Liver X Receptor Mediates Hepatitis B Virus X Protein–Induced Lipogenesis in Hepatitis B Virus–Associated Hepatocellular Carcinoma

Tae-Young Na,1,2 Young Kee Shin,1 Kyung Jin Roh,3 Shin-Ae Kang,1,2 Il Hong,1,2 Sae Jin Oh,3 Je Kyung Seong,3 Cheol Keun Park,4 Yoon La Choi,4 and Mi-Ock Lee1,2

Although hepatitis B virus X protein (HBx) has been implicated in abnormal lipid metabolism in hepatitis B virus (HBV)–associated hepatic steatosis, its underlying molecular mechanism remains unclear. Liver X receptor (LXR) plays an important role in regulating the expression of genes involved in hepatic lipogenesis. Here we demonstrate that LXRα and LXRβ mediate HBV-associated hepatic steatosis. We have found that HBx induces the expression of LXR and its lipogenic target genes, such as sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FAS), and peroxisome proliferator-activated receptor, and this is accompanied by the accumulation of lipid droplets. RNA interference with LXR expression decreases the amount of lipid droplets as well as the expression of the lipogenic genes, and this indicates that HBx-induced lipogenesis is LXR-dependent. LXRα and HBx colocalize in the nucleus and are physically associated. HBx induces the transactivation function of LXRα by recruiting CREB binding protein to the promoter of the target gene. Furthermore, we have observed that expression of LXR is increased in the livers of HBx-transgenic mice. Finally, there is a significant increase in the expression of LXRβ (P = 0.036), SREBP-1c (P = 0.008), FAS, and stearoyl–coenzyme A desaturase-1 (P = 0.001) in hepatocellular carcinoma (HCC) in comparison with adjacent nontumorous nodules in human HBV-associated HCC specimens.

Conclusion: Our results suggest a novel association between HBx and LXR that may represent an important mechanism explaining HBx-induced hepatic lipogenesis during HBV-associated hepatic carcinogenesis. (HEPATOLOGY 2009;49:1122-1131.)

Hepatitis B, caused by hepatitis B virus (HBV) infection, is very common in Asia, Africa, and the Middle East. It is estimated that there are 280 million carriers worldwide, representing more than 5% of the global population. Chronic infection with HBV is a major risk factor for the development of hepatocellular carcinoma (HCC); however, the mechanism by which HBV induces events leading to HCC has not been clearly elucidated. The HBV genome consists of four overlapping open reading frames encoding DNA polymerase, surface antigen, core protein, and a regulatory X protein [hepatitis B virus X protein (HBx)]. Among these,
HBx, an essential factor for viral replication, is considered one of the most important determinants of HBV-induced hepatocarcinogenesis.\(^1\)\(^2\)

Abnormal hepatic function, such as hepatic cellular damage and HCC, impairs the homeostasis regulating the synthesis and degradation of lipids and lipoproteins.\(^3\) Small HCC lesions at an early stage are often hyperechoic and are composed of well-differentiated cancer cells rich in triglyceride droplets.\(^4\)\(^5\) Chronic infection with HBV and hepatitis C virus (HCV), two major causes of chronic liver disease, is frequently associated with hepatic steatosis. The frequency of steatosis in HCV infection ranges from 31% to 72%, whereas this risk in HBV infection varies from 27% to 51%.\(^6\)\(^8\) HCV infection--induced hepatic steatosis has been well characterized; it has been shown that chronic HCV infection induces histological responses, including the accumulation of lipid droplets and dysplasia of hepatocytes, the activation of sinusoidal inflammatory cells, dyslipidemia, insulin resistance, and HCV genotype 3.\(^6\)\(^8\) In experimental animals, high-level HCV replication during acute infection is associated with the modulation of multiple genes involved in lipid metabolism. In addition, drugs that control cholesterol and fatty acid biosynthesis regulate the replication of the subgenomic HCV replicon.\(^9\) Among the viral proteins, HCV core protein plays a role in the regulation of the genes related to fatty acid biosynthesis, including liver X receptor α (LXRα) and sterol regulatory element binding protein-1c (SREBP-1c).\(^10\) Aberrations of lipid metabolism are also frequently seen in chronic HBV infection; however, very few reports have addressed the steatogenic pathogenesis of HBV infection at a molecular level. A complementary DNA (cDNA) microarray analysis showed that genes involved in the biosynthesis of lipids, such as fatty acid synthase (FAS) and SREBP-2, are upregulated in the HBV-transgenic mouse liver.\(^11\) Recently, HBx was shown to cause lipid accumulation in hepatic cells, mediated by the activation of SREBP-1 and peroxisome proliferator-activated receptor γ (PPARγ).\(^12\)

