

## mGrb10 Interacts with Nedd4\*

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**We have utilized the yeast two-hybrid system to identify proteins interacting with mouse Grb10, an adapter protein known to interact with both the insulin and the insulin-like growth factor-I receptors. We have isolated a mouse cDNA clone containing the C2 domain of mouse Nedd4, a ubiquitin protein ligase (E3) that also contains a hect (homologous to the E6-AP carboxyl-terminus) domain and three WW domains. The interaction with Grb10 in the two-hybrid system was confirmed using the full-length Nedd4, and it was abolished by deleting the last 148 amino acids of Grb10, a region that includes the SH2 domain and the newly identified BPS domain. The interaction between Grb10 and Nedd4 was also reproduced *in vivo* in mouse embryo fibroblasts, where endogenous Nedd4 co-immunoprecipitated constitutively with both the endogenous and an overexpressed Grb10. This interaction was Ca<sup>2+</sup>-independent. Grb10 interacting with Nedd4 was not ubiquitinated *in vivo*, raising the possibility that this interaction may be used to target other proteins, like tyrosine kinase receptors, for ubiquitination.**

Grb10, originally isolated using the CORT technique with the epidermal growth factor receptor (1), is a member of a family of adapter proteins that include at least seven isoforms in human and mouse. Grb10 was recently identified in our laboratory and in several others as an interacting partner with the IGF-I receptor (IGF-IR)<sup>1</sup> (2, 3), the insulin receptor (4–8), or both (9). All Grb10 isoforms contain a highly conserved SH2 domain at the C terminus, a pleckstrin homology domain in the central region and a less conserved N terminus, containing proline-rich sequences considered possible binding sites for SH3 domain-containing proteins (7, 10). Recently, another functional domain of the Grb10 protein has been identified and called BPS (between the pleckstrin homology and SH2 domains) domain (11).

The function of the different Grb10 isoforms is not fully elucidated and the data available are also partially discordant. We showed an inhibitory effect of mGrb10 (1) on IGF-I-mediated mitogenesis (12). An inhibitory effect on IR signaling was also reported with a human isoform (4), but O'Neill *et al.* (9)

reported opposite results on IGF-IR and IR signaling with another human variant. The most likely explanation of these data is that different isoforms may have different functions and may compete for common substrates. Recently, Grb10 has been identified as a maternally expressed imprinted gene (Meg) on mouse chromosome 11 (13), and it has been suggested that it may be a candidate gene for the Silver-Russell syndrome in humans. More recently, the hGrb10 SH2 domain has been shown to interact with both the Raf1 and MEK1 kinases, and these interactions were phosphotyrosine-independent (14). Grb10 has been also reported to interact with the growth hormone receptor (15), the ELK receptor (16), and BCR-ABL tyrosine kinase (17).

To identify other proteins that interact with Grb10, we used the yeast two-hybrid system (18) to screen a mouse embryo library (19) with full-length mGrb10 as a bait (see "Experimental Procedures"). We isolated a cDNA encoding the C2 domain of mouse Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4) (20, 21). Nedd4 is a ubiquitin protein ligase (E3) containing also three WW domains (22) involved in protein-protein interactions with proline-rich PY motifs (23) and a hect (homologous to the E6-AP carboxyl-terminus) domain bearing homology to a ubiquitin protein ligase (E3) enzyme (24). Using the deletion mutant of Grb10 in the two-hybrid system we show here that both the SH2 domain and the BPS domain of Grb10 are responsible for the binding to the C2 domain of Nedd4, with stronger binding mediated by the SH2 domain. The interactions of mGrb10 and Nedd4 was confirmed *in vivo* in mouse embryo fibroblasts using co-immunoprecipitation experiments which showed that the endogenous Nedd4 forms a constitutive, Ca<sup>2+</sup>-independent complex with Grb10, and this interaction is phosphotyrosine-independent. Grb10 was not ubiquitinated *in vivo* (25), raising the possibility that by interacting with Grb10, Nedd4 may come in contact with other proteins such as growth factor receptors and target them for ubiquitination.

