Inhibition of Cell Growth by Conditional Expression of kpm, a Human Homologue of Drosophila warts/lats Tumor Suppressor*

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kpm is a human serine/threonine kinase that is homologous to Drosophila tumor suppressor warts/lats and its mammalian homologue LATS1. In order to define the biological function of kpm, we generated stable transfectants of wild-type kpm (kpm-wt), a kinase-dead mutant of kpm (kpm-kd), and luciferase in HeLa Tet-Off cells under the tetracycline-responsive promoter. Western blot analysis showed that high levels of expression of kpm-wt as well as kpm-kd with an apparent mass of 150 kDa were induced after the removal of doxycycline. Induction of kpm-wt expression resulted in a marked decline in viable cell number measured by both trypan blue dye exclusion and MTT assay, whereas that of kpm-kd or luciferase had no effect. We then analyzed the cell cycle progression and apoptosis upon induction of kpm expression. 2-3 days after removal of doxycycline, cells underwent G1/M arrest, demonstrated by flow cytometric analysis of propidium iodide incorporation and MPM-2 reactivity. In vitro kinase assay showed that induction of kpm-wt led to down-regulation of kinase activity of the Cdc2-cyclin B complex, which was accompanied by an increase in the hyperphosphorylated form of Cdc2 and a change of phosphorylation status of Cdc25C. Furthermore, both DAPI staining and TUNEL assay showed that the proportion of apoptotic cells increased as kpm expression was induced. Taken together, these results indicate that kpm negatively regulates cell growth by inducing G1/M arrest and apoptotic cell death through its kinase activity.

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nase-dead mutant described previously (1) were recloned into pTRE vector (Clontech) downstream of the tetracycline-responsive promoter to make pTRE-kpm-wt and pTRE-kpm-kd, respectively. pTRE-Luc control response plasmid (16) was obtained from Clontech. HeLa Tet-Off cells were transfected with pTRE-kpm-wt, pTRE-kpm-kd, or pTRE-Luc together with pTK-Hyg (Clontech) by electroporation using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA). After 2 days, transfected cells were subjected to selection with 200 μg/ml hygromycin B (Invitrogen) in the presence of 10 ng/ml doxycycline (Clontech). Hygromycin-resistant cell lines were screened for induction of kpm-wt or kpm-kd expression upon removal of doxycycline by Western blotting. Induction of luciferase activity in pTRE-Luc-transfectants was confirmed with the luciferase assay kit (Promega, Madison, WI) and luminometry (Biotek Instruments Pty Ltd., Bundorah, Australia).

**Cell Viability Analysis by MTT Assay and Trypan Blue Dye Exclusion**—Both adherent and non-adherent cells were harvested by trypsinization, and the viable cell number as well as the cell viability was measured by microscopic examination with trypan blue dye exclusion. Cellular proliferation was measured by reduction of MTT, which corresponds to living cell number and metabolic activity (17). Cells were thoroughly washed, plated at 5×10^4 cells/well in 24-well plates and incubated with or without 10 ng/ml doxycycline for various periods of time (for 1–5 days). 50 μl of 1 mg/ml MTT solution (WST-8, Nacalai Tesque, Kyoto, Japan) was added to each well. After 1 h of incubation, the absorbance of each well was measured at 492 and 630 nm using a microplate reader Benchmark (Bio-Rad Laboratories) according to the manufacturer’s protocol.

