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The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of *ras* signalling

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GRB2, a small protein comprising one SH2 domain and two SH3 domains, represents the human homologue of the *Caenorhabditis elegans* protein, *sem-5*. Both GRB2 and *sem-5* have been implicated in a highly conserved mechanism that regulates p21^{ras} signalling by receptor tyrosine kinases. In this report we show that in response to insulin, GRB2 forms a stable complex with two tyrosine-phosphorylated proteins. One protein is the major insulin receptor substrate IRS-1 and the second is the SH2 domain-containing oncogenic protein, Shc. The interactions between GRB2 and these two proteins require ligand activation of the insulin receptor and are mediated by the binding of the SH2 domain of GRB2 to phosphotyrosines on both IRS-1 and Shc. Although GRB2 associates with IRS-1 and Shc, it is not tyrosine-phosphorylated after insulin stimulation, implying that GRB2 is not a substrate for the insulin receptor. Furthermore, we have identified a short sequence motif (YV/IN) present in IRS-1, EGFR and Shc, which specifically binds the SH2 domain of GRB2 with high affinity. Interestingly, both GRB2 and phosphatidylinositol-3 (PI-3) kinase can simultaneously bind distinct tyrosine phosphorylated regions on the same IRS-1 molecule, suggesting a mechanism whereby IRS-1 could provide the core for a large signalling complex. We propose a model whereby insulin stimulation leads to formation of multiple protein–protein interactions between GRB2 and the two targets IRS-1 and Shc. These interactions may play a crucial role in activation of p21^{ras} and the control of downstream effector molecules.

Key words: GRB2/*ras* signalling/SH2/SH3/tyrosine phosphorylation

Introduction

Many polypeptide growth factors mediate their effects by activating cell surface receptors that possess tyrosine kinase activity (reviewed in Ullrich and Schlessinger, 1990; Aaronson, 1991; Heldin, 1991). For both receptor and cytoplasmic tyrosine kinases, activation of *ras* is a critical step in inducing mitogenesis or differentiation (Mulcahy *et al.*, 1985; Hagag *et al.*, 1986; Smith *et al.*, 1986; Feig and Cooper, 1988; Szeberenyi *et al.*, 1990). A prerequisite for activating *ras* and other signalling pathways is the activation of receptor tyrosine kinases. This activation leads to autophosphorylation and association of the receptor with a group of cytoplasmic targets containing a conserved non-catalytic domain of ~100 amino acids, referred to as the Src homology 2 (SH2) domain (reviewed in Cantley *et al.*, 1991; Koch *et al.*, 1991; Margolis, 1992). Association of SH2 domain-containing target proteins with an activated receptor is strictly dependent on receptor autophosphorylation; SH2 domains bind to specific short sequences that encompass a phosphotyrosine moiety (Cantley *et al.*, 1991; Mohammadhi *et al.*, 1991, 1992; Kashishian *et al.*, 1992; Rotin *et al.*, 1992). It has been postulated that binding of signalling molecules to specific autophosphorylation sites on receptors determines the specificity of signalling pathways (reviewed by Cantley *et al.*, 1991; Koch *et al.*, 1991; Schlessinger and Ullrich, 1992).

Genetic studies in *Caenorhabditis elegans* revealed that an SH2/SH3 domain-containing protein *sem-5* is critical in the signal transduction pathway that links receptor tyrosine kinases to *ras* activation (Clark *et al.*, 1992). In *C.elegans*, *sem-5* lies downstream of an epidermal growth factor receptor-like tyrosine kinase (let-23) but upstream of a *ras* homologue (let-60), implying that *sem-5* mediates *ras* activation (Aroian *et al.*, 1990; Horvitz and Sternberg, 1991). We previously reported the identification of the mammalian homologue of *sem-5* (Lowenstein *et al.*, 1992), GRB2 (growth factor receptor bound-2). The evidence that GRB2 is the human homologue of *sem-5* comes from several lines of investigation. In addition to 59% sequence homology, *sem-5* and GRB2 share several functional characteristics. It has been shown that GRB2 rescues *sem-5* mutants in *C.elegans* (Stern *et al.*, 1992) and that both GRB2 and *sem-5* are able to bind the autophosphorylated epidermal growth factor receptor (EGFR) with high affinity (Lowenstein *et al.*, 1992; M.J.Stern, R.J.Daly, E.J. Lowenstein, A.Batzer, M.Kokel and J.Schlessinger, submitted). Finally, GRB2 plays a role in *ras* activation in mammalian cells; microinjection of GRB2 together with H-*ras* protein into quiescent rat embryo fibroblasts resulted in DNA synthesis, whereas the injection of either protein alone had no effect (Lowenstein *et al.*, 1992).

In addition to associating with the activated EGFR, GRB2 binds Shc, another widely expressed tyrosine-phosphorylated SH2 domain-containing protein (Pelicci *et al.*, 1992). Overexpression of Shc leads to transformation of 3T3 cells and neuronal differentiation of PC-12 cells (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992). These findings, together with the demonstration that tyrosine-phosphorylated Shc binds the SH2 domain of GRB2, suggests that the interaction between Shc and GRB2 may be important in regulating *ras* activation and mitogenesis.

A major target for the insulin and IGF-1 receptor tyrosine kinases is a widely expressed 165–185 kDa phosphoprotein, insulin receptor substrate (IRS)-1, which is phosphorylated in response to insulin and IGF-1 stimulation (Sun *et al.*, 1991). IRS-1 contains 14 potential tyrosine autophosphorylation sites, nine of which contain YMXM or YXXM motifs (Sun *et al.*, 1991). Phosphorylation of tyrosine residues contained within these motifs results in the specific association of these phosphopeptides with the SH2 domains of p85, leading to activation of phosphatidylinositol-3 (PI-3) kinase (Backer *et al.*, 1992; Myers *et al.*, 1992). Since IRS-1 contains several potential tyrosine phosphorylation sites that do not encompass a p85 binding motif, we reasoned that IRS-1 may also bind GRB2 and thereby enable IRS-1 to link the insulin receptor with *ras* activation.

