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Journal of Cellular Biochemistry 105:1172–1182 (2008)

Journal of Cellular Biochemistry

Mitogenic Roles of Gab1 and Grb10 as Direct Cellular Partners in the Regulation of MAP Kinase Signaling

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ABSTRACT

Functions of signaling mediators Grb10 or Gab1 have been described in mitogenesis but remained disconnected. Here, we report the peptide hormone-dependent direct association between Grb10 and Gab1 and their functional connection in mitogenic signaling via MAP kinase using cultured fibroblasts as a model. In response to PDGF-, IGF-I, or insulin increased levels of Grb10 potentiated cell proliferation or survival whereas dominant-negative, domain-specific Grb10 peptide mimetics attenuated cell proliferation. This response was sensitive to p44/42 MAPK inhibitor but not to p38 MAPK inhibitor. In response to IGF-I or insulin Raf-1, MEK 1/2, and p44/42 MAPK were regulated by Grb10 but not Ras or p38 MAPK. In response to PDGF MEK 1/2, p44/42 MAPK and p38 MAPK were regulated by Grb10 but not Ras or Raf-1. Peptide hormone-dependent co-immunoprecipitation of Grb10 and Gab1 was demonstrated and specifically blocked by a Grb10 SH2 domain peptide mimetic. This domain was sufficient for direct, peptide hormone-dependent association with Gab1 via the Crk binding region. In response to PDGF, IGF-I, or insulin, in a direct comparison, elevated levels of mouse Grb10 delta, or human Grb10 beta or zeta equally potentiated fibroblast proliferation. Proliferation was severely reduced by Gab1 gene disruption whereas an elevated Gab1 gene dose proportionally stimulated Grb10-potentiated cell proliferation. In conclusion, Gab1 and Grb10 function as direct binding partners in the regulation of the mitogenic MAP kinase signal. In cultured fibroblasts, elevated levels of human Grb10 beta, zeta or mouse Grb10 delta comparably potentiate mitogenesis in response to PDGF, IGF-I, or insulin. J. Cell. Biochem. 105: 1172–1182, 2008.

KEY WORDS: CELL-PERMEANT PEPTIDE; MAP KINASE; MITOGENESIS; PRO-RICH; SH2; SURVIVAL

Growth factor receptor binding protein 10 (Grb10) has been identified as a cellular partner of a number of receptor tyrosine kinases and other signaling mediators, compatible with multiple roles in metabolic, mitogenic, and embryogenic signaling that are also supported by its wide tissue distribution [Lim et al., 2004; Riedel, 2004; Holt and Siddle, 2005]. Grb10 was originally discovered as a partner of the epidermal growth factor (EGF) receptor [Ooi et al., 1995], however, a physiologic role of Grb10 in EGF action has not been established. Grb10 interacts with the Ret receptor tyrosine kinase that has been implicated in embryonic development with a role in cell differentiation, survival, and migration as well as in carcinogenesis [Pandey et al., 1995; Durick et al., 1996]. Grb10 has been identified as a target of the Eph-related receptor tyrosine kinase ELK which is involved in axonal guidance, neuronal bundling, and angiogenesis [Stein et al., 1996] and as a target of growth hormone receptor via Janus kinase Jak2 [Moutoussamy et al., 1998]. Grb10 associates with the insulin receptor (IR) [Liu and Roth, 1995; Hansen et al., 1996; O'Neill et al., 1996; Frantz et al., 1997] and the insulin-like growth factor-I (IGF-I) receptor (IGF-IR) [Dey et al., 1996; Morrione et al., 1996; Dong et al., 1997a,b] that carry out important metabolic and mitogenic functions, respectively [Laviola et al., 1997]. Grb10 binds to low-density lipoprotein receptor related protein 6 (LRP6), the Wnt co-receptor, and inhibits the Wnt signaling pathway—implicated in developmental and other signals including the progression to various cancers [Tezuka et al., 2007]. Grb10 interacts with ubiquitin ligase Nedd 4 [Morrione et al., 1999], the oncogenic tyrosine kinase Bcr-Abl [Bai et al., 1998], tyrosine kinase Tec [Mano et al., 1998; Pillai and Moran, 2002], MEK and Raf-1 [Nantel et al., 1998], Akt and c-Kit [Jahn et al., 2002], the regulatory subunit p85 of phosphatidylinositol (PI) 3-kinase [Deng et al., 2003], and GIGYF1 and GIGYF2 [Giovannone et al., 2003]. These interactions with numerous Tyr kinases and other signaling mediators define Grb10 as an adaptor or scaffold with the potential to integrate a variety of signals.

Grant sponsor: National Institutes of Health; Grant number: R01 CA77873; Grant sponsor: National Science Foundation; Grant number: MCB-9808795; Grant sponsor: American Diabetes Association; Grant number: 7-02-RA-76; Grant sponsor: Juvenile Diabetes Foundation International; Grant number: 197048.

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Received 7 December 2007; Accepted 29 April 2008 • DOI 10.1002/jcb.21829 • 2008 Wiley-Liss, Inc.
Published online 4 November 2008 in Wiley InterScience (www.interscience.wiley.com).

Based on structural similarities Grb10 is a member of a family of signaling mediators that also includes Grb7 and Grb14 [Daly, 1998]. Members of this family share a domain structure that is represented by N-terminal Pro-rich sequences, a homology domain with Caenorhabditis elegans MIG-10 (GM) [Manser et al., 1997] that includes a Ras-associating (RA)-like domain [Wojcik et al., 1999], a pleckstrin homology (PH) region, a C-terminal Src homology 2 (SH2) domain, and a receptor binding domain located between the PH and the SH2 domains termed BPS [Dong et al., 1998; He et al., 1998]. Several features are shared with the MRL protein family represented by MIG-10 [Holt and Daly, 2005]. At least seven alternative splice variants have been identified within the Grb10 gene, a proposed candidate for some types of human Silver-Russell syndrome, located on human chromosome 7 and mouse chromosome 11, and oppositely imprinted in both species [Jerome et al., 1997; Angrist et al., 1998; Hitchins et al., 2001; Lim et al., 2004; Riedel, 2004].