The LXR plays an important role in the regulation of the expression of genes involved in the metabolism of lipids and cholesterol.\(^13\)\(^14\) Two LXR subtypes, LXRα (nuclear receptor subfamily 1, group H, member 1) and LXRβ (nuclear receptor subfamily 1, group H, member 2), have been identified that form heterodimers with the retinoid X receptor (RXR) and bind specific DNA sequences, that is, the liver X receptor response element (LXRE).\(^15\) Both LXRα and LXRβ are expressed in the liver; LXRα is also abundantly expressed in other tissues associated with lipid metabolism, including the kidneys, intestines, adipose tissue, and macrophages, whereas LXRβ is ubiquitously expressed.\(^16\) LXRαs are activated by naturally produced oxysterols and synthetic compounds, such as TO901317.\(^17\)\(^18\) LXRαs directly control the expression of SREBP-1, which regulates lipogenic enzymes in the liver, including FAS.\(^19\)\(^20\) Activation of LXRs also induces expression of PPARγ in adipocytes through an LXRE motif found in the PPARγ promoter.\(^21\) Given the critical roles of LXR in hepatic lipogenesis, we hypothesized that LXR may have a central role in HBx-induced lipogenesis in the HBV-infected liver.

Here, we have found that HBx induces the expression and transcriptional activity of LXRs. The HBx-induced activation of LXR leads to the induction of the steatogenic factors SREBP-1c, FAS, and PPAR. Furthermore, we show that expression of LXR and its lipogenic downstream target genes is increased in HBV-associated HCC. Our results suggest a novel association between HBx and LXR-induced hepatic lipogenesis, which may constitute an important molecular mechanism underlying the development of HBV-associated HCC.

**Materials and Methods**

**Cells and Cell Culture.** Chang liver and Chang X-34, in which HBx gene expression is under the control of a doxycycline-inducible promoter, and SNU-354 were described previously.\(^22\)\(^23\) HepG2 was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium or Roswell Park Memorial Institute medium containing 10% fetal bovine serum at 37°C in a 5% CO\(_2\)/95% air incubator.

**Western Blotting, Immunoprecipitation, and Immunocytochemistry.** Subcellular fractionation, western blotting, immunoprecipitation, and immunocytochemistry were basically performed as previously described with specific antibodies against LXRα (Affinity BioReagents, Golden, CO), LXRα, LXRβ, HBx, SREBP-1c, PPARα, PPARβ, PPARγ, stearoyl–coenzyme A desaturase-1 (SCD-1), hemagglutinin, β-actin (Santa Cruz Biotech-
Plasmids, Transient Transfection, and Reporter Gene Analysis. The reporters LXRE-Luc, Gal4-rk-Luc, and SRE-Luc (where Gal4 is galactosidase 4, Luc is luciferase, and SRE is sterol regulatory element) and the expression vectors for LXRα and LXRβ were as described previously. The Myc-tagged HBx and FLAG-tagged HBx were constructed by the insertion of HBx cDNA into pCMV-Myc (Clontech, Palo Alto, CA) and p3XFLAG 7.1 (Sigma), respectively. The pGAL4-LXRα was constructed by the insertion of the full-length coding region of human LXRα cDNA into the expression vector containing DNA binding domain (1-147 amino acids) of yeast GAL4. The antisense (AS)-LXR containing DNA binding domain (1-147 amino acids) of bulin (Calbiochem, San Diego, CA). Antibodies against FAS were kindly provided by Dr. K.-S. Kim at Yonsei University College of Medicine.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). RT-PCR was performed as described previously with specific primers (Supporting Table 2). Genes were analyzed under the same conditions used to exponentially amplify the polymerase chain reaction (PCR) products. qRT-PCR amplifications were performed as previously described. The small interfering RNA duplexes were transfected into cells as previously described (Supporting Table 1).

