Dominant-negative Mutants of Grb2 Induced Reversal of the Transformed Phenotypes Caused by the Point Mutation-activated Rat HER-2/Neu*

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To clarify the role of the Shc-Grb2-Sos trimer in the oncogenic signaling of the point mutation-activated HER-2/neu receptor tyrosine kinase (named p185), we interfered with the protein-protein interactions in the Shc-Grb2-Sos complex by introducing Grb2 mutants with deletions in either amino- (ΔN-Grb2) or carboxy-terminal (ΔC-Grb2) SH3 domains into B104-1-1 cells derived from NIH3T3 cells expressing the point mutation-activated HER-2/neu. We found that the transformed phenotypes of the B104-1-1 cells were largely reversed by the ΔC-Grb2. The effect of the ΔC-Grb2 was much weaker. Biochemical analysis showed that the ΔN-Grb2 was able to associate Shc but not p185 or Sos, while the ΔC-Grb2 bound to Shc, p185, and Sos. The p185-mediated Ras activation was severely inhibited by the ΔN-Grb2 but not the ΔC-Grb2. Taken together, these data demonstrate that interruption of the interaction between Shc and the endogenous Grb2 by the ΔN-Grb2 impairs the oncogenic signaling of the activated p185, indicating that (i) the ΔN-Grb2 functions as a strong dominant-negative mutant, and (ii) Shc/Grb2/Sos pathway plays a major role in mediating the oncogenic signal of the activated p185. Unlike the ΔN-Grb2, ΔC-Grb2 appears to be a relatively weak dominant-negative mutant, probably due to its ability to largely fulfill the biological functions of the wild-type Grb2.

The HER-2/neu (also known as erbB-2) protooncogene encodes an M, 185,000 transmembrane glycoprotein with intrinsic tyrosine kinase activity homologous to the epidermal growth factor (EGF)1 receptor (1–6). The transforming potential of the HER-2/neu receptor tyrosine kinase (named p185) has been well documented in both clinical analysis and experimental studies (7–11). The mechanisms of aberrant activation of p185 have also been extensively investigated (12–19). A carcinogen-induced point mutation replacing a valine residue with a glutamic acid in the transmembrane domain confers transforming ability on p185 (12). Alternatively, overexpression of the wild-type p185 can also induce neoplasia transformation (13–15). Both mutation and overexpression are believed to result in enhancing formation and stabilization of receptor dimers, which allow the p185 tyrosine kinase to maintain in its active status (16–19). However, the downstream signaling pathway relaying the oncogenic signal triggered from the abnormally activated p185 is not well defined, likely due to the absence of a consensus of its ligand (20–26).

Activation of Ras is an important convergence point in the mitogenic signaling pathway of receptor tyrosine kinases (27). A key upstream pathway leading to Ras activation by receptor tyrosine kinases has recently been established, primarily as a result of studies with the receptors for EGF, platelet-derived growth factor, and insulin (28–33). The most important components of this pathway include Shc, Grb2, and Sos. Shc stands for SH2 domain-containing α2 collagen-related proteins. The Shc family consists of three isoforms (34). The p46Shc and p52Shc isoforms come from the same transcript with different translation initiation sites. The p66Shc species most likely arises from a distinct transcript. Tyrosine phosphorylation of Shc provides a docking site for Grb2 which was originally identified as a growth factor receptor-bound protein (35), a mammalian homolog of Caenorhabditis elegans Sem-5 and Drosophila Drk (36, 37). Grb2 is a 24-kDa adaptor protein containing an SH2 domain flanked by two SH3 domains. Through the SH3 domains, Grb2 constitutively associates with Sos (named for the Son of Sevenless gene), a 150-kDa guanylnucleotide exchange factor for Ras (38–41), by targeting the proline-rich motif at its carboxyl terminus. Upon ligand stimulation, most receptor tyrosine kinases examined to date have been able to induce tyrosine phosphorylation of Shc, which subsequently binds to the SH2 domain of Grb2. The formation of the Shc-Grb2-Sos ternary complex has been proposed to play an important role in activating Ras (28–33). Alternatively, the Grb2-Sos complex can be directly recruited to the activated EGF receptor (42). Activation of Ras leads to stimulation of downstream kinase cascades, which at least include Raf-1/MEK/MAPK and MEK-1/JNK/JNK pathways (44).

