Cell Cycle-dependent Expression and Centrosome Localization of a Third Human Aurora/Ipl1-related Protein Kinase, AIK3*

(Received for publication, November 2, 1998)

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We earlier isolated cDNAs encoding novel human protein kinases AIK and AIK2 sharing high amino acid sequence identities with Drosophila Aurora and Saccharomyces cerevisiae Ipl1 kinases whose mutations cause abnormal chromosome segregation. In the present study, a third human cDNA (AIK3) highly homologous to aurora/Ipl1 was isolated, and the nucleotide sequence was determined. This cDNA encodes 309 amino acids with a predicted molecular mass of 35.9 kDa. C-terminal kinase domain of AIK3 protein shares high amino acid sequence identities with those of Aurora/Ipl1 family protein kinases including human AIK, human AIK2, Xenopus pEg2, Drosophila Aurora, and yeast Ipl1, whereas the N-terminal domain of AIK3 protein shares little homology with any other Aurora/Ipl1 family members. AIK3 gene was assigned to human chromosome 19q13.43, which is a frequently deleted or rearranged region in several tumor tissues, by fluorescence in situ hybridization, somatic cell hybrid panel, and radiation hybrid cell panel. Northern blot analyses revealed that AIK3 expression was limited to testis. The expression levels of AIK3 in several cancer cell lines were elevated several-fold compared with normal fibroblasts. In HeLa cells, the endogenous AIK3 protein level is low in G1/S, accumulates during G2/M, and reduces after mitosis. Immunofluorescence studies using a specific antibody have shown that AIK3 is localized to centrosome during mitosis from anaphase to cytokinesis. These results suggest that AIK3 may play a role(s) in centrosome function at later stages of mitosis.

Equal segregation of replicated chromosomes to daughter cells is an important event in the progression of cell cycle. The centrosomes are thought to play an important role(s) in bipolar spindle formation and maintenance at M phase. However, the molecular mechanisms of centrosome and mitotic spindle organization have not been studied well. In most cancer cells, an abnormality in chromosome number (aneuploidy) is observed (1–4). The molecular mechanisms of this chromosome number alteration have not been elucidated, but some molecules that regulate chromosome segregation were identified, and overexpression or point mutations of the genes were observed in some cancer cells (5–9). The proteins that organize centrosome and/or mitotic spindle should be identified to understand the cell cycle regulation and/or tumorigenesis.

Yeast Ipl1 (10) and fly aurora (11) gene products are known to regulate chromosome segregation and constitute a family of serine/threonine kinases. Embryos derived from aurora mutant mothers inappropriately display closely paired centrosomes and monopolar spindles (11). Conditional ipl1 mutants severely missegregate chromosomes and gain extra chromosomes at restrictive temperature (12). None of the substrates or the regulators of these kinases has been identified, but type 1 protein phosphatase was shown to act in opposition to Ipl1 protein kinase in yeast (10). Recent investigations revealed the presence of members of Aurora/Ipl1 family kinases in vertebrates, such as human AIK/BTAK (13, 14), human AIK2 (15, 16), mouse STK-1 (17), mouse AYK1/IAK1 (18, 19), rat AIM-1 (20), or Xenopus pEg2 (21), but the functions of these proteins have not been studied well.

Among the Aurora/Ipl1 kinase family, human AIK/BTAK, mouse IAK1/IAK1, and Xenopus pEg2 appear to constitute a subfamily, because these three protein kinases have closely related N-terminal domain as well as C-terminal kinase domain with each other. In HeLa cells, the endogenous levels of AIK mRNA and its protein contents are tightly regulated during cell cycle progression; they are low in G1/S, accumulate during G2/M, and reduce rapidly after mitosis. Its protein kinase activity is also enhanced at mitosis. Immunofluorescence studies using a specific antibody have shown that AIK/IAK1 protein is localized to the spindle pole during mitosis, especially from prophase through anaphase (13, 20). AIK gene was mapped to human chromosome 20q13.2–13.3 (14, 22), and IAK1 gene was mapped to the distal region of mouse chromosome 2 (23). These two chromosomal regions share homology, supporting the notion that IAK1 is a mouse homolog of human AIK. This chromosomal region is amplified in several cancer tissues including breast and colorectal cancer (24–28), and AIK gene mapped to human chromosome 20q13.2–13.3 was also amplified and overexpressed in cancer cell lines and tissues (8, 14). In a recent study, overexpression of wild type human AIK was shown capable of transforming rat fibroblasts, indicating that AIK is oncogenic (8). In addition, the overexpressed AIK protein induced centrosome amplification and aneuploidy (9).

