The PreS2 activator MHBs^t of hepatitis B virus activates c-raf-1/Erk2 signaling in transgenic mice

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The large hepatitis B virus (HBV) surface protein (LHBs) and C-terminally truncated middle size surface proteins (MHBs^t) form the family of the PreS2 activator proteins of HBV. Their transcriptional activator function is based on the cytoplasmic orientation of the PreS2 domain. MHBs^t activators are paradigmatic for this class of activators. Here we report that MHBs^t is protein kinase C (PKC)-dependently phosphorylated at Ser28. The integrity of the phosphorylation site is essential for the activator function. MHBs^t triggers PKC-dependent activation of c-Raf-1/Erk2 signaling that is a prerequisite for MHBs^t-dependent activation of AP-1 and NF- κ B. To analyze the pathophysiological relevance of these data *in vivo*, transgenic mice were established that produce the PreS2 activator MHBs^t specifically in the liver. In these mice, a permanent PreS2-dependent specific activation of c-Raf-1/Erk2 signaling was observed, resulting in an increased hepatocyte proliferation rate. In transgenics older than 15 months, an increased incidence of liver tumors occurs. These data suggest that PreS2 activators LHBs and MHBs^t exert a tumor promoter-like function by activation of key enzymes of proliferation control.

Keywords: hepatitis B virus/hepatocellular carcinoma/signal transduction/surface proteins/transgenics

Introduction

In addition to causing acute and chronic hepatitis, hepatitis B virus (HBV) is considered to be a major etiological factor in the development of human hepatocellular carcinoma (HCC). Almost all HBV-associated HCCs studied so far harbor chromosomally integrated HBV DNA (Beasley *et al.*, 1981). HBV DNA also does not integrate at specific sites of the host genome. Thus a common *cis*-effect onto flanking cellular genes can be excluded as a general mechanism of HBV-associated carcinogenesis.

However, integrated HBV DNA can encode two types of transcriptional activators: the already well studied HBx (for a recent review see Murakami, 1999) and the PreS2 activators LHBs (large hepatitis B virus surface protein) and MHBs^t (C-terminally truncated middle size surface proteins) (Kekulé *et al.*, 1990; Hildt *et al.*, 1996a).

The sequence encoding the PreS2 activators is localized in the HBV surface gene. The surface gene consists of a single open reading frame divided into three coding regions: preS1, preS2 and S, each starting with an in-frame ATG codon. Through alternate translational initiation at each of the three AUG codons, a large (LHBs; PreS1 + PreS2 + S), a middle size (MHBs; PreS2 + S) and a small (SHBs; S) envelope glycoprotein can be synthesized. The activator function of the surface protein requires the cytoplasmic orientation of the PreS2 domain (the minimal functional unit) that occurs in the case of MHBs^t (Hildt *et al.*, 1995) and in a fraction of LHBs (Bruss *et al.*, 1994). In contrast, full-length MHBs displays no transcriptional activator function: here the PreS2 domain directs into the lumen of the endoplasmic reticulum (ER). The generation of functional MHBs^t from MHBs requires deletion of the 3' end of the preS2/S gene encoding at least the last 70 amino acids corresponding to the hydrophobic region III of the S domain during the integration process (Lauer *et al.*, 1992). Deletions of 3' sequences of the preS2/S gene generating functional MHBs^t activators were found in one-third of the integrates investigated so far (Schlüter *et al.*, 1994; Zhong *et al.*, 1999). This fact and the presence of complete preS/S genes in some other systematically studied integrates indicate the biological significance of the PreS2 activators.

Previous reports have described that transgenic mice that specifically overproduce LHBs in the liver develop liver tumors (Chisari *et al.*, 1989). Up to now, intracellular retention of LHBs, resulting in a situation analogous to storage disease, and subsequent permanent inflammatory processes were considered to cause the tumors in these transgenics. The physiological situation of these mice is closely related to that of HBV carriers, who strongly overproduce HBsAg. In the livers of these patients, development of ground glass hepatocytes can be observed. In light of the recent observations that LHBs functions as a transcriptional activator (Hildt *et al.*, 1996a), the question arose of whether this activator function could confer LHBs-dependent development of HCC.

MHBs^t proteins are paradigmatic for the family of PreS2 activators. However, in contrast to LHBs, the MHBs^t activators are produced only in very small amounts even if the expression is driven by strong promoters. This permits an analysis of signal transduction pathways in the absence of effects based on overexpression and intracellular accumulation of LHBs. In this study, we used, in particular, MHBs^{t76} (truncated at amino acid 76) corresponding to the MHBs^t activator encoded by the integrate of the human hepatoma cell line huH4 (Kekulé *et al.*, 1990). MHBs^{t76} belongs to the ER-localized subtype of MHBs^t activators (Hildt *et al.*, 1993). To exclude unequivocally any effects such as ER-overload based on the ER membrane association of MHBs^{t76}, MHBs^{t63} that belongs to the non-membrane associated cytosolic-

Fig. 1. MHBs^t is a phosphoprotein. (A) Highly purified His₆-MHBs^{t76} was derived from Sf9 cells. A 40 µg aliquot was subjected to isoelectric focusing under denaturing conditions using immobililine gel strips covering a range of pH 6–10. The separation in the second dimension was performed using a 15% Laemmli gel. The gel was silver stained. (B) Western blot analysis of highly purified His₆-MHBs^{t76} derived from Sf9 cells after two-dimensional separation as described in (A) using a PreS2-specific antiserum (F124). (C) Western blot analysis of highly purified His₆-MHBs^{t76} derived from Sf9 cells using a PreS2-specific antiserum (F124). The sample were subjected to acid phosphatase treatment prior to two-dimensional separation. (D) Autoradiograph of Ni-NTA-precipitated and SDS-PAGE-separated His₆-MHBs^{t76} (lane 2), His₆-MHBs^{t63} (lane 4), His₆-MHBs (lane 7), His₆-MHBs^{t76}mutI (lane 3), His₆-MHBs^{t76}mutII (lane 6), His₆-MHBs^{t63}mutI (lane 5) and His₆-MHBs^{t63}mutII (lane 8) derived from cellular lysates of [³²P]orthophosphate-labeled, transfected HepG2 cells. Cells transfected with the cloning vector served as negative control (lane 1). The autoradiograph shows that in contrast to full-length MHBs, the functional MHBs^t activators are phosphoproteins.

localized subtype was used as an appropriate control. Elsewhere, MHBs^{t63} and MHBs^{t76} have identical functions (Hildt *et al.*, 1995). The molecular basis of MHBs^t-dependent transcriptional activation, however, remained enigmatic.