Oil Red O and Nile Red Staining. Cells (2 × 10^5 cells per dish) were seeded in 100-mm dishes and incubated overnight. After cells were transfected with si-LXRs and/or treated with doxycycline for 6 days, cells were washed twice with phosphate-buffered saline and fixed with 10% formalin. Further processes of Oil Red O staining of the frozen liver section was performed as described previously. Whole cell lysates obtained from the frozen liver sections were used for western blot analysis.

HepG2 cells were transfected with 6 µg of pCMV-Myc-HBx or empty vector with Welsect-EX Plus (WelGENE, Inc., Korea). After 24 hours of transfection, cell lysates were obtained, and CHIP assay was carried out with specific antibodies as previously described. DNA was amplified by PCR with specific primers (sense, 5’-gccagtggctccagcgaaaccagtg-3’, and antisense, 5’-ggttgtactaggcaagtggccgcgcc-3’) corresponding to the flanking region of the LXR binding sites on the human SREBP-1c promoter.

HBx-Transgenic Mice. The HBx homozygote transgenic mice at different ages and age-matched C57BL/6 wild-type mice were kindly provided by Dr. DY Yu at Korea Research Institute of Bioscience and Biotechnology. The experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals at Seoul National University according to the Guide for Animal Experiments edited by the Korean Academy for Medical Sciences. A 3-µm section of paraffin-embedded tissue was stained routinely with hematoxylin and eosin. Oil Red O staining of the frozen liver section was performed as described previously. Whole cell lysates obtained from the frozen liver sections were used for western blot analysis.

HCC Samples, qRT-PCR, and Immunohistochemistry. Six normal liver samples and 30 patients with HBV-associated HCC were retrospectively identified from the surgical pathology files of the Department of Pathology at Samsung Medical Center. Five patients with normal livers were HBV-negative and HCV-negative, and one patient was hepatitis B surface antigen-positive. All samples were collected anonymously according to Institutional Review Board guidelines. All patients had undergone a surgical operation and had received neither chemotherapy nor radiotherapy before surgical resection. The histopathological features of the examined HCCs are described in Supporting Table 3.

For total RNA extraction from formalin-fixed paraffin-embedded tissues, each tissue section was stained with hematoxylin, and nontumor or cancer regions were microdissected with a laser microdissection system (Jung-Woo International, Korea) or cut directly with a needle from sections. The Paradise whole transcript reverse-transcriptase (RT) reagent system (Arcturus, Mountain View, CA) was used for RNA isolation and RT. Because of the limited amounts of RNA extracted, half-RNA and cDNA were used for RT and qRT-PCR, respectively. PCR primers and Universal Probe Library numbers for this study are provided in Supporting Table 4. Hypoxanthine phosphoribosyltransferase 1 was chosen as the endogenous reference gene. All PCR reactions were performed in a Lightcycler 2.0 (Roche Applied Science, Mannheim, Germany) according to standard procedures. PCR efficiency for each gene was determined by the measurement of serial dilutions of diluted cDNA from HepG2 cells and calculation with Lightcycler 4.0 software.

Five HCC specimens showing differential expression of FAS in nontumor and cancer regions by qRT-PCR were selected for immunohistochemistry. A standard immunohistochemistry was performed with rabbit anti-FAS.
polyclonal antibody (dilution: 1:50; Santa Cruz Biotechnology).23

**Statistics.** Experimental values are expressed as the mean ± standard deviation of three independent experiments. The significance of any difference was determined by Student t tests and expressed as a probability value. For clinicopathological studies, the independent data (normal versus nontumor region) and the matched data (nontumor versus cancer) of gene expression were analyzed with the Wilcoxon signed rank test and Mann-Whitney test, respectively. Mean differences were considered significant at P < 0.05.

