The Function of GRB2 in Linking the Insulin Receptor to Ras Signaling Pathways

E. Y. Skolnik, A. Batzer, N. Li, C.-H. Lee, E. Lowenstein, M. Mohammadi, B. Margolis, J. Schlessinger

Insulin-induced activation of extracellular signal-regulated kinases [ERKs, also known as mitogen-activated protein (MAP) kinases] is mediated by Ras. Insulin activates Ras primarily by increasing the rate of guanine nucleotide–releasing activity. Here, we show that insulin-induced activation of ERKs was enhanced by stable overexpression of growth factor receptor–bound protein 2 (GRB2) but not by overexpression of GRB2 proteins with point mutations in the Src homology 2 and 3 domains. Moreover, a dominant negative form of Ras (with Ser^{17} substituted with Asn) blocked insulin-induced activation of ERKs in cells that overexpressed GRB2. GRB2 overexpression led to increased formation of a complex between the guanine nucleotide–releasing factor Sos (the product of the mammalian homolog of *son of sevenless* gene) and GRB2. In response to insulin stimulation, this complex bound to tyrosine-phosphorylated IRS-1 (insulin receptor substrate–1) and Shc. In contrast to the activated epidermal growth factor receptor that binds the GRB2-Sos complex directly, activation of the insulin receptor results in the interaction of GRB2-Sos with IRS-1 and Shc, thus linking the insulin receptor to Ras signaling pathways.

To determine whether GRB2 is important in mediating activation of Ras in insulin-stimulated cells, we constructed stable L6 myoblast cell lines that overexpress either wild-type GRB2 or mutant GRB2 [GRB2(R86K)] containing a lysine instead of an arginine in the conserved FLVRES motif of the SH2 domain (*1, 2*). This mutation inhibits the binding of GRB2 to phosphotyrosine-containing proteins (*3*). Activation of ERKs (also known as MAP kinases) by a variety of growth factors is mediated by Ras (*4, 5*). Overexpression of GRB2 (approximately fivefold) enhanced the ability of insulin to stimulate tyrosine phosphorylation of the $p42^{erk}$ (ERK-2) and $p44^{erk}$ (ERK-1) proteins (Fig. 1) (*6*). Such overexpression led to tyrosine phosphorylation of ERK-1 and ERK-2 at insulin concentrations one-tenth of those normally required for phosphorylation. Intact Src homology 2 and 3 (SH2 and SH3) domains of GRB2 were required for this effect. There was no increase in tyrosine phosphorylation of ERK in cells that overexpressed mutant GRB2 proteins containing either a point mutation in the SH2 domain (R86K) (*2*) (Fig. 1A) or point mutations in the SH3 domains that correspond to those found in loss-of-function Sem-5 mutants (*3, 7, 9*). The increased tyrosine phosphorylation of ERKs in cell lines overexpressing GRB2 did not result simply from a change in the kinetics of ERK phosphorylation; both GRB2-overexpressing cells and control cells exhibited similar kinetics of insulin-induced tyrosine phosphorylation of ERK-1 and ERK-2 (Fig. 1B).

The increase in ERK tyrosine phosphorylation observed in GRB2-overexpressing cells also correlated with an increase in ERK enzymatic activity (Fig. 2). Enzymatic activity of immunoprecipitated ERK-1 and ERK-2 was measured in vitro with myelin basic protein as a substrate (*10*). Enzymatic activation of ERK-1 and ERK-2 in cell lines that overexpressed wild-type GRB2 occurred with one-tenth the concentration of insulin required to activate the enzyme in control cells. Analysis of three additional cell lines showed that overexpression of GRB2 consistently enhanced the ability of insulin to activate ERKs. In agreement with the results obtained for ERK tyrosine phosphorylation, the ability of insulin to activate ERK enzymatic activity was not enhanced in cell lines that overexpressed GRB2 with mutated SH2 and SH3 domains (*8*).

