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Grb2 and Its Apoptotic Isoform Grb3-3 Associate with Heterogeneous Nuclear Ribonucleoprotein C, and These Interactions Are Modulated by Poly(U) RNA*

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Grb2 is an adaptor molecule comprising one Src homology (SH) 2 and two SH3 domains. This protein has a natural isoform named Grb3-3 with a deletion within the SH2 domain. Numerous evidence points to a functional connection between SH2- and SH3-containing proteins and molecules implicated in RNA biogenesis. In this context, we have examined the binding of Grb2 and Grb3-3 to heterogeneous nuclear ribonucleoprotein (hnRNP) C. By the use of an *in vivo* genetic approach and through *in vitro* experiments, we furnish evidence that both Grb2 and Grb3-3 interact with hnRNP C proteins. Subcellular fractionation studies clearly show that Grb2 is partially localized in the nucleus. In addition, coimmunoprecipitation experiments demonstrate that Grb2·hnRNP C complexes exist in intact hematopoietic cells. The carboxyl-terminal SH3 domains of Grb2 and Grb3-3 are primarily responsible for the association with hnRNP C. However, although the proline-rich motif of hnRNP C is involved in the interaction with Grb2, it is not in the binding to Grb3-3. Furthermore, poly(U) RNA inhibits the association of Grb2 with hnRNP C, whereas it enhances the interaction between Grb3-3 and hnRNP C. These findings suggest that the Grb2/Grb3-3-hnRNP C interactions might fulfill different biological functions.

Grb2/Ash is the mammalian homolog of Sem-5 and Drk in *Caenorhabditis elegans* and *Drosophila melanogaster*, respectively. Grb2 is a ubiquitous 25-kDa protein composed of one Src homology[1] (SH) 2 and two SH3 domains. This adaptor molecule plays an essential role in cell growth and differentiation and, in addition to other functions, connects tyrosine kinase receptors to activation of the Ras pathway. It interacts via its SH3 domains with the proline-rich regions of the mammalian Sos exchange factors. This Grb2·Sos complex exists in the cytosol of quiescent cells and upon growth factor stimulation is recruited in an SH2-dependent manner to the plasma membrane, where Sos stimulates nucleotide exchange on Ras (see Ref. 1 for review). Grb2 has also been shown to interact with the ubiquitous Ras exchange factor C3G (2) and the hematopoietic guanine nucleotide exchange factor Vav (3). Furthermore, in NRK cells, Grb2 has been shown to be involved in growth factor control of cytoskeletal structure (4). The SH2 domain of Grb2 can also bind to focal adhesion kinase (5), to the receptor protein phosphatase α (6) and to the chimeric Bcr/Abl products in human leukemias (7). Finally, several other proteins, such as Abl, Cbl, dynamin, synapsin, or 5-lipoxygenase, can bind to the SH3 domains of Grb2, suggesting that this adaptor molecule may play a role in many cellular activities (1).

Several isoforms of Ash/Grb2 have been described. Ash-m and Ash-s are two rat isoforms generated from a single gene by unusual alternative splicing events. Microinjection of Ash-m or Ash-s into Balb/c 3T3 cells inhibited DNA synthesis induced by platelet-derived growth factor (8). Grb3-3 is a human isoform of Grb2 thought to arise by alternative splicing, carrying a deleted non-functional SH2 domain but retaining functional SH3 domains (9). The residues deleted in the SH2 domain (residues 60–100 in Grb2) participate in the binding of phosphotyrosine-containing proteins. Indeed, Grb3-3 did not bind to phosphorylated epidermal growth factor receptor and inhibited epidermal growth factor-induced transactivation of a Ras-responsive element. This inhibition was overcome by Grb2, suggesting that depending on the ratio of Grb2/Grb3-3, Grb3-3 might serve as a suppressor of Grb2 functions. Several evidences implicate Grb3-3 in apoptosis (9, 10), but the mechanism remains unknown. In light of the direct interaction of Grb3-3 with hSos1, it could be argued that the apoptotic effect of Grb3-3 might be a consequence of the down-regulation of Ras GTP loading, but this alone might not be sufficient. Thus, Grb3-3 and Grb2 may have common partners besides Sos, and Grb3-3 may compete with Grb2 for vital Ras-independent pathways.

In the last several years, an increasing number of SH2- and SH3-containing proteins that interact with molecules implicated in RNA biogenesis have been described (11–16). In this context, we considered it of great interest to analyze the behavior of an exclusively nuclear RNP, hnRNP C, in regard to its ability to interact with an adaptor molecule, Grb2 (and its isoform Grb3-3), involved in transducing signals from receptors. hnRNP C proteins (C1, M_r 41,000; C2, M_r 43,000) are among the most abundant pre-mRNA binding proteins. C2 is identical to C1, except for a 13-residue insertion due to a

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[1] The abbreviations used are: SH, Src homology; AD, activation domain; GST, glutathione *S*-transferase; hnRNP, heterogeneous nuclear ribonucleoprotein; PAGE, polyacrylamide gel electrophoresis.

