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A negative-feedback loop regulating ERK1/2 activation and mediated by RasGPR2 phosphorylation

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Abstract

The dynamic regulation of ERK1 and −2 (ERK1/2) is required for precise signal transduction controlling cell proliferation, differentiation, and survival. However, the underlying mechanisms regulating the activation of ERK1/2 are not completely understood. In this study, we show that phosphorylation of RasGRP2, a guanine nucleotide exchange factor (GEF), inhibits its ability to activate the small GTPase Rap1 that ultimately leads to decreased activation of ERK1/2 in cells. ERK2 phosphorylates RasGRP2 at Ser394 located in the linker region implicated in its autoinhibition. These studies identify RasGRP2 as a novel substrate of ERK1/2 and define a negative-feedback loop that regulates the BRaf–MEK–ERK signaling cascade. This negative-feedback loop determines the amplitude and duration of active ERK1/2.

Keywords

RasGRP2; ERK1/2 signaling; negative-feedback loop; phosphorylation; GEF; CalDAG-GEFI

Introduction

The Ras-Raf-MEK-ERK signaling pathway is essential for many cellular processes, including growth, cell-cycle progression, differentiation, and apoptosis. ERK1/2 phosphorylates hundreds of substrates *in vivo*, but these activities are restricted by cell type

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Conflict of interest

The authors declare no conflicts of interest.

and subcellular compartment [1,2]. Furthermore, the complexities and dynamics of ERK1/2 signaling are modified by both positive and negative-feedback loops [3,4]. For example, Sos1 is a GEF that actives Ras isoforms. In turn, active Ras allosterically enhances the exchange capacity of Sos1 and this positive feedback loop ultimately impacts ERK1/2 activation [5]. Conversely, ERK1/2 phosphorylates both Sos1 and Raf to suppress further activation of ERK1/2 in a negative-feedback loop [6,7,8].

ERK1/2 is also activated downstream of the small GTPase, Rap1, in platelets and nexus [9,10,11,12]. However, in contrast to activation by Ras isoforms, very little is known about potential feedback regulation by ERK1/2 on Rap1 signaling. RasGRP2 (alternative name CalDAG-GEFI) is the predominant GEF that activates Rap1 in platelets. RasGRP2 specifically activates Rap1 but not Ras isoforms both *in vitro* and *in vivo* [12,13,14,15]. In this study, using purified proteins and cellular studies, we show that ERK1/2 phosphorylates RasGRP2 to limit its exchange activity. In addition, we show that this connection establishes a negative-feedback loop that ultimately controls the dynamics of active ERK1/2.

Materials and methods

Reagents

Monoclonal antibodies against ERK1/2, phosphorylated ERK1/2, and phosphorylated ERK1/2-substrates were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal antibodies against FLAG M2 were purchased from Sigma-Aldrich (St. Louis, MO). HRP-conjugated secondary antibodies and HisTrap HP affinity columns were purchased from GE Healthcare (Marlborough, MA). Epidermal growth factor (EGF) and GDP-BODIPY were from ThermoFisher Scientific (Waltham, MA). U0126 was purchased from EMD Millipore (Bellerica, MA). [^{32}P] γ-ATP was from PerkinElmer (Waltham, MA).

Cell culture

HEK293T cells were grown in Dulbecco's modified Eagles's media supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. Transfections were carried out using Lipofectamine Plus (ThermoFisher) with the manufacturer's instructions.

Constructs and protein purification

The gene for human, full-length RasGRP2 was PCR-amplified and subcloned into a modified pcDNA3.1 (+) vector encoding a N-terminus FLAG-tag. Substitutions of the RasGRP2 gene were introduced using Quikchange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. For protein purification from *E. coli*, RasGRP2(1-551) was subcloned into a modified pET15b vector.

