Interaction of the Grb7 adapter protein with Rnd1, a new member of the Rho family

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Abstract Grb7 is a member of a family of molecular adapters which are able to contribute positively but also negatively to signal transduction and whose precise roles remain obscure. Rnd1 is a member of the Rho family, but, as opposed to usual GTPases, it is constitutively bound to GTP. We show here that Rnd1 and Grb7 interact, in two-hybrid assays, in vitro, and in pull-down experiments performed with SK-BR3, a breast cancer cell line that overexpresses Grb7. This interaction involves switch II loop of Rnd1, a region crucial for guanine nucleotide exchange in all GTPases, and a Grb7 SH2 domain, a region crucial for Grb7 interaction with several activated receptors. The contribution of the interaction between Rnd1 and Grb7 to their respective functions and properties is discussed.

Key words: Grb7; Rnd1; Yeast two-hybrid; SH2

1. Introduction

Molecular adapters are involved in multiple protein/protein interactions required to form the large multi-protein complexes involved in signal transduction. In addition to the Grb2/Nck prototypic family, a new kind of adapters has emerged recently, the Grb7/10/14 family, where proteins are made of combinations of four protein interaction domains. Their Src homology-2 (SH2) domains recognize phosphotyrosines in an appropriate sequence context on activated tyrosine kinase receptors [1–3] and indeed Grb7 has been shown to interact with the EGF receptor, with erbB2, 3 and 4, the PDGF receptor and with Ret [1,4–7]. Grb7 can be phosphorylated on serines and threonines [4] and becomes tyrosine phosphorylated after ligand stimulation of erbB2 [4]. Grb7 also interacts with other signaling proteins such as Syp phosphatase [8] and Shc adapter protein [4]. Grb10 isoforms interact with Ret [9], the ELK receptor [10], the IGF-1 receptor and the insulin receptor [11,12]. Grb10 also binds non-receptor tyrosine kinases such as BCR-ABL and Tec in a phosphotyrosine-dependent manner. This interaction leads to the phosphorylation of Grb10 [13,14].

Last members of this family, Grb14 and its close relative rGrb14, were found to interact with PDGF and insulin receptors [3,15], inhibiting in the latter case insulin actions [15]. Interaction of Grb7/10/14 proteins with receptor phosphorylated on tyrosines could involve an additional region located between the SH2 domain and the central pleckstrin homology (PH) region. This domain called BPS (between PH and SH2 domains) [16] or PIR (phosphorylated insulin receptor-interacting region) [15] could help to determine the specificity of Grb7/10/14 interaction with different tyrosine kinase receptors and/or modulate the strength of interactions with those receptors.

Beside the SH2 and PIR domains, Grb7/10/14 proteins contain, as the most N-terminal domain, a consensus proline-rich SH3-binding motif. In vitro binding experiments have shown a strong binding of full-length Grb10 to Abl SH3-GST fusion polypeptide whereas no binding was detected on PI-3-kinase, Grb2 and Fyn SH3 domains [17]. Finally, Grb7/10/14 harbor a central pleckstrin homology (PH) domain. Another striking feature of these Grb proteins is their putative implication in oncogenesis. Grb7, mapped on 17q11.2-q12, was found to be co-amplified with erbB2 in breast cancer cell lines and breast cancer specimens [4]. The finding that Grb7 and ErbB2 are both overexpressed and bind tightly together suggests that they participate to a common pathway involved in breast epithelial cell transformation, contributing to a more aggressive and invasive phenotype, since ErbB2 amplification has been associated to more aggressive tumors [18,19].

Presumptions for a role of Grb7/10/14 in cell migration and invasion came recently from the finding that a splice variant of Grb7, lacking the C-terminal SH2 domain, was overexpressed in metastatic lymph nodes as compared to the primitive esophageal tumor tissue [20]. Expression of an antisense Grb7 RNA lowers endogenous Grb7 protein levels and suppresses in vitro invasive phenotype of esophageal carcinoma cells.

