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Insulin receptor substrate 1 is required for insulin-mediated mitogenic signal transduction

(growth factors/cell cycle progression)

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ABSTRACT **Insulin treatment of mammalian cells immediately stimulates the tyrosine phosphorylation of a cellular protein of 185 kDa referred to as pp185 or IRS-1 (insulin receptor substrate 1). The potential role of the IRS-1 protein in insulin signaling has been examined by microinjecting affinity-purified antibodies into living cells. Stably transfected Rat-1 fibroblasts, which overexpress the human insulin receptor, were microinjected and subsequently stimulated with insulin or other growth factors. Progression through the cell cycle was monitored by using a single-cell assay, which employs bromodeoxyuridine labeling of DNA and analysis with immunofluorescence microscopy. Microinjection of anti-IRS-1 antibody completely inhibited incorporation of bromodeoxyuridine into the nuclei of cells stimulated with insulin or insulin-like growth factor I but did not affect cells stimulated with serum or a variety of purified growth factors. These studies indicate that IRS-1 is a critical component of the insulin and insulin-like growth factor I signaling pathways, which lead to DNA synthesis and cell growth.**

In insulin-responsive mammalian cells, intracellular signal transduction is initiated by hormone binding to the subunit of the insulin receptor, which activates the tyrosine kinase activity of the β subunit (1, 2). One of the earliest detectable events after this activation is tyrosine phosphorylation of pp185, a minor cytoplasmic protein with a wide tissue distribution (3). A cDNA encoding this protein has recently been isolated and is referred to as insulin receptor substrate 1 (IRS-1) (4). The physiological relevance of IRS-1 phosphorylation and the potential role of this protein in insulin signaling is still not well understood. It is known, however, that the protein contains at least 10 potential tyrosine phosphorylation sites, 6 of which are in the Tyr-Met-Xaa-Met (YMXM) motifs, which interact with Src homology 2 (SH2) domains of various signal transducing molecules (reviewed in refs. 5 and 6). Indeed, it has recently been shown that tyrosine phosphorylated IRS-1 binds the 85-kDa regulatory subunit of phosphatidylinositol (PI) 3-kinase both *in vitro* (7) and in living cells (8, 9).

The biological responses following insulin stimulation of cells are pleiotropic and can be generally subdivided into two distinguishable categories: mitogenic responses and metabolic responses (10). The role, if any, of IRS-1 in either of the signaling pathways used by cells to achieve these responses has not been directly demonstrated. It has been suggested that SH2 domain-containing proteins interact with tyrosine-phosphorylated IRS-1 (8), which may serve as a docking protein linking the insulin receptor kinase and downstream targets that regulate cellular growth and metabolism via protein–protein interactions (6) or activation of phosphorylation cascades (11). In the present study, we have examined the role of IRS-1 in insulin-mediated mitogenic signaling in intact mammalian cells and conclude that the growth stimulatory effect of both insulin and insulin-like growth factor I (IGF-I) is dependent on IRS-1.

MATERIALS AND METHODS

Cell Cultures. Rat-1 fibroblasts that had been stably transfected with the human insulin receptor (12) (HIRcB cells) were maintained at 37°C in a humidified incubator under a 5% CO_2/95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium/high glucose supplemented with 10% fetal bovine serum, gentamicin, and 500 nM methotrexate. These cells express $\approx 1.5 \times 10^6$ human insulin receptors per cell, rendering them mitogenically responsive to insulin. The parental cell line was maintained in the same medium without added methotrexate. Cells were plated on sterile glass acid-washed coverslips at subconfluent density. Before microinjection, the cells were rendered quiescent by incubation in serum-free DMEM for 24–36 h.

Microinjection. Antibodies to IRS-1 were raised by immunization of rabbits with a recombinant glutathione *S*-transferase fusion protein (amino acids 1–224 of rat IRS-1). IgG was purified from the resulting antisera by affinity chromatography on protein G-Sepharose resin (Pharmacia). The homogeneous IgG preparation thus obtained was then concentrated to 5 mg/ml using a Centricon concentration unit (Amicon). The buffer was also exchanged during this step to 5 mM sodium phosphate (pH 7.2) containing 50 mM KCl. Control injections of preimmune rabbit IgG (Sigma) were carried out in all cases and were found not to affect the cell proliferation assay described below. Samples were microinjected into the cells at room temperature using an Eppendorf microinjector and micromanipulator (models 5242 and 5171, respectively) mounted on an Axiovert 100 microscope (Zeiss). Cytoplasmic injections were performed at a typical needle pressure of $\approx$100 hPa resulting in the introduction of 10^5–10^6 IgG molecules per cell. Cells were stimulated with mitogens where indicated $\approx$2 h after microinjection.

