

Functional Implication of Human Serine/Threonine Kinase, hAIK, in Cell Cycle Progression

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Key Words

Kinase · Mitosis · Cell cycle · Centrosome

Abstract

Protein phosphorylation is involved in many biological activities and plays important roles in cell cycle progression. In the present study, we identified a serine/threonine kinase, hAIK, from human hepatic cells using degenerated polymerase chain reactions with a pair of primers derived from the highly conserved sequence in the catalytic domain of kinases. The full-length hAIK cDNA was then obtained, which contained 403 amino acids and was homologous to *Drosophila Aurora2* and yeast Ipl1 proteins. Northern blotting analysis revealed that hAIK was highly expressed in the testis but not in other tissues. Expressions of hAIK drastically increased in cancer tissues/cell lines but not in fibroblasts or nontumorigenic cell lines. The recombinant hAIK protein phosphorylated itself and histone H1; this phosphorylation activity was totally abolished after a point mutation at the catalytic domain (hAIKm). During the interphase cell, hAIK was found mainly in the cytoplasm; during mitosis hAIK accumulated at the centrosomes. In addition, overexpression of hAIK in cancer cell lines (HEK293T and HeLa) appeared to inhibit cell cycle progression. None of

these phenomena were observed in hAIKm whose kinase activity was rendered inactive. Our results suggest that hAIK protein/activity might modulate cell cycle progression by interacting with the centrosomes and/or proteins associated with these structures.

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Cells divide by duplicating their chromosomes and segregating one copy of the duplicated chromosomes to each of two daughter cells. Losing control of cell cycle progression, e.g. disruption of mitotic checkpoints, overexpression or mutational activation of oncogenes, and inaccurate segregation of chromosomes, may lead to growth abnormalities, or even tumorigenesis [4, 17, 23].

It has been shown that protein phosphorylation, an important posttranslational modification process, is involved in modulation of cell cycle progression [13, 14, 23]. For example, cyclin-dependent kinases (Cdks) have been implicated in the control of cell division through a complex network of cyclin interactions and phosphorylation events [22]: during the G1 phase, cyclin D1/cdk4 complex may be phosphorylated by cyclin H/cdk7 and imported from cytoplasm to nucleus [1, 18]. During G2-M transition, phosphorylation of cyclin B1 may promote its nuclear accumulation, then increase the binding effi-

ciency of *cdc2* and entry of mitosis [19, 31]. In mitotic cells, the initiation of chromosome segregation depends on the activation of anaphase-promoting complex/cyclosome (APC/C) [16, 25]. Phosphorylated APC may turn on the ubiquitination pathway to degrade the B-type cyclins, and thereby advance mitosis into anaphase. Although both *cdk2* and the polo-like kinases (PLKs) have been suggested to modulate centrosomal activity during mitosis [21, 30], detailed mechanisms regarding how these and other kinases regulate the process of chromosome segregation are still unclear [7].

Identification and characterization of protein kinases typically involve complicated purification procedures and functional assays; recent progress in the human genome project, however, greatly facilitated these tasks by providing more complete genetic databases and ways to annotate their potential functions. Using homologous alignment among many known protein kinase sequences, we previously identified several highly conserved sequence stretches at the catalytic domain of the kinases [12]. In our present study, we used the degenerate-oligonucleotide-primed polymerase chain reaction (PCR) method to identify a human serine/threonine kinase from human hepatoma cells. This kinase was highly homologous to aurora and *ipl* kinase gene of *Drosophila* [27] and yeast [5, 9], respectively, and was therefore named hAIK. The hAIK gene was highly expressed in the testis and cancer cells. The localization and gene expression studies both suggested that hAIK might play a role in modulating chromosome segregation and cell cycle progression.

Material and Methods

Cloning of hAIK

Total RNA was prepared from a human hepatoma cell line HuH-7 using the guanidinium isothiocyanate (GITC) method [10]. Rapid-Amplify-cDNA-End (RACE) procedures were then performed following the protocols from the user manual of Marathon cDNA amplification kit (Clontech, Inc., Calif., USA). Briefly, the first strand cDNA was synthesized from 10 µg total RNA extracted from HuH-7 cells using MMLV-reverse transcriptase with adapter-linked oligo-dT as primers. The first PCRs were performed using forward primers F1 5'-AGTCTAGAAA(A/G)(G/A)TNTGNGA(T/C)T(T/C)GG-3', F2 5'-AGTCTAGAAA(A/G)(G/A)TNGGNGA(T/C)TT(T/C)GG, F3 5'-AGTCTAGAAA(A/G)(G/A)TN(A/G)CNGA(T/C)TT(T/C)GG and backward primer 5'ATCTGCAGNCC(G/A)ANG(C/A)CCCANAGGTC-3', carried out for 5 cycles at 94°C for 1 min, 42°C for 2 min, and 72°C for 1 min and then 25 cycles at 94°C for 40 s, 55°C for 30 s, and 72°C for 45 s. The resulting 170-bp fragment was cloned into PCRII (Invitrogen Co., Calif., USA) and sequenced. Two primers (5'-TCCAGGACCACTCTTTGTGGC-3' and 5'-TCCGACCTTC AATCATTTTCAGGGG-3') derived from the above-mentioned DNA fragment were applied with the adapter

primers for the 5'- and 3'-RACE PCR. The PCR condition was carried out for 25 cycles at 94°C for 30 s, 60°C for 30 s, and 68°C for 4 min [13]. The PCR products were again sub-cloned into PCRII and sequenced to obtain the full-length hAIK cDNA clone.

