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Grb2 Dominantly Associates With Dynamin II in Human Hepatocellular Carcinoma HepG2 Cells

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Abstract The two SH3 domains and one SH2 domain containing adaptor protein Grb2 is an essential element of the *Ras* signaling pathway in multiple systems. The SH2 domain of Grb2 recognizes and interacts with phosphotyrosine residues on activated tyrosine kinases, whereas the SH3 domains bind to several proline-rich domain-containing proteins such as Sos1. To define the difference in Grb2-associated proteins in hepatocarcinoma cells, we performed coprecipitation analysis using recombinant GST-Grb2 fusion proteins and found that several protein components (p170, p125, p100, and p80) differently associated with GST-Grb2 proteins in human Chang liver and hepatocarcinoma HepG2 cells. Sos1 and p80 proteins dominantly bind to Grb2 fusion proteins in Chang liver, whereas p100 remarkably associate with Grb2 in HepG2 cells. Also GST-Grb2 SH2 proteins exclusively bound to the $p46^{Shc}$, $p52^{Shc}$, and $p66^{Shc}$ are important adaptors of the *Ras* pathway in HepG2 cells. The p100 protein has been identified as dynamin II. We observed that the N-SH3 and C-SH3 domains of Grb2 fusion proteins coprecipitated with dynamin II besides Sos1. These results suggest that dynamin II may be a functional molecule involved in Grb2-mediated signaling pathway on *Ras* activation for tumor progression and differentiation of hepatocarcinoma cells. J. Cell. Biochem. 84: 150–155, 2002.

Key words: Grb2/SH3 domain-binding proteins; Dynamin II; hepatocarcinoma HepG2 cell

Growth factor receptor-bound protein 2 (Grb2), a 25 kDa protein, is a ubiquitously expressed adapter protein composed of one SH2 domain flanked by amino- and carboxy-terminal SH3 domains (N-SH3 and C-SH3, respectively) [Lowenstein et al., 1992]. The Grb2 SH2 domain binds to several tyrosine-phosphorylated receptor-type molecules, including *erbB2* [Janes et al., 1994], EGFR [Lowenstein et al., 1992], PDGFR [Arvidsson et al., 1994], and M-CSFR [Kharbanda et al., 1995] as well as other phosphotyrosine containing proteins like Shc [Rozakis-Adcock et al., 1992]. The major function of Grb2 SH3 domains is thought to be binding the *Ras*-guanine nucleotide exchange factor Sos1 [Li et al., 1993; Warnock et al., 1995]. This Grb2/Sos complex activates *Ras* in response to growth factors in many different kinds of cells so that the activated *Ras* in turn leads to activation of Raf and MAP kinase [Joneson et al., 1996; Khosravi-Far et al., 1996]. The recruitment of Grb2/Sos complex to the plasma membrane is due to the phosphorylation of Shc proteins in *Ras* activation. In addition to the signaling pathway through *Ras* activation, it has been reported that Grb2 can associate with at least five different proteins besides Sos1 in human breast cancer cells [Sastry et al., 1997]. This report has shown a possibility that each of these Grb2 binding complexes may mediate in different cellular

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functions or other functions for tumor progression and differentiation.

Dynamin is a 100-kDa GTPase that mediates in late stages of endocytosis in both neuronal and nonneuronal cells [McClure and Robinson, 1996]. Although several past studies suggested the neuronal restricted existence of dynamin, it is now clear that dynamin isoforms are expressed in all tissues [Scaife and Margolis, 1990; Chen et al., 1992]. So far, three different isoforms of dynamin have been reported with their tissue-specific expression: neuronal specific dynamin I [Cook et al., 1994; Sontag et al., 1994]; ubiquitously expressed dynamin II [Cook et al., 1994; Kelly, 1995]; and testis-specific dynamin III [Nakata et al., 1993]. Although there is considerable evidence that dynamin is involved in the endocytic process at plasma membrane, most of the studies have been done in neuronal cells, which probably represent dynamin I. Dynamin II is highly homologous to dynamin I with an overall sequence identity of 79% [Sontag et al., 1994]. Recently, dynamin II has been reported to be localized in the trans-Golgi network (TGN) rather than plasma membrane [Maier et al., 1996] and participated in the formation of distinct transport vesicle from the TGN [Jones et al., 1998], implying that its function is distinct form that of dynamin I.