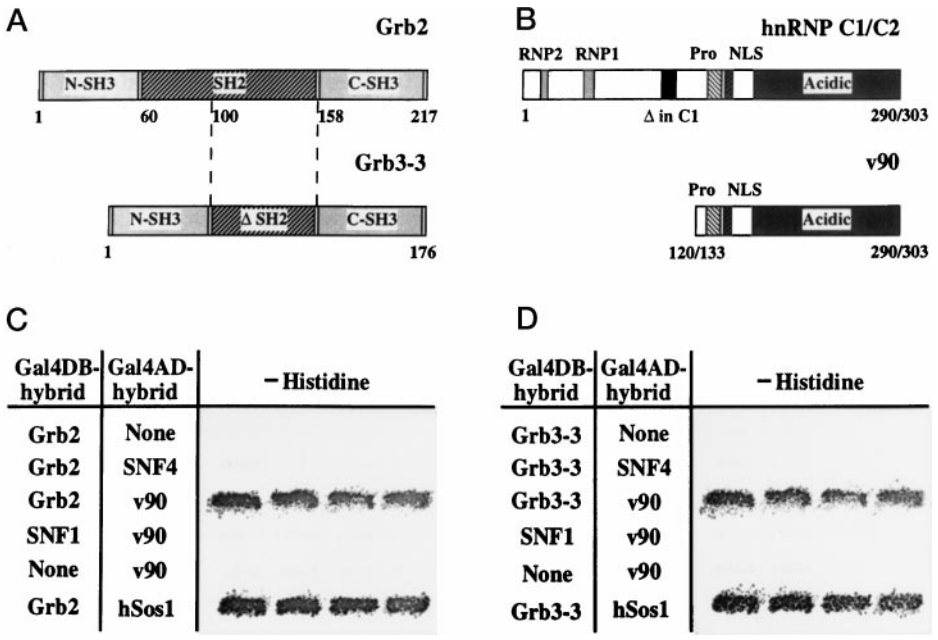

FIG. 1. **Interaction of Grb2 and Grb3-3 with hnRNP C in the two-hybrid system.** *A*, diagram of the domains of Grb2 and Grb3-3. *N*, amino; *C*, carboxyl; Δ*SH2*, deleted SH2 domain of Grb3-3 lacking the first 40 residues of the SH2 domain of Grb2. *B*, diagram of the domains of the hnRNP C proteins and v90 polypeptide. *RNP*, consensus RNA-binding domain; *Pro*, proline-rich motif; *NLS*, nuclear localization site; *Acidic*, acidic domain; Δ, region deleted in hnRNP C1. *C*, interaction of Grb2 with v90 polypeptide. Hf7c reporter strain was cotransformed with the indicated plasmids. Growth in the absence of histidine and with 5 mM 3-amino-1,2,4-triazole indicates the interaction between hybrid proteins. Cotransformations with pGAD-hSos1 (residues 1131–1333) and pGAD-SNF4 were used as controls. Each patch represents an independent transformant, and there are four patches for every strain. *D*, interaction of Grb3-3 with v90 polypeptide (see *C* for details).

39-nucleotide insert in the corresponding mRNA, probably derived from a common pre-mRNA by alternative splicing. C proteins are found in the nucleus during interphase, but during mitosis they are dispersed throughout the cell. Antibody inhibition and immunodepletion experiments implicated hnRNP C in pre-mRNA splicing (see Ref. 17 for review). Our results demonstrate that (i) Grb2 and Grb3-3 interact with hnRNP C, (ii) both isoforms partially localize in the nucleus, and (iii) while the Grb3.3-hnRNP C association is stimulated by poly(U) RNA, Grb2-hnRNP C interaction is completely inhibited. The implications of these results are discussed.

EXPERIMENTAL PROCEDURES

Cloning and Site-directed Mutagenesis—The wild types *grb2* and *grb3-3* (9) and the mutants *grb2 P49L*, *grb2 G203R*, *grb3-3 P49L* and *grb3-3 G162R* (9, 18) were cloned in frame with *gal4-DB* (DNA-binding) in pGBT10 (19) to yield pGBT10-Grb2, pGBT10-Grb3-3, pGBT10-Grb2 P49L, pGBT10-Grb2 G203R, pGBT10-Grb3-3 P49L and pGBT10-Grb3-3 G162R, respectively. The mutants *Grb2 (P49L-G203R)*, *Grb3-3 (P49L-G162R)* and *v90 (5PA)* were made in the yeast two-hybrid vectors pGBT10-Grb2 G203R, pGBT10-Grb3-3 G162R, and pGAD-v90 (residues 120–290 of hnRNP C1), respectively, using the Transformer site-directed mutagenesis kit from CLONTECH. The mutagenic primers were 5′-GGA AAA GAC GGC TTC ATT TTA AAG AAC TAC ATA GAA ATG-3′, for P49L mutations, and 5′-CCA GCA CGT GTA GCT GCT GCA GCT GCT ATT GCT CGG GC-3′, for 5PA mutation. The mutated bases are underlined. v90.1 (residues 120–167 of hnRNP C1), v90.2 (residues 168–290 of hnRNP C1), v90.3 (residues 120–209 of hnRNP C1), v90.4 (residues 148–187 of hnRNP C1), v90.5 (residues 148–209 of hnRNP C1), and v90.6 (residues 137–187 of hnRNP C1) were polymerase chain reaction-amplified from pGAD-v90 and cloned in frame with *gal4-AD* (activation domain) in pGAD1318 (20). Sequencing of polymerase chain reaction fragments and point mutations was performed on both strands with an automatic sequencer (Amersham Pharmacia Biotech) using the Sanger dideoxy-termination method (21).

