MOLECULAR AND CELLULAR BIOLOGY, July 2005, p. 5789–5800 Vol. 25, No. 14
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.14.5789–5800.2005

Phosphorylation and Stabilization of HURP by Aurora-A: Implication of HURP as a Transforming Target of Aurora-A

Chang-Tze Ricky Yu,[1] Jung-Mao Hsu,[1] Yuan-Chii Gladys Lee,[1,2] Ann-Ping Tsou,[3] Chen-Kung Chou,[1,4] and Chi-Ying F. Huang[1,3,5]*

Division of Molecular and Genomic Medicine, National Health Research Institutes, Miaoli County 350,[1] Graduate Institute of Medical Informatics, Taipei Medical University, Taipei 110,[2] Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei 112,[3] Department of Life Science, Chang Gung University, Tao-Yuan 333,[4] and Department of Computer Science and Information Engineering, National Taiwan University, Taipei 106,[5] Taiwan, Republic of China

Received 18 January 2005/Returned for modification 16 February 2005/Accepted 11 April 2005

Aurora-A, a mitotic serine/threonine kinase with oncogene characteristics, has recently drawn intense attention because of its association with the development of human cancers and its relationship with mitotic progression. Using the gene expression profiles of *Aurora-A* as a template to search for and compare transcriptome expression profiles in publicly accessible microarray data sets, we identified *HURP* (encodes hepatoma upregulated protein) as one of the best *Aurora-A*-correlated genes. Empirical validation indicates that *HURP* has several characteristics in common with *Aurora-A*. These two genes have similar expression patterns in hepatocellular carcinoma, liver regeneration after partial hepatectomy, and cell cycle progression and across a variety of tissues and cell lines. Moreover, Aurora-A phosphorylated HURP in vitro and in vivo. Ectopic expression of either the catalytically inactive form of Aurora-A or the HURP-4P mutant, in which the Aurora-A phosphorylation sites were replaced with Ala, resulted in HURP instability and complex disassembly. In addition, HURP-wild-type stable transfectants were capable of growing in low-serum environments whereas HURP-4P grew poorly under low-serum conditions and failed to proliferate. These studies together support the view that the ability to integrate evidence derived from microarray studies into biochemical analyses may ultimately augment our predictive power when analyzing the potential role of poorly characterized proteins. While this combined approach was simply an initial attempt to answer a range of complex biological questions, our findings do suggest that HURP is a potential oncogenic target of Aurora-A.

Human Aurora kinases have three gene family members, *Aurora-A*, *Aurora-B*, and *Aurora-C* (30). The Aurora kinases have a conserved catalytic domain at the C terminus and are distinguished by a noncatalytic region at the N terminus (reviewed in references 4, 7, and 12). Aurora-A is highly expressed in mitosis and localized to the centrosome in prophase; subsequently it spreads to the mitotic spindles/centrosomes, where it stays until the end of mitosis (4). Aurora-B, on the other hand, is at the centromere during G_2 and completely relocalizes to the midzone at anaphase (13, 36). Finally, Aurora-C is localized to centrosomes from anaphase to cytokinesis (22). Aurora-A has recently drawn intense attention because of its association with the development of human cancers and its relationship with mitotic progression (reviewed in references 4 and 12). *Aurora-A* maps to chromosome region 20q13.2 (37), in which DNA amplification is frequently observed in various human cancers (34, 43, 44). Ectopic expression of wild-type but not catalytically inactive Aurora-A transforms Rat-1 and NIH 3T3 cells, and the transformants can grow into tumors in nude mice, suggesting that the kinase possesses oncogenic potential (5, 54). In human tumors, gene amplification and/or elevated expression of *Aurora-A* has been detected in a significant proportion of pancreatic (26), breast (29, 42), prostate (3), gastric (33), bladder (35), ovarian (15), and colorectal (41) cancers; hepatocellular carcinoma (38); and non-Hodgkin's lymphoma (53). In short, Aurora-A has been implicated as involved in a range of cell cycle regulation processes and cell transformation processes; however, it is not yet established what signals are relayed by its activity.

To fully understand how a protein kinase regulates biological processes, it is imperative to first identify its substrate(s) or interacting protein(s). However, using the traditional biochemical approaches, this still remains a significant challenge. Recently, the development of microarray technology, which permits us to monitor transcriptomes on a genome-wide scale, has dramatically expedited a more comprehensive understanding of the gene expression profiles, for example, how the transcription profiles of genes vary across the cell cycle (for an example, see reference 51). There are a growing number of cases where cell cycle-regulated genes are typically involved in cell cycle-specific processes and are expressed at peak levels at the time that they are needed for their particular function(s). To illustrate this point, we have used the gene expression profiles of *Aurora-A*, which is highly expressed in various cancer cells and during mitosis, as a template to search for and compare transcriptome expression profiles in publicly accessible microarray data sets through clustering analysis. This resulted in the identification of *HURP* (encodes hepatoma upregulated protein) as one of the best *Aurora-A*-correlated genes. This data-mining approach may be one of the easiest available means of gaining

* Corresponding author. Mailing address: National Health Research Institutes, Division of Molecular and Genomic Medicine, No. 35, Keyan Road, Zhunan Town, Miaoli County 350, Taiwan, Republic of China. Phone: (886) 37-246-166, ext. 35305 or 35306. Fax: (886) 37-586-459. E-mail: chiying@nhri.org.tw.

insight into the functional concordance of coexpressed genes, particularly when studying novel, poorly characterized genes.

Initially identified through bioinformatic analysis performed to identify potentially important regulatory genes involved in growth control of human hepatocellular carcinoma (HCC) (50), *HURP* is a cell cycle-regulated gene characterized by the fact that it is highly expressed during the G_2/M phase in HeLa cells and this is followed by a sharp decline in early to middle G_1 phase. Immunolocalization studies indicate that HURP localizes to the spindle fibers during mitosis (50). Elevated *HURP* gene expression is highly associated with HCC (50), colon and breast cancers, and urinary bladder transitional-cell carcinoma (8, 18), suggesting that *HURP* may play a role in carcinogenesis. In fact, *HURP* overexpression in 293T cells results in enhanced cell growth at low serum levels and under polyhema-based, anchorage-independent growth conditions, implying that HURP, when abnormally upregulated, possesses transforming activity. In addition to transcriptional regulation, HURP quantity is also controlled by Cdk1/cyclin B at the posttranslational level. Cdk1/cyclin B-mediated multiple-site (Ser^{67}, Thr^{329}, Thr^{401}, Thr^{402}, Ser^{618}, Thr^{639}, Ser^{642}, Thr^{759}, and Ser^{839}) phosphorylation of HURP promotes its association with SCF^{Fbx7} ubiquitin ligase, leading to HURP ubiquitination and subsequent proteolysis by the proteasome (17). Accordingly, it appears very likely that the function and regulation of the quantity of HURP in cells are important to normal cell physiology, but the exact mechanisms involved remain to be determined.

The purpose of this study was to evaluate data-mining methods by investigating the functional correlation between *Aurora-A* and *HURP* in terms of common expression-profiling clusters in various tissues and cell lines and in a panel of synchronized cell cycles, with the aim of determining whether HURP may act in concert with Aurora-A in silico. Subsequent examination by quantitative reverse transcription-PCR (Q-RT-PCR) confirms that *Aurora-A* and *HURP* have similar expression patterns in HCC tissues and in regenerating mouse liver after partial hepatectomy. Partial colocalization of the proteins they encode to spindles has fueled further studies and identified HURP as a substrate of Aurora-A in vitro. Phosphorylation of HURP by Aurora-A may modulate HURP function at the level of HURP stability, complex assembly, and cell survival in low-serum environments. We propose that Aurora-A may be involved in the regulation of HURP function, possibly by altering its stability or its ability to interact with other associated molecules.

MATERIALS AND METHODS

Liver regeneration procedures. Male BALB/c mice, 6 to 8 weeks old and weighing 20 to 25 g, were purchased from the animal center of the National Science Council, Taiwan, Republic of China. They were fed a standard diet and water ad libitum, and treatment followed the regulations in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996). These studies were approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University. Under ketamine anesthesia (50 mg/kg given intraperitoneally), a midline incision was made and a 70% partial hepatectomy was performed as described by Higgins and Anderson (16). Resected livers were weighed and frozen in liquid nitrogen before storage at −80°C. Mice were allowed to recover with free access to food and water until they were sacrificed at 0.25, 4, 24, 36, 48, 72, 96, 120, 168, and 240 h after partial hepatectomy (six or more in each group at each time point). Liver samples were immediately frozen in liquid nitrogen and stored at −80°C.

RNA preparation and Q-RT-PCR. Total RNA was isolated from the five pairs of HCC tissues and regenerated mouse livers as described earlier (50). RNA was quantified by spectrophotometry at 260 nm. cDNA was prepared from the total RNA of the HCC samples and regenerated mouse livers using SuperScript RNase H reverse transcriptase (BRL). Expression of *Aurora-A* and *HURP* in tissue samples was analyzed by the SYBR Green quantitative PCR according to the manufacture's protocol (PE Applied Biosystems). The primers for *HURP* were as follows: forward, 5′-AAAGTTGTGCAGCCTGTAATGC-3′; reverse, 5′-CTGTTCTGGGAACCTGCTTTG-3′. Those for *Aurora-A* were as follows: forward, 5′-TTCCAGGAGGACCACTCTCTGT-3′; reverse, 5′-TGCATCCGA CCTTCAATCATT-3′. All of the primers were tested for nonspecific amplicons and primer dimers by visualizing the PCR products on 2% agarose gels before performing Q-RT-PCR. The primers used did not generate any nonspecific or primer dimer products. The cDNA product from each sample was diluted 200-fold and then used to perform a quantitative PCR. Each 10 μl of the quantitative PCR mixture for the SYBR Green system contained 5 μl of 2x SYBR Green Master Mixture, 4 μl of 200-fold-diluted cDNA product mixture, and 0.5 μl each of the 6 μM PCR forward and reverse primers (with a final reaction concentration of 300 nM). The values from the Q-RT-PCR were normalized to the housekeeping genes for glyceraldehyde-3-phosphate dehydrogenase and β_2-microglobulin. A $2^{\text{delta-CT}}$ (where CT is the threshold cycle) method was used. Briefly, the CT value of the internal control gene was used to calculate normalized target gene expression, referred to as $2^{\text{delta-CT}}$, in order to correct for differences between samples. Assays were performed in triplicate using an Applied Biosystems model 7700 instrument.