### EXPERIMENTAL PROCEDURES

**Yeast Two-hybrid System**—pRK5-Grb10 plasmid (a kind gift from Dr. Ben Margolis) was cut with *Hind*III, treated with Klenow, and then cut with *Sac*I. The purified fragment, containing the full-length coding sequence for mGrb10, was cloned in the GAL4 DNA-binding domain of the pAS2-1 yeast cloning and expression vector, digested with *Sma*I (CLONTECH). The Y190 yeast strain (26) was first transformed with pAS2-1/Grb10 plasmid, tested for the expression of the hybrid protein by Western blotting using antibodies against the Gal4 DNA-binding domain (CLONTECH), and then transformed with a mouse embryo cDNA library, cloned in pVP16 vector (a kind gift of Drs. Stanley Hollenberg and Ann Vojtek (19)). Co-transformants were plated onto -Trp-Leu-His selective medium, supplemented with 25 mM 3-aminotriazole. His<sup>+</sup> colonies were then assayed for  $\beta$ -galactosidase activity by a filter assay as described (19). Segregation of the bait plasmid was performed by cyclohexamide selection (as described in CLONTECH instructions) and confirmed by replica plating on plates lacking Leu only or lacking both Trp and Leu, and  $\beta$ -galactosidase filter assay. Trp<sup>-</sup>

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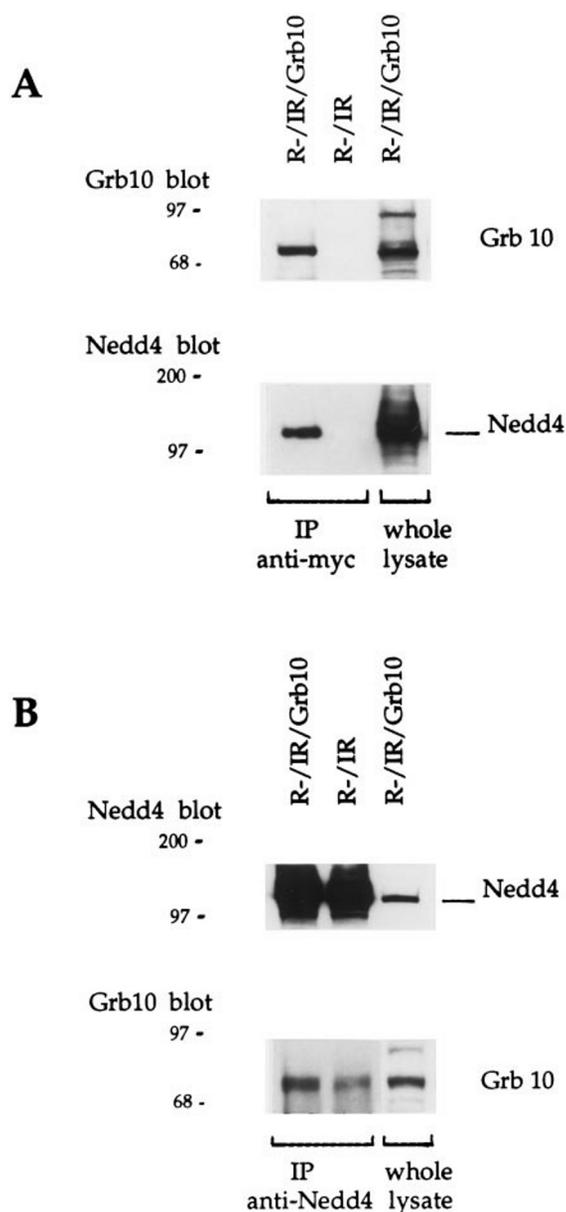
<sup>1</sup> The abbreviations used are: IGF, insulin-like growth factor; IGF-IR, IGF receptor 1; SH2, Src homology domain 2; IR, insulin receptor; PAGE, polyacrylamide gel electrophoresis; ENaC, epithelial Na<sup>+</sup> channel.