Yeast Two-hybrid Methods—Saccharomyces cerevisiae strain Hf7c was cotransformed with the indicated plasmids by the lithium acetate method (22). Double transformants were plated on yeast drop-out medium lacking Trp and Leu (22). They were grown for 3 days at 30 °C, and then colonies were patched on the same medium and replica-plated on Whatman 40 filters to test for β-galactosidase activity (23) and on yeast drop-out medium lacking Trp, Leu, and His, and supplemented with 5 mM 3-amino-1,2,4-triazole (22) (the Gal4-Grb3-3 fusion protein had a weak transcriptional activity if yeasts grew only in selective medium). Plasmids pGBT-SNF1 and pGAD-SNF4 (24), carrying unrelated proteins, and pGAD-hSos1 (residues 1131–1333) (19), a known partner of Grb2, were used as controls.

Cell Culture and Subcellular Fractionation—Jurkat T cells (clone J77.6.8) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim), 2 mM L-glutamine, penicillin, and streptomycin, in a 5% CO_2 humidified atmosphere at 37 °C. UT7-S Epo cells (a growth factor-dependent human megakaryoblastic cell line) (25) were maintained in α-medium (Life Technologies, Inc.) with 10% fetal calf serum and erythropoietin at 2 units/ml. Jurkat and UT7-S Epo cell cytosol and nuclear fractions were prepared essentially as described (26).

In Vitro Binding Studies—The *Escherichia coli* BL21 strain was transformed with pGEX-derived plasmids and bacteria incubated with 1 mM isopropyl-1-thio-β-D-galactopyranoside. GST (glutathione *S*-transferase) fusion proteins were recovered and purified by affinity chromatography with glutathione-agarose beads (Sigma).

Nuclear extracts from 10^7 Jurkat cells (150 μg of protein) were diluted to 150 mM NaCl and supplemented with 1% Nonidet P40, and the supernatant incubated for 2 h at 4 °C with fusion proteins (0.2–3 μg) bound to glutathione-coupled agarose beads. The washed beads were dissolved in sodium dodecyl sulfate (SDS)-sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), electroblotted, and probed with different antibodies.

For the RNA competition experiments, nuclear extracts were preincubated with poly(U) RNA (Amersham Pharmacia Biotech) at 50 μg/ml for 30 min at 4 °C before adding GST fusion proteins.

Coimmunoprecipitation Experiments—Nuclear extracts from 3×10^7 Jurkat cells (450 μg of protein) were diluted to 150 mM NaCl and supplemented with 0.05% Brij 96 (polyoxylethylene-10-oleylether); after centrifugation, supernatants were incubated with preimmune serum for 30 min and then with protein A-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. After centrifugation, the supernatants were incubated for 12–14 h with polyclonal anti-Grb2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies, monoclonal anti-hnRNP C (4F4) antibodies (provided by Dr. G. Dreyfuss, University of Pennsylvania) (27) or preimmune serum, and then with protein A-Sepharose beads for 1 h and centrifuged. The beads were washed, dissolved in SDS-sample buffer, and subjected to SDS-PAGE. Filters were developed with different antibodies.

For blocking experiments, the antigenic peptide (residues 195–217) of anti-Grb2 (Santa Cruz Biotechnology, Inc.) was used at 25 μg/ml.

RESULTS

Interaction of Grb2 and Grb3-3 with hnRNP C Proteins in the Two-hybrid System—Functional connections between SH2- and SH3-containing proteins and molecules implicated in RNA biogenesis have been described. Therefore, it seemed of interest to study the potential interactions of Grb2 and Grb3-3 (Fig. 1*A*), adaptor molecules involved in signaling events, with hnRNP C, an exclusively nuclear RNP. We have investigated the binding of these proteins to v90, a hnRNP C subclone

isolated in our laboratory from a Jurkat T cell oligo(dT) cDNA library constructed in frame with *gal4-AD*. This clone spans residues 120/133 to 290/303 of hnRNP C1/C2, respectively (Fig. 1*B*). We have used the yeast two-hybrid system to analyze these associations. For this purpose, the full-length *grb2* and *grb3-3* were cloned in frame with *gal4-DB* in pGBT10 and then cotransformed with pGAD-v90 in Hf7c cells. If the two hybrid proteins interact, the reporter strain is expected to grow in the absence of histidine and to produce β-galactosidase (28). Hf7c carrying pGBT10-Grb2 and pGAD-hSos1 (residues 1131–1333) (19) was used as a positive control. Fig. 1 (*C* and *D*) shows that pGBT10-Grb2/pGAD-v90 and pGBT10-Grb3-3/pGAD-v90 conferred on Hf7c yeast cells the ability to grow in the absence of histidine. Furthermore, full-length Csk, another protein with SH2 and SH3 domains, and lamin, an unrelated protein, did not interact with the v90 polypeptide (data not shown). Therefore, both Grb2 and Grb3-3 specifically bind to hnRNP C proteins in the two-hybrid system.