Results

The PreS2 activator MHBs^t is a phosphoprotein

In a recent report, mass spectrometry of MHBs^{t76} provided preliminary evidence for the existence of a phosphorylated

fraction of MHBs^t proteins (Hildt *et al.*, 1996b). To analyze this in more detail, highly purified hexahistidine (His₆)-tagged MHBs^{t76} derived from Sf9 cells was subjected to two-dimensional gel electrophoresis. The two-dimensional gel shows that Sf9 cell-derived His₆-MHBs^{t76} consists of at least three differently charged populations (Figure 1A). The identity of these three spots with His₆-MHBs^{t76} was confirmed by western blot analysis (Figure 1B). To investigate whether the more negatively charged spots represent phosphorylated His₆-MHBs^{t76}, acid phosphatase treatment was carried out. Subsequent western blot analysis of the two-dimensional separation showed that the two more negatively charged spots disappeared, reflecting their phosphatase sensitivity (Figure 1C). To prove unequivocally that phosphorylation of MHBs^t takes place in a liver cell system *in vivo*, labeling of His₆-MHBs^t- or His₆-MHBs-producing HepG2 cells with [³²P]orthophosphate was performed. The autoradiograph of the affinity-precipitated proteins shows that both the ER-localized MHBs^{t76} (Figure 1D, lane 2) and the cytoplasmic MHBs^{t63} (Figure 1D, lane 4) are phosphoproteins (Figure 1D). In the case of full-length MHBs, no specifically phosphorylated forms were detectable (Figure 1D, lane 7). Further mutants analyzed in this experiment are described below. These results demonstrate that MHBs^t activator proteins, in contrast to MHBs, are phosphorylated.

The PreS2 activator MHBs^t is phosphorylated at Ser27/28

Western blot analysis using a phosphoserine- and a phosphotyrosine-specific antibody demonstrated the presence of phosphoserine in MHBs^t (data not shown), but provided no evidence for tyrosine phosphorylation.

To study phosphorylation of various MHBs^t proteins in more detail, an *in vitro* system was used. Purified *Escherichia coli*-derived proteins were incubated with isolated cytoplasm and [³²P]ATP. Analysis was performed by SDS-PAGE followed by autoradiography. His₆-MHBs^{t76} characterized by a mol. wt of ~6.5 kDa (Figure 2A, lanes 1 and 3) is phosphorylated efficiently under these conditions. In the case of the negative control (Figure 2A, lane 4), His₆-MHBs^{t76} was replaced by an unrelated His₆-tagged protein (His₆-grb2). To exclude any autophosphorylation activity of His₆-MHBs^{t76}, the cytoplasm was replaced by homogenization buffer. The autoradiograph indicates that His₆-MHBs^{t76} possesses no autophosphorylation activity (Figure 2A, lane 2) and excludes phosphorylation by contaminating bacterial kinases. A series of C- or N-terminal deletion mutants His₆-MHBs^{t63}; His₆-MHBs^{t52}, His₆-MHBs^{t11–76} (starts at amino acid 11 of the PreS2 region, truncated at amino acid 76) and His₆-MHBs^{t23–76}, and a structural mutant MHBs^{t52*} (the amphipathic α-helix between amino acids 41 and 52 was converted to a β-sheet conformation; Hildt *et al.*, 1995) were purified from a eubacterial expression system and used for mapping the phosphorylation site. The autoradiograph (Figure 2B) shows that neither deletion of the hydrophobic transmembrane region I (MHBs^{t63}, Figure 2B), further C-terminal deletion up to amino acid 52 (MHBs^{t52}, Figure 2B) nor mutation of the amphipathic α-helix (MHBs^{t52*}, Figure 2B) abolishes the phosphorylation of MHBs^t. Even N-terminal deletions in the case of

Fig. 2. MHBSt is phosphorylated between amino acids 27 and 31. (A) Left: autoradiograph of eubacteria-derived MHBSt⁷⁶, incubated with cytoplasm derived from CCL13 cells and [γ -³²P]ATP (lane 1); as negative control, MHBSt⁷⁶ was incubated in the absence of lysate, which was replaced by an equal volume of homogenization buffer (lane 2). The molecular weight marker is given on the right. Right: autoradiograph of eubacteria-derived MHBSt⁷⁶, incubated with cytoplasm derived from CCL13 cells and [γ -³²P]ATP (lane 3); as negative control, MHBSt⁷⁶ was replaced by His₆-grb2 (lane 4). (B) Autoradiograph of *in vitro* phosphorylated MHBSt fragments (MHBSt¹⁻⁶³, MHBSt¹¹⁻⁷⁶, MHBSt¹¹⁻⁵², MHBSt^{11-52*}; here the amphipathic α -helix between amino acids 41 and 52 was replaced by a β -sheet conformation) isolated from the eubacterial expression system. The experiments were performed in duplicate. In the case of MHBSt¹¹⁻⁷⁶, only one-fifth of the amount of MHBSt⁷⁶ employed in the other reactions was used. (C) Autoradiograph of *in vivo* phosphorylated and Ni-NTA-agarose-precipitated MHBSt-specific proteins: lane 1, MHBSt⁶³ mutIa; lane 2, MHBSt⁶³ mutIb; lane 3, MHBSt⁶³ mutIc; lane 4, MHBSt⁶³ mutId; lane 5, wild-type MHBSt⁶³; and lane 6, control transfected cells. (D) CAT assay after co-transfection of HepG2 cells with reporter plasmid p3xAP-1-CAT and expression plasmid pKSVMHBSt⁷⁶, encoding the wild-type, or plasmids pKSVMHBSt⁷⁶ mutI, pKSVMHBSt⁷⁶ mutII, pKSVMHBSt⁷⁶ mutID28, or cloning vector pKSVM10 as negative control. Fold inductions are mean values from three independent transfection experiments, calculated as the ratio of the induced values to the vector control. The standard deviation is shown in the diagram.

