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DNA-dependent protein kinase 在細胞有絲分裂的功能
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成果報告

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中文摘要

許多的實驗顯示 DNA-PKcs 在細胞內具有多重且廣泛的功能，包括在 DNA 傷害修復、V(D)J 重組、DNA 複製、細胞週期檢查點 (checkpoint) 的調控和細胞凋亡等。但由於 DNA-PKcs 不易操作且缺乏適當的 *in vivo* 研究工具，因此在這些作用機制內，DNA-PKcs 扮演的角色和作用的模式仍然是不清楚的。本計劃實行中，我們成功的製備了數種能辨識 DNA-PKcs 的專一性抗體，其中包括能辨識於 T2609 胺基酸呈磷酸化的 DNA-PKcs 之單株抗體，而得以在 *in vivo* 的情況下，研究活化態的 DNA-PKcs 於細胞週期運轉和外在環境壓力下所扮演的生理角色。首先我們在 DNA 複製時，發現磷酸化 DNA-PKcs 會位於複製後染色體的中心體(centrosome)上，且對於微小管的形成(microtubule nucleation)扮演正面調控的角色。另外，我們亦發現磷酸化 DNA-PKcs 在細胞分裂前期會標第至中心粒(centromere)，與 AuroraB 發生交互作用，並影響其磷酸激酶活性。這些發現賦予 DNA-PKcs 前所未見的功能，並可望對細胞核質協調及細胞週期調控提供新的解釋。

Abstract

Since its first description in 1990, DNA-PK catalytic subunit has been one of the most attractive and intriguing molecules in the research field. The bulk of studies has implicated its participation in a wide array of cellular functions, including DNA damage repair, V(D)J recombination, DNA replication, cell-cycle checkpoint and apoptosis. Nevertheless, the role and mode of action of DNA-PKcs in these pathways still remain obscure, mainly because of its difficulty in manipulation, as well as the lack of reagents for tracking the active molecule *in vivo*. In the course of our study we raised several antibodies against different portions of DNA-PKcs, and most importantly, antibodies that can recognize specifically phosphorylated DNA-PKcs, which represents the active kinase *in vivo*. These antibodies enable us to describe for the first time the behavior of activated DNA-PKcs in physiological conditions, as well as during cell cycle progression. We detected the presence of phosphorylated DNA-PKcs on duplicated centrosomes, and described its positive role in microtubule nucleation. Furthermore, we found that phosphorylated DNA-PKcs localize to the prometaphase kinetochores to interact with the AuroraB complex, modulating its kinase activity. These findings reveal unforeseen functions for DNA-PKcs, and may offer the key to unravel new insights into cell nuclear/cytoplasmic coordination and cell cycle progression checkpoints.

Keywords: DNA-PK; centrosome; kinetochore; AuroraB; cell cycle progression.

Introduction

The DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase that is activated upon binding to DNA. DNA-PK is composed of Ku proteins and a catalytic subunit (DNA-PKcs) that belongs to member of the phosphatidylinositol (PI) 3-kinase (PI3-K) superfamily which include *S. cerevisiae* Tor1p, Tor2p and Tel1p, mammalian FRAP and ATM, *Drosophila* Mei41, and *S. pombe* Mec1p/ESR1. Proteins of this superfamily play pivotal roles in regulating cell cycle progression (Tor1p, Tor2p, and FRAP), telomere length (Tel1p), responding to DNA damage and coupling mitosis to the completion of DNA synthesis (Mec1p, Mei41 and ATM). DNA-PK is required for V(D)J recombination, and repairing DNA double-strand breaks and implicated in transcription, DNA replication and immunoglobulin class switch. Despite of this fact, it was also shown that DNA-PKcs *per se* makes contacts with the DNA and can be stimulated slightly *in vitro* by DNA in the absence of Ku. These results lead to the hypothesis that Ku helps to target DNA-PKcs to DNA. DNA-binding proteins other than Ku may regulate DNA-PK activity by mechanisms different from that mediated by Ku proteins. Poly(ADP ribose)polymerase and RNA polymerase II transcriptional activator could also stimulate DNA-PK activity. Herpes simplex virus type 1 transactivator ICP0 could attenuate DNA-PK activity. Heat shock transcription factor 1 and antigen receptor response element binding proteins NF90 and NF45 were shown to interact with DNA-PK. It is likely that many more proteins with DNA-PK modulating activities may be added to this growing list (for review, Lee and Kim,2002).

