Inhibition of FGF receptor signalling in Xenopus oocytes: differential effect of Grb7, Grb10 and Grb14

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Abstract The role of Grb7 adapters, Grb7, Grb10, and Grb14, was investigated in Xenopus oocytes expressing fibroblast growth factor receptors (FGFR). FGF-induced maturation of FGFR-expressing oocytes was blocked by previous injection of Grb7 or Grb14, but not Grb10. This effect correlated with Grb7/14 binding to the receptor, and inhibition of the Ras-dependent pathway. Interestingly, the phosphorylated insulin receptor interacting region (PIR) and Src 2 homology domains (SH2) of Grb7 and Grb14 were differently implicated in the inhibition of FGF signalling. This study provided further evidence for specificity of the biological action of the Grb7 adapters on receptor tyrosine kinase signalling.

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Key words: Fibroblast growth factor receptor; Grb7 family; ERK2; PI3-kinase; Xenopus oocyte; MDA-MB-231 cell line

1. Introduction

Fibroblast growth factor receptors (FGFRs) are involved in the regulation of a wide variety of cellular processes [1]. Four FGFR genes have been cloned to date, each coding for a transmembrane protein with a cytoplasmic tyrosine kinase domain. As for many growth factor receptors, binding of an appropriate ligand results in receptor dimerisation, which leads to the activation of the cytoplasmic tyrosine kinase domain [2]. Following these events, intracellular proteins are recruited by the receptor and a complex network of transduction cascades is elicited. The multiprotein complexes docked by the receptor include adapter proteins such as Shc, FGFR-substrate 2 (FRS2) and Grb2, and enzymes, such as phosphatidylinositol-3 kinase (PI3-kinase), phospholipase C gamma (PLCγ) [3–6].

The growth factor receptor-bound protein 7 (Grb7) family of adapters comprises three members, Grb7, Grb10 and Grb14, which interact with numerous receptor tyrosine kinases upon activation [7], including insulin-like growth factor-1 receptor (IGF-1R), insulin receptor (IR) [8–10], platelet-derived growth factor receptor (PDGFR) [11], epidermal growth factor receptor (EGFR) [12] and the Ret proto-oncogene [13]. Grb10 [14] and Grb14 [15] have also been shown to bind to FGFR in cell lines. Members of the Grb7 family of adapters interact with the activated tyrosine kinase domain of the receptors using their C terminal region which contains two binding domains, the PIR/BPS (for phosphorylated IR interacting region/between plextrin-homology and Src homology 2 (SH2)), and the SH2 domain. Participation of these two domains in receptor binding differs considering the various partners. The SH2 domain is involved in most interactions with receptor tyrosine kinases, at the exception of the IR-Grb14 binding [10,14–16]. In contrast, the PIR is likely to be implicated in the association between the members of the Grb7 family of proteins and the related receptors IR and IGF-1R [10,16–20]. A critical role for the PIR in the interaction with other receptors has not yet been reported.

The function of the Grb7 family members is not fully understood. Despite controversial findings, each member is likely to act as a negative regulator in several physiological processes, and is deregulated in cancers. Grb7 may have a role in kidney development [21], in breast and oesophageal carcinoma progression [22–25]. In addition, an overexpression is noticed for Grb7 in numerous breast tumours [25], and for Grb10 and Grb14 in several breast and prostate cancer cell lines [26,27]. Despite contradictory results obtained on Grb10 isoforms [14,28,29], their overexpression is consistent with an inhibitory role in insulin signalling [17,30,31]. Moreover, growing evidences are also emerging for an inhibitory role of Grb14 in insulin signalling: Grb14 inhibits IR catalytic activity, phosphorylation of its downstream targets IRS-1, Akt and ERK1/2, and insulin stimulation of DNA and glycogen synthesis [18,19].

