Endocrine (1995) 3, 475-479

Mechanism of activation of guanine nucleotide exchange factor by insulin

John Langlois[2], J. Wayne Leitner[1], Jheem Medh[2], Toshiyasu Sasaoka[2], Jerrold M. Olefsky[2] & Boris Draznin[1]

[1]*Medical Research Service and the Department of Medicine, Veterans Affairs Medical Center and the University of Colorado Health Sciences Center, Denver, Colorado 80220;* [2]*Medical Research Service and the Department of Medicine, Veterans Affairs Medical Center and the University of California, San Diego, California 92093, USA*

Insulin increases activity of the guanine nucleotide exchange factor (GEF) in Rat-1 fibroblasts transfected with human insulin receptors (HIRc cells), thereby promoting formation of the active form of p21Ras (p21Ras•GTP). In order to identify the upstream molecules mediating this aspect of insulin action, we selectively removed some of these molecules by immunoprecipitation and examined GEF activity in the post-immunoprecipitation lysates of the insulin-treated HIRc cells. The removal of Shc or Grb-2 depleted GEF activity from the cell lysates, whereas immunoprecipitation of the insulin receptors, IRS-1, PLCγ and GAP, were without effect. In summary, the current data demonstrate that a majority of cellular Ras GEF activity after insulin stimulation is associated with Shc and involves interactions among Shc, Grb-2 and Sos.

Keywords: insulin action; signal transduction

Introduction

Activation of p21Ras is one of the early post-receptor steps in the mechanism of insulin signaling (Baltensperger *et al.*, 1993; Draznin *et al.*, 1993; Medema *et al.*, 1993; Skolnik *et al.*, 1993). Insulin stimulates formation of the active form of p21Ras (p21Ras•GTP) by stimulating the activity of guanine nucleotide exchange factor (GEF) that facilitates dissociation of GDP from the Ras protein allowing the latter to associate with GTP (Draznin *et al.*, 1993; Medema *et al.*, 1993; Pronk *et al.*, 1994; Sasaoka *et al.*, 1994). The ras GEF is the mammalian homologue of the drosophila Son of Sevenless protein (Sos) (Baltensperger *et al.*, 1993; Buday & Downward, 1993a,b; Draznin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). Sos forms complexes with Grb-2, an adaptor protein containing one SH2 and two SH3 domains. The Grb-2 SH3 domains interact with a proline-rich region of Sos, and the SH2 domain binds to specific phosphotyrosine motifs (YV/IN) on various signaling molecules (Buday & Downward, 1993a; Egan *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993; Skolnik *et al.*, 1993; Sun *et al.*, 1993). In the case of insulin signaling, Grb-2 binds to the insulin receptor substrate-1 (IRS-1) and Shc, two docking proteins that undergo rapid tyrosine phosphorylation following insulin binding to its cell surface receptor (Sun *et al.*, 1993, 1994). Although both IRS-1 and Shc are crucial for the intracellular flow of the insulin signal, their relative importance in the Ras pathway remains unclear.

In this study, we attempted to identify the upstream molecules that interact with the Grb-2/Sos complex, mediating stimulation of GEF activity in response to insulin. Using immunoprecipitation, we selectively removed various upstream signaling molecules and examined GEF activity in the post-immunoprecipitation lysates of insulin-treated Rat-1 fibroblasts transfected with human insulin receptors (HIRc). The results of these experiments demonstrate that removal of Shc or Grb-2 depletes GEF activity from the cell lysates, whereas immunoprecipitation of the insulin receptor, IRS-1, phospholipase Cγ (PLCγ) and GTPase activating protein (GAP) were without effect.

Results and discussion

Insulin treatment for 10 min significantly enhanced GEF activity in HIRc cells (Figure 1A). Treatment with other growth factors, including EGF, also enhances Ras GEF activity*, and EGF and insulin induce binding of the Grb-2/Sos complex to the EGF receptor, Shc, or IRS-1 respectively (Buday & Downward, 1993a; Egan *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993; Sun *et al.*, 1993). In this way, Grb-2, which does not undergo phosphorylation, serves as an adaptor protein connecting upstream signaling elements with Sos. The Grb-2 SH2 domain binds to the YV/IN motifs of IRS-1, Shc, or the EGF receptor, while its two SH-3 domains interact with the proline-rich region of Sos (Buday & Downward, 1993a; Egan *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993; Sun *et al.*, 1993). GAP and PLCγ also possess SH2 domains and may be involved in growth factor signaling (Koch *et al.*, 1991). The role of these molecules in transmitting the insulin signal to Sos, as well as the relative importance of IRS-1 and Shc in this process, were addressed in the current studies.

