Communication

Grap Is a Novel SH3-SH2-SH3 Adaptor Protein That Couples Tyrosine Kinases to the Ras Pathway* (Received for publication, March 5, 1996, and in revised form, March 22, 1996)
Gen-Sheng Feng‡, Ying-Bin Ouyang‡, Dong-Ping Hu‡, Zhong-Qing Shi‡, Reiner Gentz‡, and Jian Ni‡

From the 1Department of Biochemistry and Molecular Biology, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202-5121 and 2Human Genome Sciences, Inc., Rockville, Maryland 20850

A human cytoplasmic signaling protein has been cloned that possesses the same structural arrangement of SH3-SH2-SH3 domains as Grb2. This protein is designated Grap for Grb2-related adaptor protein. The single 2.3-kilobase (kb) grap transcript was expressed predominantly in thymus and spleen, while the ubiquitously expressed grb2 gene produced two mRNA species of 3.8 and 1.5 kb. Grap and Grb2 consist of 217 amino acids and share 59% amino acid sequence identity, with highest homology in the N-terminal SH3 domain. The GrapSH2 domain interacts with ligand-activated receptors for stem cell factor (c-kit) and erythropoietin (EpoR). Grap also forms a stable complex with the Bcr-Abl oncoprotein via its SH2 domain in K562 cells. Furthermore, Grap is associated with a Ras guanine nucleotide exchange factor mSos1, primarily through its N-terminal SH3 domain. These results show that a family of Grb2-like proteins exist and couple signals from receptor and cytoplasmic tyrosine kinases to the Ras signaling pathway.

Grap, the mammalian homologue of Caenorhabditis elegans Sem-5 and Drosophila Drk, is composed of a single Src homology 2 (SH2)1 domain flanked by two SH3 domains (1–5). Genetic analyses of sem-5 in C. elegans and of drk in Drosophila and biochemical dissection of Grb2 in mammalian cells revealed a highly conserved pathway governing the stimulation of Ras proteins by receptor protein-tyrosine kinases (PTKs) (4–11). Sem-5 functions in a signaling pathway that involves the Let-23/receptor tyrosine kinase and Let-60/Ras (1). Similarly, Drosophila Drk was found to be essential for signaling by Sevenless receptor PTK to Son of Sevenless (Sos), an activator of Ras guanine nucleotide exchange, in the development of R7 photoreceptors in Drosophila eye (4–5). Mammalian Grb2 physically interacts with a number of autophosphorylated receptor PTKs through its SH2 domain, while its SH3 domains associate with the proline-rich motifs in the C-terminal tail of mSos1 (6–11). These interactions apparently mediate the growth factor stimulation of Ras proteins by promoting the conversion of inactive GDP-bound Ras to the active GTP-bound form (8, 9). Human grb2 and Drosophila drk genes specifically rescue the defects of sem-5 mutations in C. elegans, indicating a high degree of conservation of this signaling pathway throughout evolution (12). In addition to direct interaction with receptor PTKs, Grb2 binds through its SH2 domain to several cytoplasmic proteins, including Shc, Syp, and Bcr-Abl, in a tyrosine phosphorylation-dependent manner (13–15).

In this paper, we identify a novel human signaling protein Grap that has the Grb2-like architecture (SH3-SH2-SH3 domains) and is highly expressed in thymus and spleen. Our experiments suggest that Grap and Grb2 are members of the same adaptor protein family with overlapping yet distinct expression patterns and possibly functions.

MATERIALS AND METHODS

Cloning of the cDNA—Several cDNA clones highly homologous to grb2 were identified from human cDNA libraries made from a 6-week-old embryo or tonsils. A data base containing approximately 500,000 human partial cDNA sequences (expressed sequence tags) has been established in a collaborative effort of The Institute for Genomic Research and Human Genome Sciences Inc., using high throughput automated DNA sequence analysis of randomly selected human cDNA clones (16–18). Sequence homology comparison of each expressed sequence tag was performed with the blastn and tblastn algorithms against the GenBank™ data base.