We next determined whether GRB2 also potentiated the phosphorylation of ERKs on Ser and Thr residues in addition to Tyr. The activation of ERKs is unusual in that it requires phosphorylation on both Tyr and Ser-Thr residues (*11*) by a dual functional kinase, MAP kinase kinase or ERK kinase (MEK) (*12*). To assess whether the residues that were phosphorylated in the GRB2-overexpressing cells were similar to those phosphorylated in untransfected cells, we labeled cells with [^{32}P]orthophosphate, and ERK-2 was immunoprecipitated from lysates of control and insulin-stimulated cells (*13*). ERK-2 was not phosphorylated in unstimulated cells (Fig. 3). Stimulation with insulin resulted in the phosphorylation of ERK-2 in the parental cells and was enhanced in cells overexpressing GRB2. Phosphoamino acid analysis showed that similar proportions of Tyr, Thr, and Ser residues were phosphorylated on ERK-2 in both cell lines (Fig. 3, B and C). These results are consistent with the idea that MEK, albeit more active in the GRB2-overexpressing cells, is responsible for phosphorylating the ERKs in both cell lines and support the notion that GRB2 functions upstream from ERK to increase activation of Ras.

The activation of MEK and ERKs by receptors with intrinsic tyrosine kinase activity depends on activation of Ras (*4, 5, 14*). To verify that enhancement of ERK activation by GRB2 was mediated by an increase in the activity of Ras, we constructed cell lines that expressed GRB2 and a dominant inhibitor of Ras, Ras(S17N) (*2, 15*). This mutant Ras protein has a reduced affinity for guanosine triphosphate and inhibits wild-type Ras activity by interfering with the guanine nucleotide–releasing factor (GNRF) that is normally involved in Ras activation (*16*). Cell lines that overexpressed GRB2 were transfected with a plasmid containing Ras(S17N) under the control of an inducible promoter. Expression of Ras(S17N) abrogated the ability of insulin to stimulate tyrosine phosphorylation of ERK-1 and ERK-2 (Fig. 4). The ability of Ras(S17N) to block ERK tyrosine phos-

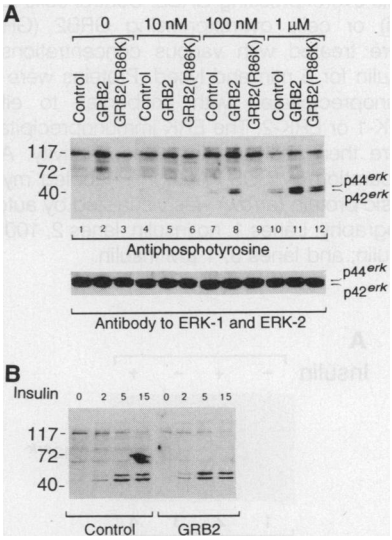

Fig. 1. GRB2 overexpression enhances tyrosine phosphorylation of ERKs by insulin. (**A**) Stable L6 cell lines that overexpressed wild-type GRB2 or GRB2 containing a point mutation in the SH2 domain (R86K) were stimulated with increasing concentrations of insulin (top) for 5 min. Equal amounts (50 μg) of total cell lysates were immunoblotted with antibodies to phosphotyrosine (antiphosphotyrosine). ERK-1 ($p44^{erk}$) and ERK-2 ($p42^{erk}$) are indicated. To control for equal amounts of protein in each lane, we processed the samples in duplicate and probed them (lower panel) with an antibody that recognizes both ERK-1 and ERK-2. (**B**) Time course of insulin-induced tyrosine phosphorylation of ERK-1 and ERK-2 in cells overexpressing GRB2 and in control cell lines. After stimulation with 1 μM insulin for various times (top, in minutes), cell lysates were processed and probed with antiphosphotyrosine. Size markers are indicated on the left in kilodaltons.

Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, NY 10016.

