RasGAP is involved in signal transduction triggered by FGF1 in *Xenopus* oocytes expressing FGFR1

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Abstract The role of RasGAP was investigated in the model system of *Xenopus* oocytes expressing fibroblast growth factor receptor 1 (FGFR1) stimulated by fibroblast growth factor 1 (FGF1). The injection of the SH2-SH3-SH2 domains of RasGAP suppressed Ras activity, extracellular signal-regulated protein kinase 2 (ERK2) phosphorylation and Mos synthesis. The SH2 domain of Src, and PP2, an inhibitor of Src, also abolished Ras activity, ERK2 phosphorylation and Mos synthesis. In addition, Src activity was blocked by the SH2-SH3-SH2 domains of RasGAP. Immunoprecipitation of a chimera composed of the extracellular domain of the platelet-derived growth factor (PDGF) receptor and the intracellular domain of FGFR1 stimulated by PDGF-BB demonstrates the recruitment of phosphorylated RasGAP. This study shows that the transduction cascade induced by the FGFR1–FGF1 interaction in *Xenopus* oocytes involves RasGAP as a co-activator of Src to stimulate the Ras/mitogen-activated protein kinase cascade and Mos synthesis. It emphasises a new positive regulatory role for RasGAP in FGFR transduction. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: RasGAP; Fibroblast growth factor receptor 1; Extracellular signal-regulated protein kinase 2; Mos; *Xenopus laevis* oocyte

1. Introduction

Fibroblast growth factors (FGFs) exert pleiotropic effects including cell growth and differentiation. Their responses are triggered by the activation of four structurally related transmembrane tyrosine kinase receptors (FGFRs) [1–3]. Under ligand stimulation FGFRs transphosphorylate each other and elicit a pattern of signal transduction dependent on or independent of Ras [4–6]. Molecular effectors of the Ras-dependent pathway are the adapter proteins Shc, FRS2 and Grb2 [7,8]. Grb2 is recruited to the membrane in complex with the Ras-activating nucleotide exchange factor SOS which in turn activates Ras. Ras is a prototype of a superfamily of conserved proteins that act as molecular switches and trigger the activation of mitogen-activated protein kinase (MAPK). Ras activity is controlled by its bound nucleotide with the GTP form being the active form competent for cellular signaling. Ras deactivation is ensured by GTPase-activating proteins (GAPs) which greatly speed up GTP hydrolysis [9,10]. RasGAP is a major regulator of cellular Ras activity through its carboxy-terminal domain that contains the catalytic domain which bind Ras-GTP and accelerates GTP hydrolysis. RasGAP possesses one SH3 domain flanked by two SH2 domains in the amino-terminal region that allows its interaction with tyrosine kinase receptors and signaling proteins [11,12]. First thought of as merely a downregulator of Ras in tyrosine kinase receptor transduction, RasGAP turns out to have some intrinsic effector function and to mediate some of the biological effects of Ras [13–17]. Though RasGAP function has become clearer for many receptor tyrosine kinases (RTKs) [18–20], its involvement in FGFR transduction is poorly understood. A report mentioned RasGAP was phosphorylated and co-immunoprecipitated with FGFR1 in *Xenopus* blastula. Also RasGAP could behave as a main effector during *Xenopus* development [6]. Others reports on cell lines showed that RasGAP was not associated with FGFR1 and did not participate in its transduction cascade [21].

To get further insight into the function played by RasGAP in FGFR1 signaling, we used a model system devoid of FGFRs, *Xenopus* oocytes, where FGFRs can be expressed and stimulated by exogenous FGFs [22–24]. *Xenopus* oocytes are physiologically arrested at the G2 stage of the first meiosis prophase. They serve as a convenient model system to study transduction cascades initiated by progesterone [25] and factors such as insulin-like growth factor (IGF) [26,27] or FGF [24]. Their binding to appropriate receptors induces the entry of oocytes into the M phase, which leads to germinal vesicle breakdown (GVBD), used as an indicator of their meiosis reinitiation. The transduction cascade involved in meiosis reinitiation includes a protein kinase A (PKA)-dependent pathway after progesterone stimulation and a Ras/MAPK-dependent pathway after growth factor addition [27].

In the present study, we analyse the relevance of RasGAP in the Ras/MAPK transduction cascade and Mos protein synthesis triggered by FGF1 in *Xenopus* oocytes expressing FGFR1.