To better understand the role played by the Grb7 family of adapters in FGF signalling, we have used a model system devoid of FGFRs, the Xenopus oocyte, where FGFRs can be expressed and stimulated by exogenous FGFs [32–34]. Xenopus oocyte offers a variety of powerful experimental approaches to question cascade transduction regulation. They are physiologically arrested at the G2 stage of the first meiosis prophase. Progesterone [35] and growth factors such as insulin [36,37] or FGF [5,32–34], when binding to appropriate receptors, induce the entry of oocytes into the M phase, which leads to a germinal vesicle breakdown (GVBD), used as an indicator of the meiosis reinitiation (maturation). The transduction cascades involved in meiosis reinitiation include a PKA-dependent pathway after progesterone stimulation, and

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Ras-dependent and -independent pathways after growth factor addition [5, 38]. The mitogen-activated protein (MAP) kinase cascade is activated in a all-or-none fashion, which allows a very sensitive detection of oocyte signalling pathways activation [39]. In the present study, we have analysed the effects of Grb7, Grb10, and Grb14 on FGFR1-induced oocyte maturation in oocytes expressing different FGFRs: FGFR1 or FGFR4 isofoms, and mRNAs from the breast cancer cell line MDA-MB-231 [40], which mainly expresses FGFR1 and FGFR4 [41]. Furthermore, we have analysed the involvement of the PIR and the SH2 domains on oocyte maturation. These results were further compared to the effect of Grb7 proteins on insulin-induced oocyte maturation, since Xenopus oocytes naturally possess receptors for insulin [38].

2. Materials and methods

2.1. Oocyte handling

After anaesthesia with MS 222 (1 g/l, Sandoz, Vienna, Austria), Xenopus laevis ovarian fragments were surgically removed and placed in ND96 medium (in mM: NaCl 96, KCl 2, MgCl₂ 1, CaCl₂ 1.8, HEPES 5, adjusted to pH 7.4 with NaOH), supplemented with streptomycin/penicillin (50 μg/ml, Eurobio, Les Ulis, France), sodium pyruvate (225 μg/ml, Sigma, Saint Quentin Fallavier, France) and soybean trypsin inhibitor (30 μg/ml, Sigma) [5.32–34]. Stage VI oocytes were harvested by using 1 h treatment with collagenase A (1 mg/ml, Roche Boehringer, Meylan, France). Complete defolliculation of the oocytes was achieved by manual dissection. The oocytes were kept at 19°C in the ND96 medium.

2.2. RNA and fusion protein preparation

FGFR1 and FGFR4 from Pleurodeles waltl, highly homologous to the human receptors [42], and the chimera composed of the human extracellular domain of the PDGF receptor and the intracellular domain of P. waltl FGFR1 (gift from Dr D.L. Shi) inserted into vector pSP64T [42] were used to generate capped cRNAs (mMESSAGE mMACHINE kit, Ambion Austin, USA). PolyA mRNAs from MDA-MB-231 breast cancer cell line were extracted by the guanidinium thiocyanate/cesium chloride gradient, using RNA plus reagent from Bioprobe followed by polydT columns (Pharmacia Biotech, Orsay, France) [40]. Grb7 adapter members, PIR, SH2, and PIR-SH2 domains were produced as glutathione S-transferase (GST) fusions as described before [10,18,19].

2.3. Microinjections, and GVBD analysis

All microinjections were performed in the equatorial region of the oocyte. Microinjection of 60 ng of FGFR mRNAs was performed 48 h before the injection of the Grb7 family members, Grb7, Grb10, or Grb14, or of their PIR, SH2, or PIR-SH2 domains. One hour after the injection of the fusion proteins, oocytes were stimulated with 5 nM FGF1. GVBD was determined by the appearance of a white spot at the centre of the animal pole, 15 h after the FGF1 treatment. Student’s t-test was used to assess the significance of the observed differences. For each experiments, 20–30 oocytes were removed from at least two animals.

