Gab2, a New Pleckstrin Homology Domain-containing Adapter Protein, Acts to Uncouple Signaling from ERK Kinase to Elk-1*

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We describe a novel human adapter molecule containing a pleckstrin homolgy (PH) domain at the N terminus that is closely related to human Grb2-associated binder 1, Gab1, and Drosophila daughter of sevenless. We designate this protein as Gab2. Northern blot analysis indicates that Gab2 is widely expressed and has an overlapping but distinctive expression pattern as compared with Gab1, with high levels of Gab2 mRNA detected in the heart, brain, placenta, spleen, ovary, peripheral blood leukocytes, and spinal cord. Upon tyrosine phosphorylation, Gab2 physically interacts with Shp2 tyrosine phosphatase and Grb2 adapter protein. Strikingly, Gab2 has an inhibitory effect on the activation of Elk-1-dependent transcription triggered by a dominant active Ras mutant (RasV12) or under growth factor stimulation, whereas Gab1 acts to potentiate slightly the Elk-1 activity in the same system. In contrast to the reciprocal effects of Gab1 and Gab2 in mediating Elk-1 induction, these two molecules have a similar function in extracellular signal-regulated kinase activation induced by either oncogenic Ras or growth factor stimulation. Taken together, these results argue that Gab1 and Gab2, two closely related PH-containing adapter proteins, might have distinct roles in coupling cytoplasmic-nuclear signal transduction. This is the first evidence that an intracellular molecule with a PH domain operates as a negative effector in signal relay to the regulation of gene expression.

*Molecular dissection of cytoplasmic signal transduction mechanisms has been greatly facilitated by the identification of a large group of signaling molecules with distinct structural motifs, such as Src homology 2 (SH2), SH3, phosphotyrosine binding (PTB), and pleckstrin homology (PH) domains (1, 2). These modules apparently mediate specific protein-protein or protein-lipid interactions and therefore orchestrate cellular responses to various extracellular stimuli. The PH domain was originally identified as a protein module of around 120 amino acids in a number of cytoplasmic signaling proteins that display homology to a region repeated in the protein pleckstrin (3–5). Our current view on the function of PH domains stands on the point that they are involved in the recruitment of proteins to plasma membranes, possibly through binding to phosphatidylinositol polyphosphates (6).

PH domains have been found in a variety of enzymes, such as Btk, Akt, βARK kinases, and phospholipase Cγ, as well as in adapter molecules with no enzymatic activity that include Grb7 and insulin receptor substrate (IRS) 1–4 (7–9). In the latter subgroups, one attractive member identified recently is the Grb2-associated binder 1, Gab1 (10). Structurally, Gab1 contains a PH domain at the N terminus and multiple tyrosyl residues at the C-terminal part, which presumably serve as docking sites for SH2-containing proteins in a tyrosine phosphorylation-dependent fashion. Overexpression of Gab1 stimulates cell growth and transformation (10). Accumulating biochemical data suggest that Gab1 functions as a major adapter molecule downstream of receptors for nerve growth factor, hematocyte growth factor, epidermal growth factor, insulin, and B cell antigen (10–14).

Genetic screening for suppressor mutations of a dominant active Sevenless protein in Drosophila led to the identification of the daughter of sevenless gene that also encodes for a PH-containing molecule, structurally related to mammalian Gab1 (15). Interestingly, daughter of sevenless seems to be a putative substrate for Corkscrew, a tyrosine phosphatase with two SH2 domains (16), because a catalytically inactive mutant of Corkscrew trapped the daughter of sevenless protein in its highly tyrosine-phosphorylated form (17). It remains to be defined whether Gab1 is a physiological substrate of Shp2, the mammalian homologue of Corkscrew. However, it was demonstrated that Shp2 physically interacts with Gab1, and this interaction might play a critical role in transducing signals into the Ras pathway (10, 14, 18).

In this paper, we report the identification of another Gab1-related molecule, designated as Gab2, that also contains a PH domain at the N terminus. Evidence is presented that although it acts to promote mitogenic stimulation of ERK kinase activity, Gab2 appears as a negative effector in the modulation of cytoplasmic signaling into the nucleus. These results allow us to make an intriguing proposal that a family of PH-containing adapter proteins function in both negative and positive ways to control intracellular signaling cascades.