Human AIK2, rat AIM-1, and mouse STK-1 have high amino acid sequence identities with each other along their full length, and these three proteins constitute the second subfamily of the Aurora/Ipl1 protein kinase family. The protein level of AIK2 elevated at G2/M phase (15). AIM-1 protein localizes at the equator of central spindles during late anaphase and at the midbody during telophase and cytokinesis (20). Overexpression of a kinase-inactive AIM-1 disrupts cleavage furrow formation without affecting nuclear division. In these cells, cytokinesis frequently fails, resulting in multinucleation and subsequent cell death. Thus, AIM-1 was shown to be required for proper...
progression of cytokinesis in mammalian cells (20). Recently, STK1 was mapped to mouse chromosome 11 and linked to TRP53 and GLUT4 (16, 23). AIK3 gene was mapped to human chromosome 17p13.1 which is a homologous region to the STK1 locus of the mouse chromosome (15). This chromosomal region is the most frequently deleted region in several cancer tissues (29–31), but mutation or down-regulation of AIK3 has not yet been identified.

We have cloned a cDNA encoding a third human Aurora/Ipl1-related kinase, AIK3, which has a high homology with catalytic domain of Aurora/Ipl1-related kinase members. AIK3 gene is mapped to human chromosome 19q13.43, a region that was reported to be rearranged and deleted in several cancer cells. AIK3 is expressed highly in testis and several cancer cell lines. AIK3 protein level of M phase was higher than that of S phase cells. AIK3 is localized at centrosome from anaphase to cytokinesis in HeLa cells.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of a Third Human Aurora/Ipl1-related Kinase (AIK3) cDNA—To identify human cDNA sequences related to AIK or Aurora kinases, a humanBank™ cDNA database was searched, and a cDNA encoding a novel kinase (AIK3) was identified (AA412825). This cDNA clone contains only the C-terminal region of kinase domain, and the full length of cDNA clones was obtained from testis cDNA library using PCR.1 Primers used were derived from nucleotide sequences of agt11 (5′-TATGGGGATTGGTGGCGACGAC-3′ or 5′-ACTGCTAATGTGATTGACGACCAGGC-3′) and the 3′-untranslated region of AIK3 cDNA (5′-GGGAACAGAGAGGACTGGAGCACC-3′). The cDNA was sequenced using a combination of dideoxy and primer extension methods. The obtained immune sera were affinity purified with anti-biotin antibody (Vector Laboratories) at a 1:500 dilution in 1% bovine serum albumin, 4% SSC for 1 h at 37 °C. The blots were washed with 4× SSC, 0.1% Nonidet P-40 in 4× SSC, 4× SSC for 5 min each, and then stained with fluorescein anti-goat IgG (Nordic Immunology) at 1:500 dilution for 1 h at room temperature. After washing with 4× SSC, the slides were incubated under a coverslip with anti-biotin antibody (Vector Laboratories) at a 1:500 dilution in 4× SSC for 30 min. The blots were exposed to UV light after staining with Hoechst 33258. The chromosome slides were hardened at 65 °C for 3 h, denatured at 70 °C in 70% formamide, then stained with 2× SSC and stained with 0.75 µg/ml propidium iodide. Excitation at 450–490-nm wavelength (Nikon filter set B-2A) and near 365 nm (UV-2A) was used for observation. Kodak Ektachrome ASA100 film was used for microphotography.