Staining and Fluorescence Microscopy. To quantitate new DNA synthesis in injected cells, a solution of BrdUrd (Amersham) was added to the culture medium for 20 h after microinjection. After labeling, coverslips were washed in phosphate-buffered saline (PBS), and the cells were then fixed in ethanol/acetic acid/H_2O (90:5:5) for 20 min at room temperature. After washing in PBS, cells were incubated with a monoclonal mouse anti-BrdUrd antibody solution (Amer-

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Abbreviations: IRS-1, insulin receptor substrate 1; IGF-I, insulin-like growth factor I; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PI, phosphatidylinositol.

sham). Staining was visualized using rhodamine-conjugated donkey anti-mouse IgG for DNA synthesis and fluorescein-conjugated donkey anti-rabbit IgG to detect injected cells (Jackson ImmunoResearch). Cells were viewed and the results were analyzed on an Axiophot epifluorescence microscope (Zeiss) equipped with rhodamine and fluorescein fluorescence filters. Photomicrographs were taken on Technical Pan 100 film (phase contrast) or Ektachrome 400 film for fluorescence (Eastman Kodak).

RESULTS AND DISCUSSION

To assess the role of IRS-1 in intracellular signal transduction, antibodies to IRS-1 were injected into quiescent living cells to abrogate the function of the protein, followed by stimulation with insulin, IGF-I, or other growth factors. The IRS-1 antibodies were derived from a polyclonal rabbit antiserum raised against recombinant protein expressed in *Escherichia coli*. The antigen used for preparation of the antiserum was a glutathione *S*-transferase fusion protein

