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Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin I

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ABSTRACT **Grb2 is a 25-kDa adaptor protein composed of a Src homology 2 (SH2) domain and two flanking Src homology 3 (SH3) domains. One function of Grb2 is to couple tyrosine-phosphorylated proteins (through its SH2 domain) to downstream effectors (through its SH3 domains). Using an overlay assay, we have identified four major Grb2-binding proteins in synaptic fractions. These proteins interact with wild-type Grb2 but not with Grb2 containing point mutations in each of its two SH3 domains corresponding to the loss of function mutants in the *Caenorhabditis elegans* Grb2 homologue sem-5. Two of the proteins, mSos and dynamin, were previously shown to bind Grb2. The third protein of 145 kDa is brain specific and to our knowledge has not been previously described. The fourth protein is synapsin I. Dynamin is required for synaptic vesicle endocytosis and synapsin I is thought to mediate the interaction of synaptic vesicles with the presynaptic cytomatrix. These data suggest that Grb2, or other proteins containing SH3 domains, may play a role in the regulation of the exo/endocytotic cycle of synaptic vesicles and therefore of neurotransmitter release.**

Src homology 2 (SH2) and Src homology 3 (SH3) domains are protein modules involved in protein–protein interactions including those mediating regulatory cascades from cell surface receptors to intracellular effector proteins. SH2 and SH3 domains, which were originally recognized in c-Src (1), interact with phosphotyrosine-containing sequences and proline-rich sequences, respectively. SH2 and SH3 domains often coexist in the same protein, including members of a group of signaling molecules that do not have any detectable intrinsic enzyme activity and that are referred to as adaptor proteins (2). One example of an adaptor molecule is growth factor receptor-bound protein 2 (Grb2), which is composed of a single SH2 domain and two flanking SH3 domains (3) and is homologous to the product of the *Caenorhabditis elegans* gene *sem-5* (4). A convergence of genetic studies in *C. elegans* and *Drosophila*, coupled with biochemical studies in mammalian cells, have demonstrated that one of the functions of Grb2 is to link tyrosine-phosphorylated receptors to Ras activation via the binding of Grb2 to Sos, a guanine nucleotide exchange factor for Ras (4–12). In addition to its role in the Ras signaling pathway, Grb2 appears to be involved in other pathways, including the regulation of membrane ruffle formation, possibly through its interactions with the cytoskeleton (13, 14).

Dynamin is a GTPase (15), which is required for synaptic vesicle endocytosis as indicated by its identity with the product of the *Drosophila shibire* gene (16, 17). A mutation of the dynamin gene in *Drosophila* leads to a temperature-sensitive block of neurotransmitter release due to a lack of synaptic vesicle membrane endocytosis and recycling. Dynamin is also involved in clathrin-mediated endocytosis in nonneuronal cells (18, 19). Neuronal dynamin is identical to dephosphin, a protein that undergoes stimulus-dependent dephosphorylation in the nerve terminal (20). It has recently been demonstrated that the proline-rich tail of dynamin binds the SH3 domains of Grb2, and that Grb2 stimulates dynamin's GTPase activity (21–23). Interestingly, a proline-rich C terminus similar to that present in dynamin is also present in synapsin I (24), a synaptic vesicle-associated nerve terminal protein (25, 26) that interacts with actin and is thought to cross-link synaptic vesicles to the actin-based cytomatrix of nerve terminals (28). Synapsin I is phosphorylated in an activity-dependent manner and its phosphorylation is thought to modulate the availability of synaptic vesicles for exocytosis (28). Synapsin I is highly homologous to synapsin II, which appears to have a similar function but lacks the proline-rich C-terminal region (24). This region of the molecule, which also contains the phosphorylation sites for Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) (28) and proline-directed protein kinase (29), is thought to have a regulatory role on synapsin I function. The presence of similar C termini on two proteins that appear to play crucial roles in the exo/endocytosis of synaptic vesicles raises the possibility that Grb2, or another SH3-containing protein, may regulate some aspects of the vesicle cycle. We have investigated here whether Grb2 binds synapsin I and/or other synaptic proteins.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies against synaptophysin (30) and synapsin I (31) have been described. A polyclonal antibody against mSos was from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies against dynamin (18, 20) were generous gifts of Sandra Schmid (Scripps Research Institute, La Jolla, CA) and Thomas Südhof (Southwestern Medical Center, Dallas). Rabbit polyclonal antibody against recombinant glutathione *S*-transferase (GST) was prepared by standard techniques.