In this report we demonstrate that tyrosine phosphorylation of IRS-1 by the insulin receptor results in the physical association of IRS-1 with GRB2. Athough GRB2 forms a stable complex with IRS-1 in insulin-stimulated cells, it does not become phosphorylated in response to insulin. In addition, we have observed that activation of the insulin receptor stimulates tyrosine phosphorylation of Shc, resulting in stable association of GRB2 with Shc. A short phosphopeptide corresponding to Tyr895 of IRS-1 prevents the binding of GRB2 to IRS-1. Interestingly, the sequence of this phosphopeptide is similar to the sequence around Tyr1068 of the EGFR, which is the high affinity binding site of GRB2 with the EGFR (A.G.Batzer, J.Urena, D.Rotin, R.Daley and J.Schlessinger, in preparation). While the biochemical mechanisms associated with the role of GRB2 in *ras* activation remain to be defined, we show that insulin-stimulated tyrosine phosphorylation initiates the formation of multiple protein–protein interactions between the SH2 domain of GRB2 and tyrosine-phosphorylated IRS-1 and Shc. We suggest that the net result of these interactions is the control of *ras* signalling leading to the many pleiotropic responses mediated by insulin.

Results

GRB2 associates with tyrosine-phosphorylated IRS-1 in living cells

IRS-1 undergoes rapid tyrosine phosphorylation in cells after insulin stimulation (White *et al.*, 1985; Sun *et al.*, 1991). To assess whether insulin stimulation results in the stable association of IRS-1 with GRB2, Chinese hamster ovary (CHO) cells, overexpressing the insulin receptor, were stimulated with insulin and the association of IRS-1 and GRB2 was assessed by determining whether the two proteins co-immunoprecipitate. In unstimulated cells there is neither detectable tyrosine-phosphorylated IRS-1 nor association between GRB2 and IRS-1. However, insulin stimulation results in IRS-1 phosphorylation and formation of an IRS-1–GRB2 complex, as demonstrated by the co-immunoprecipitation of these proteins by anti-IRS-1 (Figure 1A, lanes 5 and 6) or anti-GRB2 antibodies (Figure 1A, lanes 1–4). The requirements for the binding of GRB2 to IRS-1 parallel the requirements for interaction of GRB2 with the EGFR (Lowenstein *et al.*, 1992). In both systems, association is strictly dependent on ligand activation and requires tyrosine phosphorylation. We have previously shown that GRB2 is not phosphorylated in response to EGF

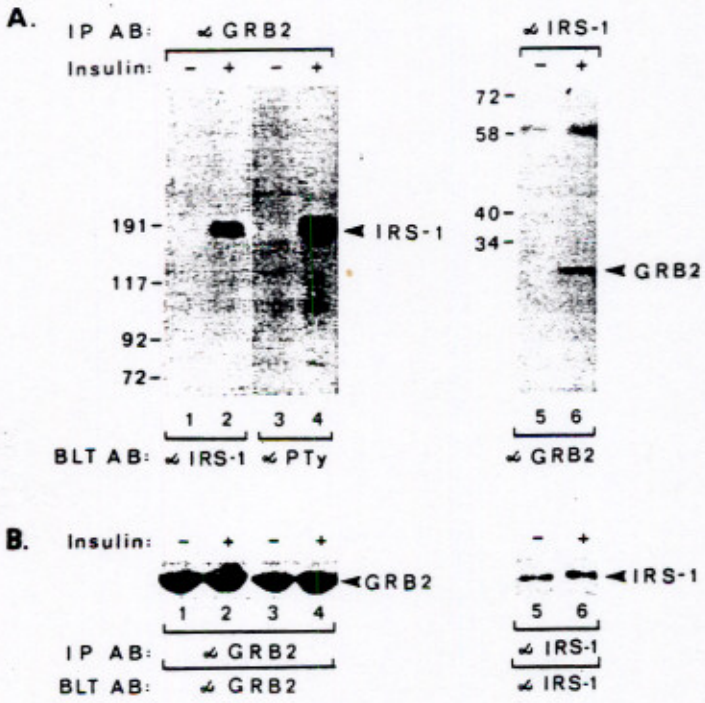

Fig. 1. Association of GRB2 with IRS-1 in insulin-stimulated CHO/IR cells. **A.** CHO/IR cells were incubated in the absence (lanes 1, 3 and 5) or presence of 100 nM of insulin (lanes 2, 4 and 6), lysed and immunoprecipitated with antibodies to GRB2 (lanes 1–4) or IRS-1 (lanes 5 and 6). The immunoprecipitated proteins were then separated by SDS–PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-IRS-1 antibodies (lanes 1 and 2), anti-phosphotyrosine antibodies (lanes 3 and 4) or anti-GRB2 antibodies (lanes 5 and 6). Bound antibodies were visualized with [^{125}I]protein A. **B.** To control for equal amounts of immunoprecipitated protein, after transfer, the nitrocellulose filter was cut, the GRB2 immunoprecipitates were immunoblotted with anti-GRB2 antibodies (lanes 1 and 2) and the IRS immunoprecipitates were immunoblotted with anti-IRS-1 antibodies (lanes 3 and 4). The mobilities of IRS-1 and GRB2 are indicated.

or platelet-derived growth factor (PDGF) stimulation (Lowenstein *et al.*, 1992). Similarly, we were unable to detect tyrosine-phosphorylated GRB2 in lysates from insulin-stimulated cells.

It is unlikely that GRB2 and the insulin receptor associate directly; we have been unable to co-immunoprecipitate GRB2 using anti-insulin receptor antibodies, and GRB2 immunoprecipitates contained only a minor phosphoprotein that co-migrated with the 95 kDa β-subunit of the insulin receptor. Since the 95 kDa subunit of the insulin receptor is the major phosphoprotein present in lysates from insulin-stimulated cells, the 95 kDa protein co-precipitating with GRB2 most likely represents an indirect association of GRB2, via IRS-1, with the insulin receptor β-subunit. This is consistent with the recent observation that a small fraction of IRS-1 associates with the insulin receptor (X.J.Sun and M.F.White, unpublished data).