**Western Blot Analysis**—Cells were harvested and lysed in TG-V04 solution (18) (1% Triton X-100, 10% glycerol, 0.198 trypsin inhibitor units (TIU) of aprotinin per milliliter of Dulbecco’s phosphate-buffered saline lacking divalent cations with fresh 100 mM Na2VO4 containing 0.1% phenylmethylsulfonyl fluoride, 1% Complete protease inhibitors (Roche Applied Science). After centrifugation, the supernatants were collected, and the protein concentration of each cell lysate was measured. Adjusted amounts of cell lysates were separated on 7.5 or 12.5% SDS-polyacrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking, the membranes were incubated with the first antibody followed by incubation with peroxidase-conjugated secondary antibody for 1 h. The protein bands were detected using the ECL detection system (Amer sham Biosciences) according to the manufacturer’s instructions. The antibodies used for Western blotting were mouse anti-HA monoclonal antibody (12CA5) (Roche Applied Science), rabbit anti-Cdc25C polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-cyclin B polyclonal antibody (Santa Cruz Biotechnology), a specific anti-phospho-Cdc2-Y15 rabbit polyclonal antibody (Cell Signaling, Beverly, MA), rabbit anti-Cdc25C polyclonal antibody (Cell Signaling, Beverly, MA), and goat anti-actin polyclonal antibody (Santa Cruz Biotechnology).

**Cell Cycle Analysis**—kpm-wt, kpm-kd, and Luc-inducible HeLa Tet-Off cell lines were cultured in medium without doxycycline for the indicated periods of time, harvested and washed twice with ice-cold phosphate-buffered saline (PBS) containing 0.1% glucose. Cells were then fixed with 70% ethanol for 1 h and incubated in 1 ml of PBS containing 50 μl of propidium iodide (Sigma), and 66 units/ml RNase (Invitrogen) on ice for 30 min. DNA content analysis was performed by a FACScan with CellQuest software (BD Biosciences). Cells populations at G2 and S phases were distinguished by the reactivity with mitotic protein monoclonal 2 (MPM-2 mouse monoclonal antibody) (Upstate, Waltham, MA) as described (19–21). In brief, cells were fixed in 70% methanol and stained with MPM-2 antibody followed by Alexa Fluor 488-labeled goat anti-mouse IgG (BIOSOURCE, Camarillo, CA). After washing, cells were incubated with propidium iodide for DNA staining and then analyzed by two-color flow cytometry using the FACScan (BD Biosciences). MPM-2 reactive cells were considered to be at the mitotic phase, and the percentage of the population represented the mitotic index.

**Establishment of Stable Transfectants of kpm-wt, kpm-kd, and Luciferase under the Control of the Tetracycline-responsive Promoter**—HeLa Tet-Off cells were transfected with either pTRE-kpm-wt, pTRE-kpm-kd, or pTRE-Luc together with pTK-Hyg and subjected to selection with hygromycin B. Several stable transfectant lines of the three genes were expanded and screened for efficient gene induction by the removal of doxycycline. Representative transfectants of each of the three genes were compared with parental cells for the gene expression in the absence or presence of doxycycline. Western blot analysis showed that the representative lines of HeLa Tet-Off-kpm-wt and -kpm-kd were induced to express a large amount of kpm protein when cultured without doxycycline (Fig. 1A). Expression of kpm was detected after 12 h and reached maximal levels after 48 h, dependent on the concentrations of doxycycline (Fig. 1B). Based on scanning densitometry, removal of doxycycline resulted in more than 100-fold induction of kpm-wt or kpm-kd by probing with anti-kpm polyclonal antibody, which recognize both endogenous and exogenous kpm (data not shown). Likewise, high levels of luciferase activity were in-

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**Fig. 1. Conditional expression of kpm protein in HeLa Tet-Off-kpm-wt and -kpm-kd** A. HeLa Tet-Off-kpm-wt and -kpm-kd cells were cultured without doxycycline for 48 h and subjected to Western blotting using anti-HA mAb. B. Western blot analysis of kpm expression in HeLa Tet-Off cells after culture with serial dilutions of doxycycline for 48 h. The same membrane was reprobed with anti-β-actin Ab as an internal protein control.
duced in a representative HeLa Tet-Off-Luc upon the removal of doxycycline (data not shown).