Unlike EGF receptor and other receptor tyrosine kinases, the mutation-activated p185 tyrosine kinase is constitutively active in the absence of exogenously added ligand. Although activation of Ras has been proposed to play an important role in the oncogenic signaling of the mutation-activated p185 (45), coupling of p185 to Ras via Shc-Grb2-Sos or Grb2-Sos or both has not been yet determined. Our recent data and that of others indicated that tyrosine phosphorylation of Shc and formation of the Shc-Grb2 complex occurred in transformed NIH3T3 cells that express the mutation-activated p185 and human breast cancer cells that overexpress p185, which suggests that the Shc/Grb2/Sos/Ras pathway may be responsible for transmit-
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Cell Lines and Culture—B104-1-1 cells are transformed NIH3T3 cells generated by transfection with the neu oncogene, originally derived from a neuroglioblastoma cell line (49). Cells were grown in Dulbecco’s modified Eagle’s medium (Ham’s F-12 medium supplemented with 10% calf serum under an atmosphere of 5% CO2 at 37°C, supplemented with 1% penicillin, 100 U/ml streptomycin, and 10 μg/ml of fungizone). Cultures were subcloned by limiting dilution, and the subclones were expanded for further characterization.

Establishment of Stable Transfectants of Grb2 Deletion Mutants—B104-1-1 cells were transfected by the calcium phosphate precipitation method as described previously (50) with the plasmid pCGN-Bam or the expression vectors encoding ΔGrb2 or ΔGrb2ΔN. After transfection, cells were subject to hygromycin (300 μg/ml) selection for 10–14 days. Individual colonies were expanded and characterized for expressing truncated Grb2 products. Vector, Grb2AC-11, and Grb2AN-11 are stable transfectants derived from B104-1-1 cells, expressing vector alone, ΔGrb2, and ΔGrb2ΔN, respectively.

Focus Forming Assays—Focus forming assays were performed as described previously with some modifications (51). cNeu-104 (1 μg) was cotransfected into NIH3T3 cells with pSV2neo (0.1 μg) and plasmids encoding vector alone, ΔGrb2, or ΔGrb2ΔN. The plasmids pSV2neo, pGEM, was used to ensure that equal amounts of DNA were transfected into cells. Two days after transfection, cells were split 1:4, and duplicate plates were cultured in regular medium for 3–4 weeks while the other set of duplicates were grown in medium containing G418. Foci and G418-resistant colonies were stained in crystal violet solution (1% crystal violet, 20% ethanol in H2O). The resulting number of foci from each transfection was corrected for transfection efficiency by dividing by the number of G418-resistant colonies created by the same transfection. Results are expressed as percentage of foci in control transfection with cNeu-104 (100%). Shown here is the average of three individual experiments. Standard deviation is shown by an error bar.

Microfocus Forming Assays—Microfocus forming assays were performed as described previously (52) with some modifications. Exponentially growing NIH3T3, B104-1-1, Vector, Grb2AC-11, and Grb2AN-11 cells were trypsinized and counted. One-hundred-fifty cells from each cell line were combined separately with 3.0 × 104 NIH3T3 cells and gently mixed in a 6-cm tissue culture plate containing regular medium. Medium was replaced every 3 days over the 2–3-week period of focus formation. Foci were counted as described above. The diameters of individual foci in a random sampling from each plate were also measured.

RESULTS

Suppression of the Transforming Ability of the Mutation-activated HER-2/neu by SH3 Domain Deletion Mutants of Grb2—To observe whether the SH3 domain deletion mutants of Grb2 (Fig. 1A) can interfere with the oncogenic signaling pathway of the mutation-activated HER-2/neu receptor tyrosine kinase, we first examined the effect of the Grb2 mutants on the transforming activity of the HER-2/neu oncogene by focus forming assays in which we cotransfected cNeu-104 (a cosmids clone containing mutation-activated rat HER2/neu, see
Amino-terminal SH3 domain deletion Grb2 (Ref. 3) together with expression vectors encoding either the cNeu-104 (Fig. 1B) backbone alone did not affect the number of foci caused by control experiments cotransfection of cNeu-104 plus vector crease the number of foci but only by approximately 25%. In section of the expression vector for the cause of cNeu-104 decreased by more than 60%. Cotransfection with cNeu-104 (100%). Data shown here are the average from three individual experiments. Standard deviations are shown by error bars.