Localization of Grb2/Grb3-3 and hnRNP C Binding Sites—We next sought to identify the specific Grb2 and Grb3-3 sequences implicated in the interaction with hnRNP C proteins. Mutations in the amino-terminal SH3 (N-SH3) or in the carboxyl-terminal SH3 (C-SH3) domains of Grb2 and Grb3-3 greatly decrease the affinity of binding to the proline-rich region of hSos1 (9, 19, 29–31). We tested the effect of point mutants in the N-SH3 (P49L) and in the C-SH3 (G203R and G162R, respectively) domains of Grb2 and Grb3-3 (9, 18) on the interaction with v90 in the yeast two-hybrid system. In addition, we generated double N- and C-SH3 mutants of Grb2 (P49L-G203R) and Grb3-3 (P49L-G162R) and assessed their ability to associate with hnRNP C proteins. Strain Hf7c was cotransformed with pGAD-v90 and pGBT10 containing the *grb2* and *grb3-3* mutants in frame with *gal4-DB*. Visual comparison of growth in the absence of histidine indicates that mutations in either of the SH3 domains of both Grb2 and Grb3-3 decreased the interaction with hnRNP C (Fig. 2, *A* and *B*), as reported for Grb2/hSos1 (19). However, the P49L mutations were less effective than G203R or G162R, and the double mutations (P49L-G203R or P49L-G162R) completely abolished the interaction. Moreover, we confirmed by Western blot experiments that wild-type and mutant proteins were produced in similar amounts in Hf7c (data not shown). These results indicate that the association of Grb2 and Grb3-3 with hnRNP C proteins is primarily mediated by the C-SH3 domains.

SH3 domains are known to bind proteins through rather specific proline-rich sequences (32). Human hnRNP C proteins have a region rich in prolines (YPARVPPPPPIARAVVPS, residues 126/139 to 143/156 (hnRNP C1/C2, respectively)) (33), which is contained in the v90 subclone. To ascertain whether the binding between Grb2/Grb3-3 and hnRNP C proteins involves the interaction between a SH3 domain and a proline-rich motif, we substituted alanines for the underlined five prolines of v90 to yield v90 (5PA). Strain Hf7c was cotransformed with pGBT10-Grb2 or pGBT10-Grb3-3 and pGAD-v90 (5PA) and the interaction tested in the yeast system. Fig. 2*C* shows that Grb2 does not bind to v90 (5PA) whereas the Grb3-3-v90 (5PA) interaction is clearly detected. Therefore, the Grb2-hnRNP C association is a classical SH3-proline-rich interaction, which is not the case for the Grb3-3-hnRNP C interaction.

To localize the Grb3-3 binding site in hnRNP C, we subcloned several v90 fragments in pGAD1318 (Fig. 3*A*). We tested these constructions with pGBT10-Grb3-3 in the two-hybrid system, and only v90.3 continued to interact with Grb3-3 (Fig. 3*B*). This limits the interacting region to residues 120–209 of hnRNP C1, although this portion does not have any known binding motifs.

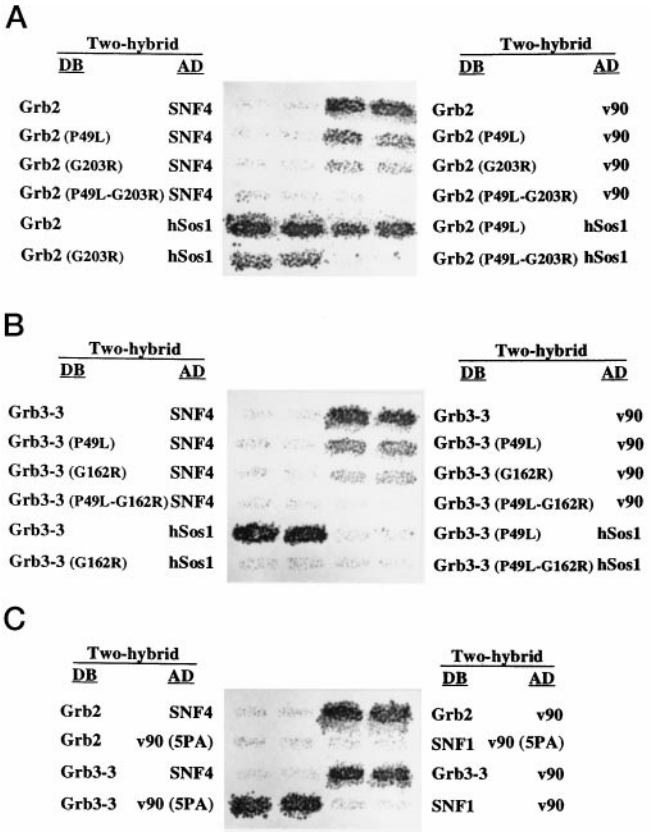

FIG. 2. **Sites of binding of Grb2, Grb3-3 and hnRNP C proteins in the two-hybrid system.** *A* and *B*, interaction of v90 polypeptide with point mutants of Grb2 and Grb3-3. hSos1 includes residues 1131–1333. *C*, interaction of Grb2 and Grb3-3 with a v90 mutant (*v90 (5PA)*). See the legend of Fig. 1*C* for details. There are two patches for every strain. *DB*, fusion with the DNA-binding domain of Gal4; *AD*, fusion of the activation domain of Gal4.