EXPERIMENTAL PROCEDURES

DNA Constructs and Other Reagents—A human cDNA clone was identified in the GenBank™ that shows high homology to the human Gab1 sequence (10). This sequence was deposited by the Kazusa DNA Research Institute, Chiba, Japan and named as KIAA 0571 (clone: HH2388). A 6052-base pair fragment inserted at the Sall-NotI sites of pbBluescript II SK+ was kindly supplied to us by Dr. T. Nagase. In a comparison with the Gab1 sequence, this cDNA clone seemed to be incomplete at the 5′-end. Accordingly, a Human Testis Marathon-
Gab2, a Novel Adapter Protein with a PH Domain

| Number of clones | Length |
|------------------|--------|
| 1                | 447**  |
| 2                | 431    |
| 3                | 405    |
| 4                | 360    |
| 5                | 318    |
| 6                | 62     |

**The nucleotide sequence of the longest clone is shown with the region overlapping with KIAA0571 underlined, and the initiation codon ATG is printed in bold.

Using the Human Testis Marathon-ready cDNA kit from Clontech, we performed 5'-RACE to search for more sequences at the 5'-end of Gab2 cDNA and obtained PCR products at different length. The six conserved subdomains are also indicated.

**Maung Magwe, Yein Sok, Hmawng Khaing, Ngaing Zaw, and D. H. Talbot**

**Department of Biological Sciences, University of Northern Iowa**

**Summary for 5'-RACE results of Gab2 cDNA**

| Number of clones | Length |
|------------------|--------|
| 26               | 2      |
| 7                | 318    |
| 7                | 360    |
| 1                | 447**  |
| 2                | 431    |
| 3                | 405    |

**Reamstown, PA.**

**Immunoprecipitation and Immunoblotting—**Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and mouse recombinant interleukin-3 (IL-3) (0.1 ng/ml, R & D Systems). Cells were washed with serum-free medium twice and starved in RPMI 1640 plus 0.8% bovine serum albumin for 8–10 h before being treated with IL-3 (1 ng/ml) for 10 min at 37 °C. Cells were washed with ice-cold phosphate-buffered saline and lysed in modified radiolmune precipitation buffer (20). Lysates were centrifuged at 15,000 × g for 10 min, and supernatants were subjected to immunoprecipitation with specific antibodies at 4 °C for 2–4 h. Resulting immune complexes were incubated with protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech) at 4 °C for 1–2 h. Immune complex beads were washed three times in HNTG buffer (20). Lysates were centrifuged at 15,000 × g for 10 min, and supernatants were subjected to immunoprecipitation with specific antibodies at 4 °C for 2–4 h. 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Gab2, a Novel Adapter Protein with a PH Domain

Gab2 shares the highest homology to Gab1. The high sequence homology and the similarity of molecular architecture between Gab1, Gab2, and daughter of sevenless strongly suggest that these proteins constitute a subfamily of signal transducers with a PH domain and multiple potential SH2 binding sites. The IRS family of proteins also contain a PTB domain, in addition to the PH domain and multiple tyrosine phosphorylation sites (7–9). It remains to be determined, however, whether Gab1 and/or Gab2 are mammalian homologues of Drosophila daughter of sevenless. Also conserved between Gab1 and Gab2 are the potential Tyr phosphorylation sites over the C-terminal part of the molecule that have consensus sequences for binding SH2 domains (25). Notably, there are three YXXM motifs that are presumably involved in binding the p85 SH2 domains of phosphatidylinositol 3-kinase, the YXIP motif for phospholipase C-γ, the YYXR motif for Nck, and the YLAL motif for src. Another striking feature that is shared by Gab1, Gab2, and IRS proteins is the enrichment of Ser and Thr residues. For example, there are 135 Ser/Thr residues in Gab1 and 143 in Gab2. Like Gab1, Gab2 possesses two proline-rich motifs that are presumably engaged in binding SH3-containing proteins. Another striking feature that is shared by Gab1, Gab2, and IRS proteins is the enrichment of Ser and Thr residues. For example, there are 135 Ser/Thr residues in Gab1 and 143 in Gab2. This would raise an interesting possibility that these adapter proteins might function to integrate or coordinate signaling pathways between Ser/Thr, Tyr kinases, and/or phosphatases.