GRB2 associates with tyrosine-phosphorylated IRS-1 via its SH2 domain

To determine which region of GRB2 is responsible for interacting with IRS-1, fusion proteins were produced that contain either GRB2 or individual domains of GRB2, and the binding of these proteins to IRS-1 was assessed. IRS-1 produced in Sf9 cells (IRS-1^{bac}) was phosphorylated using the partially purified insulin receptor and [γ-^{32}P]ATP. Radiolabelled IRS-1^{bac} was then incubated with GRB2 fusion proteins that had been immobilized on to glutathione–agarose beads and bound IRS-1 was detected by SDS–PAGE and autoradiography. Figure 2A shows that a full-length GRB2 fusion protein binds tyrosine phosphorylated IRS-1 (lane 2). The binding is mediated by the SH2 domain of GRB2, since tyrosine-phosphorylated IRS-1 was able to bind a GST fusion protein containing only the SH2 domain of GRB2 (Figure 2A, lane 3). In addition, a point mutation (R86K) in the SH2 domain, affecting the highly conserved FLVRES motif (Mayer *et al.*, 1992; Waksman *et al.*, 1992), markedly abrogated the ability of GRB2 to bind IRS-1 (Figure 2A, lane 6). Moreover, IRS-1 did not bind to GST alone or to fusion proteins containing either the amino- or carboxy-terminal SH3 domains (Figure 2A, lanes 1, 4 and 5), indicating that the binding is specific.

IRS-1 contains 14 potential tyrosine phosphorylation sites and tyrosine-phosphorylated IRS-1 can bind with high affinity the SH2 domains of PI-3 kinase-associated p85 (Backer *et al.*, 1992). To determine whether IRS-1 exhibits specificity in its interaction with different SH2 domains, GST fusion proteins were produced containing various SH2 domains. The fusion proteins were separated by SDS–PAGE, transferred to nitrocellulose membranes and incubated with radiolabelled IRS-1. While IRS-1 specifically binds GRB2 and the SH2 domains of p85, it does not interact with the SH2 domains of GTPase activating protein (GAP) or Shc (Figure 2B). The specificity of IRS-1 binding is consistent with recent data demonstrating that SH2 domains recognize with high affinity a phosphotyrosine moiety within the context of a specific amino acid sequence (Cantley *et al.*, 1991; Koch *et al.*, 1991; Schlessinger and Ullrich, 1992). This experiment indicates that IRS-1 interacts with a subset of SH2 domain-containing proteins.

GRB2 and p85 bind the same tyrosine-phosphorylated IRS-1 molecule

PI-3 kinase associates with IRS-1 in insulin-stimulated cells and this interaction is a critical step in the regulation of PI-3 kinase activation (Backer *et al.*, 1992). Since different SH2 domains bind to different tyrosine phosphorylation sites, it is conceivable that multiple SH2 domain-containing signalling proteins could bind to a single IRS-1 molecule. To determine whether GRB2- and PI-3 kinase-associated p85 could interact with the same IRS-1 molecule, we assessed whether p85 and PI-3 kinase activity co-immunoprecipitate using antibodies to GRB2. Anti-GRB2 and anti-IRS-1 immune complexes from insulin-stimulated cells contained p85 (Figure 3, lanes 4 and 6) and PI-3 kinase activity (Figure 3B and data not shown). This experiment indicates that the same IRS-1 molecule is able to bind simultaneously two different SH2 domain-containing proteins, p85 and GRB2. These findings suggest a mechanism whereby IRS-1 could provide the core of a large signalling complex.

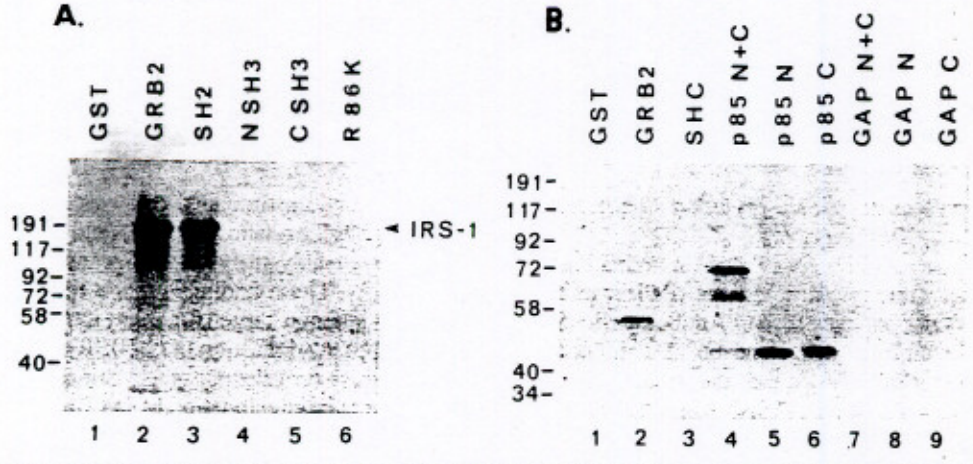

Fig. 2. *In vitro* binding of IRS-1 to GST fusion proteins. **A.** 1 μg of GST fusion protein bound to glutathione–agarose beads was incubated with [^{32}P]IRS-1^{bac} for 1 h at 4°C. After washing proteins were separated by SDS–PAGE and bound IRS-1 was visualized by autoradiography. The following fusion proteins were constructed and correspond to GST alone (GST), full-length GRB2 (GRB2), the SH2 domain of GRB2 (SH2), the amino-terminal or carboxy-terminal SH3 domain of GRB2 (N SH3 and C SH3) or full-length GRB2 with a substitution of lysine for arginine in the FLVRES motif of the SH2 domain (R86K). **B.** 10 ng of GST fusion protein corresponding to GST alone (GST), full-length GRB2 (GRB2), the Shc SH2 domain (SHC), the amino- and carboxy-SH2 domains of p85 expressed together (p85 N + C) or separately (p85 N and p85 C), or the amino- and carboxy-SH2 domains of GAP expressed together (GAP N + C) or separately (GAP N and GAP C), were separated by SDS–PAGE, transferred to nitrocellulose and then probed with [^{32}P]IRS-1^{bac}. Bound IRS-1 was visualized by autoradiography.

