, J.I., Essex, M., 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell 47, 37–47.
Tur-Kaspa, R., Teicher, L., Laub, O., Itin, A., Dagan, D., Bloom, B.R., Shafritz, D.A., 1990. Alpha interferon suppresses hepatitis B virus enhancer activity and reduces viral gene transcription. J. Virol. 64, 1821–1824.
Uddin, S., Sassano, A., Deb, D.K., Verma, A., Majchrzak, B., Rahman, A., Malik, A.B., Fish, E.N., Platanias, L.C., 2002. Protein kinase C-delta (PKC-delta) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727. J. Biol. Chem. 277, 14408–14416.
Uddin, S., Yenush, L., Sun, X.J., Sweet, M.E., White, M.F., Platanias, L.C., 1995. Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3'-kinase. J. Biol. Chem. 270, 15938–15941.
Vannice, J.L., Levinson, A.D., 1988. Properties of the human hepatitis B virus enhancer: position effects and cell-type nonspecificity. J. Virol. 62, 1305–1313.
von Hahn, T., Schulze, A., Chicano Wust, I., Heidrich, B., Becker, T., Steinmann, E., Helfritz, F.A., Rohrmann, K., Urban, S., Manns, M.P., Pietschmann, T., Ciesek, S., 2011. The novel immunosuppressive protein kinase C inhibitor sotrastaurin has no pro-viral effects on the replication cycle of hepatitis B or C virus. PLoS One 6, e24142.
Wang, W.X., Li, M., Wu, X., Wang, Y., Li, Z.P., 1998. HNF1 is critical for the liver-specific function of HBV enhancer II. Res. Virol. 149, 99–108.
Wang, Y., Chen, P., Wu, X., Sun, A.L., Wang, H., Zhu, Y.A., Li, Z.P., 1990. A new enhancer element, ENII, identified in the X gene of hepatitis B virus. J. Virol. 64, 3977–3981.
Yee, J.K., 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science 246, 658–661.
Yuh, C.H., Ting, L.P., 1990. The genome of hepatitis B virus contains a second enhancer: cooperation of two elements within this enhancer is required for its function. J. Virol. 64, 4281–4287.
Yuh, C.H., Ting, L.P., 1991. C/EBP-like proteins binding to the functional box-alpha and box-beta of the second enhancer of hepatitis B virus. Mol. Cell. Biol. 11, 5044–5052.