RasGRP2 protein was expressed in the Rosetta strain (EMD Millipore). Cell cultures were grown at 37°C in LB, 1 μM $ZnSO_4$, 50 μg/ml ampicillin and chloramphenicol and induced with 500 μM IPTG for 12 hours at 20°C. The cell pellet was resuspended in 20 mM PIPES, pH 6.8, 300 mM NaCl, 1 μM $ZnCl_2$, 10 mM β-mercaptoethanol, 10 mM imidazole, 5% glycerol, and protease-inhibitors (Roche, Basel, Switzerland), lysed using an Emulsiflex C5

cell homogenizer (Avestin, Ottawa, ON) and clarified by ultracentrifugation at 45,000 rpm and 4°C. Filtered supernatant was applied to HisTrap HP affinity column equilibrated with buffer A (20 mM PIPES, pH 6.8, 300 mM NaCl, 8 mM imidazole, and 5% glycerol). Protein was eluted from the column with buffer A plus 400 mM imidazole and treated with tobacco-etch virus protease overnight at 4°C. A second pass over the HisTrap HP affinity column removed the His-tag and protease. Purified protein fractions were loaded onto a Superdex 200 10/300 size exclusion column. The monomeric RasGRP2 proteins were concentrated, flash frozen in liquid nitrogen and stored at −80°C.

Human H-Ras(1-181) and Rap1b(1-181) were subcloned into a modified pET15b vector encoding an N-terminal His-tag. The proteins were induced with 200 μM IPTG for 10 hours at 20°C in *E. coli*. Proteins were purified by immobilized metal affinity chromatography similar as other small GTPases [16].

Constitutively active ERK2 (ERK2-CA) was purified using a single plasmid pBB131 co-expressing ERK2 and mitogen-activated protein kinase kinase (MEK1) in *E.coli* using previously methods [17].

In vitro kinase assays

In vitro kinase assays were performed as described previously [18,19]. Five micrograms of H-Ras, Rap1b, or RasGRP2 proteins were incubated with ERK2-CA (0.5 μg) in the presence of 10 μCi [^{32}P] γ-ATP in kinase buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM NaF, and 1 mM PMSF) for 10 min at 30 °C. Samples were analyzed by SDS-PAGE and autoradiography.

Real-time guanine nucleotide exchange assays

Nucleotide exchange was measured using a fluorescence-based assay as previously described [20]. Equal amounts (400 nM) of Rap1b and BODIPY-GDP were incubated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 5% glycerol prior to addition of wild-type (WT) or mutant RasGRP2 (40 nM) in a final volume of 1 ml. Four reactions were monitored simultaneously in quartz cuvettes (Hellma) using a Fluorolog-3 spectrometer (HORIBA Scientific, Edison NJ) using 2 nm slits and $\lambda_{ex/em}$ = 500/510 nm.

GTP-Rap1 pull-down assays

HEK293T cells were co-transfected with plasmids encoding FLAG-tagged RasGRP2 (50 ng) and Rap1b (1.5 μg) in a 6-well plate. After 24 hrs, cells were lysed in buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM DTT) augmented with a cocktail of inhibitors of serine/threonine phosphatases and proteases. Clarified cell lysates were incubated with 4 μg of GST-RalGDS-RBD pre-bound to glutathione beads at 4 °C for 1 h and then washed four times with lysis buffer. Beads were boiled in sample loading buffer prior to analysis by SDS-PAGE and western blotting to quantify levels of GTP-bound Rap1.

Immunoprecipitations

HEK293T cells were lysed in 25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM NaF, and 1 mM $NaVO_4$ containing protease inhibitor cocktail. Lysates were cleared by centrifugation at 13,000 rpm for 10 min prior to incubation with anti-FLAG M2 antibody for 2 hrs at 4°C. Immunoprecipitated complexes were washed four times with lysis buffer and analyzed by SDS-PAGE and western blotting.

Image quantification

The density of the bands in the western blots was measured and quantified using Image J. Images are representative of at least three independent experiments.