Cell migration involves diverse rearrangements of the cell cytoskeleton. A class of proteins involved in these events belongs to the extensively studied Rho/Rac subfamily of small GTP-binding proteins (reviewed in [21]). Micro-injection studies in Swiss 3T3 cells have indicated that Cdc42Hs, Rac1 and RhoA proteins act in sequence to control the extension of exploratory filopodia (Cdc42), the larger extensions of lamellipodia (Rac1) and attachment to substrate for traction by

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Abbreviations: p85 PI-3K, p85 phosphatidyl-inositol-3-kinase; PLCγ, phospholipase Cγ; IR, insulin receptor; IRS, insulin receptor substrate; PTP-1, phosphotyrosine phosphatase 1; PTB, phosphotyrosine-binding domain

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focal adhesions and stress fibers (RhoA) in motile mammalian fibroblasts [22–25]. Rho proteins have been suggested to be involved in invasive and metastatic capacities of tumor cells [26,27].

Like Ras, Rho/Rac proteins cycle between a GDP bound state and a GTP bound state; cycling between these two conformations is tightly regulated. The recently identified Rnd proteins do not fit in this model: they are constitutively bound to GTP [28]. Rnd1 shares 45% identity with RhoA, but key amino acid substitutions are most likely the molecular basis for Rnd1 extremely low intrinsic GTPase activities and its presumed resistance to any GAP activity [28]. Interestingly, overexpression of Rnd1 or of Rnd3/RhoE induces loss of polymerized actin structures and focal adhesion, causing loss of cell-matrix adhesion [28], and Rnd3/RhoE stimulates cell migration speed of MDCK cells in the presence of HGF [29].

In this paper, we bring together Grb7 and Rnd1 by documenting an interaction between these two proteins, raising the question of a possible biological role of such an interaction in growth factor-regulated cell motility and adhesion.

2. Materials and methods

2.1. Yeast strains and media

The *Saccharomyces cerevisiae* yeast reporter strain L40 (MATa ade2 trp1 leu3 his3 LYS2::lexA-HIS3 URA3::lexA-lacz) and the MATα yeast strain AMR70 [34] were handled according to standard procedures [34,35]. The two-hybrid screen was performed essentially as previously described by using a full-length Grb7 cDNA fused to LexA-binding domain as bait, and a 9.5 day mouse embryo cDNA library [36].

2.2. Plasmids

pRK5-Rnd1, pRK5-myc-Rnd1 and pRK5-myc-Rnd2 constructs [28] and pVP16-Grb7 [8] were previously described. Grb7 cDNA was subcloned in frame in pACTII two-hybrid vector at the *Bam*H1 site (Clontech, Palo Alto, CA, USA). pGEX2T-SH2-Grb7, pGEX2T-Grb7, Grb10, rGrb14 [15] and various deletants of Grb7 cDNA in pACTII vector (Grb7αPH-PIR-SH2, Grb7αSH2, isolated SH2-Grb7, Grb7αPIR-SH2, isolated PIR and isolated PIR-SH2) were kindly provided by Dr. A.F. Burnol. Rnd1 and Rnd2 were cloned in frame with LexA-binding domain in pVJL10 two-hybrid plasmid.

2.3. Immunoblotting and antibodies

Proteins were resolved by 10% or 12.5% SDS–PAGE, transferred to nitrocellulose sheets by electroblotting, blocked with 5% non-fat milk in phosphate-buffered saline (PBS), containing 0,1% Tween 20 overnight at +4°C, subsequently probed with primary antibodies, then incubated with horseradish peroxidase-conjugated secondary antibodies as described [37]. After extensive washing, membranes were developed using a chemiluminescent reaction (ECL, Amersham corp.) according to manufacturer's instructions.

Rabbit polyclonal affinity-purified anti-Grb7 antibodies sc-606 (C-20) and sc-607 (N20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against a 20-mer C- or N-terminal, respectively, were used at 1/200 dilution. Polyclonal rabbit anti-Rnd1 antibody 'Pegase' raised against the whole Rnd1 protein was previously described [28] and used at 1/250 dilution. A rabbit polyclonal anti-Rnd1 antibody raised against peptide 214-SELISSTFKKEKAKC-228 was used at 1:200 dilution and shown not to react with purified Rnd2 and Rnd3 proteins (G. Zalcman, personal data). A goat polyclonal anti-Rnd2 antibody (Santa-Cruz #sc-1945) was used at the same dilution and was shown to only react with Rnd2 protein without any cross-reactivity with purified bacterial or eukaryotic Rnd1 or Rnd3 proteins (data not shown). Monoclonal anti-phosphotyrosine antibody PY20 and anti-myc 9E10 antibody were from Transduction Laboratories and Boehringer Mannheim, respectively.