Generation of the Recombinant hAIK Proteins

The full-length hAIK cDNA was subcloned into GEX-2T vector (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and named pGST-hAIK. The kinase-dead hAIK mutant (hAIKm) was constructed by QuickChange™ Site-directed Mutagenesis Kit (Stratagene, Ltd., Calif., USA) with a pair of primers (5'-GCAAGTTTATTTTGGCTTTTATAGTGTTATTTAAAGC-3' and 5'-GCTTTAATAAACAATAAAAAGCCAAAATAAACTTGC-3'). Both pGST-hAIK and pGST-hAIKm were transformed into bacteria strain BL-21 using the calcium phosphate method. IPTG was then added to induce the production of fusion proteins. After 1 h induction, the transformed cells were washed and resuspended in PBS containing 5 mg/ml leupeptin, 2 mM phenyl-methyl-sulfonyl-fluoride, 5 mg/ml aprotinin, and lysed by French Press. The fusion proteins were affinity-purified using glutathione beads (Amersham Pharmacia Biotech AB) and eluted by adding 3 mg/ml glutathione in 50 mM Tris-HCl, pH8.0.

Northern Blot Analysis

The 2,045-bp hAIK cDNA inserts were obtained by *Bam*HI and *Xba*I digestion, and labeled with [γ -³²P]-dCTP using random priming (Amersham Pharmacia Biotech AB). The isotope-labeled probes were hybridized to either human multiple tissue RNA blots as described in the manufacturer's manual (Clontech), or with mRNA extracted from the cell lines [10].

The in vitro Phosphorylation Assay

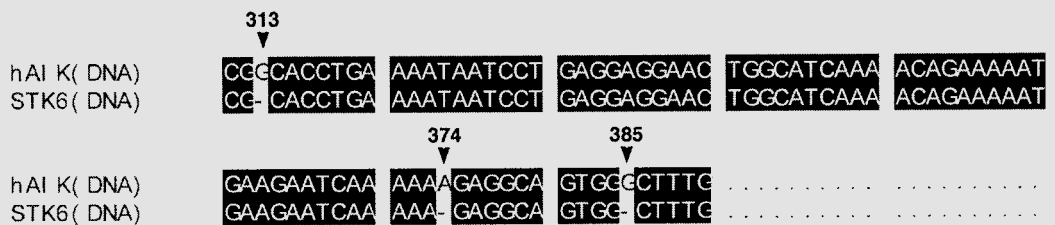
The purified hAIK fusion proteins were resuspended in 20 µl kinase buffer (20 mM HEPES, pH 7.2, 2 mM DTT, 0.1 mM EGTA, 0.1 µg/ml BSA, 0.1 mM sodium vanadate, 10 mM MgCl₂, 10 mM MnCl) containing 20 µM ATP, 5 µCi [γ -³²P]-ATP. Histone (1 µg/µl) was added into the reaction mixture as substrate for the in vitro phosphorylation assay. The reaction was carried out at 30°C for 30 min, terminated by boiling in Laemmli-SDS-PAGE sample buffer, and analyzed by 10% SDS-polyacrylamide gel electrophoresis. These amounts of reacting proteins were measured by Coomassie blue staining and the degrees of phosphorylation were visualized using autoradiography.

Immunofluorescence Staining

Immunofluorescence staining was performed according to previously recorded methods [20]. Polyclonal anti-hAIK antibody was produced by injecting a hAIK-specific synthetic peptide (NCQNKE-SASKQS) into rabbits. The hAIKm gene was cloned into pEGFP-N1 (Clontech) and transfected into HeLa cells using the lipofectamine method (Gibco BRL, New York, N.Y., USA). HeLa cells cultured on cover-slips for 24 h were fixed with 4% formaldehyde for 30 min, permeabilized with 0.5% Triton X-100/fixative for 5 min, and then stained with anti-hAIK antibody and anti- α - or γ -tubulin antibody (Sigma, St. Louis, Mo., USA) at 4°C overnight, followed by addition of fluorochrome-labeled secondary antibodies (Jackson Immuno-research Co., Penn., USA). The stained slides were observed under a confocal laser-scanning microscope (Leica TCS NT, Heidelberg, Germany).