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phorylation was correlated with an inhibition of ERK enzymatic activity. These results suggest that in insulin-stimulated cells, GRB2 links the insulin receptor to activation of Ras. Furthermore, activation of ERKs in an insulin-responsive target cell, such as a muscle cell, is mediated by Ras. Because L6 cells contain insulin-like growth factor receptors (IGF-1 receptors) in addition to insulin receptors, the insulin effects described above may be mediated in part by the IGF-1 receptor.

The interaction between GRB2 and GNRF, Sos (the product of the *son of sevenless* gene), appears to function in the control of Ras signaling by receptor tyrosine kinases (*9, 17–19*). The SH3 domains of several signaling molecules bind proline-rich stretches of nine or ten amino acids in target proteins (*20*). It has been recently shown that GRB2 binds through its SH2 domain to the epidermal growth factor (EGF) receptor and through its SH3 domains to Sos, thereby coupling receptor tyrosine kinases to Ras signaling pathways (*19, 21, 22*). To understand the mechanism whereby overexpression of GRB2 potentiates activation of Ras in response to insulin, we measured the amount of Sos associated with GRB2. GRB2 was immunoprecipitated from both control cells and cells overexpressing GRB2, and the amount of Sos associated with GRB2 was assessed (*23*). Overexpression of GRB2 led to the immunoprecipitation of more GRB2, and the increased amount of immunoprecipitated GRB2 was associated with an increased amount of co-immunoprecipitating Sos (Fig. 5A). Insulin stimulation resulted in a mobility shift of Sos in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) that was a result of phosphorylation of Sos on Ser and Thr residues (*8*). The increased GRB2-Sos complex detected in cells overexpressing GRB2 was not a result of differences in Sos expression in the two cell lines (Fig. 5B). This finding suggests that under normal conditions, the amount of Sos bound to GRB2 is limited by the amount of GRB2 present in the cell. Thus, overexpression of GRB2 apparently potentiates insulin activation of Ras and ERKs by enhancing the formation of an Sos-GRB2 complex, thereby allowing a larger pool of Sos to be recruited to the insulin signaling pathway.

After insulin stimulation, the SH2 domain of GRB2 binds two tyrosine-phosphorylated proteins: insulin receptor substrate–1 (IRS-1) (a major target for the insulin and IGF-1 receptors) and Shc (an SH2 domain-containing protein that is a target for many tyrosine kinases) (*3, 24, 25*). Tyrosine-phosphorylated IRS-1 binds the SH2 domain of p85-associated phosphatidylinositol-3 kinase (PI-3 kinase), thereby activating PI-3 kinase, and cellular overexpression of Shc transforms NIH 3T3 cells and activates Ras in PC-12 cells (*24, 25*). The interactions between GRB2, IRS-1, and Shc are likely to be important in the activation of Ras by the insulin receptor. Because insulin-induced stimulation of Ras is mediated through activation of a GNRF (*26*), one reasonable hypothesis is that the interaction of GRB2 with IRS-1 or Shc or both enables Sos, bound to the SH3 domain of GRB2, to activate Ras. This is supported by our finding that overexpression of wild-type GRB2, but not of a GRB2 SH2 mutant that is unable to bind IRS-1 or Shc, potentiates the ability of insulin to activate Ras. Furthermore, GRB2

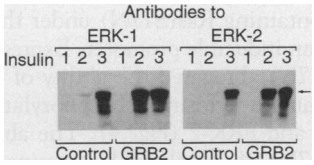

Fig. 2. Increased ERK enzymatic activity in cells overexpressing GRB2. Control cells (control) or cells overexpressing GRB2 (GRB2) were treated with various concentrations of insulin for 5 min and lysed. Proteins were immunoprecipitated with antibodies to either ERK-1 or ERK-2. The ERK immunoprecipitates were then analyzed for kinase activity. After separation by SDS-PAGE, ^{32}P-labeled myelin basic protein (arrow) was visualized by autoradiography. Lanes 1, no insulin; lanes 2, 100 nM insulin; and lanes 3, 1 μM insulin.