2.4. Cell culture and transfections

HeLa cells were grown in Dulbecco's modified Eagle's medium (Life Technologies Inc.) supplemented with 10% fetal calf serum, 1 mM glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin. SK-BR3 human breast cancer cells overexpressing Grb7 by gene amplification [4] were kindly provided by Dr. P. de Crémoux and grown in the same way. Cells were transfected with 10 μg of pRK5-myc (mock transfection), pRK5-myc-Rnd1 or pRK5-myc-Rnd2 constructs/100 mm dish using a modified calcium phosphate co-precipitation method as described previously [37]. After transfection, cells were grown for 36 h, after which cells from three 100 mm equivalent culture dishes were lysed in 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 0.5% NP-40 with a cocktail of protease and phosphatase inhibitors, and centrifuged at $600 \times g$. The supernatants were used for Rnd expression analysis, GST fusion protein binding and pull-down assays. Bacterial Rnd1 and Rnd2 proteins were expressed as non-fusion proteins using pET11a vector and produced as described [28], snap-frozen and stocked at −80°C until being used. GST-Grb7, GST-SH2-Grb7 were produced as previously described. SK-BR3 cell lysates were pre-cleared 30 min at 4°C on protein A/G Sepharose beads, and supernatants incubated with either polyclonal anti-Rnd1 or anti-Rnd2 antibodies, in the presence of 100 ng of either bacterial Rnd1 or Rnd2 protein for 1 h at 4°C. Immunocomplexes were incubated 1 h on protein A/G Sepharose columns and then collected by centrifugation, extensively washed three times in lysis buffer, and beads with bound proteins or supernatants containing unbound proteins, resolved on SDS–PAGE and immunoblotted with anti-Grb7 antibodies.

3. Results

3.1. Grb7 two-hybrid interaction with Rnd1

In an attempt to further characterize partners of Grb7 adapter protein, a two-hybrid screen was performed with a full-length Grb7 protein fused to the LexA DNA-binding domain as a bait. One of the identified cDNAs encoded a truncated form of Rnd1 [28], from amino acid 61 to amino acid 144. Although such a polypeptide would not bind guanine nucleotides, it contains a loop present on the surface of Rho proteins [30], the so-called switch II region, and the 'rho insert region', both of which are involved in several effector and regulator interactions. In an attempt to check that the full-length Rnd proteins could actually interact with Grb7, we made a two-hybrid construct to express Rnd1 deleted only of its C-terminal CSIM membrane localization peptide. This construct gives a huge background in two-hybrid assays. An accidentally generated mutant expressing the first 146 amino acids of Rnd1 (Rnd1*) did not have any background in the assay and did interact specifically with Grb7. Such a mutant, again, displays the switch II region and the so-called 'rho insert region'.

3.2. Mapping the Rnd-interacting domain on Grb7 protein

Since this interaction between a truncated Rnd1 protein and Grb7 was puzzling, we tested the specificity of this interaction. In a first series of assays, we mapped the region of Grb7 that was involved. Two-hybrid constructs that express various domains of Grb7 were tested. Surprisingly, the absence of only the SH2 domain in Grb7 prevents Grb7 interaction with Rnd1, while the SH2 domain of Grb7 alone binds to Rnd1 (Fig. 1). This result suggests that the SH2 domain of Grb7 is necessary and sufficient for the interaction with Rnd1. Notice that all positive interactions are detected with the mutated Rnd1* and with wild-type full-length Rnd1, even if these latter interactions are weaker.

Secondly, we questioned the specificity for the SH2 domain of Grb7 by testing several SH2 domains in the two-hybrid assay.