Cell Lines—The M07e megakaryocytic cell line, TF1 erythroleukemia cell line, the M07eR cell overexpressing erythropoietin receptor (EpoR), and the K562 chronic myelogenous leukemia cell line were obtained from Dr. H. E. Broxmeyer (Indiana University School of Medicine, Indianapolis, IN) and maintained according to standard protocols. Purified anti-mSos1, anti-c-kit, anti-Grb2, and anti-CAN antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-EpoR antibodies were obtained from Upstate Biotechnology Inc. Monoclonal anti-phosphotyrosine (anti-PY) antibodies were from Santa Cruz Biotechnology, Inc. (PY20) and Upstate Biotechnology Inc. (4G10).

Northern Blot Analysis—Two Northern blot filters containing approximately 2 µg of poly(A)1 RNA per lane from various human tissues were purchased from Clontech. Northern blotting was performed with the ExpressHyb Hybridization Solution (PT1190-1) from Clontech following the manufacturer’s manual. DNA probes for grap and grb2 were labeled with 32P using a randomly primed kit from Boehringer Mannheim.

Generation of GST-Fusion Proteins and Production of Rabbit Antibodies—The corresponding cDNA fragments were isolated by polymerase chain reaction and inserted into the appropriate sites of pGEX-4T-1 (Pharmacia Biotech Inc.). All of the constructs were reconfirmed by sequencing and transformed into the bacterial strain BL21 (Pharmacia). Glutathione S-transferase (GST)-fusion proteins were purified from cell lysates with glutathione-Sepharose beads (Pharmacia). Purified GST-GrapSH2 fusion protein was injected into New Zealand female rabbits for production of specific anti-Grap antisera according to standard protocols.

Assays for Protein–Protein Interaction—Immunoprecipitation and immunoblotting experiments were conducted as described previously (39, 40). Briefly, specific antibodies were incubated with cell lysates containing 1 mg of total proteins in the presence of protein A-Sepharose.
were found during sequencing of cDNA libraries derived from a 217-amino acid protein (Fig. 1) was resequenced on both strands by the dideoxy method using a kit from United States Biochemical Inc. Based on the deduced amino acid sequence, this protein is comprised of a central SH2 domain flanked by two SH3 domains. This Grb2/Sem-5/Drk-like arrangement is characteristic of an adaptor protein apparently lacking catalytic activities. Therefore, we suggest a name Grap for Grb2-related adaptor protein. The amino acid sequence of Grap was compared with Grb2, Sem-5, and Drk, and the percentage of identical amino acids is shown in Table I. Overall, Grap is most closely related to Grb2. The SH2 domain of Grap shows high homology to Grb2/Sem-5/Drk and contains all the conserved amino acid sequences that form the phosphotyrosine-binding pocket. Highest homology between these molecules is in the SH3N domain, while the SH3C domain appears less conserved. The SH2 domain of Grap shows high homology to Grb2/Sem-5/Drk and contains all the conserved amino acid residues between the two proteins. The deduced amino acid sequence of Grap is shown in an alignment with Grb2. Identical and conserved amino acid residues are printed in bold capital letters, and the other amino acid residues are shown in small letters. The SH2 domain is indicated with a solid line, and the two SH3 domains are pointed out with a dashed line. The mammalian Grap rather than Grap is the mammalian homologue of Sem-5/Drk. Indeed, Stern and his collaborators (12) have shown that Grap is predominantly expressed in thymus and spleen; lower levels were detected in placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte.

Table I

| SH3N | SH2 | SH3C | Overall |
|------|-----|------|---------|
| Grap/Grb2 | 68 | 60 | 49 | 59 |
| Grap/Drk | 61 | 56 | 45 | 53 |
| Grap/Sem-5 | 58 | 54 | 34 | 49 |
| Grb2/Drk | 69 | 62 | 56 | 63 |
| Grb2/Sem-5 | 61 | 57 | 47 | 55 |
| Sem-5/Drk | 58 | 62 | 50 | 57 |