Somatic Cell Hybrid and Radiation Hybrid Cell Panel—PCR was performed to assign AIK3 gene to human chromosome, using a DNA panel of human/rodent somatic cell hybrids (NIHMS Human/Rodent) purchased from Coriell Cell Repositories (Camden, NJ). A pair of oligonucleotide primers was constructed from the coding region (5′-AGAGAAGGAAGACTGGAGCACC-3′ and 5′-ATCTGCAATCTCTCATCCTACC3′), corresponding to nucleotides 237–259 and 530–552, respectively. PCR was carried out using the LA PCR kit (TaKaRa, Kyoto, Japan) for 38 cycles at 94 °C (30 s), 65 °C (1 min), and 72 °C (1.5 min), and amplified products were electrophoresed on 2% agarose gel in TAE buffer.

The G3 radiation hybrid cell panel was purchased from Research Genetics and used as a DNA template for PCR. The same primers and conditions were used for PCR as in the experiments with the somatic cell hybrid panel.

Northern Hybridization—Blotted filters with 2 µg of poly(A)1 RNA per lane from various human tissues were purchased from CLONTECH. The blot was probed with a 1.0-kb cDNA fragment of AIK3. Hybridization was performed as described (36). The blots were washed at 55 °C for 1 h in a buffer containing 2× SSC and 0.5% SDS, and signals were visualized by autoradiography using Kodak BioMax film.

Western Blotting—Polyclonal anti-sera against AIK3 were generated against synthesized peptide (RYQPLERPLAQILK) corresponding to the C-terminal region of AIK3 which has little homology with AIK and Ipl1. The peptide sequence was determined by affinity purification with the peptide and used for Western blotting. For the control experiment, anti-AIK3 antibody was preincubated with glutathione S-transferase-AIK3 protein produced in E. coli and then used for Western blotting. Antibody against AIB was generated and purified as described (33). Antibodies against cyclin B and ERK2 were purchased from Santa Cruz Biotechnology.

The synthesized cells were washed with ice-cold PBS and harvested by scraping and then lysed in lysis buffer (50 µM Tris, pH 7.5, 100 mM NaCl, 10 mM sodium diphosphate, 10 mM EGTA, 1 mM EDTA, 1% (v/v) Nonidet P-40). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (37) and transferred to Immobilon (Millipore) by standard methods. Membranes were blocked for 2 h in TBST (0.1% Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.6) with 3% non-fat dry milk (blocking buffer). Probing with the specific antibodies was carried out for 1 h at room temperature in the blocking buffer containing 0.02% sodium azide. After washing 4 times in TBST, the membranes were probed with 200 ng/ml peroxidase-labeled secondary antibodies for 1 h in TBST. After extensive washing in TBST, the membranes were processed for enhanced chemiluminescence using ECL reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech). ECL exposures were carried out using Kodak BioMax film.

Immunofluorescence Microscopy—For indirect immunofluorescence experiments, HeLa cells cultured on cover glasses were fixed by placing them directly into absolute methanol for 30 s. Extraction was done by incubating the cells with PBS for 10 min at room temperature. Cells were incubated with the anti-AIK3 antibody for 1 h at 37 °C, washed extensively in PBS, followed by incubation with the secondary antibody conjugated with Oregon Green (Molecular Probe) for 1 h at 37 °C. DNA was visualized by adding 4,6-diamidino-2-phenylindole at a concentration of 0.1 µg/ml.

1 The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase pair; PBS, phosphate-buffered saline; FISH, fluorescence in situ hybridization; FCS, fetal calf serum.
RESULTS

Cloning of a cDNA Encoding a Third Human Aurora/Ipl1-related Kinase, AIK3—Homology search of AIK2 cDNA with GenBank™ data base revealed the presence of a third Aurora/Ipl1-related kinase, AIK3. Since this clone did not contain initiation methionine, a full-length cDNA was obtained by PCR method with human testis cDNA library. The nucleotide sequences of several clones were determined and were identical with each other, and the nucleotide sequence was deposited in DDBJ/EMBL/GenBank™ (accession number AB017332). This cDNA encodes 309 amino acids with a predicted molecular mass of 35.9 kDa. The deduced amino acid sequence contains 11 conserved regions characteristic of protein kinase catalytic domain at C terminus of the protein (Fig. 1). Eleven conserved subdomains known in various protein kinases are indicated by roman numerals (Fig. 2). AIK3 has K PEN in subdomain VIB and does not have PXXWXXAPE but only PE in subdomain VIII which are the consensus sequences of serine/threonine kinases, suggesting that AIK3 must be a serine/threonine kinase but not a tyrosine kinase.

