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27. It is not clear why previous studies of potentiation by NO have not required paired activity (*2, 3*). Potentiation of evoked EPSPs by sodium nitroprusside in hippocampal slices (*2*) may have involved another substance released by the drug or a frequency of test stimulation that itself provided sufficient activity. Potentiation of spontaneous release by NO in culture (*3*) may have involved better access of NO to the cells or cells in a different state of development or with a different resting metabolic state.
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Binding of the Ras Activator Son of Sevenless to Insulin Receptor Substrate–1 Signaling Complexes

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Signal transmission by insulin involves tyrosine phosphorylation of a major insulin receptor substrate (IRS-1) and exchange of Ras-bound guanosine diphosphate for guanosine triphosphate. Proteins containing Src homology 2 and 3 (SH2 and SH3) domains, such as the p85 regulatory subunit of phosphatidylinositol-3 kinase and growth factor receptor–bound protein 2 (GRB2), bind tyrosine phosphate sites on IRS-1 through their SH2 regions. Such complexes in COS cells were found to contain the heterologously expressed putative guanine nucleotide exchange factor encoded by the *Drosophila son of sevenless* gene (dSos). Thus, GRB2, p85, or other proteins with SH2-SH3 adapter sequences may link Sos proteins to IRS-1 signaling complexes as part of the mechanism by which insulin activates Ras.

Insulin receptors as well as other tyrosine kinases can elicit biological actions by increasing the steady-state level of guanosine triphosphate (GTP)–bound Ras proteins (*1–4*). The mechanism of Ras activation by insulin appears to involve the stimulation of guanine nucleotide exchange activity, causing release of bound guanosine diphosphate (GDP) followed by GTP binding to Ras (*2*). Genetic studies of *Drosophila melanogaster* have identified a protein, Son of sevenless (dSos), as a putative activator of Ras. Its function is required downstream of the sevenless tyrosine kinase and upstream of Ras in a signaling pathway necessary for eye development (*5, 6*). The dSos protein and two mouse homologs denoted as mSos1 and mSos2 (*7*) each contain a region with amino acid sequence similarity to the catalytic domain of the yeast Ras exchange factor CDC25. The mSos1 and mSos2 proteins are widely distributed in mouse tissues, in contrast to brain-specific Ras exchange factors (*8*). Thus, the Sos proteins are excellent candidates for mediating the effects of the insulin receptor on GTP binding to Ras.

In our studies here the dSos complementary DNA (cDNA) was modified to encode a hemagglutinin (HA) epitope extension at its COOH-terminus to allow its immunoprecipitation and immunoblotting (*9*). Cotransfection of this dSosHA cDNA with that encoding human H-Ras increased the amount of GTP-bound Ras in ^{32}P-labeled COS-1 cells to 10 times that found in cells transfected with human H-Ras alone (Fig. 1). Co-expression of human insulin receptors with human H-Ras in COS-1 cells led to a threefold increase in GTP-bound Ras, even in the absence of added insulin. This is a result of the large amounts of the receptor expressed under these conditions, although

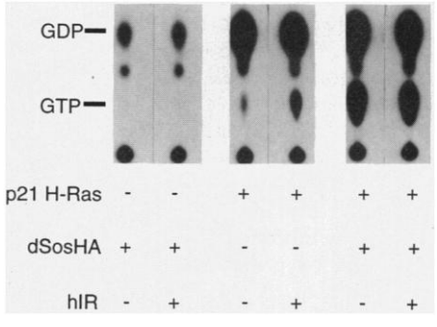

Fig. 1. Stimulation of Ras GTP-GDP exchange in intact cells by expression of recombinant dSosHA. COS-1 cells were transiently transfected (*25*) with the cDNAs encoding H-Ras (*26*), dSosHA (*9*), or the human insulin receptor (hIR) (*24*) subcloned into the mammalian expression vector pCMV (*27*) as indicated. Two days after transfection, the cells were labeled with carrier-free [^{32}P]orthophosphate (1.0 mCi/ml) for 4 hours in phosphate-free medium. The cells were lysed, and Ras was immunoprecipitated (*4*) with mAb to Ras [cell supernatant of the hybridoma cell line Y13-259 (American Type Culture Collection)]. Guanine nucleotides bound to Ras were dissociated and subsequently separated on polyethylenimine-cellulose thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) (*28*). The positions of the GTP and GDP standards on the same TLC plate are indicated.