Grb2 and Grb2 Mutants. Grb2 was cloned from a human cerebellar λgt11 library by PCR using oligonucleotide primers (30-mers) from human Grb2 (3) and was subcloned into pGEX-2t. The loss of function N-terminal SH3 mutant P49L (Pro-49 to Lys) and C-terminal SH3 mutant G203R (Gly-203 to Arg) were prepared in pGEX-2t as described (3). A construct containing both SH3 mutations (double mutant) was prepared by digestion of the N-terminal and C-terminal Grb2 mutants in pGEX-2t with *Eco*RI and *Sty* I, with the

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Abbreviations: GST, glutathione *S*-transferase; EGF, epidermal growth factor; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; SH2 and SH3, Src homology 2 and 3.

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ligation of the insert containing the C-terminal mutation into the vector containing the N-terminal mutant. DH5α cells were transformed to ampicillin resistance with pGEX-2t alone or pGEX-2t containing wild-type or mutant forms of Grb2. Large-scale cultures of LB with carbenicillin were inoculated with small overnight cultures, grown at 37°C, and induced for 2.5 hr with 0.5 mM isopropyl β-D-thiogalactopyranoside, and the GST fusion proteins or GST alone were purified on glutathione-agarose beads as described (32).

Subcellular Fractionation. Synaptic fractions were prepared from rat brain as described (25). To obtain S_3 and P_3, the S_2 fraction from Huttner *et al.* (25) was centrifuged at 165,000 × g_{av} for 2 hr. Rat tissues were homogenized in buffer A (0.3 M sucrose/0.83 mM benzamidine/0.23 mM phenylmethylsulfonyl fluoride/10 mM Hepes·OH, pH 7.4) (1:10, wt/vol) with a Polytron (Brinkman) for ≈30 sec and centrifuged at 800 × g_{max} for 5 min to obtain postnuclear supernatants. Lysates from HER14 cells treated with or without epidermal growth factor (EGF) were prepared as described (3).

Grb2 Overlay. Protein fractions on nitrocellulose membranes were blocked for 1 hr in Blotto [150 mM NaCl/5% (wt/vol) nonfat dry milk/50 mM phosphate, pH 7.5], rinsed with water, and incubated for 1 hr in buffer B (3% bovine serum albumin/0.1% Tween 20/1 mM dithiothreitol/20 mM Tris·HCl, pH 7.4) containing 4.5 μg of GST fusion proteins per ml. The nitrocellulose was then washed in Blotto, rinsed with water, and incubated for 1 hr in buffer B minus dithiothreitol containing a rabbit anti-GST antibody (1:500). Bound anti-GST antibodies were revealed as described (33).

Synapsin I/Grb2 Solution Assay. Grb2–GST and Grb2 double-mutant–GST were coupled to glutathione-Sepharose (2 μg of fusion protein per μl of Sepharose beads). After equilibration in binding buffer (150 mM NaCl/1% Triton X-100/10 mM Hepes·OH, pH 7.4), 12.5 μl of fusion protein-coupled beads were incubated with 500 ng of purified synapsin I in 50 μl of binding buffer for 3 hr at 4°C. The beads were then pelleted by microcentrifugation, and the supernatant fraction was collected. The beads were then washed by resuspending two times in binding buffer before being resuspended in SDS sample buffer.

Synapsin I/Grb2 Cosedimentation Assay. Grb2–GST fusion proteins (1 μM) were incubated for 20 min at room temperature in 225 mM glycine/40 mM NaCl/7.75 mM Tris·HCl/3.75 mM Hepes, pH 7.7 (final vol, 100 μl) in the absence or presence of purified synapsin I (46) (1 μM). After the incubation, samples were centrifuged in a Beckman TL-100 at 132,000 × g for 20 min. Pellets were resuspended in SDS sample buffer and subjected to SDS/PAGE. Gels were stained with Coomassie brilliant blue and analyzed with a laser scanning densitometer (LKB Ultrascan XL). Absolute amounts of Grb2 in the pellet were calculated by interpolation from a Grb2–GST standard curve.

Miscellaneous Procedures. For dynamin immunoprecipitation, the S_3 fraction from rat brain (60 μg) was incubated with 10 μl of anti-dynamin rabbit anti-serum or PBS for 4 hr at 4°C, followed by the addition to each tube of 25 μl of protein A-Sepharose. After 2 hr, the beads were pelleted by centrifugation and the unbound material was prepared for SDS/PAGE. Synapsin I was phosphorylated in the presence of protein kinase A and CaM kinase II and digested with *Staphylococcus aureus* V8 protease as described (34).

