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Cloning, Expression and Characterization of NIP2, A Novel Nek2 Interacting Protein

Yeon Tae Jeong, Jae Cheal Yoo and Kunsoo Rhee
School of Biological Sciences, Seoul National University, Seoul 151-742, Korea

Nek2 is a mammalian ser/thr kinase that is closely related to *Aspergillus* NIMA. The structural homology between *Nek2* and *nimA* suggests that, like NIMA, Nek2 is involved in the cell cycle regulation. When we carried out the yeast two hybrid screening Nek2 as a bait, we cloned a novel gene which has a total sequence of 3.6kb and encodes a protein of 535 amino acids, the clone named as *NIP2* (Nek2-Interacting-Protein 2). Northern blot hybridization analysis using human tissue blot showed ubiquitous expression of *NIP2* mRNA but predominantly in the testis. Immunofluorescence staining using various human cells and mouse cells indicated that NIP2 was localized in diverse places within a cell, such as centrosomes, mitotic spindle pole during mitosis, and endoplasmic reticulum.

Furthermore, various *Nek2* constructs directly phosphorylated NIP2 bacterially purified as well as immunoprecipitated from cell lysates in vitro. These results suggest the functional relationship between Nek2 and NIP2.

INTRODUCTION

The mammalian cell cycle is regulated by highly complicated mechanisms, which involve multiple cell cycle regulators. Among these cell cycle regulators, Cdc2/Cyclin B complex is most extensively studied and is known for its critical roles in G2/M progression of cell cycle. However, it has recently been known that induction of Cdc2 activity alone may not be sufficient to progress cell into M phase in certain organisms, such as yeasts and *Aspergillus nidulans* (reviewed by Gallant et al., 1995). NIMA was identified as another critical cell cycle regulator for the M phase progression (Osmani et al., 1998, 1991). NIMA is a Ser/Thr protein kinase, but distinct from Cdc2 structurally as well as biochemically (reviewed by Fry and Nigg, 1995). Recently several lines of evidence supported the existence of NIMA like pathway in mammalian cell. Ectopic expression of NIMA has been shown to cause germinal vesicle breakdown in *Xenopus* oocytes (Lu an Hunter, 1995) and premature mitotic events in HeLa cells (Lu and Hunter, 1995). However, dominant negative mutants of NIMA protein cause HeLa cells arrested at G2 phase (Lu and Hunter, 1995).

Several putative mammalian homologues of nimA gene were identified by their structural homology. Among mammalian homologues of NIMA, Nek2 protein shares the strong structural homology with *Aspergillus* NIMA. Nek2 has been shown to exhibit cell cycle specific expression (Schultz et al., 1994; Fry et al., 1995). Interestingly, Nek2 is highly expressed in germ cells (Letwin et al., 1992; Rhee and Wolgemuth, 1997; Tanaka et al., 1997) and shown to be associated with meiotic chromosomes (Rhee and Wolgemuth, 1997), suggesting that it might be important during meiosis and could be involved in meiotic chromosome condensation.

However, overexpression of Nek2 in mammalian cells did not show any effect on chromosome condensation, instead, it caused a drastic alteration of centrosomal structure (Fry et al., 1998). Furthermore, evidence from immunofluorescencestaining and immunoblotting

demonstrated that endogenous Nek2 associated with the centrosomes (Fry et al., 1998). These observations led to propose a possible implication of Nek2 in the centrosome cycle (Fry et al., 1998).

In this study, we report a putative Nek2-interacting gene, *NIP2* (Nek2 Interacting Protein 2). NIP2 is a centrosomal protein. In vitro kinase assay has shown that Nek2 phosphorylate NIP2 in vitro. This facts suggested that NIP2 might cooperate with Nek2 during the regulation of cell cycle.

