

RasGAP Shields Akt from Deactivating Phosphatases in Fibroblast Growth Factor Signaling but Loses This Ability Once Cleaved by Caspase-3*

Received for publication, February 10, 2015, and in revised form, June 15, 2015. Published, JBC Papers in Press, June 24, 2015, DOI 10.1074/jbc.M115.644633

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Background: RasGAP and its stress-activated caspase-3-generated fragments N and N2 have the potential to regulate receptor tyrosine kinase (RTK) signaling.

Results: RasGAP and fragment N favor FGF1-mediated signaling via different mechanisms, whereas fragment N2 blocks it.

Conclusion: Stress, via the cleavage of RasGAP, modulates RTK signaling.

Significance: This work provides a mechanistic framework of how stress impacts on cell signaling.

Fibroblast growth factor receptors (FGFRs) are involved in proliferative and differentiation physiological responses. Deregulation of FGFR-mediated signaling involving the Ras/PI3K/Akt and the Ras/Raf/ERK MAPK pathways is causally involved in the development of several cancers. The caspase-3/p120 RasGAP module is a stress sensor switch. Under mild stress conditions, RasGAP is cleaved by caspase-3 at position 455. The resulting N-terminal fragment, called fragment N, stimulates anti-death signaling. When caspase-3 activity further increases, fragment N is cleaved at position 157. This generates a fragment, called N2, that no longer protects cells. Here, we investigated in *Xenopus* oocytes the impact of RasGAP and its fragments on FGF1-mediated signaling during G₂/M cell cycle transition. RasGAP used its N-terminal Src homology 2 domain to bind FGFR once stimulated by FGF1, and this was necessary for the recruitment of Akt to the FGFR complex. Fragment N, which did not associate with the FGFR complex, favored FGF1-induced ERK stimulation, leading to accelerated G₂/M transition. In contrast, fragment N2 bound the FGFR, and this inhibited mTORC2-dependent Akt Ser-473 phosphorylation and ERK2 phosphorylation but not phosphorylation of Akt on Thr-308. This also blocked cell cycle progression. Inhibition of Akt Ser-473 phosphorylation and entry into G₂/M was relieved by PHLPP phosphatase inhibition. Hence, full-length RasGAP favors Akt activity by shielding it from deactivating phosphatases. This shielding was abrogated by fragment N2. These results highlight the role played by RasGAP in FGFR signaling and how graded stress intensities, by

generating different RasGAP fragments, can positively or negatively impact this signaling.

The fibroblast growth factor (FGF) family comprises a large number of members (up to 22) that regulate embryonic development and, in adult life, control cell cycle, proliferation, differentiation, migration, and survival (1–3). FGF binds to high affinity transmembrane tyrosine kinase receptors (receptor tyrosine kinases) called FGF receptors (FGFRs).³ The signaling and biological responses elicited by FGF/FGFR are dictated by their intracellular domain (4). FGFR deregulation is frequently observed during cancer development and progression (5). Of note, FGFR1 alterations or amplifications have been detected in 8–10% of breast cancers (6). FGFR1 is expressed by hormone-independent breast cancer cells, such as MDA-MB-231 cells, in which it provides an autocrine proliferative response characterized by a succession of anarchic cell cycles (7, 8, 10–12). Moreover, the activation of the FGFR pathways may provide the means for resistance against available anti-cancer drugs and ionizing radiations (6). Consequently, increasing efforts are currently focusing on the development of strategies to target FGF/FGFR signaling for cancer therapy.

FGF binding to FGFRs promotes their phosphorylation and activation, which initiates various intracellular signaling pathways, including the PI3K/Akt and the Ras/ERK MAPK pathways (13). The recruitment and the activation of proteins implicated in the PI3K/Akt signaling cascade occur in an ordered sequence of events through the successive docking of FRS2, Grb2, and PI3K to the autophosphorylated FGFR (4). PI3K activation leads to the recruitment of Akt to the plasma membrane through its pleckstrin homology (PH) domain, allowing Akt phosphorylation on two key activating residues, Thr-308 and

* This work was supported by the "Ligue Nationale Contre le Cancer, Comité du Nord" and by Swiss National Science Foundation Grant 31003A_141242/1 (to C. W.). The authors declare that they have no conflicts of interest with the contents of this article.

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³ The abbreviations used are: FGFR, FGF receptor; PH, pleckstrin homology; SH2 and SH3, Src homology 2 and 3, respectively; oligo, oligonucleotide; PP, polyproline; GVBD, germinal vesicle breakdown.

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Ser-473 (14). The activation loop of Akt is phosphorylated on Thr-308 by another FGFR-recruited kinase, PDK1, and on Ser-473 by the mTORC2 complex (15). The phosphorylation of Thr-308 is a prerequisite for kinase activation, whereas the phosphorylation of Ser-473 seems to further increase Akt activity. However, Akt-dependent responses can be activated in the absence of Ser-473 phosphorylation (15). Akt further interacts with other cell cycle partners and anti-apoptotic substrates to promote cell survival. Akt is deregulated in a large variety of cancers. Radiotherapy relapse has been associated with persistent Akt phosphorylation (16, 17). The activation of the Ras/ERK MAPK pathway is brought about by the recruitment of FRS2, Grb2, and the Ras guanine nucleotide exchange factor Sos to trigger cell cycle progression (13). Similar to Ras activators, the Ras modulator p120 RasGAP associates, through its N-terminal part, with the phosphorylated FGFR (18–20). Once recruited by the FGFR, RasGAP, via its C-terminal GTPase-activating protein (GAP) domain, stimulates the intrinsic GTPase activity of Ras and hence favors its deactivation. The binding of RasGAP to the FGFR also results in its phosphorylation, and this provides potential docking sites for additional signaling proteins (18, 21, 22). Regulation of Ras activity by RasGAP is complex, however, because its N-terminal moiety positively participates in Ras signaling (21, 23). Consistent with a positive effect of RasGAP on Ras signaling is the observation that RasGAP, downstream of FGFR activation, is a co-activator of the Src kinase to stimulate the Ras/MAPK cascade and cell cycle progression (24).

The p120 RasGAP protein was shown to be sequentially cleaved at two cleavage sites by graded increases in caspase-3 activity (25–27). The first cleavage of RasGAP occurs at very low caspase-3 activity, generating two fragments, including an N-terminal fragment (amino acids 1–455), called fragment N, that exerts a potent anti-apoptotic signal mediated by the activation of the Ras/PI3K/Akt pathway (22, 27, 28). However, the manner in which fragment N activates this pathway remains poorly understood. The increase of caspase activity above a certain threshold leads to further cleavage of fragment N at position 157 that generates two fragments called N1 (amino acids 1–156) and N2 (amino acids 157–455). The cleavage of fragment N abrogates its capacity to activate Akt (29). This inactivates the anti-apoptotic function of fragment N so that caspases are no longer hampered in their ability to kill cells (28). Fragment N2 strongly potentiates cell death and sensitizes tumor cells, but not normal cells, to the killing action of several genotoxins (30, 31). Fragment N2 also inhibits cell adhesiveness and cell migration in a DLC1-dependent manner (32) as well as hampering breast cancer metastatic progression (31). The rationale for optimal targeting of the FGF/FGFR pathway in cancers must incorporate the notion that the therapeutic treatment needs to discriminate as much as possible between normal cells and tumor cells.

Studies on the regulation of cell division by growth factors are sometimes difficult to interpret due to the other cellular actions that these receptors exert. Biological systems where specific FGFR responses on cell cycle can be analyzed are therefore useful to overcome this difficulty. The *Xenopus* oocyte has long been used for this purpose (33–35). It offers the advantage

of a large cell in which specific growth factor receptors and cytosolic regulators can be expressed and analyzed for signaling using biochemical techniques. *Xenopus* oocytes express no endogenous FGFR and only one tyrosine kinase receptor for insulin. They are naturally arrested in the G₂ phase of the cell cycle. Their activation by the physiological inducer progesterone, by insulin, or by ligands of heterologously expressed growth factor receptors allows their synchronized progression into the M phase of the cell cycle (also called the maturation process) (36). Using this model, the signaling complexes and the various cascades elicited by different FGFR isoforms and by FGFR from breast cancer cells have been successfully characterized (24, 37–39). In the present study, we used this powerful integrated cellular system to investigate the impact of RasGAP and its caspase-3-generated fragments on the signaling events triggered by FGFR stimulation.

Experimental Procedures

Estrogen-independent Breast Cancer Cell Culture and Oocyte Handling—MDA-MB-231 cells were cultured at confluence in a humidified atmosphere of 5% CO₂ in Eagle's medium containing 10% fetal calf serum, 20 mM HEPES, 2 g/liter sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamycin sulfate. After anesthesia with MS 222 (1 g/liter; Sandoz), *Xenopus laevis* ovaries were surgically removed and placed in ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, adjusted to pH 7.4 with NaOH), supplemented with streptomycin/penicillin (50 μg/ml; Eurobio), sodium pyruvate (225 μg/ml; Sigma), and soybean trypsin inhibitor (30 μg/ml; Sigma). Stage VI oocytes were harvested by using a 1-h treatment with collagenase A (1 mg/ml; Roche Applied Science) followed by a manual defolliculation. The oocytes were kept at 19 °C in the ND96 medium. For each experiment, 20 oocytes isolated from three animals were used.

