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IQGAP1 interacts with Aurora-A and enhances its stability and its role in cancer

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ABSTRACT

IQGAP1, a ubiquitously expressed scaffold protein, has been identified in a wide range of organisms. It participates in multiple aspects of cellular events by binding to and regulating numerous interacting proteins. In our present study, we identified a new IQGAP1 binding protein named Aurora-A which is an oncogenic protein and overexpressed in various types of human tumors. *In vitro* analysis with GST-Aurora-A fusion proteins showed a physical interaction between Aurora-A and IQGAP1. Moreover, the binding also occurred in HeLa cells as endogenous Aurora-A co-immunoprecipitated with IQGAP1 from the cell lysates. Overexpression of IQGAP1 resulted in an elevation of both expression and activity of Aurora-A kinase. Endogenous IQGAP1 knockdown by siRNA promoted Aurora-A degradation whereas IQGAP1 overexpression enhanced the stability of Aurora-A. Additionally, we documented that the IQGAP1-induced cell proliferation was suppressed by knocking down Aurora-A expression. Taken together, our results showed an unidentified relationship between Aurora-A and IQGAP1, and provided a new insight into the molecular mechanism by which IQGAP1 played a regulatory role in cancer.

1. Introduction

IQGAP1 is an important member of the IQGAP (IQ-domain GTPase-activating protein) protein family [1–3]. It is the best characterized member, which contains many functional binding domains for F-actin, calmodulin, S100B, Rac/Cdc42, β-catenin, E-cadherin, CLIP-170, adenomatous polyposis coli (APC), ERK1/2 and MEK1/2. By interacting with these proteins, IQGAP1 plays a crucial role in multiple fundamental cellular activities, such as cytoskeletal regulation, cadherin-mediated cell to cell adhesion, cell polarization and actin reorganization [4–7]. IQGAP1 also enhances cell proliferation and differentiation through modulating signal transduction pathways, such as the MAPK (mitogen-activated protein kinase) cascade and Wnt pathway [8–10].

Aurora-A which belongs to the Aurora kinase family, was first discovered in the screening for Drosophila mutations affecting the poles of the mitotic spindle function [11,12]. Human Aurora-A is located on the chromosome 20q13 and found overexpressed in several human cancers. Many research groups have proved that the overexpression of Aurora-A induces several cancer-associated phenotypes, including enhanced cell proliferation and colony formation, and inhibition of apoptosis [13–15]. In mammalian cells, degradation of Aurora-A depends mainly on the Anaphase Promoting Complex/Cyclosome (APC/C) with its auxiliary subunit CDH1 [16]. As a multi-subunit ubiquitin ligase, APC/C is composed of at least 13 subunits, including APC2, a structural component, and CDC27, which regulates activation of APC/C by association with CDH1 and CDC20. APC/C is activated by its association with CDH1 through recognizing either a D or a KEN box, whereas APC/C-CDC20 is activated by direct binding to the D box of the substrate protein [17,18].

In this study, we report for the first time that IQGAP1 interacts with Aurora-A, and reveal how the level of Aurora-A is regulated by IQGAP1 in cancer cells.

2. Materials and methods

2.1. Cell cultures and treatment

MCF-7, HEK293 and HeLa were obtained from the Cell Resource Center of Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). All the cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin at 37 °C in 5% CO_2. For transfection, Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) and Attractene Transfection Reagent (Qiagen, Valencia, CA, USA) were used. For analyzing protein stability, cells were treated with 100 μg/ml CHX (cycloheximide) (Sigma, St. Louis, MO, USA) or 10 μM MG132 (Z-Leu-Leu-Leu-al)

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(Sigma, St. Louis, MO, USA). In order to synchronize cells at G2/M phase, cells were treated with 400 ng/ml nocodazole (Sigma, St. Louis, MO, USA) for 16 h.