Control and insulin-treated HIRc cells (10 min at 37°C) were lysed and the specific upstream elements were immunoprecipitated with the appropriate specific antibodies. GEF activity was then measured in the post-immunoprecipitated supernatants. Immunoprecipitations of the insulin receptor (insulin receptor antibody displays nearly 100% efficiency – not shown) did not diminish GEF activity in the lysates (Figure 1B), arguing against the possibility that Sos associates with the insulin receptor either directly or as part of a larger complex. Similarly, immunoprecipitation of GAP, or PLCγ (Figure 1C and D) did not decrease insulin-stimulated GEF activity, suggesting that these elements also are not involved in mediating insulin signaling to p21Ras.

Previous studies have demonstrated that Grb-2/Sos complexes may bind to both IRS-1 and Shc (Skolnik *et al.*, 1993; Sasaoka *et al.*, 1994; Sun *et al.*, 1994). To examine the role of these two coupling molecules in mediating insulin stimulation of GEF activity, the effects of IRS-1 and Shc precipitation on GEF activity were examined. Removal of IRS-1 from the cell lysates did not deplete GEF activity (Figure 1E). To confirm

Correspondence: Dr Boris Draznin
Received 7 February 1995; accepted 14 March 1995

*EGF translocates GEF activity from the cytosol to the plasma membrane (Buday & Downward, 1993a,b; Langlois *et al.*, 1994).