hAI K_aa	-----MDR	SKENCI SGPV	KATAPVGGPK	RVLVTCQFP	CONPLPVNS-	41
STK15_aa	-----MDR	SKENCI SGPV	KATAPVGGPK	RVLVTCQFP	CONPLPVNS-	41
ARK1_aa	-----MDR	SKENCI SGPV	KATAPVGGPK	RVLVTCQFP	CONPLPVNS-	41
STK6_aa	-----MDR	SKENCI SGPV	KATAPVGGPK	RVLVTCQFP	CONPLPVNS-	41
aur or a_aa	MSHPSDHVL	RKENAPHRMP	EKSAALNMQ	KNLLLGKPN	SENMAFDSKP	50
i pl 1_aa	MGRNSLVNI K	LNANSPSKKT	TTRPNTSRI N	KPWRI SHS-	-CGRNENSK-	47
hAI K_aa	--GOAQRVLC	PSNSSQRI PL	QACKLVSS--	-----FK	PVGNQKQKQL	79
STK15_aa	--GOAQRVLC	PSNSSQRI PL	QACKLVSS--	-----FK	PVGNQKQKQQL	79
ARK1_aa	--GOAQRVLC	PSNSSQRI PL	QACKLVSS--	-----FK	PVGNQKQKQQL	79
STK6_aa	--GOAQRVLC	PSNSSQRI PL	QACKLVSS--	-----FK	PVGNQKQKQQL	79
aur or a_aa	LPSSGALI R	SAATTVPAT	KPGLGCSNSI	ASSEGNFGK	EMVPSVKITTT	100
i pl 1_aa	---IPSPVR	EKLN-RLV	NNKK-----	-----	-----FLDM	69
hAI K_aa	QATSVPHPV	RPLNNTQKSL	QPLPSAPENN	PEEELASKQK	NEESK-----	124
STK15_aa	QATSVPHPV	RPLNNTQKSK	QPLPSAPENN	PEEELASKQK	NEESK-----	124
ARK1_aa	QATSVPHPV	RPLNNTQKSK	QPLPSAPENN	PEEELASKQK	NEESK-----	124
STK6_aa	QATSVPHPV	RPLNNTQKSK	QPLPSHLKI I	LRRNWHQNRK	MKNCK-----	124
aur or a_aa	SEFAAPVIA	PI KKPESLSK	CK-LTAAASSE	SSKELGAASS	SAEKEKTKTE	149
i pl 1_aa	ESSKI PSL R	K-----	---ATSSKM	HE--NKKLFR	-----	95
hAI K_aa	-----KROM	ALEDDEFI GRP	LGGKFGNVY	LAREKQSKFI	LALKVLFKAC	168
STK15_aa	-----KROM	ALEDDEFI GRP	LGGKFGNVY	LAREKQSKFI	LALKVLFKAC	168
ARK1_aa	-----KROM	ALEDDEFI GRP	LGGKFGNVY	LAREKQSKFI	LALKVLFKAC	168
STK6_aa	-----EAV	ALEDDEFI GRP	LGGKFGNVY	LAREKQSKFI	LALKVLFKAC	167
aur or a_aa	TOPQKPKTV	ELNNFDI GRF	LGGKFGNVY	LAREKESGVY	VALKVLFKFC	199
i pl 1_aa	-----FKSL	SLDFEELCKK	LGGKFGNVY	CVFHRSTGYI	CALKVMEKEE	139
hAI K_aa	LEKAGVEHQ	RREVEI QSHL	RHPNI LRLYG	YFHDATRVYL	I LEYAPLGTV	218
STK15_aa	LEKAGVEHQ	RREVEI QSHL	RHPNI LRLYG	YFHDATRVYL	I LEYAPLGTV	218
ARK1_aa	LEKAGVEHQ	RREVEI QSHL	RHPNI LRLYG	YFHDATRVYL	I LEYAPLGTV	218
STK6_aa	LEKAGVEHQ	RREVEI QSHL	RHPNI LRLYG	YFHDATRVYL	I LEYAPLGTV	217
aur or a_aa	I GESNVHECV	RREVEI QSHL	RHPNI LRLYG	YFHDATRVYL	I LEYAPLGTV	249
i pl 1_aa	I I KYNLQKCF	RREVEI QSHL	RHPNI LRLYG	YFHDATRVYL	I LEYAPLGTV	189
hAI K_aa	YRELQKLL--S	KFDEQRTATY	I TELANALSY	CHSKRVI HRD	I KPENLLLGS	266
STK15_aa	YRELQKLL--S	KFDEQRTATY	I TELANALSY	CHSKRVI HRD	I KPENLLLGS	266
ARK1_aa	YRELQKLL--S	KFDEQRTATY	I TELANALSY	CHSKRVI HRD	I KPENLLLGS	266
STK6_aa	YRELQKLL--S	KFDEQRTANL	YNRI ANALSY	CHSKRVI HRD	I KPENLLLGS	265
aur or a_aa	FNALGACPMS	RFDBRGSATY	I QALCSALLY	I HERDI I HRD	I KPENLLIGH	299
i pl 1_aa	YKLLRLRH--G	RFNDI LASCY	I YQI ANALDY	MEKRN I HRD	I KPENLLIGF	237
hAI K_aa	AGELKI ADFG	WSVHAP--FFR	RTTLCGTLDY	LPPEM EGRM	HDEKVDLWSL	315
STK15_aa	AGELKI ADFG	WSVHAP--SSR	RTTLCGTLDY	LPPEM EGRM	HDEKVDLWSL	315
ARK1_aa	AGELKI ADFG	WSVHAP--SSR	RTTLCGTLDY	LPPEM EGRM	HDEKVDLWSL	315
STK6_aa	AGELKI ADFG	WSVHAP--SSR	RTTLCGTLDY	LPPEM EGRM	HDEKVDLWSL	314
aur or a_aa	KEVLKI ADFG	WSVHAP--NSM	RTTLCGTLDY	LPPEM VGGPK	HTKNVDLWSL	348
i pl 1_aa	NNVI KLTDFG	WSIT NPPENR	RTTLCGTLDY	LPPEM VESRE	YDHTI LAVAL	267
hAI K_aa	GVLCYEFVLC	KPPFEANTYC	ETYKRI SRVE	FTFPDFITEG	ARDLI SRLLK	365
STK15_aa	GVLCYEFVLC	KPPFEANTYC	ETYKRI SRVE	FTFPDFITEG	ARDLI SRLLK	365
ARK1_aa	GVLCYEFVLC	KPPFEANTYC	ETYKRI SRVE	FTFPDFITEG	ARDLI SRLLK	365
STK6_aa	GVLCYEFVLC	KPPFEANTYC	ETYKRI SRVE	FTFPDFITEG	ARDLI SRLLK	364
aur or a_aa	GVLCYEFVLC	HAPFYSKND	ETYKRI LKVD	YKLEHESKA	ASTLI SKLLV	398
i pl 1_aa	GVLAPELLTG	APPFEEMKD	TIYKRI AALD	I KMSNLSOD	ACDLI LKLLK	337
hAI K_aa	HNPSQRPMLK	EVLEHPW TA	NSSKPSNCOQ	KESASKQS	403
STK15_aa	HNPSQRPMLR	EVLEHPW TA	NSSKPSNCOQ	KESASKQS	403
ARK1_aa	HNPSQRPMLR	EVLEHPW TA	NSSKPSNCOQ	KESASKQS	403
STK6_aa	HNPSQRPMLR	EVLEHPW TA	NSSKPSNCOQ	KESASKQS	402
aur or a_aa	LNPCQFLPLD	GVMMHPW LA	HTQ	421
i pl 1_aa	YDFKCFMRLG	GVMMHPW LR	NKPFVENKRL	367