Overexpression of kpm Inhibits Cell Growth—In order to explore the biological function of kpm, we first examined the effects of overexpression of kpm on cell viability and proliferation. HeLa Tet-Off-kpm-wt, -kpm-kd, and -Luc cells were switched into the culture without doxycycline, and the viable cell number was counted daily by trypan blue dye exclusion. The relative values of induced cells to those of non-induced cells were plotted in the graph. Three independent experiments were done, and the data of a representative experiment are shown.

Overexpression of kpm Induces G2/M Arrest—Since overexpression of kpm-wt resulted in inhibition of cell proliferation and a decline in viable cell number, a cell cycle arrest was suspected to have occurred. To determine at which stage of the cell cycle cells were arrested, we performed the cell cycle analysis in the three transfectant lines upon removal of doxycycline. As shown in Fig. 2A, induction of kpm-wt expression resulted in an increase in cell population in G2/M phase and a decrease in cell population in G1 phase compared with non-induced culture. In contrast, cell growth of HeLa Tet-Off-kpm-kd as well as -Luc was not affected by expression of these genes. In accordance with this, the MTT assay also showed that overexpression of kpm-wt suppressed cell proliferation while that of kpm-kd or luciferase had no effect (Fig. 2B). These results indicate that kpm is involved in either cell cycle progression or cell viability, and negatively regulates cell growth. Since overexpression of kpm-kd had no effect on cell viability or proliferation as that of luciferase, it is suggested that anti-proliferative effect of kpm is dependent on its kinase activity and kd mutant does not function as a dominant negative form at least in this particular assay system.

Overexpression of kpm Induces G1/M Arrest—Since overexpression of kpm-wt resulted in inhibition of cell proliferation and a decline in viable cell number, a cell cycle arrest was suspected to have occurred. To determine at which stage of the cell cycle cells were arrested, we performed the cell cycle analysis in the three transfectant lines upon removal of doxycycline. As shown in Fig. 3A, overexpression of kpm-wt induced an increase in cell population in G1/M phase and a decrease in cell population in G2 phase compared with non-induced culture. In contrast, there was no difference in the profile of cell cycle progression between non-induced and induced overexpression of kpm-kd or that of luciferase. To further analyze the kpm-induced cell cycle arrest and determine whether it was a...
G_{2}/M transition arrest or a mitotic arrest, we performed scoring of mitotic index by MPM-2 assay that had long been used to identify mitotic cells. Overexpression of kpm-wt increased the cell proportion in G_{2}/M phase as has been shown but with no significant changes in cell proportion in mitotic phase expressing MPM-2 antigen (Fig. 3B), indicating that overexpression of kpm-wt resulted in a G_{2}/M transition arrest.

**Kpm Negatively Regulates the Kinase Activity of the Cdc2-Cyclin B Complex**—Since the overexpression of kpm induced cell cycle arrest at the G_{2}/M boundary, we next examined whether overexpression of kpm negatively regulated the kinase activity of the Cdc2-cyclin B complex. In parallel with the gene induction, cells were synchronized in prometaphase and metaphase by the nocodazole method. Western blotting with whole cell lysates showed that induction of kpm-wt, kpm-kd, or luciferase did not affect the total amounts of Cdc2 or cyclin B (Fig. 4A). Likewise, there was no particular difference in the amount of Cdc2 co-immunoprecipitated with cyclin B between non-induced and induced cells. However, the Cdc2-cyclin B complex of kpm-wt-induced cells showed much lower histone H1 phosphorylation activity than that of non-induced cells, while induction of kpm-kd or luciferase had no effect on the kinase activity (Fig. 4B). We repeated these experiments three times and obtained similar results. Synchronization by the double thymidine block did not work well with HeLa Tet-Off cells and gave only incomplete results (data not shown), which were nevertheless consistent with what we observed by the nocodazole method. As has been described elsewhere (26, 27), Cdc2 co-immunoprecipitated with cyclin B consisted of a doublet of bands of which the upper one represented the hyperphosphorylated inactive form, and the lower one represented the dephosphorylated active form. As shown in Fig. 4B, the proportion of the hyperphosphorylated inactive form of Cdc2 was increased in kpm-wt-induced cells, which was also demonstrated by Western blotting using a specific anti-phospho-Cdc2-Y15 antibody. These data suggest that Cdc2 bound to cyclin B remained or was rendered inactive by the phosphorylation at Thr-14 and Tyr-15, which seems to be the major mechanism of the G_{2}/M arrest.