The results of focus forming assays indicated that the SH3 domain deletion mutants of Grb2 were able to suppress the transformingability of the mutation-activated neu. Focus forming assays were performed as described under “Experimental Procedures.” The resulting number of foci from each transfection was corrected for transfection efficiency by dividing by the number of G418-resistant colonies created by the same transfection. Results are expressed as percent of foci in control transfection with cNeu-104 (100%).

Stable Transfection of Grb2 Mutants with SH3 Domain Deletion—To do so, we stably transfected expression vectors encoding by the number of G418-resistant colonies created by the same transfection. Results are expressed as percent of foci in control transfection with cNeu-104 (100%).

Phenotypic Reversion of the Transformed B104-1-1 Cells by ΔN-Grb2. A, immunoblot analysis of expression of the Grb2 deletion mutants stably transfected into B104-1-1 cells. B104-1-1 is a transformed cell line derived from NIH3T3 by stable transfection of the point mutation-activated HER-2/neu. Vector, Grb2ΔC-11, and Grb2ΔN-11 are stable transfectants derived from B104-1-1 cells, expressing vector alone, ΔC-Grb2, and ΔN-Grb2, respectively. Fifty micrograms of cell extracts from various cell lines as indicated were subject to 10% SDS-PAGE. After transfer, the top portion of the nitrocellulose filter was probed with c-Neu-Ab3, a monoclonal anti-p185 antibody, the lower part was incubated with either 12CA5, a monoclonal antibody specific to the HA1 tag (middle panel), or monoclonal anti-Grb2 antibody (bottom panel). Endogenous Grb2 is indicated by an arrowhead. B, morphology of various cell lines. a, NIH3T3; b, B104-1-1; c, Vector; d, Grb2ΔC-11; and e, Grb2ΔN-11.

FIG. 1. A, schematic representation of Grb2 deletion mutants. ΔN-Grb2 represents the Grb2 mutant containing only the SH2 and the carboxyl-terminal SH3 domains. ΔC-Grb2 stands for the Grb2 mutant carrying only the SH2 and the amino-terminal SH3 domains. B, SH3 domain deletion Grb2 mutants suppress the transforming ability of the mutation-activated neu. Focus forming assays were performed as described under “Experimental Procedures.” The resulting number of foci from each transfection was corrected for transfection efficiency by dividing by the number of G418-resistant colonies created by the same transfection. Results are expressed as percent of foci in control transfection with cNeu-104 (100%). Data shown here are the average from three individual experiments. Standard deviations are shown by error bars.

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...transfected with cNeu-104, the number of foci...
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### Table I

| Cell lines     | Number of foci | Size of foci | Soft agar colonies |
|---------------|---------------|-------------|-------------------|
| NIH3T3        | 100           | 2.5–5.0     | 100               |
| B104-1-1      | 92.6 ± 8.5    | 2.5–5.0     | 95.1 ± 9.6        |
| Grb2C-11      | 70.2 ± 7.4    | 2.0–4.0     | 74.5 ± 5.8        |
| Grb2N-11      | 23.7 ± 2.8    | 1.0–2.0     | 20.1 ± 2.9        |

*a* One hundred fifty cells from each line to be tested were mixed with 3.0 × 10⁶ NIH3T3 cells in 6-cm tissue culture plate and grown as described under “Experimental Procedures.” The number of foci was counted after 2–3 weeks. The sizes of foci were also measured. The resulting number of foci from various cell lines in each experiment was standardized against that of the parental B104-1-1 cells (set at 100%). Data shown here are the average of three separate experiments. Standard deviation is also indicated.

*b* Cells were seeded at 1 × 10⁵ in 0.35% agarose containing Dulbecco’s minimal essential medium/F12 with 10% calf serum. Colonies were counted 4–6 weeks later. The resulting number of colonies from various cell lines was standardized against that of the parental B104-1-1 cells (set at 100%). Data shown here represent the average of quadruplicate experiments. Standard errors are also shown.

### Fig. 3. Association of Shc or p185 with the truncated Grb2 products.

Two milligrams of cell extracts from different cell lines were used in immunoprecipitation with monoclonal anti-HA1 antibody. Immunocomplexes or 50 μg of lysates from B104-1-1 cells were separated on a 6–12% gradient SDS-PAGE. The filter was cut into three pieces after transfer. The top portion was probed with c-Neu-Ab3 for associated p185, the middle was incubated with polyclonal anti-Shc antibody for associated Shc, and the bottom portion was incubated with monoclonal anti-HA1 antibody, in order to evaluate equal loading. The band above the truncated Grb2 products was most likely from IgG light chain.