2.2. Plasmid construction

To obtain myc-tagged human IQGAP1 (amino acids 1–1657), PCR was performed on human cDNA using primers flanking nucleotides 1 and 4971. The forward primer 5′-CCGCTCGAGATGT-CCGCCGCAGACGAGGTTG-3′ and the reverse primer 5′-TCCGGGC-CCCTTCCCGTAGAACTTTTTGTTGAGA-3′ were designed to generate a 4971-bp DNA product containing an XhoI site at the 5′ end and ApaI site at the 3′ end. Digestion with XhoI and ApaI generated a fragment that was subcloned into the XhoI-ApaI site of pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) yielding a plasmid containing the cDNA for the 1657-amino acid residues of IQGAP1 fused in-frame at its N terminus to a myc epitope tag. A similar cloning strategy was employed to construct IQGAP1-C (amino acids 864–1657), IQ-GAP1-N (amino acids 1–863), IQGAP1-ΔCT (amino acids 1–1502) and IQGAP1-CT (amino acids 1503–1657) using primers flanking nucleotides 2289 and 4974, 1 and 2588, 1 and 4506, and 4506 and 4974, of IQGAP1 respectively. The forward primers 5′-CCGCTCGAGATGGAATTCCGATCCAGGATGAAT-3′, 5′-CCGCTCGA-GAT GTCCGCCGCAGACGAGGTTG-3′, 5′-CCGCTCGAGATGTCCGCCG-CAGACGA GGTTG-3′ and 5′-CCGCTCGAGATGCTA-GTGAAACTGCA ACAGACAT-3′ and the reverse primers 5′-TCCGGGCCCCTTCCCGTA-GAACTTTTT GTTGAGA-3′, 5′-TCCGGGCCCATCCTCAGCATTGATGAG AGTCTTG-3′, 5′-TCCGGGCCCTT CGGCCTTTCTCCTCTGTCG-3′ and 5′-TCCGGGCCCTTCGGCCTTTCTCCTCT GTCG-3′ were used to gener-ate the IQGAP1-(864–1657), IQGAP1-(1–86 3), IQGAP1-(1–1502), and IQGAP1-(1503–1657), respectively. Sequences were confirmed by DNA sequencing. All deletion mutants and wild type migrated to the expected positions on SDS–PAGE.

2.3. RNA interference

siRNA were purchased from Santa Cruz Technology. For MCF-7, 0.8×10^6 cells were transfected with control siRNA (sc-37007), IQ-GAP1 siRNA (sc-35700), or ARK-1 siRNA (sc-29731) using Lipofect-amine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. All siRNA products generally consist of pools of three to five target-specific 19–25 nt siRNAs designed to knockdown gene expression.

2.4. Reverse transcription and Real-time quantitative PCR

Total RNA was purified and reverse transcription was carried out as described previously [22]. Real-time PCR was performed with ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex Taq II Kit (TaKaRa, Dalian, China). The specific primer pairs used were as follows: IQGAP1, 5′-CACTGGCT AAGACGGAAGTGTC-3′ (forward), 5′-TCCTGGCTGGAACCGGAT-3′ (reverse); Aurora-A, 5′-ACTCAGCAATTTCCTTGTCAGA-3′ (forward), 5′-GATTATTTTC AGGTGCCGATG-3′ (reverse) and GAPDH, 5′-ATGA-CATCAAGAAGGTGGTG-3′ (forward), 5′-CATACCAGGAAA TGA-GCTTG-3′ (reverse).

2.5. Co-immunoprecipitation

Cellular extracts were precleared with protein A/G Sepharose beads (Santa Cruz, CA, USA) and incubated with indicated antibod-ies for 4 h at 4 °C. Then, protein A/G Sepharose beads were added and samples were incubated overnight at 4 °C. The beads were washed five times with lysis buffer followed by western blot assay.

2.6. GST pull-down assay

GST-pull down assay was performed essentially as previously described [19]. Briefly, MCF-7 cells were lysed and equal amounts of protein lysate were incubated with GST-Aurora-A on glutathi-one-Sepharose beads for 6 h at 4 °C. GST alone was used as a control.

2.7. BrdU incorporation and flow cytometry

To label MCF-7 cells *in vitro,* 10 μl BrdU (1 mM) were added di-rectly to each milliliter of cell culture media. After 4 h, cells were harvested and treated by using the BrdU Flow Kits (BD Biosciences Pharmingen, San Diego, CA, USA) according to user's manual. The stained cell samples were then used for flow cytometry analysis.

