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Phosphorylation of synaptic GTPase-activating protein (synGAP) by polo-like kinase (Plk2) alters the ratio of its GAP activity toward HRas, Rap1 and Rap2 GTPases

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ABSTRACT

SynGAP is a Ras and Rap GTPase-activating protein (GAP) found in high concentration in the postsynaptic density (PSD) fraction from mammalian forebrain where it binds to PDZ domains of PSD-95. Phosphorylation of pure recombinant synGAP by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) shifts the balance of synGAP's GAP activity toward inactivation of Rap1; whereas phosphorylation by cyclin-dependent kinase 5 (CDK5) has the opposite effect, shifting the balance toward inactivation of HRas. These shifts in balance contribute to regulation of the numbers of surface AMPA receptors, which rise during synaptic potentiation (CaMKII) and fall during synaptic scaling (CDK5). Polo-like kinase 2 (Plk2/SNK), like CDK5, contributes to synaptic scaling. These two kinases act in concert to reduce the number of surface AMPA receptors following elevated neuronal activity by tagging spine-associated RapGAP protein (SPAR) for degradation, thus raising the level of activated Rap. Here we show that Plk2 also phosphorylates and regulates synGAP. Phosphorylation of synGAP by Plk2 stimulates its GAP activity toward HRas by 65%, and toward Rap1 by 16%. Simultaneous phosphorylation of synGAP by Plk2 and CDK5 at distinct sites produces an additive increase in GAP activity toward HRas (~230%) and a smaller, non-additive increase in activity toward Rap1 (~15%). Dual phosphorylation also produces an increase in GAP activity toward Rap2 (~40–50%), an effect not produced by either kinase alone. As we previously observed for CDK5, addition of Ca^{2+}/CaM causes a substrate-directed doubling of the rate and stoichiometry of phosphorylation of synGAP by Plk2, targeting residues also phosphorylated by CaMKII. In summary, phosphorylation by Plk2, like CDK5, shifts the ratio of GAP activity of synGAP to produce a greater decrease in active Ras than in active Rap, which would produce a shift toward a decrease in the number of surface AMPA receptors in neuronal dendrites.

1. Introduction

The post synaptic density (PSD) of neurons harbors several cytosolic signaling complexes that are associated with glutamate receptors at the postsynaptic membrane of excitatory synapses [1]. synGAP, a dual Ras and Rap GTPase Activating Protein (GAP), is unusually highly concentrated in the PSD of excitatory synapses [2–4]. It binds tightly to the PDZ domains of PSD-95 [2,5] which serves to position it in close proximity to NMDA-type and AMPA-type glutamate receptors (NMDARs and AMPARs, respectively), and allows it to regulate the composition of the PSD by restricting binding of other proteins to PSD-95 [6].

SynGAP stimulates the intrinsic GTPase activity of Rap ~100-fold and that of Ras, which has a higher intrinsic rate, about 3–7-fold

Abbreviations: synGAP, synaptic GTPase activating protein; r-synGAP, soluble recombinant synGAP fragment comprising residues 103–1293; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CDK5, cyclin-dependent kinase 5; Plk2, polo-like kinase 2; HRas, p21 Ras; Rap1/2, Ras-related protein-1 or 2.

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[4,7,8]. Stimulation of Ras and Rap by various signaling pathways [9–11] modulates AMPAR trafficking in opposite directions, with active Ras increasing insertion (exocytosis) of AMPARs at the dendritic membrane, while active Rap increases their removal (endocytosis) [9].

Activation of NMDARs in cultured CNS neurons leads to phosphorylation of synGAP by CaMKII [2,11,12]. We showed that a recombinant, purified, soluble synGAP-α1 that lacks 102 residues at the N terminus (r-synGAP) can be expressed in soluble form and phosphorylated by purified Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and cyclin-dependent kinase 5 (CDK5) [4]. Phosphorylation by CaMKII accelerates the rate of inactivation of Rap1 more potently than the rate of inactivation of HRas, whereas phosphorylation by CDK5 has the opposite effect [4].

Within postsynaptic spines, spine-associated RapGAP (SPAR), also regulates Rap [13]. SPAR acts differently than synGAP because it stimulates the activity of Rap2 more potently than Rap1. CDK5 and polo-like kinase 2 (Plk2/SNK) act in concert to regulate SPAR; CDK5 primes phosphorylation by Plk2, ultimately tagging SPAR for degradation [14,15]. During homeostatic down-regulation of synapses, loss of SPAR activity increases the steady-state level of active Rap, thus increasing endocytosis of surface AMPA receptors.

We previously showed that Plk2 phosphorylation of r-synGAP decreases its affinity for PSD-95 [6] and that CDK5 phosphorylation of r-synGAP accelerates its HRas GAP activity [4]. Here we extend those studies by examining the effect of phosphorylation by Plk2 on GAP activity of r-synGAP and the effect of simultaneous phosphorylation of r-synGAP by Plk2 and CDK5. We determine the location and stoichiometry of Plk2 phosphorylation sites in r-synGAP, and the effect of their phosphorylation on HRas and Rap1/Rap2 GAP activity. We show that, as for CDK5, the addition of Ca^{2+}/CaM produces a substrate-directed increase in phosphorylation. Finally, we show that simultaneous phosphorylation by CDK5 and Plk2 results in a large additive increase in GAP activity toward HRas and a much smaller increase in GAP activity toward both Rap1 and Rap2.