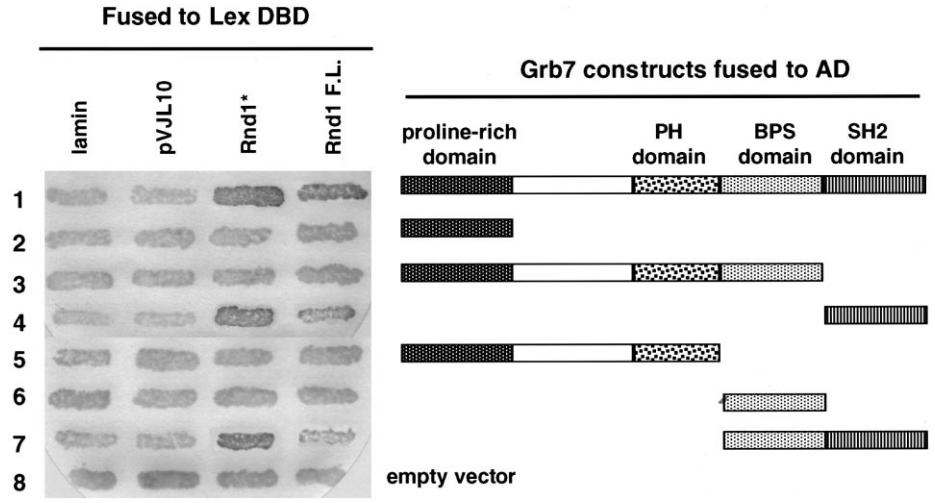

Fig. 1. Mapping the interacting domain on Grb7. Various regions of Grb7 corresponding to the proline-rich domain, the proline-rich domain plus pleckstrin homology (PH) domain and Between pleckstrin and SH2 (BPS) domain, the SH2 domain, the proline-rich domain plus the PH domain, the BPS domain and the BPS plus the PH domains were assayed for interaction with the Rnd1 fusion protein in a yeast two-hybrid assay. All constructs containing the SH2 domain were able to interact with Rnd1 (lanes 1, 4 and 7). Constructs without SH2 domain were unable to interact with Rnd1 (lanes 2, 3, 5 and 6). The SH2 domain of Grb7 was necessary and sufficient for interaction with Rnd1 (lane 4). Rnd1 F.L. refers to wild-type full-length Rnd1; Rnd1* refers to the mutated Rnd1 containing the first 146 amino acids.

The Grb10 and Grb14 proteins, or their isolated SH2 domains, failed to interact with Rnd1, despite approximately 70% amino acid identity with Grb7 in the SH2 domain. These proteins, in the same two-hybrid test, retained the ability to interact with the insulin receptor (Table 1).

SH2 or PTB domains from p85 phospho-inositol-3-kinase (PI-3K), Phospholipase Cγ (PLCγ), Src, Abl, Shc Nck, Grb2, Aps-1, PTP-1D (Syp) and IRS-2 failed also to interact with Rnd1 (Table 1). All of them were properly expressed as verified by their ability to interact with their respective natural ligands in two-hybrid tests (cf. [31] and data not shown).

3.3. *In vitro binding of full-length bacterially expressed Rnd1 to Grb7 is direct and specific*

Rnd1 and Rnd2 proteins were expressed in bacteria as non-fusion proteins as previously described, using the pET11a vector. This procedure allows the purification of bacterially expressed Rnd proteins at $>80\%$ purity in a lysis buffer containing GTP but without EDTA [28]. In such conditions, half of the purified bacterial Rnd proteins were shown to bind GTPαS [28]. GST protein, GST-Grb7 and GST-SH2-Grb7 fusion proteins were coupled to glutathione Sepharose beads and then incubated with either Rnd1 or Rnd2 proteins. As shown in Fig. 2, full-length Rnd1 protein but not Rnd2 interacts with full-length Grb7 and with SH2-Grb7. None of the Rnd proteins interacts with GST. These results confirm that Rnd1 and Grb7 can interact and that most likely this interaction is direct. The interaction between Rnd1 and Grb7 appears also very specific as under the same conditions Grb7 does not interact with the closely related Rnd2 protein.