2.8. Antibodies

Antibody against IQGAP1 was purchased from BD Biotechnol-ogy (610612). Antibodies for Aurora-A (sc-25425), GST (sc-80004), APC2 (sc-20984), CDC27 (sc-5618), CDC20 (sc-8358), Ubiq-uitin (sc-9133), and β-actin (sc-8432) were obtained from Santa Cruz Technology. Antibodies for CDH1 (c-7855) were purchased from Sigma. Antibodies for Phospho-Aurora A (#3079) were pur-chased from Cell Signaling Technology.

3. Results

3.1. IQGAP1 interacts with Aurora-A

To investigate the role of IQGAP1 in the tumorigenesis, we first examined the interaction between Aurora-A, a key mitotic regula-tor and strong oncogenic protein, and IQGAP1. Using the immuno-precipitation approach, we found that IQGAP1 was able to bind specifically to Aurora-A. Neither of the proteins was detected in samples precipitated with non-specific IgG (Fig. 1A and B). GST-pull down experiment also proved that IQGAP1 could bind to GST-Aurora-A fusion protein (Fig. 1C and D). The binding was specific, as no IQGAP1 was present in the samples incubated with GST alone.

3.2. IQGAP1 increases Aurora-A protein expression

To determine whether the abnormal expression of IQGAP1 has an effect on Aurora-A, we measured the protein levels of Aurora-A. Two complementary strategies were adopted. First, we tran-siently overexpressed IQGAP1 in MCF-7 cells, pcDNA3.1 (vector) as a control. It was shown in Fig. 2A that Aurora-A was up-regu-lated by IQGAP1 overexpression, whereas it was dramatically down-regulated when IQGAP1 was knocked down (shown in Fig. 2B). Additionally, the level of Aurora-A mRNA was examined via semi-quantitative RT-PCR and Real-time quantitative PCR, and there was no evidence of a decrease in Aurora-A mRNA level following IQGAP1 RNAi (Supplementary Fig. 1). This result suggests that IQGAP1 may regulate Aurora-A expression through a post-transcriptional mechanism.

3.3. Overexpression of IQGAP1 delays the degradation of Aurora-A

We employed CHX (cycloheximide), a protein synthesis inhibi-tor, to treat HeLa cells transfected with myc-tagged IQGAP1 or pcDNA3.1. The protein levels of Aurora-A were detected at differ-ent time points (0, 2, 4, 6 and 8 h) by western blot. It was shown in Fig. 3A that when transfected with myc-tagged IQGAP1, Aurora-A became more stable and had a longer half-life. Several