RESULTS

Proteins from a crude synaptic vesicle fraction (LP_2) were separated by SDS/PAGE, transferred to nitrocellulose, and overlaid with Grb2 expressed as a GST fusion protein. Binding of the fusion protein was then revealed with antibodies against GST. Five major Grb2-binding proteins of approximate molecular mass 170, 145, 100, 80, and 75 kDa were detected (Figs. 1 and 2). The proteins of 170, 145, and 100 kDa were also the major Grb2-binding proteins in total brain homogenate (refs. 21 and 23; data not shown). GST alone or GST fusion protein encoding Grb2 with single amino acid mutations in both the N-terminal and C-terminal SH3 domains (corresponding to the loss of function mutants in the Grb2 homologue sem-5) (3, 4) did not exhibit any binding (Fig. 2). These results demonstrate that the observed interactions are specific and are mediated through the SH3 domains of Grb2.

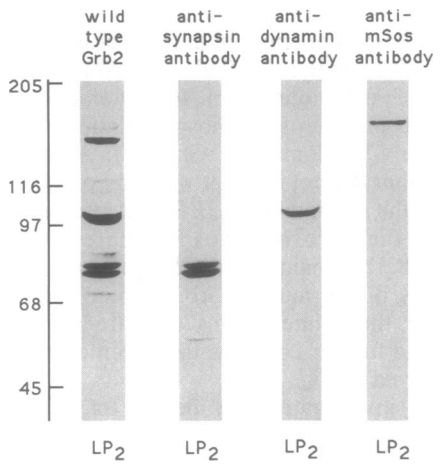

FIG. 1. Grb2-binding proteins in a synaptic fraction from rat brain. Proteins from a crude synaptic vesicle fraction (LP_2; 200 μg) were separated by SDS/PAGE on 3–12% acrylamide gradient gels, transferred to nitrocellulose, and overlaid with wild-type GST–Grb2 fusion protein. Fractions from the same gel were reacted with polyclonal antibodies against dynamin and mSos or with a monoclonal antibody specific for synapsin I. The migration of molecular mass markers (kDa) is indicated on the left.

To test that the mutant Grb2 was expressed in a functional form, we performed overlay assays on HER14 cells, which express high levels of EGF receptor (3). Wild-type Grb2 interacted with a number of proteins in these cells in the absence of EGF stimulation, including a protein at 170 kDa, which has been identified as mSos (Fig. 3) (refs. 3 and 5; data

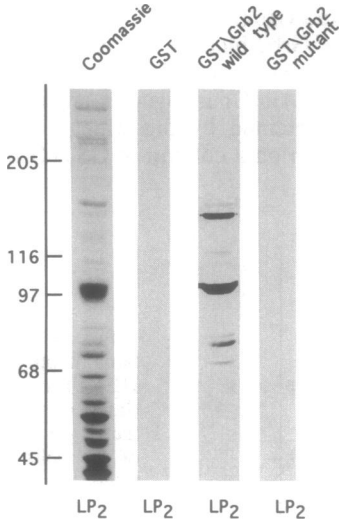

FIG. 2. Grb2 interactions are mediated through SH3 domains. Proteins of a crude synaptic vesicle fraction (LP_2; 200 μg) were separated by SDS/PAGE on 3–12% acrylamide gradient gels, stained by Coomassie brilliant blue (Coomassie), or transferred to nitrocellulose and overlaid with GST fusion protein, wild-type GST–Grb2 fusion protein, or GST–Grb2 fusion protein containing single amino acid mutations in both the Grb2 N-terminal and C-terminal SH3 domains. Numbers on left are kDa.

not shown). When the cells were stimulated with EGF, the wild-type Grb2 interacted with an additional protein of 180 kDa, which has been identified as the EGF receptor (Fig. 3) (refs. 3 and 5; data not shown). This interaction is dependent on EGF receptor tyrosine phosphorylation and is mediated through the SH2 domain of Grb2 (3). Although the Grb2 double mutant did not interact with any of the Grb2-binding proteins in the unstimulated HER14, it retained the ability to recognize the tyrosine-phosphorylated EGF receptor through its SH2 domain (Fig. 3). These data demonstrate that the inability of the mutant Grb2 to bind target proteins is specific for those interactions that are mediated by SH3 domains and further confirms the specificity of the interactions observed in Fig. 1.