Construction of expression vectors and site-directed mutagenesis. All restriction enzymes were purchased from New England BioLabs. Human full-length *Aurora-A* (23), *Aurora-B* (46), *Aurora-C* (22), and *HURP* (50) cDNAs (or their mutant variants) were subcloned into pGEX4T, pET32a, FLAG-CMV2 (Kodak), or a modified version of pcDNA3.0 (Invitrogen), with an HA epitope tag for mammalian expression and a T7 promoter for an in vitro coupled transcription-translation reaction. Various *HURP* phosphorylation site and deletion mutants and a catalytically inactive (kinase-dead [KD]) mutant form of *Aurora-A* were generated by PCR-based mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene) employing *HURP* or *Aurora-A* as the template.

Preparation of recombinant protein, in vitro kinase reactions, and phosphorylation site determination. Human *HURP* cDNA was subcloned into expression vector pET32a (Novagen), expressed as a His-tagged fusion protein, and purified by nickel-agarose chromatography (QIAGEN) as described earlier (50). Human *Aurora-A* and *-B* cDNAs were subcloned into the expression vector pGEX4T, expressed in *Escherichia coli* strain BL21(DE3) as a glutathione *S*-transferase (GST) fusion protein, and purified as described previously (49). The purified recombinant HURPs, histone H1, histone H2A, and myelin basic protein were incubated, respectively, with *E. coli*-expressed, GST-tagged Aurora-A/B or baculovirus-expressed His-tagged Aurora-C (49) in kinase buffer A (25 mM Tris HCl, pH 7.4, 10 mM $MgCl_2$, 10 μM ATP, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol) coupled with 2.5 μCi [γ-^{32}P]ATP (Perkin-Elmer Life and Analytical Sciences) at 30°C for 30 min. Cell lysates of TM4 were isolated from the Sertoli cell line containing an uncharacterized p16 protein as the substrate of Aurora-C (49). The kinase reaction volumes were 40 μl and were terminated by adding sodium dodecyl sulfate (SDS) sample buffer; they were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography. Alternatively, various hemagglutinin (HA)-tagged *HURP* constructs were used as templates for in vitro transcription and translation (TNT Quick Coupled Transcription/Translation Systems from Promega) in the presence of [^{35}S]methionine. The procedures were done according to the manufacture's instructions. [^{35}S]methionine-labeled HURP was incubated with recombinant GST–Aurora-A-WT or -KD in kinase reaction buffer B (25 mM Tris HCl, pH 7.4, 10 mM $MgCl_2$, 100 μM ATP, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol) at 30°C for 30 min. In general, the reaction volumes were 40 to 50 μl. For phosphorylation site determination, *E. coli*-expressed recombinant His-tagged HURPs were incubated with GST-tagged recombinant Aurora-A in kinase buffer B. After reaction at 30°C for 30 min, samples were subjected to SDS-PAGE and stained with Coomassie blue. HURPs were carefully sliced from the gel, and the phosphorylation sites were determined by liquid chromatography-tandem mass spectrometry (LC MS/MS) analysis as previously described (48).

Cell culture, cell cycle synchronization, and transient transfection. All cell lines were purchased from the American Type Culture Collection, and all cell culture-related reagents were purchased from Invitrogen. Cells were maintained in a humidified incubator at 37°C in the presence of 5% CO_2 and grown in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal bovine

serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transient transfection of the cells was performed with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Mixed stable transfectants of HURP were generated by transfecting 293T cells with HA-tagged HURP-WT or HURP-4P plus a plasmid containing a puromycin resistance marker and then isolating and culturing them in selective culture medium with puromycin (1 μg/ml; Sigma). To synchronize 293T cells in mitosis, exponentially growing cells were incubated with 50 ng/ml nocodazole (Sigma) or 100 μM monastrol (Sigma) for 14 h. Cells were released into cell cycle progression by removing the nocodazole or monastrol, followed by incubation with fresh medium containing cycloheximide (50 μg/ml; Sigma), which blocks de novo protein synthesis. Continuous time point samples were collected for the various assays.

Preparation of cell extracts, dephosphorylation assay, and Western blot analysis. Cell-free lysates were prepared as described earlier (17). Briefly, cells were harvested, washed with phosphate-buffer saline, and lysed in extraction buffer, which was composed of (i) 50% lysate buffer, consisting of 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.2), 100 mM NaCl, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10% sucrose, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, aprotinin, chymostatin, and pepstatin, and (ii) 50% immunoprecipitation washing buffer (50 mM HEPES, pH 7.6, 2 mM $MgCl_2$, 50 mM NaCl, 5 mM EGTA, 0.1% Triton X-100, 40 mM β-glycerolphosphate). After incubation at 4°C for 30 min, cellular debris was removed by centrifugation at 13,000 rpm for 10 min in an Eppendorf centrifuge. Protein concentrations were determined using the Bradford assay (Bio-Rad). To perform the protein dephosphorylation experiment, lysates (50 μg) were incubated with 400 U of λ phosphatase (New England Biolabs) in λ phosphatase reaction buffer (50 mM Tris, pH 7.8, 2 mM $MnCl_2$, 5 mM dithiothreitol) at 30°C for 30 min. To increase the separation of the phosphorylated and unphosphorylated forms of the protein, equal amounts of treated and untreated total lysates were loaded side by side on a 20-cm SDS-PAGE gel, and the acrylamide-to bisacrylamide ratio was adjusted from 29:1 to 100:1. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) and detected with antibodies by Western blotting analysis. The antibodies used were anti-FLAG monoclonal antibody (M2; 1:2,000) and antiactin monoclonal antibody (1:5,000) purchased from Sigma, anti-HA monoclonal antibody (3F10; 1:2,000) purchased from Roche Diagnostics, anti-Aurora-A polyclonal antibody (ARK-1 [H130]; 1:1,000) purchased from Santa Cruz Biotechnology, anti-HURP monoclonal antibody raised against whole molecular recombinant HURP, and anti-cyclin B1 monoclonal antibody purchased from BD Transduction (1:2,000). The PVDF membrane was blocked with 5% bovine serum albumin–phosphate-buffered saline–0.1% Tween 20. The appropriate antibody was incubated with the membrane at 4°C overnight. The membrane was then washed with phosphate-buffered saline–Triton X-100 at room temperature for 30 min, and this process was repeated three times. The primary antibodies were detected using alkaline phosphatase-conjugated or horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody, as appropriate (1:2,000 to 1:5,000; Perkin-Elmer Life Sciences), and Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIP; Promega) or Western Lighting (Perkin-Elmer Life Sciences) detection reagents.

Small interfering RNA (siRNA). To generate functional siRNA, we designed a gene-specific insert that was specific for a 19-nucleotide stretch of the Aurora-A sequence (ATGCCCTGTCTTACTGTCA) (24) or the HURP sequence (CGAGAGTGATGTTCGAGCA), and each was separated by a 9-nucleotide noncomplementary spacer (TTCAAGAGA) from the reverse complement of the same 19-nucleotide sequence. This insert was then subcloned into the pSUPER vector system described previously (6) to direct the synthesis of siRNA in mammalian cells. Briefly, pSUPER vector was digested with BglII and HindIII, and annealed oligonucleotides (pSUPER-Aurora-A, 5′gatccccATGCCCTGTCTTACTGTCAttcaagagaTGACAGTAAGACAGGGCATttttggaaa3′; pSUPER-HURP, 5′gatccccCGAGAGTGATGTTCGAGCAttcaagagaTGCTCGAACATCACTCTCGtttttggaaa3′ [where uppercase letters indicate siRNA sequences]) ligated into the vector.

Gel filtration chromatography. Cell lysates were clarified by centrifugation at 14,000 rpm for 1 h in a Beckman F2402H rotor at 4°C, followed by filtration through a 0.45-μm-pore-size membrane. Samples (100 μl containing roughly 2.5 mg of total protein) were subjected to gel filtration chromatography at a flow rate of 0.2 ml/min on a Superdex 200 column (HR 10/30; Pharmacia) equilibrated in buffer (50 mM Na_2HPO_4, 0.15 M NaCl, pH 7.0). The size of the fractions collected was 250 μl. The molecular size standards (Pharmacia) were blue dextran (2,000 kDa), thyroglobulin (670 kDa), ferritin (440 kDa), aldolase (158 kDa), and ovalbumin (44 kDa).

MTT assay for low-serum growth. Various 293T cell pooled stable clones (5×10^3) were seeded into 96-well plates with either 10% or 0.5% FBS for 1 to 4 days, and this was followed by an MTT assay (optical density at 570 nm [OD_{570}]) to quantify cell growth. Briefly, 100 μl of 2-mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) solution was added and incubated with the cells at 37°C for 3 h to allow the color to develop. Subsequently, the medium was removed and 100 μl of dimethyl sulfoxide was added for 5 min at room temperature to extract the colored product produced by living cells. The purple end product was quantified using a spectrophotometer (OD_{570}). Data were normalized against the OD_{570} value on day 1 of each pooled stable clone.

RESULTS

HURP and Aurora-A have similar expression patterns. Genome-wide microarray analysis has been increasingly employed to monitor global gene expression and provide insights into the molecular mechanisms of gene function. We used the unique expression profile of *Aurora-A*, which is highly expressed in cancer cell lines and during mitosis, as a template to search for and compare the transcriptome expression profiles stored in publicly accessible microarray data sets. Using this approach, we have identified a number of potential *Aurora-A*-related genes. The aim was to identify genes that share their expression profiles with *Aurora-A* and thus are classified in the same cluster as *Aurora-A*. No matter which clustering algorithm is used, *HURP* is one of the candidate genes. The *HURP* expression profile exhibits a correlation with that of *Aurora-A* in the Normal Tissue Atlas database (http://expression.gnf.org; Fig. 1B) (39) and in the HeLa cell cycle profile database (http://genome-www.stanford.edu/Human-CellCycle/HeLa; Fig. 1A) (51). Expression of the two genes oscillates during the cell cycle, and upregulation and downregulation occur at the same times (Fig. 1A). Additionally, both Aurora-A (7) and HURP (17) protein expression levels also undergo periodic change corresponding to cell cycle progression, where both protein expression levels are low in exponentially growing cells but accumulate in G_2/M-arrested cells. Moreover, a resemblance between the expression profiles of *Aurora-A* and *HURP* was also observed across a variety of tissues and cell lines (Fig. 1B). Taken together, these findings raise the possibility that these two genes are functionally linked.