Based on their multi-domain structure and capacity to interact with numerous proteins simultaneously, signaling adaptors or scaffolds such as Grb10 or Gab1 are expected to assist in the formation of various multi-protein signaling complexes. Regarding the Grb10 domain structure considerable various information is available on the SH2 and BPS domains that have been implicated in the association with receptor tyrosine kinases including IR and IGF-IR [Dong et al., 1998; He et al., 1998; Wang et al., 1999]. A role of Grb10 N-terminal sequences has also been implicated in insulin action [Mori et al., 2005]. In addition, the Grb10 SH2 domain has been shown to associate with activated platelet-derived growth factor (PDGF) receptor beta (PDGFR), hepatocyte growth factor receptor (c-Met), and fibroblast growth factor receptor, but not with EGF receptor or nerve growth factor receptor TrkA in a direct comparison [Wang et al., 1999]. Grb10 or its SH2 domain are dimeric in solution and the crystal structure of the Grb10 SH2 domain reveals a non-covalent dimeric conformation unique to this protein family that will favor binding of dimeric, turn-containing phospho-Tyr sequences typical for IR and IGF-IR [Stein et al., 2003]. The Grb10 SH2 domain has been reported to associate, independently of phospho-tyrosine, with Raf1 constitutively, and with MEK1 in response to insulin [Nantel et al., 1998; Nantel et al., 1999]. Grb10 promotes Bad-dependent cell survival in association with active Raf-1 [Kebache et al., 2007].

Protein phosphorylation on Tyr, Ser, or Thr represent a key mechanism in signal transduction, however, its role in Grb10 regulation remains largely obscure. Grb10 phosphorylation has been reported in response to EGF (on Ser), PDGF, fibroblast growth factor [Ooi et al., 1995] or insulin, [Dong et al., 1997a]. Grb10 Ser phosphorylation by MAP kinase [Langlais et al., 2005], or in response to insulin or vanadate Tyr phosphorylation by Src family kinases such as Src and Fyn has been observed [Langlais et al., 2000]. Grb10 interaction with 14-3-3 proteins has been shown to be regulated by Grb10 phosphorylation [Urschel et al., 2005]. Grb10 has been described to play a role in insulin, IGF-I, VEGF action or malignant cell transformation in a variety of studies that sometimes appear conflicting. These studies have been cited and addressed in the Discussion also in the context of the results presented in this study and have not been duplicated here. Accordingly, a brief introduction to Gab1 signaling with relevant references has been provided in the Discussion.

While a role of Grb10 and the related adaptor protein Gab1 has been established in cellular signaling, a functional connection between both proteins has not been described. In this study we address their functional relationship with various strategies. We are evaluating the association of Grb10 with Gab1, the involved protein domains, the functional connection of both proteins in mitogenesis, and compare the regulation of individual components of the MAP kinase signaling pathway specifically in response to various peptide hormones. In conclusion, Gab1 and Grb10 function as direct binding partners in the regulation of the mitogenic MAP kinase signal. In cultured fibroblasts elevated levels of human Grb10 beta, zeta or mouse Grb10 delta comparably potentiate mitogenesis in response to PDGF, IGF-I, or insulin.

MATERIALS AND METHODS

All presented data are based on repeated experiments with the error between multiple measurements shown in bar graphs or with one representative experiment shown for immunoblots.

FUSION PEPTIDES, ANTIBODIES, PEPTIDE HORMONES, AND CELL LINES

Cell-permeant Grb10 domain-specific peptide mimetics [Deng et al., 2003] and a GST-Grb10 SH2 domain fusion peptide [Wang et al., 1999] were prepared as described earlier. Rabbit polyclonal antibodies against phospho-p44/42 MAPK, p38 MAPK, Ras, or Raf-1 were obtained from Cell Signaling Technology, Grb10 antibody from Santa Cruz Biotechnology, Gab1 rabbit polyclonal antibody from Upstate Cell Signaling Solutions, phospho-Tyr antibody from BD Biosciences, and horseradish peroxidase-coupled immunoglobulin G (IgG) antibody from Kirkegaard and Perry Laboratories. Human recombinant PDGF-BB, IGF-I, or insulin were obtained from Upstate Cell Signaling Solutions. In most experiments, NIH 3T3 mouse fibroblasts were used and for samples involving insulin stimulation a cell line stably expressing human IR was consistently employed [McClain et al., 1988]. Alternatively as presented in Figure 7, immortalized embryonic fibroblast cell lines Gab1 −/− and Gab1 +/+ derived from mice carrying Gab1 gene disruptions were kindly provided by Toshio Hirano (Osaka University, Japan) [Yamasaki et al., 2003]. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% (v/v) penicillin/streptomycin solution in a 5% CO_2 environment.

PLASMIDS AND cDNA EXPRESSION

Mouse Grb10 delta was transiently expressed under control of a constitutive cytomegalovirus transcriptional promoter from plasmids pHook2 (Invitrogen) [Deng et al., 2003] or CVN [Yousaf et al., 2001] that had been described earlier. Using a proposed consensus nomenclature [Daly, 1998] in this study, human Grb10 beta was transiently expressed from plasmid pBEX/Grb10 alpha [Liu and Roth, 1995] and human Grb10 zeta from plasmid pBEX/Grb10 gamma [Dong et al., 1997a], both kindly provided by Feng Liu,

University of Texas, San Antonio, based on a different nomenclature. Expression plasmids carrying cDNA encoding complete Gab1 or deletion mutants lacking the Gab1 Crk binding region (ΔCBR) or lacking additional amino terminal sequences (ΔN), the Gab1 PH domain (ΔPH) or Grb2 binding region (ΔGrb2) were kindly provided by Ute Schaeper, Max Delbrueck Center for Molecular Medicine, Berlin, Germany [Schaeper et al., 2000]. Subconfluent cell cultures in 8-cm plates were rinsed with antibiotic-free medium before 3 ml of transfection mixture including 5–6 μg of expression plasmid, 20 μl of Plus reagent, and 30 μl of Lipofectamine were added according to the instructions of the manufacturer (Invitrogen). After 5 h the transfection medium was replaced with complete cell culture medium including 10% FBS.

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Typically 10^6 NIH 3T3 mouse fibroblasts were transfected with expression plasmid and were propagated to quiescence for 20 h in serum-free DMEM supplemented with 0.1% BSA. Cell-permeant Grb10 domain-specific peptide mimetics at 10 μg/ml were added to the culture medium for 1 h before 100 ng/ml insulin, 100 ng/ml IGF-I, or 25 ng/ml PDGF were supplemented for 15 min. Cells were rinsed twice with ice-cold PBS and detergent cell extracts were prepared with lysis buffer containing 1% Triton X-100, 50 mM HEPES, pH 7.4, 10% glycerol, 137 mM NaCl, 10 mM NaF, 100 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 2 mM EDTA, 10 μg/ml leupeptin, and 1 mM PMSF. Cell extract containing 200–500 μg of total protein was incubated at 4°C with specific antibody and subsequently with 25 μl of protein A-Sepharose slurry (50% in the same buffer). Immune complexes were collected and rinsed three times by centrifugation at 4°C. Proteins were re-suspended in Laemmli loading buffer, boiled, separated by SDS–PAGE and transferred to a nitrocellulose membrane. Proteins were identified by immunoblotting with specific antibodies. For "far western" analysis [Vidal et al., 1999] a Gab1 blot was incubated with GST-Grb10 SH2 fusion protein, followed by GST-specific antibody (Santa Cruz Biotechnology), and finally with goat anti-mouse IgG horseradish peroxidase as tertiary antibody (Santa Cruz Biotechnology). Proteins were visualized with the ECL (enhanced chemiluminescence) detection system (Amersham Biosciences).