Fig. 3. Functionality of PKC is essential for phosphorylation of MHBSt. (A) Competitive inhibition of *in vitro* phosphorylation of MHBSt using increasing concentrations (2×10^{-5} M, lane 2, to 2×10^{-4} M, lane 5) of a PKC-specific peptide. In lanes 4 and 5, the lower band represents the phosphorylated peptide. Lane 1 represents the phosphorylation of MHBSt⁶³ in the absence of the peptide. (B) *In vivo* labeling using [³²P]orthophosphate of HepG2 cells producing His₆-MHBSt⁷⁶ (lanes 1 and 2) grown in the absence (lane 2) or presence of the PKC inhibitor Goe6976 (lane 1). His₆-MHBSt⁷⁶ was enriched by Ni-NTA precipitation.

MHBSt¹¹⁻⁷⁶ and MHBSt²³⁻⁷⁶ did not result in a loss of MHBSt phosphorylation (Figure 2B). From these data, it can be concluded that MHBSt is phosphorylated between amino acids 23 and 41. The corresponding peptide harbors two S/T clusters, one between amino acids 27 and 31 (PreS2aa 23–34: PAGGSSSGTVNP) and the other between amino acids 37 and 40 (PreS2aa 34–41: PVLTTASP). To identify the phosphorylation site more precisely, two mutants were generated. In the case of mutant I, Ser27, Ser28, Ser29 and Tyr31 were replaced by alanine (MHBSt⁷⁶ mutI; MHBSt⁶³ mutI); in the case of mutant II, Tyr37, Tyr38 and Ser40 were replaced by alanine (MHBSt⁷⁶ mutII; MHBSt⁶³ mutII).

In vivo labeling experiments with [³²P]orthophosphate of HepG2 cells producing MHBSt mutI/II demonstrated that mutation of the S/T cluster at amino acids 27–31 abolished the phosphorylation of MHBSt⁷⁶ mutI (Figure 1D, lane 3) or MHBSt⁶³ mutI (Figure 1D, lane 5), whereas mutation of the S/T cluster at amino acids 37–40 had no influence on the phosphorylation of MHBSt⁷⁶ mutII (Figure 1D, lane 6) or MHBSt⁶³ mutII (Figure 1D, lane 8). Western blot analysis revealed that MHBSt⁶³ mutI and

Fig. 4. PKC is activated in MHBs^t-producing cells: inhibition abolishes MHBs^t-dependent activation of AP-1 and NF-κB. (A and B) Immunofluorescence of HepG2 cells stably transfected with the expression vector pCMVMHBs^{t76} (A). Untransfected cells served as the negative control (B). For detection, a mixture of PKCα- and PKCβ-specific antisera was used. Micrographs were taken at a 400× magnification. (C) CCL13 cells were transfected with the MHBs^{t76} expression plasmid pKSMHBs^{t76} or with pKSMHBs^{t76}mutI. Transfection with pKSV10 served as the control. The activation was determined by the ratio of membrane-bound to cytosolic PKC activity. In the case of control-transfected cells, this ratio was set arbitrarily as 1. The given factors are mean values of four independent experiments. The standard deviation is shown in the diagram. (D) Transient transfection of HepG2 cells with MHBs^{t76} expression plasmid pCMVMHBs^{t76} and the reporter constructs p3xAP-1-CAT or p2xNF-κB-CAT. Transfection with pCDNA.3 served as the negative control. Classic PKC isoforms were inhibited by the presence of 10 nM Goe6976. Cells were harvested 40 h after transfection. CAT activity was determined as described in Materials and methods. Fold inductions are mean values from four independent transfection experiments, calculated as the ratio of the induced values to the vector control. The standard deviation is shown in the diagram.

MHBs^{t76}mutII were produced in similar amounts as the respective wild-type proteins (data not shown).

To identify the exact phosphorylation site, a series of mutants were generated in which the following residues were replaced by alanine: Ser27 in MHBs^{t63}mutIa, Ser28 in MHBs^{t63}mutIb, Ser29 in MHBs^{t63}mutIc, and Tyr31 in MHBs^{t63}mutId. *In vivo* phosphorylation experiments using these mutants (Figure 2C) revealed that mutation of Ser28 to alanine abolishes the phosphorylation, indicating that the phosphorylation of MHBs^t takes place at Ser28.

Destruction of the phosphorylation site abolishes activator function of MHBs^t

The PreS2 activators are known to activate transcription factor AP-1 (Hildt *et al.*, 1995). To investigate whether the integrity of the identified phosphorylation site is a prerequisite for the functionality of the MHBs^t activators, reporter gene assays were performed. HepG2 cells were co-transfected with the expression vectors pKSMHBs^{t76} (positive control), pKSMHBs^{t76}mutI or pKSMHBs^{t76}mutII and with the reporter construct p3xAP-1-CAT (Lauer *et al.*, 1992). The vector pKSV10 served as negative control. Destruction of the phosphorylation site in the case of MHBs^{t76}mutI abolishes the activator function of MHBs^{t76} (Figure 2D) as compared with cells transfected with pKSMHBs^{t76} (positive

control) or pKSMHBs^{t76}mutII; here, an ~10-fold activation of the reporter gene was observed (Figure 2D). Co-transfection of pKSMHBs^{t76}mutID28 (Ser28 was replaced by aspartate to mimic a constitutive phosphorylation) failed to induce activation of the reporter gene.

These data demonstrate that the integrity of the identified phosphorylation site is a prerequisite for the functionality of the MHBs^t activators.

PKC phosphorylates MHBs^t

The identified phosphorylation site showed a weak homology to protein kinase C (PKC)-dependent phosphorylated sequences. To investigate a possible implication of PKC isoforms in phosphorylation of MHBs^t proteins, *in vitro* phosphorylations were performed in the presence of a PKC-specific substrate peptide. The autoradiograph (Figure 3A) shows that increasing concentrations of the peptide compete phosphorylation of His₆-MHBs^{t63}. This indicates that MHBs^t is PKC-dependently phosphorylated.