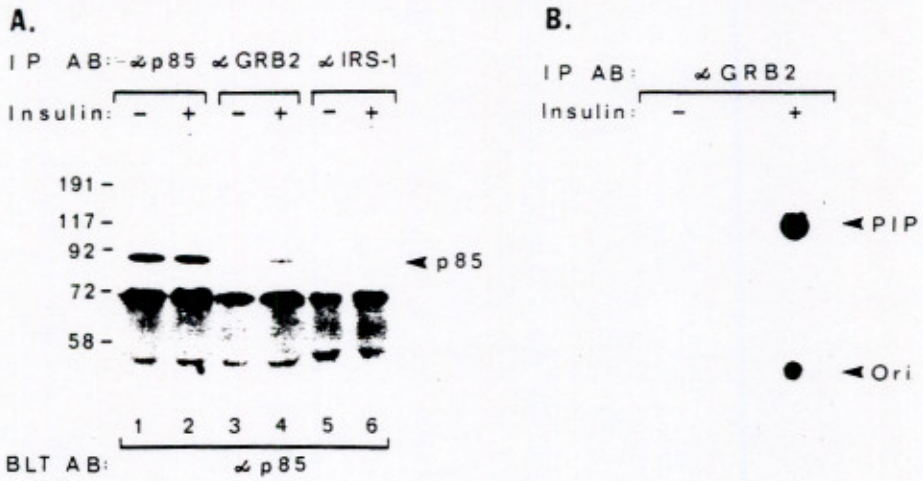

Fig. 3. Association of GRB2 with p85 and PI-3 kinase activity. **A.** Quiescent CHO/IR cells were either treated or untreated with insulin for 5 min at 37°C, lysed and immunoprecipitated with either anti-p85 antibodies (lanes 1 and 2), anti-GRB2 antibodies (lanes 3 and 4) or anti-IRS-1 antibodies (lanes 5 and 6). The immunoprecipitated proteins were then separated by SDS−PAGE and immunoblotted with anti-p85 antibodies. **B.** Immunoprecipitates were subjected to a PI-3 kinase assay as described in Materials and methods.

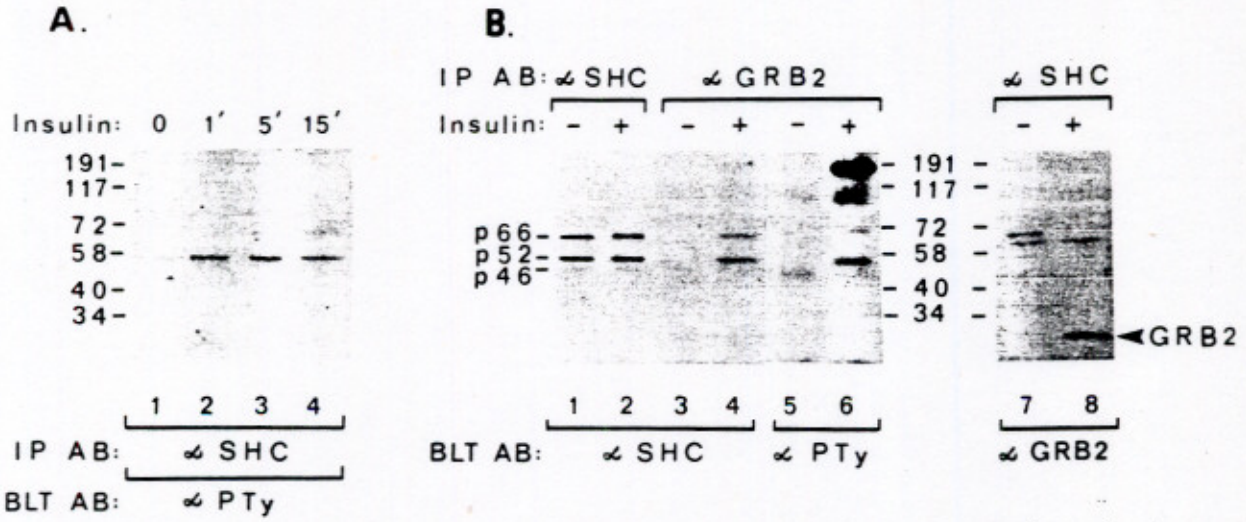

Fig. 4. Insulin stimulation of CHO/IR cells results in tyrosine phosphorylation of Shc and the formation of Shc−GRB2 complex. **A.** Quiescent CHO/IR cells were untreated or treated with 100 nM insulin for 1, 5 or 15 min, lysed and immunoprecipitated with antibodies to Shc. The immunoprecipitates were then subjected to Western blot analysis with anti-phosphotyrosine antibodies. **B.** Cells were stimulated for 5 min as in panel A and immunoprecipitated with anti-Shc or anti-GRB2 antibodies. The Shc immunoprecipitates were then immunoblotted with antibodies to Shc (lanes 1 and 2) or GRB2 (lanes 7 and 8), while the GRB2 immunoprecipitates were subjected to immunoblot analysis with anti-phosphotyrosine (lanes 3 and 4) or anti-Shc antibodies (lanes 5 and 6). The mobilities of the Shc proteins (p66, p52 and p46) and GRB2 (GRB2) are indicated.

Insulin stimulates tyrosine phosphorylation of Shc, resulting in a Shc−GRB2 complex

Shc has been found to be phosphorylated on activation of receptor and cytoplasmic tyrosine kinases (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992). Antibodies to Shc recognize three proteins of 46, 52 and 66 kDa molecular weights. It was previously shown that the 46 and 52 kDa proteins arise from a common cDNA, while the 66 kDa protein is encoded by a distinct Shc transcript (Pelicci *et al.*, 1992). Therefore, we examined whether insulin stimulates tyrosine phosphorylation of Shc in living cells. Lysates from insulin-stimulated CHO/IR cells were immunoprecipitated with anti-Shc antibodies, separated by SDS−PAGE, transferred to nitrocellulose and probed with either antibodies to Shc or anti-phosphotyrosine antibodies. While an equivalent amount of Shc protein was immunoprecipitated from both stimulated and unstimulated cell lysates (data not shown), tyrosine phosphorylation of the 66 and 52 kDa Shc proteins was detected only in lysates from insulin-stimulated cells (Figure 4A). These findings support the general notion that Shc is a common target for many tyrosine kinases.