2. Materials and methods

2.1. Oocytes

After anaesthesia with MS 222 (1 g/l, Sandoz), *Xenopus laevis* ovarian fragments were surgically removed and placed in ND96 medium (in mM: NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, 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) [23,24]. Stage VI oocytes were harvested by 1 h treatment with collagenase A (1 mg/ml, Boehringer

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Mannheim). Complete defolliculation of the oocytes was achieved by manual dissection. The oocytes were kept at 19°C in the ND96 medium.

2.2. cRNA and fusion protein preparation

FGFR1 and a plasmid-derived growth factor receptor (PDGFR)–FGFR1 chimera inserted into vector pSP64T [28] were used to generate capped cRNAs (mMESSAGE mMACHINE kit, Ambion). The SH2-SH3-SH2 domains of RasGAP and the SH2 domain of Src [15] were expressed in *Escherichia coli*. The production of glutathione *S*-transferase (GST) proteins was induced by addition of 0.1 mM isopropyl thio-β-D-galactoside for 3 h. Cells were centrifuged and lysed by sonication in the following solution: 0.01 M phosphate buffer pH 7.5, containing 0.15 M NaCl, 100 mM EDTA, 1% Triton X-100, 1 mM aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF). After addition of 1% Triton X-100 the lysate was centrifuged at $10\,000\times g$. GST proteins were bound to glutathione-Sepharose 4B (Pharmacia), washed four times, eluted with 20 mM glutathione and concentrated using Centricon 3 filters (Amicon).

2.3. Microinjection, drug treatment of oocytes and GVBD analysis

Microinjection were performed with 60 ng of FGFR1 or PDGFR–FGFR1 cRNAs and 150 ng of SH2-SH3-SH2 of RasGAP or 100 ng of SH2 of Src in the equatorial region of the oocyte. The SH2-SH3-SH2 of RasGAP and the SH2 of Src were injected 2 h before addition of FGF1 (5 nM, R&D system, UK), progesterone (2 μg/ml) or insulin (1 μM, Sigma) to the oocytes. PP2, an inhibitor of Src (10 μM) (Calbiochem), was added to the medium 2 h before FGF1 treatment.

GVBD was determined by the appearance of a white spot at the centre of the animal pole. Student's *t*-test was used to assess the significance of the observed differences. For each experiments, 20–30 oocytes were removed from different animals (n = number of animals).

2.4. Immunoprecipitation of RasGAP

Thirty oocytes expressing PDGFR–FGFR1 for 40 h were stimulated or not with PDGF-BB (5 nM) for 5 min. These oocytes were lysed in buffer A: 50 mM HEPES, pH 7.4, 1% Triton X-100, 500 mM NaCl, 0.05% SDS, 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin (BSA), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, 1 mM PMSF, 1 mM sodium vanadate. After centrifugation, $10\,000\times g$ for 5 min at 4°C, the supernatants were incubated with an anti-PDGFR antibody (1/500, Transduction Laboratories) at 4°C for 2 h. Protein A-agarose (50%, Transduction Laboratories) was added for 1 h at 4°C. The immune complexes were collected by centrifugation, rinsed three times, resuspended in Laemmli sample buffer, and subjected to 7.5% SDS–PAGE.

2.5. Electrophoresis and Western blot analysis

For extracellular signal-regulated protein kinase 2 (ERK2) phosphorylation and Mos synthesis analysis, oocytes were homogenised in ice-cold buffer as previously described [29]. Electrophoresis was performed on 15% modified polyacrylamide gels (30% acrylamide and 0.2% bisacrylamide). Proteins were transferred to a Hybond ECL membrane (Amersham life Sciences) in Tris/NaCl/Tween/BSA pH 8 (15 mM Tris–HCl, 150 mM NaCl, 0.1% Tween, 10% BSA, Sigma). The membranes were incubated with anti-ERK2 (1/2500, Santa Cruz Biotechnology) for 1 h or with an anti-Mos^{Xe} (1/500, Upstate Biotechnology) overnight. For RasGAP analysis, the membranes were incubated with an anti-RasGAP antibody for 1 h (1/750, Transduction Laboratories), stripped and reblotted overnight with an anti-phosphotyrosine antibody (1/1000, Transduction Laboratories). Antibody complexes were detected by the enhanced chemoluminescence Western blotting detection system (Amersham).