A computer search of the protein sequence data base (GenBank™) revealed that the kinase domain of AIK3 shares 71, 83, 69, 60, and 47% identity with those of human AIK (13, 14), human AIK2 (15, 16), Xenopus pEg2 (21), Drosophila Aurora (11), and budding yeast Ipl1 (10), respectively. A multiple alignment of the kinase domains is shown in Fig. 2. Amino acids that are the same as AIK3 are indicated by asterisks. However, the N-terminal domain of AIK3 shares little homology with other Aurora/Ipl1-related kinases. These results indicate that AIK3 is a novel member of the Aurora/Ipl1-related protein kinase family.

Chromosomal Localization of AIK3—The chromosomal assignment of human AIK3 was made by direct R-banding fluorescence in situ hybridization (FISH) using a 1.0-kb AIK3 cDNA fragment as a probe. The positive signals of AIK3 gene were observed at chromosome 19q13.43 (Fig. 3). We examined 150 typical R-banded (pro)metaphase spreads. 6% of them exhibited complete twin spots on both homologues and 32% had incomplete signals and/or twin spots on either or both homologues. No consistent fluorescent signals were observed on other chromosomes.

To confirm the chromosomal localization of AIK3 gene, we used the somatic cell hybrid panel (Coriell Cell Repositories). PCR amplification was performed as described under “Experi-
mental Procedures” using oligonucleotides specific for AIK3 cDNA. A band of 1.2 kb was amplified from DNAs containing human chromosome 19 and in human genomic DNA (H), but no product was seen in recipient Chinese hamster (CH) or mouse (M) DNAs (Fig. 4).

To refine the chromosomal localization of AIK3, we used the Stanford G3 radiation hybrid mapping panel (Research Genetics). PCR amplification was performed under the same conditions as in the somatic cell hybrid panel. Bands (1.2 kb) of PCR product were detected in lanes 6, 20, 31, 39, 45, 54, 65, 77, and 85 and in human genomic DNA, but no product was seen in recipient Chinese hamster DNA (data not shown). Two-point maximum likelihood analysis, calculated by the Stanford Human Genome Center radiation hybrid web server, indicated linkage to a genetic marker D19S828 with a lod score of 8.645295. There was no evidence of linkage of AIK3 to other genomic regions. The PCR product was subcloned into pCR2.1 (Invitrogen) and sequenced both strands, and the product has the same nucleotide sequence as AIK3 over 100 bases and contains an intron(s) (data not shown). The results obtained by in situ hybridization and radiation hybrid panel were in accord, indicating that AIK3 is localized to human chromosome 19q13.43.

AIK3 Expresses in Testis—Expression of AIK is high in testis, and AIK2 expression is observed in several proliferating tissues, suggesting that the two proteins have a function in cell proliferation. The tissue distribution of AIK3 was also examined by Northern hybridization. 32P-Labeled random-primed full-length AIK3 cDNA hybridized with a single transcript of 1.3 kb. AIK3 expression was limited to testis (Fig. 5). The expression of b-actin was examined using the same blot, and almost identical intensities of the bands were observed (38). These results suggest that AIK3 may have a role(s) in cell proliferation.

Western Blotting Analyses of AIK3 in Cancer Cell Lines—AIK is reported to be amplified and overexpressed in several cancer cell lines and tumor tissues (8, 14). To learn whether AIK3 is expressed in cancer cells, its protein level was examined in some cancer cell lines by Western blotting analyses. Elevated expression levels of AIK3 were observed in several of these cell lines.
lines such as HepG2, HuH7, MDA-MB-453, and HeLa cells, but in the other cancer cells examined the protein was not detected (Fig. 6). AIK was overexpressed in most of the examined cancer cell lines compared with normal fibroblast (Fig. 6). The amounts of ERK2 and cyclin B were almost identical in most of the cells, indicating that the protein amount used was almost identical, and the cells used were at log phase. A subset of cancer cells expressed AIK3 highly, but other cancer cells expressed an undetectable level of the protein, suggesting the different properties of these cell lines.