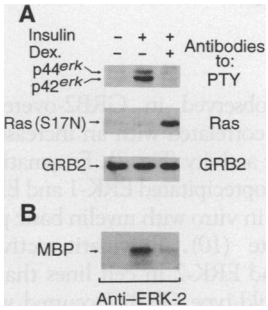

Fig. 4. Inhibition of insulin-induced activation of ERK in cells overexpressing Ras(S17N). (**A**) Cell lines overexpressing GRB2 and Ras(S17N) were treated with dexamethasone (Dex.) as indicated to induce expression of Ras(S17N) and then stimulated with or without insulin (100 nM). Cell lysates were separated by SDS-PAGE and probed with antibodies to either phosphotyrosine (PTY), Ras, or GRB2. (**B**) Cells were stimulated as in (A), except cell lysates were immunoprecipitated with antibodies to ERK (anti–ERK-2) and an in vitro kinase reaction was done. MBP, myelin basic protein.

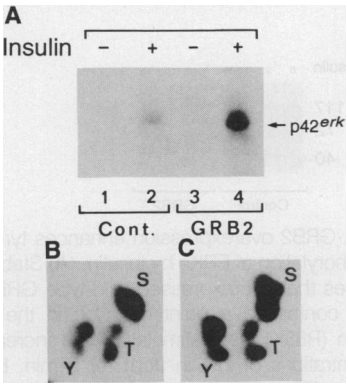

Fig. 3. Phosphorylation of ERK-2 in cells overexpressing GRB2. (**A**) Control cells (Cont.) and cells overexpressing GRB2 were labeled with [^{32}P]orthophosphate, and ERK-2 (p42^{erk}) was immunoprecipitated from either insulin-treated or untreated cells. Immunoprecipitates of ERK-2 antibodies of labeled cell lysates were then analyzed by SDS-PAGE and autoradiography. (**B**) Phosphoamino acid analysis of ERK-2 isolated from control cells. (**C**) Phosphoamino acid analysis of ERK-2 isolated from GRB2-overexpressing cells. Phosphotyrosine, phosphothreonine, and phosphoserine residues are denoted Y, T, and S, respectively.

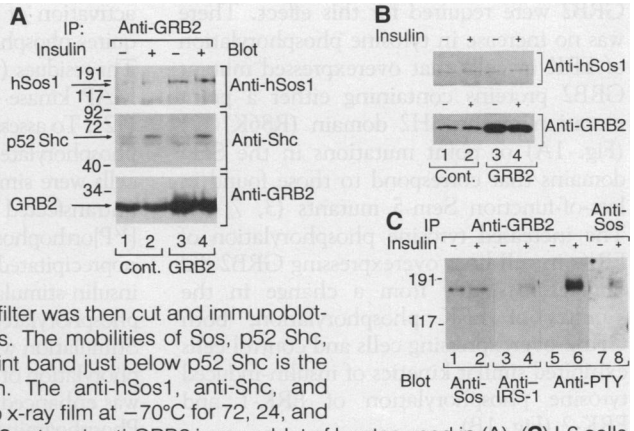

Fig. 5. Increased formation of a GRB2-Sos complex in cells overexpressing GRB2. (**A**) Control cells (Cont.) and cells overexpressing GRB2 were incubated in the absence or presence of insulin (1 μM) for 5 min and lysed. Proteins were immunoprecipitated (IP) with antibodies to GRB2 (anti-GRB2). After SDS-PAGE, the samples were transferred to nitrocellulose. The nitrocellulose filter was then cut and immunoblotted with the indicated antibodies. The mobilities of Sos, p52 Shc, and GRB2 are indicated. The faint band just below p52 Shc is the immunoglobulin G heavy chain. The anti-hSos1, anti-Shc, and anti-GRB2 blots were exposed to x-ray film at −70°C for 72, 24, and 12 hours, respectively. (**B**) Anti-hSos1 and anti-GRB2 immunoblot of lysates used in (A). (**C**) L6 cells overexpressing GRB2 were stimulated with or without insulin (1 μM) for 5 min, and cell lysates were immunoprecipitated (IP) with antibodies to GRB2 or hSos1. The immunoprecipitated proteins were then immunoblotted with anti-Sos, anti–IRS-1, or anti-PTY as indicated (*3*). Size markers are indicated to the left in kilodaltons.

