Mitotic Aberration Coupled With Centrosome Amplification Is Induced by Hepatitis B Virus X Oncoprotein *via* the Ras-Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase-Mitogen-Activated Protein Pathway

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Abstract

Multinucleated cells have been noted in pathophysiological states of the liver including infection with hepatitis B virus (HBV), the status of which is also closely associated with genomic instability in liver cancer. Here, we showed that hepatitis B virus X oncoprotein (HBx) expression in Chang cells results in a multinuclear phenotype and an abnormal number of centrosomes ($n \geq 3$). Regulation of centrosome duplication in HBx-expressing ChangX-34 cells was defective and uncoupled from the cell cycle. HBx induced amplification of centrosomes, multipolar spindle formation, and chromosomal missegregation during mitosis and subsequently increased the generation of multinucleated cells and micronuclei formation. Treatment with PD98059, a mitogen-activated protein/extracellular signal-regulated kinase (MEK) 1/2 inhibitor, significantly reduced the number of cells with hyperamplified centrosomes and decreased the multinucleated cells and micronuclei formation. Consistently, the phospho-ERK level during cell progression was substantially higher in ChangX-34 cells than that of Chang cells. In contrast, neither wortmannin, an inhibitor of phosphoinositide-3 kinase, nor SB203589, an inhibitor of p38 mitogen-activated protein kinase (MAPK), showed any effects. Introduction of Ras dominant-negative (D/N) and MEK2 D/N genes into ChangX-34 cells significantly alleviated centrosome amplification, whereas introduction of the PKC D/N and PKB D/N genes did not. Thus, our results demonstrate that the HBx induced centrosome hyperamplification and mitotic aberration by activation of the Ras-MEK-MAPK. Intervention of this signaling pathway could suppress the centrosome amplification as well as mitotic aberration. These findings may provide a possible mechanism by which HBx promotes phenotypic progression by predisposing chromosomal alteration in HBV-infected liver.

Introduction

Hepatitis B virus (HBV) infection is a major world health problem in that approximately 2 billion people have been infected at one point in their lives and over 350 million people are estimated to be chronic carriers of HBV (1, 2). Persistent HBV infection causes chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). During chronic infection, the HBV genome often became integrated into the host chromosome (3). The *HBx* gene along with the *HBs* gene most frequently remains integrated in HCC. The hepatitis B virus X oncoprotein (HBx), a small oncoprotein of M_r 16,500, is required for viral replication (4, 5) and has been implicated in HBV-mediated HCC development (for review, see Ref. 6). It has been shown that HBx can induce liver cancer in transgenic mice (7, 8) and sensitize against hepatocarcinogenic agents (9, 10). HBx is also shown to transform cultured cells, some of which acquire the ability to form tumors in nude mice (11, 12).

HBx does not bind DNA directly but activates various signaling cascades. HBx has been shown to activate the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway (13, 14), the phosphoinositide-3-kinase (PI3-K), the p38 MAPK pathway, and the stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) pathway, leading to different cellular fates such as transformation, differentiation, survival, and apoptosis (15, 16). Activation of the PI3-K and SAPK/JNK pathways by HBx exerts an anti-apoptotic

Received 11/19/03; revised 1/7/04; accepted 1/15/04.
Grant support: 2000 Research Grant from Department of Medical Sciences, the Graduate School, Ajou University.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Note: G. Chan is supported by the Alberta Cancer Board, Alberta Cancer Foundation New Investigator Award, Petro-Canada Young Investigator Award, and the Canadian Institute for Health Research.
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function against transforming growth factor β (TGF-β; Ref. 17) and Fas signaling (18). On the other hand, sustained activation of the Ras-Raf-MAPK pathway was found in cells undergoing HBx-mediated transformation (15) although the mechanism by which HBx transforms cells has not been discussed.

Genetic instability is frequently accompanied with the acquisition of transformation ability and malignant progression of tumors. It has been suggested that the integration of HBV genome at the stages of chronic viral hepatitis and cirrhosis facilitated the genetic alterations of the host genome, thereby in part increasing the risk of HCC development (19). Similarly, allelotype analysis on primary liver tumors defined two different groups according to chromosome stability status, in which the one with frequent allelic losses was strongly associated with HBV infection (20). Moreover, a recent report showed that the ectopic expression of HBx protein in hepatoma cells induced chromosomal aberration such as chromosome rearrangement and micronuclei formation (21), disrupting genome integrity. It is also of note that HBx expression in some cells tends to increase formation of multinucleated cells (22, 23), which can be generated by aberrant progression of mitosis. Formation of multinucleated cells was also found in human T-cell lymphotropic virus type I Tax-expressing cells (24) as well as in EBV EBNA3C-expressing cells (25) and has been suggested as a model for viral transformation interfering with the mitotic checkpoint (24). All these observations suggest that chromosomal aberration resulting from aberrant progression of mitosis might be an important step during multi-stage carcinogenesis.

Recently, centrosomes have attracted much attention because of their fundamental role in mitosis. The centrosome functions as a microtubule-organizing center (MTOC) and plays a pivotal role in proper alignment and segregation of chromosomes during mitosis. Centrosome abnormalities, such as excess number or variable shape and size, have been found in human cancer of multiple origins (for review, see Ref. 26). Multiple centrosomes organize multipolar mitotic spindles, which pull the chromosomes in multiple directions, consequently increasing the chance of aneuploidy. Meanwhile, abnormal shape and size of centrosomes also result in aberrant mitotic spindle formation. Interestingly, E6 and E7 oncoproteins from the high-risk type of human papillomavirus (HPV) actively induced numerical and structural centrosome abnormalities, increasing the propensity for chromosome missegregation (27, 28). Moreover, the adenovirus E1A oncoprotein and the SV40 small-T antigen were both found to interfere with centrosome duplication (29, 30). These findings suggest that the centrosome can be a common target of these viral oncoproteins.

In this study, we took an advantage of using Chang liver cell line and two sublines expressing HBx protein (31, 32), all of which were found to express albumin mRNA and demonstrated that ectopic expression of HBx protein in Chang cells stimulated centrosome duplication, which is uncoupled from the cell division cycle. In addition, HBx not only induced multipolar spindle formation and chromosomal missegregation during the mitotic phase, but also increased multinucleated cells as well as cells with micronuclei. We also revealed that the Ras-mitogen-activated protein/extracellular signal-regulated kinase (MEK)-MAPK pathway works as a downstream effector of HBx mediating centrosome amplification. We further demonstrated that the intervention of the Ras-MEK-MAPK signaling pathway blocked the HBx-dependent induction of centrosome amplification as well as the formation of multinucleated cells.