The culture time for these two cell lines in the above assays did not result in extra numbers of foci or colonies (data not shown). Taken together, our data indicated that the transformed phenotypes of B104-1-1 cells could be largely reversed by stable transfection of the ΔN-Grb2 mutant. The ΔC-Grb2 mutant had a relatively weak effect.

Both ΔN-Grb2 and ΔC-Grb2 Compete with the Endogenous Grb2 for Shc—To investigate the molecular basis of the phenotypic reversion caused by the SH3 domain deletion Grb2 mutants, we first compared the tyrosine phosphorylation profiles between B104-1-1 and its stable transfectants. Immunoblotting analysis indicated that no obvious difference for the profiles of tyrosine phosphorylation was detected between B104-1-1 and Vector, Grb2C-11, or Grb2ΔC-11 (data not shown). In particular, expression of ΔN-Grb2 or ΔC-Grb2 did not affect the expression and tyrosine phosphorylation of p185 and Shc (data not shown). We next tested the possibility that the SH3 domain deletion Grb2 mutants may compete with the endogenous Grb2 for Shc and p185. We first examined whether ΔN-Grb2 and ΔC-Grb2 could be associated with Shc and p185 by co-immunoprecipitation Western analysis. Anti-HA1 antibody was used to precipitate the ΔN-Grb2 and ΔC-Grb2 proteins along with their associated proteins. As shown in Fig. 3, comparable amounts of p52Shc were detected in the anti-HA1 immunoprecipitates in both Grb2ΔN-11 and Grb2ΔC-11 cells. Immunoblot analysis with anti-HA1 antibody indicated that comparable amounts of ΔN-Grb2 and ΔC-Grb2 were precipitated, which suggests that the ΔN-Grb2 and the ΔC-Grb2 have a similar affinity for Shc. In contrast, co-precipitation of p185 by the anti-HA1 antibody was only detected in the Grb2ΔC-11 cells. The reciprocal co-immunoprecipitation experiment with anti-p185 antibody also failed to detect a physical association between p185 and the ΔN-Grb2 in the Grb2ΔN-11 cells (data not shown). These results suggest that the ΔN-Grb2 may not be able to associate with p185. However, we cannot rule out the possibility that the association is transient and undetectable under our experimental conditions.

Detecting the association between Shc and the truncated
Grb2 proteins prompted us to ask whether the interaction of Shc and the endogenous Grb2 is impaired in Grb2ΔN-11 and Grb2ΔC-11 cells. To address this issue, we used anti-Shc antibody to precipitate Shc and associated proteins, followed by immunoblotting with anti-Grb2 or anti-Shc antibodies. As shown in Fig. 4, the endogenous Grb2 co-precipitated by the anti-Shc antibody dramatically decreased in both Grb2ΔN-11 and Grb2ΔC-11 cell lines, as compared to that in the parental B104-1-1 and the vector control cell lines. Consistently, co-immunoprecipitation of the ΔC-Grb2 and ΔN-Grb2 by anti-Shc antibody was detected by the anti-Grb2 antibody. Equal loading was confirmed by Western analysis with the anti-Shc antibody. These results are consistent with those seen in Fig. 3, which showed that both ΔN-Grb2 and ΔC-Grb2 products bound to p52HSH. We, therefore, concluded that both ΔN-Grb2 and ΔC-Grb2 were able to compete with the endogenous Grb2 for Shc.

The association between Shc and the endogenous Grb2 was impaired by the ΔC-Grb2 but not the ΔN-Grb2. Two milligrams of lysates from each cell line were immunoprecipitated with a polyclonal anti-Shc antibody. Immunocomplexes were dissected by 10% SDS-PAGE, followed by immunoblotting analysis with either monoclonal anti-Shc antibody (top panel) or polyclonal anti-Shc antibody (lower panel). Immunoprecipitated Shc proteins and co-immunoprecipitated endogenous Grb2 are indicated.