Many proteins could be the target of DNA-PK. For example, transcription factor p53, replicating protein A), Sp1, SV40 T antigen, serum response factor (SRF), c-Jun, c-Fos and c-Myc, the CTD domain of RNA polymerase II large subunit, the Ku proteins, XRCC4, nuclear receptors for steroids and high mobility group proteins 1 and 2 were phosphorylated by DNA-PK. These proteins are involved in DNA replication, transcription and DNA repair. It is generally believed that the functions of these proteins could be modified upon phosphorylation by activated DNA-PK. How does DNA-PK get activated *in vivo*? DNA-PK can be activated *in vivo* by DNA damaging agents e.g., ionizing radiation. It is likely that DNA-PK may be involved in DNA damage-induced DNA repair by modulating the activities of proteins involved, albeit the molecular mechanism remains unclear. Alternatively, DNA-PK may interact with components of the DNA repair machinery to target them to sites of DNA damage. The exact function of DNA-PK on transcription of specific genes *in vivo* has yet to be elucidated. Much of the earlier reports on DNA-PK and Ku focused on their potential roles in transcriptional regulation, either by phosphorylation of transcription factors or the transcription machinery or as transcription factor (i.e., Ku). The identification of physiological targets (e.g., factors phosphorylated *in vivo*) for DNA-PK would greatly facilitate investigations into the mechanisms and consequences of DNA-PK activation. However, recent progress has raised the possibility that DNA-PK may be a key link between DNA damage and p53 activation. It has been reported that p53 is incapable of binding to DNA in the absence of DNA-PK, that DNA-PK is necessary but not sufficient for activation of p53 sequence-specific DNA-binding, and that this activation occurs in response to DNA damage (Woo *et al.* 1998). Another report concluded that DNA-PK is not required for the p53-dependent response to DNA damage (Jimenez *et al.* 1999). This together with the results of direct phosphorylation of p53 by ATM *in vitro* or in response to ionizing radiation (Canman *et al.* 1998) suggested that DNA-PK is most likely not involved in p53-dependent response to DNA damage. How does DNA-PK activation trigger DNA damage signaling cascades that ultimately impinge on the transcription, DNA replication, cell cycle and apoptotic machineries remain to be investigated.

DNA-PK may interact with chromosomal DNA through direct binding to the DNA (with no free ends) or through chromatin components. Recent investigations

have established that the chromatin-associated high mobility group (HMG) proteins 1 and 2 have been shown to stimulate DNA-PK activity in vitro (Yumoto *et al.* 1998). Furthermore, certain proteins required for heterochromatin-mediated gene silencing in yeast interact with Ku and are required for DNA DSB repair and telomere maintenance (Boulton and Jackson 1998). In yeast, Ku protein has been shown to associate with telomere and contribute directly to telomere function. Recent work suggests that Ku is a central player in processes that regulate telomere structure, replication, recombination, and telomeric silencing. Although Ku proteins are the essential subunits of DNA-PK, ample evidence indicate that they have functions other than DNA-PK (Gao *et al.*, 1998). It remains unclear if yeast homolog of DNA-PKcs participates in these processes.

DNA-PK may have functions other than the nuclear events such as the DNA DSB repair, V(D)J recombination and transcription. The identification of DNA-PKcs as a component of centrosome by mass spectrometry (Andersen *et al.* 2003) prompted us to look into its identity and functions associated with this organelle. DNA-PKcs may be autophosphorylated when the cells were exposed to stressed signals (e.g., irradiation) (Douglas *et al.*, 2002; Chan *et al.*, 2002). Whether the autophosphorylated DNA-PKcs has distinct subcellular localization/function other than the established paradigm remains an open question. We have employed a panel of anti-DNA-PKcs antibodies for probing the subcellular localization and functions of DNA-PKcs. In particular, monoclonal antibody to autophosphorylated Thr-2609 epitope demonstrates that DNA-PKcs is localized to the centrosomes and centromeres during mitosis. In this report, we present results on the functions of DNA-PKcs in regulating the nucleation of microtubule spindles by centrosomes and in regulating the aurora kinase B activity associated with its checkpoint function in the kinetochores.

Materials and Methods

Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1mM L-glutamine, 100 units/ml penicillin, 100µg/ml streptomycin and 10% (v/v) fetal bovine serum (Hyclone), in a humidified atmosphere of 5% CO₂ at 37°C. Molt4 cells were maintained in spinner culture flasks with RPMI1640 medium, supplemented with 2mM L-glutamine, 100 units/ml penicillin, 100µg/ml streptomycin and 10% (v/v) fetal calf serum, at 37°C in 5% CO₂. All the above mentioned reagents, with the exception of fetal calf serum, were purchased from Invitrogen.

