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CDK5 REGULATES RAP1 ACTIVITY

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Abstract

Rap1 signaling is important for migration, differentiation, axonal growth, and during neuronal polarity. Rap1 can be activated by external stimuli, which in turn regulates specific guanine nucleotide exchange factors such as C3G, among others. Cdk5 functions are also important to neuronal migration and differentiation. Since we found that pharmacological inhibition of Cdk5 by using roscovitine reduced Rap1 protein levels in COS-7 cells and also C3G contains three putative phosphorylation sites for Cdk5, we examined whether the Cdk5-dependent phosphorylation of C3G could affect Rap1 expression and activity. We co-transfected C3G and tet-OFF system for p35 over-expression, an activator of Cdk5 activity into COS-7 cells, and then we evaluated phosphorylation in serine residues in C3G by immunoprecipitation and Western blot. We found that p35 over-expression increased C3G-serine-phosphorylation while inhibition of p35 expression by tetracycline or inhibition of Cdk5 activity with roscovitine decreased it. Interestingly, we found that MG-132, a proteasome inhibitor, rescue Rap1 protein levels in the presence of roscovitine. Besides, C3G-serine-phosphorylation and Rap1 protein levels were reduced in brain from Cdk5^{-/-} as compared with the Cdk5^{+/+} brain. Finally, we found that p35 over-expression increased Rap1 activity while inhibition of p35 expression by tetracycline or roscovitine decreased Rap1 activity. These results suggest that Cdk5-mediated serine-phosphorylation of C3G may control Rap1 stability and activity, and this may potentially impact various neuronal functions such as migration, differentiation, and polarity.

2. Introduction

Ras-related protein 1 (Rap1) belongs to the Ras superfamily of small GTPases and regulates downstream proteins implicated in various cellular processes, including migration, proliferation, differentiation, and cell polarity (Bos et al., 2001; Gloerich and Bos, 2011; Schwamborn and Puschel, 2004). Rap1 is expressed as two isoforms, Rap1a and Rap1b (Bos et al., 2001) and it is activated upon GTP binding and inactivated due to GTP hydrolysis. This process of activation and inactivation is regulated by guanine nucleotide-exchange factors (GEF), which enhance the exchange of GDP for GTP, leading to Rap1 activation; and for GTPase-activating proteins (GAPs) that promote the hydrolysis of the bound GTP to GDP promoting Rap1 inactivation. RapGEF1, also known as C3G, is a specific Rap1 modulator (Tanaka et al., 1994). Antibodies against C3G identify a protein with molecular weight of 130–140 kDa (Tanaka et al., 1994). C3G protein has a catalytic domain in charge of exchanging guanine-nucleotides, and also a proline-rich sequence that

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associate with SH3 domain of the proto-oncogene adaptor protein Crk. C3G-Crk association increases C3G guanine nucleotide exchange activity increasing Rap1 activity (Gotoh et al., 1995; Ichiba et al., 1997). Crk facilitates the phosphorylation of tyrosine 504 on C3G mediated by Src tyrosine kinases (Radha et al., 2004) enhancing its nucleotide exchange activity towards Rap1 (Ichiba et al., 1999). Y504F phosphorylation defective mutant of C3G has only partially compromised its ability to generate neurites in the human neuroblastoma cell line IMR-32, suggesting the existence of additional molecular mechanisms to control C3G functions (Radha et al., 2008). Protein degradation is also involved in C3G functions, since the ubiquitination of Crk perturbs its binding to C3G, leading to decreased Rap1 activity (Shao et al., 2003). Likewise, it was reported that activation of Rap1 protects it from ubiquitination and proteasome degradation (Schwamborn et al., 2007).

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase belongs to the family of Cdks. Cdk5 activity is mainly detected in post-mitotic neurons where its activators, p35 and p39, are predominantly expressed (Dhavan and Tsai, 2001). However, recently several non-neuronal functions for Cdk5 had been described (Contreras-Vallejos et al., 2012). Thus, Cdk5 play key molecular roles in brain development and neuronal functions (Dhavan and Tsai, 2001; Utreras et al., 2009a). Because C3G protein contains three putative phosphorylation sites for Cdk5 and since one peptide of C3G containing the Ser233 is phosphorylated in mouse brain (Huttlin et al., 2010), we decided to investigate whether Cdk5 regulates Rap1 stability and activity through phosphorylation of C3G. We found that roscovitine, a Cdk5 inhibitor, decreased Rap1 protein expression and it was rescued by using MG-132, a proteasome inhibitor. Moreover C3G-serine-phosphorylation and Rap1 protein levels were reduced in the Cdk5^{-/-} brain as compared with the Cdk5^{+/+} brain. Additionally, C3G-serine-phosphorylation was increased in COS-7 cells over-expressing p35, a co-activator of Cdk5, using an inducible trans-activator system dependent on tetracycline (Utreras et al., 2009b). Furthermore, p35 over-expression increased Rap1 activity whereas inhibition of Cdk5 activity decreased it. These results suggest that Cdk5 can regulate Rap1 stability and activity through C3G phosphorylation.