Fusion Protein and RNA Preparations—Fragment N, fragment N2, and glutathione S-transferase (GST) were produced as described previously (26), using the corresponding bacterial expression vectors, and purified as described (37). The RasGAP SH2-SH3-SH2-GST and SH3-GST fusion proteins were gifts from Dr. C. Garbay. Plasmids encoding GST-tagged SH2 domains of either RasGAP or Grb2 were a gift from Dr. S. Courtneidge. Capped cRNAs were transcribed using the mMessage mMachine kit (Ambion). Poly(A) mRNAs from MDA-MB-231 cells were extracted by the guanidium thiocyanate/cesium chloride gradient method (40), using RNA plus reagent from Bioprobe followed by poly(dT) columns (Amersham Biosciences). The hormone-independent human mammary MDA-MB-231 cell line, derived from a highly invasive tumor, expresses FGFR1 and FGFR4 and several types of FGFR1 splice variants (12, 42, 43).

Plasmids—HA-hRasGAP.dn3[118] is a eukaryotic expression vector encoding the N-terminally HA-tagged version of human p120 RasGAP. It was described previously (44) as HA-GAP.dn3.

GST-hRasGAP[158–455].pgx[205] is a bacterial expression vector encoding fragment N2 of RasGAP tagged at its N terminus with GST. It was constructed by PCR-amplifying

HA-hRasGAP[158–455].dn3[145], previously called HA-N2.dn3 (44), with the sense oligo 239 (TTGGTT (feeder) GGATCC (BamHI) TCTCTGGATGGACCAG (nucleotides 590–605 of human p120 RasGAP; NCBI entry M23379)) and the antisense oligo 240 (ACACAC (feeder) GAATTC (EcoRI) TCA (stop codon) ATCCACTGTGTCATTG (nucleotides 1483–1468 of human p120 RasGAP; NCBI entry M23379)). The PCR fragment was cut with BamHI and EcoRI and subcloned into pGEX-KG[88] (LGC Standards, Middlesex, UK) opened with the same enzyme.

SjGST26.cmv[574] is a eukaryotic expression vector encoding the *Schistosoma japonicum* class Mu 26-kDa GST isozyme (GST26) (Uniprot entry: P08515, GST26_SCHJA).

GST-hRasGAP[158–455].cmv[907] is a eukaryotic expression vector coding for an N-terminally GST-tagged version of human RasGAP fragment N2. It was generated by subcloning the BamHI/XhoI insert of GST-hRasGAP[158–455].pgx[205] into SjGST26.cmv[574] opened with the same enzyme.

GST-hRasGAP[1–455](D157A).pgx[234] is a prokaryotic expression vector coding for an N-terminally GST-tagged version of the caspase-3-resistant form of human RasGAP fragment N. It was described previously (30) as N(D157A).pgx.

GST-hRasGAP[1–455](D157A).cmv[578] is a eukaryotic expression vector coding for an N-terminally GST-tagged version of the caspase-3-resistant form of human RasGAP fragment N. It was produced by subcloning the BamHI/XhoI insert of GST-hRasGAP[1–455](D157A).pgx[234] into the SjGST26.cmv[574] plasmid opened with the same enzyme.

GST-hRasGGST-hRasGAP[1–119,146–455](D157A).cmv[905] is a eukaryotic expression vector coding for an N-terminally GST-tagged version of the caspase-3-resistant form of human RasGAP fragment N lacking a polyproline-rich sequence within its PP domain (Δ 120–145). It was constructed with the QuikChange II XL mutagenesis kit (Agilent Technologies, Santa Clara, CA) using GST-hRasGAP[1–455](D157A).cmv[578] as the template amplified with the sense oligo 1287 (nucleotides 459–475 of human p120 RasGAP; NCBI entry M23379), TTGGGGGCGGGC (nucleotides 554–565 of human p120 RasGAP; NCBI entry M23379), and the antisense oligo 1288 (complementary sequence of oligo 1287).

GST-hRasGAP[1–455](D157A,W317D).cmv[906] is a eukaryotic expression vector coding for an amino-terminally GST-tagged version of the caspase-3-resistant form of human RasGAP fragment N bearing an amino acid substitution (W317D) expected to disrupt the capacity of its SH3 domain to interact with its partners (45, 46). It was constructed with the QuikChange II XL mutagenesis kit using GST-hRasGAP[1–455](D157A).cmv[578] as the template amplified in separate tubes with the sense oligo 1286 (G TTC ATT GTT CAT AAT GAA TTA GAA GAT GGA GAT ATG TGG GTT ACA AAT TTA AGA ACA GAT GAA C (nucleotides 1036–1100 of human p120 RasGAP; NCBI entry M23379); the underlined nucleotides correspond to the TGG to GAT mutation converting the tryptophan residue at 317 into an aspartate residue) and the antisense oligo 1285 (complementary sequence of oligo 1286). After 30 amplification cycles, the two PCRs were mixed, denatured, and allowed to cool down slowly so that the two

mutated strands could anneal. The template DNA was then digested with DpnI.

Microinjection, Drug Treatments, and Germinal Vesicle Breakdown (GVBD) Analysis (G₂/M Checkpoint Assay)—Microinjection, in the equatorial region of the oocyte cytoplasm, of 60 ng of FGFR1, PDGF-FGFR1 (human chimeric PDGF receptor extracellular domain coupled to FGFR1 intracellular domain), or FGFR mRNAs from the breast cancer cell line MDA-MB-231 was performed 48 h before the addition of FGF1 to the extracellular medium (5 nM; R&D Systems, Abingdon, UK) as described (37–39). Sixty ng of RasGAP SH2-SH3-SH2, SH2 (Nter), or SH3 constructs of RasGAP or SH2 domains of Grb2, GST, or Nter HA-tagged RasGAP or 60 ng of each anti-AktPH domain (SKB1, Calbiochem), anti-PHLPP (Abcam, Cambridge, UK), anti-Sin1 (Millipore), and anti-IgG (Santa Cruz Biotechnology, Inc.) antibodies were injected 1 h before the addition of FGF1. Extracellular medium incubation with cycloheximide (15 μ M; Sigma) or staurosporine (Sigma) was also performed 1 h before FGF1 treatment. Control oocytes were microinjected into their cytoplasm with 60 nl of water unless specified with 60 ng of GST. For insulin time course experiments, 10 μ M was used to stimulate naive oocytes. Nuclear microinjection with 20 ng of plasmid was realized in oocytes expressing FGFR mRNAs from the breast cancer MDA-MB-231 cells for 36 h, and stimulation with FGF1 was performed 18 h after. In all cases, GVBD, a surrogate of oocyte cell cycle progression from G₂ to M, was assessed by the appearance of a white spot at the animal pole.

Immunoprecipitations of FGFR, Insulin Receptor, and RasGAP—Oocytes expressing FGFR1, PDGF-FGFR1 (a chimeric FGFR1 receptor composed of the extracellular part of the human PDGF receptor and the intracellular part of the human FGFR1 receptor), or FGFR(MDA) (FGFR derived from the estrogen-independent breast cancer cell line MDA-MB-231) for 48 h were microinjected with various fusion proteins (RasGAP SH2-SH3-SH2-GST, RasGAP SH2-GST, Grb2-SH2-GST, RasGAP SH3-GST, GST) or antibodies (anti-PHLPP (Abcam, UK), anti-Sin1 (Millipore), anti-Akt PH domain (SKB1, Calbiochem), anti-IgG (Santa Cruz Biotechnology)) for 1 h, before stimulation by FGF1 or PDGF. Both of these oocytes and MDA-MB-231 cells were lysed in 200 μ l of buffer A (25 mM MOPS, pH-adjusted to 7.2, 60 mM β glycerophosphate, 15 mM *para*-nitrophenyl phosphate, 15 mM EDTA, 15 mM MgCl₂, 2 mM DTT, 1 mM *ortho*-sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml soybean trypsin inhibitor, 10 μ M benzamidine). Extracts were centrifuged at 4 °C for 15 min at 10,000 \times g. Some samples were directly used as total cell fraction and submitted to Western blot analysis. Cytosolic fractions were separated from membrane pellets that were resuspended and mechanically homogenized with a pestle at 4 °C in buffer A containing 1% Triton X-100 and then centrifuged under the same conditions. For competition experiments on MDA-MB-231 cell extracts, RasGAP SH2-SH3-SH2-GST and Grb2-SH2 were added 1 h before immunoprecipitation was performed. For immunoprecipitations, supernatants were precleared with protein A-Sepharose (50%; Sigma) for 1 h at 4 °C before the addition of anti-FGFR antibodies (anti-FGFR1; Upstate Biotechnology) or anti-Ras-

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GAP (Santa Cruz Biotechnology) for 2 h. Immunocomplexes were pulled down using protein A-Sepharose (50%; Sigma) for 1 h at 4 °C, rinsed three times, resuspended in Laemmli sample buffer, and subjected to SDS-PAGE analysis. For insulin receptor immunoprecipitations, the same protocol was applied on naive oocytes using anti-insulin receptor (gift from Prof. D. Boujard, Université de Rennes) and anti-insulin-like growth factor receptor (Cell Signaling) antibodies.