RESULTS AND DISCUSSION

The structure of Grap—Several cDNA inserts homologous to grb2 were found during sequencing of cDNA libraries derived from a 6-week-old human embryo or tonsils. One of the clones (1.7 kb, HGS code: 285076) containing an open reading frame was found during sequencing of cDNA libraries derived from a 6-week-old human embryo or tonsils. One of the clones (1.7 kb, HGS code: 285076) containing an open reading frame was resequenced on both strands by the dideoxy method using a kit from United States Biochemical Inc. Based on the deduced amino acid sequence, this protein is comprised of a central SH2 domain flanked by two SH3 domains. This Grb2/Sem-5/Drk-like arrangement is characteristic of an adaptor protein apparently lacking catalytic activities. Therefore, we suggest a name Grap for Grb2-related adaptor protein. The amino acid sequence of Grap was compared with Grb2, Sem-5, and Drk, and the percentage of identical amino acids is shown in Table I. Overall, Grap is most closely related to Grb2. The SH2 domain of Grap shows high homology to Grb2/Sem-5/Drk and contains all the conserved amino acid sequences that form the phosphotyrosine-binding pocket. Highest homology between these molecules is in the SH3N domain, while the SH3C domain appears less conserved. The similarity of Grb2 to Sem-5 and Drk is higher than that of Grap to the two lower eukaryotic molecules, suggesting that Grb2 rather than Grap is the mammalian homologue of Sem-5/Drk. Indeed, Stern and his collaborators (12) have shown that these domains are pointed out with a dashed line. Expression of the grap Gene—The mammalian grb2 gene is widely expressed in developing mouse embryos and various adult tissues (2, 3). To examine the expression of the grap gene, we performed Northern blot analysis on various human tissues. As shown in Fig. 2, a 2.3-kb transcript was detected using 32P-labeled grap cDNA as a probe. The grap gene was primarily expressed in thymus and spleen, although lower levels of transcript were detected in other tissues (Fig. 2). When the filters were reprobed with 32P-labeled grb2 cDNA, two grb2 transcripts of 3.8 kb and 1.5 kb were observed in all the tissues (Fig. 2), as reported previously (2, 3). By immunizing rabbits with a purified GST-fusion protein containing the GrapSH2 domain, we have successfully produced specific antiserum against Grap that works very well in immunoprecipitation and immunoblotting. The antibody specifically recognized a 27-kDa polypeptide in human cells, as compared to 23-kDa Grb2 (Fig. 3). It should be noted that most commercially available anti-Grb2 antibodies cross-react with Grap (Fig. 3). Conversely, a little cross-reaction of our anti-Grap antiserum with Grb2 was observed only after a long exposure of the immunoblot filters. The slower migration of Grap than Grb2 distinguishes the two proteins despite their identical predicted size. Highest amounts of Grap protein were detected in mouse thymus and spleen; lower levels were detected in lung, liver, kidney, heart, and brain (data not shown). These results suggest that Grap is predominantly expressed in hematopoietic and lymphocytic cells, and, indeed, Grap was easily detected in M07e, M07eR, TF1, and K562 cells by immunoblot analysis (Fig. 3).

Binding to Phosphoproteins via its SH2 Domain—The structure of Grap suggests that it functions as an adaptor molecule mediating protein-protein interaction. Purified GST-fusion proteins containing the GrapSH2 or Grb2SH2 domain were mixed with lysates of M07e or M07eR cells that were stimulated with or without stem cell factor or Epo, respectively. The SH2-binding proteins were subjected to immunoblot analysis with antibodies against phosphotyrosine (anti-PY) or to specific receptors. As shown in Fig. 4, A and B, the SH2 domains of Grap and Grb2 bound to the ligand-activated c-kit and EpoR; however, the binding affinity of GrapSH2 was apparently lower than that of Grb2SH2 (Fig. 4, A and B). Similar results were obtained by immunoblot analysis of the specific anti-Grap or anti-Grb2 immunoprecipitates (data not shown). Bcr-Abl fusion protein is an up-regulated PTK implicated in the pathogenesis of chronic myelogenous leukemia and acute myelogenous leukemia. We investigated the reciprocal effect of Grap and anti-Grb2 antibodies, respectively. However, the results were not conclusive.

FIG. 1. Deduced amino acid sequence of Grap. The deduced amino acid sequence of Grap is shown in an alignment with Grb2. Identical and conserved amino acid residues are printed in bold capital letters, and the other amino acid residues are shown in small letters. The SH2 domain is indicated with a solid line, and the two SH3 domains are pointed out with a dashed line.