**Cell Cycle-dependent Expression of AIK3—AIK and AIK2 protein contents increased at G2/M phase compared with G1/S phase (13, 15).** Western blotting was performed to learn cell cycle dependence of AIK3 expression. Anti-AIK3 antibody recognized a single band of 36 kDa, but an antibody preincubated with glutathione S-transferase-AIK3 protein did not recognize the protein (Fig. 7A). To learn whether AIK3 expression is also dependent on cell cycle progression, Western blotting was performed using the lysates of HeLa cells arrested at S or M phase (Fig. 7B). AIK3 protein level of the cells arrested at M phase was higher than that of S phase arrested cells (Fig. 7B). This M phase arrest was induced by nocodazole, indicating that AIK3 was induced by metaphase. Expression of cyclin B was also higher at M phase than at S phase. However, the ERK2 protein level did not change significantly during the cell cycle progression. We also performed Western blotting using HeLa cell lysates of S phase released cells (Fig. 7C). AIK3 protein level peaked at 12–20 h after S phase release and decreased to the basal level at 24 h (Fig. 7C). Expression of cyclin B was studied to monitor the cell cycle progression, and the level changed in a similar manner to AIK3, suggesting the predominant expression of AIK3 at M phase. However, the ERK2 protein level did not change significantly during the cell cycle progression.

**Subcellular Localization of AIK3 at Centrosome during M Phase—**AIK is localized to the spindle pole region from prophase to anaphase, and AIM-1 is localized to midbody at cytokinesis. The functions of AIK3 were also sought by examining subcellular localization in HeLa cells using indirect im-
mumofluorescence microscopy (Fig. 8). AIK3 protein localized at centrosome from anaphase to cytokinesis in dividing HeLa cells. However, AIK3 was not detected at centrosome in interphase or pro- to metaphase of HeLa cells, even though we looked at well over several hundred dividing and interphase cells. The cell cycle stages of HeLa cells monitored by staining with 4,6-diamidino-2-phenylindole. These results suggest that AIK3 plays a role(s) in centrosome function at later stages of mitosis.

**DISCUSSION**

We have identified a third human gene closely related to members of the Aurora/Ipl1 family protein kinases, which are known to regulate chromosome segregation in *Drosophila* (11) and yeast (10). Temperature-sensitive mutants of IPI1 cause chromosome number alteration (12). Temperature-sensitive mutants of aurora form a monopolar spindle and cannot segregate the chromosomes (11). A single Aurora/Ipl1-related kinase is identified in yeast and fly, but at least three kinases are present in mammalian cells. It is intriguing to clarify the functional difference of these three human Aurora/Ipl1-related kinases. The three kinases have distinct N-terminal domains, which may contribute to their functional difference in human cells. AIK and AIK2 have Aurora boxes 1 and 2 that share limited amino acid sequence identities (8), but AIK3 does not have a related amino acid sequence (Fig. 1). The roles of Aurora boxes must be further studied to elucidate the functions of the proteins.

The tissue distribution of AIK3 examined by Northern blotting showed that expression was exclusively high only in testis (Fig. 5). The expression of AIK/AYK1 is also high in testis (13, 18). However, the expression of AIK2/STK-1/AM-1 is high in the proliferating tissues of thymus, testis, spleen, small intestine, and colon (15, 17, 20), suggesting that AIK2 may have some specialized function in these cells. All of the mammalian Aurora/Ipl1-related kinases are rich in several cancer cells and tissues with proliferating cells, suggesting that these have functions in cell proliferation.