To gather further functional correlations for *Aurora-A* and *HURP*, we first explored the expression profiles of these two genes in HCC tissues and in regenerating mouse liver after partial hepatectomy using real-time Q-RT-PCR. In agreement with previous observations (38, 50), both *Aurora-A* and *HURP* were co-upregulated in five pairs of HCC tissues relative to their adjacent nontumor analogs (Fig. 1C). Moreover, BALB/c mice were subjected to partial hepatectomy and total RNA was subsequently extracted from regenerating liver tissues at various time points, where 36 and 48 to 72 h represent the S and G_2/M phases, respectively, of the regenerating mouse liver (50). The expression patterns of the genes for p21, cyclin B2, and PCNA were determined by real-time Q-RT-PCR as a reference to separate the distinct cell cycle phases. This RT-PCR analysis indicated a significant induction of both *Aurora-A* and *HURP* in the regenerating mouse livers at S phase (36 h), and this reached a maximum level at G_2/M phase, 48 h after partial hepatectomy (Fig. 1D). This is in agreement with previous reports that the expression of *Aurora-A* (7) and *HURP* (50) is cell cycle regulated. Together, analysis of the data sug-

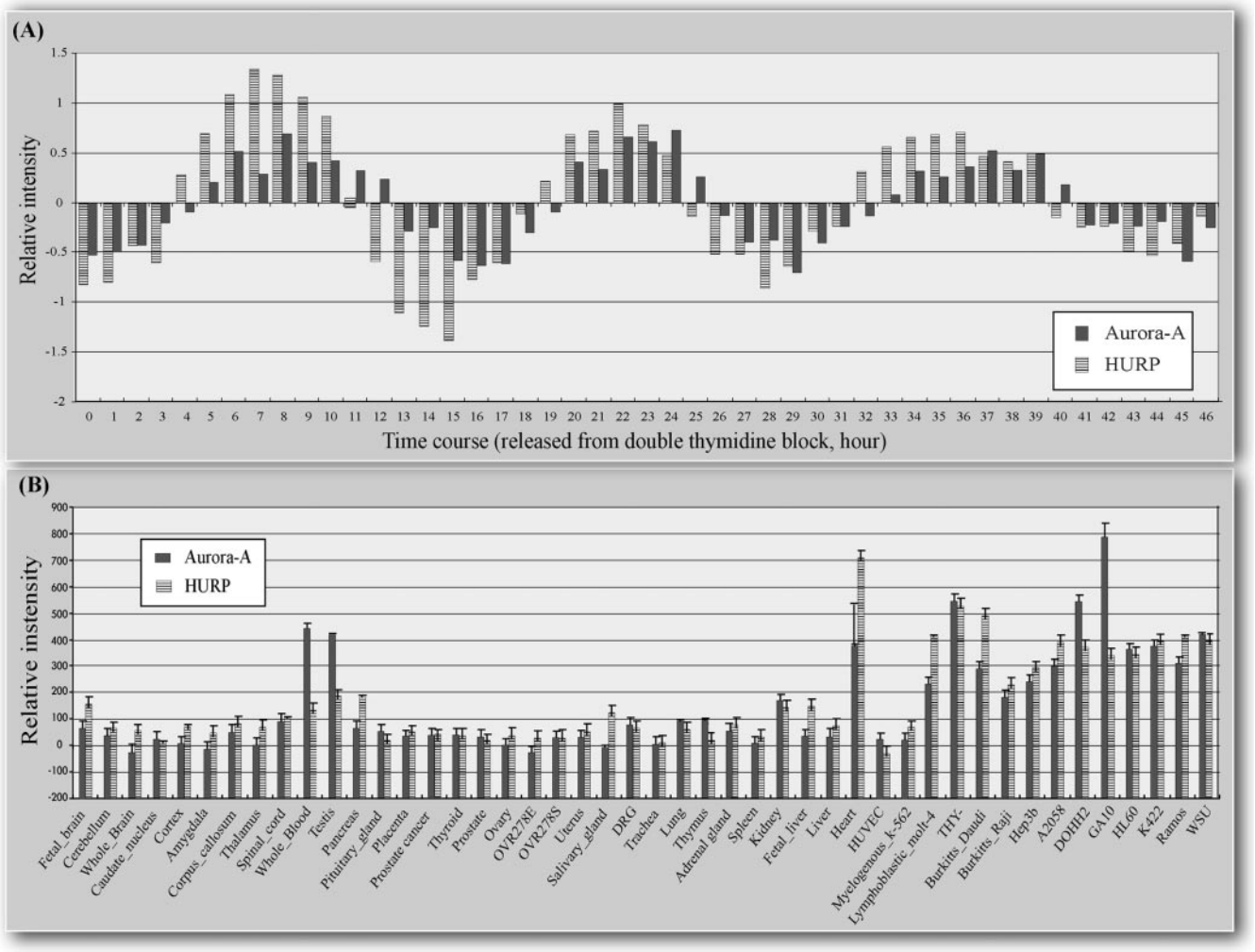


FIG. 1. Similar expression patterns for *Aurora-A* and *HURP* from analysis of various data sets and regenerating mouse livers. The expression profiles for *Aurora-A* and *HURP* in a HeLa cell cycle panel (A) and in different cell lines and tissues (B) were downloaded from publicly accessible databases (two and one specific primers, respectively). The data were plotted according to the relative expression levels of the two genes. (C) Upregulation of *Aurora-A* expression parallels that of *HURP* in HCC. Q-RT-PCR was conducted with specific primers for either *Aurora-A* or *HURP* in tumor tissue or adjacent nontumorous analogs obtained from five HCC patients. The data were then normalized with the internal control, glyceraldehyde-3-phosphate dehydrogenase. TT, primary liver tumor tissue; NT, corresponding adjacent nontumorous liver tissue. (D) Expression of mouse *Aurora-A* and *HURP* during mouse liver regeneration was detected by Q-RT-PCR. β_2-Microglobulin served as an internal control. Time indicates intervals after partial hepatectomy (h, hours; d, days). The left ordinate represents the *n*-fold change in mouse cyclin B2 mRNA. The right ordinate represents the *n*-fold change in mouse *HURP* and *Aurora-A* transcripts. The data were normalized with the expression value at time zero. The cell cycle stages, as determined by Q-RT-PCR of several markers (p21, PCNA, and cyclin B2) of each cell cycle stage, are shown at the bottom.

gests that the expression of these two genes is linked to cell proliferation or related to hepatocyte growth control.

HURP serves as a substrate of Aurora-A. As HURP is a mitotic phosphoprotein (17), the similarities between the gene expression profiles (Fig. 1) and subcellular localization in mitosis (4, 50) suggest that Aurora-A is a HURP kinase. To investigate the ability of Aurora-A to phosphorylate HURP, bacterially expressed and purified HURP was incubated in kinase reaction buffer containing [γ-^{32}P]ATP with recombinant Aurora-A. Analysis of the resulting time course by SDS-PAGE (Fig. 2A) was able to detect at 5 min the presence of a small amount of HURP with slower migration relative to the control HURP (lower part) by the presence of weak phospho-HURP radioactivity (upper part). This suggests that HURP phosphorylation may cause an electrophoretic upshift on SDS-PAGE (see later). The phosphorylation signal and electrophoretic upshift became more apparent at 20 min after commencement of incubation (Fig. 2A). In addition, in order to explore the issue of whether any or all of the three Aurora kinases possess specificity toward HURP, recombinant HURP was incubated in kinase buffer containing [γ-^{32}P]ATP with each of the three respective human Aurora members in turn. Autoradiography revealed that HURP serves as a substrate for Aurora-A but not for Aurora-B or Aurora-C under the conditions used (Fig. 2B). The levels of histone H1 and histone 2A kinase activity of the Aurora-A and Aurora-B used in the assay were comparable, and in addition, Aurora-C activity was detected and monitored using p16 protein as a substrate (46).

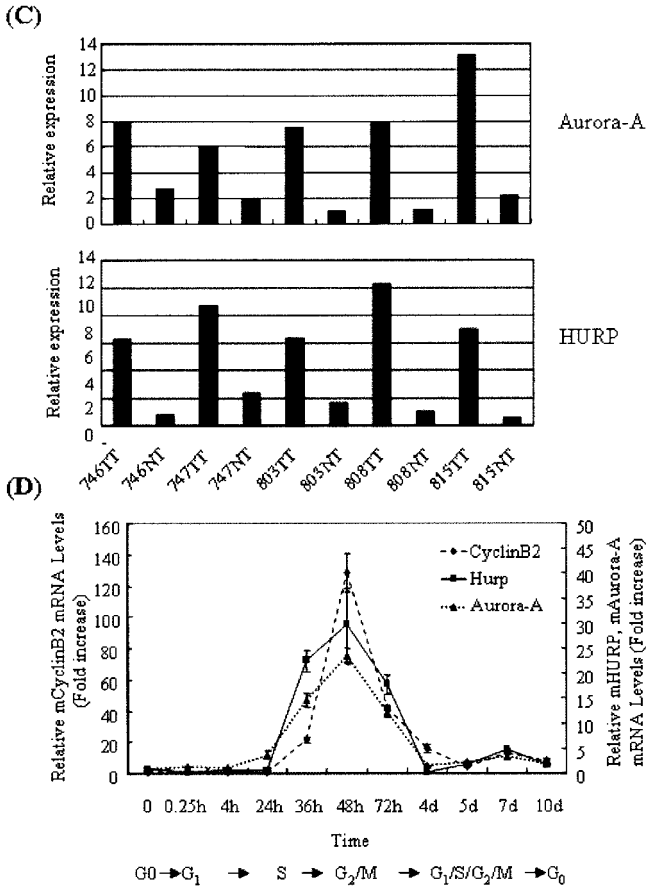


FIG. 1—*Continued*.

Only Aurora-A was able to catalyze HURP phosphorylation, suggesting that, despite sharing considerable sequence homology in their kinase domains at the C terminus, the Aurora family kinases have distinct substrate specificities involved in the propagation of various signaling pathways. These results, however, do not rule out the possibility that Aurora-B and Aurora-C need accessory proteins to enhance their kinase activity with HURP and to allow efficient phosphorylation of the protein substrate.

HURP exhibited various degrees of electrophoretic mobility shift on SDS-PAGE when cells underwent mitotic synchronization (Fig. 2C; see Fig. 4D). These mobility upshifts were abolished when λ phosphatase was added to the lysates, which is a characteristic of protein phosphorylation (17; data not shown). To further characterize HURP phosphorylation by Aurora-A in cells, we used plasmid-based (pSUPER) RNA interference (6) to deplete 293T cells of endogenous Aurora-A, followed by examination of HURP phosphorylation status during mitosis, based on the protein's electrophoretic mobility shift on SDS-PAGE as described earlier (17). The 293T cells were transiently transfected with pSUPER vector (vehicle) or pSUPER-Aurora-A and then treated with nocodazole to determine the phosphorylation status of HURP in the mitotic cells. As shown in Fig. 2C, ectopic expression of pSUPER-Aurora-A, which reduced the endogenous Aurora-A protein level, also abolished the mobility shift of HURP on SDS-PAGE. It appears that hypophosphorylation of HURP did not cause a disturbance of cell cycle progression in the cells with reduced Aurora-A due to the partial endogenous Aurora-A knockdown, because there was not a failure to enter mitosis. This was supported by approximately equal protein levels of cyclin B1, which reaches a plateau when the cell cycle progresses to mitosis (Fig. 2C). Taken together, these data indicate that mitotic phosphorylation of HURP is dependent, at least in part, on the presence of Aurora-A.