Results

Uncoupled Centrosome Duplication From the Cell Division Cycle in HBx-Expressing Cells

Various human malignancies accompany cytologic and genetic alterations. We have previously established two HBx-expressing sublines, ChangX-31 and ChangX-34, which were originated from the Chang cell lines [CCL-13 from American Type Culture Collection (ATCC), Manassas, VA] by stably transfecting the plasmids of pTetX and pUHD172-1 (31). The ChangX-31 and ChangX-34 cells expressed a significant amount of HBx protein (31, 32), and expression of HBx in these cells was accompanied with different morphological and phenotypic changes partly similar to those previously reported (33, 34). One of them is the increase of giant cells. When these giant cells were stained with antibody for centrosome-specific γ-tubulin, some of them contained more than three centrosomes (Fig. 1A) with multiple nuclei. Interestingly, we noticed that HBx expression in these giant cells was very strong and granulated (Fig. 2A), whereas HBx in most of ChangX-34 cells localized to cytoplasm as we previously reported (31). Centrosome duplication is known to be tightly linked to the cell division cycle. Centrosome duplication starts at the G_1-S transition and yields two mature centrosomes in the late G_2 phase. We, therefore, traced the number of centrosomes in different phases of the cell division cycle by staining cells with antibodies for γ-tubulin or pericentrin in two representative cell lines, Chang and ChangX-34. Cells were synchronized using the double thymidine block (DTB) method, and fluorescence-activated cell sorting (FACS) analysis showed that about 70–80% of Chang resided at the G_1 phase while 65% of ChangX-34 cells resided at the G_1 phase and about 25% of cells were at the S phase (Fig. 1B, *upper panel*). When cells were released from the DTB, 60% of Chang cells progressed into the G_2-M phase in 6 h (Fig. 1B, *lower panel*). On the other hand, the major portion (45%) of ChangX-34 cells still stayed at the S phase, showing a delayed mitotic entry. During the transition from the S phase to the G_2-M phase, the centrosome duplication in Chang cells appeared complete because about 90% of Chang cells contained two centrosomes (Fig. 1, A and C). When we treated cells with 50 ng/ml of nocodazole for 12 h to induce a prometaphase arrest, over 90% of Chang cells still retained two centrosomes (Fig. 2B). In HBx-expressing ChangX-34 cells, only 23% of cells in G_1-S phase contained one centrosome and the rest of them already possessed two to three centrosomes (Fig. 1C, *right panel*). Noticeably, the proportion of ChangX-34 cells with multiple (≥3) centrosomes increased to over 25% when these cells progressed into G_2 and mitotic phase (Fig. 1C), and the intensity of centrosome staining in these cells was often variant (Fig. 2C). The frequencies of supernumerary centrosomes in mitotic phase of ChangX-31 cells (Fig. 2D) were also similar

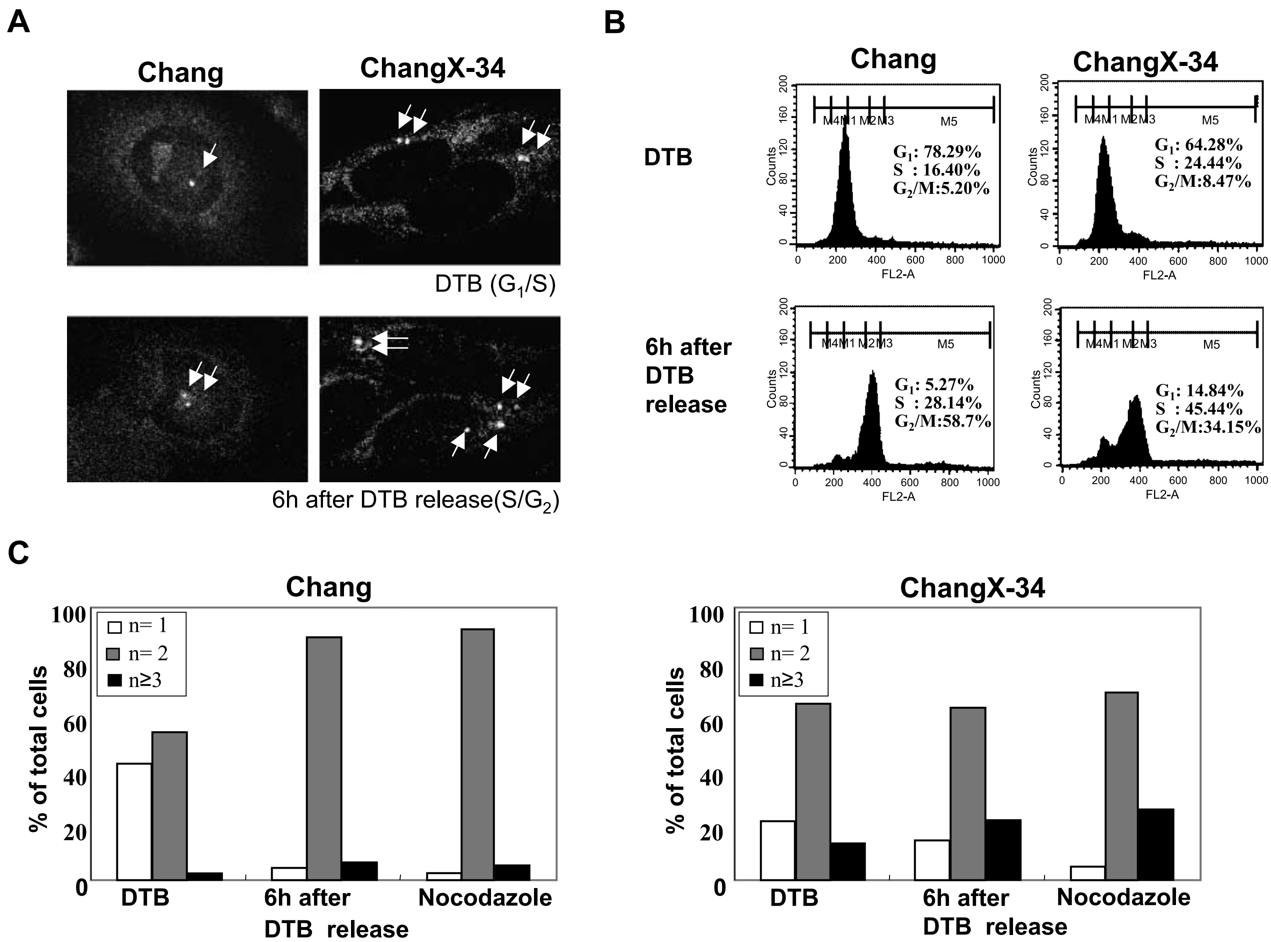

FIGURE 1. Centrosome duplication in HBx-expressing cells is uncoupled from the cell division cycle. The parental Chang cells and HBx-expressing ChangX-34 cells were cultured on cover glasses, synchronized by the DTB method. Cells were then released from the DTB and allowed to progress into G_2 phase. Or cells were treated with 100 ng/ml of nocodazole for 12 h (*Nocodazole*). **A.** The number of centrosomes at different phases of the cell cycle was visualized with rabbit anti-γ-tubulin antibody. *Arrows* indicate the centrosome. **B.** Cell cycle analysis by flow cytometry. **C.** The number of centrosomes at the different phases of the cell cycle was plotted after immunofluorescence staining against γ-tubulin and pericentrin. *Columns,* mean of two independent experiments.

to those in ChangX-34 cells. To count the number of centrosomes in a single mitotic cell, cells were stained with relatively high concentration of anti-γ-tubulin antibody, subsequently with cyc3-conjugated anti-mouse IgG antibody (Fig. 2, B–D), resulting in a strong centrosome staining with yellow spot and nonspecific red staining of the entire cells, which allowed to identify the boundary of a mitotic cell. Therefore, these results indicate that regulation of centrosome duplication in HBx-expressing cells is defective and uncoupled from the cell cycle.