Grb2 and Grb3-3 Interact with hnRNP C Proteins in Vitro—To confirm the results obtained with the yeast two-hybrid system, the interactions of Grb2 and Grb3-3 with hnRNP C proteins were studied by *in vitro* binding experiments. The cDNAs of *grb2*, *grb3-3*, *grb2 P49L*, *grb2 G203R*, *grb3-3 P49L*, and *grb3-3 G162R* subcloned into pGEX-derived plasmids (9, 18) were used. The chimeric fusion proteins were purified from bacterial lysates on glutathione-agarose beads (see "Experimental Procedures"). Since C1 and C2 hnRNP proteins are confined to the nucleus, we used nuclear extracts to carry out the binding experiments. Nuclear extracts from a T lymphoma cell line (Jurkat, J77 clone) were incubated with GST fusion proteins bound to glutathione-agarose beads. Protein complexes were resolved by SDS-PAGE, and the presence of hnRNP C was determined by using anti-hnRNP C (4F4) antibodies. As shown in Fig. 4, the chimeric GST-Grb2 and GST-Grb3-3 bind the endogenous human hnRNP C proteins and, although these interactions were usually performed with 3 μg of GST fusion proteins, GST-Grb2 and GST-Grb3-3 at concentrations as low as 0.2 μg were still able to associate with hnRNP C. Moreover, by silver staining and [^{35}S]methionine labeling of nuclear proteins from Jurkat and UT7-S Epo cells (a growth factor-dependent human megakaryoblastic cell line), we assessed that hnRNP C proteins are among the few proteins that bind to GST-Grb2 and GST-Grb3-3 (data not shown). Fig. 4 also shows that the P49L mutation of the N-SH3 domains of Grb2 and Grb3-3 did not modify the interaction with hnRNP C, whereas no binding was observed with C-SH3 mutants of Grb2 and Grb3-3. The interactions were also observed when nuclear extracts were previously treated with RNase (data not shown),

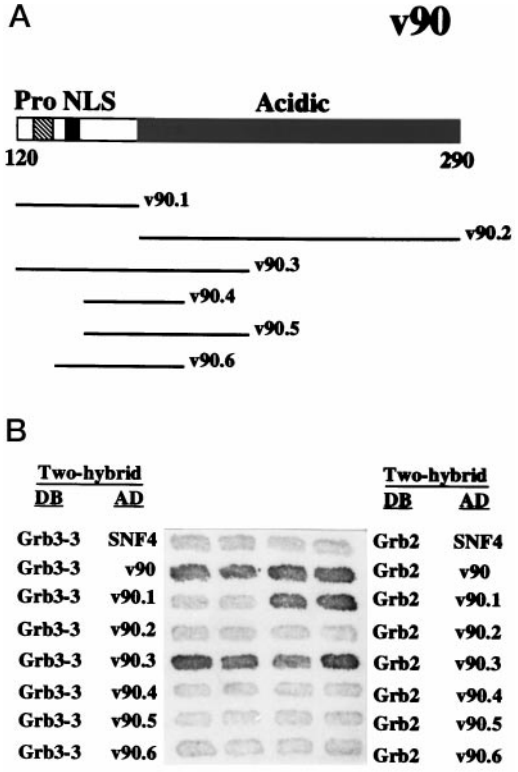

FIG. 3. **v90 polypeptide binding site to Grb3-3 in the yeast two-hybrid system.** *A*, different subclones of v90. See the legend of Fig. 1*B* for abbreviations. *B*, interaction of Grb3-3 with various v90 subclones. The Hf7c reporter strain was cotransformed with the indicated plamids. The interaction between the two hybrid proteins is indicated by the induction of *lacZ* expression (*dark gray patches*). Associations with Grb2 were used as controls. Each patch represents an independent transformant, and there are two patches for every strain. *DB*, fusion with the DNA-binding domain of Gal4; *AD*, fusion of the activation domain of Gal4.