To analyze the relevance of classic PKC isoforms for phosphorylation of MHBs^t, their activity was blocked using the cPKC-specific inhibitor Goe6976. *In vivo* phosphorylation experiments under these conditions revealed that phosphorylation of MHBs^t depends on the functionality of cPKC isoforms (Figure 3B).

Fig. 5. MHBs^t induces activation of c-Raf-1 and Erk2. (A and B) The activity of c-Raf-1 and Erk2 was determined by immunocomplex assays using recombinant MEK or basic myeloglobulin (MBP), respectively, as substrates. HepG2 cells were transfected with pCMVMHBs^{t76} or with the phosphorylation-deficient mutant pCMVMHBs^{t76}mutI. TPA stimulation was used as a positive control. The activity of c-Raf-1 or Erk2 in control-transfected cells was set arbitrarily as 1. The given factors are mean values of three independent experiments. The standard deviation is shown in the diagram.

PKC is activated in MHBs^t-producing cells

Next, we analyzed whether MHBs^{t76} affects the activity of PKC. Activation of the classic isoforms of PKC such as the liver-specific PKC α/β isoforms is associated with a translocation of PKC from the cytoplasm to the cell membrane. Immunofluorescence microscopy of a clonal mixture of HepG2 cells stably transfected with the expression plasmid pCMVMHBs^{t76} revealed in these cells a translocation of PKC α/β from the cytoplasm to the cell membrane. In the case of control-transfected HepG2 cells, PKC α/β was found in the cytoplasm (Figure 4A and B).

For a quantification of PKC activation, the ratio of membrane-bound to cytosolic PKC activity was determined in MHBs^{t76}- or MHBs^{t76}mutI-producing CCL13 cells. pKSV10-transfected cells served as negative control. An ~5-fold activation of PKC was measured in the case of MHBs^{t76}-producing cells as compared with the negative control (Figure 4C). The phosphorylation-deficient mutant MHBs^{t76}mutI did not activate PKC.

MHBs^t proteins induce activation of the transcription factors AP-1 and NF- κ B (Lauer *et al.*, 1992; Meyer *et al.*, 1992). To study the relevance of PKC activation for MHBs^{t76}-dependent induction of AP-1 and NF- κ B, the PKC inhibitor Goe6976 was employed. A complete loss of the MHBs^{t76}-dependent activation of the AP-1- or NF- κ B-dependent reporter gene was observed (Figure 4D). These data indicate that (i) MHBs^t triggers activation of PKC and (ii) the functionality of PKC is essential for MHBs^t-dependent activation of AP-1 and NF- κ B.

MHBs^t induces PKC-dependent activation of c-Raf-1 and Erk2

The activation of PKC can be mediated by the c-Raf-1/Erk2 signal transduction pathway (Carroll and May, 1994; Marais *et al.*, 1998). In the case of MHBs^{t76}-producing cells, an ~4-fold increase of c-Raf-1 activity was found (Figure 5A) as compared with the c-Raf-1 activity in control-transfected cells. As a positive control, 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) stimulation resulted in an ~9-fold activation.

Fig. 6. Functionality of c-Raf-1 kinase is a prerequisite for MHBs^t-dependent activation of AP-1 and NF- κ B. HepG2 cells were transiently transfected with the MHBs^{t76} expression plasmid pCMVMHBs^{t76}, the reporter constructs p3xAP-1-CAT or p2xNF- κ B-CAT and a transdominant-negative mutant of Ras (RasN17) or c-Raf-1 (HCR13.1) or with an equal amount of the vector pMNC to equalize the total amount of DNA. Transfection with the cloning vector pCDNA.3 served as negative control. At 36 h after transfection, CAT activity was determined. Fold inductions are mean values from three independent transfection experiments, calculated as the ratio of the induced values to the vector control. The standard deviation is shown in the diagram.

In addition, the phosphorylation-deficient mutant MHBs^{t76}mutI, which fails to activate PKC, also caused no activation of c-Raf-1. Activation of neither B-Raf nor A-Raf could be observed in MHBs^t-producing cells (data not shown). To confirm that the observed MHBs^t-dependent activation of c-Raf-1 is triggered through PKC, Goe6976 was used to inhibit cPKC. Here, determination of c-Raf-1 activity revealed a complete loss of MHBs^t-dependent activation of c-Raf-1, whereas the epidermal growth factor (EGF)-dependent activation of c-Raf-1 was not significantly affected by inhibition of PKC (data not shown).

In the case of MHBs^{t76}-producing cells, Erk2 activity was increased ~2.5-fold as compared with the lysate of control-transfected cells. Again, the phosphorylation-deficient mutant MHBs^{t76}mutI causes no activation of Erk2. TPA-stimulated cells served as a positive control. Here, an ~6-fold activation was determined (Figure 5B).

In a recent report (Marais *et al.*, 1998), it was revealed that in contrast to the receptor tyrosine kinase-mediated activation, the PKC-dependent activation of c-Raf-1 is not inhibited by a transdominant-negative mutant of Ras (pRasN17). Reporter gene assays revealed that MHBs^{t76}-dependent activation of AP-1 or NF- κ B is not affected by the co-expression of RasN17 (Figure 6). Moreover, expression of transdominant-negative Ras failed to influence the MHBs^{t76}-dependent activation of c-Raf-1 or Erk2 (data not shown). The functionality of pRasN17 was demonstrated by the inhibition of HBx-dependent activation of AP-1 and NF- κ B (data not shown). These results show that expression of the MHBs^{t76} activator results in PKC-dependent activation of c-Raf-1 and Erk2.