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binds Sos through its SH3 domains and tyrosine-phosphorylated IRS-1 and Shc through its SH2 domain, and after insulin stimulation the GRB2-Sos complex associates with IRS-1 and Shc (Fig. 5) (*8*, *27*).

It is not clear how binding of GRB2 to IRS-1 or Shc potentiates the ability of GRB2 to activate Sos. The interaction of GRB2 with IRS-1 or Shc or with both molecules simultaneously may serve to reposition Sos adjacent to Ras, which is located in the plasma membrane. In support of this mechanism, IRS-1 has been shown to relocate to the plasma membrane in insulin-stimulated cells (*28*). Alternatively, the binding of GRB2 to Shc or IRS-1 may cause a conformational change in GRB2, leading to activation of Sos. Another possibility is that the binding of GRB2 to IRS-1 or Shc promotes the phosphorylation of Sos, thus stimulating guanine nucleotide–releasing activity. The studies reported here demonstrate that, as for EGF and other growth factors, insulin stimulation of Ras signaling pathways is mediated by GRB2. However, in contrast to the EGF receptor that interacts directly with the GRB2-Sos complex, stimulation of the insulin receptor leads to phosphorylation of the two docking proteins IRS-1 and Shc. The ensuing interaction of the GRB2-Sos complex with IRS-1 and Shc in insulin-stimulated cells provides an additional level of control by the insulin receptor of this pivotal signaling pathway.

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6. GRB2 and GRB2(R86K) were subcloned into the eukaryotic expression vector PMJ30 (*29*) and cotransfected with a neomycin resistance plasmid into an L6 myoblast cell line by calcium phosphate precipitation (*30*). Stable cell lines overexpressing GRB2 and the GRB2 mutant were isolated. Mutation in the SH2 domain of GRB2 was done as described (*3*). Cell lines overexpressing GRB2 were selected for further study. To analyze for phosphotyrosine, we incubated cells for 24 hours in medium containing 0.5% fetal bovine serum. Cells were then either unstimulated or stimulated with insulin as indicated and lysed in lysis buffer [50 mM tris (pH 7.4), 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40]. Cell lysates were separated by SDS-PAGE (10% gel). Bound antibodies were visualized with ^{125}I-labeled protein A (ICN, Irvine, CA). The antibodies to phosphotyrosine were rabbit polyclonal antibodies (*15*). Antibodies to ERK-1 and ERK-2 were from Zymed (South San Francisco, CA), clone Z033.
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Dissociation of Object and Spatial Processing Domains in Primate Prefrontal Cortex

Fraser A. W. Wilson,* Séamas P. Ó Scalaidhe, Patricia S. Goldman-Rakic

Areas and pathways subserving object and spatial vision are segregated in the visual system. Experiments show that the primate prefrontal cortex is similarly segregated into object and spatial domains. Neurons that code information related to stimulus identity are dissociable, both by function and region, from those that code information related to stimulus location. These findings indicate that the prefrontal cortex contains separate processing mechanisms for remembering "what" and "where" an object is.

In the primate visual system, segregated pathways convey information pertaining to an object's identity, such as color and shape, and to its location in space (*1*). Although segregation and regional specialization are clearly established within the sensory processing areas of the cortex, they may also exist in the prefrontal cortex, which mediates cognitive and executive functions (*2*). Physiological recordings in the principal sulcus and arcuate regions of monkeys trained to perform delayed-re-