## RESULTS

**Grb10 Interacts with Nedd4 in the Two-hybrid System**—We used mGrb10 (1) as a bait to screen a mouse embryo library (19) in the yeast two-hybrid system (18). One of the cDNA clones isolated (see “Experimental Procedures”) encoded the N-terminal portion of mouse Nedd4 (20), spanning the entire C2/CalB (calcium lipid binding) domain (31, 32), from amino acid residue 65 to 237. A full-length Nedd4 was then used to confirm this interaction: as shown in Fig. 1, Grb10 strongly interacts with the full-length Nedd4 (amino acids 1–887), confirming the specificity of the interaction. In addition, no  $\beta$ -galactosidase activation was detected with the bait alone, or after co-transformation of the bait with the AD vector alone, or between an unrelated bait (laminin) and Nedd4 (data not shown). To localize the domain of Grb10 responsible for the interaction, we constructed a mGrb10 bait lacking the C-terminal 148 amino acids, a region that includes the newly identified BPS domain (11) and the SH2 domain (1). This deletion (Fig. 1) completely abolished the interaction with the C2 domain of Nedd4, while with the full-length Nedd4 the interaction was barely above a detectable level. This lack of binding was not due to lack of expression of the truncated bait, because immunoblotting with antibodies against the GAL4-binding domain revealed stronger expression of the truncated than of the full-length Grb10 (data not shown). To further narrow down the domain of Grb10 responsible for the interaction with Nedd4, we constructed three additional mGrb10 deletion mutants: one expressing the BPS domain alone (amino acids 475–518), one expressing the SH2 domain alone (amino acids 518–621), and one expressing both the BPS + SH2 domains (amino acids 475–621). As shown in Fig. 1, both the BPS (amino acids 475–518) and SH2 (amino acids 518–621) domains can independently bind Nedd4, but the SH2 domain seems to bind more strongly. The BPS+SH2 Grb10 mutant (amino acids 475–621) is interacting with Nedd4 with an affinity comparable with the one of the SH2 alone, confirming that the SH2 domain is likely the domain mostly responsible for the interaction. As a control, we tested the level of expression of the deletion mutant fusion proteins by Western blot using antibodies against the GAL4-binding domain; our results show that the mutant proteins were all expressed at comparable levels to the full-length mGrb10 (data not shown). In addition, no  $\beta$ -galactosidase activation was detected with any of the mutant baits alone, nor following co-transformation of the baits with the AD vector alone (data not shown).

**mGrb10 and Nedd4 Interact in Mammalian Cells**—To confirm these data in mammalian cells, we performed a series of co-immunoprecipitation experiments in mouse embryo fibroblasts (R-IR) or in mouse embryo fibroblasts overexpressing mGrb10 fused to a Myc tag (R-IR/Grb10) (12). In addition to Grb10, these cells express high levels of endogenous Nedd4 (Fig. 2, panel A). Thus, exponentially growing cells were lysed and immunoprecipitated with an anti-Myc antibody. As shown in Fig. 2, panel A, Grb10 was immunoprecipitated by Myc antibodies in R-IR/Grb10 cells, while no Grb10 protein was detectable in R-IR cells used as a control. Grb10 was clearly able to co-immunoprecipitate the endogenous Nedd4 expressed in R-IR/Grb10 cells. No Nedd4 protein was detectable in immunoprecipitated R-IR cells, confirming the specificity of Myc antibodies for the tagged Grb10. The same experiment was then repeated using Nedd4 antibodies: the endogenous Nedd4 (Fig. 2, panel B, overexposed to visualize the co-immunoprecipitated proteins), is co-immunoprecipitating both the overexpressed Myc-tagged Grb10 in R-IR/Grb10 cells and the endogenous Grb10 present in the R-IR cells. This experiment demonstrates that endogenous Nedd4 forms a complex with