We next investigated the identity of the Grb2-binding proteins present in the LP_2 fraction. mSos, a known Grb2-binding protein, is present in brain (35) and migrates on SDS/polyacrylamide gels with an apparent molecular mass of 170 kDa (5). Immunoblot analysis of brain fractions with an antibody directed against mSos revealed a band of the same molecular mass as the 170-kDa Grb2-binding protein, suggesting that this protein is mSos (Fig. 1). Consistent with this result, the 170-kDa Grb2-binding protein had a widespread tissue distribution (Fig. 4*A*), similar to mSos (35).

A 100-kDa protein that binds Grb2 in solution is dynamin (21–23). Immunoblot analysis of the LP_2 fraction with anti-dynamin antibody labeled a band with the same electrophoretic migration as the 100-kDa Grb2-binding protein (Fig. 1). The identity of this protein as dynamin was confirmed by its depletion from brain extracts by immunoprecipitation with anti-dynamin antibodies (Fig. 4*B*). Dynamin is present in mammals in at least three forms: one that is highly expressed in brain (15, 36), a second that is specifically expressed in testis (36), and a third that has a broad tissue distribution (37, 38). Grb2-binding proteins of 100 kDa were detected in a broad range of tissues including testis (Fig. 4*C*). This is in contrast to the results obtained with a polyclonal antibody specific to neuronal dynamin (20), which recognized a band in brain but showed no reactivity in the 100-kDa region with the other tissues (Fig. 4*C*). These results suggest that the nonneuronal dynamin isoforms, similar to the neuronal form of dynamin, bind Grb2.

The 145-kDa Grb2-binding protein appears to be brain specific and to our knowledge has not been previously described (Fig. 4*A*). Unambiguous amino acid sequences from peptides obtained by digestion of the protein after partial purification on a column of GST–Grb2 were used to search the protein sequence data bases, revealing no significant homologies (data not shown).

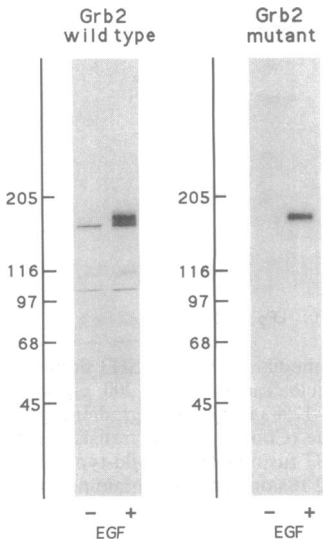

FIG. 3. SH3 mutant contains functional SH2 domains. HER14 cells were treated (lanes +) or mock treated (lanes –) with EGF. Lysates of the cells were then separated by SDS/PAGE on 3–12% acrylamide gradient gels, transferred to nitrocellulose, and overlaid with wild-type GST–Grb2 fusion protein or the GST–Grb2 fusion protein containing point mutations in both SH3 domains. For both gels, the migration of molecular mass markers (kDa) is indicated on the left.

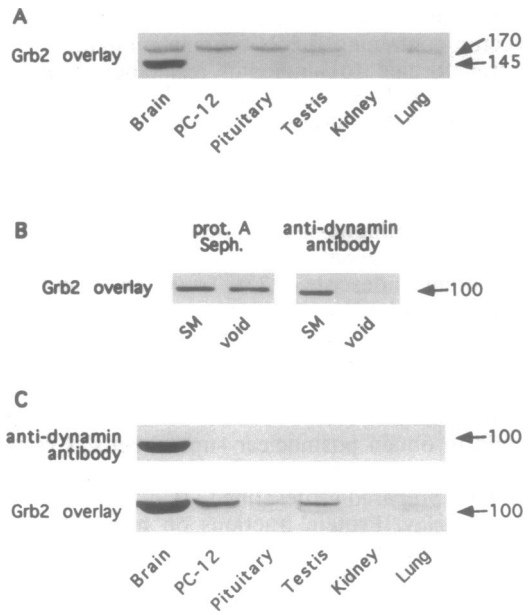

FIG. 4. Identification and tissue distribution of Grb2 binding proteins. (*A*) Proteins from postnuclear supernatants (200 μg) were overlaid with wild-type GST–Grb2 fusion protein. Arrows on right indicate migratory positions of the 170-kDa (mSos) and the 145-kDa Grb2-binding proteins. (*B*) A soluble brain fraction was subjected to immunoprecipitation with anti-dynamin polyclonal antibody followed by protein A-Sepharose or protein A-Sepharose alone. The supernatant (void) was processed along with an equal amount of starting material (SM) for Grb2 overlay. (*C*) Proteins from postnuclear supernatants were overlaid with wild-type GST–Grb2 fusion protein or reacted with polyclonal antibody against dynamin. Arrows on right indicate migratory positions of the 100-kDa Grb2-binding protein (dynamin).