Expression of the Gab2 Gene—To determine the expression pattern of the human Gab2 gene, we performed Northern blot analysis on various human tissues. As shown in Fig. 1B, alignment of the Gab2 PH domain with that of Gab1, daughter of sevenless, and IRS1 revealed that Gab2 shares the highest homology to Gab1. The high sequence homology and the similarity of molecular architecture between Gab1, Gab2, and daughter of sevenless strongly suggest that these proteins constitute a subfamily of signal transducers with a PH domain and multiple potential SH2 binding sites. The IRS family of proteins also contain a PTB domain, in addition to the PH domain and multiple tyrosine phosphorylation sites (7–9). It remains to be determined, however, whether Gab1 and/or Gab2 are mammalian homologues of Drosophila daughter of sevenless. Also conserved between Gab1 and Gab2 are the potential Tyr phosphorylation sites over the C-terminal part of the molecule that have consensus sequences for binding SH2 domains (25). Notably, there are three YXXM motifs that are presumably involved in binding the p85 SH2 domains of phosphatidylinositol 3-kinase, the YXIP motif for phospholipase C-γ, the YYXR motif for Nck, and the YLAL motif for src. Another striking feature that is shared by Gab1, Gab2, and IRS proteins is the enrichment of Ser and Thr residues. For example, there are 135 Ser/Thr residues in Gab1 and 143 in Gab2. This would raise an interesting possibility that these adapter proteins might function to integrate or coordinate signaling pathways between Ser/Thr, Tyr kinases, and/or phosphatases.

RESULTS

Molecular Structure of Gab2—By searching the GenBank™ data base, we identified a human cDNA sequence (KIAA 0571) that shows a high homology to Gab1. However, this 6-kilobase cDNA fragment contains an open reading frame for a polypeptide consisting of 638 amino acid residues that seemed to lack a portion of the N-terminal part when compared with Gab1. By performing 5′-rapid amplification of cDNA ends, we isolated a cDNA fragment that contains the missing part of Gab2 to different extents (Table I). Upon recombining one 5′-rapid amplification of cDNA ends product with the KIAA0571 sequence, we obtained a cDNA fragment that encodes a protein of 676 amino acid residues. Based on the deduced amino acid sequence, this protein is comprised of a PH domain at the N terminus and multiple potential tyrosine phosphorylation sites at the C-terminal part, without any known enzymatic domains. Therefore, the architecture of this molecule is closely related to human Gab1 and Drosophila daughter of sevenless (10, 15). We suggest Gab2 for the name of this protein.

Expression of Gab2 in various human tissues. Three filters that contain 2 μg of poly(A) mRNA on each lane were purchased from CLONTECH. Northern blot analysis was performed using a specific probe for Gab2 and also a probe for human β-actin. Lane 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, peripheral blood leukocyte; 17, stomach; 18, thyroid; 19, spinal cord; 20, lymph node; 21, trachea; 22, adrenal gland; 23, bone marrow.

Gab2 tyrosine phosphorylation and interaction with Shp2 and Grb2. A, control or IL-3-stimulated Ba/F3 cell lysates were immunoprecipitated by anti-Gab2 antibody and subjected to immunoblot analysis with anti-phosphotyrosine antibody (pTyr) or immunoblotted with anti-Shp2 and, after stripping, re-blotted with anti-Gab2 antibody. B, Ba/F3 cell lysates were precipitated with anti-Grb2 antibody. The precipitates were immunoblotted first with anti-Gab2 and, after stripping, re-blotted with anti-Grb2. IP, immunoprecipitate.