**Results**

**HBx Induces Expression and Transcriptional Activity of LXR.** The observation that hepatic steatosis frequently occurs in HBx-transgenic mice attributes an important role to HBx in hepatic lipogenesis. We therefore hypothesized that HBx may trigger LXR-mediated lipogenic signaling because LXRα and LXRβ are the major regulators of hepatic lipogenesis via the modulation of the expression of lipogenic genes, such as SREBP-1c, FAS, SCD-1, and ChREBP.18-20,29 To address this hypothesis, we first examined whether expression of LXRα and LXRβ was altered by HBx in the Chang X-34 cell line, in which the expression of HBx gene is under the control of an inducible doxycycline promoter.22,23 HBx expression, induced by doxycycline treatment, led to a significant up-regulation of the expression of LXRα, LXRβ, and their downstream target genes at both the protein and messenger RNA levels (Fig. 1A,B). In the case of SREBP-1c, both the precursor form (128 kDa) and the active form (68 kDa) of proteins were increased (Fig. 1A and Supporting Fig. 1). Similar results were obtained when HBx was exogenously introduced into Chang cells by transient transfection of the vector encoding HBx cDNA (Fig. 1A,B). To examine whether the HBx-induced LXRα was transcriptionally active, we carried out reporter gene analysis in Chang X-34 cells using a reporter gene containing LXRE sequences.25 Doxycycline induced the reporter activity in a dose-dependent manner, which was further enhanced by the addition of an LXR ligand, TO901317 (Fig. 1C). A similar observation was reported in a recent article30 that was published during the processing of this article.

**LXR Mediates HBx-Induced Lipogenesis in Liver Cells.** Next, we carried out an RNA interference experiment to examine whether the induction of LXR plays a role in HBx-induced lipogenic gene expression and lipogenesis. Transfection of either si-LXRα or si-LXRβ largely decreased the levels of SREBP-1c and FAS pro-
teins (Fig. 2A). When the expression of LXRα was decreased by AS-LXRα, HBx-induced PPAR expression was dramatically decreased (Fig. 2B). Consistently, the reporter gene containing LXRE or SRE that was activated by doxycycline and TO901317 was largely repressed by si-LXRα (Fig. 2C). Importantly, transfection of si-LXRα and si-LXRβ effectively blocked the lipid droplet accumulation induced by doxycycline treatment in Chang X-34 cells when they were examined by microscopy or flow cytometry (Fig. 2D,E).

**HBx Interacts with LXRα and Increases the Transactivation Function of LXRα.** To characterize cross talk between LXRα and HBx, we examined whether these proteins were physically associated. First, immunofluorescence studies were carried out with SNU-354 cells in which the HBV genome was integrated. HBx was expressed in both the cytoplasm and the nucleus, whereas LXRα was mainly expressed in the nucleus. The expression of HBx in the nucleus was overlayed with that of LXRα (Fig. 3A). Results from the subcellular fractionation further support this observation. When HBx was exogenously introduced into HepG2 cells, HBx was mainly expressed in the nucleus, and it induced the nuclear expression of LXRα (Fig. 3B). Second, LXRα and HBx reciprocally coimmunoprecipitated, and this indicated that these factors physically interact (Fig. 3C). Next, we examined whether HBx enhances the transactivation function of LXRα, using a Gal4-driven luciferase reporter system. TO901317 and doxycycline enhanced the Gal4-LXRα-induced reporter activity by about 8-fold and 2-fold, respectively. Doxycycline together with TO901317 synergistically activated the reporter. Similar results were obtained when HBx was transiently expressed in Chang cells (Fig. 4A). Consistent with this result, CHIP assay showed that LXRα and the coactivator, CREB binding protein, were able to bind to the SREBP-1c promoter in the presence of HBx (Fig. 4B). Together, these results suggest that HBx interacts with LXRα and that this interaction may enhance the transactivation function of LXRα by recruiting CREB binding protein onto the promoter of the LXR target genes.

**Increases in the Expression Level of LXRα in Liver Samples of HBx-Expressing Transgenic Mice and HCC Patients.** We next examined the expression of LXRα in liver samples obtained from HBx-transgenic mice. Consistently with previous observations, lipid droplet accumulation increased in the liver sections from the HBx-transgenic mice (Fig. 5A). Expression of LXRα and LXRβ was strongly elevated in the HBx-transgenic livers in comparison with wild-type tissues. Similarly, the expression of SREBP-1c, FAS, SCD-1, and ChREBP was dramatically increased in the HBx-trans-
genic liver, and this indicated a strong correlation between the activation of LXR and HBx expression (Fig. 5B).