a



1

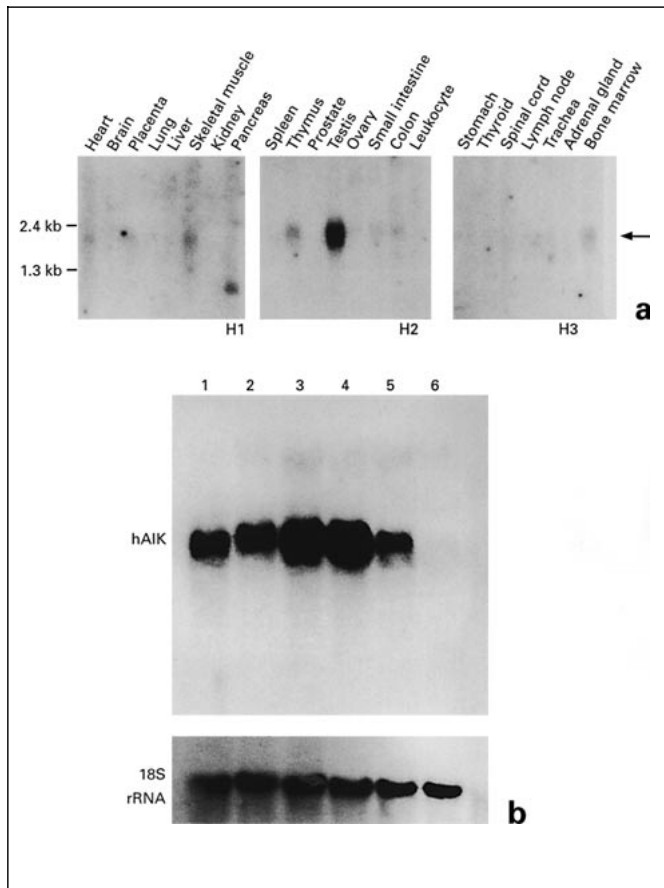


Fig. 2. Northern blotting analyses of hAIK gene in various tissues and cell lines. **a** A representative example of Northern blotting analyses using hAIK cDNA probes on human multiple tissue RNA blots. The 2.1-kb hAIK transcript is indicated (arrow). **b** Northern blotting analyses were performed in various cell lines. Note hAIK was highly expressed in all cancer cell lines, including three well-differentiated human hepatoma cells, HuH-7 (lane 1), Hep 3B (lane 2), and Hep G2 (lane 3), the poorly differentiated HA22T hepatoma cell, (lane 4), and the human cervical carcinoma HeLa cells (lane 5). Expression of hAIK, however, was barely detectable in non-tumorigenic human fibroblasts (lane 6). 18S rRNA was used as a reference for equal loading of the samples.

Fig. 1. Sequence alignment among various *aurora/ipl*-related genes. **a** The protein sequences are presented in single-letter codes, with number of amino acid residue shown along each line. The stretches of identical sequences among these genes are shaded. The highly conserved catalytic domain obtained using degenerated PCR is also marked (asterisks). **b** The partial DNA sequences of hAIK (No. 311–390) and STK6 (No. 311–387) are aligned. Three additional nucleotides in hAIK are located at positions 313, 374, 385 (arrowheads).

The Cell Proliferation Assay

HeLa or HEK293T cells were transfected with hAIK using the lipofectamine method (Gibco BRL). After 24–48 h, the cells were harvested and washed twice with PBS at 4 °C, fixed with 2% formaldehyde in PBS for 20 min, and 70% ethanol at 4 °C. To measure the DNA contents, the fixed cells were stained with propidium iodide (PI) solution (50 µg/ml propidium iodide, 100 µg/ml ribonuclease A in PBS) for 20 min on ice, pre-filtrated with Nylon Mesh, and assayed using a flow cytometer (FACSVantage, Becton Dickinson). These results were analyzed using the CellQuest and ModFit-LT software.

Results

Molecular Cloning of hAIK Gene from Human Hepatoma Cells

To investigate the potential roles of protein kinases in the carcinogenesis processes of human liver cancers, we have used degenerate oligonucleotide-primed PCR to identify various protein kinase genes from HuH-7 hepatoma cells. From one of the resulting 170-bp PCR fragments (asterisks, fig. 1), we have used 5'- and 3'-RACE experiments to obtain a 2,045-bp cDNA clone which contained a putative N-terminal methionine, an un-translated region, and a poly-A tail. As shown in figure 1a, this gene encoded a product of 403 amino acid residues and was named *hAIK* since this human gene was highly homologous to the *Drosophila aurora2* [11] and yeast *ipl* kinase genes [5]. Amino acid sequence comparison revealed that *hAIK* was a member of the *aurora/ipl*-related gene family (fig. 1a). It shared 98% sequence identity with *STK15* [32] and *ARK1* [18] originally identified from human colon cancer tissues and Jurket T-cell line, respectively, and 95% sequence identity with *STK6* from a human B-cell line [15]. When comparing the stretch of 105–123 amino acid among four human *aurora/ipl* genes (fig. 1a), we found that hAIK was identical to both *STK15* and *ARK1*, but different from *STK6*. While examining the published DNA sequence of these genes (fig. 1b), we noticed that the difference might actually have resulted from three single-nucleotide insertions at the position 313 (G), 374 (A), and 385 (G), which caused a short stretch of frame-shift during peptide decoding.

hAIK Was Highly Expressed in the Testis and Various Cancer Cell Lines

Northern blotting analysis showed that hAIK was highly expressed in the cells of testis, and moderately expressed in bone marrow, skeletal muscle and the thymus (fig. 2a). Only trace amounts of hAIK mRNA were found in other tissues, including the liver. Interestingly, hAIK