MATERIALS AND METHODS

cDNA library screen

Phage plates containing 50,000 plaques per 150mm plate are prepared for blotting of hybridization membranes. At this time, hybridization membranes are duplicated, first one, blotted for 1 minute, second one, blotted for 2 minutes. Blotted hybridization membranes are soaked serially into washing solution (2XSSC) for 1 minute, denaturation solution (1M NaOH, 1.5M NaCl) for 5 minutes, neutralization solution (0.5M Tris-HCl pH7.5, 1.5M NaCl) for 5 minutes. Soaked hybridization membrane is dried onto 3MM papers. Dried membranes are baked at 80℃ for 2 hours. Prepared hybridization membranes are pre-washed at room temperature for 1 hours with shaking in prewashing solution (50mM Tris-HCl pH8.0, 1M NaCl, 1mM EDTA pH8.0, 0.1% SDS), Then pre-hybridized at 65℃ for 1 hour in prehybrid solution (6X SSC, 5X Denhardt s solution, 0.5% SDS, 80μg/ml salmon sperm DNA). DNA probe is acquired from yeast two hybrid screen and labeled by random-prime DNA labeling system (Amersham Pharmacia). ^{32}P labeled DNA probe is added into prehybrid solution containing hybridization membranes, then hybridized for 16 hours at 65℃. After hybridization, hybridization solutions are saved for secondary screen and stored at -20℃. Hybridization membranes are washed with washing solution I (2X SSC, 0.1% SDS) at room temperature for 30 minutes, 2 times, then washed with washing solution II (0.2X SSC, 0.1% SDS) at 65℃ for 30 minutes, 2 times. After washing, hybridization membranes are exposed onto X-ray film at -70 ℃ overnight. Positive colonies are picked up and suspended in 1ml SM buffer/30$\mu\ell$ chloroform at room temperature for 4 hours or 4℃ overnight. Secondary screen is a repeat of primary screen. Final positive clones of secondary screen are taken into in vivo excision.

Northern blot hybridization

Human tissue hybridization blot is purchased from commercial company and Total RNA of human cell line is prepared following Chomczynski methods (Chomcznski and Sacchi, 1987). Prepared RNA is loaded onto 0.85% agarose/6.6% formaldehyde gels and run at 70V constant voltage. Separated RNA in gels is transferred onto nylon membrane overnight and baked 2 hours at 80℃ in vacuum oven. Prepared hybridization membranes are prehybridized for 1 hour at 65℃ in hybridization solution (0.5M $NaPO_4$, 1mM EDTA, 1%BSA, 7% SDS, 2X Denhardt s solution).

After prehybridization, ^{32}P labeled DNA probes are added into hybridization solution and incubated at 65℃ overnight with shaking. Hybridized membranes are washed serially with washing solution I (2X SSC, 0.1% SDS) at room temperature for 30 minute, 2 times and washing solution II (0.2X SSC, 0.1% SDS) at 65℃ for 30 minute, 2 times. After washing, hybridization membranes are exposed onto X-ray film at -70℃ overnight or a week.

Antibody production

Full open reading frame of NIP2 is subcloned into pGEX 4T-1 via EcoR I and Xho I sites. AD494 DE3 bacterial strain is used as a expression host of pGSTNIP2. Optimal time course are tested to acquire a maximum output of soluble GST-NIP2 fusion proteins. AD494

DE3 containing pGSTNIP2 construct are grown until OD600 reaches to 1.0, then induce with 1mM IPTG for 1 hour. After induction, bacteria are harvested by centrifugation and suspended in 1X PBS, then lysed with French Pressure. Soluble fraction of Prepared bacterial lysates are separated from insoluble pellet by centrifugation at 39,000 X g . Separated soluble fractions are poured onto top of glutathione-Sepharose 4B bead column. After pouring, fusion proteins are eluted with elution buffer (10mM reduced glutathion) following 3 times 1X PBS washing. Acquired fusion proteins are used as antigen injected into rabbit. Before first injection, preimmune serum is bled. 100μg fusion proteins are injected into rabbit at first injection. 3 weeks after primary injection, 50μg fusion proteins are injected as a secondary injection. 3 weeks after secondary injection, 50μg fusion proteins are injected as a tertiary injection. 2 weeks after secondary injection, test immune serum is bled. After testing the correct boosting, rabbit injected with GSTNIP2 fusion proteins are sacrificed. Acquired anti serum is purified by affinity column chromatography. For preparing affinity column, 100μg purified fusion proteins are coupled with CNBr-Sepharose 4B bead in coupling buffer 4℃ overnight. After coupling, fusion protein-bead are blocked with 0.1M Tris-HCl pH8.0, and then washed with washing solution. Antiserum diluted 1:5 by 0.1% TBST are poured onto top of antigen-coupled columns. After pouring of antiserum, antigen column is washed with 0.1% TBST extensively. After washing, antibodies interacting with antigens are eluted with 100mM Glycine pH2.5 followed by neutralizing with 1M Tris-HCl pH8.0.