MHBs^t fails to promote activation of AP-1 and NF- κ B in cells producing a transdominant-negative mutant of c-Raf-1

In the next set of experiments, we investigated whether activation of c-Raf-1 is a prerequisite for MHBs^{t76}-dependent activation of AP-1 and NF- κ B. For this

purpose, reporter gene assays were performed and endogenous c-Raf-1 was inhibited by co-expression of a transdominant-negative mutant (pHCR13.1; Köhlch *et al.*, 1991) in MHBs^{t76}-producing HepG2 cells. Transdominant-negative c-Raf-1 abolished MHBs^{t76}-dependent activation of AP-1 or NF- κ B (Figure 6). These results indicate that the integrity of c-Raf-1 is essential for triggering the activation of AP-1 and NF- κ B in MHBs^t-producing cells.

Liver-specific expression of the transgene encoding MHBs^{t76}

To study the pathophysiological relevance of the detected PKC-controlled signaling pathway, two lines of transgenic mice were established that specifically produce the PreS2 activator MHBs^{t76} in the liver. To direct the expression of the transgene to the liver, the transgene was placed under the control of the albumin promoter. Due to the expected very low expression level of the transgene, the β -globin

intron was inserted to increase the expression. To permit an efficient enrichment of MHBs^t from tissue lysates, MHBs^t was produced as a His₆-tagged fusion protein. For stabilization of the mRNA, a poly(A) site was introduced at the 3' end. A schematic representation of the transgene is given in Figure 7A.

The expression of the transgene was analyzed by immunofluorescence microscopy of liver sections derived from transgenic animals and the respective wild-type littermates stained with a PreS2-specific antiserum. An inhomogenous, specific staining of the cytoplasm can be observed, while the nucleus remains unstained, reflecting the ER localization (Hildt *et al.*, 1993) of MHBs^{t76} (Figure 7B).

To verify that (i) the observed staining is specific for authentic MHBs^{t76} and (ii) the expression is liver specific, lysates derived from the liver or kidney of transgenic animals were analyzed by western blotting using the PreS2-specific antibodies HBV-25-19 or F124 (Neurath *et al.*, 1987; Mimms *et al.*, 1990). Under these conditions, the MHBs^t protein was not detectable (data not shown). Therefore, the lysates were loaded on metal-chelating columns to enrich the His₆-tagged MHBs^t-specific proteins. The eluate was analyzed by western blotting using a PreS2-specific antiserum [monoclonal antibody (mAb) HBV25-19].

The western blot shows that MHBs^{t76} was only detectable in the lysate derived from the liver of the transgenic animals (Figure 7C/I), confirming the liver specificity of the transgene expression. The liver-derived MHBs^{t76} was also analyzed for phosphorylation using a phosphoserine-specific antiserum. The western blot (Figure 7C/II) confirms that MHBs^{t76} is a phosphoprotein.

Determination of JNK2 activity and of the hsp72 level revealed that production of MHBs^{t76}, in contrast to LHBs, does not result in unspecific activation of signal

Fig. 7. Liver-specific expression of the transgene encoding MHBs^{t76}. (A) Schematic structure of the transgene encoding MHBs^{t76}. To direct the expression to the liver, the albumin promoter that was taken from the plasmid pAlb-PSX (Chisari *et al.*, 1985) was used. To increase the expression level, the β -globin intron, derived from plasmid pSWbglob (Werner *et al.*, 1993), was inserted. The HBV-specific fragment nt3174–3221 was fused to the sequence coding for an N-terminal His₆ tag. The poly(A) site was taken from SV40, subcloned in plasmid pAK2 (Lauer *et al.*, 1992). The HBV-specific fragment nt3174–3221 codes for MHBs^{t76}. (B) Immunofluorescence microscopy of liver sections derived from 5-month-old female MHBs^{t76}-producing mice (line 2) (tg). Sections derived from sex-matched wild-type littermates served as control (wt). The PreS2-specific mAb F124 (Neurath *et al.*, 1987) was used for detection of MHBs^{t76}. The staining was performed using a Cy3-conjugated donkey-derived secondary antibody. The micrographs were taken at a 400 $\times$ magnification. (C) I: western blot analysis of the eluates from Ni-loaded Hitrap chelating columns. The columns were loaded with lysates derived from the liver or kidney of MHBs^{t76}-producing transgenic mice or their respective littermates (5-month-old, female mice, line I). Elution was performed by a gradient of increasing imidazole concentration. The PreS2-specific mAb HBV 25-19 (Mimms *et al.*, 1990) was used for detection of MHBs^{t76}. II: western blot analysis, using a phosphoserine-specific antiserum (Sigma), of the eluates from Ni-loaded Hitrap chelating columns. The columns were loaded with lysates derived from the liver of wild-type mice (lanes 1 and 3) or MHBs^{t76}-producing transgenic mice (5-month-old, female mice, line I, lane 2; line II, lane 4). The western blot shows that the mouse-derived MHBs^{t76} is a phosphoprotein.

transduction (see Supplementary data available at *The EMBO Journal* Online).

A comparable amount of MHBs^{t76} was observed in the transgenic lines I and II. Here, the expression level seems

to be sex independent (data not shown). In the case of lines I and II, no significant reduction of transgene expression was detectable over the investigated time period of 12 months.

Fig. 8. MHBs^{t76} triggers *in vivo* specific activation of the c-Raf-1/Erk2 signal transduction pathway resulting in an increased proliferation rate of the hepatocytes. **(A)** Immunocomplex assay of c-Raf-1 activity in liver-derived lysates of MHBs^{t76}-producing mice (lines 1 and 2), of LHBs-producing transgenic mice, or of the corresponding littermates as negative control. In the case of the lysates derived from LHBs- or MHBs^{t76}-producing mice, the autoradiograph shows a significant activation of c-Raf-1 as compared with the control. The activation factors (f.a.) are mean values of three independent experiments. Both f.a. and standard deviation (s.d.) are shown at the bottom of the figure. **(B)** Immunocomplex assay of Erk2 activity in liver-derived lysates of MHBs^{t76}-producing mice (lines 1 and 2), of LHBs-producing transgenic mice or of the corresponding littermates as negative control. In the case of the lysates derived from LHBs- or MHBs^{t76}-producing mice, the autoradiograph shows a significant activation of Erk2 as compared with the control. The f.a. are mean values of three independent experiments. Both f.a. and s.d. are shown at the bottom of the figure. **(C)** Western blot analysis of lysates derived from the liver of the two lines of MHBs^{t76}-producing transgenic mice (lanes 2 and 3) and of the corresponding littermates (lane 1) using a PCNA-specific antiserum. The western blot shows that in the case of the lysates derived from the MHBs^{t76}-producing transgenics, an elevated amount of PCNA as compared with the control was detectable. The induction factors (f.i.) are mean values of three independent experiments. Both f.i. and s.d. are shown at the bottom of the figure. **(D)** Immunofluorescence microscopy of liver cryosections derived from a male 6-month-old MHBs^{t76}-producing mouse (a and b). A sex- and age-matched wild-type littermate served as control. For detection of PCNA (a and c), a PCNA-specific monoclonal antibody (Santa Cruz) was used and was visualized using a Cy3-conjugated anti-mouse secondary antiserum (Dianova). The nuclei were visualized by DAPI staining (b and d).