Synapsins Ia and Ib migrate on SDS/polyacrylamide gels as a doublet with an apparent molecular mass of 75 and 80 kDa. Several antibodies directed against synapsin I recognized a protein doublet in the LP_2 fraction with the same electrophoretic mobility as the 75- and 80-kDa Grb2-binding proteins (Fig. 1; data not shown). Synapsins IIa and IIb did not interact with Grb2 in this assay (data not shown). The interaction of Grb2 with synapsins Ia and Ib was confirmed by using purified synapsin I in solution assays. Synapsin I specifically bound to glutathione-Sepharose beads containing wild-type Grb2 fusion protein but did not interact with beads containing double-mutant Grb2 fusion protein (Fig. 5).

Several similar, yet distinct, proline-rich consensus binding sequences for SH3 domains have been proposed (5, 21, 39–41). Synapsin I has two proline-rich regions containing several sequences that are similar or identical to proposed consensus sequences. One proline-rich region, located in domain B in the N terminus of synapsin I, is relatively well conserved in synapsin II. A second region, in domain D of the C terminus, is specific to synapsin I (24). To determine which region of synapsin I binds Grb2, V8 protease digestion of synapsin I was used to generate a 10-kDa N-terminal fragment containing a single phosphorylation site for CaM kinase I and protein kinase A and a 35-kDa C-terminal fragment containing two CaM kinase II-specific sites (42) (Fig. 6). Grb2 overlay of V8-digested synapsin I demonstrated that Grb2 bound only to the C-terminal fragment of synapsin I (Fig. 6). This finding is consistent with the lack of interaction of Grb2 with synapsin II.

Purified synapsin I was overlaid with GST–Grb2 or GST–Grb2 containing point mutations in its SH3 domains. Wild-

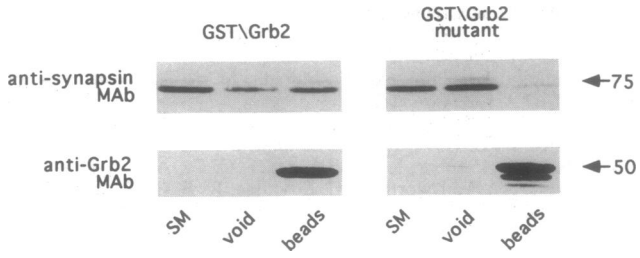

FIG. 5. The 75-kDa Grb2-binding protein is synapsin I. Purified synapsin I was incubated with GST–Grb2 or GST–double-mutant Grb2 coupled to glutathione-Sepharose. The glutathione-Sepharose (beads) were subjected to SDS/PAGE along with the supernatants (void) and equal aliquots of the starting material (SM). Samples were then reacted with anti-synapsin I (*Upper*) or anti-Grb2 (*Lower*) monoclonal antibodies (mAb).

type Grb2 interacted strongly with synapsins Ia and Ib, whereas the double mutant showed no interaction (Fig. 7 *Upper*). Grb2 containing single point mutations in either its N-terminal or C-terminal SH3 domains interacted with synapsin I but in a greatly reduced manner (Fig. 7 *Upper*), demonstrating that both of Grb2's SH3 domains participate in its interaction with synapsin.

Since the proline-rich C terminus of synapsin I has several potential binding sites for SH3 domains, and both SH3 domains of Grb2 can interact with synapsin I, we tested the possibility that Grb2 may crosslink synapsin I into large, macromolecular complexes by centrifugation experiments. The addition of purified synapsin I (1 μM) to wild-type GST–Grb2 fusion protein (1 μM) increased the precipitation of the fusion protein to >4-fold over control values in the absence of synapsin I (Fig. 7 *Lower*). Addition of synapsin I to the C-terminal mutant, the N-terminal mutant, or the double-mutant GST–Grb2 fusion proteins did not lead to a significant increase in sedimentation over control values.