Ras Activity—Oocytes, expressing FGFR1 for 48 h and microinjected with various fusion proteins (RasGAP SH2-SH3-SH2-GST and N and N2 RasGAP) 1 h before stimulation by FGF1, were lysed in 200 μ l of buffer A. Total extracts were centrifuged at 4 °C for 15 min at 10,000 \times *g* to remove vitelline platelets. For membrane fractions, cytosols were discarded, and membranes were resuspended and mechanically homogenized with a pestle at 4 °C in buffer A containing 1% Triton X-100 and then centrifuged under the same conditions. Active Ras was determined using a Ras activation assay kit (Upstate Biotechnology) as per the manufacturer's instructions. This kit uses the Raf1 Ras-binding domain to pull down RasGTP and an anti-pan-Ras antibody to reveal the immobilized fraction of active Ras on Western blot.

Electrophoresis, Western Blot Analysis, and Stripping—Samples were resuspended in Laemmli buffer and subjected to SDS-PAGE electrophoresis and transferred to a Hybond ECL membrane (Amersham Biosciences) in Tris/NaCl/Tween/BSA, pH 8 (15 mM Tris-HCl, 150 mM NaCl, 0.1% Tween, 10% bovine serum albumin; Sigma). The membranes were immunorevealed with the following antibodies: anti-FGFR1 antibodies (clone 19B2, Upstate Biotechnology), anti-RasGAP (171, Santa Cruz Biotechnology), anti-Akt (sc1618, Santa Cruz Biotechnology), anti-phospho-Akt (Ser-473, Upstate Biotechnology; Thr-308, New England Biolabs), anti-ERK2 (D2, Santa Cruz Biotechnology), anti-phospho-ERK2 (Santa Cruz Biotechnology), anti-Grb2 (Santa Cruz Biotechnology), anti-Mos (Santa Cruz Biotechnology), anti-caspase 3 (BD Biosciences), anti-HA (Santa Cruz Biotechnology), anti-Aurora A (Santa Cruz Biotechnology), anti-phospho-Aurora A (Transduction Laboratories), and anti-GST (gift from Dr. C. Dissous). Detection of the immunocomplexes was performed with the advanced ECL detection system (Amersham Biosciences). Before they were reprobbed, Western blots were treated with antibody stripping buffer from (Gene Bio-Application Ltd.) using instructions from the manufacturer.

Results

The RasGAP N and N2 Fragments Differentially Affect the Akt and ERK2 FGFR-mediated Signaling Pathways—To decipher the role played by fragment N and fragment N2 (see Fig. 1 for a schematic representation of the RasGAP-derived constructs used in this study) in proximal FGFR signaling, activation of the Akt and ERK2 MAPK pathways was analyzed over time after FGFR stimulation. We initially used the wild-type FGFR1 and FGFRs derived from the estrogen-independent breast cancer cell line MDA-MB-231 (37), hereafter referred to as FGFR(MDA), to perform these experiments. The rationale to do so was to gather information on the signaling deregulations that can occur in breast cancers. Fig. 2A (top blots) shows that

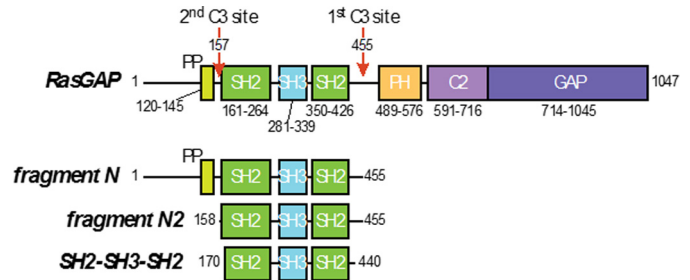


FIGURE 1. RasGAP, fragment N, fragment N2, and the closely related SH2-SH3-SH2 construct used in the study. The p120 RasGAP protein, encoded by the *Rasa1* gene, is a GAP specific for Ras that bears, from the N-terminal end to the C-terminal end, a PP-rich domain, an SH3 domain flanked by two SH2 domains, a PH domain, a C2 domain, and the GAP domain. RasGAP displays two cleavage sites that are used sequentially as caspase-3 (C3) activity increases. RasGAP is cleaved at low caspase-3 activity at site 1 (after amino acid 455). This generates an N-terminal product called fragment N. At higher caspase-3 activity, fragment N is further cleaved at site 2 (after amino acid 157), and this generates fragment N2. SH2-SH3-SH2, used in previous experiments by some authors (18), corresponds to a fragment that is slightly shorter than fragment N2. The domains and the fragments are drawn to scale.

fragment N and fragment N2 did not activate Akt or the ERK MAPK pathways on their own. However, upon FGF1 stimulation, fragment N induced an earlier phosphorylation of ERK2, both in wild-type FGFR- and in FGFR(MDA)-expressing oocytes (Fig. 2A). On the other hand, fragment N did not modulate FGF1- or insulin-mediated Akt activation (Fig. 2A). Fragment N was also able to accelerate insulin-mediated ERK2 phosphorylation and G_2/M transition (Fig. 2A), suggesting that fragment N does not need to engage a specific receptor tyrosine kinase to exert this effect. Fragment N2, in contrast to fragment N, potentially inhibited ERK activation, G_2/M transition, and the phosphorylation of Akt on serine 473 (but not on threonine 308) (Fig. 2A). This effect was specific to FGF1 signaling because it was not observed when oocytes were stimulated with insulin (Fig. 2A). A fragment slightly shorter than fragment N2, called SH2-SH3-SH2 (amino acids 170–440), also blocked ERK activation, G_2/M transition, and phosphorylation of Akt on residue 473 (Fig. 2B) (24). The SH3 domain of RasGAP (amino acids 279–341) blocked ERK2 phosphorylation and G_2/M transition triggered by FGF1 but had no effect on Akt phosphorylation (Fig. 2B). Because the signaling behavior of the wild-type FGFR1 and FGFR(MDA) receptors in the presence or in the absence of RasGAP-derived fragments was identical (Fig. 2A), we generally used only one of these FGFR receptor subtypes (the receptors from MDA-MB-231 cells) in subsequent experiments.

Sequential cleavage of active caspase-3 in the presence of graded concentrations of active caspase-3 generates fragment N and then fragments N1 and N2 (44). The results presented in Fig. 2, A and B, predict that caspase-induced cleavage of RasGAP mediated by increasing stress conditions should result in inhibition of Akt phosphorylation on residue 473, ERK activation, and G_2/M progression when fragment N2 is produced but not when fragment N is generated. To test this hypothesis, a version of full-length RasGAP HA-tagged fused at its N terminus was injected into oocytes. The oocytes were then subjected to graded concentrations of staurosporine, which generated increasing activation of caspase-3 and a sequential cleavage of