Results

ERK2 phosphorylates RasGRP2 at Ser394 in vitro

RasGRP family members have four conserved functional domains: i) an N-terminal Ras-exchange motif (REM), ii) a catalytic CDC25 domain, and a C-terminal regulatory region comprised of iii) two EF hands and iv) an atypical C1 domain (**Fig. 1A**) [15]. Post-translational modifications to RasGRP2 have previously been identified and are available from several databases that provide mass spectral data [21,22]. From these databases, we noted that RasGRP2 is phosphorylated at Ser394 within the linker between the CDC25 domain and EF hands. In particular, this region occludes the Ras-binding region of RasGRP1 in its autoinhibited state [23] and is conserved in other RasGRP isoforms. Furthermore, sequence adjacent to Ser394 matches a consensus motif of many ERK1/2 substrates defined by P-X-S/T-P, where “X” indicates any amino acid and S/T is the site of phosphorylation (**Fig. 1A**). Thus, we hypothesized that ERK1/2 phosphorylates RasGRP2 at Ser394 to potentially regulate its capacity to activate Rap1.

To test this idea, we initially assessed the capacity of constitutively active ERK2 (ERK2-CA) to phosphorylate purified RasGRP2. Notably, ERK2-CA robustly phosphorylated RasGRP2 but not other proteins including GST, H-Ras and Rap1b (**Fig. 1B**). Moreover, ERK2-CA failed to phosphorylate RasGRP2(S394A) indicating that Ser394 is the major site of phosphorylation under these conditions.

The ERK1/2 pathway is required for phosphorylation of RasGRP2 in cells

HEK239T cells are competent to activate ERK1/2 downstream of EGF activation. Consequently, we transfected these cells with either WT RasGRP2 or RasGRP2(S394A) prior to stimulation with EGF. Without EGF addition, RasGRP2 is basally phosphorylated based on an antibody specific for ERK1/2 substrates (**Fig. 2A**). Consistent with our *in vitro* data, RasGRP2(S394A) is poorly phosphorylated under these conditions. Upon stimulation with EGF, phosphorylation of RasGRP2 is increased and conversely, this phosphorylation is dramatically decreased upon inhibition of MEK1 with the small molecule, U0126 (**Fig 2B**).

Phospho-mimetic mutation of RasGRP2 impairs its nucleotide exchange activity

A fluorescence-based, guanine nucleotide exchange assay was used to assess the capacities of purified RasGRP2 proteins to activate Rap1b (**Fig. 3A-B**). The intrinsic rate of nucleotide

exchange of Rap1b is low and this rate was increased dramatically by the addition of RasGRP2. A similar increase in exchange activity was seen upon addition of RasGRP2(S394A). However, the phospho-mimetic mutant, RasGRP(S394E), was unable to activate Rap1b to the same degree. Consistent with these in vitro findings, the expression of RasGRP2(S394E) in cells led to reduced levels of active Rap1 when compared to cells expressing RasGRP2 or RasGRP2(S394A) (**Fig. 3C-D**).

RasGRP2 phosphorylation negatively regulates ERK1/2 activation

ERK1/2 activation by Rap1 requires BRaf [10]. The previous experiments indicate that ERK1/2 phosphorylates and inhibits the Rap1 GEF, RasGRP2. Thus it is possible that these proteins participate in a negative-feedback loop whereby active ERK1/2 leads to decreased active Rap1 through RasGRP2. Since HEK293T cells also express BRaf in addition to being competent to activate ERK1/2 as shown above, we used this cell line to delineate potential linkages between RasGRP2 and ERK1/2. Consistent with a negative-feedback loop, expression of RasGRP2(S394A), which is not able to be phosphorylated, increased levels of phos-ERK1/2. (**Fig. 4A-B**). Expression of the phospho-mimetic mutant, RasGPR2(S394E), did not substantially reduce phos-ERK1/2 levels, but this situation might arise due to the already high basal levels of phosphorylated WT RasGRP2 in these cells (**Fig.2**).