Grb2 mutants were tested for their ability to compete with the endogenous Grb2 for Shc. The association between Shc and the endogenous Grb2 is inhibited in Grb2ΔN-11 and Grb2ΔC-11 cells. To address this issue, we used anti-Shc antibody to precipitate Shc and associated proteins, followed by immunoblotting with anti-Grb2 or anti-Shc antibodies. As shown in Fig. 4, the endogenous Grb2 co-precipitated by the anti-Shc antibody dramatically decreased in both Grb2ΔN-11 and Grb2ΔC-11 cell lines, as compared to that in the parental B104-1-1 and the vector control cell lines. Consistently, co-immunoprecipitation of the ΔC-Grb2 and ΔN-Grb2 by anti-Shc antibody was detected by the anti-Grb2 antibody. Equal loading was confirmed by Western analysis with the anti-Shc antibody. These results are consistent with those seen in Fig. 3, which showed that both ΔN-Grb2 and ΔC-Grb2 products bound to p52HSH. We, therefore, concluded that both ΔN-Grb2 and ΔC-Grb2 were able to compete with the endogenous Grb2 for Shc.

Endogenous Grb2 was used to co-precipitate endogenous Grb2 from NIH3T3, B104-1-1, Vector, Grb2ΔN-11, and Grb2ΔC-11 cell lines. As expected, the association of Grb2 and Sos was comparable in NIH3T3, B104-1-1, and Vector cells (Fig. 5). In contrast, the association of Sos and the endogenous Grb2 was inhibited in the Grb2ΔC-11 cells. This interference was most likely due to the competition for Sos between the endogenous Grb2 and the ΔC-Grb2 since the anti-Shc antibody was able to co-precipitate ΔC-Grb2. In contrast from the ΔC-Grb2, the ΔN-Grb2 did not significantly affect the association of Sos and the endogenous Grb2 in the Grb2ΔN-11 cells (Fig. 5). Converse immunoprecipitation with the anti-HA1 antibody also failed to co-precipitate appreciable amounts of Sos in the Grb2ΔN-11 cells (data not shown), suggesting that the association of ΔN-Grb2 with Sos is very weak. Our results are consistent with a previous report which demonstrated that substitution of Gly-203 with Arg in the carboxyl-terminal SH3 domain of Grb2 had little effect on its binding to Sos in vitro, whereas replacement of Pro-49 with Leu in the amino-SH3 domain (a mutation causing loss of function in C. elegans Sem-5) abrogated this binding (39). However, some previous reports claimed that binding of Grb2 to Sos depends on cooperative interactions of the two SH3 domains (38, 41). This discrepancy may be an issue of binding affinity.

Effect of Grb2 Mutants on Ras Activation—It has been proposed that formation of the Shc-Grb2-Sos ternary complex plays an important role in Ras activation triggered by activation of the EGF receptor, platelet-derived growth factor receptor, or insulin receptor (28–33). Given that both Grb2ΔN-Grb2 and ΔN-Grb2 mutants can compete with the endogenous Grb2 for Shc and that ΔC-Grb2 can also interact with p185 and Sos (Figs. 3–5), we examined whether signaling from the activated p185 to Ras was affected in Grb2ΔN-11 and Grb2ΔC-11 cells. Cells were labeled with 32Pi, and the guanine nucleotides bound to Ras were analyzed. As expected, the Vector control cells had a similar percentage of GTP-Ras, as compared to the B104-1-1 cells (Fig. 6). In contrast, the percentage of GTP-Ras in the Grb2ΔN-11 cell lines was less than 35% of that in the parental B104-1-1 cells while the percentage of GTP-Ras decreased to about 75% in the Grb2ΔC-11 cells. These results indicate that interruption of the interaction between Shc and the endogenous Grb2 can inhibit Ras activation by the activated p185 and that the ΔN-Grb2, which does not bind to p185 or Sos, acts as a strong dominant-negative analog of the endogenous Grb2.

The ΔC-Grb2 mutant appears to be a relatively weak dominant-negative mutant, probably because it is still able to form complexes with Sos, Shc, and p185. The differential inhibition effect of ΔN-Grb2 and ΔC-Grb2 on Ras activation is consistent with the different extent of phenotypic reversion of Grb2ΔN-11 cells caused by these two Grb2 mutants. These observations support the idea that Ras activation is a key event in the process of transformation induced by the mutation-activated p185.