After insulin stimulation of CHO/IR cells, Shc, in addition to undergoing tyrosine phosphorylation, also associates with GRB2. Immunoprecipitation of insulin-stimulated CHO/IR cell lysates using anti-GRB2 antibodies co-precipitated the 52 and 66 kDa tyrosine-phosphorylated Shc proteins (Figure 4B, lanes 3−6). The association between GRB2 and Shc was strictly dependent on ligand activation and required tyrosine phosphorylation of Shc. Further evidence supporting these results is the finding that GRB2 was also co-immunoprecipitated using antibodies to the Shc proteins (Figure 4B, lanes 7 and 8).

Since tyrosine-phosphorylated GRB2 was not detected after insulin stimulation, it seems most likely that the interaction between GRB2 and Shc is mediated by binding of tyrosine-phosphorylated Shc to the SH2 domain of GRB2. Indeed, we have recently shown that the SH2 domain of GRB2 binds to tyrosine-phosphorylated Shc (Rozakis-Adcock *et al.*, 1992). Consistent with this hypothesis, we were able to detect tyrosine-phosphorylated Shc in GRB2 immunoprecipitates (Figure 4, lane 6), while only non-tyrosine-phosphorylated GRB2 was found in the anti-Shc immunoprecipitates. It is unlikely that GRB2 and Shc are interacting indirectly by binding via their SH2 domains to tyrosine-phosphorylated IRS-1, since tyrosine-phosphorylated IRS-1 was not detected in Shc immunoprecipitates from living cells (Figure 4A). Moreover, we could not detect *in vitro* binding of IRS-1 to Shc (Figure 2B, lane 3).

Synthetic phosphotyrosine-containing peptides interfere with GRB2 binding to IRS-1

It is now well established that different SH2 domains bind to phosphotyrosine residues that are flanked by different short peptide sequences (Cantley *et al.*, 1991; Koch *et al.*, 1991; Schlessinger and Ullrich, 1992). The finding that the SH2 domain of GRB2 interacts with tyrosine-phosphorylated EGFR, IRS-1 and Shc led us to search for a common motif responsible for binding of GRB2 to these otherwise diverse substrates. We have recently identified Tyr1068 of the EGFR as a high affinity binding site for the SH2 domain of GRB2 (Batzer *et al.*, in preparation). Furthermore, the SH2 domain of GRB2 binds a synthetic tyrosine-phosphorylated peptide derived from the sequence around Tyr1068 of the EGFR (Batzer *et al.*, in preparation). Interestingly, a similar motif is found around Tyr895 of IRS-1 and Tyr317 of Shc (Table IB). To determine whether Tyr895 of IRS-1 could function as a binding site for GRB2, several synthetic phosphopeptides were synthesized and the ability of these phosphopeptides to inhibit the binding of IRS-1 to GRB2 was determined. While several tyrosine-phosphorylated peptides did not block the binding of IRS-1 to a GRB2 fusion protein immobilized on to nitrocellulose, a seven amino acid phosphopeptide, which encompassed the amino acid sequence around Tyr895 of IRS-1, consistently resulted in a >50% inhibition in IRS-1 binding to GRB2 (Figure 5, lane 3). In agreement with these findings, real-time kinetic analysis and direct binding experiments utilizing the biosensor system (BIAcore, Pharmacia; Felder *et al.*, 1992) demonstrated that of the IRS-1 peptides tested, only the Y895 phosphopeptide bound GRB2 with a high affinity (K_d = 35 nM, Table I). Further support for the idea that phosphorylated Tyr895 and its flanking sequence represents a specific binding site for GRB2 are the findings that the related phosphopeptides corresponding to Tyr1068 of the EGFR and Tyr317 of Shc bind GRB2 with an affinity similar to that observed for GRB2 binding Tyr895 (Table I). Moreover, addition of the Tyr1086 phosphopeptide resulted in a >50% inhibition in the binding of IRS-1 to GRB2 (Figure 5). Finally, when a tryptic digest of tyrosine-phosphorylated IRS-1 was added to a GRB2–SH2 fusion protein, only a single peptide containing Tyr895 of IRS-1 was found to bind GRB2 (M.F.White, data not shown). Comparison of the GRB2 binding sites on IRS-1, Shc and the EGFR suggests that an isoleucine or valine at the +1 position and an asparagine at the +2 position are important in mediating binding (Table IB).

Table I.

	Peptide	Inhibition (Figure 5)	K_d[a]
A			
IRS-1			
Y895*		–	no binding
pY895	S P G E **Y** V N I E F G S	+	35 nM
pY939	S E E **Y** M N M D L G P	–	no binding
pY809	G G G **Y** C G A R P E S	–	ND
pY628	G D G **Y** M P M S P K S	–	ND
pY727	T G D **Y** M N M S P V G	–	no binding
EGFR			
pY1068	P V P E **Y** I N Q S V P K	+	30 nM
SHC			
pY317	D P S **Y** V N V N Q N L	ND	23 nM

B

IRS-1 895	P G E Y V N I E F
EGFR 1068	V P E Y I N Q S V
SHC 317	D P S Y V N V N Q
Consensus	X P X Y V N V
	I I

A. Sequence of the various peptides used for competition experiments. (*peptide was not tyrosine phosphorylated.) Also included is a summary of the binding data obtained in Figure 5. Inhibition was defined as >50% of control value. The K_d values for GRB2 binding the various peptides were measured using a Biosensor device (BIAcore, Pharmacia). **B.** The consensus binding sequence for the SH2 domain of GRB2 derived from the GRB2 binding sites on IRS-1, Shc and EGFR.