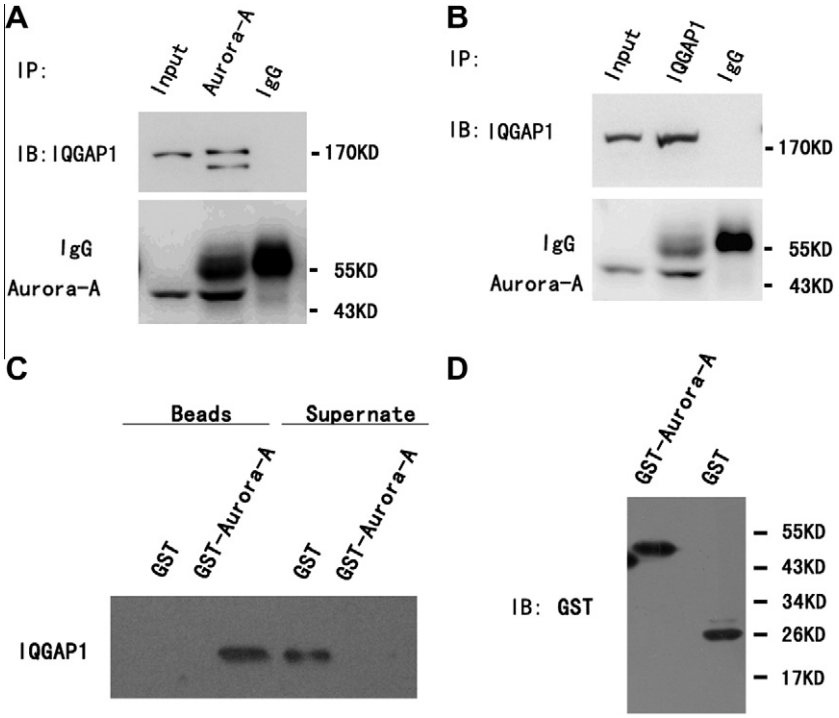

Fig. 1. Interaction between IQGAP1 and Aurora-A. (A and B) *In vivo* interaction of IQGAP1 and Aurora-A. Immunoprecipitation of IQGAP1 and Aurora-A from Hela cell lysates was carried out as described in Section 2.5 using antibodies against IQGAP1 and Aurora-A. Then, immunoblotting was performed, using IgG as a negative control. (C and D) *In vitro* interaction between IQGAP1 and Aurora-A. GST and GST-Aurora-A fusion proteins were expressed in *E. coli* and purified using glutathione agarose beads. Pull-down assays were performed as described in Section 2.6 using whole-cell extracts of Hela cells. Samples were analyzed by western blot. GST was used as a negative control.

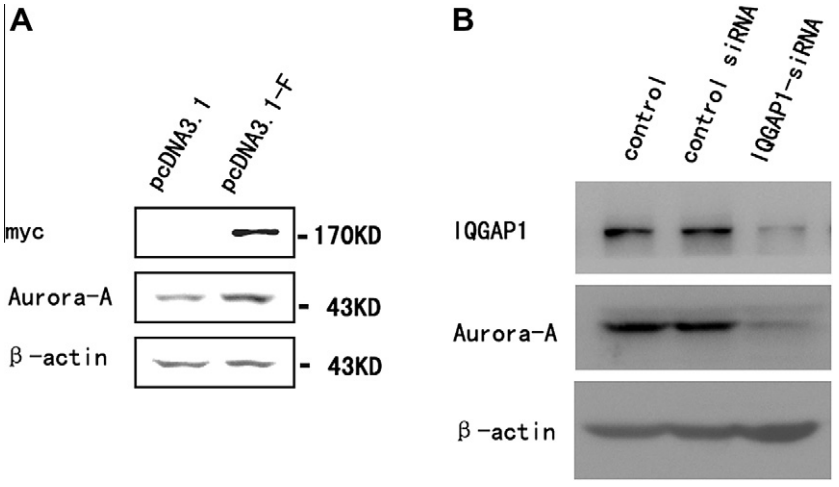

Fig. 2. Role of IQGAP1 in regulating Aurora-A protein stability. MCF-7 cells were transiently transfected with pcDNA3.1 (vector), myc-tagged IQGAP1 (A) or IQGAP1 siRNA (B). Twenty-four hours later, Protein expression was analyzed by western blot with indicated antibodies.

groups have shown previously that human Aurora-A is turned over through the anaphase promoting complex/cyclosome (APC/C) ubiquitin proteasome pathway [16], so we suspected that IQGAP1 may inhibit the degradation of Aurora-A by disrupting the interactions between Aurora-A and the proteins involved in its degradation. We performed co-immunoprecipitation experiments by using Aurora-A antibody to investigate this hypothesis. As expected, after incubation with MG132, a selective inhibitor of the proteasome, the level of ubiquitinated Aurora-A was lower in IQGAP1 over-expressing cells than those in control cells (shown in Fig. 3B). Additionally, reduced amounts of APC2, CDC27 and CDH1 were also detected in Aurora-A immune-complexes from IQGAP1 over-expressing cells compared to control cells (shown in Fig. 3C). These results suggest that the interactions between Aurora-A and its degradation associated proteins are weakened by IQGAP1 overexpression, which lead to a suppression of ubiquitin-mediated degradation of Aurora-A.