CELL PROLIFERATION

NIH 3T3 mouse fibroblasts (5×10^5) were plated and cultured on 24-well plates for 24 h in normal DMEM with 10% FBS. Cells were rinsed once and incubated with 100 ng/ml insulin, 100 ng/ml IGF-I, 100 ng/ml EGF, or 25 ng/ml PDGF in DMEM containing 1% FBS, and simultaneously with cell-permeant Grb10 domain-specific peptide mimetics at 10 μg/ml and with 20 μM MEK1/2 inhibitor PD098059 or 20 μM p38 MAP kinase inhibitor SB203580. Three days later 200 μl of 0.5 mg/ml 2-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was added for 4 h. The resulting product formazan was dissolved in isopropyl alcohol and quantified colorimetrically at OD_{570} [Denizot and Lang, 1986]. The obtained value has been presented as a measure of relative cell number. Sub-confluent mouse embryonic fibroblasts carrying Gab1 gene disruptions or control cells cultured in 6-well plates were transiently transfected by adding 4 μg Grb10 variant-specific and/or Gab1 expression plasmid and 10 μl Lipofectamine 2000 in 500 μl Opti-MEM I according to the instructions of the manufacturer (Invitrogen) for 24 h. Cells were subsequently trypsinized, transferred to 96-well plates and were incubated with peptide hormones as described above. Three days later 20 μl of MTT solution was added to evaluate relative cell numbers as described above.

CELL SURVIVAL

NIH 3T3 fibroblasts were transiently transfected with mouse Grb10 delta expression plasmid or control plasmid and cultured on 6-well plates. Cells were propagated in DMEM with 0.5% FBS for 4 days in various concentrations of insulin, IGF-I, or PDGF. Detached cells and adherent cells after trypsinization were collected, and both were quantified microscopically. The number of detached cells was displayed as the percent of the total cell number [Webb et al., 2000].

RESULTS

ATTENUATION OF INSULIN-, IGF-I-, OR PDGF-MEDIATED CELL PROLIFERATION BY CELL-PERMEANT Grb10 PEPTIDE MIMETICS OF THE SH2 DOMAIN OR PRO-RICH REGION

We had earlier reported the potentiation of DNA synthesis by mouse Grb10 delta cDNA transfection of fibroblasts while Grb10 domain-specific peptide mimetics interfered with the same response [Wang et al., 1999]. In the current study we show that these earlier observations fully extend to cell proliferation. For this purpose NIH 3T3 mouse fibroblasts were cultured with increasing doses of cell-permeant peptide mimetics either representing the Grb10 SH2 domain (Fig. 1a) or amino terminal Pro-rich region (Fig. 1b) in minimal serum and in the presence of specific peptide hormones. We found that either peptide attenuated cell proliferation in response to IGF-I or insulin in a dose-dependent fashion (Fig. 1a,b). However, only the SH2 domain peptide mimetic specifically attenuated the PDGF response (Fig. 1a), whereas for none of the peptides any impact was observed on the EGF response (Fig. 1a,b). These findings are reminiscent of our earlier reported observations at the level of DNA synthesis [Wang et al., 1999] and indicate the peptide hormone-specific role of Grb10 and the selectivity of the involved, individual protein domains [Deng et al., 2003].

Grb10-POTENTIATED CELL PROLIFERATION INVOLVING P44/42 MAP KINASE

In a complementary, positive experimental strategy we demonstrated potentiation of cell proliferation by Grb10 delta cDNA transfection in response to insulin, IGF-I, or PDGF (Fig. 2). Grb10 delta transfection in the absence of hormone (not shown) resulted in a basal level of cell proliferation similar to the level observed in the absence of Grb10 transfection (Fig. 2, solid bars). This is also supported by a comparison of NIH 3T3 and baby hamster kidney fibroblasts where the mitogenic response remained unaffected by increasing levels of Grb10 in the absence of a stimulatory hormone [Wang et al., 1999]. The use of specific MAP kinase inhibitors showed an impact of p44/42 inhibitor PD098059 but not of p38 inhibitor SB203580 (Fig. 2) for any of the hormones. As a result a role specifically of the MAP kinase p44/42 pathway was implicated in the regulation of cell proliferation by Grb10 as well as an

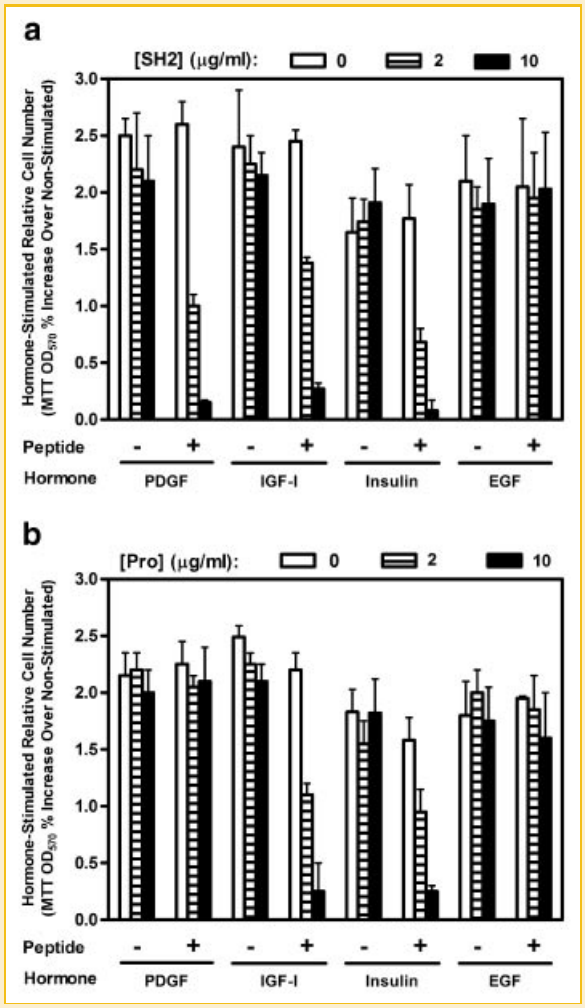

Fig. 1. Attenuation of insulin-, IGF-I-, or PDGF-mediated cell proliferation by cell-permeant Grb10 peptide mimetics of the SH2 domain or Pro-rich region. Mouse NIH 3T3 fibroblasts cultured in DMEM containing 1% FBS were incubated with 100 ng/ml insulin (using cells stably expressing human IR), 100 ng/ml IGF-I, 25 ng/ml PDGF, or 100 ng/ml EGF. In parallel, peptide mimetics of either (a) the Grb10 SH2 domain or (b) the amino terminal Pro-rich region were added to the culture medium at the three indicated doses (0, 2, or 10 μg/ml). After 3 days cell proliferation was evaluated by quantifying cell numbers biochemically based on mitochondrial succinate dehydrogenase activity through the colorimetric change of MTT. The OD_{570} was measured and the observed increase in response to each hormone over basal cell proliferation has been presented in percent. All data points were measured in duplicate as represented by the error bar.