Studies show disruption of other ERK1/2 negative-feedback loops increase the amplitude and duration of ERK1/2 activation upon stimulation [3]. If phosphorylation of RasGRP2 at Ser394 is critical for a negative-feedback loop involving active ERK1/2, then we might expect to see an increase in the amplitude and duration of ERK1/2 activation upon expression of RasGRP2(S394A). Indeed, ERK1/2 activation was increased and prolonged in EGF-stimulated cells expressing RasGRP2(S394A) when compared to cells expressing WT RasGRP2 (**Fig. 4C**).

Discussion

The equivalent of Ser394 and surrounding residues of RasGRP2 are conserved in all members of the RasGRP family suggesting that the negative-feedback loop described here for RasGRP2 is also relevant for signaling by other RasGRP family members. Consistent with this hypothesis, phosphorylation of human RasGRP3 at Ser391, the equivalent of Ser394 in RasGRP2, was identified in two independent phosphoproteomics studies [24,25].

In addition to ERK1/2, PKA has also been shown to phosphorylate RasGRP2 at multiple sites [26,27,28]. However, these studies have produced conflicting results. In the original studies [26,27], phosphorylation by PKA resulted in the inhibition of RasGRP2 in HEK293T cells, while the most recent study showed PKA-mediated activation of RasGRP2 in Cos-7 cells [28]. It is difficult to reconcile these results and perhaps future studies using purified proteins will clarify this issue. It seems likely that the RasGRP proteins are directly targeted by diverse kinases and that the regulation of RasGRP2 by phosphorylation is crucial for a range of cellular processes.

Signaling cascades leading to the activation of ERK1/2 are subject to stringent homoeostatic control through both positive and negative-feedback loops. A number of inhibitors that target

these cascades are used to treat cancer. However, drug-resistant tumors frequently emerge leading to disease progression [29]. Alteration in the feedback regulation of ERK1/2 pathway is one potential cause of acquired drug resistance. Our study has defined a new negative-feedback loop from ERK1/2 to RasGRP2 and this feedback contributes to the dynamic regulation of ERK1/2 activity that likely has clinical ramifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinases
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GST	glutathione-S-Transferase
HRP	horseradish peroxidase
IPTG	isopropyl β-D-1-thiogalactopyranoside
MEK	mitogen activated protein kinase kinase
PKA	protein kinase A
PLC	phospholipase C
RalGDS-RBD	Ral guanine disassociation stimulator Rap1-binding domain

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Research Highlights

1. ERK2 phosphorylates the guanine nucleotide exchange factor RasGRP2 at Ser394.
2. Phosphorylated RasGRP2 has decreased capacity to active Rap1b *in vitro* and in cells.
3. Phosphorylation of RasGRP2 by ERK1/2 introduces a negative-feedback loop into the BRaf-MEK-ERK pathway.