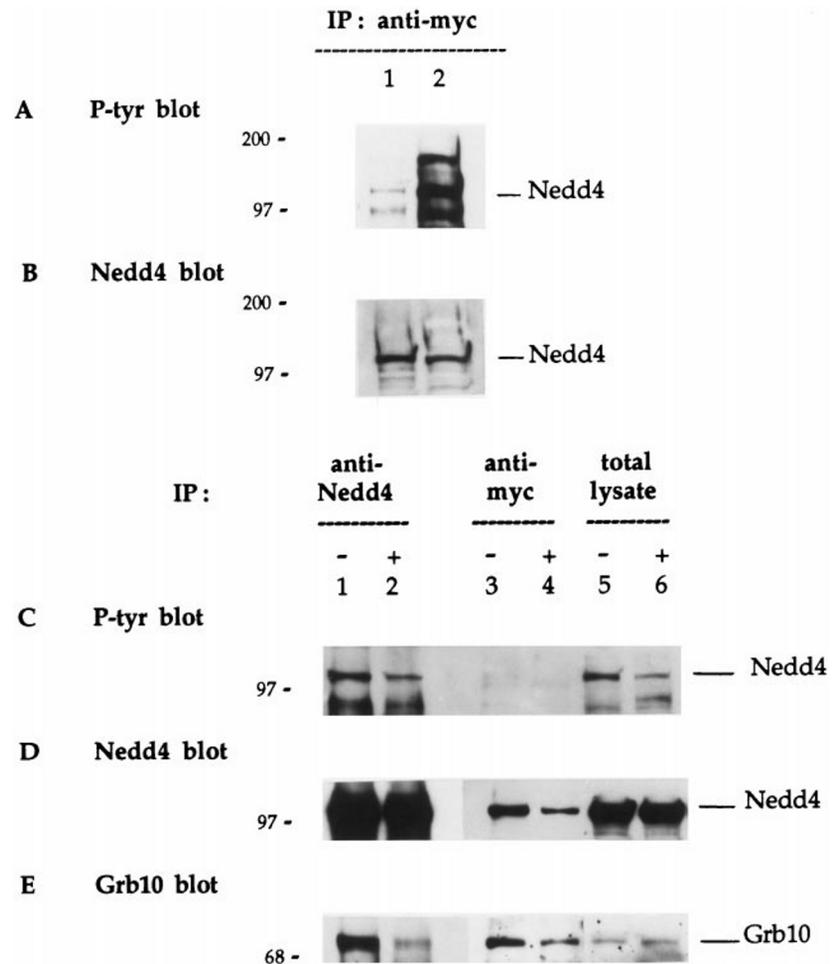


**FIG. 2. Grb10 co-immunoprecipitates *in vivo* with Nedd4.** Lysates from R-IR cells or R-IR-Grb10 cells overexpressing Grb10 fused to a Myc tag (see “Experimental Procedures”) were immunoprecipitated (IP) with either antibodies for the Myc tag (panel A) or anti-Nedd4 antibodies (panel B) and probed with Grb10 and Nedd4 antibodies. Whole cell lysates from a sample of R-IR/Grb10 cells was used as a control, for the correct size of the co-immunoprecipitating proteins.

endogenous Grb10 (or with heterologously expressed Grb10) in living cells, suggesting the two proteins likely interact with each other under physiological conditions.

**The Nedd4/Grb10 Interaction is  $Ca^{2+}$ -independent and Phosphorylation-independent**—Because the C2 domain of Nedd4 has been demonstrated to target Nedd4 to the plasma membrane in response to  $Ca^{2+}$  (27), we investigated whether  $Ca^{2+}$  also plays a role in the interaction between Grb10 and the Nedd4-C2 domain. To this end, we performed a series of co-immunoprecipitation experiments in R-IR cells overexpressing Grb10, starved in  $Ca^{2+}$ - and serum-free medium and then stimulated with  $Ca^{2+}$  in the presence of ionomycin (see “Experimental Procedures”). We routinely observed a slightly decreased immunoprecipitation of both Grb10 and Nedd4 by the respective antibodies in the presence of increasing  $Ca^{2+}$  concentrations, correlating with a decrease in the amount of the co-immunoprecipitating proteins (for Nedd 4 protein in panel