2.4. Immunoprecipitation of the PDGFR–FGFR1 chimera

20 oocytes expressing for 48 h the PDGFR–FGFR1 chimera were injected with Grb7, Grb10 or Grb14 (100 ng), 1 h before stimulation by PDGF-BB (5 nM). After 5 min, these oocytes were lysed in 200 μl of buffer A: 50 mM HEPES, pH 7.4, 1% Triton X-100, 500 mM NaCl, 0.05% SDS, 5 mM MgCl₂, 1 mg/ml bovine serum albumin, 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. A centrifugation of 10000 g for 15 min at 4°C was then performed. Supernatants were incubated with anti-PDGFR antibodies (that react with the extracellular domain of the chimera, R&D system, Abingdon, UK), for 2 h at 4°C. Protein A-Sepharose (50%, BD Biosciences, Erembodegem, Belgium) was added for 1 h at 4°C. Immunocomplexes were collected by centrifugation, rinsed three times, resuspended in Laemmli sample buffer, and subjected to a 7.5% SDS-PAGE.

2.5. Electrophoresis and western blot analysis

For western blot analysis, oocytes were homogenised in ice cold buffer A as previously described and electrophoresis was performed [5]. Proteins were transferred to a Hybond ECL membrane (Amer- sham Biosciences, Freiburg, Germany) in Tris/NaCl/Tween/BSA pH 8 (15 mM Tris-HCl, 150 mM NaCl, 0.1% Tween, 10% bovine serum albumin, Sigma). To analyse the association of Grb7 members with receptors in the immunoprecipitation experiments, the membranes were first immunorevealed with anti-phosphotyrosine antibodies (BD Biosciences). The membranes were then stripped and reprobed with anti-PDGFR antibodies (BD Biosciences). The lower part of the membrane were cut and blotted with anti-Grb7 antibodies (BD Transduction Laboratories) for the oocyte samples injected with Grb7, with anti-Grb10 antibodies (BD Biosciences) for the oocyte samples injected with Grb10 or with anti-Grb14 antibodies [18] for the oocyte samples injected with Grb14.

For transduction analysis, samples were prepared as described [33,34], and electrophoresis was performed on 15% modified polyacrylamide gels (30% acrylamide, and 0.2% bisacrylamide). The membranes were incubated either with anti-ERK2 (Santa Cruz Biotechnol- ogy, Santa Cruz, USA), with anti-Raf (Santa Cruz Biotechnology), with anti-phosphorylated Akt (BD Biosciences), or with anti-β-catenin (BD Biosciences) antibodies. Antibodies complexes were detected by the enhanced chemiluminescence western blotted detection system (Amer sham).

3. Results

3.1. Effects of Grb7 family members on FGF-induced oocyte maturation

Oocytes were injected with FGFR1 or FGFR4 cRNA, or MDA-MB-231 polyA mRNA. After 48 h of expression, the addition of 5 nM FGF1 triggered meiosis reinitiation, moni- tored by GVBD observation (Fig. 1). GVBD appearance takes an average of 15 h after FGF1 addition, as previously described [5,40]. When injected with 100 ng of Grb7 or Grb14 1 h before the addition of FGF1, oocytes expressing FGFRs did not present any GVBD (Fig. 1), even 48 h after FGF1 stimulation (data not shown). In contrast, Grb10 did not block oocyte maturation. Control oocytes injected with GST.
alone (100 ng) displayed FGF1-induced maturation (Fig. 1), and Grb7 adapters had no effect on naive oocytes or on oocytes expressing FGFRs when left unstimulated. In naive oocytes, that naturally express the IR, stimulation with 1 μM insulin induced oocyte maturation, as previously described [43]. Injections of either Grb7, Grb10 or Grb14 blocked GVBD appearance (Fig. 1, right part).

3.2. Role of PIR and SH2 domains of Grb7 members

The PIR and PIR–SH2 domains of Grb7 injected in oocytes expressing FGFRs totally blocked FGF1-induced maturation, whereas the SH2 domain alone was inefficient to inhibit it (Fig. 2A). Control experiments using naive oocytes demonstrated that the PIR, the SH2 or the PIR–SH2 domains of Grb7 blocked insulin-induced GVBD. For Grb10, none of these domains had any effect on FGF1-induced GVBD, but all domains blocked insulin-induced oocyte maturation (Fig. 2B). Concerning Grb14, the PIR and the SH2 domains as well as the PIR–SH2 domain totally suppressed FGF1-induced GVBD. In contrast, only the PIR or the PIR–SH2 domains of Grb14, but not the SH2 domain, inhibited insulin-induced oocyte maturation (Fig. 2C).