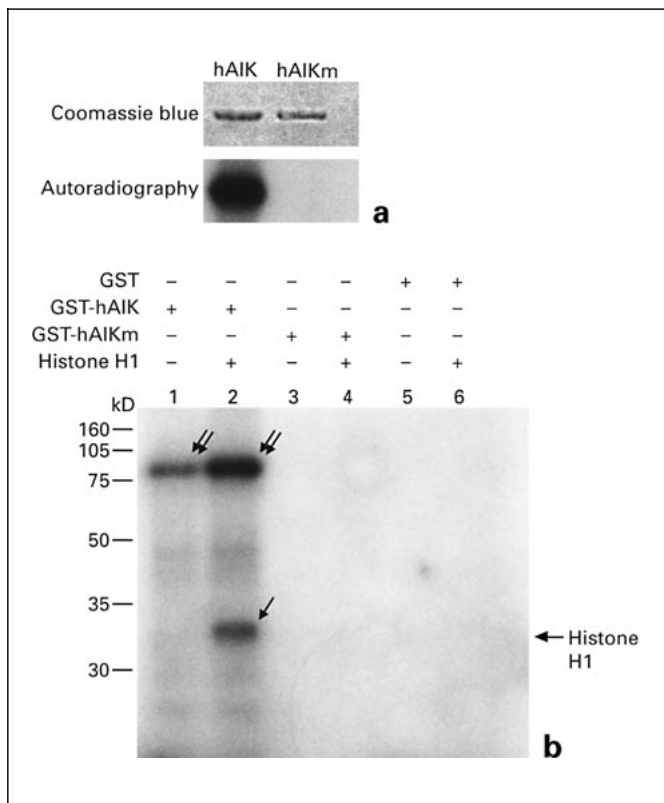


Fig. 3. The recombinant hAIK protein, but not the point mutation construct, possessed active kinase activities. **a** In vitro phosphorylation assays were performed on the *E. coli*-expressed hAIK fusion proteins, GST-hAIK and GST-hAIKm. With equal amounts of proteins (Coomassie blue-stained panel), only GST-hAIK exhibited statistically significant autophosphorylation activity (autoradiograph panel). **b** In vitro phosphorylation assays were performed using histone H1 as the substrate. Note only GST-hAIK (lanes 1–2), but not GST-hAIKm (lanes 3–4) or GST alone (lanes 5–6), could phosphorylate itself (double arrow) and histone H1 substrate (arrow).

was highly expressed in all human hepatoma cell lines examined (fig. 2b), including well-differentiated HuH-7, Hep 3B and Hep G2 (lane 1–3), and poorly differentiated HA22T cells (lane 4). Note HeLa cells, from human cervical carcinoma, also exhibited high hAIK expression (lane 5), while non-tumorigenic human fibroblast did not contain significant amounts of transcript (lane 6).

Recombinant hAIK Exhibited Active Phosphorylation Activities

We then addressed whether the recombinant hAIK proteins were active in kinase activity. To this end, we have performed in vitro phosphorylation assays on both recombinant wild-type hAIK (GST-hAIK), and a kinase-

inactive mutant, GST-hAIKm, whose lysine 163 was changed into isoleucine (see ‘Materials and Methods’). A typical example of this experiment is shown in figure 3. Given the same amount of proteins (measured by the Coomassie blue staining), note GST-hAIK, but not GST-hAIKm, was able to phosphorylate itself (autoradiograph, fig. 3a), and this autophosphorylation activity appeared to be constitutively active. The histone H1 (fig. 3b) or MBP proteins (data not shown) were also used as substrates to measure the phosphorylation function of the recombinant hAIK proteins. While GST-hAIK could phosphorylate itself (lanes 1–2, double arrow) and histone H1 (lane 2, arrow), no (auto)phosphorylation activity was detected when using GST-hAIKm (lane 3–4) and GST proteins (lane 5–6) as the catalysts. It is interesting to note that in the presence of histone H1, there appeared to be a slight increase of GST-hAIK auto-phosphorylation (comparing lane 2 with 1); the nature of this phenomenon, however, is not clear.

Redistribution of hAIK Proteins during Mitosis

We then examined the localization of endogenous hAIK proteins using immunofluorescence techniques (fig. 4). During interphase, hAIK proteins were found mainly in the cytoplasm (asterisks). When the cell started to divide during early prophase, significant amounts of hAIK had already accumulated at the centrosomes (arrows). During the pro-metaphase, metaphase and anaphase, prominent hAIK staining was noted to associate with not only centrosomes (arrows) but also spindle fibers (arrowheads) at the peri-centrosomal regions. When the dividing cell entered the telophase, the above-mentioned centrosomal staining pattern gradually disappeared; hAIK resumed the cytoplasmic distribution when the mitosis completed. Similar localization results were found with exogenous hAIK proteins in cells transfected with recombinant wild-type hAIK genes (data not shown). Note, however, that this protein redistribution event was not observed with the mutant hAIKm (fig. 5) whose (auto)phosphorylation function was rendered inactive by a specific point mutation at the catalytic kinase domain (fig. 3). The hAIKm proteins were found mainly in the cytosol during the interphase and remained so (not associated with centrosomes) even when the cell entered different mitotic stages (fig. 5).