**FIG. 3. Grb10 preferentially associates *in vivo* with the unphosphorylated Nedd4 and the interaction is  $\text{Ca}^{2+}$ -independent.** Exponentially growing R-IR-Grb10 (1 mg) cells were immunoprecipitated with anti-Myc antibodies (panels A and B, lane 1) and probed with anti-phosphotyrosine antibodies (panel A) or anti-Nedd4 antibodies (panel B).  $\text{Ca}^{2+}$ - and serum-starved R-IR-Grb10 cells (panels C, D, and E, lanes 1, 3, and 5) were stimulated with  $\text{Ca}^{2+}$  + ionomycin (panels C, D, and E, lanes 2, 4, and 6) and immunoprecipitated with anti-Nedd4 antibodies (lanes 1 and 2) or anti-Myc antibodies (lanes 3 and 4), and probed with anti-phosphotyrosine (panel C), Nedd4 (panel D) or Grb10 (panel E) antibodies. Lane 2 in panels A and B, lanes 5 and 6 in panels C, D, and E are total cell lysates loaded as a control.



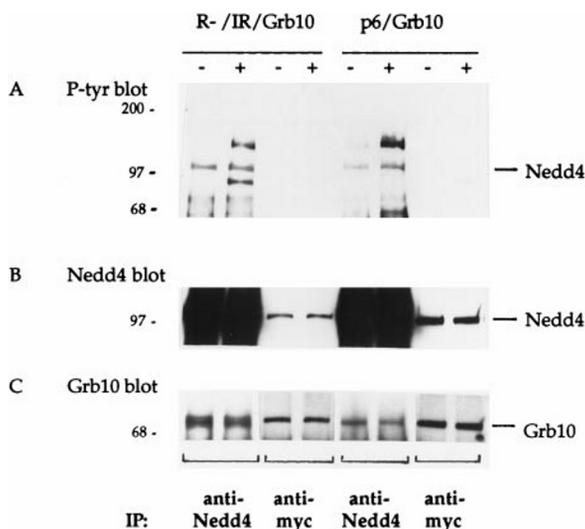
D, this is more evident in a shorter exposure of the film). This is probably due to a reduced affinity of the antibodies or reduced efficiency of the immunoprecipitation in the presence of increasing  $\text{Ca}^{2+}$  concentrations. Taking that into account our results show that there was no significant difference in the level of Grb10 and Nedd4 co-immunoprecipitating in the presence or absence of  $\text{Ca}^{2+}$  plus ionomycin treatment (Fig. 3 panels C, D, and E). Moreover, tyrosyl-phosphorylation of Nedd4 did not seem to be affected by increasing  $\text{Ca}^{2+}$  concentrations (Fig. 3C).

To further characterize the interaction between Grb10 and the C2 domain of Nedd4, we tested whether Nedd4 co-immunoprecipitating with Grb10 is tyrosyl-phosphorylated: thus, exponentially growing R-IR cells overexpressing Grb10 were immunoprecipitated with anti-Myc antibody to precipitate Grb10, and the blot was stained with anti-phosphotyrosine antibodies. As shown in Fig. 3 (panel A), there is a tyrosyl-phosphorylated band migrating at the expected size of Nedd4 (lanes 1 and 2). To confirm that this band is indeed Nedd4, the blot was stripped and reprobed with anti-Nedd4 antibodies (panel B); the Nedd4 protein (lane 1), clearly detectable in the co-immunoprecipitation, is perfectly overlapping with the tyrosyl-phosphorylated band shown in panel A (lane 1), suggesting that Nedd4 was tyrosyl-phosphorylated. However, only a small fraction of the total phosphorylated Nedd4 protein appears to co-immunoprecipitate with Grb10, suggesting that Grb10 may preferentially associate with the unphosphorylated Nedd4.