2.6. Src assay

Oocytes expressing FGFR1 were lysed 5 min after FGF1 addition at 4°C in buffer A. The material was harvested, centrifuged at $10\,000\times g$ for 15 min at 4°C and 200 μl of oocyte supernatant was immunoprecipitated with anti-Src (5 μg/μl, Santa Cruz) for 1.5 h at 4°C followed by addition of 40 μl of protein A-agarose (50%, Transduction Laboratories) agitated for 1.5 h at 4°C. The immune complexes were collected by centrifugation and rinsed three times with buffer A. Src immunoprecipitates were incubated with 2 μg of acid-denatured enolase (Sigma), 20 mM HEPES pH 7.2, 10 mM $MnCl_2$, 20 μg/ml aprotinin and 20 μCi of [γ-^{32}P]ATP (2 μM) in a total reaction volume of 20 μl. The reaction was stopped after 10 min at 30°C by adding Laemmli buffer. Samples were boiled for 2 min and subjected to electrophoresis and autoradiography.

2.7. Ras assay

Oocytes expressing FGFR1 for 36 h were incubated at 19°C in ND96 with 0.5 mCi/ml [^{32}P]orthophosphate (NEM). They were lysed in buffer A 5 min after FGF1 addition. After centrifugation, immunoprecipitation was carried out on supernatants by addition of anti-v-H-Ras (Y13-259, Calbiochem) coupled to CNBr-activated Sepharose 4B, 3 h under rotation at 4°C, as previously described [24]. The beads were washed with buffer A. Elution was carried out for 20 min at 65°C with: 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM GTP, 0.5 mM GDP. The supernatants were spotted onto a polyethyleneimine cellulose plate and chromatography was carried out with 1 M KH_2PO_4 at pH 3.4. GTP and GDP were analysed using a UV lamp and the ratio of GTP to GDP was determined by quantification with a phosphoimager. The results are expressed as the percentage of GTP relative to total GTP plus GDP detected. Each column represents the average ± S.E.M. from two animals (20 oocytes per animal).

3. Results

3.1. RasGAP is involved in RTK-induced GVBD

The external addition of FGF1 (5 nM) to oocytes expressing FGFR1 induced 85.2 ± 14.9% (n = 14) of GVBD which took place 15–24 h after FGF1 addition. The oocyte meiosis reinitiation was specific for FGF1R activation since oocytes expressing FGFR1 unstimulated or treated with genistein (10 μM, an inhibitor of tyrosine kinase activity) or naive oocytes injected with water 2 h prior to FGF1 stimulation never underwent GVBD. When oocytes expressing FGFR1 were treated with SH2-SH3-SH2 of RasGAP (150 ng) 2 h prior to FGF1 addition, GVBD never occurred (Fig. 1).

Xenopus oocytes express endogenous IGF1R. Insulin treatment (10 μM) of naive oocytes induced GVBD (97.3 ± 4.6%, n = 3) after the same delay as oocytes expressing FGFR1 stimulated with FGF1. The same inhibition of the GVBD was detected when SH2-SH3-SH2 of RasGAP (150 ng) were microinjected 2 h before insulin treatment (Fig. 1).

Addition of progesterone, the natural inducer, in immature oocytes induced GVBD (83.4 ± 7.4%, n = 3) with a shorter delay, 8–15 h. In contrast, SH2-SH3-SH2 of RasGAP were not able to lower the percentage of GVBD (Fig. 1).

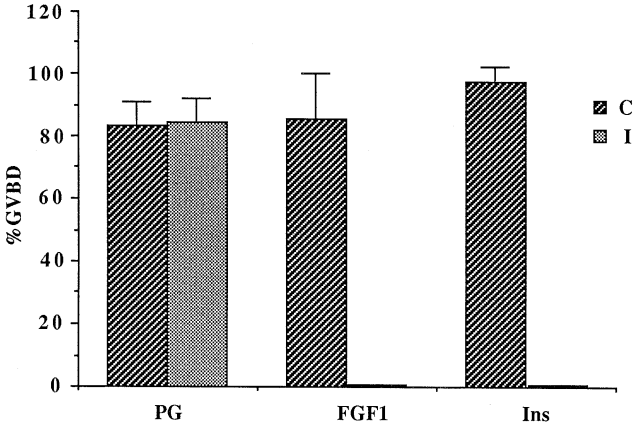

Fig. 1. Percentage of GVBD observed after 12–15 h in immature *Xenopus* oocytes stimulated with progesterone 2 μg/ml (PG) or insulin 1 μM (Ins) or in oocytes expressing FGFR1 (60 ng) stimulated with FGF1 (5 nM). Two hours before stimulation, the oocytes were injected with SH2-SH3-SH2 of RasGAP (150 ng) (I) or not (C).