We performed dose–response experiments to test the sensitivity of the inhibition of oocyte maturation by the different Grb fusion proteins. However, oocyte maturation is an all or none type of response [39]. A given amount of injected protein is or is not sufficient to block GVBD, in a similar way in all oocytes tested, avoiding a classical dose–response curve. Thus, as shown in Fig. 3, microinjection of 50 ng of Grb7 or Grb14 did not affect FGF1 induced oocyte maturation, whereas 75 ng blocked FGF1 effect in 100% of the oocytes. By comparison, microinjection of 50 ng of PIR, SH2 or PIR–SH2 domains Grb7 or Grb14 were sufficient to block FGF1-induced oocyte maturation. When these results are expressed in pmol of proteins injected in each oocyte, it appears that 0.85 pmol of Grb7 or Grb14 were inhibitory, but about 1.4 pmol of the isolated domains PIR or SH2 were needed for a similar effect.
Interestingly, 0.87 pmol (40 ng) of the Grb14 PIR–SH2 appeared to represent the lower amount of protein able to inhibit FGF-induced oocyte maturation, showing that this region is as effective as the full length protein. In contrast, 1.1 pmol (50 ng) of Grb7 PIR–SH2 are required to block GVBD.

3.3. Association of FGFR1 with the members of the Grb7 family

We then tested whether the inhibitory effect of Grb7 and Grb14 on FGFR1-induced oocyte maturation depended on an association with FGFRs. We used the PDGFR–FGFR1 chimera since available antibodies directed against FGFR did not cross react with FGFR1 from *Pleurodeles* chimeric receptor is efficiently precipitated by anti-PDGFR antibodies [34]. Furthermore, PDGFR-BB induced maturation of oocytes expressing this chimera [5]. Naive oocytes and oocytes expressing the PDGFR–FGFR1 chimera for 48 h, were injected with either Grb7, Grb10 or Grb14 (100 ng). One hour after these injections, oocytes were stimulated or not by PDGFR-BB for 5 min, and oocyte lysates were immunoprecipitated using anti-PDGFR antibodies. As shown in Fig. 4, PDGFR-BB induced tyrosine phosphorylation of the chimeric receptor (upper blots). In addition, ligand stimulation induced interaction between the phosphorylated receptor and Grb7 or Grb14, whereas no interaction could be detected with Grb10 (middle blots). We verified that receptors were expressed at similar levels in oocytes injected with the different Grb proteins (lower blots). Control experiments performed on naive oocytes stimulated with PDGFR-BB, showed that immunoprecipitation of Grb7 and Grb14 with anti-PDGFR antibodies was dependent on PDGFR expression (Fig. 4, lanes 1, 4 and 7). These results provided evidence that the inhibitory effect of Grb7 and Grb14 on FGF-induced oocyte maturation correlated with their ability to bind to activated FGFR1.

3.4. Grb7 and Grb14 act on two main FGFR1 signalling cascade

FGF-induced oocyte maturation is associated with the activation of the MAP kinase pathway, characterised by the phosphorylation of Raf and ERK2, the activation of the PI3-kinase pathway seen by the phosphorylation of Akt, and the accumulation of β-catenin (Fig. 5, lanes 1). We thus tested whether Grb7 and Grb14 injection altered these pathways in FGFR-expressing oocytes. As reported in Fig. 5, the injection of Grb7 or Grb14 suppressed Raf, ERK2 and Akt phosphorylation, and enhanced β-catenin degradation in FGFR1-stimulated oocytes expressing FGFR1 (Fig. 5, lanes 3 and 7). As expected, the injection of Grb10 had no effect (Fig. 5, lane 5). Control experiments performed on oocytes let unstimulated, displayed no Raf, ERK2 or Akt phosphorylation, and only a low level of β-catenin (Fig. 5, lanes 2, 4, 6).