Western blot analysis

Protein lysates were subjected to SDS-PAGE electrophoresis and blotted onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 1%BSA in TBS/0.1% Tween 20 for one hour at room temperature and hybridized with the primary antibody overnight at 4°C. After three times of wash with TBS/0.1% Tween 20, membranes were incubated with the corresponding HRP conjugated secondary antibody diluted in TBS/0.1% Tween 20 for 1hr at room temperature. The blots were visualized using the enhanced chemiluminescence system (ECL, DuPont-NEN).

Immunofluorescence staining

Cells were typically seeded at 50% confluency on glass coverslips. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilized for 5 minutes with 0.2% Triton X-100, then blocked in PBS containing 0.1% bovine albumin and 0.1% Triton X-100 for 30 minutes, then incubated with the indicated antibodies for 1 hour. Cells were washed three times with PBS and then incubated with goat

anti-mouse IgG or goat anti-rabbit IgG secondary antibody coupled to Alexa[®] 488, Alexa[®] 594 (Molecular Probes) or Texas red-conjugated goat anti-human IgG (Jackson Laboratory) for 40 minutes. Cells were counter stained with DAPI to highlight the nuclei. Cells were examined under Leica DM RD immunofluorescence microscope and images were captured with a cool CCD, processed by CoolSNAP software (Roper Scientific Inc.).

Isolation and analysis of centrosomes

Nocodazole-treated Molt4 cells ($\sim 10^9$) were used for centrosome isolation. About 150 mg of protein was applied to a discontinuous sucrose gradient using the procedure described in Moudjou, M., and M. Bornens, 1998. Gradient fractions were immunoblotted with the indicated antibodies. For immunofluorescence analysis of centrosomes, the centrosome-containing fractions were spun onto acid-treated coverslips and probed with the indicated antibodies.

Microtubule nucleation test

Microtubule nucleation activities of isolated centrosomes were analyzed according to Mitchison and Kirchner, 1984. The centrosome-containing fractions ($\sim 5 \mu\text{l}$) were incubated in 60 μl of RG1 solution (80 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.8], 1 mM MgCl_2 , 1 mM EGTA, and 1 mM GTP) containing 125 μg of bovine brain tubulin (Molecular Probes) for 8 min at 37°C. Microtubules were fixed by adding glutaraldehyde (1%), sedimented onto acid-treated coverslips, and then subjected to immunofluorescence analysis as described.

***In vitro* kinase assays**

For DNA-PK kinase assays, 0.1 μg of DNA-PK (Promega) were used in each reaction. The assay buffer is 13.75 mM HEPES (pH 7.5); 1.3 mM spermidine; 7.28 mM MgCl_2 ; 11 % glycerol; 0.055 % NP-40; 27.5 mM KCl; 0.55 mM DTT and 0.2 mM ATP, in a final reaction volume of 20 μl . After incubation for the indicated times at 30 °C, reactions were stopped by adding 6 μl of SDS-sample buffer. The reaction products were analyzed by Western Blot.

AuroraB kinase assays were performed by incubating 25 ng of purified histone H3 proteins in a total volume of 15 μl with recombinant kinase. Incubations were carried out for 30 min at 30 °C in kinase buffer (50 mM Tris [pH 7.4], 10 mM MgCl_2 , 6 mM EGTA, and 10 μM ATP). Recombinant AuroraB complex proteins were purified from insect Sf21 cells by co-infection of recombinant baculoviruses encoding GST-fused human AuroraB and His-tagged hINCENP and His-tagged hSurvivin using standard Glutathion column techniques.

Chromosome spreads

HeLa cells were treated with 0.5 $\mu\text{g}/\text{ml}$ colcemide for 60 min, harvested by trypsinization, hypotonically swollen in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 5 mM MgCl_2 , and sedimented onto cover slips for 15 s at 900x g. Chromosomes were swollen for an additional 15 min in 25 % PBS, fixed in 3.7 % formaldehyde in 25 % PBS for 10 min, and permeabilized with 0.5 % NP-40 in 25 % PBS for 10 min. Samples were blocked with 1 % bovine serum albumin (BSA) in PBS, and then processed as described in immunofluorescence staining.