3.4. Identification of Aurora-A binding domain in IQGAP1

IQGAP1 contains many binding domains, such as calponin-homology domain, coiled coil, predicted α-helical structure, poly-proline protein–protein domain, four IQ motifs, Ras GTPase-activating protein related domain and RasGAP C-terminus [6]. To

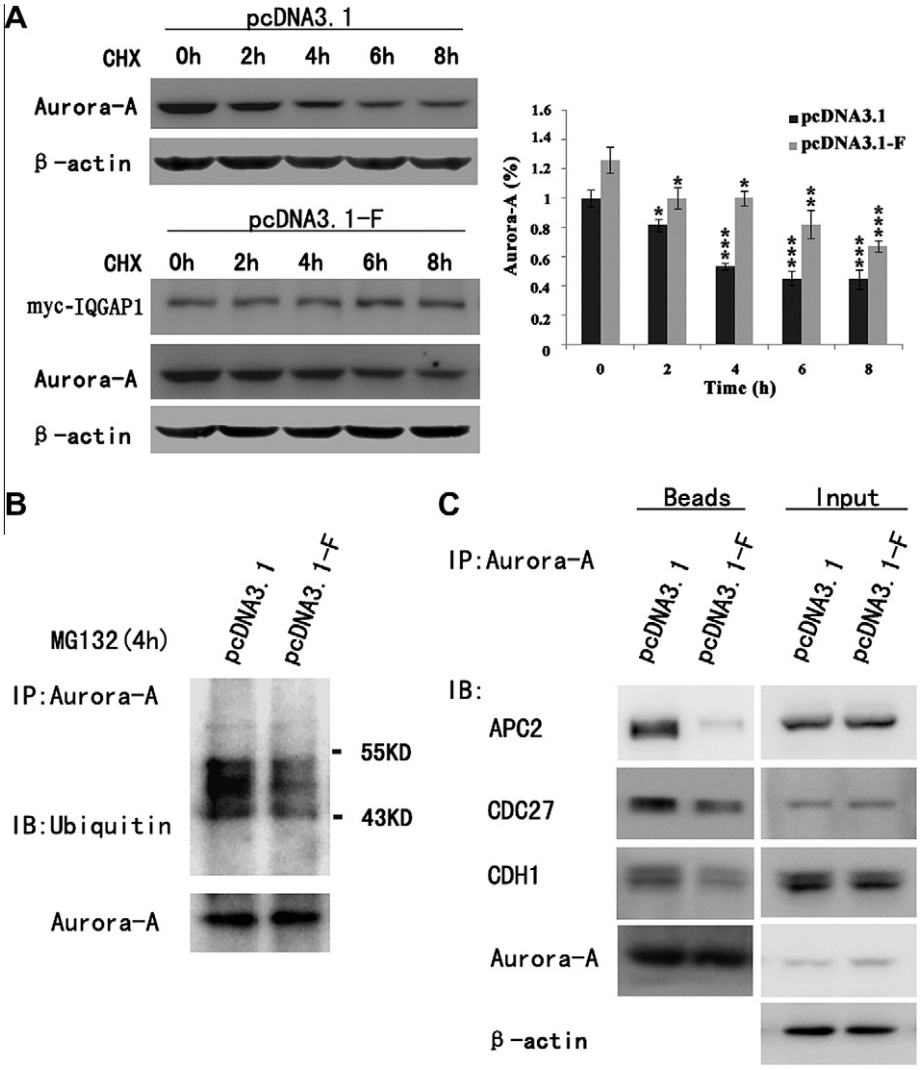

Fig. 3. Overexpression of IQGAP1 delays the degradation of Aurora-A. (A) Hela cells transfected with pcDNA3.1 (vector) or myc-tagged IQGAP1 were treated with CHX, then harvested at the indicated time points (0, 2, 4, 6 and 8 h) followed by western blot assay. Aurora-A protein levels were quantified using QuantityOne software and normalized to β-actin level. (B) MCF-7 cells transfected with pcDNA3.1 or myc-tagged IQGAP1 were treated with MG132 (10 μM) for 4 h, then collected for co-immunoprecipitation with anti-Aurora-A antibody. Equal amounts of immunoprotein complex were loaded and resolved by SDS–PAGE. The presence of Aurora-A in the immunocomplex was further verified by blotting the same membrane with anti-Aurora-A antibody, as shown in the lower panel. Polyubiquitinated Aurora-A appears in multiple forms with a molecular mass range 48 kDa. (C) MCF-7 cell lysate was immunoprecipitated with anti-Aurora-A antibody, followed by immunoblotting using antibodies against ANAPC2, CDC27, CDH1 and Aurora-A. Immunoprecipitation with IgG was used as a negative control.

investigate the Aurora-A binding region in IQGAP1, we constructed several expression vectors encoding IQGAP1 fragments (764–1657aa, 1–863aa, 1503–1657aa and 1–1503aa). All the fragments were transfected into MCF-7 cells, after 48 h, co-immunoprecipitation experiments were performed by using myc antibody. The results showed that the fragments harboring 764–1657aa and 1503–1657aa were able to precipitate by Aurora A antibody, suggesting that Aurora-A might bind to the RGCt domain of IQGAP1.