Furthermore, the observation suggests that the interaction is not dependent on tyrosine phosphorylation as all proteins were purified from *Escherichia coli*.

Table 1
Interaction of Rnd1 with various SH2-containing proteins

SH2 or PTB domains fused to activation domain	RTK fused to LexA (positive control)	LexA-Rnd1
p85 PI-3K	+(c-Met)	−
PLCγ	+(c-Met)	−
Src	+(c-Met)	−
Abl	+(c-Met)	−
Shc	+(TrkA)	−
Nck	+(IR)	−
Aps-1	+(IR)	−
PTP-1(Syp)	+(c-Met)	−
IRS-2	+(IR)	−
Grb2	+(c-Met)	
Grb10	+(IR)	−
Grb14	+(IR)	−
Grb7	+(c-Met)	+

All SH2- or PTB-containing proteins used in this experiment were checked for binding to at least one receptor tyrosine kinase (named in between brackets) in a two-hybrid assay.

3.4. *In vitro binding of HeLa cell-expressed Rnd1 to Grb7 and SH2-Grb7 does not require Rnd1 tyrosine phosphorylation*

To further document the Grb7-Rnd1 interaction, we trans-

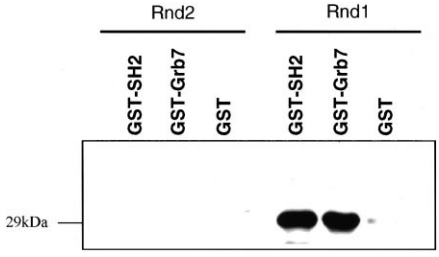

Fig. 2. Specific binding of Rnd1 to Grb7 is direct. To verify the interaction observed in two-hybrid system, we produced in vitro GST-Grb7 and GST-SH2 fusion proteins corresponding to the entire protein and to the SH2 domain of Grb7. Immobilized GST fusions were incubated with purified Rnd1 or Rnd2 proteins expressed in bacteria as non-fusion proteins using the pET11a vector. Proteins bound to the beads were resolved by SDS–polyacrylamide gel electrophoresis and subsequently immunoblotted with anti-Rnd antibodies. No interaction was detected between Rnd2 protein and Grb7 constructs (left panel). Rnd1 was retained as well on GST-Grb7 as on GST-SH2 beads (right panel). The specific interaction between Rnd1 and Grb7 is direct since both proteins are highly purified.

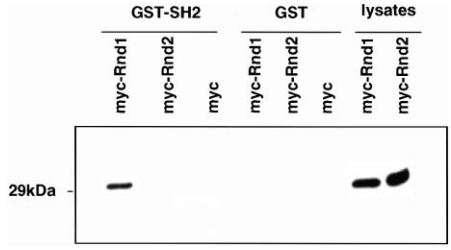

Fig. 3. The SH2 domain of Grb7 interacts with Rnd1 in vitro. We tested the ability of the GST fusion proteins GST-Grb7 and GST-SH2 to interact with Rnd proteins expressed in mammalian cells. Immobilized GST fusions were incubated with lysates derived from HeLa cells that were transfected with either control pRK5-myc or pRK5-myc-Rnd1 or pRK5-myc-Rnd2. Proteins bound to the beads were resolved by SDS–polyacrylamide gel electrophoresis and subsequently immunoblotted with anti-myc antibodies. The GST protein alone showed no in vitro interaction with myc-Rnd1 or myc-Rnd2 (middle panel). Rnd1 interacts with GST-SH2 (first lane) whereas no interaction was detected with Rnd2 (second lane). Expression of myc-Rnd1 and myc-Rnd2 was checked in total lysates (right two lanes).