**DISCUSSION**

Grb2 consists of a single SH2 domain and two SH3 domains. Previous studies have indicated that Grb2 is a key component of the pathway leading to Ras activation by receptor tyrosine kinases (56). In the present study we tested whether deletion of the amino- or carboxyl-SH3 domain of Grb2 could create dominant-negative mutants which are capable of binding to tyrosine-phosphorylated Shc or mutation-activated p185 but are unable to associate with Sos. We speculated that these mutants could interfere with the recruitment of the Sos-Grb2 complex to Shc or p185, leading to inhibition of Ras activation. Our data demonstrated here that the ΔN-Grb2 functioned as a dominant-negative mutant that suppressed by more than 65% the activation of Ras by the mutation-activated p185 and largely reversed the transformed phenotypes of B104-1-1 cells. The
∆C-Grb2 appears to be a weak dominant-negative mutant. It down-regulated Ras activation, by only 25%, and slightly induced phenotypic reversion of the B104-1-1 cells. Similar results have been recently obtained for the oncogenic Bcr-Abl tyrosine kinase (57). A ∆N-Grb2 mutant suppresses Bcr-Abl-mediated Ras activation and reverses the transformed phenotype. As shown here, the ∆C-Grb2 mutant is less effective in reversing Bcr-Abl-induced transformation as compared to the ∆N-Grb2.

It is of interest to note that the dominant-negative effect of the ∆C-Grb2 is much weaker than that of the ∆N-Grb2 even though the ∆C-Grb2 is able to compete with the endogenous Grb2 for Shc, p185, and Sos while the ∆N-Grb2 can only bind to Shc. One possible model to explain this phenomenon is shown in Fig. 7. Wild-type Grb2 (wt-Grb2) constitutively binds to Sos mainly through its amino-terminal SH3 domain. The wt-Grb2:Sos complex is recruited to the tyrosine-phosphorylated Shc, subsequently resulting in Ras activation. The wt-Grb2:Sos may also be directly recruited to the activated p185, which is not shown here in order to simplify the model (discussion seen in the text). When introduced into the B104-1-1 cells, the ∆N-Grb2 can bind to Shc but not Sos. The Shc/∆N-Grb2 complex by itself is unable to trigger Ras activation. On the other hand, ∆N-Grb2 sequesters Shc. Therefore, the endogenous wt-Grb2 cannot be recruited to Shc. Thus, ∆N-Grb2 is a dominant-negative mutant of Grb2. In contrast, the ∆C-Grb2 binds to Sos and Shc. The Shc/∆C-Grb2:Sos complex can largely fulfill the functions of the Shc:wt-Grb2:Sos complex, leading to Ras activation. Since the recruitment efficiency of ∆C-Grb2:Sos by Shc is relatively lower as compared to that of the wt-Grb2:Sos, Ras activation is slightly reduced in B104-1-1 transfected expressing the ∆C-Grb2. In order to simplify the model, direct recruitment of wt-Grb2 or ∆C-Grb2 to the mutation-activated p185 is not included in this model, which is described in the text instead.

had a much weaker phenotypic effect in C. elegans than the mutation (sem-5 allele n1619) corresponding to the P49L Grb2 mutant (N-terminal SH3) (59). However, other studies suggest that both P49L and G203R Grb2 were loss-of-function mutants (35). For example, co-microinjection of either P49L or G203R Grb2 protein together with the H-ras protein did not stimulate DNA synthesis in quiescent rat embryo fibroblast cells while co-injection of the wild-type Grb2 and H-ras proteins enhanced DNA synthesis, suggesting that the two SH3 domains of Grb2 constitute an essential functional component of the protein. These conflicting observations may be explained by the use of different assays in different biological systems. The functional difference between ∆C-Grb2 and G203R Grb2 is not known currently. We speculate that the ∆C-Grb2 possesses, at least in part, the biological functions of the wild-type Grb2 since it can bind to Shc, p185, and Sos. The slight down-regulation of Ras activation and partial reversal of transformed phenotypes in Grb2∆C-11 cells may be due to the relatively lower efficiency of recruitment of the Sos:∆C-Grb2 complex to Shc, as compared to the complex of Sos and the endogenous Grb2. This is supported by the observation seen in Fig. 4 in which comparable amounts of the ∆C-Grb2 in the Grb2∆C-11 cells and the endogenous Grb2 in the B104-1-1 cells were co-precipitated by the anti-Shc antibody even though the expression level of the ∆C-Grb2 was much higher in the Grb2∆C-11 cells than that of the endogenous Grb2 in B104-1-1 cells (Fig. 2A). This finding suggests that the ∆C-Grb2 may have a lower affinity for Shc than does the endogenous Grb2. Alternatively, the nucleotide exchange activity of Sos may be relatively weaker in the Sos:∆C-Grb2 complex than in the Sos-endogenous Grb2 complex, probably because of an unknown allosteric effect. Therefore, Ras activation in the Grb2∆C-11 cells is not as efficient as that in the B104-1-1 cells, leading to slight reversal of the transformed phenotypes caused by the mutation-activated p185.