Dynamin is known to bind to the several signal molecules containing the SH3 domains such as Grb2, PLCγ [Seedorf et al., 1994], subunit of PI3K (p85) [Miki et al., 1994], amphiphysin [Grabs et al., 1997], IRS1 [Ando et al., 1994] and Src [Foster-Barber and Bishop, 1998]. These results suggested that dynamin II might have diverse functional roles, which link signal transduction mechanisms to the endocytic pathway. Recently, most studies on this field have previously been done in neuronal cells with dynamin I, the interest raises the possibility that dynamin II might have more functionally significant roles involved in growth factor receptor signaling. We have previously reported that dynamin II dominantly associated with Grb2 SH3 domain in *Ras* overexpressed NIH3T3 cells [Yoon et al., 1997]. This result suggested that dynamin II might be a functional molecule on *Ras* signaling pathway.

In this study, to find the difference in signals mediated by Grb2 bound proteins between normal and tumor cells, we investigated Grb2 associated proteins in human normal Chang liver and hepatocarcinoma HepG2 cells by the coprecipitation method using GST fusion proteins. We also found that several proteins differently associated with Grb2 between Chang liver and HepG2 cells, and dynamin II is a dominant protein associated with Grb2 SH3 domains in HepG2 cells. Our results suggest a possibility that the tumor promotion and progression in human liver cancer may be closely related with these complexes, Grb2-dynamin II.

MATERIALS AND METHODS

Cell Culture and Cell Lysis

Chang liver and HepG2 cells were maintained in the Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin G (100 IU/ml), streptomycin sulfate (100 μg/ml), amphotericin B (0.25 μg/ml), and 2-mercaptoethanol (50 μM) at 37°C in a 5% CO_2 humidified incubator.

Chang liver and HepG2 cells grown to 80–90% confluent in T-185 flask were replenished with fresh methionine-free DMEM medium and were incubated for 15 min in a CO_2 incubator. After washing the cells twice with 10 ml of labeling medium (methionine-free DMEM, 5% fetal bovine serum), 0.25 mCi of [^{35}S]-methionine (Amersham, Arlington Heights, IL) was added into the culture and incubated for 4 h in a humidified incubator. The labeled cells were washed and replenished with 10 ml of ice-cold phosphate buffered saline (PBS). The cells were then scrapped and collected by centrifugation at 300*g* for 5 min at 4°C. The supernatant was removed and the cells were resuspended in lysis buffer (10 mM Tris–HCl [pH 7.4], 0.5% Nonidet P-40, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml aprotinin) for 1 h on ice. The crude cell extracts were obtained after removing cell debris by centrifugation at 14,000*g* for 20 min at 4°C.

In vitro Binding of GST-Grb2 Fusion Proteins

The GST fusion proteins of Grb2 (1-217 amino acids), Grb2 SH2 domain (Grb2/SH2, 60–158 amino acids), Grb2 N-terminal SH3 domain (Grb2/SH3(N), 1–54 amino acids), and Grb2 C-terminal SH3 domain (Grb2/SH3(C), 163–217 amino acids) have been previously described [Yoon et al., 1997] and were purified on glutathione sepharose beads (Pharmacia, USA) as described previously [Smith and Johnson,

1988]. Ten micrograms of each GST fusion protein was incubated with the lysates of HepG2 cells pulsed with [^{35}S]-methionine for 3 h at 4°C with gentle rotating. Then glutathione sepharose beads were incubated with each sample. The beads were washed with PBS to remove unbound proteins to GST fusion proteins and GST alone. After washing, the bound proteins to beads were analyzed by SDS-PAGE using 6–15% gradient gel, transferred to polyvinylidene difluoride (PVDF) membrane, and exposed to X-OMAT film for 24 h at room temperature.