4. Discussion

The role of Grb7 family members was assessed using an integrated biological system, the *Xenopus* oocyte, which is devoid of endogenous FGFRs. In this system a specific expression of FGFRs is obtained after injection of FGFR1, FGFR4 or FGFRs from a highly invasive hormono-independent breast cancer cell line, MDA-MB-231. *Xenopus* oocytes react as an all or none system after FGFR activation, through a Ras/MAP kinase cascade that lead the oocyte to enter into the M phase of the cell cycle [39]. This allowed us to analyse in a single experimental model the effect of the Grb7 family members on transduction cascades activated by FGFRs. In addition, a comparison of these results with other RTKs expressed in the oocyte, the endogenous IR and IGF-1R, was achieved.

The present study provided evidence that members of Grb7 family adapters can interfere with FGFR signalling, with a given specificity for each protein. Grb7 and Grb14 inhibited FGF-induced oocyte maturation, whereas Grb10 had no effect. Similar results were obtained for FGFR1, FGFR4, and FGFRs from MDA-MB-231. This study reports the role of all members of the Grb7 family in FGF signal transduction, and is in agreement with a previous work reporting an inhibition of FGF-stimulated DNA synthesis in a stable cell line over-
Grb14 was already established, in cell lines and in *Xenopus* was efficient, as previously reported[43]. Thus, the isolated amount of injected Grb proteins. In such conditions, Grb7 or Grb14 were required for the same effect (data not shown). These results suggest that FGFR signalling exhibits similar sensitivity towards Grb7 and Grb14 but is insensitive to Grb10, whereas IR signalling is sensitive to Grb7, Grb10, and Grb14 inhibitory action.

The inhibitory effect of the Grb proteins on FGF-induced oocyte maturation nicely correlates with their binding ability to the receptors in this biological system. An interaction between Grb7 and FGFR was already reported in the two-hybrid system [10], and Grb14 was also shown to bind to FGFR1 in the two-hybrid system and in vitro binding assays [15]. Grb10 did not bind to phosphorylated FGFR1 after ligand stimulation of receptor expressing oocytes, and it was also unable to alter oocyte GVBD. This is in contrast with a previous study reporting an in vitro interaction between the SH2 domain of Grb10 expressed in fusion with GST and activated FGFR1 [14]. However, huge amounts of proteins can be used in GST pull-down experiments, allowing the detection of weak interactions. In contrast, in the present work, similar amounts of receptors were expressed in presence of the same amount of injected Grb proteins. In such conditions, Grb7 and Grb14, but not Grb10, bound to activated FGFR, and inhibited oocyte maturation. All together, these results suggest that Grb10 is unlikely to play a physiological role in FGFR signalling.