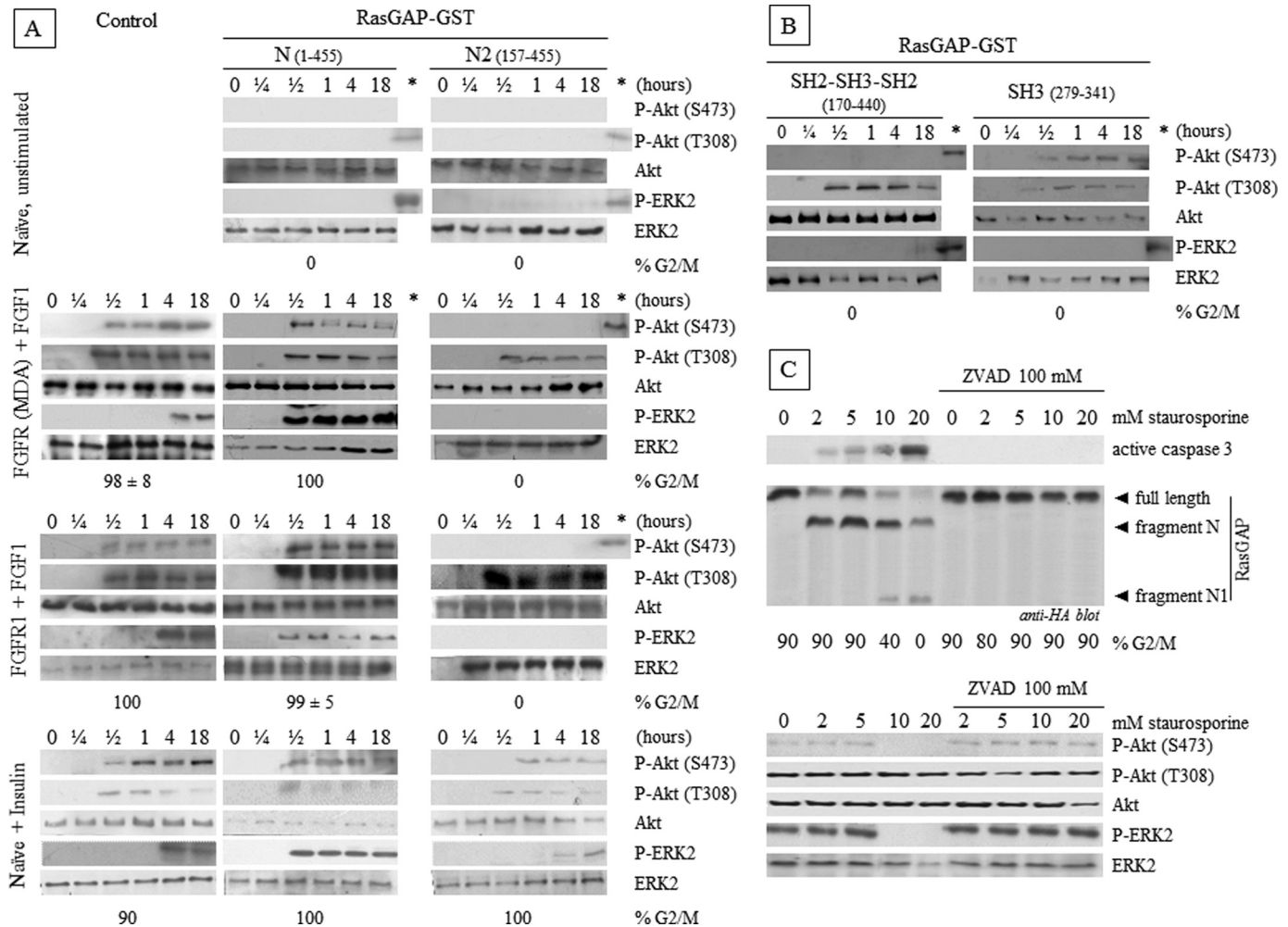


FIGURE 2. Signaling cascades activated by insulin and FGF receptors are differentially regulated by RasGAP fragments. A, GST-tagged RasGAP fragment N or N2 was microinjected into naive oocytes or into oocytes where FGF receptors, either from the breast cancer cells MDA-MB-231 (FGFR(MDA)) or wild type FGFR1, had been expressed for 48 h. Insulin receptors, naturally expressed in naive oocytes, were then stimulated by 10 μ M insulin, while FGF receptors were stimulated with 5 nM FGF1. Extracts were prepared at the indicated times following FGF1 stimulation and analyzed by Western blotting using the indicated antibodies. B, GST-tagged RasGAP SH2-SH3-SH2 or its SH3 domain was microinjected into oocytes expressing FGFR(MDA) and then stimulated with 5 nM FGF1 and analyzed as in A. In conditions where no signals are detected, positive controls (FGFR(MDA) receptors stimulated with FGF1 for 18 h) are included (indicated by asterisks). C, a plasmid encoding the HA-tagged RasGAP was injected into oocytes 36 h after FGFR(MDA) mRNA. Oocytes were treated or not for 1 h with benzoyloxycarbonyl-VAD (ZVAD) and then submitted to gradually increasing concentrations of staurosporine for 1 h before stimulation with FGF1. The G₂/M progression was assessed as described. Total proteins were extracted and subjected to SDS-PAGE and Western blot analysis. The G₂/M transition was assessed by the appearance of a GVBD at the animal pole.

RasGAP (Fig. 2C). The oocytes were finally stimulated with FGF to induce G₂/M transition. Fragment N was produced at low caspase-3 activity, and this fragment was further cleaved into fragments N1 and N2 at high caspase-3 activity (because fragment N2 does not bear the HA tag, only fragment N1 is detected in the blot shown). Low concentration staurosporine-induced fragment N production did not hamper Akt phosphorylation, ERK activation, or G₂/M transition, but further cleavage of fragment N into fragments N1 and N2 at higher staurosporine concentrations did. The benzoyloxycarbonyl-VAD caspase inhibitor blocked RasGAP processing and fully prevented high staurosporine concentration-induced inhibition of G₂/M transition and Akt and ERK phosphorylation (Fig. 2C). These results are compatible with the notion that stress-induced caspase-mediated cleavage of RasGAP differentially modulates FGF receptor signaling in a manner that depends on the extent of RasGAP processing.

Role of Translation in the Signaling-inhibitory Functions of RasGAP Fragments—The cycloheximide translation inhibitor mimicked the capacity of the SH3 domain of RasGAP to inhibit FGF1-induced ERK activation and G₂/M transition (Fig. 3A). This suggests that this SH3 domain blocks the expression of proteins (e.g. Mos; see Fig. 11) required for subsequent ERK activation and G₂/M transition. Fig. 3A also shows that fragment N2 does not require new protein synthesis to block Akt phosphorylation on Ser-473 because this was unaffected by cycloheximide.

Synchronized FGF-mediated Signaling in Xenopus Oocytes—The data presented so far indicate that there is a sharp activation of Akt upon FGF1 stimulation of FGF receptor-expressing oocytes. To more precisely map the timing of Akt and ERK stimulation by FGF1, we repeated the time course analyses of activation of these proteins. Fig. 3, B and C, shows that there is a synchronized and apparently maximal activation of Akt and

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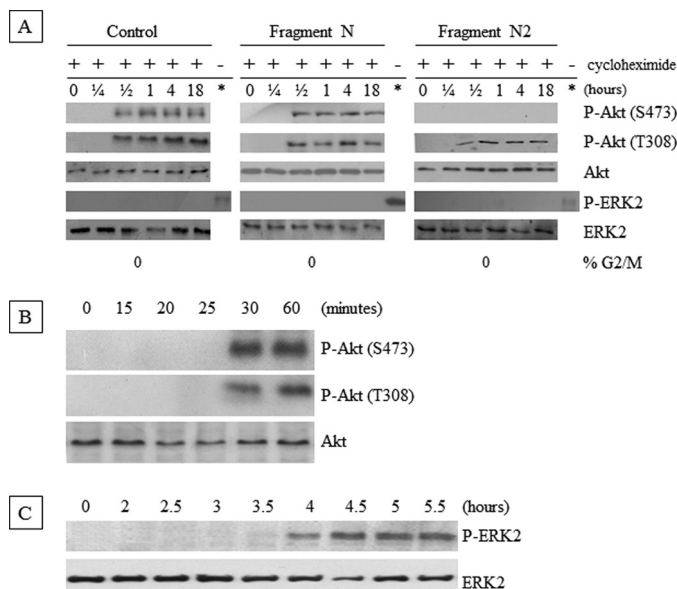


FIGURE 3. Protein synthesis requirement for Akt and ERK phosphorylation and kinetics of these responses. *A*, oocytes expressing FGFR(MDA) for 48 h were microinjected or not with fragment N or fragment N2 and then treated with cycloheximide (15 μ M) for 1 h before stimulation with FGF1. Total cellular extracts were collected after 15 min, 30 min, 1 h, 4 h, and 18 h; SDS-PAGE and Western blot analysis and G₂/M cell cycle progression were performed as described. In conditions where no signals are detected, positive controls (FGF(MDA) receptors stimulated with FGF1 for 18 h) are included (indicated by asterisks). The G₂/M transition was assessed by the appearance of a GVBD at the animal pole. *B* and *C*, oocytes where FGFR(MDA) had been expressed for 48 h were stimulated with 5 nM FGF1. A time course analysis was performed after FGF1 addition at early time points (0–60 min) for Akt phosphorylation (*B*) or later time points (2–5.5 h) for ERK2 phosphorylation (*C*). Total proteins were extracted and subjected to SDS-PAGE and Western blot analysis.

ERK mediated by FGF1 occurring between 25 and 30 min and at around 4 h, respectively. Hence, both proteins undergo very precisely timed apparently maximal phosphorylation modifications in growth factor-stimulated *Xenopus* oocytes.

Akt Binding to the FGFR-RasGAP Complex Is Disrupted by the RasGAP-derived N2 and SH2 Fragments—We next analyzed the formation of signaling complexes on FGF receptors. In oocytes expressing FGFR(MDA), immunoprecipitation experiments using anti-FGFR antibodies revealed that RasGAP and Grb2 were recruited to the FGFR 15 min after ligand addition. This was followed by Akt recruitment 15 min later (Fig. 4A). The differential recruitment of RasGAP, Grb2, and Akt to the FGFR was also observed when RasGAP was immunoprecipitated from membrane fractions (Fig. 4B). The cytosolic pools of Akt and RasGAP were fully recruited to the FGFR signaling complex upon ligand stimulation (Fig. 4, C and D). Similar results were observed in oocytes expressing wild-type FGFR1 (data not shown). In contrast, neither RasGAP nor Akt could be detected in insulin receptor immunoprecipitates upon insulin stimulation (Fig. 5A).

When the SH2-SH3-SH2 fragment (a fragment N2-like construct; see Fig. 1 and 2) or the first SH2 domain of RasGAP (SH2(1)) was microinjected into FGFR(MDA)-expressing oocytes, it fully competed with endogenous RasGAP for binding to the FGF receptors. This also prevented Akt recruitment, but not Grb2 recruitment, to the FGFR complex (Fig. 4, A and B). These RasGAP fragments exerted a similar effect in MDA-

MB-231 breast cancer cell extracts (Fig. 4E). These results indicate that Grb2 can be recruited to FGFR complexes independently of RasGAP (*i.e.* in conditions where RasGAP does not bind to FGFR due to competition with smaller fragments). As a control, Grb2 recruitment to the FGFR complex was abolished by overexpression of the SH2 domain of Grb2 (Fig. 4, A and B). Alone, the SH3 domain of RasGAP was unable to prevent RasGAP and Akt recruitment to the FGFR complex (Fig. 6A). Although the SH2-SH3-SH2 and (SH2(1)) constructs prevented Akt from being recruited to the FGFR complex, they did not inhibit Akt membrane translocation after FGFR signaling was triggered by FGF1 addition (Fig. 4C). As expected, the membrane translocation of Akt was prevented by injection of an antibody directed against the Akt PH domain (Fig. 6).