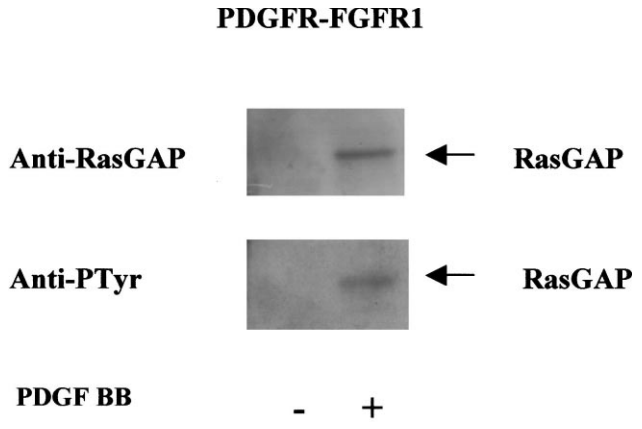

Fig. 2. Recruitment of RasGAP by PDGFR–FGFR1. Oocytes expressing PDGFR–FGFR1 were stimulated (+) or not (−) with PDGF-BB for 5 min and immunoprecipitated with an anti-PDGFR. After SDS–PAGE, Western blotting was performed with an anti-RasGAP followed by an anti-phosphotyrosine antibody.

3.2. RasGAP is recruited by the PDGFR–FGFR1 chimera

A chimera composed of the extracellular domain of PDGFR and the intracellular domain of FGFR1 was expressed in oocytes. No differences in GVBD percentage were observed between FGFR1 stimulated with FGF1 (5 nM) and PDGFR–FGFR1 activated by PDGF-BB (5 nM), as previously shown [24]. Using an antibody against RasGAP, we demonstrated that RasGAP is immunoprecipitated with PDGFR–FGFR1, while it is not in unstimulated controls. RasGAP was phosphorylated on tyrosine only when oocytes were stimulated with PDGF-BB (Fig. 2).

3.3. RasGAP is involved in Ras/ERK2 activation and Mos synthesis

FGF1 stimulation of oocytes expressing FGFR1 (Fig. 3A), insulin treatment of naive oocytes (Fig. 3B), or progesterone addition to naive oocytes (Fig. 3C) induced phosphorylation of ERK2 and synthesis of Mos.

No ERK2 phosphorylation and no Mos synthesis were detected in oocytes expressing FGFR1, and in insulin-treated oocytes that had received SH2-SH3-SH2 of RasGAP prior to stimulation with FGF1 (Fig. 3A,B). In contrast, SH2-SH3-SH2 of RasGAP allowed ERK2 phosphorylation and Mos synthesis in progesterone-treated oocytes (Fig. 3C).

In addition, Ras activity in oocytes expressing FGFR1 treated with FGF1 was lowered significantly from 41.4 ± 0.07% to 21.3 ± 0.3% in oocytes treated with SH2-SH3-SH2 of RasGAP (Fig. 4).

3.4. Src kinase is a target for RasGAP in FGFR1 transduction

FGFR1 oocytes stimulated with FGF1 2 h after their treatment with SH2 of Src (100 ng) or PP2 (10 μM), an inhibitor of Src activity, displayed no GVBD, no ERK2 phosphorylation and no synthesis of Mos (Fig. 5). As control, GVBD, ERK2 phosphorylation and Mos synthesis of oocytes treated with progesterone were not abolished by PP2 addition.

Src activity, present in oocytes expressing FGFR1 stimulated with FGF1, was suppressed by injection of SH2-SH3-SH2 of RasGAP (Fig. 6).