**Overexpression of kpm Affects the Phosphorylation Status of Cdc25C**—We next investigated the possible involvement of Cdc25C in the down-regulation of the Cdc2-cyclin B kinase. It is known that in SDS-PAGE Cdc25C consists of an 85-kDa band of the hyperphosphorylated form with the highest phosphatase activity and a doublet of 60- and 57-kDa bands, which represent the Ser-216-phosphorylated inactive form and the dephosphorylated form with weak phosphatase activity, respectively (28, 29). In parallel with gene induction, cells were synchronized in prometaphase and metaphase by the nocodazole method. Western blotting with whole cell lysates showed that the 85-kDa hyperphosphorylated active form as well as the doublet of Cdc25C were present in luciferase- or kpm-kd-induced cells as described above. In contrast, it was noted that the mitotic hyperphosphorylated form was almost undetectable and conversely the Ser-216-phosphorylated inactive form (the upper band of the doublet) was increased in kpm-wt-induced cells (Fig. 4C). These data suggest that Cdc25C remains or is rendered inactive by overexpression of kpm-wt resulting in the decrease in the activity of dephosphorylating Cdc2, which seems to be one of mechanisms of the inactivation of the Cdc2-cyclin B kinase.

**Overexpression of kpm Induces Apoptosis after G_{2}/M Phase Arrest**—Because many tumor suppressor genes are known to inhibit cell growth by inducing apoptosis as well as cell cycle arrest, we examined whether this was also the case with kpm. In fact, induction of apoptosis by kpm was already suggested by cell cycle analysis in which an increase in sub-G_{1} phase cells with a DNA content less than 2 N was observed after an elongated induction of kpm-wt for more than 4 days (Table I). To demonstrate that this population was generated as a result of apoptosis, cells after kpm induction were first subjected to TUNEL assay. As shown in Fig. 5A, an increase in TUNEL-positive cells was clearly detectable in kpm-wt-induced but not non-induced cells. Induction of kpm-kd or luciferase elicited no change in TUNEL-positive cells. Presence of apoptotic cells was
confirmed by DAPI staining and immunofluorescence microscopy. Induction of kpm-wt resulted in chromatin condensation and segregation characteristics of apoptotic cells whereas that of kpm-kd or luciferase did not (Fig. 5B).

**DISCUSSION**

We previously reported the molecular cloning of kpm, which encodes a putative human serine/threonine kinase homologous to warts/lats, a Drosophila tumor suppressor (1). Prior to our article, Tao et al. (11) described a human as well as mouse homologue of warts/lats named LATS1 that could functionally compensate the defect of warts/lats in Drosophila. In contrast to LATS1 that has been extensively studied, the function of kpm remains largely unknown. It is to be determined whether kpm has similar or unique function compared with LATS1. In the present study, we established HeLa-derived stable transfectants of wild-type kpm (kpm-wt), a kinase-dead mutant of kpm (kpm-kd), and luciferase under the control of tetracycline-responsive promoter in order to define the biological function of kpm. Using this system (16, 30), we demonstrated that overexpression of kpm-wt resulted in suppression of cell proliferation due to cell cycle arrest in G2/M phase and subsequent apoptotic cell death.