The results presented above indicate that fragment N2 and the nearly identical SH2-SH3-SH2 fragment bind to the FGFR once stimulated. Surprisingly, fragment N, which contains the SH2-SH3-SH2 domains, was unable to be recruited to the FGFR complex and hence was unable to compete with endogenous RasGAP and Akt (Fig. 5, B and C). One possibility to explain this finding is that the putative intramolecular interaction between the SH3 domain and the polyproline (PP)-rich domain of fragment N affects the conformation of the fragment so that it cannot interact with the FGF receptor complex. To disrupt such putative intramolecular interaction, a mutation of the SH3 domain known to prevent its interaction with PP domains (45, 46) was introduced in fragment N. Alternatively, a 25-amino acid stretch within the PP domain of fragment N was deleted. Fig. 7B shows that both mutants, in contrast to wild-type fragment N, were recruited to stimulated FGF receptors. This occurred at the expense of Akt and full-length RasGAP recruitment to the FGF receptor, resulting in the inhibition of Akt phosphorylation at position 473 and blockage of the G₂/M transition (Fig. 7A). Hence, abrogating the putative intramolecular interaction between the SH3 and PP domains of fragment N made it behave as fragment N2 in terms of its capacity to be recruited to the FGF receptor. Intriguingly, the W317D SH3 mutation, but not the PP mutation, prevented the FGF receptor recruited fragment N to inhibit ERK activation. It is therefore possible that a functional SH3 domain within fragment N or N2 is necessary to exert a repressive activity on the Mos/ERK pathway (see Fig. 12). However, the W317D fragment N mutant was still able to block G₂/M transition (Fig. 7A), suggesting that it acted downstream of ERK to block G₂/M transition. Additionally, despite the fact that it bears the functional SH3 and PP domains, full-length RasGAP is recruited to the activated FGF receptor complex, and it does not inhibit G₂/M transition. Thus, there is obviously more complexity in the signaling of the RasGAP-derived fragments than depicted in the model shown in Fig. 12.

Modulation of Ras Activation Upstream of ERK2 Phosphorylation in Cells Expressing Fragment N and Fragment N2—Ras activity was detected in oocytes expressing FGFR(MDA) following FGF1 activation. This activity was increased upon injection of fragment N in the cytosol of oocytes starting at 30 min and reaching a maximal level at 18 h post-stimulation (Fig. 8A). Fragment N also induced a similar increase of Ras activity in oocytes stimulated with insulin (Fig. 8B). Fragment N2 (and the

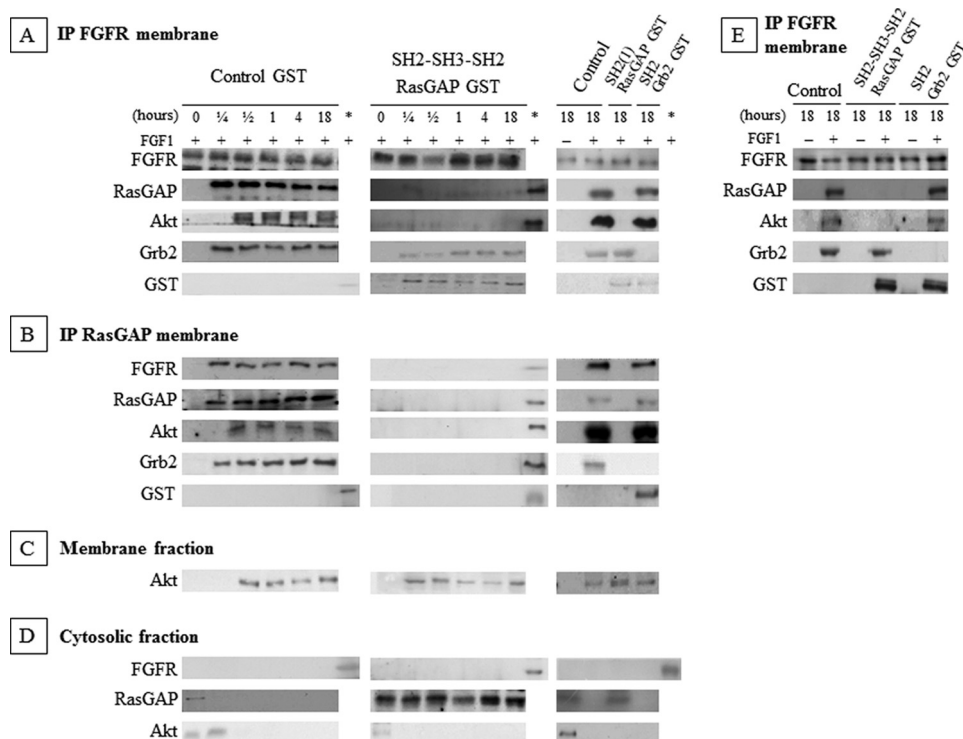


FIGURE 4. RasGAP uses SH2 domains to bind FGF1-stimulated FGFR. *A–D*, oocytes expressing FGFR(MDA) were microinjected with or without the following constructs: GST, GST-tagged SH2 domain of Grb2, or one of the following GST-tagged fragments of RasGAP: SH2-SH3-SH2 or SH2(1). A time course was performed after FGF1 stimulation (15 min, 30 min, 1 h, 4 h, and 18 h). *E*, MDA-MB-231 cell extracts were stimulated or not with FGF1 for 18 h and then submitted for 1 h to various constructs. Immunoprecipitations (*IP*) were performed with anti-FGFR (*A* and *E*) or anti-RasGAP antibodies (*B*). Whole membrane extracts (*C*) or whole cytosolic fractions (*D*) were analyzed by SDS-PAGE and Western blotting using the indicated antibodies. In conditions where no signals are detected, positive controls (FGF(MDA) receptors stimulated with FGF1 for 18 h) are included (indicated by asterisks).

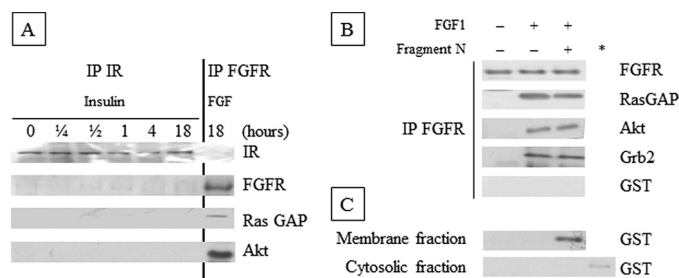


FIGURE 5. Full-length RasGAP, but not fragment N, is recruited in FGFR complexes. *A*, naive oocytes that naturally express the insulin receptor (*A*, lanes on the vertical line) and oocytes expressing FGFR(MDA) for 48 h (*A* (lane on the right of the vertical line), *B*, and *C*) were stimulated with 10 μ M insulin and 15 nM FGF1, respectively. Thereafter, they were injected (*B* and *C*) or not (*A*) with fragment N. Then the samples were collected at the indicated times (*A*) or 18 h later (*B* and *C*). Following extraction, the samples were submitted to immunoprecipitation (*IP*) as indicated (*A* and *B*) or separated into membrane and cytosolic fractions (*C*). The samples were finally analyzed by Western blotting using the indicated antibodies. In the condition where no signal was detected, a positive control (GST) is included (indicated by the asterisk).

SH2-SH3-SH2 construct) did not affect Ras activity at the 30 min time point but totally suppressed Ras activity 18 h post-stimulation (Fig. 8A). However, none of these constructs were able to inhibit Ras activity induced by insulin (Fig. 8B). Levels of Ras protein stayed constant regardless of the fragments expressed or length of stimulation, suggesting that these fragments modulate Ras activity at the post-transcriptional level.

The PHLPP Phosphatase Dephosphorylates Akt at Position Ser-473 in Cells Expressing the RasGAP SH2-SH3-SH2 Domains—An anti-PHLPP antibody directed against active PHLPP phosphatase, one of the major phosphatases able to

dephosphorylate Akt on serine 473 (39), was microinjected into oocytes expressing FGFR(MDA) (Fig. 9, *A* and *B*) or a chimeric FGFR receptor composed by the extracellular part of the human PDGF receptor and the intracellular part of the human FGFR1 receptor (Fig. 9C), 1 h before stimulation by PDGF or FGF1. This chimera, originally used to access the role played by the intracellular domain *versus* the extracellular domain (involvement of extracellular cofactor, such as low affinity heparin sulfates), triggers similar signaling pathways as wild-type FGFR1 (33). As a positive control for an antibody that could disrupt a signal leading to Akt phosphorylation, we used an antibody directed against Sin1, a component of the mTORC2 complex that phosphorylates Akt on serine 473. Injection of the anti-PHLPP antibody led to an earlier FGF1-stimulated phosphorylation of Akt Ser-473 but did not affect Akt phosphorylation on Thr-308. As expected, the anti-Sin1 antibody blocked Akt Ser-473, but not Akt Thr-308, phosphorylation (Fig. 9A).