As shown in Fig. 1B, alignment of the Gab2 PH domain with that of Gab1, daughter of sevenless, and IRS1 revealed that Gab2 shares the highest homology to Gab1. The high sequence homology and the similarity of molecular architecture between Gab1, Gab2, and daughter of sevenless strongly suggest that these proteins constitute a subfamily of signal transducers with a PH domain and multiple potential SH2 binding sites. The IRS family of proteins also contain a PTB domain, in addition to the PH domain and multiple tyrosine phosphorylation sites (7–9). It remains to be determined, however, whether Gab1 and/or Gab2 are mammalian homologues of Drosophila daughter of sevenless. Also conserved between Gab1 and Gab2 are the potential Tyr phosphorylation sites over the C-terminal part of the molecule that have consensus sequences for binding SH2 domains (25). Notably, there are three YXXM motifs that are presumably involved in binding the p85 SH2 domains of phosphatidylinositol 3-kinase, the YXIP motif for phospholipase C-γ, the YYXR motif for Nck, and the YLAL motif for src. Another striking feature that is shared by Gab1, Gab2, and IRS proteins is the enrichment of Ser and Thr residues. For example, there are 135 Ser/Thr residues in Gab1 and 143 in Gab2. This would raise an interesting possibility that these adapter proteins might function to integrate or coordinate signaling pathways between Ser/Thr, Tyr kinases, and/or phosphatases.

Expression of the Gab2 Gene—To determine the expression pattern of the human Gab2 gene, we performed Northern blot analysis on various human tissues. As shown in Fig. 2, Gab2 is widely expressed, with high levels detected in the heart, brain, placenta, spleen, ovary, peripheral blood leukocytes, and spinal cord. Compared with the Gab1 expression pattern described previously (10), there is an overlap for the expression of Gab1 and Gab2 genes in tissues, such as heart, brain, spinal cord and placenta. Distinct expression of Gab1 and Gab2 was also ob-
served in several other tissues. For example, Gab1 was highly expressed in the small intestine and colon where there were low levels of Gab2 mRNA detected. On the other hand, Gab1 expression was almost undetectable in peripheral blood cells, in which Gab2 was expressed at a high level. This differential expression pattern might reflect the participation of Gab1 and Gab2 in overlapping yet distinct signaling pathways in different cell types.

**Putative Roles of Gab2 in Cytoplasmic Signaling**—To explore the Gab2 function in cell signaling, we generated a specific antibody against Gab2 by immunizing rabbits with a GST fusion protein containing the C-terminal part of Gab2. This antibody recognized a protein of around 85 kDa (Fig. 3). Gab2 became tyrosine-phosphorylated and associated with Shp2 tyrosine phosphatase and Grb2 adapter protein in Ba/F3 cells treated with IL-3, as detected by co-immunoprecipitation (Fig. 3). We also examined the response to EGF of HA-Gab1 and HA-Gab2 exogenously expressed in human 293 cells. EGF treatment of cells for 10 min induced a significant increase in the tyrosine phosphorylation of Gab1 and Gab2 (data not shown).

As demonstrated in Fig. 1A, there are two proline-rich motifs in Gab2, which might be engaged in SH3-mediated protein interaction. To test this hypothesis, we performed an in vitro binding assay on the interaction of HA-Gab2 expressed in 293 cells with purified GST fusion proteins that contain individual SH3 domains. These were isolated from a number of signaling molecules, including Grb2, Grap, c-Abl, Crk, c-Src, RasGTPase-activating protein, and phospholipase Cγ, as well as full-length Nck. As shown in Fig. 4, the SH3-C domain of Grb2 has the highest affinity to bind Gab2, whereas there were modest levels of binding for Gab2 with the Grb2 SH3-N, Grap SH3-N, c-Src SH3, phospholipase C-γ SH3, and Nck. This result suggests that Gab2 might be a main partner of Gab2 in a cell-signaling scheme that involves SH3 function. It is interesting to note that the binding of Grb2 to Gab2 is apparently different from the Grb2-Sos1 interaction, which is primarily mediated through the SH3-N domain of Grb2 (26–28).