FIG. 2. Grap is predominantly expressed in thymus and spleen. Northern blotting was performed on two human multiple tissue Northern blots as described in the text. Lanes 1–16, heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte.
lysoblastic leukemia. It has been demonstrated that Bcr-Abl forms a physical complex with Spr and Grb2 (15, 21, 22), and binding to Grb2 is required for activation of Ras proteins and the transforming activity of Bcr-Abl (15). To detect the interaction between Grap and Bcr-Abl, Grap was immunoprecipitated from a K562 cell lysate and the precipitates were blotted with anti-c-Abl. As shown in Fig. 4C, Grap co-precipitated from K562 cells with Bcr-Abl and purified GST-GrapSH2 bound Bcr-Abl in vitro. These results indicate that Grap, like Grb2, forms a stable complex through its SH2 domain with the Bcr-Abl oncoprotein in leukemia cells.

Grap binding to c-Abl forms a physical complex with Spr and Grb2 (15, 21, 22), and binding to Grb2 is required for activation of Ras proteins and the transforming activity of Bcr-Abl (15). To detect the interaction between Grap and Bcr-Abl, Grap was immunoprecipitated from a K562 cell lysate and the precipitates were blotted with anti-c-Abl. As shown in Fig. 4C, Grap co-precipitated from K562 cells with Bcr-Abl and purified GST-GrapSH2 bound Bcr-Abl in vitro. These results indicate that Grap, like Grb2, forms a stable complex through its SH2 domain with the Bcr-Abl oncoprotein in leukemia cells.

Binding to mSos1 through its SH3N Domain—The SH3 domain of mSos1, particularity the SH3N domain, of Grb2/Sem-5/Drk bind the C-terminal tail of the mSos1 protein (4, 9, 23). Co-immunoprecipitation shows that Grap is constitutively associated with mSos1 in TF1 cells (Fig. 4D). In vitro binding assay using purified GST-fusion proteins containing different Grap motifs demonstrates that the SH3N domain is responsible for Grap-mSos1 interaction (Fig. 4D). There is no detectable binding of the GrapSH2 and -SH3C domains to mSos1. The physical complex of Grap and mSos1 was also easily detected in M07e and several other cell lines (data not shown).

In summary, we have described a human cytoplasmic signaling protein that shares structural and functional similarity with Grb2. Both proteins contain a central SH2 domain sandwiched by two SH3 domains. The SH2 domains directly recognize phosphotyrosine-containing sites on activated receptor PTKs and cytoplasmic proteins. Its binding specificity is often dictated by the three amino acids C-terminal to phosphotyrosine (the +1 to +3 positions). A Trp residue in the EF1 position of the +3 binding pocket in Grb2SH2 was critical in defining specificity (24). In this regard, it is of great interest that the GrapSH2 has a Trp at the same position (Fig. 1) in addition to its high homology with Grb2SH2. Results from Fig. 4 demonstrate that the SH2 domains of Grap and Grb2 bind to tyrosine-phosphorylated c-kit, EpoR, and Bcr-Abl. We also observed that the two SH2 domains bind to a similar group of phosphoproteins in v-src transformed cells in vitro (data not shown). Further experiments are needed to determine whether the two SH2 domains recognize and compete for the same phosphotyrosine motifs in these activated receptors in vivo and select identical phosphopeptide sequences (25).

Grap appears to form a physical complex with mSos1 through its SH3N domain (Fig. 4D). This agrees with previous observations that the SH3N domain is primarily involved in association of Drk and Grb2 to mSos1 and mSos2, respectively (7, 23). Notably, higher similarities between these proteins are often seen in the SH3N region, and the SH3C domains appear less conserved. It seems reasonable that the SH3C domain of Grap may couple to other signaling pathways by binding yet-to-be identified proteins. For example, Grap may couple to other guanine nucleotide exchange factors for Ras, Rac, or Rho. The SH3 domains of Grb2 are also reported to associate with c-Cbl (26), a p75 protein (27), the p85 subunit of the phosphatidylinositol 3-kinase (28), β-dystroglycan (29), and Vav (30), although the physiological significance of these interactions has not been documented. It will be worthwhile to examine whether the GrapSH3 domains form complexes with these proteins. Finally, generation of Grb2 and Grap knockout mice will give important clues to their overlapping yet distinct functions in vivo.

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