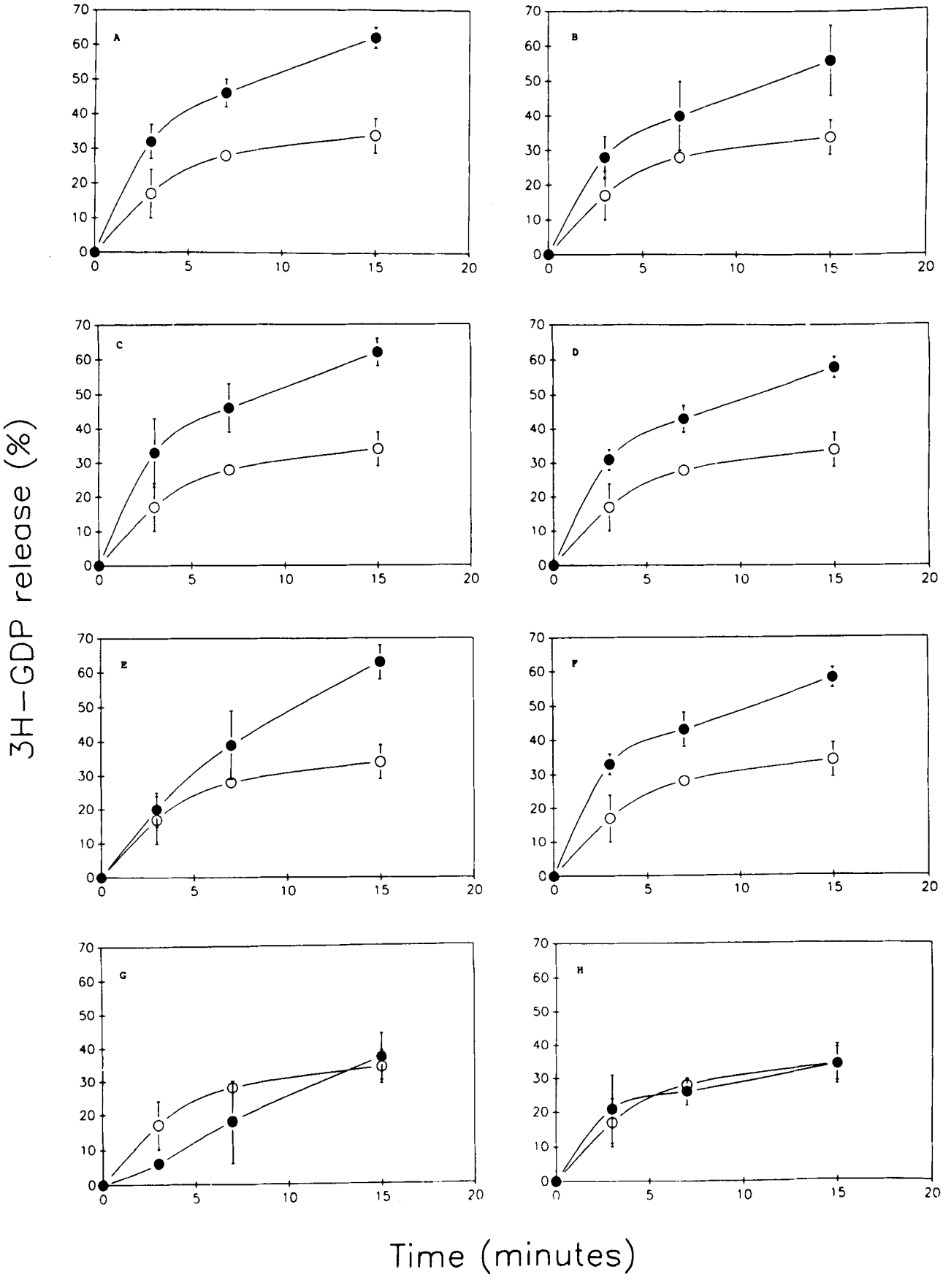

Figure 1 Effect of insulin (**A**) and removal by immunoprecipitation of the insulin receptor (**B**, $n = 4$), GAP (**C**, $n = 8$), PLCγ (**D**, $n = 6$), IRS-1 (**E**, $n = 8$), precipitation of phosphorylated IRS-1 by the GST fusion protein of the N-terminal SH2 domain from p85 subunit of PI-3 kinase (**F**, $n = 4$), or immunoprecipitation of Shc (**G**, $n = 10$), or Grb-2 (**H**, $n = 10$) on GEF activity in the lysates of HIRc cells. The cells were treated with insulin (17 nM) for 10 min, lysed, and immunoprecipitations were carried out as described in the Methods section. GEF activity was assessed by the rate of [^{3}H]-GDP release from the Ras●GDP complex at 3, 7 and 15 min of incubation with the lysates. Results represent mean±SEM of four to 10 independent experiments with either control non-precipitated (○----○) or insulin-treated cell lysates precipitated as indicated (●----●)

this finding, we also used a GST fusion protein of the N-terminal SH2 domain from the p85 subunit of PI-3 kinase. Incubation of this fusion protein with lysates from insulin stimulated cells, with subsequent precipitation with GST-Sepharose beads, quantitatively precipitated phosphorylated IRS-1 (Jhun *et al.*, 1994). However, depletion of the lysates with this SH2 domain (Figure 1F) also did not reduce GEF activity. In contrast, immunoprecipitation of Shc depleted GEF activity to levels seen in unstimulated cells (Figure 1G). These results indicate the relative importance of Shc compared to IRS-1 as adapter molecules connecting the IR to p21ras stimulation, and is consistent with our previous findings on the importance of Shc in signaling insulin action to p21Ras (Sasaoka *et al.*, 1994a,b). In light of the tight association between Grb-2 and Sos, it was not surprising to find that immunoprecipitation of Grb-2 greatly reduced GEF activity in the lysates (Figure 1H).

In order to appropriately interpret the results of these experiments, two control points had to be addressed. First, immunodepletion of various intermediates must be confirmed by demonstrating their absence in the post-immunoprecipitate supernatants. Second, to attribute GEF activity to Sos, one must demonstrate that immunodepletion of Sos removes GEF activity from the cell lysate.

Experiments quantitating immunoprecipitations of Shc, Grb-2 and IRS-1 are depicted in Figure 2. Lysates of the insulin-treated cells were analysed by Western blotting before and after immunoprecipitation with anti-Shc (Figure 2A) or anti-Grb-2 (Figure 2B) antibodies. Immunoprecipitations depleted the cell lysates of Shc and Grb-2 by more than 98% and 90% respectively (determined by densitometry of Western blots). Similarly, post-IRS-1 and post-P85 SH2 GST precipitation supernatants showed a loss of over 90% and 95% of phosphorylated IRS-1 (Figure 2C).