Fig. 9. Increased incidence of liver tumors in transgenics producing the activator MHBs^{t76}. van Gieson-stained (A and C) and HE-stained (B and D) sections of paraffin-embedded liver tissue derived from 15-month-old MHBs^{t76}-producing mice. These sections encompass healthy tissue and tumor tissue. In (C), infiltration in surrounding liver tissue can be observed in the case of a MHBs^{t76} mouse with a primary liver tumor. These photographs were taken at 400× magnification. No significant histological change can be observed in the case of (D), which shows HE-stained liver tissue from a 4-month-old MHBs^{t76}-producing mouse at 100× magnification.

These results demonstrate that in the two investigated transgenic mice lines, detectable, comparable, liver-specific expression of the transgene occurred.

Specific activation of the c-Raf-1/Erk2 signal and increased proliferation rate in the liver of transgenic mice producing the PreS2 activator MHBs^{t76}

The results described above demonstrate that this system is suitable to perform an *in vivo* analysis of signal transduction pathways specifically activated by the PreS2 activator. The activity of c-Raf-1 and Erk2 was determined by immunocomplex assays in liver-derived lysates from MHBs^{t76}- or LHBs-producing mice or the respective controls. The autoradiographs (Figure 8A and B) show a strong phosphorylation of MEK or MBP, respectively, in the case of lysates derived from LHBs- or MHBs^{t76}-producing mice as compared with the control. These results confirm that the PreS2 activator is able to trigger a significant specific activation of the c-Raf-1/Erk2 signaling pathway *in vivo* that is not subjected to depletion. Activation of c-Raf-1/Erk2 signaling can result in increased cellular proliferation. The proliferating cell nuclear antigen (PCNA) is a well established marker of cellular proliferation. Western blotting of liver-derived lysates (Figure 8C) as well as immunofluorescence microscopy (Figure 8D) of cryosections derived from 6-month-old transgenics using a PCNA-specific antiserum revealed an increased amount of PCNA in the case of the transgenics as compared with their wild-type littermates. These results indicate that the Pres2 activator MHBs^{t76} indeed causes an increased cellular proliferation *in vivo*.

Fig. 10. Schematic representation of the interference of the PreS2-activators with intracellular signal transduction pathways.

Table I. Tumor development in wild-type and transgenic mice killed after 15 months

Genotype	<i>n</i>	Sex	Tumor development
Transgenic	23	male	16
Transgenic	7	female	11
Wild type	14	male	2
Wild type	11	female	1

Increased incidence of liver tumors in transgenic mice producing the PreS2 activator MHBs^{t76}

The results described above indicate that the PreS2 activator MHBs^{t76} is able to trigger the activation of a tumor promoter pathway *in vivo*. However, according to the literature, it is well established that the HBV genome does not harbor a classic oncogene. To analyze whether expression of the transgene coding for the PreS2 activator MHBs^{t76} confers a transforming potential, we performed histological analysis of liver tissue. The analysis was performed over a time period of 18 months. Transgenics younger than 1 year old did not develop tumors. However, in the case of transgenics killed at the age of between 15 and 18 months, a significant number of mice with liver tumors was found (Figure 9). From 23 investigated male mice, 16 mice (69%) displayed liver tumors, while in the case of 17 killed female mice, a liver tumor was observed in 11 animals (65%). There were 2–8 tumors per animal, with a size between 2 and 6 mm. Moreover, the histological analysis revealed the existence of hyperplastic noduli. In the case of the corresponding wild-type controls, two of 14 male mice developed a tumor (14%), as did one of 11 female mice (9%). Immunohistochemistry of six tumors derived from male mice and five from female mice revealed that MHBs^t was detectable in each of these tumors (data not shown). These results are summarized in

Table I. In contrast to the LHBs transgenic mice, there is no significant difference in the percentage of tumor development between male and female mice and the transgene is expressed in the tumor cells.

These results indicate that the continuous activation of the signaling cascade promotes the development of liver tumors in an age-dependent process.

Discussion

MHBs^t- or LHBs-encoding sequences are found in many integrates subcloned from HBV-associated HCCs (Schlüter *et al.*, 1994; Zhong *et al.*, 1999). To evaluate the putative relevance of PreS2 activator function for the process of HBV-associated HCC development, a detailed *in vitro* and *in vivo* analysis of PreS2 activator function is of major biological significance.

Based on the *in vitro* and *in vivo* experiments, we propose that in the first step PreS2 interferes with PKC, resulting in activation of PKC and phosphorylation of PreS2.

The PreS2-dependent activation of PKC requires the structural integrity of the phosphorylation site. Conversion of the essential serine residues 27, 28, 29 to alanine abolishes the activator function, as does mutation of Ser28 to aspartate, mimicking phosphoserine. From this, it can be concluded that the conversion from the unphosphorylated to the phosphorylated form is not essential for the activator function. However, the proper interaction of the PreS2 domain with PKC is crucial to trigger activation of PKC. Co-expression of RasN17 does not affect the PreS2-dependent activation of c-Raf-1. This is in accordance with the described model of PKC-dependent activation of c-Raf-1 (Marais *et al.*, 1998).