Aurora-A regulates protein complex formation of HURP. It is known that changes in protein phosphorylation sometimes bring about, or are brought about by, changes in protein-protein interactions. Therefore, we set out to determine whether overexpression of the dominant negative form of Aurora-A (FLAG–Aurora-A-KD) in 293T cells would be accompanied by a change in the association of HURP with other proteins. Lysates were prepared from transfectants that contained the vector control (vehicle), FLAG–Aurora-A-WT, or FLAG–Aurora-A-KD, and these were subjected to gel filtration chromatography on a Superdex 200 column. Fractions were analyzed by Western blotting with anti-HURP antibody. The endogenous HURP exhibited a broad size distribution, roughly corresponding to 2,000 to 150 kDa (tubes 32 to 48; Fig. 3, top panel), with a range of electrophoretic mobility shifts on SDS-PAGE (Fig. 3). Treatment with λ phosphatase abolished the slowly migrating mobility shift bands on SDS-PAGE, indicating that the electrophoretic mobility shifts were caused by protein phosphorylation (data not shown). Overexpression of FLAG–Aurora-A-WT resulted in two HURP populations which differed markedly from each other. Typically, about half of the HURP was found at a molecular mass of ~670 kDa and the other half was centered at ~200 kDa (Fig. 3, middle panel). Interestingly, the gel filtration profile for the ectopic expression of FLAG–Aurora-A-KD was different from either of the previous profiles for HURP. In this case, the high-molecular-weight HURP peak disappeared and only the low-molecular-weight form was found at ~200 kDa. This was not caused by a variation in the amount of protein loaded because the gel filtration profiles of c-jun and actin were about the same in three different samples (data not shown). The reason for the disappearance of the high-molecular-weight HURP or its conversion to a low-molecular-weight version is not clear. However, one plausible explanation is HURP instability and another is degradation of the high-molecular-weight fraction (see Fig. 5). Taken together, these observations indicate that Aurora-A-elicited HURP phosphorylation may cause complex formation or oligomerization of HURP into the high-molecular-weight fractions, suggesting that an alteration of HURP phosphorylation may, at least in part, be accompanied by changes in the association and/or stabilization state.

Aurora-A phosphorylates HURP at four residues as determined by LC MS/MS. To investigate the kinase relay signals, it is essential to identify the phosphorylation site(s) for a given substrate to allow studies that use phosphorylation site mutants. A combination of two approaches was adopted to further characterize HURP phosphorylation by Aurora-A. First, we had previously set up a sensitive in vitro kinase assay using in vitro transcribed and translated [^{35}S]methionine-labeled protein substrates (52). Figure 4A shows that the [^{35}S]methionine-

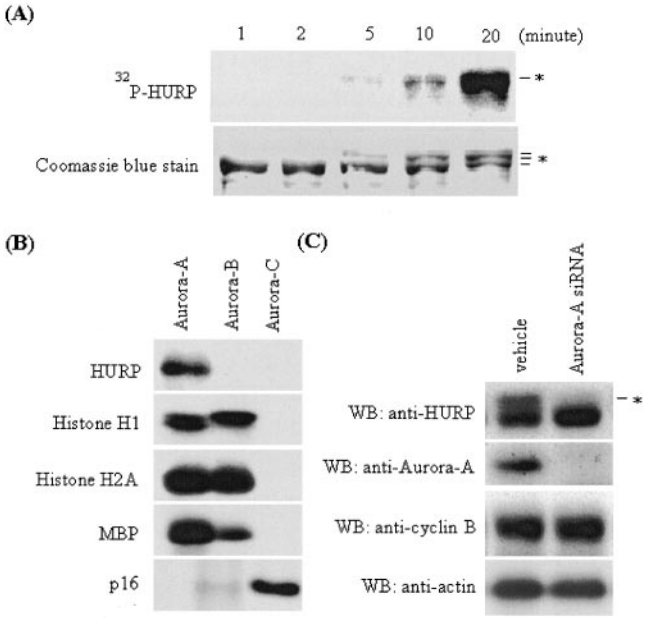


FIG. 2. HURP is a potential substrate of Aurora-A. (A) Recombinant Aurora-A was incubated with recombinant His-tagged HURP in kinase reaction buffer in the presence of [γ-^{32}P]ATP for different time intervals. The samples were separated by SDS-PAGE and transferred to a PVDF membrane, and this was followed by autoradiography (top) or membrane staining with Coomassie blue (bottom). An asterisk indicates multiple phosphorylation forms of HURP. (B) Recombinant Aurora-A, Aurora-B, and Aurora-C were incubated with recombinant His-tagged HURP in the presence of [γ-^{32}P]ATP. Analysis of the autoradiography data demonstrates that Aurora-A efficiently phosphorylated the recombinant HURP; however, no such phosphorylation was observed when Aurora-B or Aurora-C was present. Histone H1, histone H2A, myelin basic protein (MBP), and p16 were also incubated with the Aurora kinases to serve as substrate controls and ensure that the input of Aurora-A kinase activity was similar to that of Aurora-B and that the Aurora-C used was active. (C) 293T cells were transfected with pSUPER vector (vehicle) or pSUPER-Aurora-A for 48 h and then synchronized in mitosis using nocodazole treatment for 16 h. Equal amounts of extracts were prepared and analyzed by Western blotting (WB) with anti-Aurora-A, anti-HURP, anti-cyclin B1, or anti-actin antibodies. Actin was used as a loading control. The similar cyclin B1 expression level indicates a comparable synchronization effect for pSUPER-Aurora-A transfectants during mitosis. The asterisk indicates the phosphorylation form of HURP.

labeled HURP exhibited an electrophoretic mobility upshift on SDS-PAGE when incubated with recombinant Aurora-A-WT, but not Aurora-A-KD. Using this assay, we were able to generate a series of HURP deletion mutants to narrow down the possible Aurora-A phosphorylation region in HURP. A total of six deletion constructs were generated, and these constructs were then transcribed in vitro and translated in the presence of [^{35}S]methionine. Their relative extent of phosphorylation was determined by incubation with recombinant Aurora-A-WT and Aurora-A-KD. Of these constructs, HURP(324-846) exhibited electrophoretic mobility shifts on SDS-PAGE when incubated with Aurora-A-WT, but not Aurora-A-KD, whereas HURP(1-575) did not have an analogous mobility upshift when incubated with Aurora-A-WT (Fig. 4A). The results indicate that Aurora-A may phosphorylate HURP in the C-terminal region (positions 576 to 846). It should be noted that phosphorylation does not always lead to an electrophoretic mobility shift. Therefore the assay may only detect a subset of the potential phosphorylation sites in HURP, and some may have been missed.

Second, recombinant HURP was incubated with Aurora-A in an in vitro kinase reaction and subjected to SDS-PAGE analysis. The carefully excised band containing HURP was subjected to LC MS/MS analysis as previously described (48). Results of the various analyses are shown in Fig. 4B. Four phosphorylated residues were identified, namely, HURP-S627, -S725, -S757, and -S830, with 65% amino acid sequence coverage (Fig. 4B, lower part). This result is in agreement with the mobility shift assay described in Fig. 4A and indicates that Aurora-A may phosphorylate HURP at its C terminus (positions 576 to 846).

To provide additional support for the assignment of the four phosphorylated residues, each phosphorylation site was initially replaced individually with alanine and various combinations of two phosphorylation site mutations on HURP were also created. These mutants were assayed for the ability to serve as the Aurora-A substrate. However, none of these tested mutants showed a significant reduction in ^{32}P incorporation compared to HURP-WT on incubation with Aurora-A (data not shown). Therefore, we constructed a HURP mutant in which all four phosphorylation sites had been replaced with alanine (designated HURP-4P). Indirect immunofluorescence

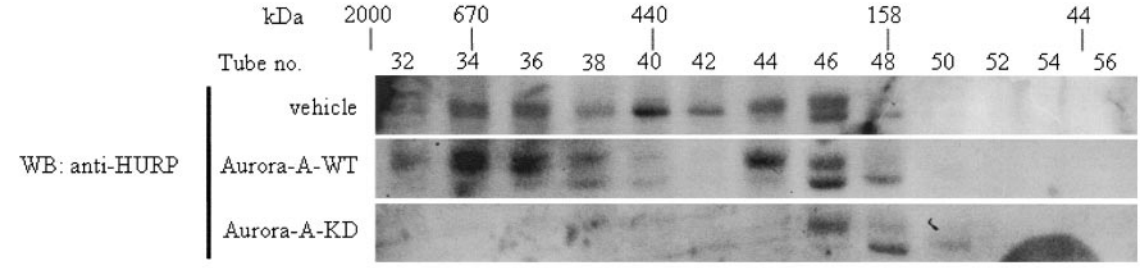


FIG. 3. Aurora-A regulates protein complex formation of HURP. 293T cells were transiently transfected with the pCMV2 vector (vehicle), FLAG–Aurora-A-WT, or FLAG–Aurora-A-KD. The cells were harvested and lysed. Equal amounts of lysates were subjected to protein liquid chromatography (FPLC). Fractions (tubes 32 to 56) were analyzed by SDS-PAGE, followed by Western blotting (WB) with anti-HURP antibody. Higher- and lower-molecular-weight fractions were also analyzed, and they contained essentially no HURP (data not shown). The lack of HURP signal in Aurora-A-KD-transfected cell lysates from tubes 32 to 44 was not due to differences in protein loading. The relative loading of each sample in the corresponding tubes was similar, as determined by visualization with Coomassie blue. The migration of the molecular size (MW) standards is shown at the top. Similar results were obtained for two independent transfections.

analysis indicated that the subcellular localization of the HURP-4P mutant was the same as for HURP-WT in the various cell types assayed (data not shown). The retained spindle-binding ability of the HURP-4P mutant not only suggests that serine-to-alanine mutations did not cause any dramatic conformational changes in HURP, but it also reveals that Aurora-A does not seem to be involved in the regulation of HURP spindle binding. Subsequently, 293T cells were transiently transfected with HA–HURP-WT or HA–HURP-4P. The cells were then lysed and immunoprecipitated with anti-HA antibody, and the immunocomplexes were incubated with recombinant Aurora-A in the presence of [γ-^{32}P]ATP. Reaction mixtures were resolved by SDS-PAGE and analyzed using Western blotting with anti-HA antibody or autoradiography. HURP-WT, but not HURP-4P, expressed in 293T cells, was Aurora-A phosphorylated, as detected by [γ-^{32}P]ATP incorporation (Fig. 4C, right) and mobility upshift (Fig. 4C, left), both representing phosphorylation of HURP (17).