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insulin treatment further increases Ras activation about twofold (*10*). Insulin receptor expression in the absence or presence of insulin did not further augment the effect of dSosHA on the amounts of GTP-bound Ras (Fig. 1) unless submaximal amounts of dSosHA were used (*10*). These results indicate that when overexpressed, the dSosHA protein catalyzes GDP release from human Ras in intact COS-1 cells; such results are consistent with the involvement of Sos proteins in insulin-stimulated Ras activation.

The hypothesis that the insulin receptor might regulate Sos proteins by direct tyrosine phosphorylation in intact cells was examined (Fig. 2). COS-1 cells expressing the dSosHA protein with or without insulin receptors were labeled with ^{32}P, and proteins in cell lysates were immunoprecipitated with the monoclonal antibody (mAb) to HA, 12CA5. Separation of the immune complexes by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) revealed a phosphorylated 180,000-kD band that was absent in untransfected cells (Fig. 2A). Labeling of the dSosHA protein with ^{32}P was unaffected by expression of insulin receptors in the presence of insulin. Phosphoamino acid analysis of the ^{32}P-labeled dSosHA protein from cells with or without expressed insulin receptors showed similar amounts of phosphoserine and phosphothreonine, but no phosphotyrosine was detected (Fig. 2B).

Another way by which the insulin receptor may regulate Ras exchange proteins is by physical association (*11*). Complexes between a major cellular substrate protein denoted as the insulin receptor substrate–1 (IRS-1) (*12*) and other proteins are formed in response to tyrosine phosphorylation of IRS-1 by the insulin receptor. Tyrosine-phosphorylated IRS-1 associates with the Src homology 2 (SH2)–containing proteins phosphatidylinositol-3 kinase (PI-3 kinase) and growth factor receptor–bound protein 2 (GRB2) (*13*), which also contain SH3 domains. Importantly, GRB2 is a human homolog of Sem-5, identified in genetic studies as a key element in Ras activation in *Caenorhabditis elegans* (*14*). GRB2 has been shown to stimulate DNA synthesis when microinjected into rat fibroblasts with Ras (*15*). The Sos proteins are devoid of SH2 and SH3 domains. However, recent reports identified a protein, 3BP1, that binds SH3 domains through a proline-rich motif similar to those in the Sos proteins (*16*). The dSos protein binds directly to a *Drosophila* homolog of Sem-5 and GRB2, Drk (*17*). This information suggests the hypothesis that Sos proteins may associate with IRS-1 signaling complexes indirectly through SH3 domains of adapter proteins such as GRB2 or p85 that in turn bind tyrosine phosphate sites through their SH2 domains.

To test this hypothesis, we immunoprecipitated lysates from COS-1 cells expressing dSosHA and native insulin receptor or a catalytically inactive mutant receptor with antiserum to IRS-1 (anti–IRS-1). When cells were transfected with dSosHA or cotransfected with the catalytically inactive receptor and dSosHA, anti–IRS-1 immunoprecipitates contained small amounts of dSosHA as detected with the HA antibody (anti-HA) (Fig. 3A). The association of IRS-1 and dSosHA in the absence of the transfected native insulin receptor may reflect the presence of residual tyrosine-phosphorylated IRS-1 after 2 hours of serum starvation. Importantly, cotransfection of cells with the native insulin receptor and dSosHA increased the amount of dSosHA protein found in IRS-1 immune complexes, as detected by immunoblot analysis with the HA antibody (Fig. 3A). Moreover, tyrosine-phosphorylated IRS-1 was observed in anti-HA immunoprecipitates from cells cotransfected with the insulin receptor and dSosHA (*18*). These results suggest that tyrosine phosphorylation of IRS-1 by a catalytically active insulin receptor is required to promote the interaction of dSosHA with IRS-1. Endogenous guanosine triphosphatase activating protein (GAP), which contains SH2 and SH3 domains, as well as the expressed dSosHA were present in total cell lysates, but GAP