DISCUSSION

We have examined the interaction of Grb2 with rat brain proteins and have identified the major Grb2-binding proteins in crude synaptic vesicle fractions. Grb2 fusion proteins containing point mutations in their SH3 domains were used to confirm the SH3 domain specificity of the interactions. Two of the Grb2-binding proteins identified here are mSos and neuronal dynamin, both of which are known Grb2-

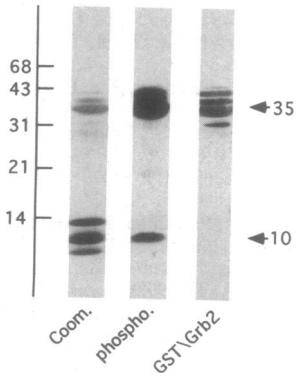

FIG. 6. Grb2 binds to the proline-rich C terminus of synapsin I. Purified synapsin I was digested with V8 protease and the digested protein was analyzed by SDS/PAGE on a 15% gel. Gels were stained with Coomassie (Coom.) or transferred to nitrocellulose and overlaid with wild-type GST–Grb2 fusion protein. Purified synapsin I was phosphorylated with [^{32}P]ATP by CaM kinase II before V8 protease digestion. Phosphorylated, digested synapsin I was analyzed on SDS/polyacrylamide gel, and the gels were dried and used for autoradiography (phospho.). Numbers on left and right are kDa.

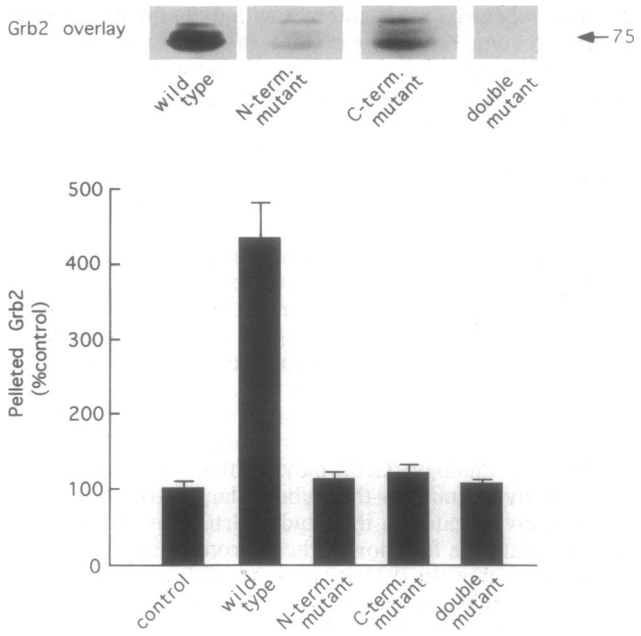

FIG. 7. Characterization of the Grb2–synapsin I interaction. (*Upper*) Purified synapsin I was overlaid with wild-type GST–Grb2 fusion protein or with various GST–Grb2 fusion proteins containing point mutations in either their N-terminal or C-terminal SH3 domains or in both SH3 domains. (*Lower*) Purified synapsin I was incubated with GST–Grb2 fusion protein or various mutant forms of GST–Grb2 fusion proteins. Samples were then subjected to centrifugation, and the amount of Grb2 in the pellet was quantitated by scanning densitometry of Coomassie-stained polyacrylamide gels. Amount of Grb2 in the pellet is expressed as percentage control, with 100% representing the amount of GST–Grb2 fusion protein in the pellet after incubation and centrifugation in the absence of synapsin I (100% equals 226 ng per sample; ≈10% of total Grb2).

binding proteins containing proline-rich regions (5–12, 21–23). Nonneuronal forms of dynamin have recently been identified (36–38). Our results suggest that both neuronal and nonneuronal forms of dynamin bind Grb2. The proline-rich tail of dynamin is the most divergent region among the three isoforms, but the conservation of the Grb2-binding activity indicates an important role for this property in the regulation of dynamin's function. The third Grb2-binding protein, of 145 kDa, to our knowledge has not been previously described and is enriched in nerve terminals (P.S.M. and P.D.C., unpublished data).

The fourth protein is synapsin I. Our data suggest that synapsin I can bind both SH3 domains of Grb2 via at least two distinct sites within its C-terminal proline-rich domain. This region is thought to be involved in the regulation of synapsin I function since its phosphorylation induces a conformational change in the molecule, which affects its binding to synaptic vesicles and actin (43, 44). Targeted deletion of the synapsin I gene was found to be compatible with nearly normal synaptic function, possibly due to redundant function of synapsin I and synapsin II (45). However, an increase in paired-pulse facilitation was observed (45), consistent with an involvement of the proline-rich C terminus of synapsin I in the modulation of neurotransmitter release. Also, in the case of dynamin, the proline-rich C terminus appears to have a regulatory role in the function of the molecule. Phosphorylation of the C terminus or the binding of Grb2 to this region both stimulate the GTPase activity of dynamin (20–22).