3. Material and Methods

3.1. Antibodies

-tubulin and total phospho-serine antibodies, protein G, tetracycline and MG-132 were obtained from Sigma. C3G, Cdk5, and p35 antibodies were obtained from Santa Cruz Biotechnology, and secondary antibodies (HRP-conjugated goat anti-mouse and anti-rabbit) were obtained from Jackson ImmunoResearch. Rap1 antibody was obtained from BD Transduction Laboratories. Roscovitine was obtained from Cell Signaling Technology.

3.2. Animals

Cdk5^{-/-} mice were generated as previously reported (Ohshima et al., 1996). All of the animal procedures were conducted in agreement with NIH Standards, and approved by the Bioethics Committee of the Faculty of Science, University of Chile for the care and handling of laboratory animals.

3.3. Cell line culture

The COS-7 cell line was cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone Laboratories).

3.4. Transient transfection of cell lines

COS-7 cells were transiently co-transfected with C3G vector (provided by Dr. Vegesna Radha, Centre for Cellular and Molecular Biology, Andhra Pradesh, India), pBI-p35/EGFP

vector (Utreras et al., 2009b) and tTA vector (provided by Dr. Jose Javier Lucas, Universidad Autonoma de Madrid, Spain) in Opti-MEM with Lipofectamin 2000 (Invitrogen) in DMEM. After 4 h, transfection medium was changed for DMEM+10% FBS, and cells were treated with tetracycline (2 µg/ml) or roscovitine (20 µM) during 20 h.

3.5. Immunoprecipitation assays

Briefly, 800 µg of protein extract from transfected COS-7 cells were immunoprecipitated with 4 µg of rabbit C3G antibody and after incubations and washed protein samples were separated in SDS-PAGE gels. Immunoprecipitates assays were done as previously reported (Utreras et al., 2009b).

3.6. Western Blot Analysis

Protein were separated on 10% or 7.5% SDS-PAGE gels and transferred to nitrocellulose membranes. Western blot analysis was performed as described (Utreras et al., 2012).

3.7. GST fusion protein preparation

Purification and expression of GST-RalGDS was made in BL21 (DE3) strain. Bacteria treated with IPTG (0.1 mM) were lysed in lysis buffer A (50 mM Tris-HCl pH 8.0, 1% Triton X-100, 1 mM EDTA, 0.15 M NaCl, 25 mM NaF, 0.5 mM PMSF and protease inhibitor complex (Roche)) and affinity-purified with glutathione-Sepharose beads (Amersham). Loaded beads were washed with lysis buffer B (lysis buffer A plus 300 mM NaCl) at 4°C and GST fusion proteins were quantified and visualized in SDS-PAGE gels stained with Coomassie brilliant blue or immunoblotted using the anti-GST antibody.

3.8. Rap1 activity pull-down assay

The Rap1-GTP binding assay was performed as described (Franke et al., 1997). Briefly, 1.5 mg of COS7 cell lysates co-transfected with C3G and tet-OFF p35 system were incubates GST-RalGDS and glutathione-Sepharose beads. Complexes were washed and separated on SDS-PAGE gel and subjected to Western blot analysis.

3.9. RNA Isolation and Real-Time RT-PCR

Real-time RT-PCR was performed as described previously (Utreras et al., 2012). Briefly, total RNA from embryonic 18.5 brains of *Cdk5^{-/-}* and *Cdk5^{+/+}* was isolated using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. The following primers were used for real time RT-PCR: Rap1b (F) 5'-CGA CTG TGC TGT GAT GGA GT-3'; Rap1b (R) 5'-GCT TGA AAT CTT GGA CAC TGC-3'; S29 (F) 5'-GGA GTC ACC CAC GGA AGT TCG G-3'; S29 (R) 5'-GGA AGC ACT GGC GGC ACA-3'. S29 primers were used to amplify a 40S ribosomal protein S29 as a housekeeping gene.

3.10. Statistical Analysis

All experiments were performed in triplicate. Statistical analysis was performed using GraphPad Prism software v5.0. Significant differences between experiments were assessed by an unpaired Student test where α was set to 0.05.