Immunodepletion of Sos completely removed insulin-stimulated GEF activity from the cell lysates (Table 1), confirming that GEF activity in the insulin-treated cells is appropriately attributed to Sos. Pre-clearing of cell lysates with pre-immune IgG had no effect on GEF activity (Table 1).

Western blotting of Shc and IRS-1 immunoprecipitates with anti-Grb-2 antibodies confirmed that after exposure to insulin for 10 min, Grb-2 was found in association with Shc and not with IRS-1 (Figure 3). Thus, Shc appears to be the principal molecule involved in conveying the insulin signal to GEF and p21Ras. The p52 isoform of Shc underwent insulin-inducible association with both Grb2 and Sos, as evidenced by Western blotting (Figure 4), supporting the role of Shc phosphorylation and its association with Grb-2/Sos complexes following insulin treatment.

Since our previous studies demonstrated that the kinetics of Grb-2/IRS-1 association were rapid, with complex formation detected after 1–2 min of insulin stimulation, and decreasing thereafter, we examined whether immunoprecipitation of IRS-1 at earlier time points (2 min of insulin treatment) would affect insulin-stimulated GEF activity. Results of these experiments are shown in Table 2. Immunoprecipitation of IRS-1 following 2 min of insulin exposure revealed a trend toward partial depletion of insulin-stimulated GEF activity, but the effect was of low magnitude and not statistically significant, whereas, immunoprecipitation of IRS-1 at 10 min had no effect on GEF activity.

Thus, these experiments confirm our previous findings that insulin stimulates an induction of Ras GEF (Sos) activity, and provides clarification of the intracellular signaling pathway involved. Firstly, there is no significant association seen between the insulin receptor and Ras GEF activity. This is in contrast to the situation with other growth factor receptors, and suggests that, despite undergoing tyrosine phosphorylation, the receptor does not bind the Grb-2/Sos complex, nor does it form part of a larger complex of signaling molecules including Grb-2/Sos. Thus, the insulin-induced activation of Sos activity must be mediated by intermediary signaling

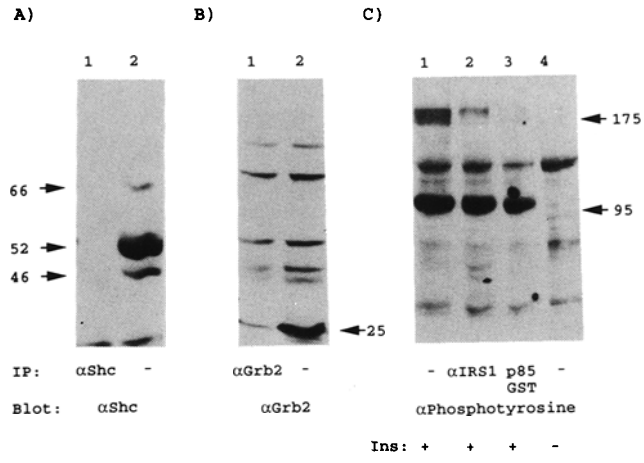

Figure 2 Immunodepletion of Shc, Grb-2, IRS-1 and PI-3 kinase: The lysates of the insulin treated cells before immunoprecipitations with polyclonal rabbit Shc (**A**, lane 2) or Grb-2 (**B**, lane 2) antibodies, and post-immunoprecipitation supernatants (**A**, lane 1 and **B**, lane 1) were Western blotted with a corresponding monoclonal antibody to quantitate Shc and Grb-2 depletion. (**C**) demonstrates that PY20 antibody detects IRS-1 in non-immunoprecipitated insulin-treated cell lysates (lane 1). Lanes 2 and 3 demonstrate depletion of IRS-1 (175 kD band) in the lysates precipitated with either anti-IRS-1 (lane 2) or the GST fusion protein of the N-terminal SH-2 domain of the p85 subunit of PI-3 kinase (lane 3). Lysates of control (non-insulin treated) cells are shown in lane 4