When the SH2-SH3-SH2 construct was co-injected with the anti-PHLPP antibody, Akt Ser-473 phosphorylation was restored (Fig. 9, *B* and *C*, lane 6). This restored phosphorylation was mediated by mTORC2 because it was prevented by the anti-Sin1 antibody (Fig. 9, *B* and *C*, lane 8). Phosphorylation of Akt on serine 473 upon injection of the anti-PHLPP antibody concerned Akt molecules that were membrane-bound but that were not associated with FGFR complexes (Fig. 9B, part 2). The regulation of Akt phosphorylation exerted by the RasGAP fragment was signal-specific because the SH2-SH3-SH2 construct did not alter the extent of insu-

Impact of RasGAP on FGF Signaling

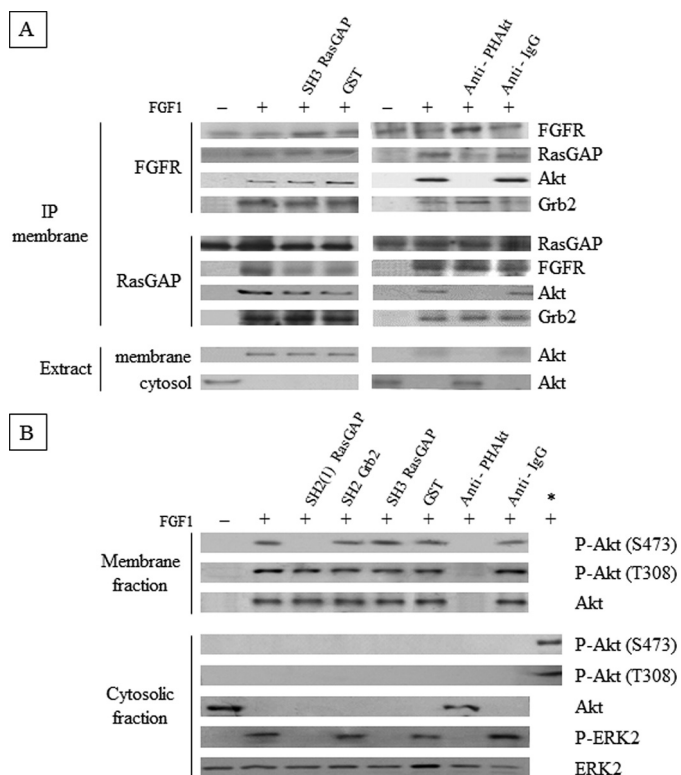


FIGURE 6. Inhibition of Akt recruitment to the plasma membrane and expression of individual RasGAP SH domains block FGF-mediated ERK2 phosphorylation. Oocytes expressing FGFR(MDA) for 48 h were treated as described in the legend to Fig. 4. They were then microinjected with or without the indicated constructs or antibodies 1 h before being stimulated (+) or not (-) with FGF1. Membrane and cytosolic extracts were immunoprecipitated (IP) with anti-FGFR or anti-RasGAP antibodies and, together with non-immunoprecipitated extracts, analyzed by Western blotting using the indicated antibodies. In conditions where no signals are detected, positive controls (FGF(MDA) receptors stimulated with FGF1 for 18 h) are included (indicated by asterisks).

lin-mediated, Sin1-dependent, Akt phosphorylation on residues 473 and 308 (Fig. 10).

Impact of RasGAP Constructs on Aurora (Eg2) Regulation by FGF—The first step in the activation of the MAPK pathway in stimulated oocytes is Mos synthesis (38), which was completely abrogated by fragment N2, SH2-SH3-SH2, or the SH3 domain (Fig. 11A). Efficient translation of Mos (47) requires phosphorylation of Aurora kinase A (Eg2) (48). We observed that fragments N2 and SH2-SH3-SH2 did indeed block Eg2 phosphorylation (Fig. 11B). Therefore, the inhibitory RasGAP fragments act upstream of Eg2 to modulate ERK2 activation and G_2/M transition.

Discussion

The investigation of the impact of fragments and domains of RasGAP on FGFR complex composition performed in the present work provides important information on the function of RasGAP during FGF1 signaling as well as the signaling properties of the caspase-3-generated N and N2 fragments. Fig. 12 summarizes schematically the main findings of our study. RasGAP was recruited to the FGFR complex with the same kinetics as the adapter Grb2 (15 min after FGF1 stimulation), although it did not depend on the presence of Grb2. Binding between RasGAP and Akt has been reported (49), and indeed after Ras-

GAP recruitment, Akt was also recruited from the cytosol into the FGFR complex. Fragment N was targeted to membranes; however, this fragment, presumably because of an intermolecular association between its SH3 and PP domains, could neither associate with the FGFR complex after activation by FGF1 nor displace full-length RasGAP or Akt from FGFR complexes.

In contrast, fragment N2 was able to displace RasGAP from the FGFR complex. It also inhibited Akt incorporation into the complex, although Akt remained membrane-bound. Blocking the PH domain of Akt prevented the incorporation of Akt, but not of RasGAP, into the FGFR complex. These results suggest that it is RasGAP that recruits Akt into the FGFR complex and not the other way around.

Phosphorylation of both ERK2 and Akt was induced after stimulation with insulin or FGF1. This phosphorylation pattern was differently affected by fragment N or fragment N2. Akt phosphorylation on the PDK1 site (threonine 308) was unaffected by the RasGAP fragments. Phosphorylation of Akt on serine 473, the site used by the mTORC2 complex, also remained unaffected by fragment N. On the contrary, fragment N2-like constructs or the first SH2 domain were found to potently inhibit FGF1-stimulated, but not insulin-mediated, phosphorylation of Akt on serine 473. Akt phosphorylation on both the 473 and 308 sites was reported to be critical for Akt activity (50). Although Akt phosphorylation on Thr-308 alone may be sufficient to activate Akt in some instances (51, 52), stronger Akt activity can be generated by additional phosphorylation on Ser-473 (14) and possibly other sites (53). In our heterologous system, however, Akt phosphorylation on serine 473 appears dispensable for the proliferative response downstream of FGFR activation because inhibition of FGF1-stimulated Akt Ser-473 phosphorylation with anti-Sin1 antibodies did not result in the blockage of G_2/M transition.

Fragment N was found to accelerate by 3 h 30 min ERK2 phosphorylation induced by FGF1 or insulin. This fragment does not need to be incorporated in receptor tyrosine kinase signaling complexes to exert its effects because it is not recruited in the FGFR complex (Fig. 5, B and C). However, it was targeted to membranes, where it can obviously regulate signaling originating from cell surface receptors. Fragment N accelerated ERK2 phosphorylation in cells stimulated with either insulin or FGF, suggesting that it did not directly act through the FGFR complex to modulate downstream signaling. Together, these observations strongly suggest that fragment N acts on activated signaling effectors that are not in the receptor tyrosine kinase complexes themselves but instead downstream in the signaling cascade. Ras is a good candidate in this context because its activity is positively modulated by fragment N during both FGFR and insulin signaling. The accelerated ERK2 phosphorylation led to an earlier G_2/M transition in the oocyte system. Surprisingly, Mos synthesis, an upstream event involved in ERK2 phosphorylation and G_2/M transition (24, 54), was delayed (Fig. 11). We have no explanation at the present time for this discrepancy.

In stark contrast to fragment N, fragment N2 hastened Ras inactivation following FGFR stimulation, impairing ERK2 phosphorylation and the G_2/M transition. The effects of fragment N2 were specific to FGFR signaling because it did not

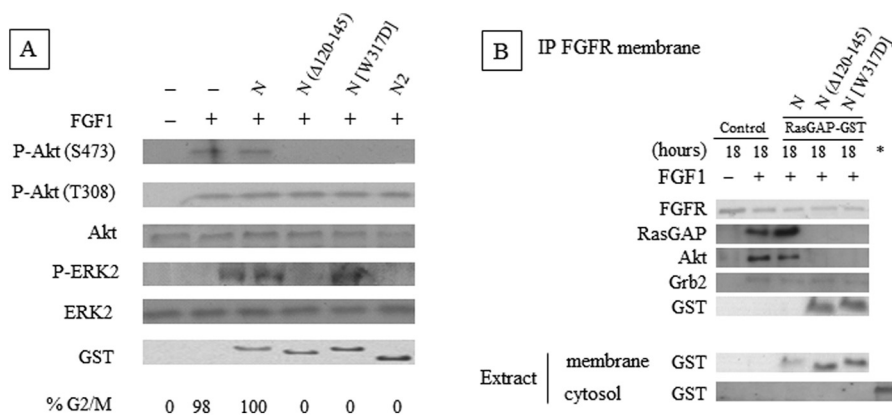


FIGURE 7. Intramolecular PP and SH3 domains of fragment N inhibits recruitment to FGF receptor complexes. Twenty ng of plasmids coding for GST-tagged fragment N, its polyproline N(Δ 120–145) and SH3 N(W317D) mutants, or fragment N2 were microinjected or not in the nucleus of oocytes expressing FGFR(MDA) for 36 h and stimulated later with FGF1 for 18 h. *A*, oocytes were scored for G₂/M transition and prepared for SDS-PAGE and Western blot analysis using the indicated antibodies. *B*, whole membrane extracts or whole cytosolic fractions were immunoprecipitated (IP) with anti-FGFR antibodies and analyzed by Western blotting using the indicated antibodies. Non-immunoprecipitated extracts were also analyzed for the presence of GST-tagged constructs. In the condition where no signal was detected, a positive control (GST) is included (indicated by the asterisk). The G₂/M transition was assessed by the appearance of a GVBD at the animal pole.