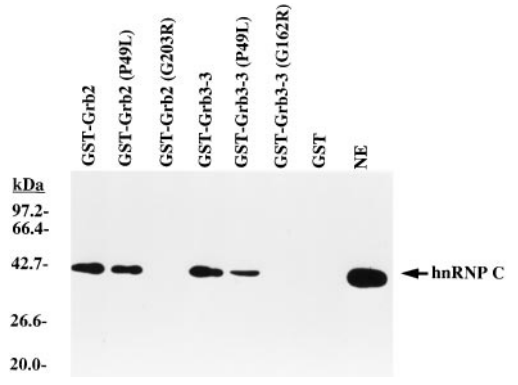

FIG. 4. **The interactions of Grb2 and Grb3-3 with hnRNP C proteins *in vitro* are mediated by the C-SH3 domains.** Expression of the GST-fusion proteins was induced by addition of isopropyl-1-thio-β-D-galactopyranoside. The bacterial lysates were purified on GST-agarose beads. GST-fusion proteins were incubated with nuclear extracts from Jurkat cells (10^7), and their associations with hnRNP C proteins were determinated by immunoblotting with anti-hnRNP C (*4F4*) antibody. *NE*, nuclear extract from 10^6 Jurkat cells. These interactions were detected with different amounts of GST fusion proteins (between 0.2 and 3 μg).

indicating that Grb2/Grb3-3-hnRNP C interactions are not mediated by RNA strands. Taken together, these findings confirm the association of Grb2/Grb3-3 with hnRNP C and localize the interaction in the Grb2/Grb3-3 C-SH3 domains.

In Vivo Binding of Grb2 and hnRNP C Proteins—Grb2 has been described to be localized mainly in membrane ruffles and in the cytoplasm (1, 34), whereas hnRNP C proteins are restricted to the nucleus (35). To evaluate the physiological relevance of the Grb2/Grb3-3-hnRNP C interactions it was important to determine whether Grb2/Grb3-3 were present in the nucleus. For this purpose, the localization of Grb2/Grb3-3 was investigated by subcellular fractionation. Western blot experiments performed on cytosol and nuclear fractions of Jurkat and UT7-S Epo cells showed that Grb2 is present in both fractions, whereas little or no Raf1 was found in the nuclear fractions and hnRNP C was absent from the cytosol fractions (Fig. 5). In addition, a similar localization for Grb3-3 was found on NIH3T3 cells stably transfected with *grb3-3* (36) (data not shown). Thus, by subcellular fractionation, we demonstrate the presence of Grb2/Grb3-3 in the nucleus.

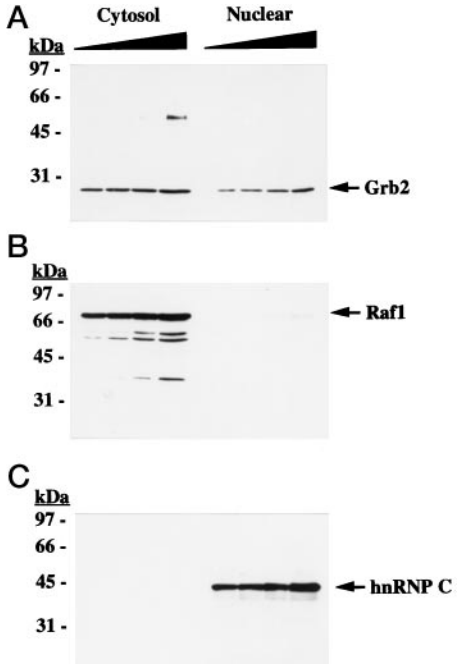

FIG. 5. **Detection of Grb2 in cytosol and nuclear fractions of UT7-S Epo cells.** *A*, subcellular fractionations of 0.5, 0.75, 1, and 2 × 10^6 UT7-S Epo cells were separated on SDS-PAGE, transferred to a nitrocellulose membrane, and then probed with anti-Grb2 polyclonal antibody. *B* and *C*, the same filter was reblotted with anti-Raf1 polyclonal antibody and anti-hnRNP C monoclonal antibody, respectively, to control the purity of the fractions. Exposures of 1 min (*A*) and 120 min (*B* and *C*) using epichemiluminescence Western immunoblotting system (ECL, Amersham Pharmacia Biotech).

Next, to investigate the interaction of Grb2 and hnRNP C proteins in the context of a living cell, we performed coimmunoprecipitation experiments with nuclear extracts from Jurkat cells. Anti-Grb2 immunoprecipitates were resolved by SDS-PAGE and the blots developed with anti-hnRNP C. Fig. 6*A* shows that hnRNP C proteins were detected in the anti-Grb2 immunoprecipitates, whereas immunoprecipitates obtained with a preimmune serum contained no detectable hnRNP C. Furthermore, the anti-Grb2 antigenic peptide blocked immunoprecipitation of hnRNP C (Fig. 6*B*). The reciprocal experiments, namely anti-hnRNP C immunoprecipitates immunoblotted with anti-Grb2, also yielded positive results (Fig. 6*C*). Similar results were obtained with nuclear extracts from UT7-S Epo (data not shown). These data clearly demonstrate that endogenous Grb2 is able to interact with hnRNP C proteins in the nuclei of intact hematopoietic cells.