Previous studies on the EGF receptor signaling pathway indicated that the Grb2:Sos complex could be recruited to tyrosine-phosphorylated Shc or directly to the activated EGF receptor (38-41). The data presented here imply that the Shc/Grb2:Sos pathway is most likely the dominant one coupling the

![Fig. 6. Effect of expression of SH3 domain deletion mutants of Grb2 on Ras activation.](image)

![Fig. 7. Hypothetical working modes of wild-type Grb2 and its SH3 domain deletion mutants.](image)
activated p185 to Ras since interference of the interaction between Shc and Grb2 by ΔN-Grb2 leads to a dramatic inhibition of Ras activation. This idea is consistent with our previous observation that deletion of most of the auto-phosphorylation sites, including the potential Grb2 binding site on the mutation-activated p185, did not affect its transforming ability, suggesting that direct binding of Grb2 to p185 is not essential for Ras activation (46). Similarly, recent studies using peptide competition and immunodepletion approaches also demonstrated that formation of a complex of EGFR receptor with Grb2 was only responsible for a minor part of EGFR-stimulated Ras activation while the formation of the ShcGrb2-Sos complex played the major role (28, 29). Indirect evidence has been shown suggesting that direct binding of Grb2 to p185 is not essential for Ras activation (46).}

"Our data, however, also suggest that the Shc/Grb2/Sos pathway may not be the sole pathway that leads to the activation of Ras by the mutation-activated p185. Disruption of the association between Shc and Grb2 by ΔN-Grb2 is unable to completely inhibit Ras activation or to completely reverse the transformed phenotypes mediated by the mutation-activated p185, suggesting the existence of multiple routes to Ras, which may not be influenced by the ΔN-Grb2. One conceivable pathway is the direct recruitment of Grb2-Sos to the activated p185 since ΔN-Grb2 appears to be unable to compete with the endogenous Grb2 for p185. Alternatively, the formation of complexes containing Grb2 and phosphorylated proteins other than Shc, which can stimulate the Ras pathway, may not be interfered with by the ΔN-Grb2. It has been shown that a complex of Syk/SH-PTP2 tyrosine phosphatase and Grb2 can couple platelet-derived growth factor receptors to Ras (60). Recently, a Ras-GAP associated protein, named p62, has been found to form a complex with Grb2 in v-src transformed NIH3T3 cells (61). Interestingly, the presence of the Grb2-p62 complex correlates with the phosphorylation of p62 and cellular transformation, suggesting that the Grb2-p62 complex may be able to lead to Ras activation. It will be of interest to test whether these complexes exist in the B104-1-1 cells and whether these complexes contain Ras guanine nucleotide exchange factors (GEF) apart from Sos. One of these, C3G (named for Crk SH3 binding GEF), can activate Ras in yeast (62). Intriguingly, via its proline-rich domain C3G binds the amino-terminal domain of the adaptor protein Crk (62, 63). The Crk-C3G complex may thus, like the Grb2-Sos complex, couple the oncogenic signal of the mutation-activated p185 to Ras. However, no data exist showing that C3G is an exchange factor for Ras in mammalian cells. On the other hand, an alternative explanation for the failure to completely reverse the transformed phenotypes of B104-1-1 cells by the ΔN-Grb2 could be that additional, perhaps less efficient, signaling pathways which do not involve Ras are not influenced by the ΔN-Grb2 and may culminate in cell transformation by the activated p185. Indeed, recent studies have shown that Raf can be activated by the Drosophila a torso receptor tyrosine kinase in a Ras-independent pathway (58). Our studies provided direct evidence to support the hypothesis that the Shc/Grb2/Sos pathway plays a major role in the oncogenic signaling of the mutation-activated p185 and may shed light on developing therapeutic agents to block the oncogenic signaling pathway of the p185 oncoprotein."
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