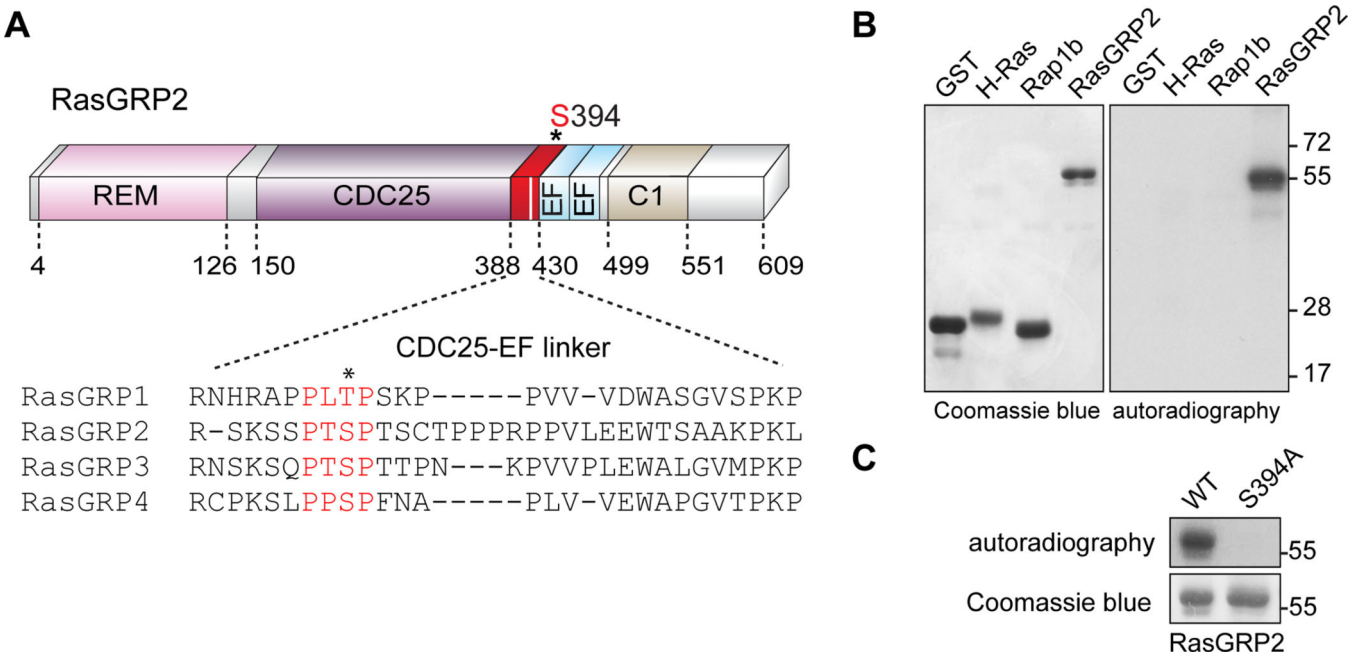

Fig. 1. ERK2 phosphorylates RasGRP2 at Ser394 *in vitro*

(A) Domain architecture of human RasGRP2. Sequence alignment for the individual RasGRP family members highlights the conserved ERK1/2 phosphorylation motif “PXSP” (red) in the linker between the Cdc25 domain and the EF hands. Asterisk identifies Ser 394 in RasGRP2. (B) ERK2 phosphorylates RasGRP2 *in vitro*. (C) ERK2 phosphorylates RasGRP2 at Ser394. For panels B and C, bacterially purified proteins (5 μg) were incubated with recombinant, constitutively active ERK2 in the presence of [^{32}P] γ-ATP for 10 min prior to SDS-PAGE and autoradiography.