Multipolar Mitotic Spindle Formation in HBx-Expressing Cells

Centrosomes play a crucial role in the assembly of bipolar spindles during the progression of mitosis. Because abnormal centrosome numbers were frequently observed in ChangX-31 and ChangX-34 cells, we investigated whether the abnormal mitotic spindles were also formed in these cells. Cells were synchronized and arrested in prometaphase by nocodazole treatment, and cells were subsequently removed from nocodazole and allowed to reform the mitotic spindles and progress into mitosis. At 60 min after nocodazole release, 70–80% of the cell population proceeded to the metaphase (data not shown). When the ChangX-34 cells at this stage were stained with an antibody to α-tubulin and 4′,6-diamidino-2-phenylindole (DAPI), tripolar (Fig. 2E) and tetrapolar mitotic spindles were easily found with multidirectional chromosomal staining (Fig. 2F). When the patterns of chromosome segregation with α-tubulin were overlaid, multipolar spindles radiating from the spindle poles as well as multidirectional segregation of chromosomes were found in the HBx-expressing ChangX-34 (Fig. 2G) and ChangX-31 cells (Fig. 2H). When these cells were further progressed into late mitotic phase, abnormal segregation of chromosomes or orphan chromosomes (Fig. 2J) were found in HBx-expressing cells with multiple mitotic spindles stained with anti-α-tubulin antibody (Fig. 2I). The data here suggest that multiple centrosomes nucleate the formation of multipolar spindles, resulting in the abnormal segregation of chromosomes.

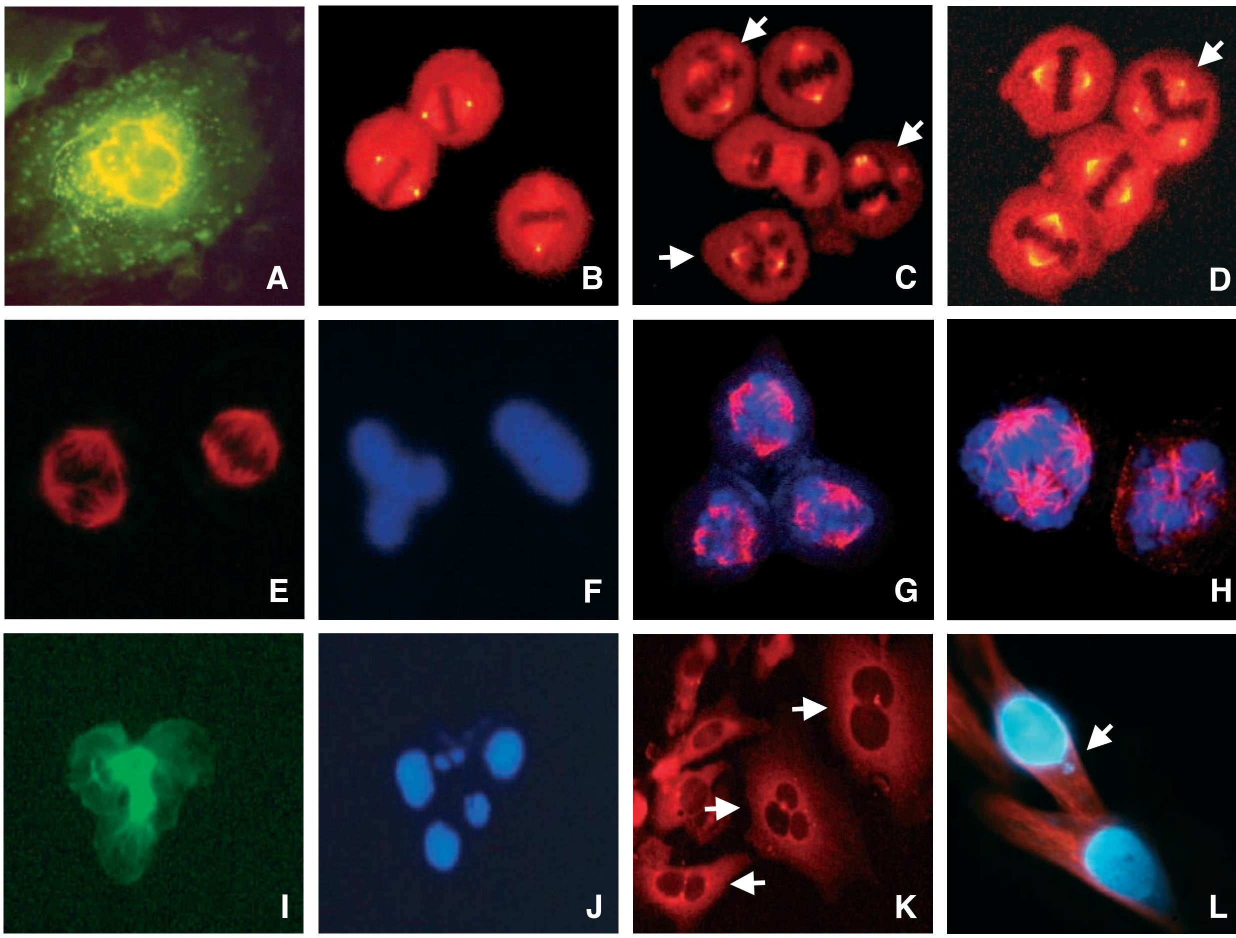

FIGURE 2. Aberrant mitotic spindle formation and chromosomal missegregation in cells with multicentrosomes. Immunofluorescence staining of HBx protein in a giant multinucleated ChangX-34 cell using anti-HA antibody (**A**). The cells released from the DTB were allowed to progress to G_2 phase and treated with 100 ng/ml of nocodazole for 12 h. Round mitotic cells were collected, washed, and seeded on cover glasses. The cells were then further progressed for 60 min (*B–H*), 90 min (*I–J*), or 24 h (*K, L*) and fixed for immunofluorescence staining. Centrosomes (*B–D*) were visualized by staining cells with anti-γ-tubulin antibody in Chang (**B**), ChangX-34 cells (**C**), and ChangX-31 cells (**D**). Mitotic defects were indicated by aberrant mitotic spindles (α-tubulin staining; **E**) and multidirectional chromosomal segregation (DAPI staining; **F**) in ChangX-34 cells. The overlaid image of mitotic spindles and chromosomal staining are shown in ChangX-34 (**G**) and ChangX-31 cells (**H**). Abnormal segregation of chromosomes or orphan chromosomes (**J**) was found with multiple mitotic spindles stained with anti-α-tubulin antibody in the late mitotic phase of ChangX-34 cells (**I**). Generation of multinucleated cells was visualized with rabbit anti-α-tubulin antibody (**K**) and micronuclei formation was visualized after staining with DAPI and anti-PKC-α antibody (**L**) to identify the boundary of ChangX-34 cells.