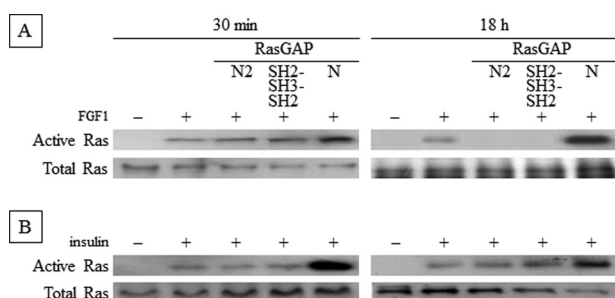


FIGURE 8. Fragment N enhances FGF1-mediated Ras activity. Oocytes expressing FGFR(MDA) for 48 h (*A*) or naive oocytes (*B*) were microinjected with the indicated RasGAP-derived fragments and then treated (+) or not (–) with (5 mM FGF1 (*A*)) or 10 μ M insulin (*B*)). Total protein extracts were collected 30 min and 18 h post-stimulation. Total Ras and pulled down active Ras proteins were visualized by Western blot.

impact insulin signaling. Fragment N2 most likely mediated its effects by competing with endogenous full-length RasGAP, avoiding its incorporation in the FGFR complex, whereas the insulin receptor does not associate with RasGAP. Either fragment N2 or the SH3 domain of RasGAP (but not full-length RasGAP) blocked Mos protein synthesis and the activation of its upstream activator Aurora kinase A, a prerequisite for downstream ERK2 phosphorylation and activation in *Xenopus* oocytes. It is unlikely that this is a consequence of its ability to prevent Akt phosphorylation because Akt phosphorylation on Ser-473 can be dissociated from Mos synthesis, ERK activation, and G₂/M transition. Rather, our data suggest that the ability of fragment N2 to prevent G₂/M transition most likely resides in its capacity to inhibit targets upstream Aurora A (Eg2) phosphorylation, Mos synthesis, and ERK activation (38, 55). Aurora A has been described as an interactor of the RasGAP SH3 domain, and it has been proposed to be inhibited by GSK3 (47, 56), which itself is inhibited by Akt. A hypothetical explanation for the role of Akt could be that it acts as a positive regulator of Aurora A and thus allows ERK2 phosphorylation. The simultaneous interaction of RasGAP with Akt and Aurora A, in the context of the FGFR complex, could enhance such positive regulation.

There have been conflicting reports regarding the interaction between RasGAP and FGFR. RasGAP has been shown to be present and tyrosine-phosphorylated in FGFR1 complexes in *Xenopus* oocytes and embryos (20, 24). RasGAP is recruited by activated FGFRs in *Drosophila* and regulates the signaling intensity of the Ras and ERK MAPK pathways (19). The interaction of RasGAP with FGFR appears dependent on the N-terminal SH2 domain of RasGAP because this domain is recruited into the FGFR complex, where it interferes with the binding of endogenous RasGAP. This was specific for this particular SH2 domain because the SH2 domain of Grb2, while being able to block the binding of Grb2 to the FGFR complex, did not abrogate the RasGAP-FGFR interaction. We show here that a single isolated RasGAP-derived SH2 domain is able to induce this displacement; however, it remains to be resolved whether, in the context of the whole N2 fragment, the displacement depends on a single SH2 domain.

Displacement of RasGAP from the FGFR complex specifically impaired Akt Ser-473 phosphorylation. Therefore, it was of interest to determine whether Akt domains could alter the binding of one of the partners in the FGFR complex. The classical function of PH domains is to interact with phosphorylated lipids in the membrane (57, 58). It is known that Akt can be recruited to the plasma membrane upon receptor tyrosine kinase activation (59), but whether the subsequent incorporation in signaling complexes such as the FGFR complex also required the PH domain is currently not known. Our results indicate that the PH domain of Akt is critical for the anchorage of Akt to the RasGAP-FGFR complex and for the phosphorylation of Akt on residues 473 and 308.

An important finding of our study is that full-length RasGAP facilitates Akt activation by shielding it, within a receptor tyrosine kinase signaling complex, from serine 473-targeted phosphatases. This was revealed through the use of RasGAP fragments, such as N2, that competed with the full-length protein for binding to the FGFR. Fragment N2 prevented the phosphorylation of Akt on serine 473 (the mTORC2 site) but not phosphorylation on threonine 308 (the PDK1 site). Several phospho-

Impact of RasGAP on FGF Signaling

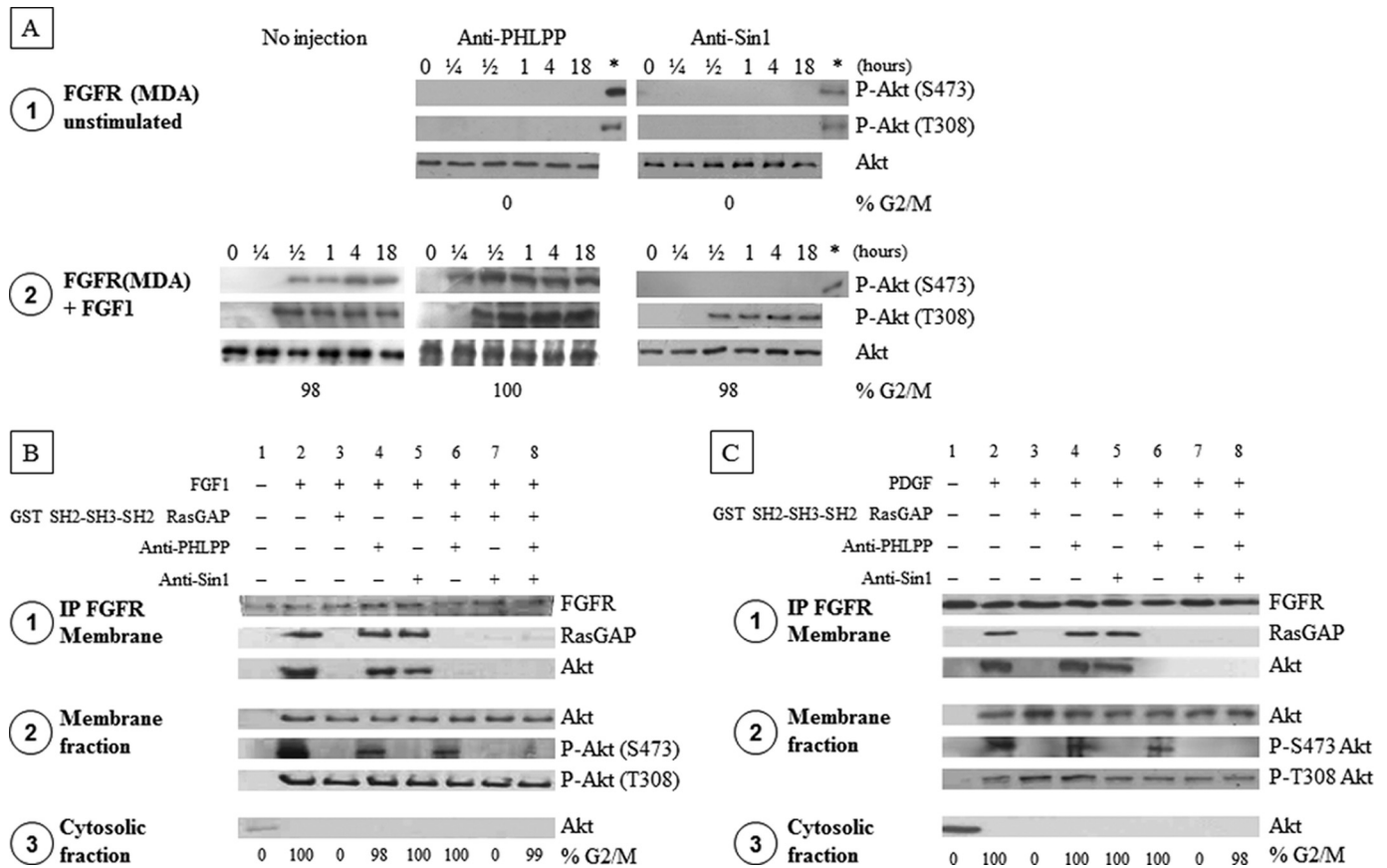


FIGURE 9. Anti-PHLPP antibodies abrogate fragment N2-mediated inhibition of Akt Ser-473 phosphorylation in oocytes expressing FGFR. *A*, anti-PHLPP and anti-Sin1 antibodies were injected into oocytes expressing FGFR(MDA). They were then stimulated or not with 5 nM FGF1, and samples were collected after 15 min, 30 min, 1 h, 4 h, and 18 h of treatment. *B*, anti-PHLPP and anti-Sin1 antibodies or SH2-SH3-SH2 RasGAP-GST were microinjected into oocytes expressing FGFR(MDA) 1 h before stimulation with 5 nM FGF1. *C*, oocytes expressing a chimeric FGFR1 receptor (composed of the extracellular part of the human PDGF receptor and the intracellular part of the human FGFR1 receptor) were treated as in *B* and stimulated with 5 nM PDGF. Total cellular extracts (*A*), FGF receptor-immunoprecipitated extracts (*B1* and *C1*), whole membrane fractions (*B2* and *C2*), and cytosolic fractions (*B3* and *C3*) were analyzed by Western blotting using the indicated antibodies. The G₂/M transition was scored for each condition. In conditions where no signals are detected, positive controls (FGF(MDA) receptors stimulated with FGF1 for 18 h) are included (indicated by asterisks). The G₂/M transition was assessed by the appearance of a GVBD at the animal pole.