4. Results

4.1. Pharmacological inhibition of Cdk5 decreased Rap1 protein levels in COS-7 cells

Rap1 protein levels were evaluated by Western blotting of protein lysates from COS-7 cells co-transfected with C3G, pBI-p35/EGFP, and tTA in the presence or absence of roscovitine. We found that roscovitine decreased significantly Rap1 protein expression whereas cells

over-expressing p35 have a slight increase in Rap1 protein expression (Figure 1A and 1B). In order to confirm the specificity of Cdk5 inhibition, we evaluated the effect of other proline-directed protein kinase inhibitors in COS-7 cells such as MEK1/2 inhibitor (PD98059 20 μ M), a P38 MAP kinase inhibitor (SB203580 20 μ M), a JNK1/2 inhibitor (SP600125 20 μ M), and a Cdk5 inhibitor (roscovitine 20 μ M) during 24 h. We found that only roscovitine affects the overall levels of Rap1 protein (Figure 1C). Because of Rap1 expression can be regulated by proteasome degradation (Schwamborn et al., 2007), we evaluated the effect of a proteasome inhibitor, MG-132, over Rap1 expression. COS-7 cells co-transfected with C3G, pBI-p35/EGFP, and tTA were treated with roscovitine with or without MG-132 (20 μ M) during 18 h. We found that Rap1 protein expression was decreased by roscovitine treatment, however it was increased by MG-132 treatment plus roscovitine (Figure 1D), suggesting that Cdk5 can be regulating Rap1 protein stability through a proteasome pathway.

4.2. Cdk5 deletion decreased Rap1 protein levels in Cdk5^{-/-} brain

We evaluated the *in vivo* effect of Cdk5 deficiency over Rap1 expression using Cdk5^{-/-} mice (Ohshima et al., 1996). The Rap1b isoform is known to be more important in neuronal functions (Schwamborn and Puschel, 2004). Hence, we determined Rap1b mRNA levels in Cdk5 deficient mice using real-time RT-PCR. Rap1b mRNA levels were unchanged in Cdk5^{-/-} as compared to Cdk5^{+/+} brains (Figure 2A). Remarkably, Rap1 protein levels were robustly decreased in Cdk5^{-/-} brains (Figure 2B and 2C, 100% v/s 32.4%, Cdk5^{-/-} as compared with Cdk5^{+/+} mice), confirming that Cdk5 may regulate Rap1 expression through a post-transcriptional mechanism.

4.3. C3G is a potential new substrate of Cdk5

C3G is a GEF protein that increase Rap1 activity. We performed a bioinformatics analysis of mouse C3G protein sequence and found three possible consensus sites of phosphorylation for Cdk5 that contain Thr46, Ser233, and Ser311 (Figure 3A). To assess whether C3G is phosphorylated by Cdk5, we co-transfected C3G and a tetracycline inducible system for p35 over-expression (Utreras et al., 2009b), which consists of pBI-p35/EGFP and tTA vectors in COS-7 cells (Figure 3B). Western blot analysis determined that p35 and C3G endogenous levels were absent in un-transfected cells and C3G levels were increased in COS-7 cells transfected with C3G cDNA. When we co-transfected pBI-p35/EGFP, tTA and C3G, we observed an increase in p35 and C3G expression, and we also detected an increase in p25 expression, a proteolytic fragment of p35 which can also activate Cdk5 (Cruz et al., 2003) whereas p35 and p25 proteins were undetectable in the presence of tetracycline (Figure 3C). To evaluate whether p35 over-expression increase C3G-serine-phosphorylation, we immunoprecipitated C3G and performed Western blot using a total phospho-serine antibody and we found that p35 over-expression increased the phosphorylation of a protein compatible with the molecular weight (130 kDa) of C3G whereas tetracycline treatment decreased it (Figure 3D, arrow). Additionally, roscovitine and tetracycline treatment reduced the C3G-serine-phosphorylation in COS-7 cells (Figure 3E). Furthermore, we found that C3G-serine-phosphorylation was diminished in Cdk5^{-/-} brain samples compared with Cdk5^{+/+} brain samples (Figure 3F), suggesting that Cdk5 can in turn regulate C3G phosphorylation in brain tissue.