Next, 293T cells overexpressing either HA–HURP-WT or HA–HURP-4P were arrested in mitosis by nocodazole or left untreated. The cell lysates were separated by SDS-PAGE, followed by Western blotting with anti-HA antibody to determine the phosphorylation status of HURP in mitotic and asynchronized cells. The results were consistent with previous observations that HURP-WT exhibited a mobility upshift in mitotic cells compared with asynchronized cells (Fig. 4D, top part) (17). By contrast, the HURP-4P mutant displayed essentially no mobility upshift in either mitotic or asynchronized cells (Fig. 4D, bottom part). The failure of HURP-4P to exhibit a mobility upshift (or phosphorylation) in mitosis was not due to the efficacy of mitotic arrest since the percentage of mitotic cells, expressing either HURP-WT or HURP-4P, was almost the same (data not shown). Taken together, these findings, even though not able to rigorously exclude the possibility that additional mitotic or Aurora-A-dependent phosphorylation sites are present in HURP, support the view that Aurora-A phosphorylates HURP at the four residues described, as well as at sites that are phosphorylated in mitosis.

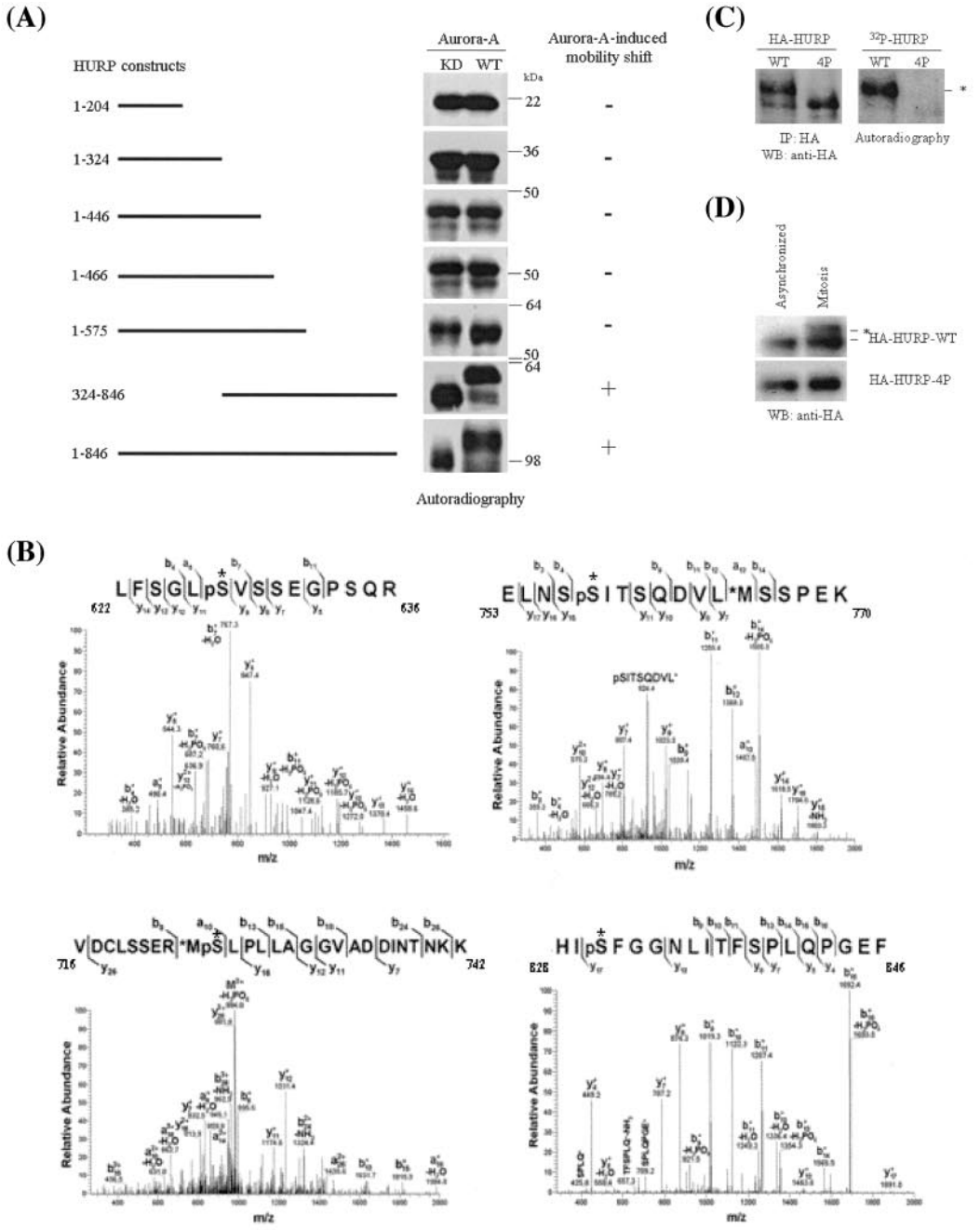
Aurora-A regulates HURP stability. Previous analysis has shown that HURP undergoes proteasome-mediated proteolysis as arrested mitotic cells are released into cell cycle progression (17). This irreversible proteolysis is often intricately connected to protein phosphorylation. To delineate this interplay and the regulation of HURP phosphorylation and degradation, 293T cells were transfected with either HURP-WT or HURP-4P and arrested in mitosis by nocodazole treatment for 16 h. Cells were then released into cell cycle progression at the indicated times by removing the nocodazole, followed by incubation with fresh medium containing cycloheximide to analyze HURP stability, as described earlier (17). Western blotting analysis indicated that while the HURP-WT level diminished steadily after the cells were released from mitotic synchronization, the overall turnover rate was higher for the HURP-4P mutant (half-life [$t_{1/2}$], ~2 h) compared to HURP-WT ($t_{1/2}$, ~7 h) (Fig. 5A). This raises the possibility that Aurora-A-elicited HURP phosphorylation is essential to the maintenance of HURP stability. To explore this possibility, 293T cells were transfected with vehicle, FLAG–Aurora-A-WT, or FLAG–Aurora-A-KD, synchronized in mitosis, and finally released from mitotic arrest to evaluate turnover rates of endogenous HURP (Fig. 5B). Overexpression of either construct did not alter the degradation rate of cyclin B1, which is known to be subjected to proteolytic downregulation when cells exit from mitosis (Fig. 5B). The levels of FLAG–Aurora-A-WT and FLAG–Aurora-A-KD expressed in the 293T cells were comparable, but overexpression of the former resulted in a longer HURP half-life relative to the FLAG–Aurora-A-KD-transfected cells ($t_{1/2}$ of ~7 versus 3 h) (Fig. 5B). Similar results were observed when the cells were synchronized and released from mitosis by treatment with monastrol (Fig. 5C), which acts to arrest cells through a mechanism that is different from nocodazole (19). These results suggest that Aurora-A kinase activity affects HURP stability.

A HURP-4P pooled clone loses its growth advantage in low-serum media. To further characterize the biological effects of Aurora-A-mediated HURP phosphorylation, we compared cells overexpressing HURP-WT or HURP-4P and determined whether either was able to confer any growth advantage on the cells. 293T cells were transfected with vehicle, HA–HURP-WT, or HA–HURP-4P. Mixed stable cell lines were selected for resistance to puromycin and analyzed for HA-tagged HURP expression by Western blotting (Fig. 6A). These cells, which retained a morphology similar to the parental 293T cells (data not shown), were cultured with either 10% or 0.5% serum for 1 to 4 days. Their relative growth was determined by MTT assay. All stable transfectants grew normally in 10% serum, but HA–HURP-WT had a slightly higher proliferation rate than those with vehicle or HA–HURP-4P (Fig. 6B). More importantly, in agreement with previous observations, HURP-WT stable transfectants were capable of growing in a low-serum environment (0.5%) (Fig. 6C). In contrast, HURP-4P, despite having slightly higher protein expression relative to the HURP-WT clone, grew poorly under low-serum conditions and failed to proliferate. Together, these results suggest that HURP overexpression promotes cell survival under low-serum conditions and HURP phosphorylation may confer the potential to exhibit serum-independent growth.

DISCUSSION

Overexpression of *Aurora-A* has been found in many tumors and has also been shown to be oncogenic in murine NIH 3T3 or Rat1 cells (5, 54), although no oncogenic potential has been demonstrated in mouse embryonic fibroblasts (2). This study aimed to correlate Aurora-A with proteins as yet unlinked to malignant transformation processes. However, to do this using traditional biochemical approaches remains difficult. In this study, therefore, we first employed bioinformatics to analyze microarray data sets, aiming to identify novel targets of Aurora-A. Using the concept of synexpression, genes belonging to the same pathway or similar function often show the same regulatory profiles under a variety of biological situations. Hence, as a hypothesis, genes of unknown function, for example, *HURP*, showing regulatory behavior similar to that of a gene of known function, for example, *Aurora-A*, may have a similar function.