4. Discussion

In the present study, the role of RasGAP was investigated in FGFR1 transduction. For this purpose, oocytes expressing FGFR1 were injected with SH2-SH3-SH2 of RasGAP devoid of its catalytic domain. This approach uses a competition which inhibits the association of the endogenous RasGAP with FGFR1 and other signaling effectors. GVBD was first scored to determine the effect of the RasGAP competitor. Meiosis was reinitiated in oocytes expressing FGFR1 stimulated with FGF1, as previously demonstrated [24], and also in naive oocytes stimulated with insulin or progesterone. Our results show that only the transduction pathways triggered by the two tyrosine kinase receptors were sensitive to the injection of SH2-SH3-SH2 of RasGAP. It is known that these receptors transduce messages through a Ras-dependent pathway, while the progesterone receptor involves a PKA-depen-

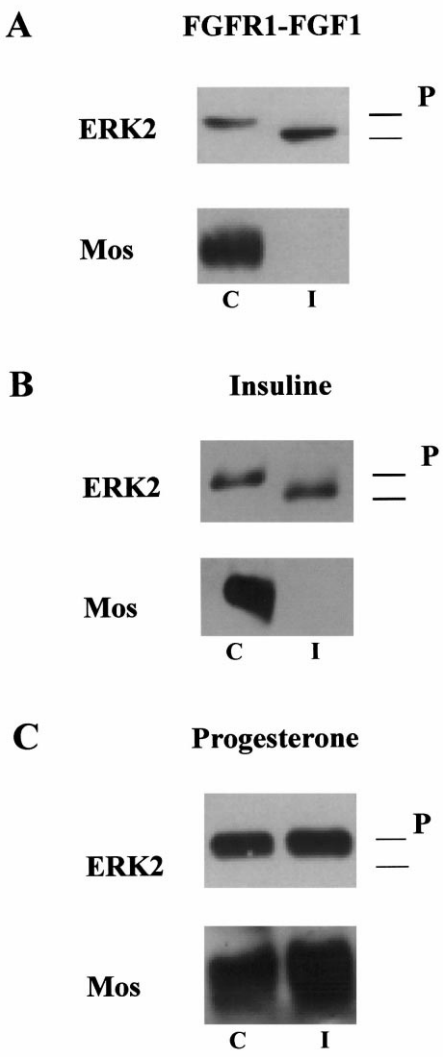

Fig. 3. Western blot analysis of ERK2 phosphorylation state (-P) and Mos synthesis. A: Oocytes expressing FGFR1 were stimulated with FGF1 (5 nM) (C) and injected with SH2-SH3-SH2 of RasGAP (I). B: Oocytes were stimulated with insulin 1 μM (C) and injected with SH2-SH3-SH2 of RasGAP (I). C: Oocytes were treated by progesterone 2 μg/ml (C) and injected with SH2-SH3-SH2s of RasGAP (I). The injection of SH2-SH3-SH2s of RasGAP (150 ng) was carried out 2 h before stimulation.

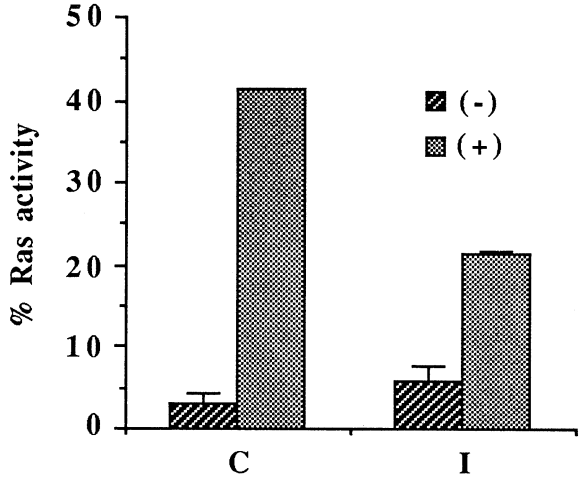

Fig. 4. Percentage of Ras activity (GTP/GDP+GTP) observed in oocytes expressing FGFR1 (60 ng) unstimulated (−) or stimulated with FGF1 (+). Oocytes were injected with SH2-SH3-SH2 of RasGAP (I) 2 h before FGF1 application. Average ± S.D. of two independent experiments.

dent pathway [27]. The lack of effect of SH2-SH3-SH2 of RasGAP on progesterone-stimulated oocytes is in agreement with its way of transducing messages in oocytes and attests to the specific effect of the RasGAP domain used. This further allowed us to dissect the role of RasGAP in the transduction cascade triggered by FGFR1–FGF1.