alternative mechanism represented by the p44/42 MAP kinase inhibitor-independent component of the Grb10 response (Fig. 2).

Grb10-POTENTIATED CELL SURVIVAL

We also addressed whether this role would extend to the potentiation of cell survival. For this purpose we induced apoptosis of NIH 3T3 fibroblasts by serum withdrawal and measured the impact of Grb10 delta cDNA transfection on cell survival in the presence of peptide hormones. As expected, we observed that increasing concentrations of either PDGF, IGF-I, or insulin enhanced

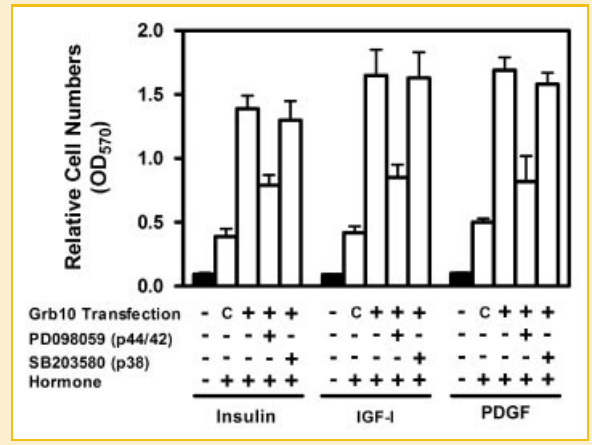

Fig. 2. Grb10-potentiated cell proliferation involving p44/42 MAP kinase. Mouse NIH 3T3 fibroblasts were transfected with mouse Grb10 delta expression plasmid (+) or control plasmid (C) or remained untransfected (−). Cells were incubated with 25 ng/ml PDGF, 100 ng/ml IGF-I, 100 ng/ml insulin (using cells stably expressing human IR), or were left untreated (Hormone:−) as indicated. In parallel, 20 μM p44/42 (PD098059) or p38 (SB203580) MAP kinase inhibitor was added as indicated (−/+). After 3 days of culture in DMEM containing 1% FBS, cell numbers were quantified biochemically based on mitochondrial succinate dehydrogenase activity through the colorimetric change of MTT. The OD_{570} is presented as a reflection of relative cell number. All data points were measured in duplicate as represented by the error bar.

cell survival in a dose-dependent fashion (Fig. 3). Grb10 delta cDNA transfection substantially potentiated cell survival up to ten-fold at the highest peptide hormone dose indicating that the mitogenic role of Grb10 in fibroblasts extends to the potentiation of cell survival. We had earlier shown that apoptosis correlates well with cell detachment in these experiments [Deng et al., 2007] and DNA fragmentation had been independently confirmed (not shown).

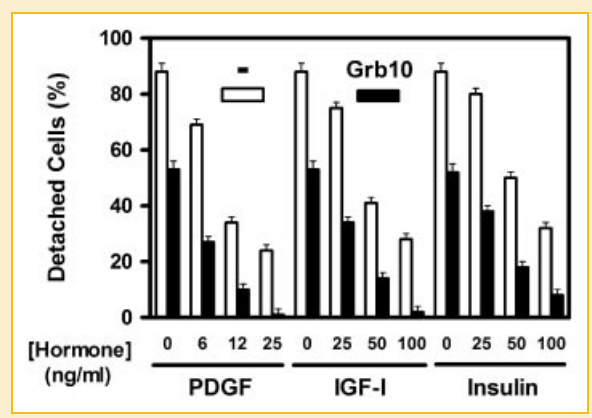

Fig. 3. Grb10-potentiated cell survival. Mouse NIH 3T3 fibroblasts were transfected with mouse Grb10 delta expression plasmid (solid bars) or control plasmid (empty bars). Cells were cultured in DMEM with 0.5% FBS and, where indicated, with increasing concentrations of PDGF (0, 6, 12, or 25 ng/ml), IGF-I (0, 25, 50, or 100 ng/ml), or insulin (0, 25, 50, or 100 ng/ml; using cells stably expressing human IR). After 4 days attached and detached cells were quantified. All data points were measured in triplicate as represented by the error bar.

REGULATION OF SPECIFIC COMPONENTS OF THE MAP KINASE PATHWAY BY COMPLETE Grb10 OR DOMAIN-SPECIFIC PEPTIDE MIMETICS

To address the participation of specific components of the MAP kinase signaling pathway, activation of individual mediators in response to PDGF, IGF-I, or insulin was measured after either Grb10 delta cDNA transfection or treatment with Grb10 peptide mimetics representing the SH2 domain or the amino terminal Pro-rich region.