Finally, the expression levels of LXR, SREBP-1c, FAS, and SCD-1 in 30 HBV-associated HCC specimens was assessed with qRT-PCR after microdissection of the HCC tissues and the adjacent nontumorous tissues (Supporting Table 3). The expression level of LXR was significantly increased \((P = 0.036)\) in the HCC cells in comparison with expression in the adjacent nontumorous region. However, the expression level of LXRα was not significantly different (Fig. 6A). Consistently, the expression levels of SREBP-1c, FAS, and SCD-1 were significantly increased in the HCC tissues (SREBP-1c, \(P = 0.008\); FAS and SCD-1, \(P = 0.001\)). Most strikingly, the induction of FAS and SCD-1 was greater than 2-fold in about 50% of the HCC samples. A strong immunoreactivity for FAS in cancerous lesions was observed in all five HCC samples, showing a strong messenger RNA induction of FAS by qRT-PCR (Supporting Table 3 and Fig. 6B). The results obtained from the HBx-transgenic mice and clinicopathological studies strongly support our hypothesis that HBx activates LXR and its downstream lipogenic genes and that this may contribute to the development of HBV-associated HCC.

Fig. 3. HBx interacts with LXRα in the nucleus. (A) SNU-354 cells were fixed, and expression of HBx and LXRα was visualized by immuno- cytochemistry with specific antibodies. DAPI was used to stain the nuclei. (B) Cytoplasmic and nuclear fractions were obtained from SNU-354 cells or HepG2 cells that were transfected with pCMV-HA-HBx or empty vector. Expression of the indicated proteins was analyzed by western blotting. (C) Whole cell lysates were prepared from SNU-354 cells or HepG2 cells that were transfected with pCMV-HA-HBx. IP was performed with the indicated antibodies, and the resulting precipitates were analyzed by WB. Abbreviations: CMV, cytomegalovirus; DAPI, 4,6-diamidino-2-phenylindole; HA, hemagglutinin; HBx, hepatitis B virus X protein; IgG, immunoglobulin G; IP, immunoprecipitation; LXR, liver X receptor; WB, western blotting.

Fig. 4. HBx enhances the transactivation function of LXRα. (A) Chang X-34 cells were transfected with Gal4-Luc and pGal4-LXRα. Chang cells were transfected with Gal4-Luc and pGal4-LXRα together with EV or pCMV-Myc-HBx. Transfected cells were treated with 2 \(\mu\)g/mL Doxy and/or 1 \(\mu\)M T17 for 24 hours. \(* P < 0.05, ** P < 0.001\) (n = 3). (B) Schematic presentation of the chromatin immunoprecipitation assay (top). HepG2 cells were transfected with EV or pCMV-Myc-HBx. DNA fragments that were immunoprecipitated with the indicated antibodies were amplified by polymerase chain reaction (bottom). Abbreviations: CBP, CREB binding protein; CMV, cytomegalovirus; Doxy, doxycycline; EV, empty vector; Gal, galactosidase; HBx, hepatitis B virus X protein; IgG, immunoglobulin G; IP, immunoprecipitation; Luc, luciferase; LXR, liver X receptor; LXRE, liver X receptor response element; SREBP, sterol regulatory element binding protein; T17, TO901317.
Discussion

In general, human cancers show high levels of lipogenesis because lipids are essential components of cellular growth and survival. Many tumor tissues, including colon, breast, and lung tissues, undergo significant fatty acid synthesis; therefore, these tumor tissues contain high levels of lipids in comparison with normal tissues.\textsuperscript{31,32} Up-regulation of lipogenic enzymes such as FAS, the key metabolic multienzyme responsible for the terminal catalytic step in fatty acid synthesis, represents a phenotypic alteration in many human malignancies, including breast cancer.\textsuperscript{31,32} Interestingly, gene expression profiles obtained by cDNA microarray analysis of HBV-infected mouse liver revealed that the expression of genes that are involved in the biosynthesis and metabolism of fatty acids and steroids together with genes associated with cell cycle progression is up-regulated after HBV infection.\textsuperscript{11} Lipogenic enzymes such as FAS and acetyl–coenzyme A carboxylase 1 are markedly induced in HCC.\textsuperscript{33} Here, we demonstrated that the transcriptional function of LXR was strongly enhanced by HBx in a cell culture and in an HBx-transgenic mouse model and that the expression of LXR\textsubscript{α} and its downstream lipogenic genes, such as SREBP-1c, FAS, and SCD-1, was significantly up-regulated in liver specimens of HBV-associated HCC. These results suggest that the HBx-induced lipogenic pathways, including the activation of the LXR–SREBP-1c–FAS axis of the lipogenic program, may play an important role in the progression of hepatocarcinogenesis and may provide diagnostic advantages as well as therapeutic targets for HBV-associated HCC.