Poly(U) RNA Modulates the Association of Grb2/Grb3-3 with hnRNP C—hnRNP proteins can bind *in vitro* to different single-stranded ribo- and deoxyribopolynucleotides, suggesting that hnRNP proteins bind to heterogeneous nuclear RNA regardless of the nucleotide sequence (37–41). Nevertheless,

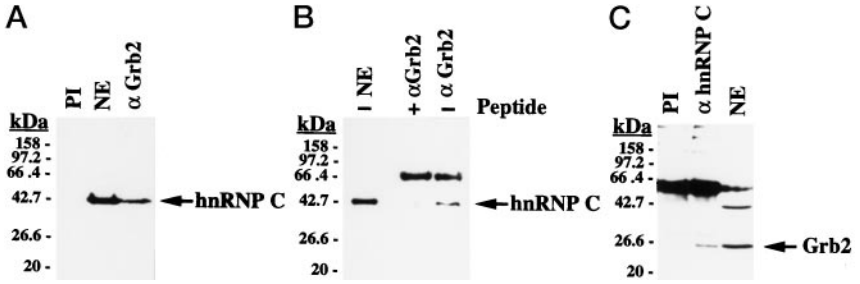

FIG. 6. **Coimmunoprecipitation of Grb2 and hnRNP C proteins.** *A*, nuclear extracts from Jurkat cells (3×10^7) were incubated with anti-Grb2 or preimmune (*PI*) serum and, after washing, resolved by SDS-PAGE, transferred to nitrocellulose filters, and incubated with anti-hnRNP C (*4F4*). *NE*, nuclear extract from 7×10^5 Jurkat cells. *B*, nuclear extracts were incubated with anti-Grb2 in absence (−) or presence (+) of antigenic peptide (25 μg/ml). Western blot was developed with anti-hnRNP C. *C*, similar experiment using anti-hnRNP C and preimmune (*PI*) serum followed by immunoblotting with anti-Grb2 polyclonal antibody. The band of about 40 kDa seen in the *NE lane* is a nonspecific band that appears sometimes when using anti-Grb2 polyclonal antibodies from Santa Cruz Biotechnology.

more stringent *in vitro* assays demonstrated that hnRNP proteins have preferences for specific sequences. For example, hnRNP C proteins have high avidity for poly(U) RNA (42). To determine whether poly(U) RNA has a role in the association of Grb2/Grb3-3 with hnRNP C, we examined their binding in the presence or absence of poly(U) RNA. Nuclear extracts from Jurkat cells were incubated with or without poly(U) RNA (50 μg/ml), followed by glutathione-agarose beads containing GST-Grb2 or GST-Grb3-3 fusion proteins. The resulting complexes were analyzed by Western blots with anti-hnRNP C. As shown in Fig. 7, poly(U) RNA abolished the Grb2-hnRNP C interaction, whereas it increased the Grb3-3-hnRNP C association. This effect was not observed with other RNA homopolymers (poly(rC), poly(rG), or poly(rA) RNA), and combination of any of these poly-RNAs with poly(U) RNA did not modify the effect induced by poly(U) RNA alone (data not shown). These findings indicate that the Grb2/Grb3-3-hnRNP C interactions are modulated by poly(U) RNA and suggest that the nature of these associations is different for Grb2 and for Grb3-3.

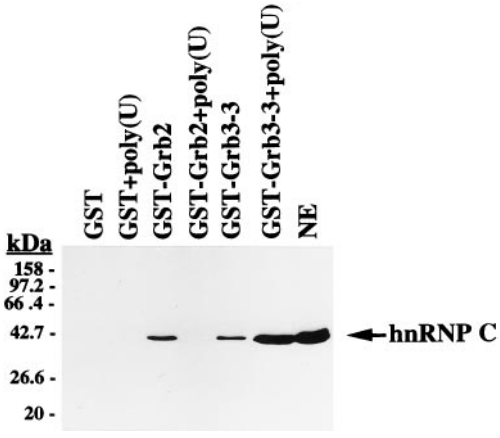

FIG. 7. **Association of Grb2 and Grb3-3 with hnRNP C proteins is modulated by poly(U) RNA.** Nuclear extracts from Jurkat cells (10^7) with or without 50 μg/ml poly(U) RNA were incubated with glutathione beads bound to either GST or GST-fusion proteins. The resulting complexes were analyzed by Western blots with anti-hnRNP C (*4F4*). *NE*, nuclear extract from 10^6 Jurkat cells.

DISCUSSION

In the last several years, the repertoire of complexes between RNPs and SH2- and SH3-containing proteins has increased (11–16, 43). To investigate new pathways involving Grb2 and Grb3-3, we studied their interactions with hnRNP proteins using different but complementary approaches. By the two-hybrid system, we demonstrated that Grb2 and Grb3-3 interact with a subclone of hnRNP C. hnRNP C1 and C2 bind strongly to sequences relevant to the processing of pre-mRNA, including the polypyrimidine stretch of introns (if uridine-rich) (44–46). The hnRNP proteins can be divided into two groups according to their nucleocytoplasmic transport properties. One group is completely restricted to the nucleus in interphase cells, whereas the other group shuttles between the nucleus and the cytoplasm. The hnRNP C proteins belong to the first group. They are restricted to the nucleus not because they lack a nuclear export signal but because they bear a nuclear retention sequence that is capable of overriding nuclear export signals (35). To evaluate the physiological relevance of Grb2/Grb3-3-hnRNP C interactions, it was critical to determine the subcellular localization of Grb2 and Grb3-3. Our subcellular fractionation studies clearly demonstrate the presence of both proteins in the nucleus and the cytoplasm. We estimated that 20% of endogenous Grb2 is found in the nucleus of Jurkat and UT7-S Epo cells. Most importantly, by coimmunoprecipitation experiments, we showed the existence of endogenous Grb2-hnRNP C complexes in nuclear extracts from hematopoietic cells. Previous work performed by microinjection of GST-Grb2 fusion protein in REF-52 cells did not describe its presence in the nuclei (34). The explanation of this discrepancy may lie in the addition of the GST, which may alter the normal behavior of the native protein and prevent its nuclear localization (47).