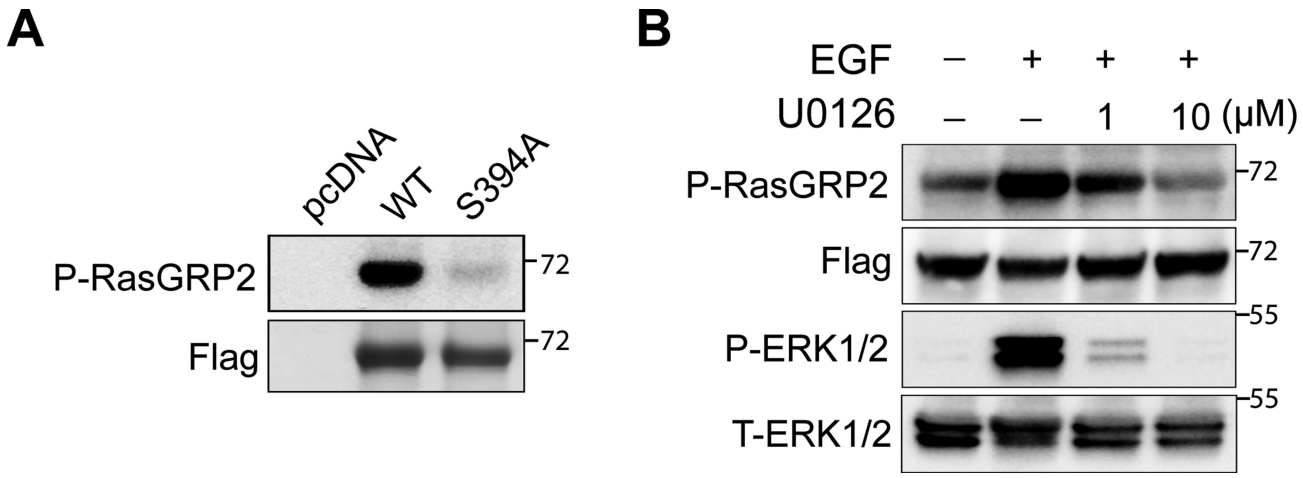

Fig. 2. RasGRP2 is phosphorylated in response to EGF stimulation in HEK293T cells

(A) RasGRP2 is basally phosphorylated at Ser394 in HEK293T cells. Plasmids encoding FLAG-tagged RasGRP2 wild-type (WT) or S394A were transfected into HEK293T cells prior to immunoprecipitation of the expressed protein and western blotting as indicated. The parental vector was used as a control and treated similarly. (B) Phosphorylation of RasGRP2 in response to EGF is eliminated by the MEK inhibitor, U0126. Cells were pretreated with the indicated amount of U0126 or DMSO for 1 hr prior to stimulation with 50 ng/ml EGF for 5 min. FLAG-RasGRP2 was immunoprecipitated from cell lysates and followed by western blotting as indicated. Cell lysates were also blotted for total ERK1/2 (T-ERK1/2) and phos-ERK1/2 (P-ERK1/2).