[a]Tyrosine-phosphorylated or non-phosphorylated peptides were immobilized, within a flow chamber, onto a carboxymethyl-dextran polymer attached to a gold-coated surface. Solutions containing recombinant SH2 domains were allowed to flow through the chamber and the amount of protein associated with the immobilized phosphopeptide was assessed by surface plasmon resonance. This detects changes in the index of refraction localized adjacent to the gold-coated surface. Dissociation constants were calculated either from steady-state binding and Scatchard analysis or from kinetic parameters. Full details concerning the methodology for measuring the dissociation constants of SH2 domain binding to tyrosine-phosphorylated peptides are described in a report by Felder *et al.* (1992). ND, not determined.

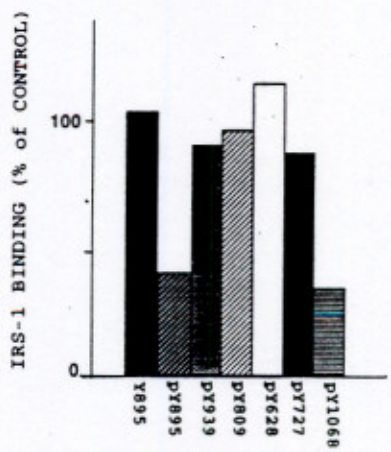

Fig. 5. Inhibition of binding of GRB2 to tyrosine-phosphorylated IRS-1 by phosphopeptides derived from IRS-1 and EGFR. 100 nM of GRB2–GST fusion protein immobilized onto glutathione–agarose beads were incubated with 100-fold excess of peptide for 1 h. ^{32}P-labelled IRS-1^{bac} was then incubated with the beads and bound IRS-1 was determined by SDS–PAGE and autoradiography. The amount of IRS-1 bound was quantified using a phosphorimager, with 100% binding defined as the amount of IRS-1 bound in the absence of competing peptide.

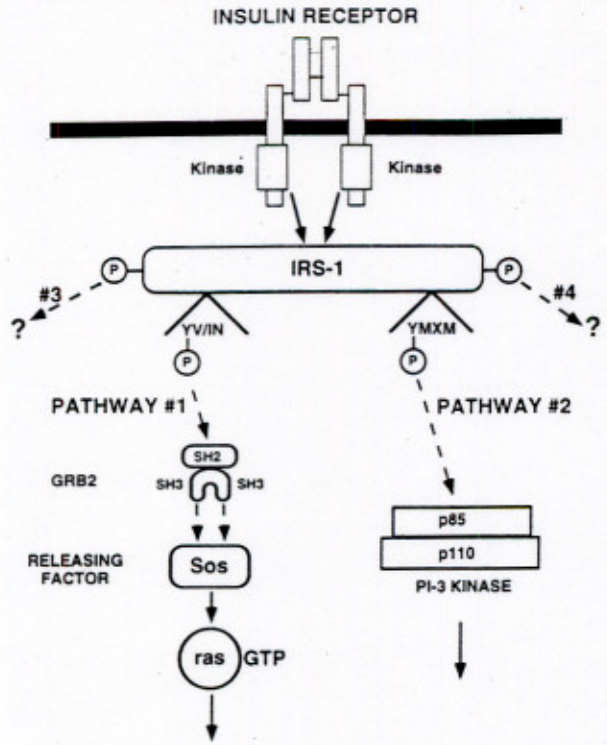

Fig. 6. Scheme for the role of IRS-1 in insulin-mediated signal transduction. Activation of the insulin receptor results in tyrosine phosphorylation of IRS-1. Phosphorylation of Tyr895 of IRS-1 creates a high affinity binding site for the SH2 domain of GRB2. GRB2 also binds to the GRF Sos via its SH3 domain, thereby linking IRS-1 to *ras* signalling (pathway 1). In addition, phosphorylation of tyrosine residues contained within the motif YMXM or YXXM results in the specific association of IRS-1 with the SH2 domains of p85, leading to PI-3 kinase activation (pathway 2). It is hypothesized that phosphorylation of other tyrosine residues will recruit other signalling molecules (pathways 3 and 4).

Discussion

Small proteins, like v-crk, Nck and GRB2/*sem-5*, which are composed primarily of SH2 and SH3 domains, have been thought to be important in signal transduction based on the fact that cellular overexpression of several proteins in this group results in transformation (Mayer *et al.*, 1988; Chou *et al.*, 1992; Li *et al.*, 1992; Pelicci *et al.*,1992). However, definitive proof that these molecules fulfil specific functions in tyrosine kinase receptor signalling was not available until the recent demonstration that GRB2/*sem-5* plays a role in *ras* activation by receptor tyrosine kinases in *C.elegans* and mammalian cells (Clark *et al.*, 1992; Lowenstein *et al.*, 1992). The finding that GRB2 requires an intact SH2 domain in order to activate *ras* suggests that physical interaction between GRB2 and phosphotyrosine-containing proteins is essential for GRB2/*sem-5* function. To identify whether GRB2 plays a role in insulin activation of *ras*, we set out to identify whether GRB2 interacts with phosphotyrosine-containing proteins in insulin stimulated cells. In this report we have demonstrated that in response to insulin stimulation, GRB2 forms stable complexes with IRS-1 and Shc. This interaction is strictly dependent on ligand activation of the insulin receptor and is mediated by the binding of the SH2 domain of GRB2 to phosphotyrosines on both IRS-1 and Shc. In addition, the ability of GRB2 to interact with these proteins exhibits specificity; IRS-1 does not bind to several other SH2 domain-containing proteins including GAP and Shc. Moreover, we have identified a short amino acid sequence present in the EGFR, IRS-1 and Shc that binds the SH2 domain of GRB2 with a high affinity.