Binding studies with GST fusion proteins localized the domain involved in interaction with the endogenous hnRNP C proteins to the C-SH3 of Grb2 and Grb3-3 since these associations were completely abolished by G203R and G162R mutants. However, in the two-hybrid system interactions were prevented only in the double mutants (P49L-G203R and P49L-G162R), suggesting that the N-SH3 may participate in the binding. It should be noted that in the two-hybrid system only a fragment of hnRNP C (residues 120–290 of hnRNP C) was tested. Therefore, the constraints for the binding imposed by the structure of the native protein may be overridden in the v90 polypeptide. Therefore, the interactions are more likely mediated by the C-SH3 domains *in vivo*.

Although Grb2 and Grb3-3 use the same domain to bind to hnRNP C proteins, our results indicate that the nature of the association must be different. As expected for a conventional SH3-mediated interaction, mutation of the only proline-rich motif in hnRNP C suppressed the binding to Grb2, but, surprisingly, interaction with Grb3-3 remained unchanged. We localized the Grb3-3 binding site to a more extended region (residues 120–209 of hnRNP C1) probably involving a conformational motif, as judged by the finding that other subclones containing the potential interaction region were unable to associate with Grb3-3. These results suggest that significant differences exist between Grb2 and Grb3-3 in their interactions with common partners. This view is supported by the results of the two-hybrid system in which point mutations in either of the SH3 domains of Grb3-3 completely disrupted the association

with hSos1 (residues 1131–1333), whereas in the case of Grb2 this phenotype was only obtained with the double mutant. Our results are in agreement with previous observations showing that, although both Grb2 and Grb3-3 bound to hSos1 in an SH3-dependent manner, there were qualitative differences in their respective binding to the exchange factor (10).

The hnRNP complexes contain at least 20 major proteins with different RNA binding specificities. hnRNP C proteins have striking avidity for poly(U) RNA (42). Therefore, we examined the role of poly(U) RNA in Grb2/Grb3-3-hnRNP C interactions. We clearly show that, whereas Grb2 only binds to hnRNP C in the absence of poly(U) RNA, Grb3-3-hnRNP C interaction is enhanced by the presence of poly(U) RNA. Other RNA homopolymers do not have this effect. Four Grb3-3 partners have been identified so far: hSos1 (10), Vav (18), adenosine deaminase (36), and now hnRNP C proteins. However, we report for the first time that an interaction of Grb3-3 is regulated.

The physiological significance of these interactions remains to be determined. The localization of Grb2 in the nucleus opens new perspectives. Up to now, Grb2 has been shown to be involved in signal transduction from tyrosine-phosphorylated receptors to cytosolic partners. Our results suggest that Grb2 might also be involved in communicating signals from the cytoplasm to the nucleus. Another possibility is that Grb2 functions as an adaptor protein in the nucleus. It would be interesting to identify tyrosine-phosphorylated partners of Grb2 in the nucleus. Very recently, Nck, another adaptor molecule without a nuclear localization signal, has been found in the nuclei of NIH3T3 and A431 cells in association with Sam68 (48). Specific binding partners have also been identified for SH2 and SH3 domains of the adaptor protein c-Crk in cytosolic and nuclear lysates (49). These studies, together with our results, suggest that there might be signal transduction mechanisms in the nucleus. The fact that Grb2/Grb3-3-hnRNP C interactions are regulated by poly(U) RNA greatly enhances the physiological relevance of these associations. Nuclear retention sequence-bearing proteins, like hnRNP C, have been suggested to retain pre-mRNA in the nucleus (35). The removal of hnRNP C from pre-mRNA/mRNA is likely to be involved in mRNA export from the nucleus. It is possible that Grb2 and Grb3-3 participate in this function. On the other hand, it has been reported that increased expression of Grb3-3 coincides temporally with extensive cell death in some tissues (9, 10). However, further experiments will be necessary to investigate the hypothetical involvement of Grb3-3·hnRNP C complexes in apoptosis.

In summary, we detected Grb2/Grb3-3 in the nucleus and identify hnRNP C proteins as nuclear partners of Grb2 and Grb3-3. Furthermore, we postulate that Grb3-3 is not only an SH2 mutated form of Grb2, but that its structure is changed such that the SH3-mediated binding to hnRNP C is modified and might participate in different functions.

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