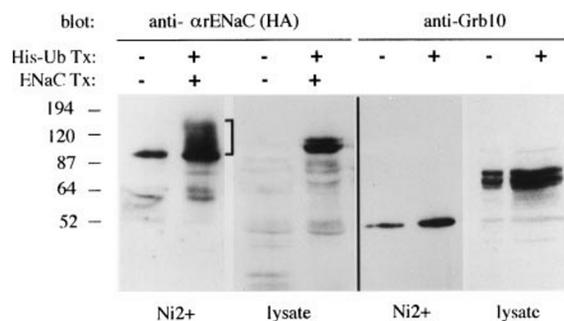
To investigate if the binding between Grb10 and Nedd4 is regulated by growth factors, we used two different cell lines overexpressing Myc-tagged mGrb10 in combination with either

the insulin receptor (R-IR/Grb10) or the IGF-IR (p6/Grb10) (12). The cells were serum-starved, stimulated with insulin (R-IR/Grb10) or IGF-I (p6/Grb10) (see "Experimental Procedures"), and then immunoprecipitated with either Nedd4 or Myc antibodies. As seen in Fig. 4, in both cell lines, we detected co-immunoprecipitation of Grb10 with Nedd4 in both stimulated and unstimulated cells. These results therefore demonstrate that the interaction is constitutive and not modulated by growth factors. The identity of the additional proteins immunoprecipitated by Nedd4 antibodies or co-precipitated with Nedd4 in either the insulin or the IGF-I-stimulated cells is currently unknown. Only a small fraction of the total Nedd4 protein appears to be tyrosine-phosphorylated either by insulin (in R-IR/Grb10 cells) or IGF-I (in p6/Grb10 cells), and a tyrosyl-phosphorylated Nedd4 is detectable in the co-immunoprecipitation by Myc antibodies only after a longer exposure of the film; it is likely therefore that the majority of the Nedd4 protein is co-immunoprecipitating with Grb10 in a phosphotyrosine-independent manner, in agreement with the data of Fig. 3, panels A and B, and the original identification of the interaction in the yeast two-hybrid system.

**Grb10 Is Not Ubiquitinated *In Vivo***—Because Nedd4 is an ubiquitin protein ligase (E3) (20, 21, 23, 24) we wanted to investigate if Grb10, which interacts with it, is a target for ubiquitination by Nedd4. We therefore tested whether Grb10 is ubiquitinated *in vivo*, using a previously described methodology (25). Thus, we transiently transfected R-IR cells overexpressing Grb10 with a plasmid encoding His-tagged multiubiquitin (30), precipitated the histidinated (hence ubiquitinated) cellular proteins with  $\text{Ni}^{2+}$ -agarose beads, and immunoblotted the precipitated proteins with anti-Grb10 antibodies. As can be



**FIG. 4. Effect of IGF-I and insulin on Grb10/Nedd4 binding.** R-/IR/Grb10 cells and p6/Grb10 cells were serum-starved for 72 h, stimulated (see "Experimental Procedures") with insulin (R-/IR/Grb10) or IGF-I (p6/Grb10), immunoprecipitated with either anti-Nedd4 or anti-Myc antibodies, and then probed with anti-phosphotyrosine, anti-Nedd4, and anti-Grb10 antibodies.



**FIG. 5. Lack of *in vivo* ubiquitination of Grb10 protein.** R-/IR/Grb10 cells stably expressing Grb10 were transiently transfected (+) or not (-) with HA-tagged  $\alpha$ ENaC (*ENaC Tx*) and/or His-ubiquitin (*His-Ub Tx*). The cells were lysed and lysates were incubated with  $\text{Ni}^{2+}$ -NTA agarose beads ( $\text{Ni}^{2+}$ ) to precipitate histidinated (ubiquitinated) proteins. These proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose, and blotted with (A) anti-HA antibodies (to detect ubiquitinated  $\alpha$ ENaC) or anti-Grb10 antibodies. Ubiquitinated species appear as a high molecular weight smear (indicated with a bar). Lysates represent expression of the protein.

seen in Fig. 5, we did not detect any ubiquitination of Grb10, despite relatively strong ubiquitination of the  $\alpha$  subunit of the epithelial  $\text{Na}^+$  channel ( $\alpha$ ENaC) used as a positive control (25). These results show that Grb10 is not ubiquitinated *in vivo* in exponentially growing R-/IR cells overexpressing Grb10. We repeated this experiment in p6/Grb10 cells and once again did not detect any ubiquitination of Grb10 (data not shown).