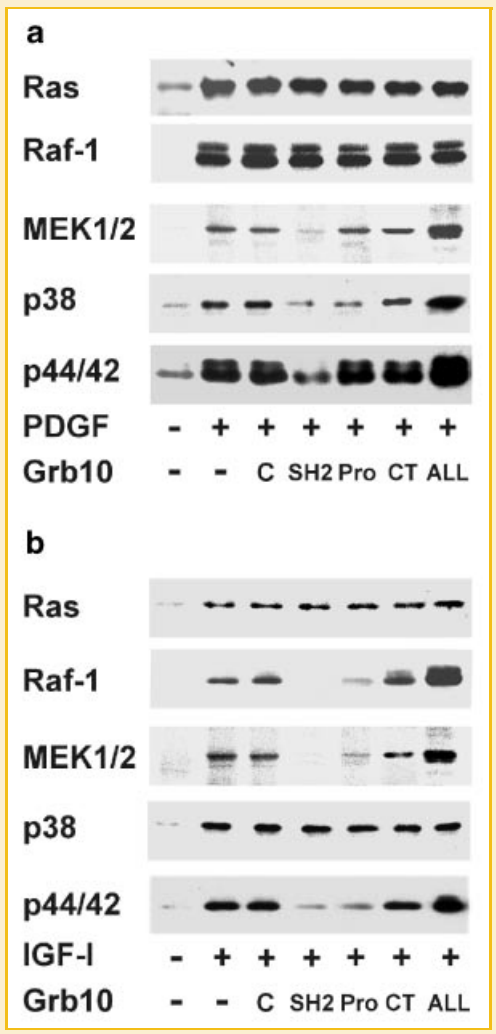

Fig. 4. Regulation of specific components of the MAP kinase pathway by complete Grb10 or domain-specific peptide mimetics. Mouse NIH 3T3 fibroblasts were transfected with mouse Grb10 delta expression plasmid (All), with control plasmid (CT) or, alternatively, were treated with peptide mimetics of the Grb10 SH2 domain, Pro-rich region, or control peptide samples (C). Cells were incubated for 15 min with (a) 25 ng/ml PDGF or (b) 100 ng/ml IGF-I. Detergent cell lysates were analyzed for activation of the various signaling mediators (indicated on the left) either by immunoblotting with phosphorylation-specific antibodies (after PAGE for Raf-1 or MEK1/2) or by using specific substrates/interacting peptides (p38: ATF-2, p44/42: Elk-1; followed by PAGE) subsequent to immunoprecipitation with phosphorylation-specific antibodies. Specific substrates were recognized by phosphorylation-specific antibodies or activated Ras-GTP (labeled as Ras) was precipitated with a peptide representing the Ras-binding domain of Raf-1. Proteins were visualized with the ECL detection system (Amersham).

Each of the peptide hormones activated any of the tested mediators and typically Grb10 delta cDNA transfection potentiated activation whereas the SH2 domain and Pro-rich peptide mimetics attenuated mediator activation with the following exceptions (Fig. 4): Unaffected by Grb10 only remained Ras and Raf-1 activation in response to PDGF (Fig. 4a) or Ras and p38 MAP kinase activation in response to IGF-I (Fig. 4b). Specifically in the PDGF response no impact was observed for the Pro-rich peptide on MEK1/2 or p44/42 activation. The insulin response (not shown) was essentially regulated in the same way as the IGF-I response (Fig. 4b). These results explain the sensitivity of the Grb10 delta potentiation of hormone-mediated cell proliferation to p44/42 inhibitor PD098059 (Fig. 2) as well as the lack of impact of the Pro-rich peptide specifically on PDGF-mediated cell proliferation (Fig. 1b). The Grb10-mediated regulation of p38 MAP kinase specifically in the PDGF response appears not to contribute to cell proliferation but instead to Grb10 regulation of cell migration (not shown).

MAPPING THE DIRECT Grb10-Gab1 INTERACTION

Looking into additional components of the Grb10 signaling network we tested for an interaction between signaling adapter Gab1 and Grb10. We identified a peptide hormone-dependent complex formed between both mediators that was demonstrated by immunoprecipitation with Gab1 antibody followed by immunoblotting with Grb10 antibody. This complex was observed in response to insulin (Fig. 5a), IGF-I or PDGF (not shown). The complex was disrupted by

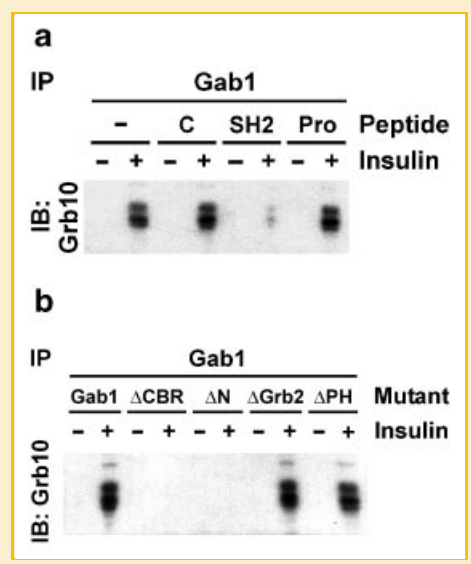

Fig. 5. Mapping the Grb10-Gab1 interaction. NIH 3T3 fibroblasts stably expressing human IR were transfected with expression plasmids encoding (a) normal Gab1 or (b) in addition with various Gab1 deletion mutants lacking the specific, indicated domains (see Materials and Methods Section). Cells were treated with 100 ng/ml insulin as indicated (+/−) and in (a) in addition with peptide mimetics of the Grb10 SH2 domain, Pro-rich region, or control peptide samples (C). Detergent cell lysates were prepared and immunoprecipitates with Gab1 antibody were analyzed in immunoblots with Grb10 antibody. Proteins were visualized with the ECL detection system (Amersham) and Grb10 is typically represented by a triplet of bands.

the addition of Grb10 SH2 domain peptide mimetic whereas no impact was observed for a peptide mimetic of the Grb10 Pro-rich region (Fig. 5a). cDNA transfection of a series of Gab1 deletion mutants defined that the amino terminal region, specifically the Crk binding region (CBR) of Gab1 was required for interaction with Grb10. Gab1 mutants lacking this region failed to associate with Grb10 in contrast to any other tested Gab1 mutant (Fig. 5b). The association was found to be direct in a ''far western'' blot (Fig. 6a). GST-Grb10 SH2 domain fusion peptide was found to bind to Gab1 in response to insulin when overlayed over a Gab1 blot. In contrast, no interaction was observed in the same blot with the Gab1 mutant lacking the Crk binding region (Fig. 6a). Association of the GST-Grb10 SH2 domain fusion peptide with Gab1 was also demonstrated in response to IGF-I or PDGF (not shown).