Recently, Kim et al.\textsuperscript{12} reported that HBx induces lipogenesis through activation of SREBP-1 and PPAR\textsubscript{γ}. In the current study, we also observed that expression of SREBP-1c and all subtypes of PPARs was enhanced in the presence of HBx (Fig. 1 and Supporting Fig. 2). Importantly, we demonstrated that knockdown of LXR\textsubscript{α} and/or LXR\textsubscript{β} dramatically inhibited the expression and transcriptional activity of SREBP-1 and PPARs. Consistently, the HBx-induced lipogenesis was largely reduced when expression of LXR\textsubscript{α} and LXR\textsubscript{β} was repressed by RNA interference (Fig. 2). These results suggest that regulation of SREBP-1c and PPARs by HBx may be indirect and mediated by LXR, and this indicates that LXR may be the main regulator of HBx-induced hepatic lipogenesis. The fact that the RXR, a heterodimer partner of LXR, directly interacts with HBx\textsuperscript{34} may further provide a tool to effectively enhance the transcriptional activity of LXR\textsubscript{α}–RXR\textsubscript{α} heterodimers on promoters of the LXR target genes.

Chronic infection with HBV has been implicated in metabolic syndromes; HBx has been shown to transactivate phosphoenolpyruvate carboxykinase, which is associated with gluconeogenesis and insulin resistance, and an increased prevalence of carotid atherosclerosis was found in HBV carriers in a large cohort study.\textsuperscript{33,35} Recently,
LXR was implicated in insulin resistance and gluconeogenesis. Therefore, the potential role of the LXR pathway in HBV-associated metabolic syndromes warrants further investigation. Importantly, the transcriptional activity of LXR can be antagonized by small molecules, such as the polyunsaturated fatty acids docosahexanoic acid and eicosapentanoic acid. Interestingly, a rapid decline in endogenous fatty acid synthesis following FAS inhibition using chemical inhibitors significantly decreases cell proliferation in several types of tumor cells. Therefore, such compounds may be useful for targeting LXRα and/or FAS to repress HBV-associated hepatic steatosis as well as the related symptoms resulting from HBV infection.

Similar to HBx, HCV core protein plays an important role in hepatic steatosis during the pathogenesis of HCV infection. It enhances the de novo biosynthesis of fatty acids and triacylglycerol in vitro and induces hepatic ste-
Atosis in core protein–carrying transgenic mice. Recently, potential mechanisms for HCV core protein–induced hepatic steatosis have been elucidated at the molecular level. HCV core protein indirectly enhances SREBP-1c promoter activity by increasing the binding of LXRα/RXRα to the LXRE, which is markedly activated by treatment with the ligands for LXRe and RXRs. PPARα activation is essential for HCV core protein–induced hepatic steatosis and HCC in mice. In particular, the genotype-3α core up-regulates the FAS promoter, which could contribute to a higher prevalence and severity of steatosis. These observations together with our results suggest that HBV and HCV use similar molecular tools for the modulation of lipogenesis. This implies that the LXR-mediated lipogenic pathway may be critical for the pathogenesis of the hepatotropic and oncogenic viruses.

In summary, in hepatocytes, HBx increases lipogenesis mediated predominantly by LXR and its lipogenic downstream target genes, such as FAS. Therefore, our study provides crucial information to further the understanding of the mechanisms that underlie HBV-mediated fatty liver, cirrhosis, and the development of HCC. LXR may thus represent a new potential therapeutic target for the prevention of hepatic steatosis and further progression to HCC after chronic HBV infection.

References
16. Lu TT, Repa JJ, Mangelsdorf DJ. Orphan nuclear receptors as eLXReRs and FIeXeRs of sterol metabolism. J Biol Chem 2001;276:37735-37738.