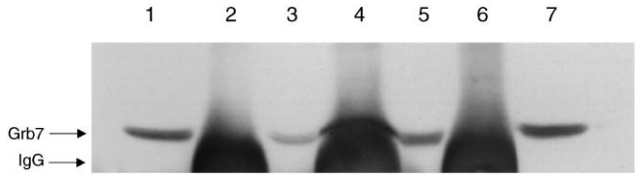

Fig. 5. Interaction of endogenous Grb7 from SK-BR3 cells with Rnd1. SK-BR3 breast cancer cell extracts were incubated with polyclonal anti-Rnd1 antibodies in the presence of 100 ng of either Rnd1 or Rnd2 bacterial proteins. Lysates were collected on protein A Sepharose columns and bound (lanes 2, 4, 6), unbound proteins (lanes 1, 3, 5) and whole cell extracts (lane 7) were resolved by SDS–polyacrylamide gel electrophoresis and subsequently immunoblotted with either anti-Grb7 antibodies. Endogenous Grb7 protein was easily detected in unbound SK-BR3 lysates (lanes 1, 3, 5) and in whole cell extracts (lane 7). Co-immunoprecipitated Grb7 was detected in lysates incubated with anti-Rnd1 antibodies and Rnd1 protein (lane 4). Co-immunoprecipitated Grb7 was not detected when Rnd2 protein was added instead of Rnd1 (lane 2) or when Rnd1 protein was omitted (lane 6).

fected HeLa cells with plasmids that allow expression of N-terminally myc-tagged Rnd1 (pRK5-myc-Rnd1) and Rnd2 (pRK5-myc-Rnd2). Cell lysates were loaded 1 h on immobilized GST fusion proteins or on immobilized GST alone as control. Rnd1 and Rnd2 were present at equal concentrations as checked by anti-myc (two right lanes) or specific anti-Rnd antibodies (data not shown).

As shown in Fig. 3, myc-Rnd1 was retained on an SH2-Grb7 column (first lane) whereas no myc-Rnd2 was found bound to a same column (second lane).

To check whether the interaction between Grb7 and Rnd1 requires Rnd1 tyrosine phosphorylation, cell extracts of HeLa cells transfected with pRK5-myc-Rnd1 (or pRK5-myc) were incubated with GST-SH2, GST-Grb7 or GST beads and resolved by immunoblotting with anti-myc antibodies (Fig. 4A). The proteins bound to GST and GST-Grb7 were submitted to immunoblotting with anti-phosphotyrosine antibodies. No tyrosine phosphorylation is revealed on Rnd1, whereas higher molecular weight phosphoproteins were detected in the GST-Grb7 bound fraction, indicating that tyrosine phosphorylation actually occurred in HeLa transfected cells under our experimental conditions (Fig. 4B). These high molecular weight phosphoproteins retained on GST-Grb7 are likely to be receptor tyrosine kinases previously shown to interact with Grb7 [1,15]. Thus, Rnd1 tyrosine phosphorylation does not seem to be required for Rnd1 to bind to the SH2 domain of Grb7.

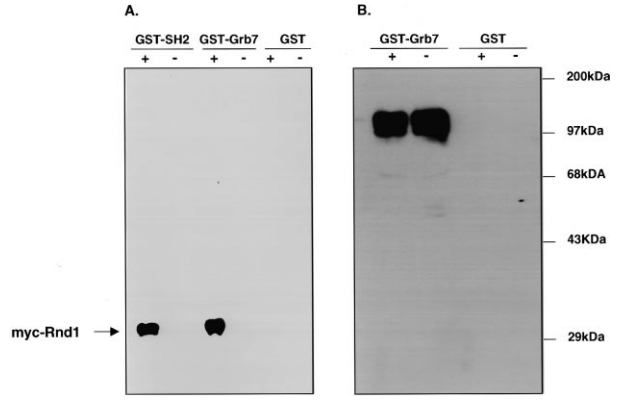

Fig. 4. Rnd1/Grb7 interaction does not require Rnd1 tyrosine phosphorylation. Immobilized GST fusions were incubated with lysates derived from HeLa cells that were transfected with either control pRK5-myc or pRK5-myc-Rnd1. Proteins bound to the beads were resolved by SDS–polyacrylamide gel electrophoresis and subsequently immunoblotted with either anti-myc (panel A) or anti-phosphotyrosine (panel B) antibodies. A: The GST protein alone showed no in vitro interaction with myc-Rnd1 (right two lanes). Rnd1 interacts as well with GST-SH2 (first lane) as with GST-Grb7 (third lane). B: The proteins bound to GST and GST-Grb7 were probed with anti-phosphotyrosine antibodies: no phosphorylation was detected on Rnd1 (first lane), despite presence of other tyrosine-phosphorylated proteins of higher molecular weight bound to GST-Grb7. Thus myc-Rnd1 interacts with GST-Grb7 in a non-phosphotyrosine-dependent manner.