In this study, we first took advantage of a range of well-organized and publicly accessible microarray data sets (e.g., SOURCE [http://source.stanford.edu]) (9) to search for genes related to Aurora-A by comparing their gene expression pro-



1 MSSSHFASRH RKDISTEMIR TKIAHRKSLS QKENRHKEYE RNRHFGLKDV NIPTLEGRIL
61 VELDETSQEL VPEKTNVKPR AMKTILGDQR KQMLQKYKEE KQLQKLKEQR EKAKRGIFKV
121 GRYRPDMPCF LLSNQNAVKA EPKKAIPSSV RITRSKAKDQ MEQTKIDNES DVRAIRPGPR
181 QTSEKKVSDK EKKVVQPVMP TSLRMTRSAT QAAKQVPRTV SSTTARKPVT RAANENEPEG
241 KVPSKGRPAK NVETKPDKGI SCKVDSEENT LNSQTNATSG MNPDGVLSKM ENLPEINTAK
301 IKGKNSFAPK DFMFQPLDGL KTYQVTPMTP RSANAFLTPS YTWTPLKTEV DESQATKEIL
361 AQKCKTYSTK TIQQDSNKLP CPLGPLTVWH EEHVLNKNEA TTKNLNGLPI KEVPSLERNE
421 GRIAQPHHGV PYFRNILQSE TEKLTSHCFE WDRKLELDIP DDAKDLIRTA VGQTRLLMKE
481 RFKQFEGLVD DCEYKRGIKE TTCTDLDGFW DMVSFQIEDV IHKFNNLIKL EESGWQVNNN
541 MNHNMNKNVF RKKVVSGIAS KPKQDDAGRI AARNRLAAIK NAMRERIRQE ECAETAVSVI
601 PKEVDKIVFD AGFFRVESPV KLFSGLSVSS EGPSQRLGTP KSVNKAVSQS RNEMGIPQQT
661 TSPENAGPQN TKSEHVKKTL FLSIPESRSS IEDAQCPGLP DLIEENHVVN KTDLKVDCLS
721 SERMSLPLLA GGVADDINTN KKEGISDVVE GMELNSSITS QDVLMSSPEK NTASQNSILE
781 EGETKISQSE LFDNKSLTTE CHLLDSPGLN CSNPFTQLER RHQEHARHIS FGGNLITFSP
841 LQPGEF

files in microarray data sets using cluster analysis (11). Such genes, even though similar in terms of expression pattern, do not reveal their biological significance without further empirical exploration and validation. To prove the concept, we were able to identify in the search several known Aurora-A substrates, such as TPX2 (24), centromere protein A (CENP-A) (25), and TACC3 (46), by the fact that they also have a gene expression pattern similar to that of *Aurora-A* in various microarray data sets. For example, both *Aurora-A* and *TPX2* are in the same cluster in NCI60 (32), the HeLa cell cycle (51), and carcinoma classification (40). Similar expression patterns for *Aurora-A* and *CENP-A* can also be found in the Normal Tissue Atlas (39) and Lymphoma (1). Together, this data-mining method may ultimately allow the use of available biochemical methods to study such identified targets and this will facilitate a greater understanding of downstream target prioritization and delineation of the cellular pathways governing Aurora-A carcinogenesis. While the current approach is just an initial attempt to answer various complex biological questions, the extent to which the coexpressed genes can be functionally correlated with each other and methods to achieve such integration have yet to be determined.

Several cellular components have been documented as Aurora-A substrates; however, the molecular significance of such phosphorylation has only been delineated for some of these. For instance, phosphorylation of protein phosphatase 1 by Aurora-A leads to its inactivation (20). Cytoplasmic polyadenylation element binding factor (CPEB), which induces translation of many maternal mRNAs by triggering polyadenylation, is phosphorylated by Aurora-A. The phosphorylation elicits the binding of cleavage and polyadenylation specificity factor (CPSF) and thereby recruits it into an active cytoplasmic polyadenylation complex (28). Phosphorylation of the *Drosophila* transforming acidic coiled coil (D-TACC) leads to its spindle targeting (14). Localization of CDC25B to the centrosome is dependent on phosphorylation by Aurora-A (10). As a substrate of Aurora-A, phosphorylation of CENP-A is essential for the concentration of Aurora-B, an important mitosis regulator, at the inner kinetochore (25). In summary, Aurora-A regulates substrates by modulating activity, downregulating stability, determining localization, and promoting protein-protein interaction. Therefore, the finding that Aurora-A is able to protect HURP from protein degradation suggests that Aurora-A not only negatively controls substrate stability but also positively regulates substrate half-life, further expanding the versatile range of functions linked to Aurora-A.

In this study, we further characterized *HURP*, which grouped into the same cluster as *Aurora-A* in the HeLa cell cycle (51) and the Normal Tissue Atlas database (39), as a downstream substrate for Aurora-A. We also investigated whether HURP might have some of the characteristics of TPX2, a substrate for Aurora-A essential for its localization to the spindle (24). However, the HURP-4P mutant still associated with the spindle (data not shown), indicating that any abnormal behavior of HURP-4P may not be due to a global conformational change caused by the serine-to-alanine mutations and that Aurora-A phosphorylation at these sites is not relevant to HURP spindle targeting. This result is in line with the observation that expression of pSUPER-Aurora-A does not alter the subcellular localization of HURP (data not shown). In addition, HURP knockdown by pSUPER-HURP also has no effect on the association of Aurora-A with spindles (data not shown), reflecting the fact that HURP does not seem to be involved in guiding Aurora-A to its location. All together, these findings suggest that although HURP is a downstream target of Aurora-A, it does not seem to participate in targeting of Aurora-A.

Both Aurora-A and cdk1/cyclin B (17) phosphorylate HURP, but at distinct phosphorylation sites. The two phosphorylation events differ in certain aspects. Thus, HURP stability is likely to be under positive and negative regulation in cells, depending on the various states of phosphorylation of HURP. On the one hand, HURP may be phosphorylated by cdk1, leading to ubiquitination and subsequent degradation when cells progress from M to G_1 phase (17). On the other hand, we propose here that Aurora-A may phosphorylate HURP and this probably attenuates the negative impact of cdk1 phosphorylation and by inhibiting subsequent proteasome activity and this will generate a longer HURP half-life. For instance, the HURP-4P mutant undergoes a more rapid degradation than its wild type version when cells are released from nocodazole blockage. It is worth pointing out that the HURP-4P mutant protein suddenly disappears at 8 h. Interestingly, HURP translocates to the nucleus when nocodazole is

FIG. 4. Aurora-A phosphorylates HURP at four residues in vitro. (A) HURP-WT and various deletion constructs were subjected to in vitro transcription and translation in the presence of [^{35}S]methionine, followed by incubation with Aurora-A-WT or -KD, respectively. The phosphorylation status of HURPs was judged by mobility shift revealed by SDS-PAGE and autoradiography. (B) Recombinant HURPs were incubated with Aurora-A in kinase reaction buffer. After reaction at 30°C for 30 min, the samples were subjected to SDS-PAGE and stained with Coomassie blue. HURP bands were carefully sliced from the gel, and phosphorylation sites were determined by LC MS/MS analysis. Four residues were determined to be Aurora-A-dependent phosphorylation sites (the residues with asterisks above them in the upper four graphs) with 65% amino acid sequence coverage (the lower sequence; underlined amino acids represent those residues resolved by LC MS/MS). An asterisk indicates a phosphorylation site. (C) The four Aurora-A in vitro phosphorylation sites on HURP, which had been determined by LC MS/MS, were replaced with alanine (designated HURP-4P). 293T cells were transiently transfected with HA–HURP-WT or HA–HURP-4P. Twenty-four hours after transfection, the cells were lysed and immunoprecipitated (IP) with anti-HA antibody. Immunocomplexes were incubated with Aurora-A in the kinase reaction buffer in the presence of [γ-^{32}P]ATP at 30°C for 30 min. Reaction mixtures were separated by SDS-PAGE and analyzed using Western blotting (WB) with anti-HA antibody (left) or autoradiography (right). The asterisk indicates the phosphorylation form of HURP. The HURP-4P mutant showed no [γ-^{32}P]ATP incorporation. (D) 293T cells were transiently transfected with HA–HURP-WT or HA–HURP-4P. Twenty-four hours after transfection, the 293T cells were either left untreated (referred to as asynchronized) or synchronized in mitosis by nocodazole treatment for 16 h. Equal amounts of extracts were prepared and analyzed by Western blotting with anti-HA antibody. A mobility upshift band (indicated by the asterisk) was found for HA–HURP-WT, but not HA–HURP-4P, suggesting differences in protein phosphorylation. Similar results were obtained in two independent experiments.

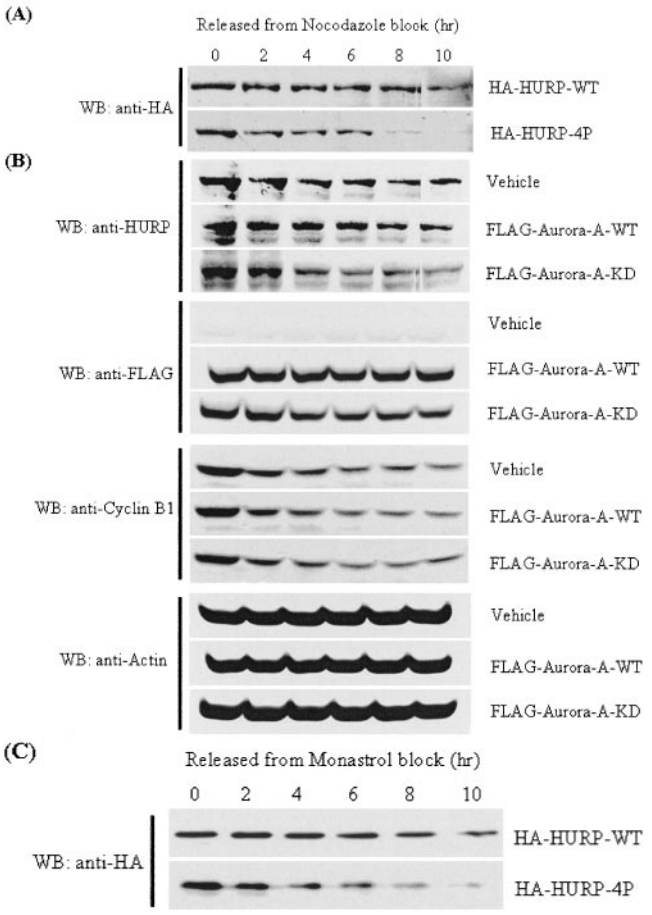


FIG. 5. Aurora-A protects HURP from protein degradation. 293T cells were transiently transfected with HA–HURP-WT or HA–HURP-4P. Twenty-four hours after transfection, the 293T cells were synchronized in mitosis by nocodazole (A) or monastrol (C) treatment for 16 h. Cells were released into cell cycle progression by removing these mitosis-synchronizing agents and subsequently incubated with fresh medium containing cycloheximide, which blocks de novo protein synthesis. At the indicated time points, cells were harvested and analyzed by Western blotting (WB) with anti-HA antibody. (B) 293T cells were transiently transfected with the pCMV2 vector (vehicle), FLAG–Aurora-A-WT, or FLAG–Aurora-A-KD; synchronized in mitosis by nocodazole treatment; and released from nocodazole blockage as described for panel A. At the indicated time points, the cells were harvested and analyzed by Western blotting with antibodies against HURP, FLAG, cyclin B1, and actin. Cyclin B1 is well known to be degraded at the M/G_1 transition, and the rapid decrease in cyclin B1 serves as a positive control showing that protein degradation under these conditions is working normally. Actin was used as a loading control. The results shown are representative of three independent experiments.