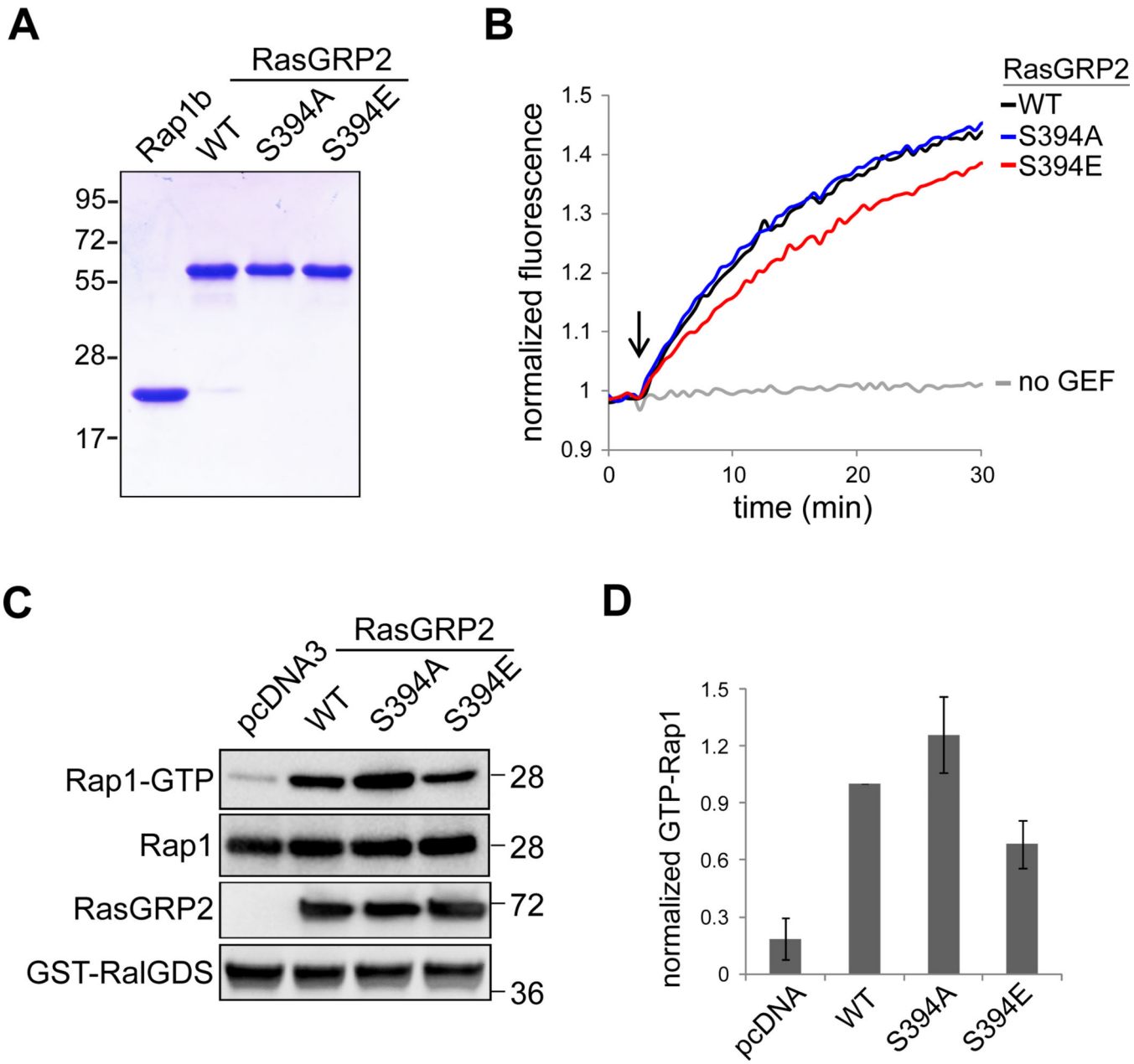

Fig. 3. Phosphorylation of RasGRP2 impairs its nucleotide exchange activity

(A) Purified proteins (3 μg) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. (B) *In vitro* nucleotide exchange assay. Equivalent amounts of Rap1b and BODIPY-FLGDP were preincubated prior to the addition (arrow) of the indicated forms of RasGRP2. (C) GST-RalGDS was used to precipitate GTP-Rap1 from HEK293T cells expressing the indicated forms of RasGRP2 prior to western blotting for proteins as indicated. (D) GTP-Rap1 levels as shown in *C* were normalized to that of WT (average ± SEM).