It is known that progesterone and insulin stimulation of *Xenopus* oocytes activate the Ras/MAPK cascade and Mos synthesis [30,31]. We have seen that oocytes expressing FGFR1, injected with SH2-SH3-SH2 of RasGAP, display a decrease in Ras activity. Moreover, in these expressing oocytes, ERK2 phosphorylation and Mos synthesis are blocked. The same effect can be seen for IGFR1-stimulated oocytes, while progesterone-stimulated oocytes were not sensitive to this injection. The involvement of RasGAP in the insulin transduction cascade was previously observed using a modified peptide from the catalytic domain of RasGAP, which inhibits GAP-stimulated RasGTPase activity. This peptide blocked insulin- but not progesterone-induced GVBD [32]. Other experiments performed with antibodies raised against the SH3 domain of RasGAP demonstrate the specific involvement of this domain in GVBD and Mos synthesis, after an activated form of Ras was injected in oocytes [14]. The fact that the intracellular part of the PDGF–FGFR1 chimera re-

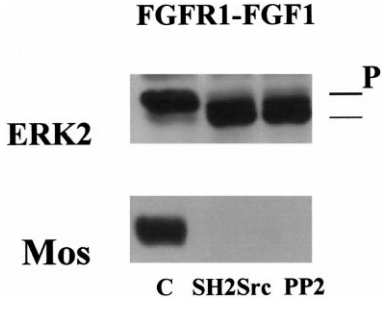

Fig. 5. Western blot analysis of ERK2 phosphorylation state (-P) and Mos synthesis in oocytes expressing FGFR1, stimulated with FGF1 (C) and injected with the SH2 domain of Src kinase (100 ng) or treated with PP2 inhibitor (10 μM) 2 h before FGF1 addition.

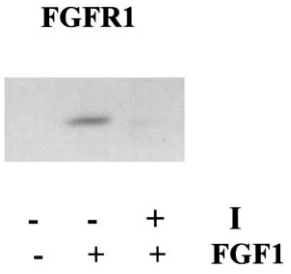

Fig. 6. Src activation of oocytes expressing FGFR1 untreated (−) or treated (+) with SH2-SH3-SH2 of RasGAP (I) 2 h before FGF1 addition. Src activity in anti-Src immunoprecipitates was measured with purified enolase and [γ-^{32}P]ATP as substrate. [^{32}P]Enolase was then subjected to electrophoresis and autoradiography.

cruits the tyrosine phosphorylated RasGAP effector supports its role in FGFR1 transduction. Others have demonstrated that tyrosine phosphorylation of RasGAP occurred after its recruitment by activated tyrosine kinase receptors, in association with a complex of various SH2-containing proteins [33,34]. RasGAP is a key element in the Ras signaling pathway initiated by tyrosine kinase receptors. It has been implicated both as a downregulator and as an effector of Ras proteins. However, its role in the Ras-mediated signal transduction pathways triggered by FGFR is unclear. Only two contradictory studies report either an interaction [6] or no interaction [35] of RasGAP with FGFR1. Our study is therefore in favour of a role for RasGAP in FGFR transduction. It suggests a novel way in which RasGAP positively regulates signals initiated by FGFR1.

We then determined how RasGAP mediates its effect. Recently, RasGAP was shown to contain binding sites for the Src family kinase [36]. In the PDGFR transduction pathway RasGAP interacts with Src to modulate PDGF mitogenic effect [18]. Furthermore, FGFR1 transduction involves Src as a mitogenic effector in somatic cells [37] and as a GVBD inducer in *Xenopus* oocytes [24]. We have shown that PP2, a Src inhibitor, and SH2 of Src blocked Ras activity, ERK2 phosphorylation and Mos synthesis, triggered by FGFR1–FGF1. According to this fact, Src is a main effector in the FGFR signaling cascade upstream of Ras in oocytes, we tested the interaction of RasGAP with Src. Using a Src kinase assay, we demonstrate Src activity is inhibited by SH2-SH3-SH2 of RasGAP. The differences in the results we obtained for ERK2 phosphorylation between SH2-SH3-SH2 of RasGAP compared to the injection of SH3 antibodies by others [14] could be explained by the specific action of RasGAP on Src activity. Effectively, in our study the transduction cascade, including RasGAP, starts from an RTK upstream of Ras while the study that uses an anti-SH3 of RasGAP only starts from activated Ras.

In conclusion, we propose that FGFR1 signaling triggered by FGF1 addition needs RasGAP as a coactivator of the Src kinase which activates ERK2 kinase and Mos synthesis. This suggests a novel role for RasGAP as a positive regulator of the FGFR1 transduction pathways.

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