This signal is mediated subsequently to the nucleus by the c-Raf-1/Erk2 signal transduction pathway. Most importantly, based on results obtained from transgenic mice, it could be clearly concluded that the PreS2-dependent activation of the c-Raf-1/Erk2 cascade is not subjected to depletion resulting in its permanent activation. In accordance with this, an increased proliferation rate of hepatocytes was indeed observed in the MHBs^t-producing transgenics. However, this increased proliferation rate was not reflected in a significantly enlarged liver. In many features, the PreS2 activators seem to resemble HBx. Both HBx (Su and Schneider, 1997; Pollicino *et al.*, 1998) and the PreS2 activator MHBs^t (E. Hildt, unpublished results) can confer a proapoptotic potential to hepatocytes. This could compensate for the increased proliferation rate, resulting in an elevated turnover of hepatocytes. On the other hand, there are reports describing an antiapoptotic potential of HBx (Su *et al.*, 2001).

The observed activation of c-Raf-1/Erk2 signaling *in vitro* and *in vivo* indicates that the PreS2 activators are able to activate a classic tumor promoter pathway. During the process of aging, a variety of critical DNA mutations can occur (initiation). Due to the production of the PreS2 activator and the resulting permanent activation of proliferative pathways, these cells are positively selected (promotion). The cooperation of both processes can result in tumor formation. Moreover, accumulation of mutations in these mice can also be increased by MHBs^t-dependent inactivation of p53 by induction of mdm2

expression and sequestration of p53 to the ER (E. Hildt, unpublished results). In the case of the LHBs-producing transgenics, critical mutations are generated with higher incidence by permanent inflammation of liver tissue due to intracellular accumulation.

In conclusion, according to the classic model of carcinogenesis (initiation and promotion), the PreS2 activators MHBs^t and LHBs might act as a tumor promoter through activation of key enzymes of proliferation control, i.e. PKC isoforms α/β or c-Raf-1/Erk2 as described here.

Materials and methods

Construction of expression plasmids

For construction of eukaryotic expression vectors pCMV-MHBs^t76C-His₆, the truncated preS2/S gene (nt_{HBV} 3174–3221) was amplified from plasmid pTKTHBV2 (Will *et al.*, 1985) by PCR employing primers with restriction sites for *Bam*HI. For plasmid pCMV-MHBs^t63C-His₆, the truncated preS2/S gene (nt_{HBV}3174–3180) was amplified. In both cases, the coding sequence for the His₆ tag fused to the C-terminus of the respective MHBs^t protein was introduced by the reverse primer. The amplified fragments were digested with *Bam*HI and ligated into the *Bam*HI-restricted and dephosphorylated expression vector pCDNA.3 (Invitrogen). For construction of pCMVMHBsC-His₆, the complete preS2/S gene was amplified using primers with restriction sites for *Bgl*II. The His₆ tag was introduced by the reverse primer. Amplified fragments were digested with *Bgl*II and ligated into *Bam*HI-restricted and dephosphorylated expression vector pCDNA.3 (Invitrogen).

Construction of eubacterial expression vectors pQeMHBs^t76, pQeMHBs^t63, pQeMHBs^t52, pQeMHBs^t11–76, pQeMHBs^t23–76 and pQeMHBs^t1–52* was described in a recent report (Hildt *et al.*, 1995). For construction of the coding sequence for the MHBs^tmutI mutants, the sequence nt_{HBV}3174–3160 was amplified. The forward primer harbored at its 5' end a *Bam*HI restriction site, and the reverse primer a *Nar*I restriction site. By a second PCR, fragment nt_{HBV}60–221 (MHBs^t76) or nt_{HBV}60–176 (MHBs^t63) was amplified. The forward primer introduced mutated codons, resulting in a replacement of Ser27, Ser28, Ser29 and Tyr31 by alanine; at the 5' end, the forward primer contained a *Nar*I site, and the reverse primer a *Hind*III site. The *Nar*I site introduced an inversion from A–G in the wild-type protein to G–A in the mutant. The amplified fragments were digested with *Nar*I, ligated and inserted into the *Bam*HI–*Hind*III-restricted, dephosphorylated vector pQe8. The remaining mutants were constructed in an analogous manner. The fragments were inserted into *Bam*HI-restricted vector pCDNA.3 or into the *Bgl*II-restricted vector pKSV10 (Pharmacia).

Cells and cell cultures

CCL13 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For transfection, cells were seeded at a density of 0.8×10^6 cells per 60 mm plate. Stimulation of PKC was performed by addition of TPA (Sigma) to a final concentration of 100 ng/ml 30 min prior to lysis of the cells. Goe6976 was used at a final concentration of 10 nM to inhibit classic PKC isoforms.

Culture of Sf9 cells (ATCC, CRL-17-11) and infection with recombinant baculovirus encoding MHBs^t76 was performed as described by Hildt *et al.* (1995).

Cell fractionation

Cell fractionation for preparation of cytoplasmic and membrane fractions was performed by differential centrifugation as described earlier (Hildt *et al.*, 1993).

Reporter gene assays

The chloramphenicol acetyltransferase (CAT) gene was used as reporter gene. The AP-1-dependent reporter plasmid p3xAP-1 CAT and the reporter plasmid p2xNF- κ B-CAT (also designated p55A2) that harbors two binding sites for the transcription factor NF- κ B were described in Lauer *et al.* (1994).

In the case of double transfections, cells were transfected with 2.5 μ g of activator plasmid or 2.5 μ g of pCDNA.3 as negative control, and 1 μ g of p3xAP-1-CAT or 2.5 μ g p2xNF- κ B-CAT reporter plasmid. In the case of triple transfections, 2.5 μ g of the transdominant-negative mutants of

c-Raf-1 (HCR13.1; Köhlch *et al.*, 1991) or Ras (RasN17; kindly provided by W.Fantl, San Francisco, CA) were used. To exclude unspecific effects, the total amount of DNA in the controls was balanced by transfection with an equal amount of the cloning vector pMNC.

All these transfections were performed by lipofection, using DOTAP (Boehringer) according to the manufacturer's instructions. The medium was changed 18 h after transfection. Cells were harvested 38 ± 4 h after transfection.