3.5. *Interaction of endogenous Grb7 from SK-BR3 cells with full-length Rnd1 protein*

To test whether binding of Grb7 to Rnd1 could occur in vivo, SK-BR3 cells, a breast cancer cell line that overexpresses Grb7, were put to contribution. However, we could not detect any endogenous Rnd1 proteins by Western blotting with specific antibodies (data not shown). Transfection of these cells with Rnd1 expression plasmids appeared to be insufficient for a robust expression of Rnd1, maybe because overexpression of Rnd1 is deleterious for these cells, as has been shown for Swiss 3T3 cells [28]. Not surprisingly, in such conditions no co-immunoprecipitation of Grb7 and Rnd1 could be detected. Therefore, we performed binding experiments with SK-BR3 cell extracts incubated with polyclonal anti-Rnd1 antibodies, in the presence of 100 ng of either bacterially expressed Rnd1 or Rnd2 protein. Lysates were collected on protein A Sepharose columns, and bound (lanes 2, 4, 6) or unbound proteins (lanes 1, 3, 5) were tested by Western blot with anti-Grb7 antibodies. As shown in Fig. 5, endogenous Grb7 protein was easily detected in SK-BR3 lysates (lanes 1, 3, 5, 7). Co-immunoprecipitated Grb7 protein was only detected in lysates incubated with anti-Rnd1 antibodies and Rnd1 bacterial protein (lane 4), but not in lysates incubated with anti-Rnd1 antibodies but no Rnd1 protein (lane 6). Incubation with anti-Rnd1 antibodies and Rnd2 protein leads to no precipitation of Grb7 (lane 2). Incubation in presence of Rnd2 protein and

anti-Rnd2 antibodies leads to no Grb7 precipitation either (data not shown).

4. Discussion

Rnd1, a member of the Rho family, has no GTPase activity and appears constitutively GTP bound. Its overexpression inhibits stress fibers formation, but to what extent this property reflects its real function in tissue is not clear. Grb7 is an adapter protein, member of the Grb7/10/14 family. The generic function of these proteins is not clear either, they have been shown to participate positively but also negatively in signal transduction (see for example [15]).

Here we show that Grb7 is able to specifically interact with Rnd1 protein. This interaction was shown to be direct and involves the SH2 domain of Grb7 and a central part of Rnd1. Surprisingly, the minimal Rnd1 region sufficient to interact with Grb7 lacks the Rho effector domain (switch I) and two of the guanine-binding motifs. Preferential interactions with truncated protein partners in the two-hybrid system have already been reported. For instance, the SH2 domain of Grb10 is unable to interact with the full-length Raf1 while it can bind to the isolated amino-terminal domain of Raf1 in a two-hybrid assay. This observation led to the hypothesis that the binding site of Raf1 is masked in the context of the whole protein [32].

Modeling of Rnd1 structure, based on the GTP-locked RhoA [30], suggests that the Grb7-interacting region contains the switch II region of Rnd1. In the full-length protein, this region could be in a conformation that does not favor the binding of Grb7 and could be better exposed upon truncation. Therefore, in a similar way to the two-hybrid Grb10/truncated Raf1 interaction, the truncation in the Rnd1 two-hybrid clone could explain the unmasking of a binding site for Grb7. In the cell, this site might be unmasked by binding of a cellular effector on the 'effector region', leading to a displacement of the 310-helix 78RPL80. Such speculations remain to be confirmed by crystallographic studies of Rnd1/Grb7 complexes. On the other hand, our biochemical experiments show that Grb7 is able to interact with full-length Rnd1 protein. This was shown in two different ways. Rnd1 purified from *E. coli* is able to bind Grb7 expressed in mammalian cells. Grb7 expressed as a fusion protein in *E. coli* is able to bind both Rnd1 made in *E. coli* and Rnd1 expressed in mammalian cells. Again, two-hybrid deletion analysis that involves the SH2 domain of Grb7 as the Rnd1-binding site were validated by in vitro binding assays.