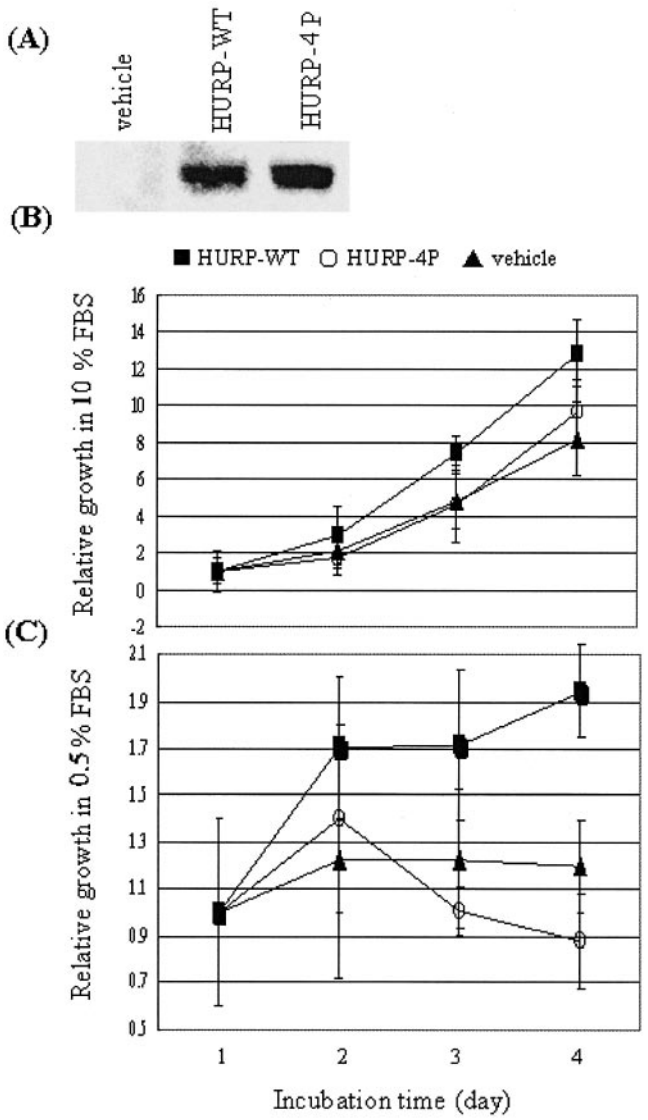


FIG. 6. HURP-4P stable transfectant cannot proliferate in a low-serum environment. (A) 293T cell pooled stable clones expressing the vehicle, HA–HURP-WT, or HA–HURP-4P were established, and the protein expression levels of these clones were determined by Western blotting using anti-HA antibody. These stable transfectants (5×10^3) were seeded into 96-well plates with either 10% (B) or 0.5% (C) FBS for 1 to 4 days, followed by MTT assay (OD_{570}) to quantify cell growth. Data were normalized against the OD_{570} value on day 1 for each pooled stable clone. The results are the averages of three independent assays.

removed, and the nuclear targeting of HURP reaches a peak at 8.5 h (data not shown). In contrast, HURP-4P fails to move to the nucleus in cells recovered from nocodazole arrest (data not shown). It can therefore be speculated that a HURP-4P mutant is subjected to protein degradation in the cytoplasm; however, some population of HURP-WT is transferred to the nucleus, thereby escaping from such degradation. What factors are important to the kinetics of degradation is still not understood. Our speculation is that more HURP remains in G_1 when *Aurora-A* expression is elevated, and this is observed in many human cancers. It is thus reasonable to speculate that an excess of deregulated HURP in G_1 phase is somehow crucial for cell transformation, and this also explains the considerable cellular energy that is expended to regulate HURP stability via both the negative cdk1 pathway and the positive Aurora-A pathway.

It appears likely that Aurora-A phosphorylation of HURP is an important determinant of cell growth promotion under low-serum conditions for the following reasons. First, Aurora-A stabilizes HURP, leading to the accumulation of intracellular

HURP, which is beneficial for cell transformation because cell lines with overexpressed HURP can grow in a serum-independent manner (50). Second, cells with the HURP-4P mutant lose the ability to grow in media containing low serum concentrations, suggesting that Aurora-A-elicited HURP phosphorylation is crucial to HURP cell transformation activity. Finally, *HURP* and *Aurora-A* are coexpressed in many cancers (Fig. 1), raising the possibility that a cancerous outcome, at least serum- and anchorage-independent growth, is potentiated by a synergistic effect of these two genes.

Aurora-A is reported to phosphorylate tumor suppressors like p53 (21, 27), BRCA1 (31), and Lats2 (47) and therefore is able to regulate their activities. For instance, Aurora-A phosphorylates p53, leading to destruction and loss of p53's DNA-binding activity (21, 27). Alternatively, Aurora-A is also capable of regulating some tumor-promoting pathways or molecules. For example, overexpression of Aurora-A potentiates HRAS-mediated cell transformation (45). Some of our recent work has pointed out that RalA, a tumor-promoting molecule, can serve as a substrate of Aurora-A and that this is essential for Aurora-A-triggered cell motility and anchorage-independent growth (52). Together, these findings suggest that Aurora-A may elicit tumor formation through multiple pathways by interacting with tumor suppressors and tumor promoters, adding even more complexity to this system and increasing the difficulty of uncovering the complete underlined mechanism of Aurora-A-induced tumorigenesis.

ACKNOWLEDGMENTS

We thank Erich A. Nigg for providing *Aurora-A* and *Aurora-B* cDNAs and Tang K. Tang for providing recombinant Aurora-C protein. We are also grateful to Hau-Chen Lin and Ming-Hong Lin for technical assistance and Yeou-Guang Tsay for LC MS/MS analysis.

This work was supported in part by grants from the National Health Research Institutes, the Taichung Veteran General Hospital (TCVGH-927312D), and the National Science Council (NRPGM: NHRI93A1-NSCMM-11-5) to C. F. Huang and a grant from the National Health Research Institutes (NHRI-EX90-9001-BL) to C. K. Chou.

REFERENCES

1. **Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. J. Hudson, L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, R. Levy, W. Wilson, M. R. Grever, J. C. Byrd, D. Botstein, P. O. Brown, and L. M. Staudt.** 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature **403:**503–511.
2. **Anand, S., S. Penrhyn-Lowe, and A. R. Venkitaraman.** 2003. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. Cancer Cell **3:**51–62.
3. **Bar-Shira, A., J. H. Pinthus, U. Rozovsky, M. Goldstein, W. R. Sellers, Y. Yaron, Z. Eshhar, and A. Orr-Urtreger.** 2002. Multiple genes in human 20q13 chromosomal region are involved in an advanced prostate cancer xenograft. Cancer Res. **62:**6803–6807.
4. **Bischoff, J. R., and G. D. Plowman.** 1999. The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. Trends Cell Biol. **9:**454–459.
5. **Bischoff, J. R., L. Anderson, Y. Zhu, K. Mossie, L. Ng, B. Souza, B. Schryver, P. Flanagan, F. Clairvoyant, C. Ginther, C. S. Chan, M. Novotny, D. J. Slamon, and G. D. Plowman.** 1998. A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J. **17:**3052–3065.
6. **Brummelkamp, T. R., R. Bernards, and R. Agami.** 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science **296:**550–553.
7. **Carmena, M., and W. C. Earnshaw.** 2003. The cellular geography of aurora kinases. Nat. Rev. Mol. Cell. Biol. **4:**842–854.
8. **Chiu, A. W., Y. L. Huang, S. K. Huan, Y. C. Wang, J. P. Ju, M. F. Chen, and C. K. Chou.** 2002. Potential molecular marker for detecting transitional cell carcinoma. Urology **60:**181–185.
9. **Diehn, M., G. Sherlock, G. Binkley, H. Jin, J. C. Matese, T. Hernandez-Boussard, C. A. Rees, J. M. Cherry, D. Botstein, P. O. Brown, and A. A. Alizadeh.** 2003. SOURCE: a unified genomic resource of functional annotations, ontologies, and gene expression data. Nucleic Acids Res. **31:**219–223.
10. **Dutertre1, S., M. Cazales, M. Quaranta, C. Froment, V. Trabut, C. Dozier, G. Mirey, J. P. Bouché, N. Theis-Febvre, E. Schmitt, B. Monsarrat, C. Prigent, and B. Ducommun.** 2004. Phosphorylation of CDC25B by Aurora-A at the centrosome contributes to the G_2-M transition. J. Cell Sci. **117:**2523–2531.
11. **Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein.** 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA **95:**14863–14868.
12. **Giet, R., and C. Prigent.** 1999. Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. J. Cell Sci. **112:**3591–3601.
13. **Giet, R., and D. M. Glover.** 2001. Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. J. Cell Biol. **152:**669–682.
14. **Giet, R., D. McLean, S. Descamps, M. J. Lee, J. W. Raff, C. Prigent, and D. M. Glover.** 2002. Drosophila Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. J. Cell Biol. **156:**437–451.
15. **Gritsko, T. M., D. Coppola, J. E. Paciga, L. Yang, M. Sun, S. A. Shelley, J. V. Fiorica, S. V. Nicosia, and J. Q. Cheng.** 2003. Activation and overexpression of centrosome kinase BTAK/Aurora-A in human ovarian cancer. Clin. Cancer Res. **9:**1420–1426.
16. **Higgins, G. M., and R. M. Anderson.** 1931. Experimental pathology of the liver. I. Restoration of liver of the white rat following partial surgical removal. Arch. Pathol. **12:**186–202.
17. **Hsu, J. M., Y. C. Lee, C. T. Yu, and C. Y. Huang.** 2004. Fbx7 functions in the SCF complex regulating Cdk1-cyclin B-phosphorylated hepatoma up-regulated protein (HURP) proteolysis by a proline-rich region. J. Biol. Chem. **279:**32592–32602.
18. **Huang, Y. L., A. W. Chiu, S. K. Huan, Y. C. Wang, J. P. Ju, and C. L. Lu.** 2003. Prognostic significance of hepatoma-up-regulated protein expression in patients with urinary bladder transitional cell carcinoma. Anticancer Res. **23:**2729–2733.
19. **Kapoor, T. M., T. U. Mayer, M. L. Coughlin, and T. J. Mitchison.** 2000. Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. J. Cell Biol. **150:**975–988.
20. **Katayama, H., H. Zhou, Q. Li, M. Tatsuka, and S. Sen.** 2001. Interaction and feedback regulation between STK15/BTAK/Aurora-A kinase and protein phosphatase 1 through mitotic cell division cycle. J. Biol. Chem. **276:**46219–46224.
21. **Katayama, H., K. Sasai, H. Kawai, Z. M. Yuan, J. Bondaruk, F. Suzuki, S. Fujii, R. B. Arlinghaus, B. A. Czerniak, and S. Sen.** 2004. Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53. Nat. Genet. **36:**55–62.
22. **Kimura, M., Y. Matsuda, T. Yoshioka, and Y. Okano.** 1999. Cell cycle-dependent expression and centrosome localization of a third human aurora/Ipl1-related protein kinase, AIK3. J. Biol. Chem. **274:**7334–7340.
23. **Kimura, M., S. Kotani, T. Hattori, N. Sumi, T. Yoshioka, K. Todokoro, and Y. Okano.** 1997. Cell cycle-dependent expression and spindle pole localization of a novel human protein kinase, Aik, related to Aurora of Drosophila and yeast Ipl1. J. Biol. Chem. **272:**13766–13771.
24. **Kufer, T. A., H. H. Sillje, R. Korner, O. J. Gruss, P. Meraldi, and E. A. Nigg.** 2002. Human TPX2 is required for targeting Aurora-A kinase to the spindle. J. Cell Biol. **158:**617–623.
25. **Kunitoku, N., T. Sasayama, T. Marumoto, D. Zhang, S. Honda, O. Kobayashi, K. Hatakeyama, Y. Ushio, H. Saya, and T. Hirota.** 2003. CENP-A phosphorylation by Aurora-A in prophase is required for enrichment of Aurora-B at inner centromeres and for kinetochore function. Dev. Cell **5:**853–864.
26. **Li, D., J. Zhu, P. F. Firozi, J. L. Abbruzzese, D. B. Evans, K. Cleary, H. Friess, and S. Sen.** 2003. Overexpression of oncogenic STK15/BTAK/Aurora A kinase in human pancreatic cancer. Clin. Cancer Res. **9:**991–997.
27. **Liu, Q., S. Kaneko, L. Yang, R. I. Feldman., S. V. Nicosia, J. Chen, and J. Q. Cheng.** 2004. Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. J. Biol. Chem. **279:**52175–52182.
28. **Mendez, R., K. G. Murthy, K. Ryan, and J. D. Richter.** 2000. Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Mol. Cell **6:**1253–1259.
29. **Miyoshi, Y., K. Iwao, C. Egawa, and S. Noguchi.** 2001. Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. Int. J. Cancer **92:**370–373.
30. **Nigg, E. A.** 2001. Mitotic kinases as regulators of cell division and its checkpoints. Nat. Rev. Mol. Cell. Biol. **2:**21–32.
31. **Ouchi, M., N. Fujiuchi, K. Sasai, H. Katayama, Y. A. Minamishima, P. P. Ongusaha, C. Deng, S. Sen, S. W. Lee, and T. Ouchi.** 2004. BRCA1 phos-