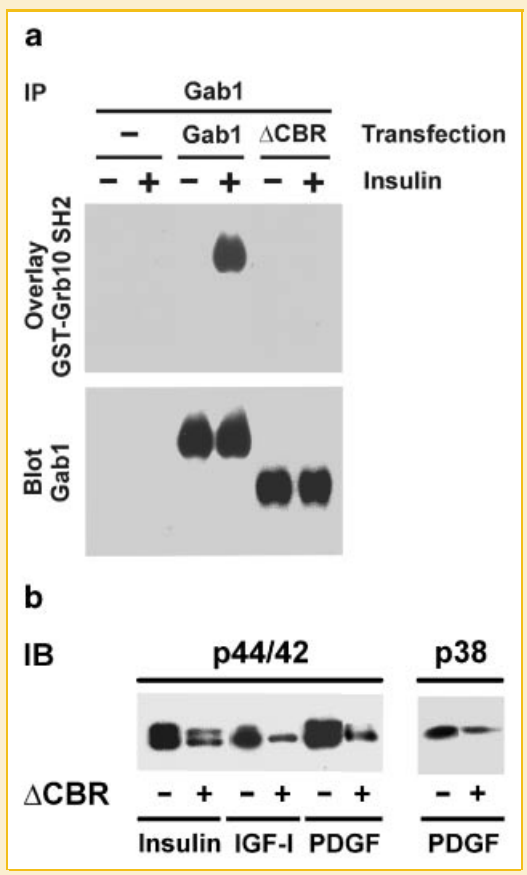

Fig. 6. Direct Gab1–Grb10 association and functional interaction. a: NIH 3T3 fibroblasts stably expressing human IR were transfected with expression plasmid encoding normal Gab1, a deletion mutant lacking the CBR domain (ΔCBR), or with control plasmid (–) as indicated and cells were subsequently treated with 100 ng/ml insulin (+/–). Detergent cell lysates were prepared and immunoprecipitates with Gab1 antibody were analyzed in immunoblots with the same antibody (lower panel: Blot Gab1) or in a ''far western'' overlay assay with GST-Grb10 SH2 domain fusion peptide (upper panel). b: NIH 3T3 fibroblasts were transfected with expression plasmid encoding a Gab1 deletion mutant lacking the CBR domain (ΔCBR) or with control plasmid (–) as indicated. Cells were incubated with 25 ng/ml PDGF, 100 ng/ml IGF-I, or 100 ng/ml insulin (using cells stably expressing human IR) as indicated. Detergent cell lysates were prepared and analyzed in immunoblots with p44/42 MAP kinase antibody (left panel) or p38 MAPK antibody (right panel). Proteins were visualized with the ECL detection system (Amersham).

Gab1–Grb10 COOPERATION IN CELL PROLIFERATION

Testing for a functional connection between Gab1 and Grb10 signaling we observed that activation of p44/42 MAP kinase in response to insulin, IGF-I, or PDGF was significantly reduced by cDNA transfection of a dominant-negative Gab1 mutant lacking the Crk binding region (Fig. 6b). This mutant also specifically reduced activation of p38 MAP kinase in response to PDGF (Fig. 6b) but not in response to insulin or IGF-I (not shown). In parallel, a role of Gab1 in the cell proliferation response to Grb10 was evaluated in fibroblasts expressing varying Gab1 levels down to zero. In these experiments three variants, mouse Grb10 delta (Fig. 7a), human Grb10 beta (Fig. 7b), and human Grb10 zeta (Fig. 7c) were directly compared by individual cDNA transfection to address any potential functional differences between the variants. All three Grb10 variants produced comparable results. Disruption of the Gab1 gene led to substantial reduction of cell proliferation that was only minimally stimulated by Grb10 in response to any hormone with little hormone preference. Normal Gab1 levels led to a strong proliferative response to Grb10 that showed a clear preference for IGF-I followed by PDGF and insulin. Elevated levels of Gab1 further potentiated basal and Grb10-stimulated cell proliferation with continued preference for IGF-I followed by PDGF and insulin that was now also observed in the absence of Grb10 transfection. These results indicate that the observed hormone-induced direct interaction between Grb10 and Gab1 reflects a functional connection between both mediators in the regulation of cell proliferation.

DISCUSSION

Gab1 and its family members are scaffolding adaptors that play a key role in cell signaling including proliferation, differentiation, development and likely oncogenesis downstream of multiple Tyr kinases including peptide hormone receptors [Liu and Rohrschneider, 2002]. Gab family interactions with receptors are typically indirect, frequently mediated via signaling mediator Grb2, and result in responses including the regulation of PI 3-kinase or phosphatase Shp2 [Gu and Neel, 2003]. Gab1 displays structural and functional similarity to insulin receptor substrate IRS-1 and both proteins like Grb10 play a role in the regulation of PI 3-kinase activity by direct association with p85 [Winnay et al., 2000; Deng et al., 2003; Gu and Neel, 2003; Nishida and Hirano, 2003]. Gab1 has also been described as a substrate of p44/42 MAP kinase [Lehr et al., 2004]. In this study we have evaluated the role of Gab1 in the context of Grb10 signaling. We observed peptide hormone-dependent association between Grb10 and Gab1 by coimmunoprecipitation (Fig. 5) that was found to be direct in a ''far western'' overlay assay (Fig. 6a). The Grb10 SH2 domain was sufficient for Gab1 association (Fig. 6a) and a Grb10 SH2 domain peptide mimetic competed with cellular Grb10-Gab1 association (Fig. 5a). Gab1 mutants lacking the Crk binding region (CBR) or lacking additional N-terminal sequences failed to associate with Grb10 suggesting that