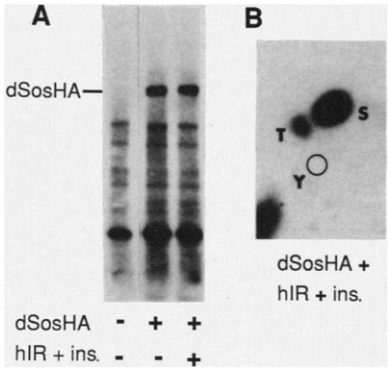

Fig. 2. Phosphorylation of dSosHA on Ser and Thr residues in intact cells. COS-1 cells were transiently transfected with the human insulin receptor (hIR) or dSosHA cDNAs and labeled with carrier-free [^{32}P]orthophosphate (6 mCi/ml) as described (Fig. 1). Cells transfected with hIR cDNA were then stimulated for 10 min with human insulin (ins.) (100 nM). A lysate was prepared, and dSosHA was immunoprecipitated (*29*). Two-dimensional phosphoamino acid analysis of dSosHA was performed after separation of the proteins by SDS-PAGE (6% gel), transfer to Immobilon-P transfer membranes (Millipore), and localization of the dSosHA band by autoradiography as described (*30*). (**A**) Autoradiograph of the Immobilon membrane. (**B**) Autoradiograph after two-dimensional thin-layer electrophoresis. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. The position of the phosphotyrosine marker is indicated by the circle.

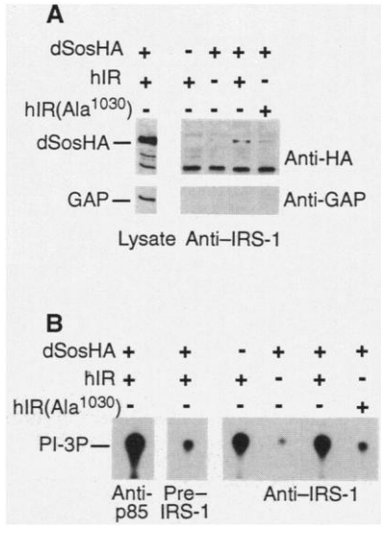

Fig. 3. Insulin receptor–dependent association of IRS-1 with transiently expressed dSosHA. Dishes (100 mm) of COS-1 cells were transiently transfected (*25*) with dSosHA, insulin receptor (hIR), or with catalytically inactive insulin receptor [hIR(Ala^{1030})] (*31*) as indicated. Forty-eight hours after transfection, immunoprecipitates were prepared from cell lysates (*3, 32*) after the cells were starved of serum for 2 hours. This treatment down-regulated basal levels of PI-3 kinase associated with tyrosine-phosphorylated proteins in cells that were not transfected with the insulin receptor. In insulin receptor–transfected cells, signaling was still fully maintained by approximately 20% of the insulin receptors that remained tyrosine-phosphorylated under these conditions (*18*). (**A**) Proteins in the immune complexes (from 45 μl of lysate) or in cleared lysates (8 μl) were separated by reducing SDS-PAGE (6% gel) and analyzed by protein immunoblotting with antibody 12CA5 (anti-HA) (Babco, Berkeley, California) or antibody to GAP (anti-GAP) (Zymed Laboratories, San Francisco, California) and detection by enhanced chemiluminescence (ECL) (Amersham). The positions of dSosHA and GAP are indicated. (**B**) Immune complexes with the indicated antibodies from COS cell lysates (200 μl) were assayed for PI-3 kinase activity with [γ-^{32}P]adenosine triphosphate and phosphatidylinositol as the substrates (*3, 33*). The products were separated by TLC (*34*). Anti-p85, antibody to PI-3 kinase subunit p85 (Upstate Biotechnology, Lake Placid, New York); Pre–IRS-1, preimmune serum to IRS-1; PI-3P, phosphatidylinositol 3′-phosphate.