4.4. Rap1 activation is dependent of Cdk5 activity

To investigate whether Cdk5 increases Rap1 activity, we performed a pull down assay by using GST-RalGDS. Protein extracts from co-transfected COS-7 cells were pulled down (PD) and analyzed by Western blot against total Rap1 protein. Interestingly, we found that p35 over-expression significantly increased bound Rap1-GTP (Figure 4A, PD in lane 2 and Figure 4B). Conversely, Rap1-GTP levels in tetracycline and roscovitine treated cells were

similar to un-transfected COS-7 cells (Figure 4A, PD in lanes 3 and 4 and Fig. 2B). These results suggest that Rap1 activity is likely correlated to Cdk5 activity through C3G phosphorylation.

5. Discussion

Here we demonstrated that Rap1 protein expression and activity are dependent on Cdk5 activity in cell lines and brain of Cdk5^{-/-} mice possibly through phosphorylation of C3G. Thus, p35 over-expression increased C3G-serine-phosphorylation and Cdk5 inhibition decreased it in COS-7 cells. Interestingly, p35 over-expression increased Rap1 activity while Cdk5 activity inhibition by tetracycline or roscovitine decreased Rap1 activity, supporting the hypothesis that Rap1 activity is dependent on Cdk5 activity. Finally, Rap1 protein levels were significantly reduced by roscovitine as well as in the Cdk5^{-/-} brain. Altogether these results suggest that Cdk5/p35 regulates Rap1 activity through C3G phosphorylation.

The small GTPases play a crucial role in neuronal morphogenesis and polarity (Gonzalez-Billault et al., 2012; Luo, 2000). In particular, Rap1 participates in the cellular mechanisms of differentiation and neuronal polarity, although its function is not fully addressed (Bos et al., 2001; Gloerich and Bos, 2011; Schwamborn and Puschel, 2004). Rap1 activity is dependent on the role of specific GEFs and GAPs that contribute to a tight control of Rap1 activity, cellular distribution, and stability (Gloerich and Bos, 2011). Specifically, C3G was the first identified Rap GEF protein (Tanaka et al., 1994). The mechanism involved in C3G-dependent Rap1 activation is related to the association of C3G with Crk, and the tyrosine phosphorylation of C3G by Src tyrosine kinase (Ichiba et al., 1999; Ichiba et al., 1997) (Radha et al., 2004; Shao et al., 2003). Tyr504 phosphorylation of C3G is the most common post-translational modification that regulates the functions of C3G. Moreover, Crk ubiquitination affects the binding to C3G and the subsequent Rap1 activation (Shao et al., 2003). Here we have identified a novel post-translational modification for C3G, the phosphorylation in serine mediated by Cdk5/p35. As a consequence of a bioinformatics analysis of the C3G sequence, we identified three consensus sites for phosphorylation mediated by Cdk5. Indeed, one peptide that contains Ser233 was recently described to be phosphorylated *in vivo* in brain tissue measured by using phosphoproteomic analysis (Huttlin et al., 2010), suggesting that some kinase could play such a function *in vivo*. Also, Cdk5 plays crucial roles in several neuronal processes in which Rap1 is also involved, such as brain development, migration, differentiation, and neuronal polarity (Arimura and Kaibuchi, 2007; Dhavan and Tsai, 2001; Ohshima et al., 1996; Schwamborn and Puschel, 2004; Utreras et al., 2009a). Additionally, was reported that Cdk5 regulates cAMP-dependent signaling supporting synaptic plasticity and memory formation (Guan et al., 2011), two processes in which Rap1 is also involved (Morozov et al., 2003). We therefore investigated whether Cdk5 could be regulating C3G phosphorylation and subsequently affecting Rap1 activity.

The important *in vivo* function of C3G was demonstrated in C3G^{-/-} mice, which display early embryonic lethality with abnormal migration and cell adhesion (Ohba et al., 2001). Moreover, a hypomorphic mutant of C3G expressing less than 5% of C3G protein (Voss et al., 2003) impacts the cortical neuron migration and preplate splitting (Voss et al., 2008; Voss et al., 2006). Neuronal migration is highly dependent on the Reelin signaling pathway, which controls the formation of most laminated structures in the brain (Frotscher, 2010; Frotscher et al., 2009). Indeed, Reelin can activate C3G in cortical neurons and C3G can also induce migration of sympathetic preganglionic neurons (Voss et al., 2008; Yip et al., 2012). Instead, the key role that Cdk5 plays in neuronal migration and preplate splitting has been widely studied (Dhavan and Tsai, 2001; Xie et al., 2006). Cdk5 and Reelin pathways participate synergistically in the positioning of the cortical neurons in the developing mouse

brain (Ohshima and Mikoshiba, 2002; Ohshima et al., 2001). Therefore, it is plausible to propose that the Reelin signaling pathway may induce C3G serine phosphorylation by Cdk5.