Increase of Multinucleated Cells and Micronuclei Formation in HBx-Expressing Cells

Several lines of recent evidence demonstrate that aberrant centrosome duplication is closely associated with the occurrence of aneuploidy and thus contributes to tumor development (26, 35). Because the multipolar spindles and missegregation of chromosomes were frequently found in HBx-expressing Chang cells, we expected to see an increase in multinucleated cells or micronuclei formation in these cells. Cells were synchronized by nocodazole treatment as before and released from nocodazole to allow the completion of mitosis. The newly divided cells were subjected to lamin B and/or α-tubulin staining to visualize the boundary of the cells. Microscopic examination of these cells revealed that the number of bi-nucleated and tri-nucleated cells (Fig. 2K) was much higher in ChangX-34 cells than in Chang cells (Fig. 3). About 25% of ChangX-34 cells at 24 h after nocodazole release were identified as multinucleated cells, whereas the number of multinucleated cells remained low in Chang cells (<5%). Similarly, a large fraction of ChangX-34 cells at this time point containing a micronucleus was observed (Fig. 2L) when these cells were subjected to DAPI and PKC-α staining (red). Approximately 15% of ChangX-34 cells contained micronuclei (Fig. 3), which were significantly more abundant than in Chang cells. To confirm whether HBx itself does cause mitotic aberrations in different cell lines, we employed another HBx-expressing cell line, NHBx1 (36), derived from NIH3T3 cells. NHBx-1 cells were previously shown to be tumorigenic in nude mice. Consistent with the observation in ChangX-34 cells, HBx expression in NIH3T3 cells significantly increased the cells with multiple (≥3) centrosomes and multi-nucleation (Fig. 4A). The frequency generating multiple centrosomes at metaphase in NIH3T3 cells was relatively high (~10%) but was significantly greater in NIHx-1 cells ($P < 0.005$, Student's t test). Similarly, the frequency of multinucleation in NIHx-1 cells was three times higher than that in the parental NIH3T3 cells. The expression level of HBx mRNA in these cells was confirmed by RT-PCR (Fig. 4B). Thus, these data suggest that HBx expression in cells is likely to provoke the generation of cells with multinuclei and micronuclei via hyperamplification of centrosomes.

The ERK Pathway Is Involved in Hyperamplification of Centrosomes

HBx is well known as a promiscuous transactivator on various DNA-responsive elements mediated through the activation of signaling cascades or through direct interactions with transcription factors in the nucleus (6). We hypothesized that the hyperamplification of centrosomes in HBx-expressing cells might be the consequence of the activation of signaling pathways by HBx. HBx is known to activate the Ras-Raf-MAPK pathway, the PI3-K, the p38 MAPK pathway, and the SAPK/JNK pathway, exerting effects of anti-apoptosis and transformation (15–17, 37). We employed various signaling inhibitors to test their effects on the amplification of centrosomes in ChangX-34 cells. The optimal concentrations of signaling inhibitors and their cytotoxicities vary greatly among different cell lines. Therefore, we first tested the cytotoxicity of signaling inhibitors in ChangX-34 cells and selected two concentrations, one of which is the maximum, causing about 10% of cell death (data not shown). Next, ChangX-34 cells were synchronized by the DTB method, treated with various signaling inhibitors for 12 h (wortmannin: 200 nM, SB203580: 0.5 μM, PD98059: 20 μM), and further incubated in the presence of 100 ng/ml of nocodazole for 12 h. The frequency of multiple centrosomes in ChangX-34 cells at 60 min after nocodazole release was analyzed after immunofluorescence staining of centrosome using anti-γ-tubulin antibody. We only analyzed the cells in mitotic phase (mainly metaphase) because these signaling inhibitor may also interfere with cell cycle progression (38). Interestingly, pretreatment of MEK1/2 inhibitor, 15 μM of PD98059, significantly reduced the number of cells with multiple (≥3) centrosomes in ChangX-34 cells at mitotic phase (Fig. 5A). In contrast, even maximum concentrations (200 nM) of wortmannin as a PI3-K inhibitor and SB203580 (10 μM) as a p38 MAPK inhibitor did not alleviate the number of cells with multiple centrosomes. These high concentrations of wortmannin and SB203580 were shown to effectively inhibit PI3-K activity and p38 kinase activity in Chang cells and other cell lines (39, 40). We have also shown that 100 nM of SB203580 was sufficient enough to inhibit mitochondrial aggregation in ChangX-34 cells.[4]

The effect of ERK inhibitor on the centrosome amplification was further examined under different concentrations of PD98059 (Fig. 5B). Consistent with the results in Figs. 1 and 2, approximately 25% of ChangX-34 cells contained amplified centrosomes at the 60 min after nocodazole release (see the actual number in Fig. 5). Addition of 5 μM of PD98059 to the ChangX-34 cells significantly inhibited the number of cells with multiple centrosomes, and the higher concentrations

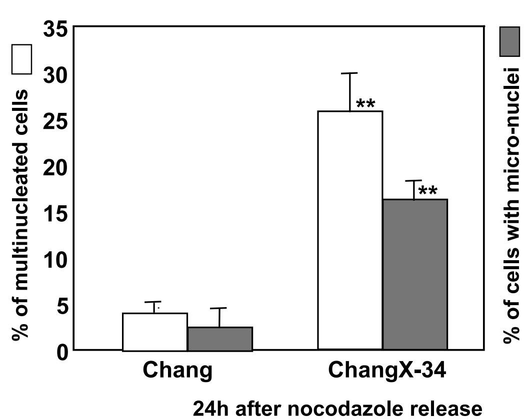

FIGURE 3. Generation of multinucleated cells and micronuclei formation by HBx. The parental Chang and HBx-expressing ChangX-34 cells were treated with 100 ng/ml of nocodazole for 12 h and released from nocodazole for 24 h. Generation of multinucleated cells as well as micronuclei formation were counted after staining cells with anti-α-tubulin antibody or DAPI and anti-PKC-α antibody, respectively. *Columns,* mean from at least three independent experiments; *bars,* SD. ** $P < 0.005$ by Student's t test.

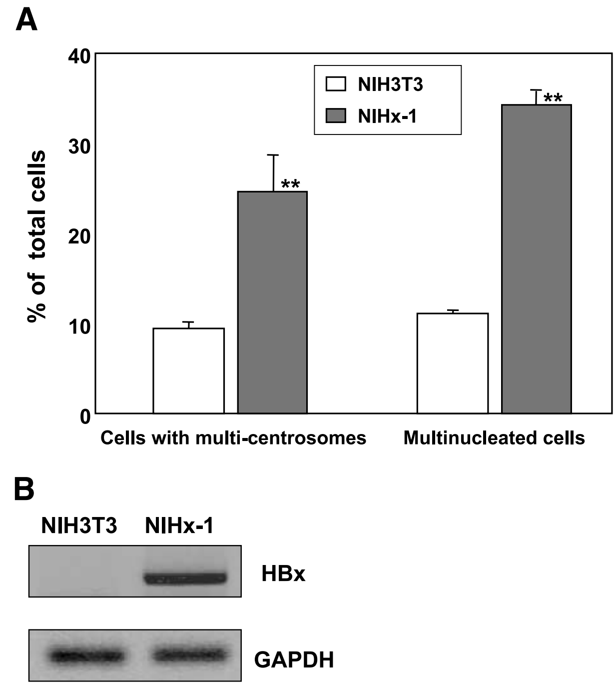

FIGURE 4. Centrosome hyperamplification and increase of multinucleated cells in NIHx-1 cells. **A.** The parental NIH3T3 fibroblasts and NIHx-1 cells stably transfected with the *HBx* gene were synchronized by treatment with 100 ng/ml of nocodazole for 12 h. After removal of nocodazole, the cells progressed either for 60 min for the determination of centrosome number or for 24 h to count the frequency of multinucleated cells. *Columns,* mean from at least three independent experiments; *bars,* SD. ** $P < 0.005$ by Student's t test. **B.** Expression of HBx mRNA in NIHx-1 cells was determined by RT-PCR analysis.