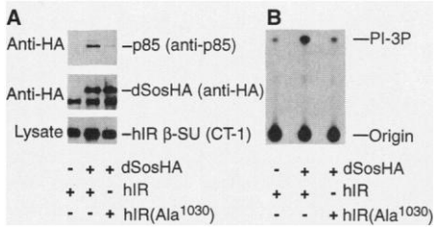

Fig. 4. Insulin receptor–dependent co-immunoprecipitation of PI-3 kinase with transiently expressed dSosHA. COS-1 cells were transiently transfected as indicated and starved of serum for 2 hours (Fig. 3). The dSosHA protein was immunoprecipitated from cell lysates (*3, 32*) and assayed for coprecipitation of PI-3 kinase. (**A**) Half of the immunoprecipitate was separated by reducing SDS-PAGE (8% gel) and analyzed by protein immunoblotting with anti-HA or polyclonal anti-p85 and detection by ECL. Total lysates were analyzed for overexpression of hIR or hIR(Ala^{1030}) with antibody CT-1 (*20*) (hIR β-SU, insulin receptor β-subunit). (**B**) The remaining half of the immunoprecipitate was assayed for PI-3 kinase activity as described (Fig. 3).

was not detected in IRS-1 immunoprecipitates, which is consistent with previous studies (*19*). Anti–IRS-1 immunoprecipitates from cells transfected with native insulin receptor cDNA but not catalytically inactive receptor cDNA contained increased PI-3 kinase activity, accounting for about 40% of that immunoprecipitated with p85 antiserum (Fig. 3B).

If dSosHA and PI-3 kinase are recruited to the same IRS-1 protein complex, specific immunoprecipitation of dSosHA should coprecipitate the regulatory subunit of PI-3 kinase, p85, and PI-3 kinase activity. We tested this by co-expressing dSosHA with native or catalytically inactive insulin receptors and immunoprecipitating cell lysates with 12CA5 antibody. When blotted with 12CA5 antibody, these immunoprecipitates revealed an immunoreactive species at about 180,000 kD, as expected in lysates of COS-1 cells transfected with dSosHA but not in untransfected cells (Fig. 4A). Expression of native or catalytically inactive insulin receptors was confirmed in lysates of COS-1 cells transfected with the respective constructs with the use of COOH-terminal insulin receptor mAb, CT-1 (*20*). Increased amounts of the p85 regulatory subunit of PI-3 kinase (Fig. 4A) and PI-3 kinase activity (Fig. 4B) were detected in the anti-HA immunoprecipitate from cells cotransfected with dSosHA and the native insulin receptor compared to the amounts in anti-HA immunoprecipitates from cells expressing the catalytically inactive insulin receptor mutant. Thus, dSosHA and a portion of the endogenous PI-3 kinase appear to be colocalized in the same IRS-1–containing protein complex in response to insulin receptor tyrosine kinase.

Results related to the insulin receptor and other tyrosine kinases (*13–15, 17*) as well as those in the accompanying paper by Skolnik *et al.* (*21*) suggest that the SH2-SH3–containing GRB2 protein is the most likely adapter that links IRS-1 to dSosHA expressed in COS-1 cells. However, IRS-1 complexes also contain p85 (Fig. 3) and potentially other, yet unidentified proteins that contain both SH2 and SH3 domains. Identification of which proteins actually serve as the putative adapter or adapters in the insulin signaling pathway will require detailed experiments on endogenous Sos proteins in primary insulin-responsive cell types, such as liver, muscle, and fat.

How might the recruitment of Sos proteins to IRS-1 complexes elicit Ras activation? Both Ras and receptor tyrosine kinases are present at the plasma membrane. Recent findings suggest that complexes of GRB2 and Sos associate with the epidermal growth factor (EGF) receptor upon cell stimulation (*22*). Although IRS-1 has been thought to be largely cytoplasmic, a subpopulation of IRS-1 proteins binds to insulin receptors in intact cells (*18, 23*). The association of Sos with IRS-1 may be a means to translocate the exchange factor to the plasma membrane in a position to interact with Ras. The binding of Sos proteins to IRS-1 signaling complexes may also lead to increased intrinsic guanine nucleotide exchange activity.

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