Expression levels of AIK3 in cancer cells were examined by Western blotting to study whether AIK3 expression level was altered in tumor cells like AIK. AIK3 protein level was high only in a subset of cancer cells such as HepG2, HuH7, and HeLa cells and was hardly detected in others, suggesting the different properties of these cell lines (Fig. 6). A computer search of the data base (GenBank™) also revealed the existence of AIK3 transcripts in proliferating cells such as testis (GenBank™ accession numbers AA421312 and AA421265) and cancer cells (GenBank™ accession number AA484664). AIK expression was high in most of the cancer cells examined. Considering the altered expression of AIK3 in several cancer cell lines, it is tempting to speculate that AIK3 may function in tumorigenesis.

AIK3 gene was mapped to human chromosome 19q13.43 by fluorescence in situ hybridization and by using somatic cell hybrid and radiation hybrid cell panel (Figs. 3 and 4). The human chromosomal region 19q13.43 was known to be deleted or translocated in some cancer tissues (39–42), suggesting that disorganization of AIK3 or another gene located in this chromosomal region is involved in tumorigenesis. AIK gene (STK6) was assigned to human chromosome 20q13.2–13.3 (13, 14). This chromosomal region is amplified in some cancer tissues including breast and colorectal cancers (24–28), and AIK overexpression was observed in several cancer cell lines and tumor specimens (8, 14). In a recent study, overexpression of AIK was shown capable of transforming rodent fibroblasts, indicating that AIK is oncogenic (8, 9). The AIK2 gene (STK12) was recently assigned to human chromosome 17p13.1 (15). This chromosomal region contains p53 tumor suppressor gene and is frequently deleted in some tumor tissues (29–31). Overexpression of kinase-inactive rat AIM-1 disrupts cleavage furrow formation without affecting nuclear division, resulting in multinucleation. Thus, rat AIM-1 was shown to be required for proper progression of cytokinesis in mammalian cells (20). Although point mutations or enhanced expressions of AIK2 have not yet been identified, it is conceivable that AIK2 may have a role(s) in tumorigenesis. Considering the chromosome localization of AIK3 gene and the altered expression of AIK3 in several cancer cell lines, it is tempting to speculate that AIK3 may function in tumorigenesis. We are now searching for an altered expression or a point mutation of AIK3 in tumor tissues, and we are examining whether AIK3 can transform fibroblasts or not.

Since all of the previously known Aurora/Ipl1 family protein kinases play roles in mitosis, it is conceivable that AIK3 may also function at M phase. The present investigation provided evidence supporting this notion. AIK3 is predominantly expressed in a subset of proliferating tissues or cells. Cell cycle-dependent expression of AIK3 was observed in HeLa cells by Western blotting. The AIK3 protein level is low in G1/S but high in G2/M (Fig. 7). Timing of the appearance and disappearance of AIK3 and cyclin B was almost identical, suggesting that
they function in M phase. AIK3 is localized at centromere from anaphase to cytokinesis (Fig. 8). This subcellular localization as well as the expression profile during cell cycle progression suggests that AIK3 has a role(s) in centrosome or spindle function at M phase. To our knowledge, no protein other than AIK3 has been reported to be localized to centrosome from anaphase to cytokinesis. Western blotting with nocodazole-treated cells revealed that AIK3 was induced by metaphase (Fig. 7B), but it was not localized to centrosome at metaphase by fluorescence microscopy, suggesting that some regulation such as phosphorylation may exist to determine the localization of AIK3 at centrosome. AIK localized to spindle pole from prophase to anaphase and AIK2 localized to midbody in cytokinesis. Both AIK and AIK3 localized to centrosome at mitosis, whereas the localization timings between the two proteins are different, suggesting that they function at different steps in mitosis. Although the subcellular localization of Aurora and Ipl1 has not been studied, the phenotypes of these mutants may play parts in organizing microtubules during mitosis. We are now examining the effect of overexpression of a dominant negative AIK3 on cell division.

Acknowledgments—We are grateful to Dr. H. Moriwaki (1st Department of Internal Medicine, Gifu University) for HepG2 and HuH7 cells. We thank Dr. Y. Nozawa (Department of Biochemistry, Gifu University) for allowing us to use a fluorescent microscope and for helpful discussion. HeLa, HBL100, WI38, NB69, SK-N-SH, NB1RGB, and MDA-MB-453 cells were obtained from RIKEN cell bank.

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