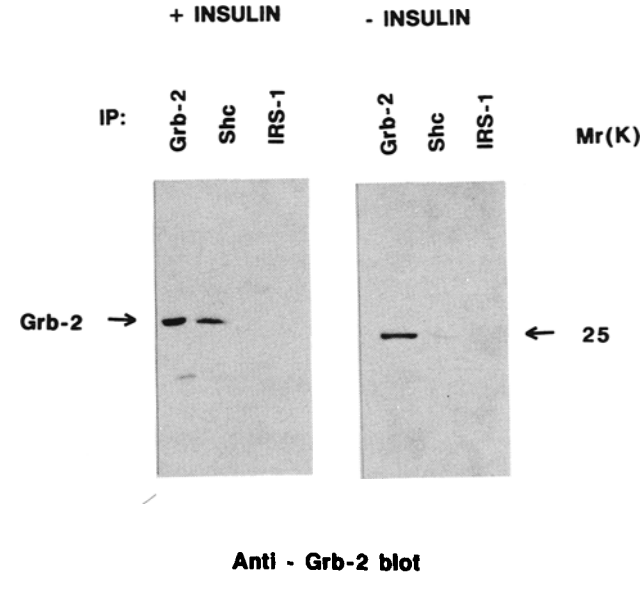

Figure 3 Association of Grb-2 with Shc in the insulin-treated HIRc cells. The lysates of control and insulin-treated cells (100 nM for 10 min) were pre-cleared and immunoprecipitated (IP) with Grb-2, Shc, or IRS-1 antibody and immunoblotted with anti-Grb-2 antibody

Table 1 Effect of Sos immunoprecipitation (IP) on insulin-stimulated GEF activity*

	*[^{3}H]-GDP released (%)***
Control	19.4 ± 2.7%
Insulin (100 nM for 10 min)	38.1 ± 3.5%
Insulin + IP with pre-immune serum	44.0 ± 3.9%
Insulin + IP with Sos antibody	14.0 ± 2.4%

*Results represent the mean ± SEM of two experiments. **At the 15th minute of the dissociation assay.

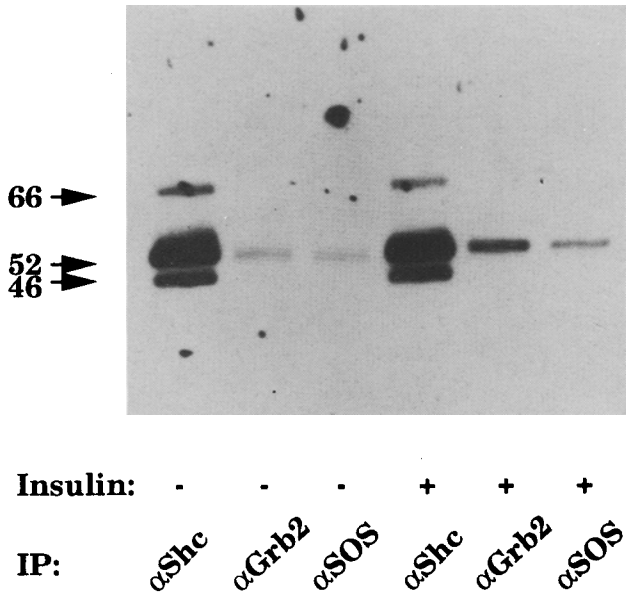

Figure 4 Association of Shc with Grb-2 and Sos in the insulin-treated (100 nM) HIRc cells. The cells were lysed, pre-cleared and immunoprecipitated (IP) as indicated with anti-Shc, anti-Grb-2 or anti-Sos antibody (all rabbit polyclonal antibodies). Immunoblotting was performed with mouse monoclonal anti-Shc. Molecular mass is indicated by the arrows

Table 2 Effect of IRS-1 immunoprecipitation (IP) on insulin-stimulated GEF activity

	Control	*Insulin (2 min)*	*Insulin (2 min) IP with IRS-1 Antibody*	*Insulin (10 min)*	*Insulin (10 min) IP with IRS-1 Antibody*
GEF activity % [^{3}H]-GDP released $n=4$	19±3	39±8	32±4	52±5	51±3

molecules. Neither GAP, PLCγ nor IRS-1 were found to be associated with GEF activity, suggesting that these do not play important roles in this signaling pathway. IRS-1 has been previously shown to interact with Grb-2, but the current data and previous findings (Sasaoka *et al.*, 1994a,b) suggest that such interactions are quantitatively minor, occur quickly after insulin stimulation, and have declined markedly by the time maximal induction of GEF activity is seen. In contrast, Shc seems to play a major role in Ras GEF activation. The current data show that a majority of cellular Ras GEF activity after insulin stimulation is associated with Shc.