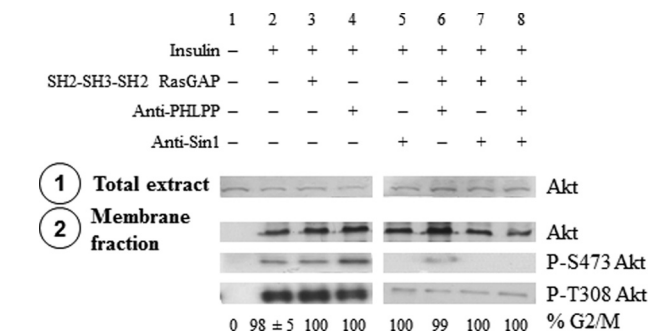


FIGURE 10. The SH2-SH3-SH2 RasGAP fragment does not inhibit phosphorylation of Akt Ser-473 in insulin-stimulated oocytes. Naive oocytes were microinjected with the SH2-SH3-SH2 construct of RasGAP alone and together with the indicated combinations of anti-PHLPP and anti-Sin1 antibodies. The oocytes were then stimulated with 10 μM insulin. Total extract (*1*) and membrane fractions (*2*) were analyzed by Western blot alone or with the indicated antibodies. The G₂/M transition was assessed by the appearance of a GVBD at the animal pole.

tases have been involved in Ser-473 dephosphorylation, including the PHLPP phosphatase (60–62). The use of an antibody against PHLPP showed that mTORC2-dependent Akt Ser-473 phosphorylation was preserved in FGF1-stimulated oocytes expressing fragment N2. This indicates that PHLPP

phosphatases are mediating the dephosphorylation of Akt on serine 473 when full-length RasGAP is displaced. One possibility is that fragment N2 facilitates the action of PHLPP within the FGFR complex. However, this does not seem to be the case because fragment N2 disrupts the association of Akt with the FGFR complex. Despite the fact that it does not associate with the FGFR complex in the presence of fragment N2, Akt is nevertheless targeted to membranes, where it can be phosphorylated by PDK1 upon FGF1 stimulation and by mTORC2, in the latter case only when PHLPP is inhibited. It can therefore be concluded that fragment N2 favors the dephosphorylation of Akt on serine 473 by PHLPP by removing Akt from the protective RasGAP-dependent environment within the FGFR signaling complex.

Our study also provides information on how the caspase-3-generated RasGAP fragments modulate receptor tyrosine kinase signaling, which can explain some of their respective cellular functions. Fragment N is known to exert potent survival functions that depend on the activation of the Ras/PI3K/Akt pathway. Previous work has shown that fragment N expression in mammalian cell lines leads to Ras and Akt stimulation (23, 26, 63). Using the *Xenopus* oocyte, it was found that fragment N

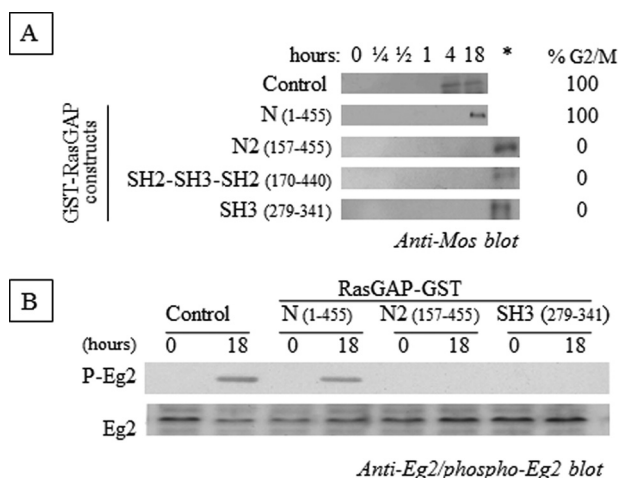


FIGURE 11. Effect of RasGAP-derived fragments on Mos synthesis and Eg2 phosphorylation. *A*, FGFR(MDA) was expressed in oocytes 48 h before injection of various RasGAP constructs (N, N2, SH2-SH3-SH2, or SH3) 1 h before stimulation with 5 nM FGF1. Total proteins were extracted at the indicated time and analyzed by Western blot analysis using anti-Mos antibodies. The G₂/M transition was assessed by the appearance of a GVBD at the animal pole. In conditions where no signals are detected, positive controls (FGF(MDA) receptors stimulated with FGF1 for 18 h) are included (indicated by asterisks). *B*, alternatively, FGFR(MDA)-expressing oocytes were microinjected with the indicated constructs and then stimulated with FGF1 as in *A* for the indicated periods of time. The samples were then analyzed by Western blotting using total and phospho-Eg2-specific antibodies.

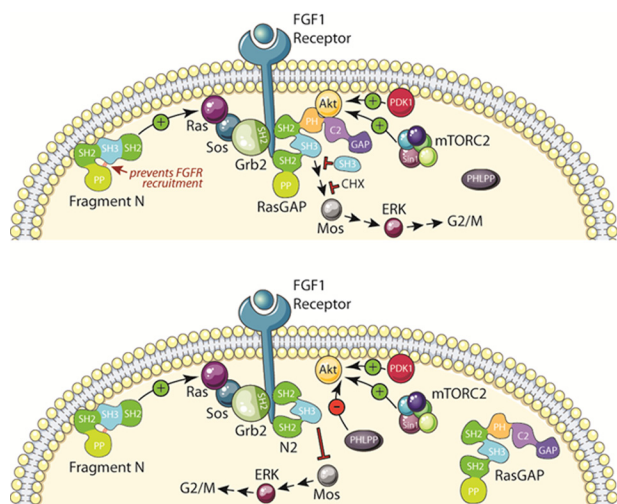


FIGURE 12. Schematic representation of the impact of full-length RasGAP and its caspase-3-generated fragments on FGFR1 signaling. *Top*, binding of full-length RasGAP to the FGF1 receptor is required for Akt to be incorporated in the FGFR signaling complex. In this condition, full-length RasGAP prevents PHLPP phosphatases from accessing Akt, which then becomes fully activated as a result of its phosphorylation on Thr-308 (PDK1 site) and Ser-473 (mTORC2 site). Full-length RasGAP also mediates FGF1-induced Mos-dependent ERK activation and cell cycle progression. Fragment N does not bind to the FGFR but contributes to Ras activation and ERK stimulation. The *three thin lines* connecting the PP and the SH3 domains of fragment N indicate a putative *cis* interaction, which would affect the accessibility of its SH2 domains for binding to FGF1 receptors. *Bottom*, fragment N2 binds to the FGFR, displacing full-length RasGAP away from the FGFR complex. Akt is no longer shielded from PHLPP phosphatases that now counteract phosphorylation on the mTORC2 site. As a result, Akt activity is inhibited. Fragment N2 is blocking Mos signaling.

alone could not activate Akt on its own. However, fragment N strongly potentiated FGF1-induced Ras stimulation in terms of intensity and more particularly so in terms of persistence. Besides the possibility of a species-dependent specificity, this

could be related to the fact that oocytes are cell cycle-arrested, whereas the mammalian cells used in previous studies are tumor cells continuously proliferating, undergoing tonic activation of growth factor and/or oncogene-dependent signaling. Based on the present finding, one could predict that inhibition of those tonic signals would prevent fragment N from favoring the activation of Akt in mammalian cells. Fragment N would therefore be unable to protect cells that are not concomitantly activating pathways that stimulate Akt to some extent.

Fragment N2 and fragment N2-derived peptides sensitize tumor cells to anti-cancer drugs (28, 64–66), but the underlying mechanisms remain obscure (67, 68). Here we show that fragment N2 potentially inhibits FGF1-induced phosphorylation of ERK2 and Akt. Because both Akt and ERK2 participate in cell survival signaling (69), one possibility to explain the capacity of fragment N2 to sensitize tumor cells to anti-cancer treatment is a shutdown of pro-survival pathways. There is, however, no evidence for such mechanisms in mammalian cells. Indeed, neither the ERK MAPK pathway nor Akt is modulated by the tumor-sensitizing fragment N2-derived peptides in tumor cell lines (41). Hence, although fragment N2 has the potential to inhibit growth factor-mediated pro-survival signaling, as shown here for FGF1, it is unlikely that this is the main mechanism underlying its broad anti-tumoral activity. Nevertheless, because dysregulation in Akt signaling is found in a majority of cancer (9), the use of a fragment N2-like compound could be particularly useful to target RasGAP in receptor tyrosine kinase signaling complex in order to render Akt accessible to inactivating phosphatases.

Author Contributions—K. C. and E. B. designed, performed, analyzed, conceived, and coordinated the study and wrote the paper. A. L. provided technical assistance and performed the experiments. C. W. and A. C. M. conceived and coordinated the study and wrote the paper. A. C. M. designed and constructed the plasmids. A. F. B. analyzed and conceived the study. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Dr. C. Garbay (Université Paris Des-cartes), Dr. S. Courtneidge (Sanford/Burnham Medical Research Institute, La Jolla, CA), Prof. D. Boujard (Université de Rennes), and Dr. C. Dissous (Institut Pasteur, Lille) for the gift of GST fusion proteins.

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RasGAP Shields Akt from Deactivating Phosphatases in Fibroblast Growth Factor Signaling but Loses This Ability Once Cleaved by Caspase-3

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J. Biol. Chem. 2015, 290:19653-19665.

doi: 10.1074/jbc.M115.644633 originally published online June 24, 2015

Access the most updated version of this article at doi: [10.1074/jbc.M115.644633](https://doi.org/10.1074/jbc.M115.644633)

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