Overexpression of hAIK Affects Cell Cycle Progression

The redistribution patterns of hAIK proteins during mitosis suggest that hAIK proteins might play a role in cell cycle progression. To this end, we introduced GFP-

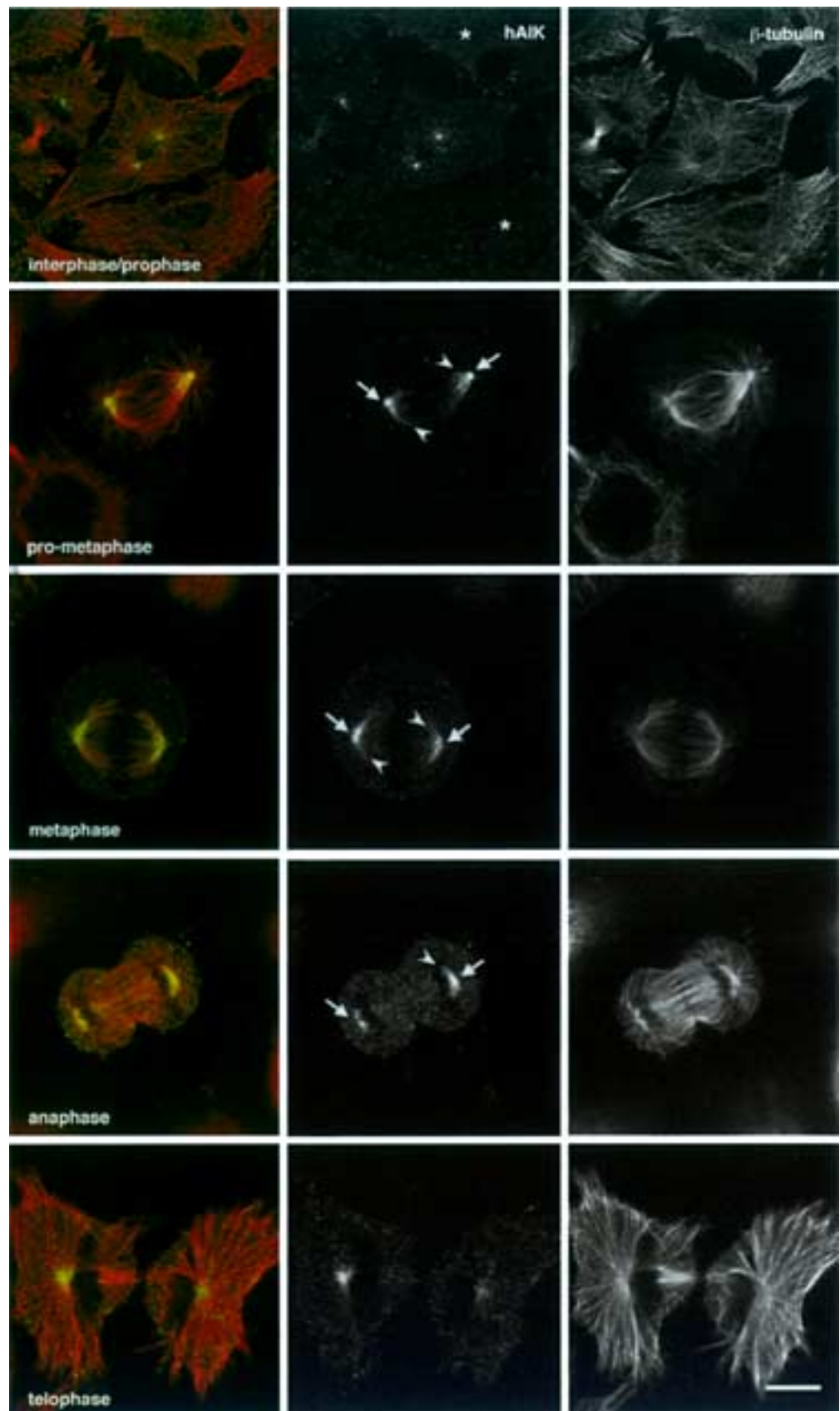


Fig. 4. Gallery of hAIK protein distributions at different mitotic stages. The distribution of endogenous hAIK protein in HeLa cells was revealed using double immunofluorescence staining with affinity-purified polyclonal anti-hAIK antibodies (in FITC channel, middle column), and monoclonal anti- β -tubulin antibodies (in rhodamine channel, right column) and examined under a confocal laser-scanning microscope. The superimposed images are also shown (left column). Bar = 10 μ m.