[4] S. Kim, C. Yun, J.-H. Lee, G. Yoon, and H. Cho. HBx triggers mitochondrial aggregation *via* the p38/MAPK signaling pathway, manuscript in preparation.

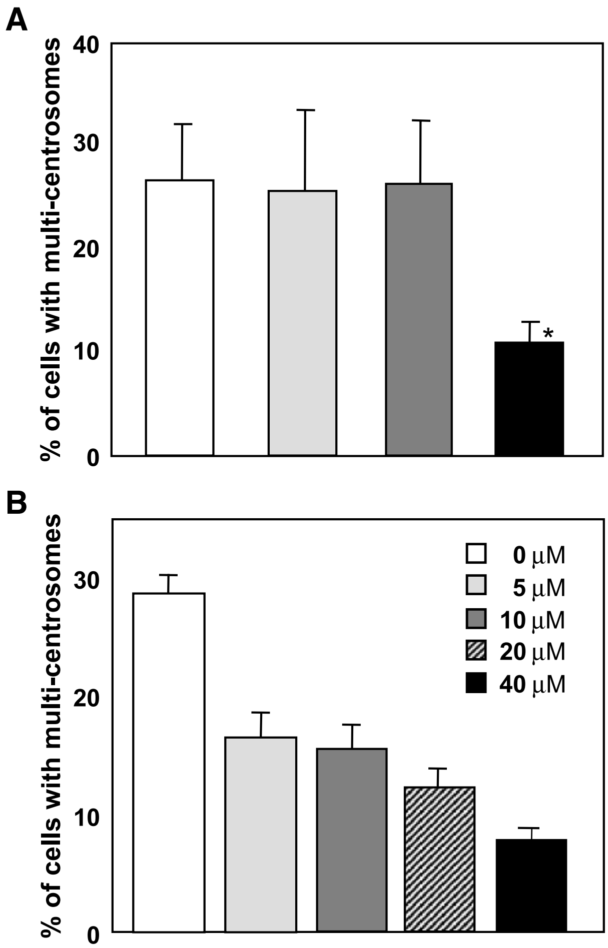

FIGURE 5. Treatment with PD98059 reduced the number of cells with hyperamplified centrosomes in a dose-dependent manner. **A.** ChangX-34 cells were synchronized by the DTB method, treated with various signaling inhibitors for 12 h (Wortmannin: 200 nM, SB203580: 0.5 μM, PD98059: 20 μM), and further incubated in the presence of 100 ng/ml of nocodazole for 12 h. The frequency of multiple centrosomes in ChangX-34 cells at 60 min after nocodazole release was analyzed after immunofluorescence staining of the centrosome using the anti-γ-tubulin antibody. □, Control; ▭, Wortmannin; ▭, SB203580; ■, PD98059. **B.** ChangX-34 cells were treated with different concentrations of PD98058 and analyzed for the frequency of multiple centrosomes. *Columns,* mean; *bars,* SD. *$P < 0.05$ by Student's t test.

of PD98059 gradually further lowered them to 7% of the whole population (Fig. 5B).

Because the activation of the ERK pathway is required for the hyperamplification of centrosomes, we expected that the endogenous ERK activity in ChangX-34 cells would be higher than in Chang cells. Indeed, the phosphorylation status of ERK at different phases of the cell cycle was found to be higher in ChangX-34 cells (Fig. 6A). At the G_1-S phase, the level of phospho-ERK is already elevated in ChangX-34 cells and remained higher to the prometaphase. The higher activity of ERK in ChangX-34 cells was well correlated with the previous observation in Fig. 1C, showing that a large fraction of ChangX-34 cells already contained two to three centrosomes at the G_1-S phase. Using immune complex kinase assay, we also observed that the ERK activity was considerably reduced in ChangX-34 cells pretreated with 20 μM of PD98059, whereas those were not affected by pretreatment with either 200 nM of wortmannin or 0.5 μM of SB204580 (Fig. 6B). Thus, the activation of ERK by HBx is involved in the amplification of centrosomes.

Suppression of the ERK Pathway Also Reduces the Formation of Both Multinucleated Cells and Micronuclei

We have shown that amplification of centrosomes by HBx protein was accompanied by the increase of both micronuclei formation and generation of multinucleated cells (Figs. 1, 2, 3). Here, we found that inhibition of ERK by PD98059 treatment abrogated increase in multinucleated cells and micronuclei formation. In these experiments, about 20% of ChangX-34 cells at 24 h after nocodazole release was identified as multinucleated cells. Addition of wortmannin or SB203580 did not reduce the generation of multinucleated cells, whereas PD98059 reduced the population of multinucleated cells significantly ($P < 0.05$ by Student's t test, Fig. 7A). We also found that PD98059 treatment inhibited micronuclei formation in ChangX-34 cells ($P < 0.05$, Fig. 7B), although the frequency was still higher than the basal level in Chang cells.

Ras Works as a Downstream Effector of HBx Mediating the Amplification of Centrosomes

The ERK activity in cells is controlled under the Ras-Raf-MEK pathway. It has been previously shown that HBx activated the Ras-Raf-MAPK pathway (13, 14). Therefore, we transfected several dominant-negative mutants into ChangX-34 cells and determined their effects on centrosome amplification in these cells. Because the transfection efficiency of ChangX-34 cells using the calcium phosphate method was determined as 60–80% using the EGFP control construct (data not shown), we directly counted cells with more than two centrosomes after transfection. About 20% of untransfected ChangX-34 cells in mitotic phase were again shown to have multicentrosomes. When ChangX-34 cells were transfected with dominant-negative mutants of PKB and PKC-α, the population with multicentrosomes remained unchanged (Fig. 8A). However, the dominant-negative mutants of Ras (RasN17) significantly reduced the number of cells with multicentrosomes to half ($P < 0.05$, Student t test). Expression of a dominant-negative form of MEK2, the upstream activator of ERK, in ChangX-34 cells further inhibited the generation of cells with multicentrosomes to less than 10% ($P < 0.005$, Student t test).

To exclude the possibility of clonal variation in ChangX-34 cells, we further investigated the effects of HBx on centrosome amplification in the parental Chang cells. Chang cells were transiently transfected with HBx expression vector along with various dominant-negative mutants for 48 h and treated with 50 ng/ml of nocodazole for 12 h. The floating mitotic cells were washed and then reseeded on coverslips and stained for γ-tubulin. Transfection with the pCDNA3 control vector revealed that about 5% of Chang cells contained supernumerary centrosomes (Fig. 8B). On the other hand, 12% of Chang cells transfected with the HBx expression vector contained supernumerary centrosomes, a significant increase compared to control ($P < 0.005$, Student's t test). The elevated percentage of cells with supernumerary centrosomes remained unchanged even after coexpressing dominant-negative mutants of PKB and PKC-α constructs. In contrast, the dominant-negative mutant of Ras reduced the population with multicentrosomes to 7% ($P < 0.05$, Student's t test). The dominant-negative MEK construct, MEK2A, was reproducibly more effective on the reduction of supernumerary centrosomes in these cells ($P < 0.005$, Student's t test). These results confirm that the Ras-MEK-ERK pathway works as a downstream effector of HBx mediating the amplification of centrosomes.