#### DISCUSSION

To identify new interacting partners for Grb10, we performed a yeast two-hybrid screen of a mouse embryo library (19) using mGrb10 as a bait, and identified Nedd4 (20, 21) as a Grb10 interacting protein. We further showed that (i) the C2 domain (31, 32) of Nedd4 is sufficient for the interaction. (ii) A deletion of the mGrb10 C-terminal 148 amino acids, a region that includes the newly identified BPS domain (11) and the SH2 domain, abolishes the interaction with Nedd4. (iii) The SH2 domain alone of Grb10 shows the strongest interaction with Nedd4 but the BPS domain alone is also able to bind Nedd4. (iv) Grb10 and Nedd4 interact *in vivo*, as assessed by co-immunoprecipitation experiments. (v) The unphosphoryl-

ated Nedd4 preferentially co-immunoprecipitated with Grb10, and this interaction is  $\text{Ca}^{2+}$ -independent. (vi) The interaction is constitutive and (vii) Grb10 is not ubiquitinated *in vivo* in mouse embryo fibroblasts over-expressing Grb10.

Mouse Nedd4 (20) has been shown to be expressed in a variety of embryonic tissues and to localize in the cytoplasm (21). Nedd4 is a ubiquitin protein ligase (E3) also containing 3 WW domains (in the mouse) (22) and a hect domain (24). Not much is known on the biological role of mouse Nedd4: the WW domains of rat Nedd 4 have been shown to interact with the epithelial sodium channel (ENaC), recognizing proline-rich PY motifs (23), while the C2 domain is mediating  $\text{Ca}^{2+}$ -dependent translocation to the plasma membrane (27).

C2 domains (31, 32) encompass about 130 residues (including the calcium lipid-binding region) (32), and usually contain 5 conserved aspartates which provide  $\text{Ca}^{2+}$ -binding sites (33–35). The domain has been shown to mediate  $\text{Ca}^{2+}$ -stimulated phospholipid and membrane binding (27, 36). Evidence is accumulating showing that some C2 domains can bind proteins as well (37–39): the fact that Grb10 is interacting with Nedd4 in a  $\text{Ca}^{2+}$ -independent manner is in agreement with increasing lines of evidence showing that some C2 domains which are  $\text{Ca}^{2+}$ -regulated can bind other molecules in a  $\text{Ca}^{2+}$ -independent manner (for a review, see Ref. 32).

The interaction between Grb10 and Nedd4 *in vivo* is phosphotyrosine-independent, and is not influenced by mitogenic agents: our finding that an SH2 domain can bind a C2 domain in a phosphotyrosine-independent manner is novel and interesting, as it suggests an association which does not involve the binding pocket in the SH2 domain reserved for phosphotyrosine. Other phosphotyrosine-independent interactions with SH2 domains have already been reported in the literature (40–43), and it has been recently shown that the Grb10 SH2 domain can bind Raf1 and MEK1 kinases in a phosphotyrosine-independent manner (14). How the SH2 domain of Grb10 binds the C2 domain of Nedd4 is unknown, and we cannot currently preclude the possibility of phospholipids involvement in this interaction, since the latter have been demonstrated to bind both SH2 and C2 domains (32, 44).

Ubiquitination of proteins usually tag them for rapid degradation (for a review, see Ref. 45) and many lines of evidence are now accumulating on the role of the ubiquitin-proteasome system in regulating and degrading a number of cytosolic proteins, including cell cycle proteins (see for review, Ref. 46). Some transmembrane proteins are ubiquitinated as well, including several tyrosine kinase receptors like the epidermal growth factor receptor and the platelet-derived growth factor receptor (47, 48), and ubiquitination seems to be involved in their subsequent degradation by the endosomal/lysosomal pathway (49). We could not detect any ubiquitination *in vivo* (25) of Grb10: however, we can speculate that the interaction between Grb10 and Nedd4 may be used to target other proteins, such as the IGF-I receptor or the insulin receptor, for ubiquitination. The biological role of the interaction between Grb10 and Nedd4 and a possible role of this complex in tyrosine kinase receptors ubiquitination is currently under investigation.

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