Western Blotting

To identify the Grb2 associated proteins the coprecipitation blots were blocked with 3% bovine serum albumin in PBS for 30 min and incubated with mouse antidynamin IgG_1 (Transduction Laboratory, Lexington, KY), rabbit anti-Sos1 IgG (Upstate Biotechnology, Lake Placid, NY) and rabbit anti-Shc IgG (Upstate Biotechnology) at 4°C overnight. The blots were then incubated with horseradish peroxidase-conjugated goat–mouse IgG (Sigma) for 2 h at 4°C. To detect the p100 protein, the blot was incubated with mouse antidynamin IgG_1 whereas we used rabbit anti-Sos1 IgG to detect the p170 bound protein. Immunoreactivity was determined using substrate solution, PBS containing 4-chloro-1-naphthol (0.018%, Sigma), H_2O_2 (0.05%), and methanol (5%).

RESULTS AND DISCUSSION

Comparison of Grb2-Associated Proteins in Human Normal Chang Liver and Hepatocarcinoma HepG2 Cells

Transformed malignant cells growing in cell culture exhibit several tumor specific changes including alteration of cytoplasmic phenotypes, growth characteristics, cell membrane structure and function, and altered cellular signal mechanisms. To explore the transformed malignant cell-derived changes, occurring around Grb2 in signaling pathways, we carried out coprecipitation experiments with the purified GST alone or with GST-Grb2 fusion proteins and compared the precipitates from Chang liver (Fig. 1A) and HepG2 cells (Fig. 1B). We observed that Grb2 coprecipitated with four proteins (p170, p125, p100, p80) in these cells. Interestingly, p170 and p80 exclusively coprecipitated

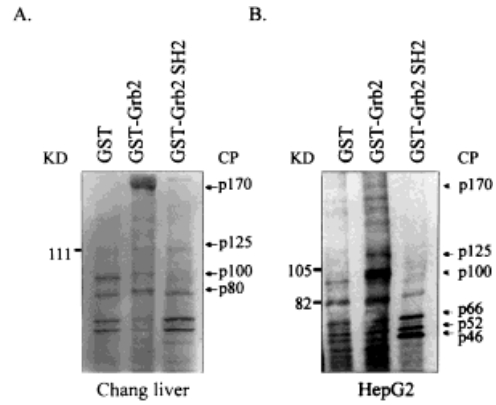

Fig. 1. Coprecipitated proteins with GST-Grb2 fusion protein in human liver and hepatocarcinoma cells. The lysates prepared from human liver cell line, Chang liver (**A**) and hepatocarcinoma, HepG2 (**B**) cells which were pulsed with [^{35}S]-methionine were incubated with 50 μg of GST, GST-Grb2 or GST-Grb2 SH2 fusion proteins immobilized on the glutathione sepharose beads (20 μl of 50% slurry) for 3 h at 4°C. The beads were washed with PBS to remove unbound proteins to GST or GST fusion proteins. The proteins bound to beads were analyzed by SDS-PAGE using 6–15% gradient gels, transferred to PVDF membrane, and exposed to X-OMAT film. Grb2 associated proteins (p170, p125, p100, and p80) are shown by arrows in Chang liver and HepG2 cells. P170, the coprecipitated protein with GST-Grb2 in Chang liver cells was dominantly shown (A). On the other hand, p100 was mainly associated with Grb2 fusion protein and p66, p52, and p46 were exclusively bound to Grb2 SH2 fusion proteins in HepG2 cells (B).