**Gab2 Functions to Suppress Ras-mediated Elk-1 Activation**—Structural and biochemical analyses on Gab2 described above would suggest that this novel PH domain-containing protein might participate in the Ras signaling pathway, with similar function to Gab1. To test this hypothesis, we compared the effect of Gab1 and Gab2 on the Ras-mediated activation of Elk-1, by monitoring the luciferase activity under the control of Elk-1, a member of the ternary complex factors family. Human 293 cells were co-transfected with RasV12, Gab1 or Gab2, Gal4-Elk, and Gal4-Luc. Surprisingly, Gab1 and Gab2 had opposite effects in the modulation of RasV12-driven Elk-1 activity. Whereas Gab1 had a modest promoting effect on Elk-1-dependent transcription in response to the dominant active Ras, Gab2 exhibited a significant suppression of RasV12-induced Elk-1 activity under the same conditions (Fig. 5A). To further examine its inhibitory effect, we examined the Elk-1-driven luciferase activity under growth factor stimulation. Cells were co-transfected with Gal4-Elk and Gal4-Luc together with Gab1 or Gab2. After 8 h, cells were serum-starved for 12 h and treated with human recombinant EGF (50 ng/ml, Becton Dickinson) for 8 h. Consistently, Gab2, in contrast to Gab1, had a dramatic suppressing activity on Elk-1 function under EGF stimulation (Fig. 5B). To determine the expression levels of HA-Gab1 and HA-Gab2 in 293 cells, total cell lysates were immunoblotted with anti-HA antibody, and similar protein amounts of Gab1 and Gab2 were detected (Fig. 5C). Taken together, these results indicate that Gab2 acts to suppress Ras signaling into the nucleus in the modulation of Elk-1 transcription.

**Fig. 5. Inhibitory effect of Gab2 on RasV12- or EGF-stimulated Elk-1 activation.** A, 293 cells in 60-mm dishes were transfected with 0.2 μg of Gal4-Elk, 2.5 μg of 5xGal4-Luc, 0.2 μg of pBp-Ha-RasV12, and 8 μg of HA-Gab2 (or HA-Gab1, vector control as indicated). Cells were starved in serum-free medium for 24 h before being harvested. pCMV/β-galactosidase (10 ng) was used in the co-transfection as an internal control. The activity of pBp vector control was 1. The data are averaged from three independent experiments. B, 293 cells were transfected with reporter plasmids, HA-Gab1, HA-Gab2, or pcDNA3 vector. Cells were kept in serum-free medium for 12 h and then treated human recombin...
tion activity.

Gab2 Acts to Enhance ERK Kinase Activation—The results described above raised a very interesting possibility that Gab1 and Gab2, two structurally related molecules, might have opposite effects in mediating the Ras signal that is presumably transmitted through ERK kinase into the nucleus. To dissect the molecular mechanism, we examined the influence of Gab1 and Gab2 on activation of the ERK kinase by RasV12. Cells were co-transfected with RasV12, HA-Gab1 or HA-Gab2, and GST-ERK1. After 48 h, GST-ERK1 was precipitated with glutathione-Sepharose beads from cell lysates, and ERK activity was measured in an in vitro kinase assay using myelin basic protein as a substrate. As shown in Fig. 6A, expression of RasV12 in 293 cells induced a significant increase in the ERK1 activity. This effect was slightly enhanced by co-transfection of either Gab1 or Gab2, suggesting that these two molecules have similar roles in mediating the Ras to ERK signaling.

To corroborate this observation, we further compared the effect of Gab1 and Gab2 on EGF-induced ERK activation. As shown in Fig. 6B, expression of Gab1 or Gab2 had a similar and modest enhancing effect on EGF-stimulated ERK1 activity. These results suggest that Gab1 and Gab2 act in the same fashion in the Ras-ERK signaling cascade activated by growth factor or oncogenic Ras. However, Gab2 apparently functions as a unique negative effector to modulate the signal output of the Ras-ERK pathway in the control of gene expression.