While there are many potential SH2 domain-containing target molecules, only a subset of these molecules are recruited to the signalling pathway by each receptor tyrosine kinase. The finding that SH2 domains recognize specific binding sites has led to the hypothesis that phosphorylation of specific tyrosine residues plays a critical role in determining which signalling molecules are recruited to a particular pathway (Cantley *et al.*, 1991; Koch *et al.*, 1991; Schlessinger and Ullrich, 1992). To understand the specificity in signalling, it is of particular interest to identify the binding site of the SH2 domain of GRB2. The interaction of GRB2 with IRS-1, Shc and the EGFR enabled us to identify a common motif that binds the SH2 domain of GRB2. We have shown in this report that Tyr895 of IRS-1 and its flanking sequence represent a high affinity binding site for the SH2 domain of GRB2. A similar motif, found around tyrosine Tyr1068 of the EGFR and around Tyr317 of Shc, is responsible for the high affinity binding of GRB2 to both the EGFR and Shc (Batzer *et al.*, in preparation; P.G.Pelicci and T.Pawson, in preparation). Comparison of the phosphopeptides that bind GRB2 suggests that an isoleucine or valine at the +1 position and an asparagine at the +2 position are important in mediating binding. This suggests that binding of GRB2 to IRS-1 is similar to the binding of other SH2 domain-containing proteins with their targets; association is absolutely dependent on tyrosine phosphorylation and the amino acid sequence around the phosphotyrosine determines specificity.

It is intriguing that after insulin stimulation, GRB2 interacts with two molecules: IRS-1, which is a target for the insulin and IGF-1 receptors, and Shc, which is a target for many tyrosine kinases. The importance of IRS-1 in insulin-stimulated signalling has been established (Figure 6). It has been shown that after tyrosine phosphorylation of IRS-1 by the insulin receptor, the SH2 domains of p85 bind YMXM motifs on IRS-1 and this interaction leads to activation of PI-3 kinase (Backer *et al.*, 1992). It is still unclear how IRS-1 regulates the function of GRB2. We envision that GRB2, like p85, will function as an adaptor (Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Skolnik *et al.*, 1991; Hu *et al.*, 1992) coupling IRS-1 to the catalytic subunit of as yet unidentified molecules that regulate *ras* activity. Our inability to detect phosphorylation of GRB2 in lysates from insulin or EGF-stimulated cells implies that GRB2 is not regulated by tyrosine phosphorylation. It is conceivable that the interaction between IRS-1 and GRB2 is analogous to the interaction between IRS-1 and p85 and that this binding alone is sufficient to alter the enzymatic activity of a downstream effector molecule (Backer *et al.*, 1992). Alternatively, these interactions may serve to combine several effector molecules into a large signalling complex, enabling different effector molecules to interact directly. This scenario is supported by our finding that a single IRS-1 molecule can interact with both GRB2 and PI-3 kinase. Finally, it is conceivable that the interaction of GRB2 with effectors is regulated by the binding of the SH2 domain of GRB2 to phosphotyrosine-containing proteins.

A more detailed understanding of the role of GRB2 in signal transduction awaits the characterization of effector

proteins and their enzymatic activities. In this regard, there is now evidence that SH3 domains interact directly with a subset of effector proteins (Cicchetti *et al.*, 1992). We have recently identified several cellular proteins that specifically bind the SH3 domains of GRB2, but fail to bind when these SH3 domains contain mutations corresponding to those in the loss-of-function *sem-5* mutants (Clark *et al.*, 1992; Lowenstein *et al.*, 1992). The human guanine nucleotide releasing factor Son of sevenless (Sos) (Simon *et al.*, 1991; Bonfini *et al.*, 1992; Bowtell *et al.*, 1992) is one protein that interacts with the SH3 domains of GRB2 (N.Li, A.Batzer, R.Daley, V.Yajnik, E.Y.Skolnik, P.Chardin, D.Bar-Sagi, B.Margolis and J.Schlessinger, in preparation). The interaction between Sos and GRB2 is thought to be important in *ras* activation because cellular overexpression of GRB2 potentiates EGF's ability to activate *ras*. Moreover, the increase in *ras*−GTP observed in cells that overexpress GRB2 is mediated through an increase in *ras* guanine nucleotide exchange activity (N.Gale, S.Kaplan, P.Chardin, J.Schlessinger and D.Bar-Sagi, in preparation). In addition, cellular overexpression of GRB2 potentiates the ability of insulin to activate MAP kinase and preliminary evidence suggests that the increase in MAP kinase activity detected after insulin stimulation is mediated by an increase in *ras* activity (E.Y.Skolnik and J.Schlessinger, unpublished observation). Thus, a reasonable hypothesis is that interaction of IRS-1 with the SH2 domain of GRB2 enables the guanine nucleotide exchange factor (GNEF) Sos, coupled to the SH3 domain of GRB2, to activate *ras* (Figure 6).

Shc tyrosine phosphorylation and association of phosphorylated Shc with GRB2 had not been previously recognized in insulin-stimulated cells. While Shc is tyrosine-phosphorylated after insulin stimulation, it may not necessarily be a direct substrate for the insulin receptor. Rather, it is more likely that Shc is a target for a downstream cytoplasmic tyrosine kinase that is activated in response to insulin stimulation. This notion is based on the findings that Shc does not bind the insulin receptor and the kinetics of Shc phosphorylation after insulin stimulation are slower than that found for another insulin receptor substrate, IRS-1 (data not shown). It is now becoming evident that Shc may be an important signalling molecule for a wide variety of cytoplasmic tyrosine kinases. Shc is ubiquitously expressed and is tyrosine-phosphorylated after activation of a large number of tyrosine kinases (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992). In addition, Shc is an oncoprotein and has been implicated in the regulation of *ras* activity through its interaction with GRB2 (Rozakis-Adcock *et al.*, 1992). Our demonstration that Shc associates with GRB2 in insulin-stimulated cells supports the idea that tyrosine phosphorylation of Shc, and its subsequent interaction with GRB2, may be an essential component of a common pathway used by many tyrosine kinases.

The functional consequences of interaction between GRB2, IRS-1 and Shc in insulin-stimulated cells are likely to be complex and to form part of a pathway branching into many options. The outcome of GRB2 binding to Shc and IRS-1 may be similar, however, it is more likely that distinct effector signals are elicited by GRB2 binding to each phosphoprotein. An appealing hypothesis is that the interaction of GRB2 with IRS-1 will mediate metabolic responses regulated by insulin, while the interaction of GRB2 with Shc will be important for insulin-stimulated mitogenesis.