with Grb2 in Chang liver, whereas Grb2 appears to have preferential interaction with p100 and Grb2 SH2 dominantly bound to p66, p52, and p46 in HepG2 cells. Protein p125 seems to interact with Grb2 in these two cells. Although several minor bands were shown, p100 was the dominantly precipitated protein in hepatocarcinoma HepG2 cells. Since we pulsed the cells with [^{35}S]-methionine, the intensities of bands shown in Figure 1 represent the amount of newly synthesized proteins. The pattern of total proteins precipitated with GST-Grb2, when detected by Coomassie blue staining, was not significantly different from that of [^{35}S]-labeled proteins (data not shown). It is already known that Grb2 interacts with focal adhesion kinase (FAK) in v-src-transformed cells [Schlaepfer et al., 1994] and with erbB2 and Shc in erbB2-overexpressing cancer cells [Sastry et al., 1997]. Recently it has been reported that Grb2 was required to stimulate the Akt pathway to propagate mitogenic signals in breast cancer cells [Lim et al., 2000]. Therefore, each of these Grb2 binding proteins may mediate in a different function in various tumor cells and our results suggest a possibility that

Grb2–p100 complex has other cellular functions in HepG2 cells.

Identification of the p100 Protein as Dynamin II

In general, dynamin II is expressed ubiquitously in the entire tissue and [Cook et al., 1994; Sontag et al., 1994] its association with Grb2 has been observed in human monocytes when stimulated with macrophage-colony stimulating factor [Kharbanda et al., 1995] and insulin [Ando et al., 1994]. We also previously reported that dynamin II dominantly associated with Grb2 in *Ras* overexpressed NIH3T3 cells. And then to define whether p100 is dynamin II, we carried out Western blot by using antidynamin antibody and found that the p100 is dynamin II (Fig. 2A).

As shown in Figure 3B probing with anti-Sos1 antibodies, 170-kDa protein was identified as Sos1 by Western blot analysis in HepG2. It is known that Sos1 interacts with Grb2 via SH3 domains in *Ras* activating signal pathway [Joneson et al., 1996; Khosravi-Far et al., 1996]. 66, 52, and 46-kDa proteins associated with Grb2 SH2 fusion protein was identified as Shc (Fig. 3C). It has been published that the phosphorylated Shc proteins associated with the Grb2 adaptor protein [Clark et al., 1992] through direct binding of the Grb2 SH2 domain to the major Shc tyrosine phosphorylation site [Rozakis-Adcock et al., 1992]. And then phosphorylated Shc proteins recruited the membrane localization of Grb2/Sos complex for *Ras* activation [Aronheim et al., 1994]. Therefore our results have shown that Shc proteins were constitutively phosphorylated in HepG2 cells as compared with normal liver cells. Pelicci et al.

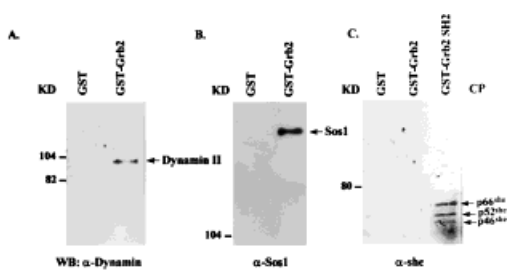

Fig. 2. Identification of coprecipitated proteins with GST-Grb2 fusion proteins. To identify the coprecipitated (CP) proteins with Grb2 fusion proteins, the membranes for this analysis by Western blotting (WB) were incubated with mouse anti-dynamin IgG (**A**), rabbit anti-Sos1 IgG (**B**), and rabbit anti-Shc IgG (**C**), respectively as described in Materials and Methods. Arrows indicates the position of dynamin II, Sos1 and Shc.