No tyrosine phosphorylation was required for this interaction as shown by the direct interaction of purified proteins expressed in *E. coli*, and the absence of detectable tyrosine phosphorylation on Rnd1 expressed in exponentially growing HeLa cells. What could be the function of such a phosphotyrosine-independent SH2 interaction? Interactions between Grb2 SH2 domain and non-phosphorylated ligands have been previously reported but were shown to require a YXN sequence [33]. In such a case, phosphorylation of the tyrosine residue increased the strength of the interaction. A similar peptide sequence (74YDN76, highly conserved in Rnd1, Rnd2 and Rnd3) is present in the Grb7-interacting domain. Thus, further experiments with Rnd1 peptides are required to precisely map the Grb7 interaction motif.

In addition, the SH2 domain of Grb10 has also been shown to interact with phosphorylated threonines or serines of both Raf1 and MEK1 kinases, in a constitutive way for Raf1, but in an insulin stimulation-dependent way for MEK1 [32]. Thus, SH2-Grb7 interaction with Rnd1 could also involve serine and threonine motifs in Rnd1, but no serine phosphorylation was detected on GST-SH2-Grb7 bound Rnd1, using an anti-phosphoserine antibody (data not shown).

Grb7 and Rnd1 mRNA are detected in liver and kidney showing a restrictive pattern of overlapping tissue expression [28]. Expression of myc-Rnd1 proteins in immortalized hepatocyte cell lines, in HeLa cells or in breast cancer cell lines (SK-BR3, BT474), led to disassembly of actin stress fibers, and longer expression to loss of cell adhesion ([28] and our unpublished data). Such events are a prerequisite for cell migration to take place. A presumptive role for Grb7 in invasive growth has been proposed [20] and one might speculate that the mechanism of invasiveness will involve a Grb7-Rnd1 link. However, co-expression of Grb7 failed to interfere with stress fiber disassembly generated by Rnd1 expression (data not shown). Thus, we could not determine yet whether Grb7 actually complexes with Rnd1 in a signalling module involved in control of the actin cytoskeleton rearrangements. The existence of a stable or transient ternary complex involving a tyrosine kinase receptor (e.g. erbB2, EGF-R, PDGF-R), Grb7 and Rnd1 remains to be established. One can only speculate about an inhibitory role for Grb7 in proliferation signalling cascades initiated by membrane tyrosine kinase activation, and to what extend this role will be played via interaction with Rnd1 and interference with a Rho signalling cascade. Such hypothesis is currently tested in cells exhibiting a migratory phenotype after growth factor stimulation or epithelial scar induction. Grb7 involvement in cell migration could be the consequence of the recruitment of Rnd1. Alternatively, Rnd1 binding to Grb7 might impair its interaction with other molecular partners by steric hindrance. However, no two-hybrid interaction was detected between Rnd1 and several known Rho/Rac effectors or activators such as citron, RhoA-interacting domain of Rock, lfc, mPak3 or rhotekin (data not shown). Alternatively, Rnd1 might act upon Grb7 by interfering the interaction of this latter adapter with a molecular partner by steric traffic jam.

We have shown by different approaches that a Rnd1/Grb7 interaction can take place in vitro. These two proteins are expressed in kidney and liver tissues. In liver, Rnd1 seems to be located at the boundary of cell-cell contacts (GZ, preliminary data) and Grb7, at least in tissue culture, was shown to be relocalized at the plasma membrane after insulin and growth factor stimulation. Unfortunately, we were unable to detect endogenous Grb7 protein directly in liver sections. However, we would like to propose that when cells are driven to divide by growth factor stimulation, Grb7 relocalizes at the membrane, in the same subcellular compartment as Rnd1, where they could interact in vivo. The low level of expression of both proteins and short life-time of Rnd1, as well as the transient nature of the interaction might explain why we have not been able to co-immunoprecipitate the endogenous proteins. Alternatively, such an interaction could take place between closely related members of these families such as Rnd3/RhoE and Grb10 or 14.

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