The ability of SH2 domain-containing proteins to interact simultaneously with many different tyrosine-phosphorylated proteins in growth factor-stimulated cells has been reported previously. It has been shown that the same site in the SH2 domain of GAP binds to both tyrosine-phosphorylated p62 and the PDGFR (Fantl *et al.*, 1992). In addition, EGF stimulation results in the formation of a GRB2−Shc and GRB2−EGFR complex (Rozakis-Adcock *et al.*, 1992). Taken together with our observation that GRB2 interacts with both Shc and IRS-1, the available evidence suggests that the functional consequences of interactions mediated by GRB2 are numerous. The exact functions regulated by GRB2, IRS-1 and Shc are likely to be determined by the enzymatic activities of the different effector proteins that couple to the SH3 domain of GRB2 upon binding to Shc or IRS-1. It is likely that these functions will include stimulation of *ras* activity and other downstream effectors crucial for the induction of the broad repertoire of pleiotropic responses mediated by insulin.

Materials and methods

Antibodies and baculovirus-produced IRS-1

Polyclonal rabbit antibodies to a peptide corresponding to amino acid residues 36−50 of GRB2 (Ab 86) and to a full length GRB2−GST fusion protein (Ab 50) were used for GRB2 immunoprecipitation and immunoblotting (Lowenstein *et al.*, 1992). IRS-1 antibodies were produced by immunizing rabbits with either an IRS-1 peptide (PEP 80) or with baculovirus-produced rat IRS-1 (IRS-1^{bac}) (Backer *et al.*, 1992; Myers *et al.*, 1992). Antibodies to p85 were raised against a GST fusion protein containing amino acids 265−523 and anti-phosphotyrosine immunoblots were performed with a rabbit polyclonal antibody (Hu *et al.*, 1992). Shc antibody was kindly provided by T.Pawson and is a rabbit polyclonal antibody raised against a Shc−GST fusion protein.

Baculovirus-expressed IRS-1 was collected from Sf9 cells and purified as described previously (Backer *et al.*, 1992; Myers *et al.*, 1992). Purified IRS-1^{bac} was then phosphorylated with [γ-^{32}P]ATP using wheat germ-agglutinated purified insulin receptor as previously described by Myers *et al.* (1992).

Cell lines, immunoblotting and immunoprecipitation

CHO/IR cells are CHO cells that overexpress the wild type human insulin receptor. After overnight starvation in F12 medium containing 0.5% bovine serum albumin (BSA) the cells were stimulated for 1 or 5 min with 100 nM bovine insulin. Cell lysis, immunoprecipitation and immunoblotting were performed as previously described by Backer *et al.* (1992).

GST fusion proteins

Oligonucleotides were synthesized in order to contain appropriate restriction sites at the 5′ end and complementary to flanking regions of various domains of GRB2. Using the GRB2 cDNA as a template, PCR was used to amplify the appropriate DNA fragments. The amplified products were cleaved with *Bam*HI and *Eco*RI, and cloned into PGEX 2T. After transformation of *Escherichia coli* HB101, large-scale cultures were grown and GST fusion proteins were purified as described by Hu *et al.* (1992). The following fusion proteins were used: GST−GRB2 full-length (amino acids 1−217), GST−SH2 (amino acids 58−159); GST−amino-terminal SH3 (amino acids 4−55); GST−carboxy-terminal SH3 (amino acids 1561−214). Site-directed mutagenesis was performed according to published procedures (Kunkel *et al.*, 1987) after subcloning GRB2 into M13 mp18. The mutagenic oligonucleotide used (5′-GGGGCCTTTCTTATCAAAGAGAGTGAGAGCGCTC-3′) resulted in a conservative substitution of lysine for arginine in the FLVRES motif of the SH2 domain of GRB2. The mutations were confirmed by sequencing and the DNA was subcloned into pGEX-2T and GST fusion proteins were produced as described above.

Binding assays

To assess the binding of IRS-1 to GST fusion proteins *in vitro*, 1 μg of GST fusion protein was immobilized on to glutathione−agarose beads and incubated with ^{32}P-labelled IRS-1^{bac} in buffer containing 50 mM Tris−HCl (pH 7.8), 250 mM NaCl, 10 mM dithiothreitol and 0.5% Triton X-100.

After a 1 h incubation at 4°C the beads were washed with HNTG [20 mM HEPES (pH 7.5), 10% glycerol, 0.1% Triton X-100 and 150 mM NaCl], and after boiling in sample buffer, the proteins were separated on an 8% SDS–polyacrylamide gel. Bound IRS-1 was then visualized by autoradiography.

For the IRS-1 protein blot, 10 ng of GST fusion protein were separated on a 10% SDS–polyacrylamide gel and then transferred to nitrocellulose. The nitrocellulose filter was blocked for 1 h at 4°C in TBS [10 mM Tris–HCl (pH 7.6) and 150 mM NaCl], containing 5% Carnation instant milk and then hybridized overnight with ^{32}P-labelled IRS-1^{bac}. The filters were then washed three times with TBS containing 0.05% Triton X-100 and bound IRS-1 was visualized by autoradiography. A duplicate immunoblot, probed with antibodies to GST, was performed to ensure that equal amounts of protein were loaded in the lanes.

Synthesis, purification of phosphopeptides and peptide inhibition studies

Peptides used in the competition and binding studies were prepared by conventional methods using a Milligen/Biosearch 9600 synthesizer. Phosphopeptides were prepared using FmocTyr(OP(OCH$_2$)$_2$ as previously described by Chatterjee *et al.* (1992). All peptides were purifed by HPLC and subjected to amino acid analysis.

For inhibition studies, 100 nM of GRB2–GST fusion protein immobilized on to glutathione–agarose beads were incubated with 100-fold excess peptide at 4°C for 1 h. ^{32}P-labelled IRS-1^{bac} was then incubated with the beads and bound IRS-1 was determined by SDS–PAGE and autoradiography. The amount of bound IRS-1 was then quantified using a phosphorimager, with 100% binding defined as the amount of IRS-1 bound in the absence of competing peptide.

PI-3 kinase assay

PI-3 kinase was assayed from immunoprecipitated proteins as described previously by Whitman *et al.* (1985).

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