Discussion

Chromosome instability is known as the most common form of genetic instability in human malignancies and can be caused by aberrant chromosomal segregation during mitosis. The cells with persistent chromosomal instability may undergo cell death, but some of them would survive and become malignant clones bearing defective genomic information. These cells can be characterized by gains or losses of whole chromosomes along with eccentric cytologic alterations such as nuclear pleomorphism and multi/giant-nucleated cells (26, 35).

HBV infection has been known as one of the most important risk factors in HCC development. Although the frequent allelic losses and loss of heterozygosity (LOH) on primary liver tumors have been shown to be closely associated with HBV infection (20, 41), the mechanism of this causal relationship with HBV infection has not been established. In this study, we demonstrated that a multinuclear phenotype with chromosome aberration in HBx-expressing cells was closely associated with the centrosome hyperamplification. Centrosome hyperamplification can trigger chromosomal gain and/or loss by forming more than two spindles. Although abnormalities in centrosomes have been found in various cancers, it is noteworthy that HBx by itself induces the supernumerary centrosome in Chang cells (Figs. 1 and 8B) as well as in NIH3T3 cells (Fig. 4). These data suggest that the ability of HBx to promote centrosome hyperamplification and mitotic aberration may contribute to genomic instability during hepatocarcinogenesis.

Here, we demonstrated that the Ras-MEK-MAPK pathway works as a downstream effector of HBx mediating the amplification of centrosomes. It has been shown that HBx activates the Ras-Raf-MAPK pathway (13, 14) and is important in the transformation of differentiated hepatocytes (15). Ras and its active mutants have been involved in many different human cancers by promoting deregulated cell cycle progression and transformation. The activated Ras is also able to override the pro-apoptotic propensity of HBx, leading to transformation of cells (42). In this study, we introduced a new oncogenic role of Ras activation by HBx on centrosome amplification and subsequent chromosomal aberration. Suppression of the downstream signaling of Ras by treatment with PD98059 (Fig. 5) and introduction of the dominant-negative mutants of Ras and MEK2 into HBx-expressing cells significantly inhibited the centrosome amplification (Fig. 8A) as well as the generation of multinucleated cells and micronuclei formation (Fig. 7). To exclude the possibility that suppression of ERK activity by treatment with PD98059 just delays cell cycle progression (38), subsequently delaying centrosome duplication, we only determined the number of centrosomes in mitotic cells released from nocodazole treatment for these experiments (Figs. 5 and 8). These results were also supported by the observation that the phosphorylation status of ERK in ChangX-34 cells remained higher than that in the parental Chang cells through the cell cycle (Fig. 6A). Using immune complex kinase assay, we also confirmed that the ERK activity was inhibited in ChangX-34 cells pretreated with 20 μM of PD98059 (Fig. 6B). These results clarified that chromosomal aberration in HBx-expressing cells is mainly driven by Ras-mediated centrosome hyperamplification. During the preparation of this manuscript, one paper regarding the supernumerary centrosomes induced by HBx was published (43), although most of their works have been done in human fibroblast cells using adenoviral vector system. They found that HBx sequestered Crm1, a nuclear exporter, in the cytoplasm, causing the supernumerary centrosomes. They proposed that Crm1 is important in maintaining centrosome integrity of which disruption by HBx resulted in abnormal centrosome number. These findings may partly explain our observation that treatment with even higher concentrations of PD98059 did not completely inhibit the generation of the supernumerary centrosome in ChangX-34 cells (Figs. 5 and 8A). Whether employment of both PD98059 and leptomycin B, a

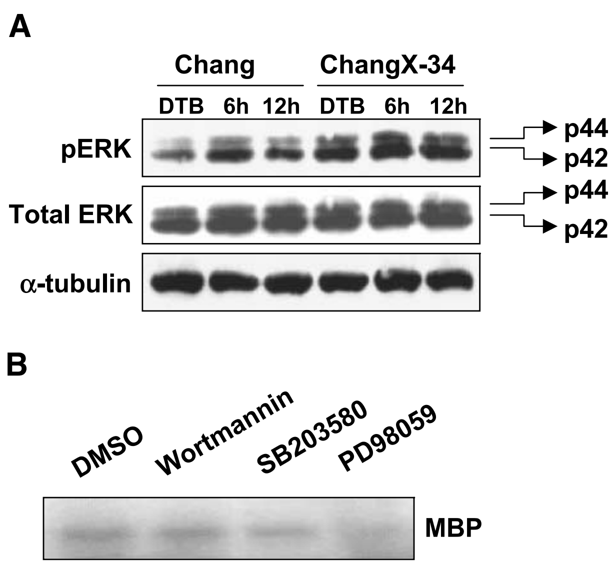

FIGURE 6. The phospho-ERK level during cell cycle progression is substantially high in ChangX-34 cells. **A.** The phospho-ERK levels in synchronized Chang and ChangX-34 cells as prepared in Fig. 1 were examined by Western blotting. *DTB,* cells at the 2nd round of the DTB; *6h,* released from the DTB for 6 h; *12h* (nocodazole), treated with 100 ng/ml of nocodazole for 12 h. Two forms of p42 and p44 ERK are shown. **B.** ChangX-34 cells as prepared in **A** were subjected to immune complex kinase assay. Whole cell lysates (500 μg) were incubated with anti-p42/44 ERK antibody at 4°C and precipitated by incubation with protein G-Sepharose. The immune complex was washed and subjected to the kinase reaction for 30 min at 30°C in the presence of 2 μCi of [γ-^{32}P]ATP and myelin basic protein (*MBP*) as a substrate. The reaction mixtures were separated on a 5% SDS-polyacrylamide gel, and the phosphorylated MBP substrate was visualized by autoradiography.