Transfections followed by an analysis of the PKC activity were performed by the calcium phosphate precipitation method, described elsewhere (Graham and van der Eb, 1973). Under these conditions, 4 μ g of plasmid were used and, after 6 h, cells were exposed for 90 s to a glycerol shock and harvested 6–8 h later.

Transfection efficiencies were ~60% as determined by analysis of enhanced green fluorescent protein (eGFP) fluorescence after transfection of pEGFP (Clontech).

Transfection efficiencies were standardized with a luciferase reporter plasmid containing the luciferase gene under the control of the non-stimulatable minimal promoter of pTK/37luc.

Each experiment was performed with at least three different preparations of CsCl gradient-purified plasmid DNA.

CAT assays

Relative CAT activity was determined with 50–100 μ g of protein from total cellular lysates as described (Gorman *et al.*, 1982). Acetylated and unreacted [14 C]chloramphenicol was quantified with an isotope scanner (Berthold). Fold inductions are mean values from at least three independent transfection experiments.

Indirect immunofluorescence labeling

Staining was performed as described (Hildt *et al.*, 1995). For detection, a mixture of PKC α/β -specific antibodies (Santa Cruz Biotechnology) diluted in phosphate-buffered saline–Tris (PBST) containing 10% bovine serum albumin (BSA) was used. Bound antibody was visualized by incubation with Cy3-conjugated anti-rabbit antibody (Dianova). A DMR fluorescence microscope (Leica) was used. In order to exclude fixation artifacts, the integrity of the fixed cells was confirmed by phase contrast microscopy of the same specimens.

Sections and staining of the paraffin-embedded, formaldehyde-fixed tissue and cryosections of the liver were obtained and processed as described previously (Saher and Hildt, 1999).

In vivo phosphorylation

HepG2 cells were seeded at a density of 0.8×10^6 cells per 60-mm-diameter plate. The cells were transfected with 6 μ g of the His₆-MHBS⁴ expression plasmids. At 20 h after transfection, the cells were washed twice with phosphate-free DMEM (Sigma) and incubated for 2 h in this medium. Medium was removed and replaced by 1.2 ml of phosphate-free medium, which contained 1 mCi of [32 P]orthophosphate. Cells were incubated in this medium for 2 h then washed twice with phosphate-free medium. Lysis and immunoprecipitation were performed as described (Hildt *et al.*, 1995).

In vitro phosphorylation assays

Highly purified, renatured proteins (0.1 μ g) derived from a bacterial expression system were added to 25 μ l (20 μ g of total protein) of the cytosolic fraction of CCL13 cells. The reaction mixture contained in addition 10 μ l of 10 $\times$ kinase buffer (20 mM Tris–HCl pH 7.5, 20 mM β -mercaptoethanol, 0.1 mM EGTA, 0.1 mM EDTA, 2% glycerol, 110 mM NaCl), 10 μ l of 100 μ M ATP and 5 μ l of 2 mM MgCl₂ containing 10 μ Ci of [γ - 32 P]ATP. By addition of water, the reaction mixture was brought to a final volume of 100 μ l. This basic assay was varied by addition of several activators or inhibitors. Competitive inhibition of the PKC activity was performed by increasing concentrations of a PKC-specific substrate peptide (AAKIQASFRGHMARKK).

Immunocomplex assays

Cells were transfected by lipofection using 2.5 μ g of pCMVMHBS⁷⁶. After 12 h, cells were harvested and lysed in 20 mM Tris–HCl pH 7.5, 137 mM NaCl, 0.2 mM EDTA, 1 mM EGTA, 10 mM sodium- β -glycerolphosphate, 50 mM sodium fluoride, 1% Triton X-100, 1 mM sodium orthovanadate, 0.25 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 0.15 U/ml aprotinin and 2 μ g/ml leupeptin. Insoluble material was removed by centrifugation at 20 000 g at 4°C for 15 min. Protein concentrations were determined by a Bradford assay. A-Raf, B-Raf, c-Raf-1 or Erk2 were immunoprecipitated from lysates by the respective polyclonal serum (Santa Cruz Biotechnology). The

subsequent procedure was described recently (Hildt and Oess, 1999). Immunocomplex assays for determination of c-Raf-1 or Erk2 kinase activity in liver tissue were performed as described recently (Saher and Hildt, 1999).

Determination of PKC activity

Cells were fractionated 6 h after the glycerol shock in the cytoplasm and membrane fractions. PKC was enriched from both fractions using ion exchange chromatography on DEAE–cellulose columns as described by Maly *et al.* (1989). PKC activity was determined using a commercial assay system (PKC assay system; Amersham).

Protein analysis

Proteins were separated by SDS–PAGE (Laemmli, 1970; Schägger and von Jagow, 1987). Western blotting was performed as described by Hildt and Oess (1999). The different MHBS⁴ fragments were detected by the PreS2-specific mAbs HBV25-19 (Mimms *et al.*, 1990) or F124 (Neurath *et al.*, 1987). A mouse monoclonal antiserum (Santa Cruz) was used for detection of PCNA, and phosphoserine- or phosphotyrosine-specific mouse mAbs (Sigma) were used for analysis of protein phosphorylation.

Generation of transgenic mice

Animals were generated at the Laboratory Animal Research Unit of the University of Ulm and bred at the Technical University Munich under strict specific pathogen-free conditions. For production of preS2/S²²¹ transgenic lineages, plasmid pTG17 was linearized with *NorI* and *EcoRV*, and the HBV fragment was microinjected into F₂(CBA/Ca $\times$ C57BL/6J) embryos according to standard procedures. Transgenic lineages were established by backcrossing hemizygous transgenic individuals to the C57BL/6J (B6) inbred strain. Transgenic animals were distinguished from their non-transgenic littermates by PCR amplification of transgene sequences using PreS2-specific primers. In all experiments, the non-transgenic, sex- and age-matched littermates served as controls.

The LHBs-producing mice (the preS/S gene is expressed under the control of the albumin promoter) were generated by F.Chisari and co-workers and described in Chisari *et al.* (1985). The expression characteristics were determined with animals of the first backcross generation. For the tumor studies, we used animals of the second and third backcross.

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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