3.5. Aurora-A siRNA attenuates IQGAP1-induced cell proliferation

It has been reported previously that IQGAP1 promotes cell division, growth and migration [19,20]. Meanwhile, in our earlier studies we have shown that Aurora-A is overexpressed in human esophageal squamous cell carcinomas (ESCC), and the abnormal increased expression of Aurora-A is associated with increased malignancy and poor prognosis of ESCC patients. Expression of exogenous Aurora-A in human KYSE 150 cells promotes cell proliferation and stimulates colony formation [14,15,21]. In view of that both IQGAP1 and Aurora-A can promote cell proliferation, we assumed that IQGAP1 might promote cell proliferation by accumulation of Aurora-A in cancer cells. To test this hypothesis, the BrdU (thymidine analog, bromodeoxyuridine) incorporation and flow cytometry assays were performed. The results showed in Supplementary Fig. 2A (1 and 2) demonstrated that DNA synthesis in MCF-7 cells was strongly enhanced by overexpression of IQGAP1 compared with the control cells. But this enhancing effect was attenuated by knockdown of Aurora-A (shown in Supplementary Fig. 2A (4)). The phosphorylation of Aurora-A in IQGAP1 overexpressing cells was also tested by western blot, the results showed in Supplementary Fig. 2B demonstrated that IQGAP1 might also modulate the kinase activity of Aurora-A.

4. Discussion

In eukaryotic cells, scaffold proteins play crucial roles in many important signaling pathways [22]. As a scaffold protein, IQGAP1 could interact with a number of proteins to enhance cell prolifera-

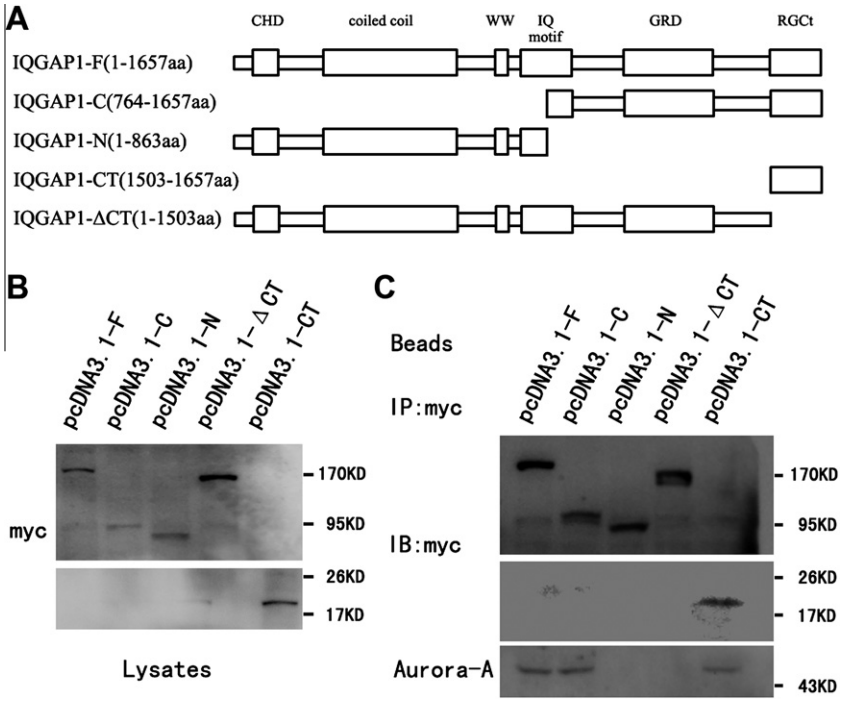

Fig. 4. Identifying the interaction region between IQGAP1 and Aurora-A. (A) Schematic representation of IQGAP1 fragments (764–1657aa, 1–863aa, 1503–1657aa and 1–1503aa). (B and C) IQGAP1 expression vectors (F, C, N, ΔCT and CT) were transfected separately into HEK293 cells. After 48 h, cells were collected for immunoprecipitation with anti-myc antibody. The precipitated complexes were analyzed by immunoblotting assay with anti-myc and anti-Aurora-A antibodies.