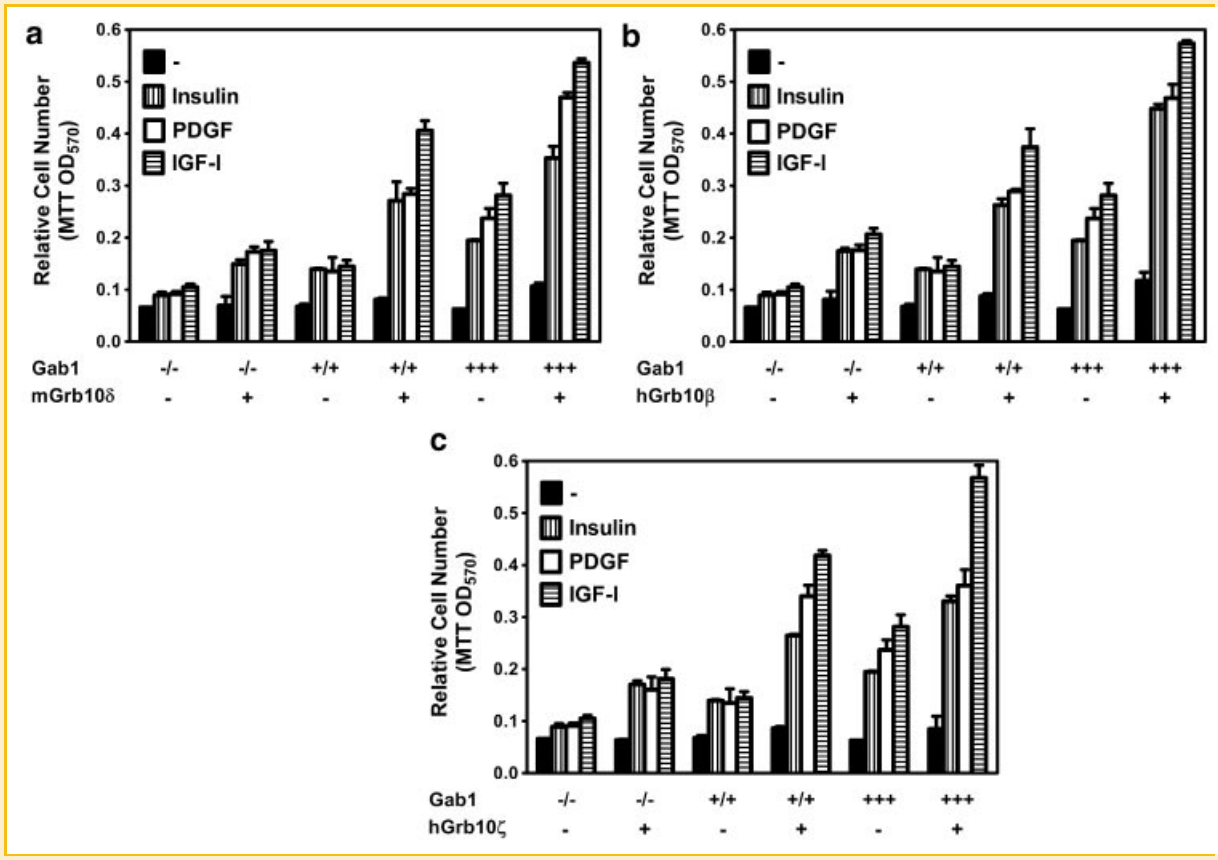

Fig. 7. Gab1 and Grb10 in cell proliferation. Fibroblasts derived from Gab1 gene disrupted mice (−/−), normal control mice (+/+), or normal control mice transfected with Gab1 expression plasmid (+++) were evaluated for cell proliferation in a comparison of three distinct transfected Grb10 variants. Each cell line was transfected with expression plasmid as indicated (+/−) encoding either (a) mouse Grb10 delta, (b) human Grb10 beta, or (c) human Grb10 zeta. Cells were cultured in DMEM with 1% FBS and either 100 ng/ml insulin, 100 ng/ml IGF-I, 25 ng/ml PDGF or no hormone (−) as indicated for 3 days. Cell numbers were quantified biochemically based on mitochondrial succinate dehydrogenase activity through the colorimetric change of MTT. The OD_{570} is presented as a reflection of relative cell number. All data points were measured in duplicate as represented by the error bar.

the CBR region may represent the site of Gab1 association with Grb10, whereas deletion of the Gab1 PH domain or Grb2 binding region did not impact on Grb10 binding (Figs. 5b and 6a). A functional connection between both mediators was indicated since expression of the dominant-negative Gab1 mutant lacking the CBR region interfered with PDGF, IGF-I, or insulin-mediated activation of p44/42 MAP kinase (Fig. 6b), a major Grb10-regulated mitogenic signal.

This functional connection was explored in fibroblasts expressing various levels of Gab1 and/or Grb10 (Fig. 7). Gab1 gene disruption materially interfered with cell proliferation in response to PDGF, IGF-I, or insulin that was only minimally increased by elevated levels of Grb10. Normal cellular levels of Gab1 restored peptide hormone stimulated cell proliferation and in combination with increased levels of Grb10 resulted in strong peptide hormone potentiation of cell proliferation with a preference for IGF-I followed by PDGF and insulin. Increased cellular levels of Gab1 significantly potentiated cell proliferation with a preference for IGF-I followed by PDGF and insulin that was substantially further potentiated by increased levels of Grb10. In a direct comparison between mouse Grb10 delta and human Grb10 beta and zeta comparable responses were observed suggesting a lack of isoform-specific functional differences in the observed mitogenic response (Fig. 7a–c). Our findings support a cooperative functional relationship between Grb10 and Gab1 in the regulation of mitogenesis. A role of Gab1 in mitogenesis extending to cell proliferation and transformation is well established [Holgado-Madruga and Wong, 2004; Mood et al., 2006].

Grb10 regulation of cell proliferation was significantly reduced by p44/42 MAP kinase inhibitor (Fig. 2) and involves p44/42 MAP kinase in response to any of the tested three peptide hormones (Fig. 4a,b). In response to insulin (not shown) or IGF-I, Grb10 regulated Raf-1, MEK1/2 and p44/42 MAP kinase without impacting on Ras activity whereas in response to PDGF Grb10 regulated MEK1/2 and p44/42 MAP kinase without impacting Ras or Raf-1. Bypassing Ras and/or Raf-1 as implicated by our results is consistent with the direct interaction reported between Grb10 and Raf1 or MEK1 [Nantel et al., 1998, 1999]. A role of Grb10 in cell survival involving Raf-1 has been described [Nantel et al., 1999; Kebache et al., 2007]. Grb10 potentiates cell migration (not shown) specifically in response to PDGF through p38 MAP kinase (Fig. 4a) potentially involving Grb10-Gab1 interaction. In contrast,

Grb10 potentiation of cell proliferation through p44/42 MAP kinase is observed in response to PDGF, IGF-I, or insulin (Fig. 2) as is Grb10 potentiation of cell survival (Fig. 3). Gab1 may participate in any of these mechanisms since it has been shown to regulate the p44/42 and p38 MAP kinase pathways and specifically in response to PDGF cytoskeletal reorganization and chemotaxis [Kallin et al., 2004; Meng et al., 2005].

A Grb10 SH2 domain peptide mimetic consistently interfered with cell proliferation (Fig. 1a) or p44/42 MAP kinase activation (Fig. 4) in response to PDGF, IGF-I, or insulin. However, in a direct comparison a Grb10 peptide mimetic of the N-terminal Pro-rich region only interfered with the IGF-I or insulin responses (Figs. 1b and 4) reminiscent of our earlier reported observations at the level of DNA synthesis [Wang et al., 1999]. These findings demonstrate the peptide hormone-specific role of Grb10 and the selectivity of the involved, individual protein domains [Deng et al., 2003] and suggest the participation of Grb10 in distinct signaling platforms. In the PDGF response a role of the N-terminal Grb10 Pro-rich region was not observed in cell proliferation (Fig. 1b) or p44/42 MAP kinase regulation (Fig. 4a) but was instead observed in p38 MAP kinase regulation (Fig. 4a). In contrast, a role of Grb10 in p38 MAP kinase regulation was not observed in the insulin (not shown) or IGF-I response (Fig. 4b).