Results and Discussion

Localization of DNA-PKcs during the cell cycle

We have developed a panel of antibodies specific to the DNA-PKcs protein, and conducted a series of cell biological studies employing these antibodies. In

particular, we obtained a monoclonal antibody specific to the T2609 phosphorylated form of DNA-PKcs, which in the current understandings of the protein, represents the activated form of DNA-PKcs (Refer to previous reports).

DNA-PKcs is a centrosomal protein

Using immunofluorescence staining techniques, we found that two of our antibodies against DNA-PKcs could stain the centrosomes, with the pattern being especially prominent during mitosis. In order to unequivocally determine the association of DNA-PKcs with centrosomes, centrosome fractions were prepared from Molt4 cells using sucrose density gradient centrifugation as described in Materials and Methods. Immunoblotting analysis revealed that T2609 phosphorylated DNA-PKcs and γ -tubulin cosedimented with the centrosomal fractions (Fig. 1A). This result is further supported by the fact that the N antibody specific to the N-terminal portion of the protein also detects DNA-PKcs in the centrosome fractions, although the N-derived signals reveal a broader distribution of the kinase. The isolated centrosomes were further analyzed by immunofluorescence experiments using anti- γ -tubulin, T2609 and Mid (recognizes DNA-PKcs middle region) antibodies. As shown in Fig. 2B, both proteins colocalized to the isolated centrosome. These results indicate that DNA-PKcs, like γ -tubulin, is an intrinsic centrosomal component.

DNA-PKcs may be involved in microtubule nucleation.

We then evaluated the possible role of DNA-PKcs in microtubule-nucleating activity. The centrosome fractions isolated from Molt4 cells were incubated with either Mid or T2609 antibody and then assayed for aster formation. As shown in Fig. 2, most centrosomes nucleated microtubule asters in the presence of control antibody (mIgG). In contrast, the number and the length of nucleating microtubules were significantly diminished when the centrosomes were incubated with Mid, T2609, or anti- γ -tubulin antibodies. Notably, both the number and the length of the microtubules were significantly affected by coincubation with Mid or T2609 antibodies in a dose dependent manner. These results suggest that DNA-PKcs is a centrosomal protein that may play a positive role in microtubule nucleation.

Taken together, our results indicate that DNA-PKcs is a component of the centrosome and may participate in microtubule nucleation. Centrosomes play an important role in maintaining the fidelity of chromosome distribution during mitosis. The centrosome must be duplicated only once during each cell cycle to ensure the fidelity of chromosomal segregation in subsequent mitosis. Loss of these functions should cause chromosomal instability and aneuploidy. It is possible that accumulation of various instabilities of chromosomes eventually leads to tumorigenesis (Assoro *et al.*, 2002). DNA-PKcs was well known for its nuclear localization, and its interaction with many nuclear proteins involved in DNA repair and cell cycle regulation. Our results show that DNA-PKcs localizes not only in the nucleus but also to the centrosomes. It is possible that DNA-PKcs in centrosomes plays roles in ensuring correct distribution of each centrosome or chromosome to daughter cells. To study these possibilities, identification of physiological substrates of DNA-PKcs on the centrosome may be of great importance in its functional characterization.

Recently, a mass-spectrometry-based proteomic analysis of centrosomes has also reported the identification of DNA-PKcs derived peptide sequences in the centrosome (Andersen *et al.* 2003). This is the only study that complements our results so far. It is conceivable because in our own observations, DNA-PKcs seems to be buried inside the compact mass of centrosome, and is quite inaccessible to antibody detection in immunofluorescence studies. Under our experimental procedures, neither N nor C antibodies could produce clear centrosomal staining of DNA-PKcs. However, the middle portion of DNA-PKcs as well as the T2609 epitope seem to be much more accessible, thus lead us to discover this undescribed phenomenon.

T2609 Phosphorylated DNA-PKcs localizes to the centromere

Our immunofluorescence studies also revealed that during prophase, foci of

phosphorylated DNA-PKcs become associated with chromosomes. As the cells progressed into metaphase, some of them loss their chromosomal T2609 signal, while others retain the pattern until late anaphase. In view of these observations, we further analyzed the localization of phosphorylated DNA-PKcs using prometaphase chromosome spread techniques (Figure 3). Interestingly, the T2609 antibody stained as pairs at the centromere region, with even doublet signal intensity suggesting that association of DNA-PKcs occurs along a same axis joining two kinetochore discs.