Cell cycle analysis combined with MPM-2 assay clearly showed that overexpression of kpm-wt induced a cell cycle arrest by blockade of G2/M transition rather than delaying progression of mitosis. Consistent with this, we showed that the histone H1 kinase activity of the Cdc2-cyclin B complex was markedly diminished in kpm-wt-induced cells. Furthermore, Cdc2 bound to cyclin B remained or was rendered phosphorylated at Tyr-15 in kpm-induced cells. It is well known that the transition between the G2 phase and mitosis is regulated through inhibitory phosphorylation of the Cdc2 kinase (31, 32). Since overexpression of kpm-wt did not change the protein levels of Cdc2 and cyclin B in the whole cell lysates as well as in the immunoprecipitates by anti-cyclin B, it seems likely that overexpression of kpm led to a cell cycle arrest at G2/M by increasing the ratio of the hyperphosphorylated inactive form of Cdc2.

We examined whether Cdc25C was involved in the inactivation of Cdc2 because it is established that phosphorylated Cdc2 is dephosphorylated by this dual-specific phosphatase. Western blot analysis showed that overexpression of kpm-wt resulted in a marked decrease in the hyperphosphorylated active form of Cdc25C and an increase in the Ser-216-phosphorylated inactive form. Considering that 14-3-3 proteins bind to phospho-serine 216 of Cdc25C and translocate it from the nucleus to the cytoplasm, the overall phosphatase activity of Cdc25C should be strongly down-regulated in kpm-wt-induced cells, which seems to be one of the mechanisms of the increase in phosphorylated inactive form of Cdc2. Although we do not exclude other possible mechanisms for the kpm-induced cell cycle arrest in G2/M phase, it is certain that the kinase activity of kpm itself plays the central role in such a putative phosphorylation-dephosphorylation cascade, because kpm-kd had no effect.

LATS1 has also been reported to inhibit cell growth and induce cell cycle arrest in G2/M (33, 34). However, the mechanism of the cell cycle arrest in LATS1 overexpression is different from that of kpm described here. According to Tao et al. (11) LATS1 could associate with Cdc2 and competitively inhibit the binding of cyclin B to Cdc2, which resulted in a decrease in kinase activity of the Cdc2-cyclin B complex. In addition, ectopic expression of LATS1 in MCF-7 cells has been reported to induce specific down-regulation of protein levels of cyclin A and cyclin B, while no effect was found on cyclin E, Cdc2, CDK2, p27Kip1, and p21Cip1 levels (34). The discrepancy between kpm
and LATS1 may be simply because these two molecules are distinct from each other. The experimental systems were also different, in which we used a tetracycline-responsive gene expression system in HeLa-derived cells (30, 35) whereas LATS1 overexpression was induced by transduction of fibroblasts and other cancer cells using adenovirus vectors (33, 34). We do not exclude the possibility that kpm has the capacity to associate with Cdc2 although we have not been able to demonstrate the association of these molecules in vivo. However, the data presented here clearly indicated that, at least in this system and in HeLa cells, the cell cycle arrest at G2/M transition is not mediated by the competitive inhibition of binding between Cdc2 and cyclin B but rather by the increase in the ratio of phosphorylated inactive form of Cdc2 bound to cyclin B. Thus, the present study has revealed the presence of a novel pathway of G2/M regulation through kpm and the Cdc2-cyclin B complex.

It is likely that the function of kpm is not restricted to the regulation of Cdc2-cyclin B kinase. In fact, we showed that overexpression of kpm induced apoptotic cell death after cell cycle arrest. With regard to this, our preliminary experiments have suggested that expression of Bcl-2 protein is specifically downregulated after 3 days of kpm-wt induction (data not shown) although the signaling cascade leading to apoptosis needs to be investigated. On the other hand, a new motif (1, 36, 37) that is predicted to interact with WW domain, containing-gene) has recently been described that interacts with molecularly define the function of kpm in terms of cell cycle arrest. With regard to this, our preliminary experiments have suggested that expression of Bcl-2 protein is specifically downregulated after 3 days of kpm-wt induction (data not shown) although the signaling cascade leading to apoptosis needs to be investigated. On the other hand, a new motif (1, 36, 37) that is predicted to interact with WW domain, containing-gene) has recently been described that interacts with kpm and the Cdc2-cyclin B complex. 

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