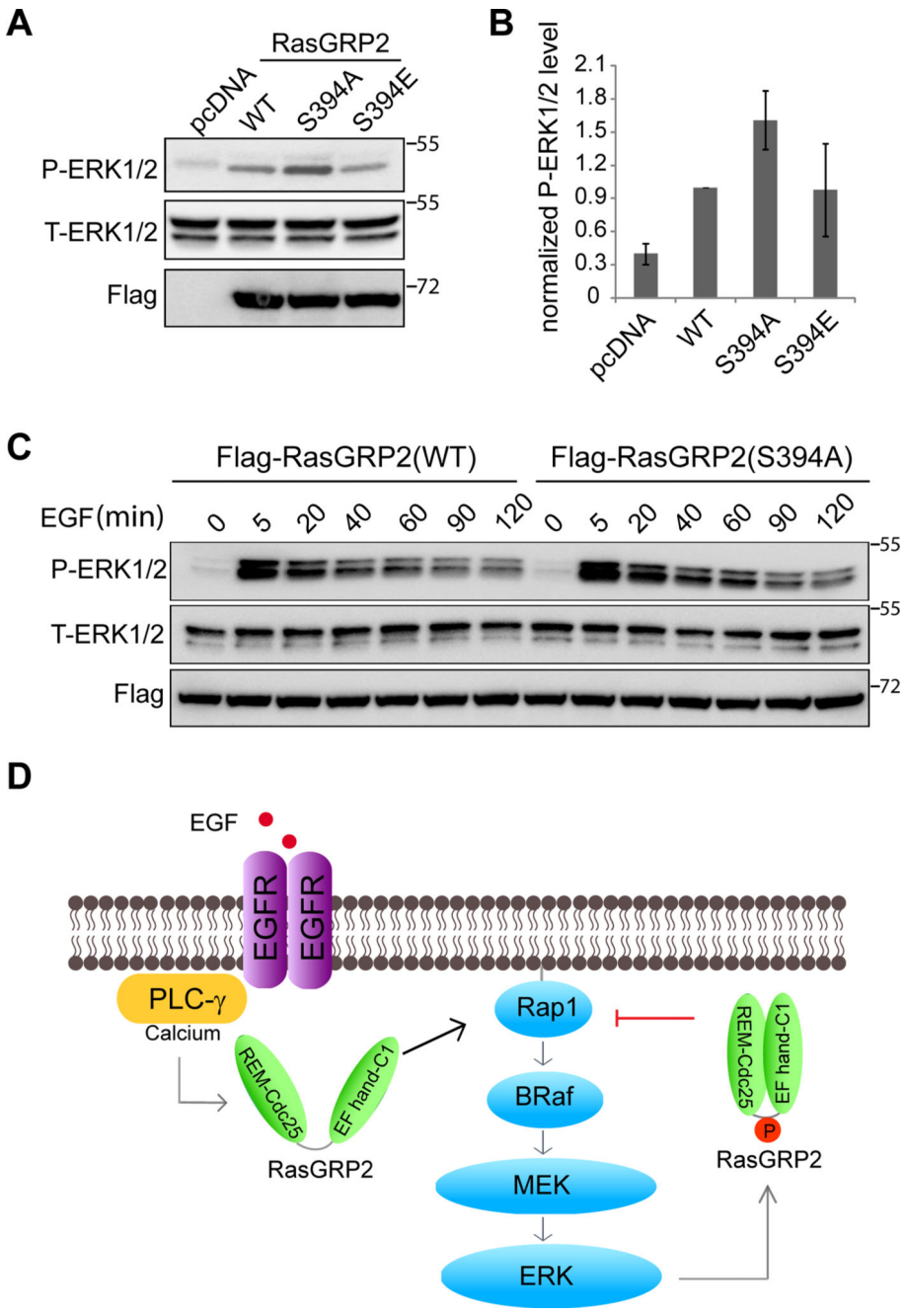

Fig. 4. Phosphorylation of RasGRP2 negatively regulates ERK1/2

(A) P-ERK1/2 levels in HEK293T cells transfected with the indicated forms of RasGRP2. Cells were lysed 24 hrs after transfection and analyzed by western blotting as indicated. (B) Levels of P-ERK1/2 as shown in *A* for three independent experiments (average ± SEM), analyzed with Image J and normalized to that of the cells transfected with WT RasGRP2. (C) RasGRP2(S394A) increases the magnitude and duration of ERK1/2 phosphorylation in response to EGF stimulation. HEK293T cells were transfected with either WT or mutant RasGRP2. Twenty four hrs after transfection, cells were starved for 2 hrs and treated with 20 ng/ml EGF for the indicated times prior to lysis. Cell extracts were subjected to SDS-PAGE

and immunoblotting as indicated. The result is representative of three independent experiments. (D) Model of the RasGRP2 mediated negative-feedback loop. Active EGFR recruits and activates PLC-γ isozymes leading to elevated levels of intracellular calcium and subsequent activation of RasGRP2. Activated RasGRP2 catalyzes the formation of GTP-bound Rap1 to initiate signaling through BRaf to ERK1/2. ERK1/2 subsequently phosphorylates RasGRP2 at Ser394 to impair the activation of Rap1 and ultimately attenuating the Rap1-ERK pathway.