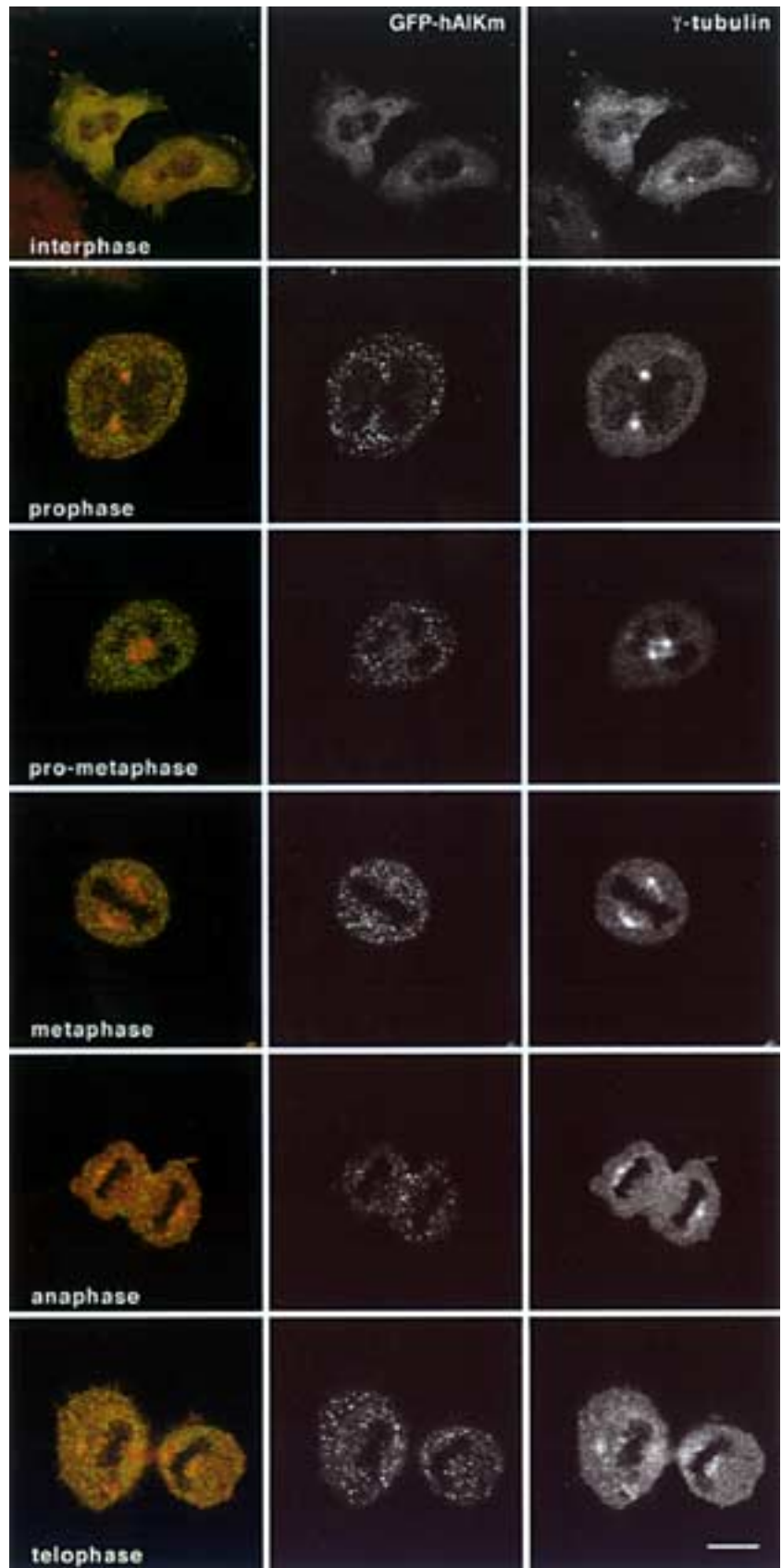
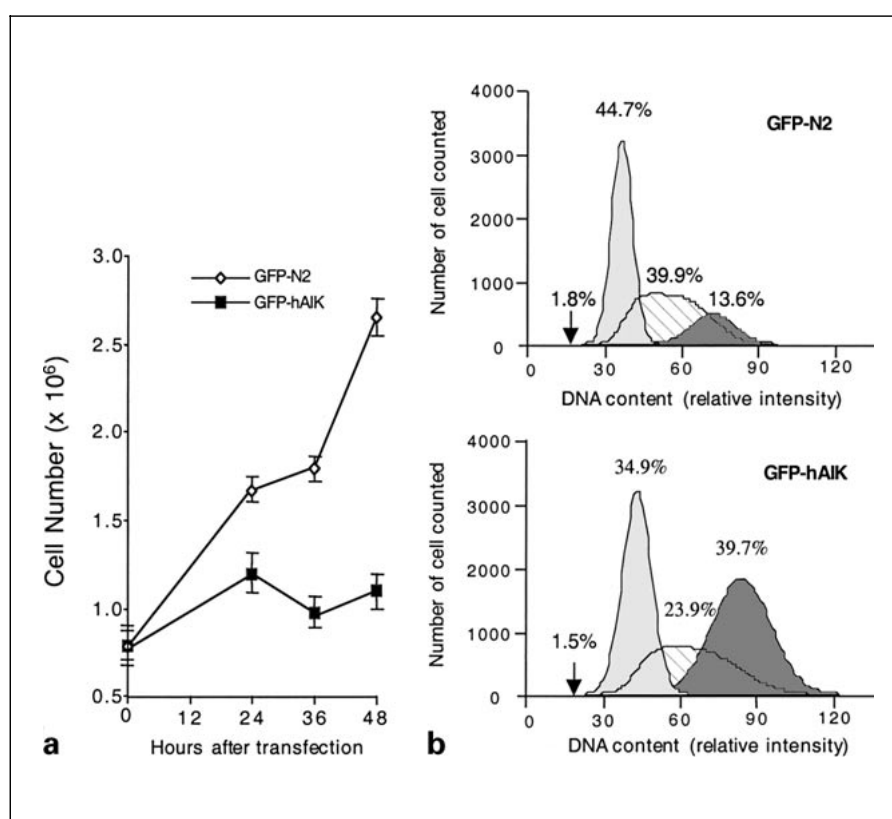


Fig. 5. Gallery of hAIKm protein distributions at different mitotic stages. HeLa cells were transfected with GFP-hAIKm for 48 h, fixed and stained with monoclonal anti- γ -tubulin antibodies, and then observed under a fluorescence microscope (middle column: FITC channel showing hAIKm; right column: rhodamine channel showing γ -tubulin; left column: merged channel). Note the kinase-inactive hAIKm was present in the cytosol, and remained so during the entire mitotic process. Bar = 10 μ m.

Fig. 6. Overexpression of hAIK in HEK293T cells decreased cell proliferation by interfering with G2/M progression. **a** HEK293T cells were transfected with either GFP vector alone (open diamonds) or GFP-hAIK (closed squares). The numbers of cells that were successfully transfected with the constructs (i.e. containing green fluorescence signals) were counted 24, 36 and 48 h after transfection. Note in the control condition HEK293T cells continued to divide, with the doubling time at about 18–20 h. The cells transfected with GFP-hAIK, on the other hand, only slightly increased during the first 24 h, but decreased during the subsequent 24-hour culture period. The mean \pm SD from three independent experiments was shown. **b** The above-mentioned transfected cells cultured for 36 h were subjected to FACS analysis. Based on their DNA content, we categorized individual cells as at different cell cycle statuses, i.e. G1 (light gray area), S (lined area), G2/M (dark gray area). The percentage of putative apoptotic cells were also indicated (arrows). Note the G2/M component drastically increased from 13.6% in the control condition to 39.7% in cells transfected with GFP-hAIK. A representative example of two such experiments is shown.