Cell culture and Transfection

All tissue culture cells used in experiments are grown at 37℃ in a 10% CO_2 atmosphere in DMEM/10%FBS with penicillin/streptomycin. For transient transfection, 293T cells are seeded onto 60mm dish at a density of $2X10^6$ cells/dish approximately 18-24 hours prior to transfection. Before adding DNA/$CaCl_2$ mixture, media are changed with fresh media containing chloroquine. 5-10μg DNA are used for transfection. DNA/$CaCl_2$ mixtrues are added onto dish drop wise. After 10 hours, transfection media are changed by fresh media. Cells are harvested at 24-48 hours after transfection. For immunofluorescence microscopy, SaOS2 and CHO cells are seeded onto coverslip at a density of $1-2X10^4$ cells/coverslip and grown to make proper morphology.

Immunoblotting and Immunofluorescence microscopy

For immunoblotting, total cell lysates are prepared with cell lysis buffer (50mM Tris-HCl pH7.3, 150mM NaCl, 0.1% SDS, 1% NP-40, 1X protease inhibitor cocktail). Concentration of cell lysates is measured by Lowry assay. Cell lysates are separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. After transfer, nitrocellulose membranes are stained with Ponseu S solution and then, blocked in blocking solution (5% skim milk in 0.1% TBST) at room temperature for 30 minutes. After blocking, nitrocellulose membranes are incubated with primary antibody at room temperature for 1 hour 30 minute. Primary antibody incubated membranes are washed with 0.1% TBST 3-4 times, 10 minutes each. After washing, nitrocellulose membranes are incubated with secondary antibody (alkaline phosphatase conjugated IgG, 1:5000) and then, washed with 0.1% TBST 3-4 times, 10 minutes each. After washing, specific band is detected by NBT/BCIP color reaction.

For immunofluorescence microscopy, SaOS2 cells are either transiently transfected with various DNA or observed directly without transfection. Lipofectamine (GibcoBRL) is used in transient transfection following manufacturer"s instruction. 24-48 hours after transfection, samples are fixed with -20℃ cold methanol for 10 minutes, then washed 1X PBS.

Fixed samples are permeablized and blocked with 3% BSA/0.5% PBST solution for 15 minutes, then washed with 1X PBS. Blocked samples are incubated in affinity purified antibody solution for 1 hour, and then washed quickly with 0.1% PBST 4 times. After primary antibody incubation, secondary antibody conjugated with FITC or TRITC are added onto samples for 30 minutes, and then washed with 0.1% PBST 4 times. After incubation, coverslips are mounted onto slideglass. The sample was observed by both fluorescence microscope and confocal microscope.

RESULTS

Cloning of NIP2

NIP2 was isolated from the yeast two hybrid screen using Nek2 as a bait. In the yeast two hybrid screen, initially a partial cDNA of 1.8kb in size was isolated. In order to clone a full open reading frame of NIP2, a cDNA library was screened. In cDNA library screen, 5″ further sequence of NIP2 was found, but when the cDNA was sequenced, any proper start codon could not be found, so we made a strategy to perform 5″ RACE (rapid amplification of cDNA end). As a result of 5″ RACE, a full cDNA sequence of 3.6kb could be isolated. NIP2 encodes a protein containing 535 amino acids and has a calculated molecular weight of 59.7 kDa. NIP2 gene has a putative coiled coil region at 398-440 a.a. (Figure 1a,b).