Since the tension on spindle microtubules is required for stabilizing the correct attachment of kinetochores to opposite poles, it is important to know whether the localization of DNA-PKcs was dependent on the integrity of spindle microtubules. HeLa cells were cultured in the presence of the microtubule stabilizing agent taxol, or the destabilizing agent demicolcemid. We also compared cells that had been grown directly on coverslips with cells that were spread onto slides by cytocentrifugation, a procedure that disrupts microtubules mechanically. In each case, the staining of T2609 antibody remained positive on the prometaphase/metaphase centromere (data not shown). Therefore, association of DNA-PKcs to the centromere did not depend on the integrity of microtubules.

AuroraB is an in vitro substrate for DNA-PKcs kinase activity

To explore the role of phosphorylated DNA-PKcs on the centromeres, we searched for candidate substrates proteins that were reported to be present on prometaphase centromeres. Interestingly, we found that two components of the AuroraB kinase complex, namely AuroraB and INCENP, possess DNA-PKcs phosphorylation sites that are conserved through all of its metazoan homologues identified to date (Figure 4a). We then prepared purified recombinant AuroraB complex proteins using the baculovirus overexpression system, and tested whether DNA-PK could phosphorylate these proteins in vitro. As shown in Figure 4b, incorporation of ^{32}P from $\gamma\text{-}^{32}\text{P}\text{-ATP}$ onto AuroraB was greatly enhanced when the DNA-PK kinase reaction was supplemented with DNA, but not if the DNA-PK inhibitor wortmannin was included. This is in good accordance with the definition of DNA-PK as a DNA activated kinase. Thus, we conclude that AuroraB can serve as in vitro substrate for DNA-PK activity in vitro.

DNA-PKcs can simulate AuroraB kinase activity in vitro

The kinase activity of AuroraB was shown to be regulated by phosphorylation. To determine if AuroraB kinase activity could also be influenced by DNA-PK, purified recombinant AuroraB complexes were subjected to DNA-PK phosphorylation, prior to incubation with its substrate histone H3. AuroraB specific kinase activity was then detected using an anti-S10 phosphorylated histoneH3 antibody. We found that at least six-fold greater activity was detected if the AuroraB complex was phosphorylated as compared with the unphosphorylated fractions (Figure 5, upper panel). To verify that the kinase stimulation was due to an increase in specific activity of AuroraB, we performed the same assay using a kinase dead mutant of AuroraB, which showed no increase in histone S10 phosphorylation along with different extent of DNA-PK phosphorylation. Similar amounts of AuroraB protein was detected by western blotting (Figure 5, lower panel). Therefore, we conclude that the presence of DNA-PK could stimulate AuroraB kinase activity in vitro. AuroraB regulates chromosome segregation, cytokinesis and also bipolar attachment of spindle microtubules to kinetochores. How does the interaction between DNA-PKcs and AuroraB implicates in the physiological scenario, as well as the detailed mechanism of these kinase-substrate relationships deserve devoted study.

A

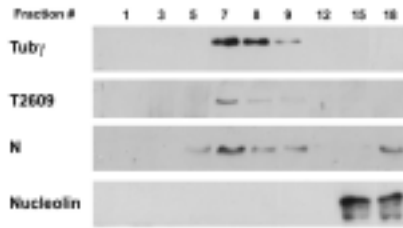
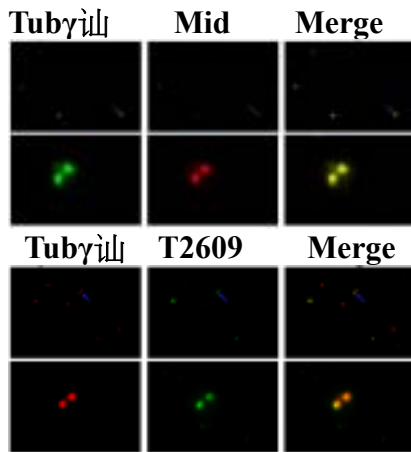


Figure 1. DNA-PKcs is a component of the centrosome.

A fraction of DNA-PKcs co-migrates with centrosomal markers. Centrosome fractions of Molt4 cells were prepared by discontinuous sucrose density gradient (70, 50, and 40% sucrose solutions) and immunoblotted with the indicated antibodies.

B



(B) Colocalization of T2609 phosphorylated DNA-PKcs and -tubulin in the isolated centrosomes. The centrosome fractions in (A) were spun onto acid-treated coverslips for immunofluorescence analysis and then double stained with the indicated antibodies. Arrows indicate the pair of centrosomes that are shown magnified in panels below.