hAIK in excessive amounts into cells that were actively dividing. The GFP-tag enabled us to separately quantify the numbers of transfected cells from non-transfected cells. As shown in figure 6a, HEK293T cells transfected with GFP vector continued to divide normally (open diamonds). The calculated cell doubling time was about 18–20 h. On the other hand, overexpression of GFP-hAIK profoundly inhibited cell proliferation (closed squares). There was only a slight increase of cells 24 h after transfection; however, the total cell number in fact decreased during the subsequent 24-hour culture period. Overexpression of hAIKm did not significantly decrease cell proliferation compared with the control (data not shown). Note these data were different from those in a previous report where overexpression of a hAIK analog, STK15, was noticed to increase cell proliferation in noncancerous NIH-3T3 cells [32, see ‘Discussion’]. To further address the mechanisms underlying the decreased cell population after hAIK overexpression, we performed FACS scan to quantify the distribution of the cells at different statuses during cell cycle (i.e. G1, S or G2/M). A representative example is shown in figure 6b. HEK293T cells transfected with either GFP vector (*GFP-N2*) or GFP-hAIK (*GFP-*

hAIK) for 36 h were subjected to DNA content analysis. The fluorescence gating method for selecting positively transfected cells was again applied (see ‘Materials and Methods’). As shown, 13.6% of cells were residing at the G2/M status when the control vector was employed, while the percentage significantly ‘shifted to the right’, increased to 39.7% when the cells were transfected with GFP-hAIK. The proportions of apoptotic cells were 1.8 and 1.5% in the control and hAIK-transfected cell population, respectively; there were no statistical differences between the two results. Taken together, our data suggest that excessive amounts of hAIK proteins could decrease cell proliferation, probably by interfering the G2/M progression, instead of inducing a profound apoptotic event.

Discussion

During our present study, we have successfully identified a member of *aurora/ipl*-related serine/threonine kinase, hAIK, from human hepatoma cells (fig. 1). This gene was highly homologous to three other human genes, STK15, ARK1, and STK6. We also showed that recombi-

nant hAIK proteins were functionally active (fig. 3). The wild-type hAIK proteins could phosphorylate themselves as well as substrates such as histone H1 and MBP. This kinase activity of hAIK was totally inhibited by a point mutation at the catalytic domain (hAIKm), and could therefore serve as a negative control for functional investigations.

The exogenous wild-type hAIK, like the endogenous protein, exhibited an interesting redistribution phenomenon during mitosis (fig. 4). The inactive hAIKm mutant, on the other hand, did not accumulate at the centrosome, but distributed relatively evenly throughout the entire cytosol (fig. 5). In a previous report, Kimura et al. [15] also showed that STK6 kinase was present in low amounts during G1/S, and increased to the maximum level during M phase. Our data suggest that the association of hAIK with centrosomes and/or spindle microtubules adjacent to the centrosome during mitosis seemed to require intact kinase activity of the protein. The immunofluorescence staining patterns also suggest that hAIK might interact with centrosomes and phosphorylate certain proteins associated with these structures. Although the native substrates of hAIK are currently unclear [29], we have evidence suggesting that hAIK may phosphorylate a centrosome-associated protein and affect microtubule kinetics and chromosomal segregation during mitosis [Yang et al., in prep.].

Among the various adult tissues, the highest expression of hAIK was found in the testis (fig. 2a), an organ undergoing active cell proliferation (spermatogenesis). All tumor cell lines tested in this report, including well- or poorly differentiated hepatoma cells and HeLa cells, also expressed high levels of hAIK. However, cell division might not be the only role played by hAIK. We noticed that noncancerous fibroblast cell line, despite being rapidly proliferating, exhibited very little hAIK, suggesting a potential role of hAIK in tumorigenesis. Our transfection results demonstrated that overexpression of hAIK in HEK293T cell lines effectively inhibited cell proliferation while mock and mutant hAIKm transfection did not have any obvious effects. The hAIKm transfectants continued to divide without notable abnormalities (data not shown), indicating no dominant-negative effect exerted by this 'kinase-dead' mutant. On the other hand, we found that overexpression of hAIK in the WI-38 fibroblast cell line did not inhibit cell cycle progression (data not shown). Consistent with our findings, the results from Zhou et al. [32] showed that stable transfectants of fibroblasts that produced excessive amounts of STK15 were able to form cell foci in soft agar. The seemingly opposite results of

overexpression of *aurora/ipl* genes in transformed and fibroblast cells imply that an optimal level of protein/activity of this kinase may be essential for keeping cell cycle progression in check. The cell cycle analysis of the transfected cell population also indicated that excessive amounts of hAIK might decrease cell proliferation by directly interfering with the mitotic process (fig. 6b). The nature of this regulation is currently unknown but may involve interactions between hAIK and the centrosome/spindle pole [2, 8, 26; Yang et al., in prep.], as suggested by our results from the protein localization (fig. 4).

Modulation of centrosome/spindle pole activity may affect chromosomal segregation [6, 17]. During our transfection studies, overexpression of hAIK not only inhibited cell division, but also resulted in various abnormal mitotic configurations, such as mitotic cells having more than two centrosomes [Yang et al., in prep.]. These results are again in accordance with a previous study showing that overexpression of STK15, another hAIK homologue, appeared to alter the copy numbers of centrosomes which led to aneuploidy [3, 32]. The mis-segregation of chromosomes is one of the major causes for chromosome instability, which in turn may underlie the sequential pathogenesis events of tumor formation [7]. Further delineation of the molecular mechanisms underlying the interactions between hAIK and centrosomes will be the exciting next chapter of this investigation.

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