Here we have shown that the SH3 domains of Grb2 bind to at least two, and possibly three, proteins that play a key role in synaptic vesicle function. These results raise the possibility that SH3 domain-containing proteins in the nerve termi-

nal, possibly Grb2 itself, interact with dynamin and synapsin I and play some role in the regulation of the exo/endocytotic cycle of synaptic vesicles. Although the interaction of SH3 domains with dynamin appears rather promiscuous *in vitro* (21, 22), dynamin has been shown to coimmunoprecipitate with Grb2 (23). A number of SH3 domain-containing proteins, including spectrin (1), SAP90 (47), $pp60^{c\text{-}src}$ (48), and Grb2 (P.S.M. and P.D.C., unpublished observation) are present in nerve terminals. Grb2 is the only full-length SH3 domain-containing protein that has been tested so far for an interaction with synapsin I. However, we have tested isolated SH3 domains from c-src and SAP90, neither of which reacted with synapsin I in our assay. It will be important to identify the relevant SH3 domain-containing proteins that bind dynamin and synapsin I *in vivo*.

The main known function of Grb2 is to recruit mSos to the plasma membrane where it can promote GDP/GTP exchange on Ras. By analogy, Grb2 may participate in presynaptic function by regulating the subcellular distribution of the proteins investigated in this study. Grb2 may also allosterically regulate the function of these proteins as suggested by its stimulation of the GTPase activity of dynamin (21, 22). It is of interest that synapsin I can be phosphorylated *in vitro* by mitogen-activated protein kinase, a downstream effector of Ras (A.J.C. and P.G., unpublished data). There is some evidence to suggest that Ras participates in the regulation of vesicular traffic. For example, brain-derived neurotrophic factor, which is known to activate a tyrosine kinase receptor, trk-B, was recently shown to modulate neurotransmitter release (49). Ras is involved in the translocation of glucose transporters to the cell surface in adipocytes (50), and microinjection of Ras into rat embryo fibroblasts stimulated an increase in membrane ruffling and pinocytosis (51). An attractive possibility is that signaling through tyrosine phosphorylated proteins may converge on synapsin I by two independent pathways: activation of mitogen-activated protein kinase and direct binding of SH2/SH3-containing adaptor proteins.