In summary, insulin-stimulated GEF activity appears to involve interactions among Shc, Grb-2 and Sos. The nature of the signaling mechanism linking the insulin receptor with Shc remains to be defined.

Materials and methods

Materials

Tissue culture media, gentamicin, methotrexate and phosphate-free Dulbecco's modified Eagle's medium were from Life Technologies, Inc (Grand Island, NY). Fetal calf serum was from Gemini Bio-Products, Inc. (Calabasas, CA). Bovine serum albumin and other biochemicals were from Sigma (St. Louis, MO). The anti-p21Ras rat monoclonal IgG (Y13-259) and Protein G-PLUS/Protein A-agarose immunoprecipitation reagents were from Oncogene Science, Inc. (Uniondale, NY). The p21Ras (c-Ha-Ras) was a gift from Dr Alan Wolfman (Cleveland Clinic Foundation). [^{32}P]-orthophosphate and [^{3}H]-GDP were obtained from DuPont-New England Nuclear (Boston, MA). Polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC) plates were purchased from J.T. Baker, Inc (Phillipsburg, NJ). Polyclonal and monoclonal anti-Shc antibodies, polyclonal and monoclonal anti-IRS-1 antibodies, monoclonal anti-Grb-2 antibody, monoclonal anti-P-TYR (PY-20) antibody, and monoclonal anti-Sos antibody were purchased from Transduction Laboratories (Lexington, KY). Polyclonal anti-Sos antibody was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Plasmids for expression of a GST fusion protein of the N-terminal SH2 domain of the p85 subunit of PI-3 kinase were a generous gift of A. Saltiel, Parke-Davis, a division of Warner-Lambert, Ann Arbor, MI. All reagents and supplies required for SDS–polyacrylamide gel electrophoresis and Immun-lite chemiluminescent assay kits were from BioRad Laboratories (Richmond, CA). Porcine insulin was from Eli Lilly and Company (Indianapolis, IN).

GEF assay

Rat-1 fibroblasts expressing human insulin receptors (1.2×10^6 receptors/cell) were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% fetal calf serum, 15 mM HEPES, 2.5 mM glutamine, 50 μg/ml gentamicin and 500 mM methotrexate. Cells were serum-starved overnight, challenged with insulin (0–100 mM) for 2 or 10 min at 37°C, washed and lysed in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT), 1 mM sodium vanadate, 1 mM Na_2PO_4, 0.1% Triton X100, 0.05% sodium dodecyl sulfate (SDS), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM GTP and 100 μM GDP. Cell lysates were pre-cleared for 30 min at 4°C with Protein G-plus/Protein A-agarose pre-coupled to rabbit anti-rat IgG. The lysates were clarified by centrifugation at 10 000 *g*. The lysate supernatants were then immunoprecipitated with the indicated antibodies, and the supernatants were stored at −80°C until use. Precipitation with the GST fusion protein of the p85 SH2 domain was performed by incubating 7.5 μg of the fusion protein with the cell lysates, followed by incubation with glutathione-sepharose. After centrifugation at 10 000 *g* for 5 min, the supernatants were stored at −80°C. Protein concentration was determined by bicinchoninic acid assay. Determination of GEF activity was performed as previously described (Draznin *et al.*, 1993). In brief, to assay the Ras GEF, purified c-Ha-Ras (550 ng, 28 pmole) was incubated with 3 μM [^{3}H]-GDP (32 Ci/mmol) in 50 μl of binding buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 400 μg/ml chain A insulin) for 15 min at 30°C. The complex was stabilized by addition of 5 μl of 127 mM $MgCl_2$ to yield 10 mM. Ras●GDP complex (5 μl, 50 ng) was added to 200 μl of lysate (200 μg of protein) or lysis buffer and incubated at 23°C. At intervals, 50 μl aliquots were removed and filtered through 0.45 μM Millipore nitrocellulose filters which were washed three times with 1 ml of ice-cold buffer containing 50 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 100 μM ATP. The radioactivity of [^{3}H]-GDP bound to p21Ras was quantitated by scintillation counting. A value of 0% release represents 10 325 ± 442 c.p.m. bound. Background c.p.m. were less than 1% of the total bound [^{3}H]-GDP. [^{3}H]-GDP binding to p21Ras was confirmed by immunoprecipitation and integrity of dissociated [^{3}H]-GDP by thin layer chromatography.