Inhibition of FGF induced oocyte maturation by Grb7 and Grb14 was reproduced by their C-terminal region, containing the PIR and SH2 domains. Interestingly, testing separately these two domains, gave evidence that Grb7 and Grb14 seemed to interfere with FGFR signalling through specific mechanisms. The inhibitory action of Grb7 was only due to the PIR, whereas both PIR and SH2 of Grb14 were able to block GVBD induced by FGF–FGFR signalling. By comparison, the PIR and SH2 domains of Grb7 and Grb14 exhibited also different inhibitory effects on insulin-induced reinitiation of meiosis: both PIR and SH2 domains of Grb7 could block insulin-induced oocyte maturation, but only the PIR of Grb14 was efficient, as previously reported [43]. Thus, the isolated domains of Grb7 and Grb14 able to inhibit insulin signalling in *Xenopus* oocyte are those responsible for the interaction with the IR [10,18]. However, such a correlation is not so clear for FGFR. Grb14 binding to activated FGFR1 is mainly mediated by the SH2 domain, and no participation of the PIR was reported [15]. Furthermore, two-hybrid experiments suggested that the SH2, but not the PIR, was implicated in the FGFR1-Grb7 interaction (V.B. and A.F.B., unpublished data). This suggests that, besides a direct interaction between the two proteins, other mechanisms should also be important for the inhibition of FGFR signal transduction by the members of the Grb7 family of adapters. A number of hypothesis can be proposed to explain this effect. First, members of the Grb7 family of adapters can act as direct inhibitors of receptor tyrosine kinase activity, as demonstrated for the IR [17,19]. Furthermore, this inhibitory effect is dependent on the PIR domain [17,19]. PIR domains of both Grb7 and Grb14 blocked reinitiation of meiosis in FGF stimulated oocytes. However, as discussed above, they do not appear to bind to FGFR, implying that their action is not linked to a direct inhibition of the receptor catalytic activity. On the other hand, the SH2 domain of Grb14 interacts with the phosphorylated tyrosyl residues Y766 and Y776 in the C-terminus of FGFR1 [15]. Y766 is phosphorylated after ligand addition, and is the binding site for PLCγ [4]. We have previously reported that FGFI-induced GVBD in FGFR1-expressing oocyte is dependent on PLCγ, and that the inhibition of PLCγ blocked ERK2 phosphorylation [5]. Thus, a competition between PLCγ and Grb14 on this binding site could inhibit GVBD through an inhibition of the Ras/Raf/ERK2 pathway. A report mention that FGFR1 mediated activation of PI3-kinase was dependent on the same Y766 site, in aortic endothelial cells under FGFI stimulation [44]. We have previously reported that FGFI-induced GVBD in FGFR1-expressing oocyte is dependent on PI3-kinase, and that the inhibition of PI3-kinase blocked ERK2 phosphorylation [5]. Finally, expression of a Grb14 SH2-mutant unable to bind to FGFR, potentiated FGF-induced cellular proliferation. This suggests that Grb14 effect is mediated by its interaction with unidentified downstream effectors [15]. Nevertheless, further studies are needed to elucidate the molecular mechanisms of Grb7 and Grb14 inhibition of FGFR signalling.

FGFI stimulation of oocytes expressing FGFR1, FGFR4 or FGFRs from MDA-MB-231 cells triggers Ras-dependent and Ras-independent transduction cascades [40]. In *Xenopus* oocytes, ERK2 is the main activated MAP kinase [45]. Raf is phosphorylated [46], and Akt is phosphorylated and activated as a downstream target of PI3-kinase [5,47]. Injection of Grb7 or Grb14 induced a decrease in phosphorylation of Raf, ERK2 and Akt, showing that two main transduction cascades activated by FGFR1, the Raf-ERK2 and the PI3-kinase cascades, were inhibited. The PI3-kinase pathway also leads to inactivation of GSK-3, and to the accumulation of β-catenin. We showed that β-catenin was no longer expressed in FGFR-expressing oocytes injected with Grb7 or Grb14 before FGFI stimulation. This suggests that injection of Grb7 or Grb14 released the inhibitory effect of GSK-3 and allowed β-catenin degradation, thus confirming that the upstream PI3-kinase was no longer activated. We verified that injection of Grb7 or Grb14 did not alter oocyte maturation induced by progesterone, the natural inducer (data not shown). In progesterone-stimulated oocytes, PI3-kinase blocked GSK-3 and β-catenin was not degraded [45,48]. Furthermore, we previously reported that PI3-kinase sets upstream from Ras in the transduction pathways activated by FGFI–FGFR1 [5]. Consequently, the action of Grb7 and Grb14 in FGFR signalling is directed towards the Ras-dependent cascade, upstream from Raf, presumably on molecular effectors such as PLCγ or PI3-kinase.

In conclusion, the *Xenopus* oocyte model provided a useful system to delineate the role of the members of the Grb7 family of adapters on signalling induced by various tyrosine kinase receptors. Using this integrated biological model, we showed that Grb7, Grb10 and Grb14 and their PIR and or SH2 domain differently interfered with FGFR and IR signalling. Furthermore, we also provided evidence that the Grb proteins inhibit upstream effectors of the Ras pathway in FGFR signalling.
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