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1. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C. & Pawson, T. (1991) *Science* **252**, 668–674.
2. Pawson, T. & Schlessinger, J. (1993) *Curr. Biol.* **3**, 434–442.
3. Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D. & Schlessinger, J. (1992) *Cell* **70**, 431–442.
4. Clark, S. G., Stern, M. J. & Horvitz, H. R. (1992) *Nature (London)* **356**, 340–344.
5. Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B. & Schlessinger, J. (1993) *Nature (London)* **363**, 85–88.
6. Simon, M. A., Dodson, G. S. & Rubin, G. M. (1993) *Cell* **73**, 169–177.
7. Oliver, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. & Pawson, T. (1993) *Cell* **73**, 179–191.
8. Chardin, P., Camonis, J. H., Gale, N. W., Van Aelst, L., Schlessinger, J., Wigler, M. H. & Bar-Sagi, D. (1993) *Science* **260**, 1338–1343.
9. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M. & Weinberg, R. A. (1993) *Nature (London)* **363**, 45–51.
10. Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J. & Bar-Sagi, D. (1993) *Nature (London)* **363**, 88–92.
11. Rozakis-Adcock, M., Fernely, R., Wade, J., Pawson, T. & Bowtell, D. (1993) *Nature (London)* **363**, 83–85.
12. Buday, L. & Downward, J. (1993) *Cell* **73**, 611–620.
13. Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V. & Schlessinger, J. (1993) *Cell* **74**, 83–91.
14. Matuoka, K., Shibasaki, F., Shibata, M. & Takenawa, T. (1993) *EMBO J.* **12**, 3467–3473.
15. Obar, R. A., Collins, C. A., Hammarback, J. A., Shpetner, H. S. & Vallee, R. B. (1990) *Nature (London)* **347**, 256–261.
16. Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A., Wadsworth, S. C. & Vallee, R. B. (1991) *Nature (London)* **351**, 583–586.
17. van der Bliek, A. M. & Meyerowitz, E. M. (1991) *Nature (London)* **351**, 411–414.
18. van der Bliek, A. M., Redelmeier, T. E., Damke, H., Tisdale, E. J., Meyerowitz, E. M. & Schmid, S. L. (1993) *J. Cell Biol.* **122**, 553–563.
19. Herskovits, J. S., Burgess, C. C., Obar, R. A. & Vallee, R. B. (1993) *J. Cell Biol.* **122**, 565–578.
20. Robinson, P. J., Sontag, J.-M., Liu, J.-P., Fyske, M., Slaughter, C., McMahon, H. & Südhof, T. C. (1993) *Nature (London)* **365**, 163–166.
21. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., Campbell, I. D. & Waterfield, M. J. (1993) *Cell* **75**, 25–36.
22. Herskovits, J. S., Shpetner, H. S., Burgess, C. C. & Vallee, R. B. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11468–11472.
23. Miki, H., Miura, K., Matuoka, K., Nakata, T., Hirokawa, N., Orita, S., Kaibuchi, K., Takai, Y. & Takenawa, T. (1994) *J. Biol. Chem.* **269**, 5489–5492.
24. Südhof, T. C., Czernik, A. J., Kao, H.-T., Takei, K., Johnston, P. A., Horiuchi, A., Kanazir, S. D., Wagner, M. A., Perin, M. S., De Camilli, P. & Greengard, P. (1989) *Science* **245**, 1474–1480.
25. Huttner, W. B., Schiebler, W., Greengard, P. & De Camilli, P. (1983) *J. Cell Biol.* **96**, 1374–1388.
26. De Camilli, P., Harris, S. M., Huttner, W. B. & Greengard, P. (1983) *J. Cell Biol.* **96**, 1355–1373.
27. Bar-Sagi, D. & Feramisco, J. R. (1986) *Science* **233**, 1061–1068.
28. Greengard, P., Valtorta, F., Czernik, A. J. & Benfenati, F. (1993) *Science* **259**, 780–785.
29. Hall, F. L., Mitchell, J. P. & Vulliet, P. R. (1990) *J. Biol. Chem.* **265**, 6944–6948.
30. Jahn, R., Schiebler, W., Ouimet, C. & Greengard, P. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4137–4141.
31. Mandell, J. W., Czernik, A. J., De Camilli, P., Greengard, P. & Townes-Anderson, E. (1992) *J. Neurosci.* **12**, 1736–1749.
32. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67**, 31–40.
33. McPherson, P. S. & Campbell, K. P. (1990) *J. Biol. Chem.* **265**, 18454–18460.
34. Llinas, R., Gruner, J. A., Sugimori, M., McGuiness, T. L. & Greengard, P. (1991) *J. Physiol. (London)* **436**, 257–282.
35. Bowtell, D., Fu, P., Simon, M. & Senior, P. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6511–6515.
36. Nakata, T., Takemura, R. & Hirokawa, N. (1993) *J. Cell Sci.* **105**, 1–5.
37. Cook, T. A., Urrutia, R. & McNiven, M. A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 644–648.
38. Sontag, J.-M., Fykse, E. M., Ushkaryov, Y., Liu, J.-P., Robinson, P. J. & Südhof, T. C. (1994) *J. Biol. Chem.* **269**, 4547–4554.
39. Cicchetti, P., Mayer, B. L., Thiel, G. & Baltimore, D. (1992) *Science* **257**, 803–806.
40. Ren, R., Mayer, B. J., Cicchetti, P. & Baltimore, D. (1993) *Science* **259**, 1157–1161.
41. Yu, H., Chen, J. K., Feng, S., Delgarno, D. C., Brauer, A. W. & Schreiber, S. L. (1994) *Cell* **76**, 933–945.
42. Huttner, W. B. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5402–5406.
43. Benfenati, F., Neyroz, P., Bähler, M., Masotti, L. & Greengard, P. (1990) *J. Biol. Chem.* **265**, 12584–12595.
44. Benfenati, F., Valtorta, F., Rubenstein, J. L., Gorelick, F., Greengard, P. & Czernik, A. J. (1992) *Nature (London)* **359**, 417–420.
45. Rosahl, T. W., Geppert, M., Spillane, D., Herz, J., Hammer, R. E., Malenka, R. C. & Südhof, T. C. (1993) *Cell* **75**, 661–670.
46. Bähler, M. & Greengard, P. (1989) *Nature (London)* **326**, 704–707.
47. Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D. & Garner, C. C. (1993) *J. Biol. Chem.* **268**, 4580–4583.
48. Linstedt, A. D., Vetter, M. L., Bishop, J. M. & Kelly, R. B. (1992) *J. Cell Biol.* **117**, 1077–1084.
49. Lohof, A. M., Ip, N. Y. & Poo, M. (1993) *Nature (London)* **363**, 350–353.
50. Kozma, L., Baltensperger, K., Klarlund, J., Porras, A., Santos, E. & Czech, M. P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4460–4464.