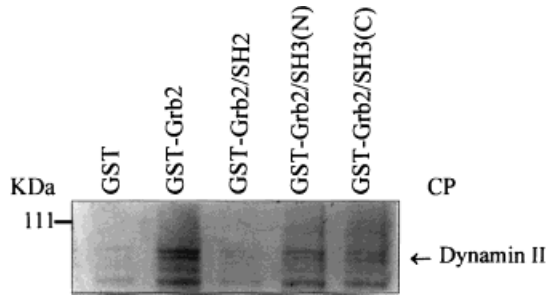

Fig. 3. The SH3 domain-mediated association of Grb2 to dynamin II. [^{35}S]-methionine labeled lysates of HepG2 cells were incubated with GST, GST-Grb2, GST-Grb2/SH2, GST-Grb2/SH3(N), or GST-Grb2/SH3(C) immobilized on glutathione sepharose beads as described in Materials and Methods. The bound proteins were separated on 6–15% gradient SDS-PAGE, transferred to PVDF membrane, and exposed to X-ray film. Arrows indicate the position of dynamin II.

[1995] has published that Shc proteins were constitutively phosphorylated in several human tumor cell lines containing hepatocarcinoma, Hep3B cell line.

Our results clearly represented that Sos1 is coprecipitated with Grb2 in both cells; however, Grb2 proteins remarkably interact with Sos1 in Chang liver rather than in HepG2. Otherwise dynamin II interacts with Grb2 in both cells, but the dynamin II in HepG2 is strongly bound to Grb2. For these results, several explanations would be possible. First, transformation by malignancy tumor might bring a structural change in dynamin II, which become more favorable to bind Grb2 and, second, the expression of dynamin II may be upregulated on malignancy tumor induced by *Ras* activation causing cancer.

The identity of the 125- and 80 kDa proteins is currently not determined yet, but similar sizes of proteins were found to complexes with Grb2 in previous data [Sastry et al., 1997].

Dynamin II Associated With Grb2 via SH3 Domains in HepG2 Cells

To determine which domain of Grb2 protein is critical for interaction with dynamin II, we performed coprecipitations with GST-fusion proteins of Grb2/SH2, Grb2/SH3 (N), and Grb2/SH3 (C). As shown in Figure 3, dynamin II coprecipitated with Grb2/SH3 (N) or Grb2/SH3(C) domain as well as Grb2, but not with Grb2/SH2 domain. Five putative SH3 domain-binding sites have been suggested at the C-terminus of dynamin by the sequence comparison with the SH3 binding sites found in

Sos1 [Gout et al., 1993]. Besides, it has been reported that dynamin I bound to several other proteins including PLCγ [Gout et al., 1993], p85 [Tuma et al., 1993], amphiphysin [Seedorf et al., 1994] and Src [Seedorf et al., 1994] in vitro, and these interactions are all mediated by a proline-rich region at the dynamin C-terminus that contains SH3 domain binding motifs. The precise role of Grb2 binding to dynamin II is presently unclear. Recently, several research groups have shown that the recruitment of Grb2-Sos1 to the membrane, triggered by epithelial growth factor (EGF) stimulation, activates the *Ras*-dependent signaling and simultaneously enhances free dynamin levels, leading to both receptor internalization and endocytotic process [Vidal et al., 1998]. These results proposed that dynamin function on endocytosis might link larger numbers of signal molecules to cellular signaling. Recently, it was proposed that dynamin II participated in the formation of distinct transport vesicle from trans-Golgi network (TGN) rather than from plasma membrane [Maier et al., 1996; Jones et al., 1998]. Generally, dynamin is involved in intracellular vesicle formation including endocytosis, synaptic transmission, and receptor internalization, the Grb2-dynamin complex can construct an intermediate messenger in signal pathway leading to vesicle formation. The C-terminal region of dynamin, which is considered as the binding site of Grb2, has also been elucidated to associate with microtubules [Shpetner and Vallee, 1992; Herskovits et al., 1993]. These data suggest a possibility that the signals triggered by ligand binding to its receptor is mediated through the Grb2-dynamin association. In addition, another report that stimulation of human monocytes with macrophage colony-stimulating factor induces a Grb2-mediated association of FAK and dynamin suggests that dynamin may be one of the early participants in the signaling triggered by exogenous stimulations [Kharbanda et al., 1995]. Therefore, our results add further evidence to suggest the importance of the association of Grb2 with dynamin II or Sos1 in carcinoma cells.

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