phorylation by Aurora-A in the regulation of G_2 to M transition. J. Biol. Chem. **279:**19643–19648.
32. **Ross, D. T., U. Scherf, M. B. Eisen, C. M. Perou, C. Rees, P. Spellman, V. Iyer, S. S. Jeffrey, M. Van de Rijn, M. Waltham, A. Pergamenschikov, J. C. Lee, D. Lashkari, D. Shalon, T. G. Myers, J. N. Weinstein, D. Botstein, and P. O. Brown.** 2000. Systematic variation in gene expression patterns in human cancer cell lines. Nat. Genet. **24:**227–235.
33. **Sakakura, C., A. Hagiwara, R. Yasuoka, Y. Fujita, M. Nakanishi, K. Masuda, K. Shimomura, Y. Nakamura, J. Inazawa, T. Abe, and H. Yamagishi.** 2001. Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. Br. J. Cancer **84:**824–831.
34. **Savelieva, E., C. D. Belair, M. A. Newton, S. DeVries, J. W. Gray, F. Waldman, and C. A. Reznikoff.** 1997. 20q gain associates with immortalization: 20q13.2 amplification correlates with genome instability in human papillomavirus 16 E7 transformed human uroepithelial cells. Oncogene **14:**551–560.
35. **Sen, S., H. Zhou, R. D. Zhang, D. S. Yoon, F. Vakar-Lopez, S. Ito, F. Jiang, D. Johnston, H. B. Grossman, A. C. Ruifrok, R. L. Katz, W. Brinkley, and B. Czerniak.** 2002. Amplification/overexpression of a mitotic kinase gene in human bladder cancer. J. Natl. Cancer Inst. **94:**1320–1329.
36. **Severson, A. F., D. R. Hamill, J. C. Carter, J. Schumacher, and B. Bowerman.** 2000. The aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis. Curr. Biol. **10:**1162–1171.
37. **Shindo, M., H. Nakano, H. Kuroyanagi, T. Shirasawa, M. Mihara, D. J. Gilbert, N. A. Jenkins, N. G. Copeland, H. Yagita, and K. Okumura.** 1998. cDNA cloning, expression, subcellular localization, and chromosomal assignment of mammalian aurora homologues, aurora-related kinase (ARK) 1 and 2. Biochem. Biophys. Res. Commun. **244:**285–292.
38. **Smith, M. W., Z. N. Yue, G. K. Geiss, N. Y. Sadovnikova, V. S. Carter, L. Boix, C. A. Lazaro, G. B. Rosenberg, R. E. Bumgarner, N. Fausto, J. Bruix, and M. G. Katze.** 2003. Identification of novel tumor markers in hepatitis C virus-associated hepatocellular carcinoma. Cancer Res. **63:**859–864.
39. **Su, A. I., M. P. Cooke, K. A. Ching, Y. Hakak, J. R. Walker, T. Wiltshire, A. P. Orth, R. G. Vega, L. M. Sapinoso, A. Moqrich, A. Patapoutian, G. M. Hampton, P. G. Schultz, and J. B. Hogenesch.** 2002. Large-scale analysis of the human and mouse transcriptomes. Proc. Natl. Acad. Sci. USA **99:**4465–4470.
40. **Su, A. I., J. B. Welsh, L. M. Sapinoso, S. G. Kern, P. Dimitrov, H. Lapp, P. G. Schultz, S. M. Powell, C. A. Moskaluk, H. F. J. Frierson, and G. M. Hampton.** 2001. Molecular classification of human carcinomas by use of gene expression signatures. Cancer Res. **61:**7388–7393.
41. **Takahashi, T., M. Futamura, N. Yoshimi, J. Sano, M. Katada, Y. Takagi, M. Kimura, T. Yoshioka, Y. Okano, and S. Saji.** 2000. Centrosomal kinases, HsAIRK1 and HsAIRK3, are overexpressed in primary colorectal cancers. Jpn. J. Cancer Res. **91:**1007–1014.
42. **Tanaka, T., M. Kimura, K. Matsunaga, D. Fukada, H. Mori, and Y. Okano.** 1999. Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast. Cancer Res. **59:**2041–2044.
43. **Tanner, M. M., M. Tirkkonen, A. Kallioniemi, C. Collins, T. Stokke, R. Karhu, D. Kowbel, F. Shadravan, M. Hintz, and W. L. Kuo.** 1994. Increased copy number at 20q13 in breast cancer: defining the critical region and exclusion of candidate genes. Cancer Res. **54:**4257–4260.
44. **Tanner, M. M., M. Tirkkonen, A. Kallioniemi, K. Holli, C. Collins, D. Kowbel, J. W. Gray, O. P. Kallioniemi, and J. Isola.** 1995. Amplification of chromosomal region 20q13 in invasive breast cancer: prognostic implications. Clin. Cancer Res. **1:**1455–1461.
45. **Tatsuka, M., S. Sato, S. Kitajima, S. Suto, H. Kawai, M. Miyauchi, I. Ogawa, M. Maeda, T. Ota, and T. Takata.** 2004. Overexpression of Aurora-A potentiates HRAS-mediated oncogenic transformation and is implicated in oral carcinogenesis. Oncogene **24:**1122–1127.
46. **Tien, A. C., M. H. Lin, L. J. Su, Y. R. Hong, T. S. Cheng, Y. C. Lee, W. J. Lin, I. H. Still, and C. Y. Huang.** 2004. Identification of the substrates and interaction proteins of aurora kinases from a protein-protein interaction model. Mol. Cell. Proteomics **3:**93–104.
47. **Toji, S., N. Yabuta, T. Hosomi, S. Nishihara, T. Kobayashi, S. Suzuki, K. Tamai, and H. Nojima.** 2004. The centrosomal protein Lats2 is a phosphorylation target of Aurora-A kinase. Genes Cells **9:**383–397.
48. **Tsay, Y. G., Y. H. Wang, C. M. Chiu, B. J. Shen, and S. C. Lee.** 2000. A strategy for identification and quantitation of phosphopeptides by liquid chromatography/tandem mass spectrometry. Anal. Biochem. **287:**55–64.
49. **Tseng, T. C., S. H. Chen, Y. P. Hsu, and T. K. Tang.** 1998. Protein kinase profile of sperm and eggs: cloning and characterization of two novel testis-specific protein kinases (AIE1, AIE2) related to yeast and fly chromosome segregation regulators. DNA Cell Biol. **10:**823–833.
50. **Tsou, A. P., C. W. Yang, C. Y. Huang, R. C. Yu, Y. C. Lee, C. W. Chang, B. R. Chen, Y. F. Chung, M. J. Fann, C. W. Chi, J. H. Chiu, and C. K. Chou.** 2003. Identification of a novel cell cycle regulated gene, HURP, overexpressed in human hepatocellular carcinoma. Oncogene **22:**298–307.
51. **Whitfield, M. L., G. Sherlock, A. J. Saldanha, J. I. Murray, C. A. Ball, K. E. Alexander, J. C. Matese, C. M. Perou, M. M. Hurt, P. O. Brown, and D. Botstein.** 2002. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol. Biol. Cell **13:**1977–2000.
52. **Wu, J. C., T. Y. Chen, C. T. Yu, S. J. Tsai, J. M. Hsu, M. J. Tang, C. K. Chou, W. J. Lin, C. J. Yuan, and C. Y. Huang.** 2005. Identification of V23RalA-Ser194 mediates cellular motility and transformation of Aurora-A through small pool expression screening. J. Biol. Chem. **280:**9013–9022.
53. **Yakushijin, Y., M. Hamada, and M. Yasukawa.** 2004. The expression of the Aurora-A gene and its significance with tumorgenesis in non-Hodgkin's lymphoma. Leuk. Lymphoma **45:**1741–1746.
54. **Zhou, H., J. Kuang, L. Zhong, W. L. Kuo, J. W. Gray, A. Sahin, B. R. Brinkley, and S. Sen.** 1998. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat. Genet. **20:**189–193.