Northern blot hybridization of NIP2

The northern blot hybridization analysis of NIP2 was carried out in human tissue blot. The estimated size of NIP2 mRNA was approximately 3.6kb, indication that the cDNA clone that we have is a full-length clone (Figure 1,2). NIP2 mRNA was expressed in all human tissues tested, but most predominantly in the testis (Figure 2a). The northern blot hybridization analysis in human tissue culture cells revealed that NIP2 mRNA is expressed in all tissue culture cells tested (Figure 2b). These results suggest that NIP2 may be a basic constituent of all cell types.

Immunoblot analysis of NIP2

A polyclonal antibody against the NIP2 protein was raised in order to study expression of NIP2 at the protein level. Immunoblot analysis using affinity column purified NIP2 antibody has shown that anti-NIP2 antibody specifically recognized a single band that migrates with position of approximately 70kDa in various tissue culture cells used in northern blot hybridization (Figure 3b). Interestingly, anti-NIP2 antibody recognized two specific bands in cell lysates transiently transfected with MycNIP2WT. The expression pattern of NIP2 protein in immunoblot analysis conforms to that of northern blot hybridization in human tissue culture cells.

Subcelluar localization of NIP2

In order to examine the subcelluar localization of endogenous NIP2 protein, I carried out immunofluorescence staining experiments using the anti-NIP2 antibody. The results showed that endogenous NIP2 proteins were localized specifically to the centrosome and ER (Figure 4). The centrosomal localization of NIP2 was confirmed by co-immunostaining with the anti-NIP2 antibody and an anti-tubulin antibody (Figure 4). Furthermore, endogenous NIP2 proteins are co-localized with Bip, a ER marker protein. Interestingly, the signal of NIP2 has always been localized to centrosome throughout mitosis (Figure 5). In order to determine whether NIP2 proteins are a microtubule-independent core component of centrosome or not, SaOS2 cells were treated with nocodazole or cold-treated for destruction of microtubule array and immunostained with the anti-NIP2 antibody. The results showed that the NIP2 antibody stained the centrosome, even if microtubule array was totally disturbed. These results indicated that the NIP2 protein was a core component of centrosome, independent of microtubule (Figure 6).

Nek2 specifically phosphorylates NIP2 in vitro.

In order to examine the character of NIP2 as a substrate of Nek2, we carried out in vitro kinase assay. we used dephosphorylted casein as a positive control. In this results, Nek2 specifically phosphorylated casein as previous reports but Nek2KD (kinase defective) did not. Nek2 also specifically phosphorylated NIP2WT. This results showed that NIP2 possibly be a good substrate of Nek2 in vitro and Nek2 regulate the mechanics of NIP2 via a phosphorylation.

DISCUSSION AND CONCLUSION

In this study, we reported a novel centrosomal protein, NIP2. *NIP2* had a total cDNA length of 3.6kb and 535 amino acid, calculated molecular weight of 59.7 kDa. NIP2 mRNA expressed in all human tissue together with all tissue culture cells tested and most predominantly in the testis. NIP2 proteins expressed in all human tissue culture cells tested. Endogenous NIP2 proteins specifically localized to centrosome. Furthermore, endogenous NIP2 proteins were always localized to centrosome throughout the mitosis and even though when microtubule array were totally disturbed by nocodazole or cold treatment.

NIP2 mRNA expressed in all human tissue tested but predominantly in the testis reminiscent of Nek2 mRNA. Predominant expression of NIP2 mRNA in the testis suggests that NIP2 might play an important role with Nek2 during spermatogenesis (Rhee and Wolgemuth 1997).

In further study of NIP2, the relationship between Nek2 and NIP2 should be further examined by co-immunoprecipitation. Also, the roles of NIP2 protein itself in centrosome regulation should be investigated. On other hand, other NIP2 interacting protein and Nek2 interacting proteins should be isolated and characterized taking advantage of yeast two hybrid screen.