2. Materials and methods

2.1. Expression and purification of recombinant proteins

R-synGAP, residues 103–1293 in synGAP A1-α1 (118–1308 in synGAP A2-α1), and full length HRas, Rap1B and Rap2A were purified as previously described [4]. We used synGAP isoform names and residue numbering from Ref. [16] with all residue numbering corresponding to synGAP A1-α1.

2.2. Stoichiometry and rate of r-synGAP or α-casein phosphorylation by Plk2

R-synGAP (286 nM) or dephosphorylated α-casein (3.7 μM) from bovine milk (Sigma-Aldrich) was phosphorylated with 110 nM Plk2 (Life Technologies). Phosphorylated proteins were detected and calculation of the stoichiometry of phosphorylation was performed as described in Ref. [4].

2.3. Phosphorylation of r-synGAP by Plk2 or CDK5 for use in GTPase assays

Phosphorylation of 725 nM r-synGAP by 230 nM Plk2, 230 nM CDK5/p35 or 230 nM Plk2 and CDK5/p35 was carried out as described in Ref. [4]. GTPase assays were carried out under conditions previously described in Refs. [4,6].

2.4. Mass spectrometry of phosphorylated r-synGAP

Mass spectrometry of phosphorylated r-synGAP using a hybrid LTQ-FT (Thermo Scientific) equipped with a nano-electrospray ion source (Thermo Scientific) was carried out by the Proteome Exploration Laboratory at the California Institute of Technology as previously described [4].

3. Results and discussion

3.1. Stoichiometry and rate of phosphorylation of r-synGAP by Plk2

Both endogenous and recombinant synGAP expressed in COS7 cells [17] can be phosphorylated by Plk2. In our assay, r-synGAP was phosphorylated by Plk2 (Fig. 1A) at a rate and stoichiometry similar to that of CDK5 phosphorylation [4]. The reaction was linear for 10 min, reaching a stoichiometry of ~0.4 mol phosphate/mol r-synGAP. After 30 min, the stoichiometry approached ~0.8 mol phosphate/mol. As for CDK5, addition of Ca^{2+}/CaM to Plk2 phosphorylation reactions doubles the stoichiometry and rate of r-synGAP phosphorylation to ~0.8 and ~1.8 mol phosphate/mol at 10 and 30 min, respectively (Fig. 1A). In contrast, phosphorylation by Plk2 of dephosphorylated α-caesin, was unaffected by Ca^{2+}/CaM (Fig. 1B). We previously showed that synGAP contains a binding site for Ca^{2+}/CaM with affinity in the nM range [4]. Thus, the simplest explanation for the effect of Ca^{2+}/CaM is that its binding to synGAP induces a conformational change that increases accessibility to CDK5 and Plk2, accelerating the kinase reaction rate and enabling the phosphorylation of additional residues.

3.2. Identification of sites in r-synGAP phosphorylated by Plk2

Recombinant synGAP expressed in COS cells was phosphorylated at nine residues by Plk2 (S364, S370, S434, S451, S821, S825, S827 and S880); however, phosphorylation at only three of them (S370, S434, S825 and S827) influenced synGAP's HRas GAP activity [17]. We identified eight additional Plk2 phosphorylation sites in r-synGAP (S140, S750, S751, S756, S765, S808, S810, T897), and confirmed phosphorylation at S821, S825 and S827 *in vitro* (Table 1, Supplemental Table 1 and Data). All of the identified Plk2 phosphorylation sites had high Mascot scores and false localization rates of less than 1%. Phosphorylation at sites S140, S750, S751, S756, S765, S808 was detected after 2 min of reaction with Plk2, whereas phosphorylation at S810, S821, S825, S827, and T897 was only detectable after 10 min of reaction with Plk2. We previously showed that addition of Ca^{2+}/CaM to reactions with CDK5/p35 results in phosphorylation of r-synGAP at S751, S765, S810, S1093 and S1123, in addition to sites S728, S773/T775, S802 and S842 [4] (Table 2, Supplemental Table 2 and Data). Similarly, here we show that addition of Ca^{2+}/CaM results in phosphorylation of S810 and T897 by Plk2 within 2 min, and phosphorylation of S1099, S1123 and S1283, which are also phosphorylated by CaMKII, after 10 min [4,12] (Table 1, Supplemental Table 1 and Data). Plk2 and Plk3 kinases prefer phosphorylation sites in which acidic amino acids (D, E) occur at positions between −4 and +4 of the target serine or threonine residue; in endogenous substrates, this preference is often extended to additional positions (e.g. −7 to +7) [18–23]. All of the Plk2 phosphorylation sites in r-synGAP, except S1123, contain one (S140, S756, S765, S808, S821, S825, S827, T897, S1099, S1283), two (S750, S751) or three (S810) acidic residues within −6 to +6 residues of the target serine or threonine residue.

3.3. Effect of Plk2 phosphorylation on GAP activity of r-synGAP

Plk2 phosphorylation increases the RasGAP activity of synGAP in

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