Immunoprecipitation and Immunoblotting

Cells were starved for 24 h in serum-free Dulbecco's modified Eagle's medium (DMEM), incubated with 100 nM insulin at 37°C for 10 min and lysed in buffer as previously described

(Sasaoka *et al.*, 1994). The precleared cell lysates were centrifuged (10 000 *g* for 10 min) and the supernatants used for immunoprecipitation with the indicated rabbit polyclonal antibodies according to manufacturer's suggested protocols. Immunoprecipitates were separated by SDS–PAGE and transferred onto nitrocellulose membranes by electroblotting. After incubation with the specified mouse monoclonal antibody, blots were incubated with alkaline phosphatase conjugated second antibody followed by chemiluminescence detection.

Acknowledgements

The authors wish to thank Ms. Gloria Smith for her excellent secretarial assistance. This work was supported in part by Grant DK 33651 from the National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health and by Veterans Administration Medical Research Service. Dr Langlois is a research fellow of the Medical Research Council of Canada.

References

Baltensperger, K., Kozma, L.M., Cherniack, A.D., Klarlund, J.K., Chawla, A., Banerjee, U. & Czech, M.P. (1993). *Science*, **260**, 1950–1952.

Buday, L. & Downward, J. (1993a). *Mol. Cell. Biol.*, **13**, 1903–1910.

Buday, L. & Downward, J. (1993b). *Cell*, **73**, 611–620.

Draznin, B., Chang, L., Leitner, J.W., Takata, Y. & Olefsky, J.M. (1993). *J. Biol. Chem.*, **268**, 19998–20001.

Egan, S., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M. & Weinberg, R.A. (1993). *Nature*, **363**, 45–51.

Jhun, B., Rose, D., Seely, B.L., Rameh, L., Cantley, A., Saltiel, A. & Olefsky, J.M. (1994). *Mol. Cell. Biol.*, **14**, 7466–7475.

Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. & Pawson, T. (1991). *Science*, **252**, 668–674.

Langlois, W.J., Sasaoka, T., Medh, J., Leitner, J.W., Olefsky, J.M. & Draznin, B. (1994). *Endocrinology*, **135**, 2412–2417.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., BarSagi, D., Margolis, B. & Schlessinger, J. (1993). *Nature*, **363**, 85–88.

Medema, R.H., deVries-Smits, A.M.M., van derZon, G.C.M., Maassen, J.A. & Box, J.L. (1993). *Mol. Cell. Biol.*, **13**, 155–162.

Olivier, J.P., Taabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. & Pawson, T. (1993). *Cell*, **73**, 179–191.

Pronk, G.J., de Vries-Smits, A.M.M., Buday, L., Downward, J., Maassen, J.A., Medema, R.H. & Bos, J.L. (1994). *Mol. Cell. Biol.*, **14**, 1575–1581.

Rozakis-Adcock, M., Fernley, R., Wade, J., Powson, T. & Bowtell, D. (1993). *Nature*, **363**, 83–85.

Sasaoka, T., Draznin, B., Leitner, J.W., Langlois, W.J. & Olefsky, J.M. (1994a). *J. Biol. Chem.*, **269**, 10734–10738.

Sasaoka, T., Rose, D.W., Jhun, B.H., Saltiel, A.R., Draznin, B. & Olefsky, J.M. (1994b). *J. Biol. Chem.*, **269**, 13689–13694.

Simon, M.A., Dodson, G.S. & Rubin, G.M. (1993). *Cell*, **73**, 169–177.

Skolnik, E.Y., Lee, C.-H., Batzer, A., Vicentini, L.M., Zhou, M., Daly, R., Myers, M.J. Jr., Backer, J.M., Ullrich, A., White, M.F. & Schlessinger, J. (1993). *EMBO J.*, **12**, 1929–1936.

Sun, X.J., Crimmins, D.L., Myers, J.G. Jr., Miralpeix, M. & White, M.F. (1993). *Mol. Cell. Biol.*, **13**, 7418–7428.

Sun, X.J., Miralpeix, M., Myers, J.G. Jr., Glasheen, E.M., Backer, J.M., Kahn, C.R. & White, M.F. (1992). *J. Biol. Chem.*, **267**, 22662–22672.