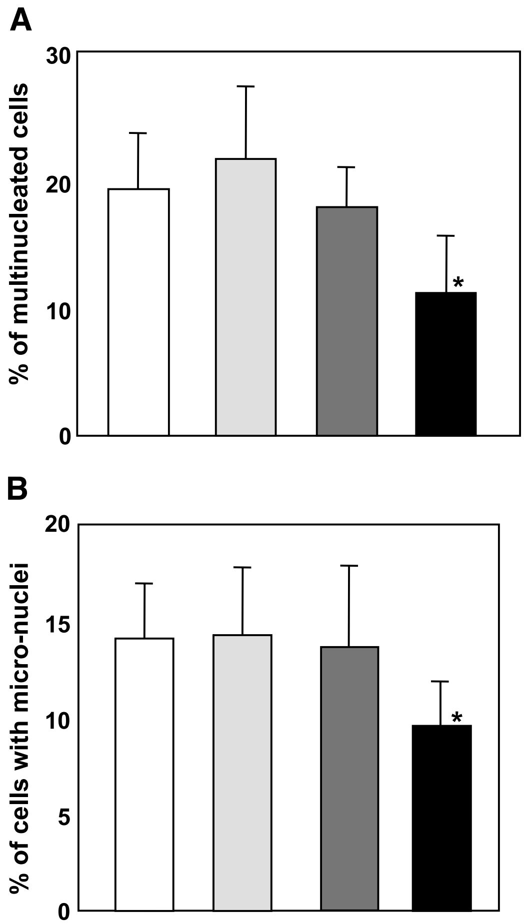

FIGURE 7. Suppression of the ERK pathway reduced the generation of multinucleated cells and micronuclei formation. ChangX-34 cells were synchronized by the DTB method, treated with various signaling inhibitors for 12 h (Wortmannin: 200 nM, SB203580: 0.5 μM, PD98059: 20 μM) and further incubated in the presence of 100 ng/ml of nocodazole for 12 h. The frequencies of multinucleated cells (**A**) and micronuclei formation (**B**) were quantified after immunofluorescence staining with anti-α-tubulin antibody, anti-PKC-α antibody, and DAPI as shown in Fig. 2. □, Control; ▭, Wortmannin; ▭, SB203580; ■, PD98059. *Columns,* mean from four independent experiments; *bars,* SD. * $P < 0.05$ by Student's *t* test.

Crm1-specific inhibitor, will completely suppress the generation of the supernumerary centrosome would be interesting for the future experiment. It is also possible that cytoplasmic sequestration of p53 protein in ChangX-34 cells may also contribute to an increase in the supernumerary centrosomes. We have previously reported that the major portion of p53 in ChangX-34 cells has been found in the cytoplasm with HBx (31). It has been shown that the loss or mutational inactivation of p53 is involved in abnormal amplification of centrosomes (44, 45).

Because genomic integrity can be severely compromised by abnormal amplification of centrosomes, cells with abnormal amplification of centrosomes may not always be eliminated under the guard of the mitotic checkpoint. Mitotic checkpoint recognizes the unattached kinetochore and the altered tension generated by aberrant mitotic spindles (46, 47). Treatment of HBx-expressing cells with nocodazole, a microtubule destabilizing drug, can activate the mitotic checkpoint. The mitotic checkpoint renders cells with unaligned chromosomes either to mitotic arrest, and if accurate chromosome segregation is impossible, to apoptosis. In HBx-expressing cells, however, the mitotic checkpoint appears to be defective because the cells underwent mitosis and yielded multinucleated cells as well as the cells with micronuclei (Figs. 2 and 3). These observations agreed with the recent observation that nocodazole-treated 4pX-1 cells, HBx-expressing de-differentiated hepatocyte cell line, exhibited formation of multinucleated cells (23). Mad1, Mad2, BubR1, and Bub3 are found to be the main mitotic checkpoint components in mammals (48, 49). It has been shown that Tax, encoded by human T-cell leukemia virus type 1 (HTLV-1), directly interacts with Mad1 and thereby abrogates the mitotic checkpoint (24), generating multinucleated cells. HBx may act like the HTLV1 Tax oncoprotein and override the action of the mitotic checkpoint by directly binding to one of these components. Alternatively, HBx may alter the phosphorylation status of these components, thereby modulating their activities because some of the mitotic checkpoint proteins are phosphoproteins. Besides, it is of a special interest that HBx along with HBxIP is shown to interact with survivin, which participates in regulation of chromosome segregation and mitotic exit (50, 51). Although the role of the mitotic checkpoint and its deregulation during carcinogenesis in human is only starting to emerge, the data presented here emphasize its importance in cancer development. The detailed mechanism of how HBx copes with the mitotic checkpoint remains to be solved.

Materials and Methods

Cell Culture

Human Chang liver cells were originally purchased from ATCC (CCL-13), and two sublines of ChangX-31 and ChangX-34 were established after stably transfecting the plasmids of pTetX and pUHD172-1 (31). Although these sublines were originally designed to express HBx tagged with HA (hemagglutinin) in a doxycyline-inducible manner, the basal expression of HBx was substantially maintained as previously shown (31, 32). The hepatocyte origin of Chang and other sublines were confirmed by RT-PCR by their ability to express albumin mRNA (data not shown). NIHx-1 cells (36) were kindly provided by Dr. Katsuro Koike (Department of Gene Research, The Cancer Institute, Japan). All these cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified CO_2 incubator. All reagents for cell culture were purchase from Life Technologies, Inc. (Gaithersburg, MD).

Antibodies and Chemicals

Rabbit anti-α-tubulin, goat anti-HA (Hemagglutin), and rabbit PKC-α antibodies were purchased from Santa Cruz (Santa Cruz Laboratory, Santa Cruz, CA); rabbit anti-γ-tubulin, rabbit anti-laminB1, DAPI, hematoxylin from Oncogene Research Products (Cambridge, MA); rabbit anti-pericentrin antibody from Covance (Princeton, NJ); goat anti-total ERK, goat anti-phospho-specific ERK (pERK) antibodies from Cell Signaling Technology (Beverly, MA); wortmannin, SB203580, PD98059 from Calbiochem (La Jolla, CA). [γ-^{32}P]ATP (specific activity, 6000Ci/mmol) was purchased from Dupont NEN (Boston, MA). Cycle test was purchased from Becton Dickinson (San Jose, CA), and ProFection kit from Promega (Madison, WI).

Cell Synchronization

For the G_1-S synchronization, cells at a density of $1-2 \times 10^5$ were plated into a 100-mm culture dish, incubated for 1 day, and treated with 20 μM of thymidine for the first synchronization according to the DTB method (52) with some modification. Eighteen hours later, cells were washed with thymidine-free medium and replaced with the complete medium for 6 h. Cells were then cultured again in the thymidine-containing medium for another 18 h for the second round of synchronization. Using FACS analysis, the extent of cell synchronization was assessed. For synchronization at the mitotic phase, the cells released from the DTB were allowed to progress to G_2 phase and treated with 100 ng/ml of nocodazole for 12 h. Round mitotic cells were collected after three times of gentle tapping and brief centrifugation at $200 \times g$ for 5 min. After washing with PBS twice, the cells in the complete medium were reseeded onto the poly-L-lysine-coated cover glasses or to culture dishes to release from the nocodazole arrest.

Indirect Immunofluorescence

Cells at interphase were grown in cover glasses while the cells released from nocodazole arrest were grown on poly-L-lysine-coated cover glasses for the indicated times. Cells were fixed with the mixture of methanol/acetone (1:1) solution and permeabilized with 0.5% Triton X-100 (31). Fixed cells were preincubated in blocking solution (5% BSA in PBS), followed by incubation with primary antibodies for overnight at 4°C. Cells were then washed three times with shaking and probed with fluorescence-conjugated secondary antibody for 1 h at room temperature. After washing, cells were mounted in the mounting solution containing DAPI and