tion and reduce cell differentiation which could lead to oncogenesis [8,10,19,23]. In the study of human primary tumors, researchers found that the alteration of IQGAP1 expression and localization correlate with cancer progression [24–27]. But, how IQGAP1 contributes to the aggressive phenotype and which interacting partner(s) enhance the tumorigenic role of IQGAP1 are still unclear.

In this report, we add Aurora-A to the broad range of IQGAP1 targets. First, we proved an *in vitro* interaction between GST-Aurora-A and IQGAP1. Moreover, co-immunoprecipitation displayed that endogenous IQGAP1 binds to endogenous Aurora-A. Interestingly, we found that when IQGAP1 was overexpressed, the half-life of Aurora-A was increased, and the degradation of Aurora-A was delayed (shown in Fig. 3A). Furthermore, we identified that IQGAP1 interacts with Aurora-A via RGCt domain (1503–1657aa) which numerous proteins can bind to, including APC, E-cadherin, CLIP-170, Dia1 and β-catenin (shown in Fig. 4C). But we found no evidence that IQGAP1 could regulate Aurora-A at the transcription level. Based on these evidences, we assumed that the upregulation of Aurora-A in IQGAP1 over-expressing cells was probably due to the post-transcriptional mechanism. Because the degradation of Aurora-A is mediated by hCDH1 through the anaphase promoting complex/cyclosome (APC/C) ubiquitin proteasome pathway, not on hCDC20, by treating cells with MG132 we found that the level of ubiquitinated Aurora-A was lower in IQGAP1 over-expressing cells. Co-immunoprecipitation showed that the interactions between Aurora-A and proteins involved in its degradation (APC2, CDC27 and CDH1) were much weaker. Taken together, these results suggest that overexpression of IQGAP1 delays the degradation of Aurora-A probably through the disruption of the interactions between Aurora-A kinase and the APC/C complex.

In early mitosis, Aurora-A begins to accumulate on centrosomes, and by mitosis, it is heavily concentrated on centrosomes at the spindle poles, and also being detectable along spindle microtubules [28,29]. Meanwhile, IQGAP1 has been detected at cytoplasmic, endoplasmic reticulum and Golgi membranes. Recently, other studies have demonstrated that IQGAP1 may associate with microtubules through CLIP-170. IQGAP1 from *Saccharomyces cerevisiae* and *Dictyostelium* have essential roles in cytokinesis. Researchers also found that IQGAP1 located in the nucleus in several mammalian cell lines [5,30–33]. In our study we found a partial co-localization between IQGAP1 and Aurora-A during mitosis by using fluorescence microscopy (data not shown). This result give us a hint that the IQGAP1 may involve in mitosis through interaction with Aurora-A. But whether the interaction between Aurora-A and IQGAP1 is mitosis-specific needs to be further investigated.

Aurora-A often exerts important functions via its kinase activity. In our study, we found that the kinase activity of Aurora-A is upregulated in IQGAP1 over-expressing cells (shown in Supplementary Fig. 2B). Knockdown of Aurora-A could attenuate IQGAP1-induced cell proliferation in MCF-7 (shown in Supplementary Fig. 2A). These data suggest that IQGAP1 might affect the cell proliferation through regulating both kinase activity and protein stability of Aurora-A. But the underlying mechanisms still need to be further investigated.

In summary, our studies document a previously undiscovered interaction between IQGAP1 and Aurora-A. Both overexpression and knockdown of endogenous IQGAP1 are able to alter the protein stability of Aurora-A significantly. IQGAP1 overexpression leads to enhanced Aurora-A kinase activity and increased cell proliferation. These findings provide a new insight into the role of IQGAP1 in the regulation of Aurora-A kinase.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.112.

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