It is known that Rap1 protein is regulated by Smurf2 ubiquitination and subsequent degradation by a proteasome pathway (Schwamborn et al., 2007). It is worth noting that Rap1 protein levels were also regulated by the Cdk5 activity, suggesting that Cdk5-mediated C3G phosphorylation stabilizes Rap1 protein against proteasome degradation. Interestingly, the effect of MG-132, a proteasome inhibitor, produced an increased in Rap1 protein level in COS-7 cells treated with roscovitine, suggesting that Cdk5 may regulated Rap1 degradation. Similarly, it has been reported that Cdk5 can block degradation by proteasome pathway of talin mediated by direct phosphorylation (Huang et al., 2009). Another possibility may be the direct phosphorylation of Cdk5 upon Rap1 and posterior stabilization, since the Rap1 protein sequence contains a threonine residue (Thr-169) adjacent to a proline, although it is not part of a canonical consensus Cdk5 motif (Dhavan and Tsai, 2001). This latter option will be further analyzed.

The regulation of Rap1 functions is dependent on many different regulator proteins displaying some redundant functions. A bioinformatics approach shows that several members of the GEF and GAP proteins involved in Rap1 regulation display Cdk5 phosphorylation consensus motifs, which include EPAC, RapGEF2, PDZ-GEF, REPAC, and RASGRP2. It may be of interest to address whether the Cdk5-dependent Rap1 regulation presented in this paper is part of a more general group of mechanisms that control more than a single Rap1-activating protein.

Altogether our results suggest that Rap1 functions are controlled by the Cdk5 signaling pathway. This invites the possibility of linking C3G and Cdk5 dependent functions, which may be responsible for migration, differentiation, and neuronal polarity.

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Figure 1.

Pharmacological inhibition of Cdk5 decreased Rap1 protein levels in COS-7 cells. (A) Western blot of Rap1 protein from COS-7 cells co-transfected with treatment indicated in each lane. α -tubulin protein levels were used as loading control. (B) Quantification of Rap1 protein levels from different treatments described. (C) Western blot of Rap1 protein levels from COS-7 cells treated with different inhibitors of serine/threonine kinases at 20 μ M during 24 h. (D) Representative Western blot of Rap1 protein from COS-7 cells co-transfected with treatment indicated in each lane and treated with MG-132 and roscovitine. Bars are the mean $\pm$ SEM of four to six independent experiments. ** $p < 0.01$ by using Student's test.

Figure 2.

Cdk5 deletion decreased Rap1 protein levels in Cdk5^{-/-} brains. (A) Real-time RT-PCR of Rap1b mRNA level from brain of E18.5-days-old Cdk5^{-/-} and Cdk5^{+/+} embryos. S29 was used as housekeeping gene. (B) Western blot analysis of Rap1 protein levels from brain of E18.5-days-old Cdk5^{-/-} and Cdk5^{+/+} embryos. α-tubulin protein levels were used as a loading control. (C) Quantification of Rap1 protein levels from brains of Cdk5^{-/-} and Cdk5^{+/+} embryos. Bars are the mean ± SEM of 6 independent mice. ** p<0.01 by using Student's test.

Figure 3.

C3G is a potential new substrate of Cdk5. (A) Bioinformatics analysis of C3G sequence showing aligned that C3G sequence of human, mouse, and rat, displaying three predicted consensus sites of phosphorylation for Cdk5 (underline) in Thr46 and Ser234 and Ser313 (boxes). (B) Tet-OFF system of over-expression of p35 and GFP under control of tTA vector [14]. (C) Western blot of p35 and C3G from protein extract of COS-7 cells co-transfected with treatment described in each lanes. (D) Immunoprecipitation assays of COS-7 cells previously co-transfected against C3G antibody, followed by Western blot analysis against total phospho-serine. Arrow showed a band 130 kDa. (E) Western blot of phospho-serine, p35, and C3G in COS-7 cells co-transfected and treated with tetracycline or

roscovitine. (F) Immunoprecipitation of C3G from brains of E18.5-days-old of Cdk5^{-/-} and Cdk5^{+/+} embryos followed by Western blot of total phospho-serine.

Figure 4.

Rap1 activation is dependent upon Cdk5 activity. (A) Pull down analysis (PD) showing Rap1 activation. Protein extract from co-transfected COS-7 cells with different vectors described in each lanes were incubated with GST-RalGDS and the Western blot on quantified Rap1-GTP (from PD) and Rap1 total (from Input (I)) were done in bead complexes. (B) Quantification of the Rap1 active form in PD from a different treatment. * $p < 0.05$ by using Student's test.