Based on the existing reports Grb10 plays important roles in the signaling mechanism of numerous Tyr kinases including peptide hormone receptors. The exact role of Grb10 appears to be carefully governed by the specific cellular context based on the reported differences. Various Grb10 isoforms have been identified as cellular partners of the insulin receptor (IR) and insulin-like growth factor-I (IGF-I) receptor that provide the best-established regulators of Grb10 signaling. A regulatory role of Grb10 has been established in the respective metabolic and mitogenic responses by numerous lines of experimental evidence. However, the specific contribution of Grb10 appears to be highly dependent on the cellular context including the balance of other signaling mediators that may define whether increased Grb10 levels will potentiate or attenuate a specific cellular response.

In the mouse Grb10 gene disruption led to disproportionate embryonal overgrowth and during postnatal life to altered body composition, glucose homeostasis, enhanced insulin signaling and sensitivity [Charalambous et al., 2003; Smith et al., 2007; Wang et al., 2007]. Ubiquitin ligase Nedd4 appears to control IGF-I and insulin signaling partly through down regulation of Grb10 function [Cao et al., 2008]. Over-expression of the Grb10 gene caused postnatal growth retardation and insulin resistance via negative modulation of the IGF-IR and IR signaling pathways [Shiura et al., 2005] as well as type 2 diabetes in a non-obese mouse model [Yamamoto et al., 2008]. Disruption of the related Grb14 gene similarly potentiated glucose homeostasis and insulin signaling [Cooney et al., 2004] and a role of Grb14 as an inhibitor of IR activity has been described [Cariou et al., 2004]. On the other hand in cultured adipocytes Grb10 was found to potentiate insulin metabolic action by interacting with PI 3-kinase [Deng et al., 2003]. Evidence for a role of Grb7 in insulin action has not been found. Instead, a major role of Grb7 has been described in cell migration including its up-regulation in various human cancers as a partner of ErbB2 that may also reflect a role of Grb7 in mitogenesis [Shen and Guan, 2004].

Independent studies in fibroblasts addressing mitogenesis consistently showed an inhibitory role of individual Grb10 protein domains such as the SH2 domain that are expected to act dominant-negatively [O'Neill et al., 1996; He et al., 1998; Wang et al., 1999] and as also observed in this study (Fig. 1). In parallel potentiation of mitogenesis by increased levels of Grb10 was observed in this study (Fig. 2), or was reflected by the over-expression of cellular Grb10 observed in cervical squamous cell carcinoma and in Adenovirus 12-transformed cells [Wang et al., 1999; Guan et al., 2003; Okino et al., 2005]. Grb10 is highly amplified in breast cancer and has been identified as a potent breast cancer antigen in a mouse model where immunization with Grb10 over-expressing fibroblasts has resulted in immunity and instances of tumor rejection [O-Sullivan et al., 2008]. In addition, Grb10 was found to prevent Nedd4-mediated VEGFR-2 degradation [Murdaca et al., 2004] and to potentiate VEGF signaling [Giorgetti-Peraldi et al., 2001]. These studies consistently point to a role of Grb10 to potentiate mitogenesis whereas a number of alternative independent studies are consistent with a role of Grb10 to attenuate mitogenesis. Grb10 has been proposed to interfere with insulin signaling [Liu and Roth, 1995; Langlais et al., 2004] by physically blocking the interaction between IR and IRS [Wick et al., 2003] and anti-Grb10 strategies have been shown to potentiate IGF-I signaling in fibroblasts [Dufresne and Smith, 2005]. Grb10 over-expression was found to interfere with IGF-I-mediated but not with insulin-mediated cell proliferation [Morrione et al., 1997]. A Grb10/Nedd4 complex was observed to reduce the half-life of IGF-IR and its internalization via multiubiquitination [Morrione et al., 1999; Morrione, 2003; Vecchione et al., 2003; Monami et al., 2008]. Grb10 negatively regulates IR by mediating its insulin-mediated degradation [Ramos et al., 2006]. It is currently difficult to reconcile the various conflicting studies that frequently involve distinct experimental approaches, cellular backgrounds, and Grb10 variants. The observation that Grb10 also attenuates Wnt signaling [Tezuka et al., 2007] reflects the complexity of Grb10 function that can be expected to impact cellular signaling at multiple distinct levels including various developmental mechanisms.

Very few studies have addressed whether functional differences between Grb10 variants could explain some of the reported differences, however, the available data support comparable activity of the tested Grb10 variants in the potentiation of metabolic insulin action [Riedel, 2004] or mitogenesis (Fig. 7a–c). The interpretation of Grb10 signaling is complicated by the observation of apparently opposing Grb10 functions even in the same experimental system. This is exemplified by Grb10 knockdown experiments that were found to potentiate the phosphorylation of downstream signaling mediators such as IRS proteins, Akt, and p44/42 MAP kinase in response to IGF-I whereas the activation of the controlling signaling step, IGF-I receptor Tyr phosphorylation was found to be attenuated [Dufresne and Smith, 2005].

When considering all available observations it is evident that Grb10 restrains IGF-I-mediated growth, insulin signaling and glucose homeostasis in experimental mice. However, the potentiation of mitogenic responses or metabolic insulin action observed by Grb10 in cell culture as well as Grb10 over-expression in cancer cells

suggests more complex functional features that depend on the specific cellular context [Riedel, 2004]. Based on its interactions with a number of signaling mediators including protein kinases, other scaffold adapters such as Gab1, and enzymes such as ubiquitin ligase Nedd4, Grb10 may act as a signaling hub to integrate multiple incoming signals and as a molecular scaffold to help assemble distinct signaling platforms. The specific contribution of Grb10 in a signaling complex may depend on the local balance of associating mediators, including the ratio of competing signaling proteins and their affinity. In this context the same cellular level of Grb10 may potentiate or attenuate a specific signaling mechanism depending on the local distribution and level of specific Grb10 signaling partners. This concept is compatible with the diverse experimental observations on Grb10 function and emphasizes the importance of the specific cellular context to define the consequences of local changes in Grb10 distribution. To simply view Grb10 as either a positive or negative signaling mediator will be inadequate in reflecting the complexity that underlies the final output of the Grb10 signal. Grb10 has been appropriately described as an enigmatic regulator of more than insulin action [Holt and Siddle, 2005] and the continued dissection of its at times paradoxical molecular actions should reveal exciting new features in cell signaling.

ACKNOWLEDGMENTS

We thank Dr. Weidan Peng and Dr. Ruchi Tandon for their help with cDNA expression and cell-permeant peptide preparation, respectively. We are grateful to Dr. Nora Riedel for the critical discussion of experimental strategies, data, and the manuscript. This work was supported (to H.R.) in part by National Institutes of Health Grant R01 CA77873, by National Science Foundation Grant MCB-9808795, by American Diabetes Association Grant 7-02-RA-76, and by Juvenile Diabetes Foundation International Grant 197048.

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