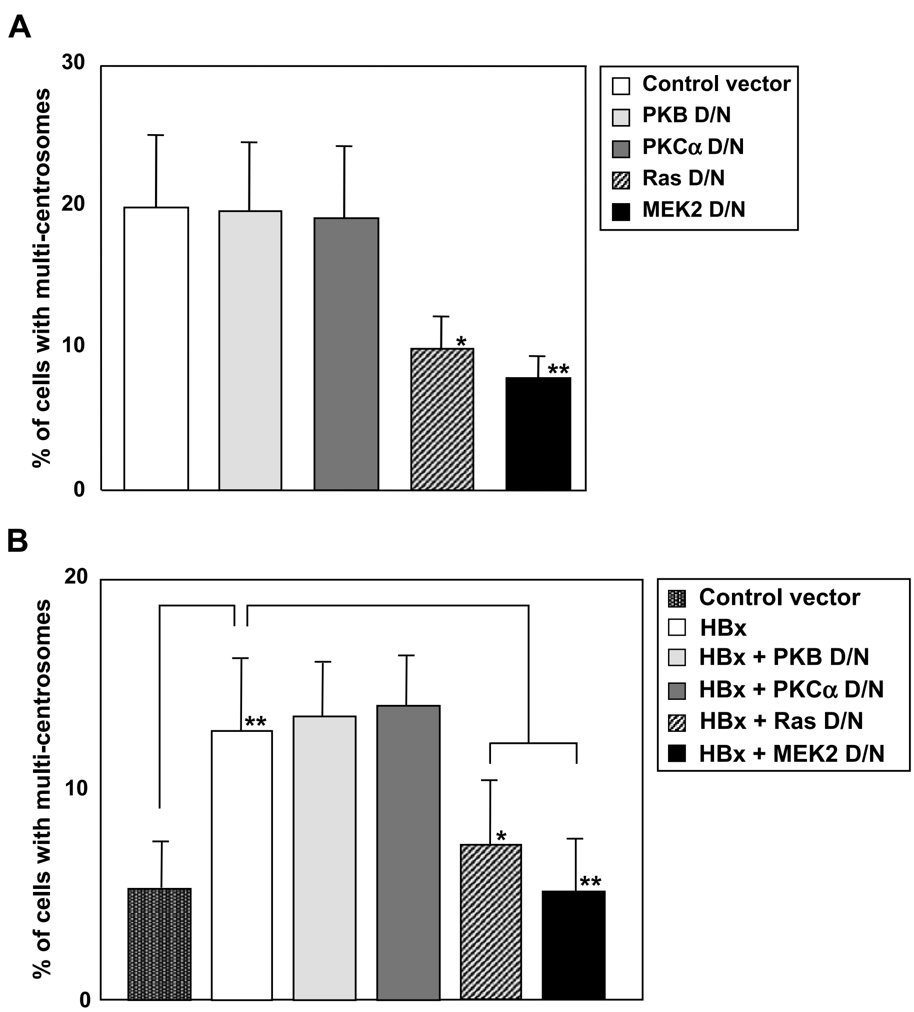

FIGURE 8. Intervention of the Ras-MEK2 pathway suppressed the centrosome hyperamplification in ChangX-34 cells. **A.** ChangX-34 cells and Chang cells (**B**) were transfected with various dominant-negative (D/N) mutant forms of expression vector with/without pCMV-HBx using the calcium phosphate precipitation method and cultured for another 24 h for the expression of protein before nocodazole treatment. The cells released from nocodazole arrest were analyzed for the frequency of multiple centrosomes by staining with anti-γ-tubulin antibody. *Columns,* mean from four independent experiments; *bars,* SD. * $P < 0.05$, ** $P < 0.005$ by Student's t test.

examined by fluorescence microscope (Zeiss) and analyzed with Aims software. For the accurate centrosome counting, antibodies against γ-tubulin and pericentrin were used and the number of centrosome was counted from 200–600 cells each time. The boundary of cells was identified by double-immunostaining with anti-PKC-α antibody, when it is necessary (Fig. 2L).

Cell Cycle Analysis by Flow Cytometry

Chang and ChangX-34 cells were synchronized by the DTB method and/or by treatment with nocodazole. Cells were trypsinized, pelleted, and fixed with 70% cold ethanol for 30 min. Samples were then resuspended in a solution containing propidium iodide and subjected to FACScan analysis using a FACS Vantage flow cytometer (Becton Dickinson).

Western Blotting

Western blotting analysis was described previously (31, 32). Briefly, harvested cell pellets were extracted with RIPA buffer, and the resultant extracts were subjected to a SDS-PAGE and probed with appropriate antibodies. For the detection of HBx, anti-HA antibody (Santa Cruz Laboratory) was used to detect HA-tagged HBx protein (31). The enhanced chemiluminescence (ECL) non-radioactive detection system was used to detect the antibody-protein complexes by exposure of the membrane to a Kodak X autoradiography film.

Reverse Transcription-PCR

Total RNA (0.8 μg) was isolated from the NIH3T3 and NIHx-1 cells and reverse-transcribed by using TaKaRa's reverse transcription system (Takara Shuzo Co., LTD., Japan) to detect the presence of HBx mRNA. The primer sets for *HBx* gene were 5′-CTG-GAT-CCT-GCG-CGG-GAC-GTC-CTT as a sense primer and 5′-ACA-GTC-TTT-GAA-GTA-TGC-CT as an antisense primer, producing 321 bp after RT-PCR. The RT-PCR product of GAPDH mRNA was shown to normalize the mRNA level in each lane.

Plasmid and Transfection

ChangX-34 cells and the parental Chang cells were seeded in 100-mm culture dishes for 1 day and transfected with 15–20 μg of various expression vectors for 16 h using the calcium phosphate precipitation method according to the manufacturer's protocol (ProFection, Promega). The remaining DNA precipitates on cells were removed after several washing with serum-free DMEM and the transfected DNAs were allowed to express proteins for 1 day. The cells were then treated with 50 ng/ml of nocodazole for 12–15 h and round mitotic cells were collected after washing. The cells were resuspended in the complete medium, reseeded onto the poly-L-lysine-coated cover glasses and allowed to progress through mitotic phase for 1 h. The cells were subjected to indirect immunofluorescence staining using antibodies against γ-tubulin and pericentrin and the number of centrosomes were counted. The eukaryotic expression vector of HBx (pCMV–HBx) was kindly provided by Dr. Schneider (University of New York). Dominant-negative expressions vectors of PKB (kinase-dead; K179A; Ref. 53), Ras (RasN17 in that Ser17 was replaced by Asn; Ref. 54), MEK2 (K101A; Ref. 55), and PKC-α (K368R; Ref. 56) have been also employed for the transfection.

Immune Complex Kinase Assay

Immune complex kinase assay was performed as previously described (57) with some modification. ChangX-34 cells in mitotic phase pretreated with various signaling inhibitors were extracted with lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA] supplemented with various protease inhibitors for 30 min at 4°C. Protein from whole cell lysate (500 μg) was reacted with anti-p42/44 ERK antibody at 4°C overnight and further incubated in the presence of protein G-Sepharose for 1 h. The immune complex was washed with the lysis buffer twice and subsequently with 20 mM HEPES (pH 7.4) three times and then resuspended in 20 μl of kinase assay buffer [10 mM $MgCl_2$, 2 mM DTT, 0.2 mM sodium orthovanadate, and 1 μg of myelin basic protein (MBP) as a substrate]. The kinase assay was carried out in the presence of 2 μCi of [γ-^{32}P]ATP for 30 min at 30°C, and stopped by addition of 5× SDS sample buffer [10 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol] and boiled for 5 min. The reaction mixtures were separated on a 5% SDS-polyacrylamide gel and the phosphorylated MBP substrate was visualized by autoradiography.

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