DISCUSSION

We have described a novel adapter protein, Gab2, that contains a PH domain at the N terminus and shares the same molecular architecture as Gab1. Gab2 is widely expressed in many human tissues. We also demonstrate a physical interaction between Gab2 and Shp2, Grb2. The SH3-C domain of Grb2 has a high binding affinity toward Gab2. Gab2 has a positive effect similar to Gab1 in mediating the induction of ERK kinase activity by growth factor or oncogenic Ras. However, Gab2 appears to function in a unique way in Ras signaling by uncoupling Elk-1 activation from ERK kinase activity, in contrast to the positive role of Gab1 in the same pathway.

Although the physiological function of Gab1 has not been fully understood, it apparently acts to promote cell growth and transformation. Overexpression of Gab1 leads to enhanced cell division and anchorage-independent cell growth in soft agar (10). Tyrosine phosphorylation of Gab1 and its association with Grb2 might also play an important role in cellular transformation by Tpr-Met, an oncprotein consisting of the catalytic domain of hepatocyte growth factor receptor tyrosine kinase fused with sequences encoded by the tpr gene (13). A mutant Tpr-Met protein (Tyr<sup>489</sup>→Phe) that fails to bind to Grb2 has significantly impaired transforming activity. Although it is unclear how Gab1 functions in cytoplasmic signaling, the molecular architecture of this protein strongly suggests that Gab1 is a tyrosine phosphorylated at numerous sites and therefore is engaged in the formation of a complex that consists of many proteins, including phospholipase C<sub>γ</sub>, Shp2, and phosphatidylinositol 3-kinase. In this regard, Gab1 and Gab2 might work in cells in a similar manner as the family of insulin receptor substrates, IRS1–4, by coupling to multiple signaling pathways. However, the difference is the possession of a PTB domain following the PH domain in the IRS proteins, which is missing from Gab1 and Gab2. Without a PTB domain, Gab1/2 might interact with growth factor receptors indirectly via other SH2-containing molecules, such as Grb2.

Gab1 and Gab2 share structural similarity with the Droso<i>phila</i> daughter of sevenless protein (15). Not only is daughter of sevenless physically associated with Corkscrew, but daughter of sevenless is the major phosphoprotein trapped by a catalytically inactive mutant of Corkscrew that was overexpressed in cells (17). Both Gab1 and Gab2 have been detected in association with Shp2, the mammalian homologue of Droso<i>phila</i> Corkscrew. Now the critical issue is to determine whether they are the physiological substrates of Shp2 and how this dephosphorylation event could contribute to the Ras signaling pathway. It is possible that these two molecules may serve as substrates of the ubiquitously expressed Shp2 phosphatase in different cell types. Further, these proteins may also be potential substrates of another Shp2-related phosphatase,
Gab2, which is predominantly expressed in hematopoietic cells (29, 30).

The most striking finding in this report is the reciprocal effects of Gab1 and Gab2 in mediating Ras-responsive Elk-1 activation in cells. Gab2 does not seem to block the ERK kinase activation by either receptor tyrosine kinase or dominant active Ras, but rather it acts to uncouple signals from activated ERK to Elk-1, a member of ternary complex factors family. While this paper was under review, Gu et al. (31) reported the cloning of mouse p97/Gab2. Consistent with our data, transient expression of p97/Gab2 in Ba/F3 cells also led to a suppression of IL-3-induced Elk-1 activity, whereas ERK1 kinase activation was not affected. This inhibitory effect of mouse Gab2 on IL-3-stimulated Elk-1 activity was exacerbated by the introduction of a C-terminal truncation mutation that abolished its binding to Shp2.

A similar uncoupling effect on ERK kinase to Elk-1 stimulation was recently observed for the kinase suppressor of Ras (32). When overexpressed, the kinase suppressor of Ras blocked EGF and the Ras induction of Elk-1-dependent transcription. Therefore, the Gab2 and kinase suppressor of Ras might represent components of a novel and poorly understood pathway that modulates the signal relay from the Ras-ERK kinase to the activation of nuclear ternary complex factors. Although the mechanism for its suppression on Elk-1 activation is to be understood, the Gab2 protein without any catalytic activities acts apparently by complexing with other enzymes, such as kinases and phosphatases, that contain SH2 and SH3 domains. Further investigation of the unique function of Gab2 will gain fundamental insight into the molecular mechanism by which the Ras-signaling scheme is concerted.

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