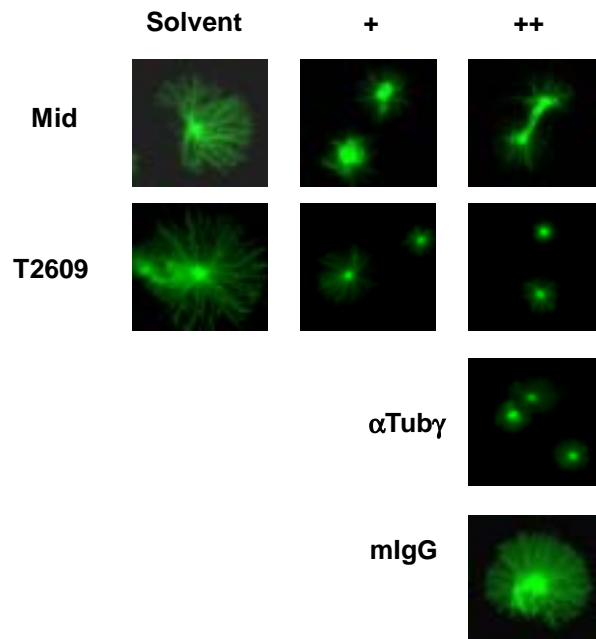


Figure 2. Inhibition of microtubule nucleation by anti-DNA-PKcs antibodies.

Isolated centrosomes were preincubated with 0.5ul (+) or 5ul (++) of the indicated antibodies for 30 min at 4°C before tubulin was added to start *in vitro* microtubule nucleation. The microtubule asters were stained with anti-tubulin α mAb. Non-specific mouse IgG was used as a negative control, while anti-tubulin γ serves a positive control for inhibitory activity.

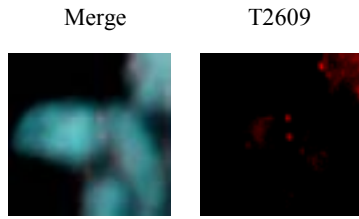


Figure 3. DNA-PKcs locates to the prometaphase centromeres

Prometaphase HeLa chromosome spreads were carried out as described in Materials and Methods. T2609 labelling (red) indicating phosphorylated DNA-PKcs is counterstained with DAPI (blue). The doublet signals are clearly seen on sites of primary constriction, i.e. the centromeres.

A

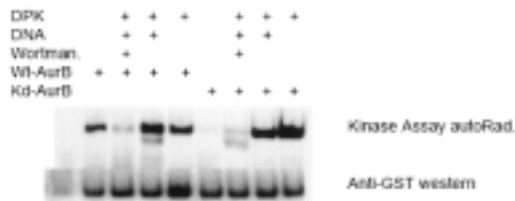
Aurora B

human: **CLFKSQIEKEG** NP_004208
 mouse: **CLFKSQIEKEG** XP_181344
 xenopus: **LFKSQLEKEG** AAM776715

INCENP

human: **ARGTPLSQAIHQY** NP_064623
 mouse: **KGTQLSQAIHQY** NP_057901
 xenopus: **ASGNLLTQAIHQY** AAC60120

B



C

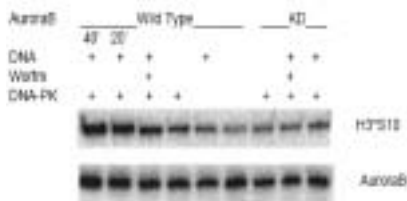


Figure 3. DNA-PKcs can phosphorylate and stimulates AuroraB kinase activity in vitro

(A) Putative DNA-PK phosphorylation sites found on AuroraB and INCENP. Note that these two sites are conserved among metazoan. (B) Baculovirus expressed GST-AuroraB recombinant proteins were subjected to DNA-PK kinase assay as detailed in Materials and Methods. The lower panel shows the loading of the GST-recombinants as visualized by western blotting using a monoclonal antibody against GST. (C) AuroraB specific kinase activity is measured through histone S10 phosphorylation as determined by western blot assay using the H3*S10 monoclonal antibody. Lower panel showing anti-AuroraB western blotting serve as kinase loading control.

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發明人/創作人	
技術說明	中文： (100~500 字)
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技術特點	
推廣及運用的價值	

1. 每項研發成果請填寫一式二份，一份隨成果報告送繳本會，一份送貴單位研發成果推廣單位（如技術移轉中心）。

2. 本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。