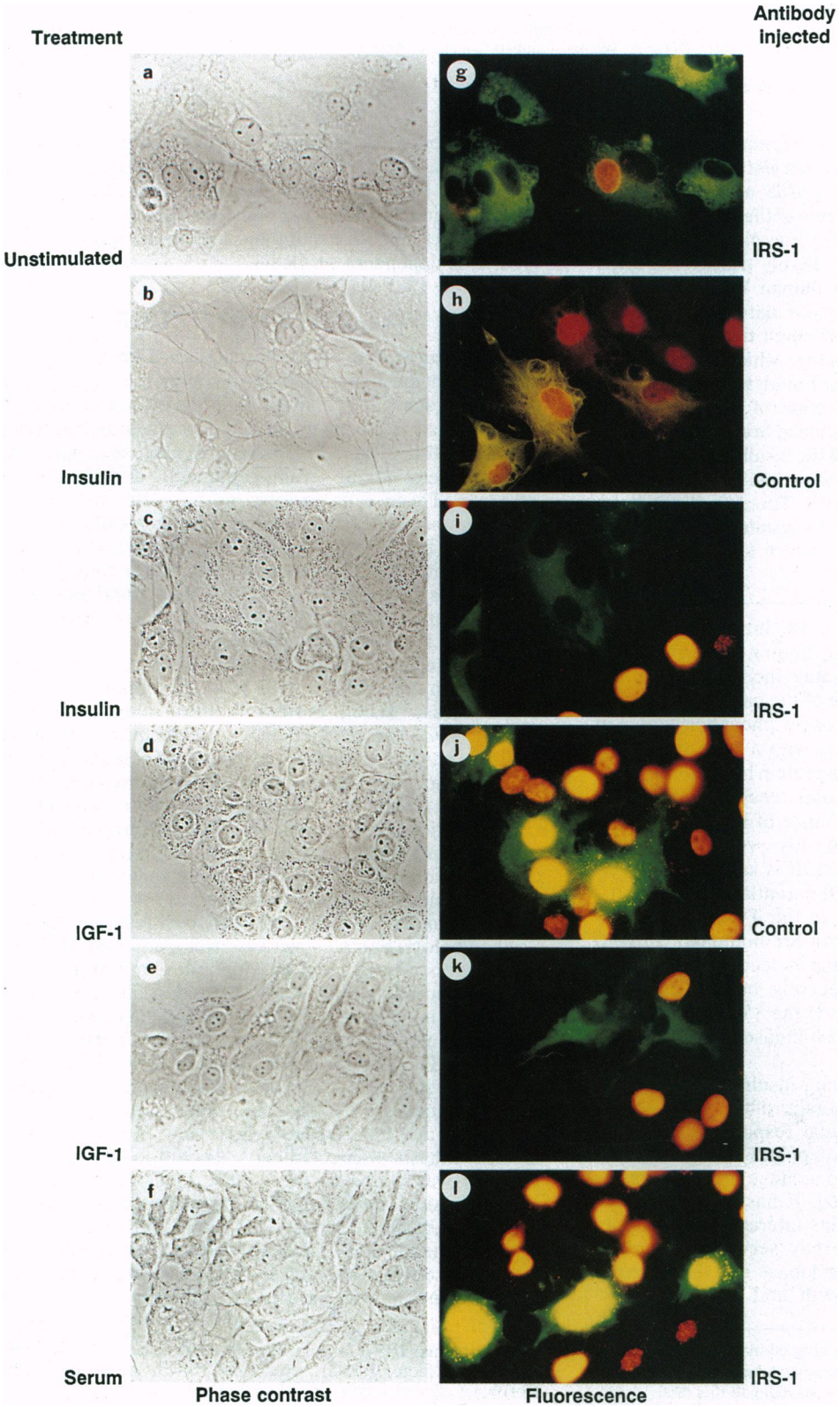

FIG. 1. Microinjection of anti-IRS-1 antibodies. Phase-contrast (*a–f*) and the corresponding double-exposure fluorescence (*g–l*) photomicrographs demonstrate staining for microinjected antibody (fluorescein) and for incorporation of BrdUrd into newly synthesized DNA (rhodamine). Microinjection was carried out with stably transfected Rat-1 cells that overexpress human insulin receptors (12). Cells were grown on glass coverslips and rendered quiescent by serum deprivation of 24–36 h. The cells were then injected with either IRS-1 antibody or preimmune IgG (control), stimulated as indicated, and then labeled with BrdUrd and processed for immunofluorescence microscopy as described (18). Results were analyzed and photography was performed on an Axiophot epifluorescence microscope (Zeiss) equipped with separate fluorescein and rhodamine filters. Injected cells were unambiguously identified by cytoplasmic fluorescein staining and then individually scored positive or negative for BrdUrd incorporation based on nuclear rhodamine staining.

containing amino acid residues 1–224 of rat IRS-1, which had been purified by glutathione resin affinity chromatography (13). After affinity purification of the IgG by protein G chromatography, the specificity and activity of the IRS-1 IgG preparation was established by demonstrating its ability to Western blot and immunoprecipitate pp185/IRS-1 (A.R.S., unpublished data) (data not shown). The IRS-1 IgG preparation was then used for microinjection of mammalian cells.

These studies were accomplished by using Rat-1 fibroblast cells (HIRcB) that had been stably transfected with a cDNA for the human insulin receptor (12). These cells express ≈1.5 × 10^6 insulin receptors per cell, rendering them sensitive to mitogenic stimulation by insulin, as measured by induction of growth response genes (14, 15), DNA synthesis (16), and cell growth. In addition, these cells retain normal responsiveness to the complement of growth factors that stimulate the parental cell line (17). After microinjection with either IRS-1 IgG or control preimmune IgG preparation, the cells were stimulated with insulin or other growth factors. The cells were then labeled with BrdUrd, fixed, immunostained, and analyzed by fluorescence microscopy to unambiguously identify cells that had been injected and/or synthesized DNA (Fig. 1). The IRS-1 IgG preparation was first tested for its potential ability to influence DNA synthesis in quiescent cells after microinjection. No effect was observed, since injected cells incorporated BrdUrd at the same basal level as uninjected cells (Fig. 1*g*). Compared to quiescent cells, stimulation with insulin (100 ng/ml) or IGF-I (100 ng/ml) led to a marked increase in the proportion of cells staining positive for BrdUrd incorporation, and this was unaffected by the control preimmune IgG microinjection (Fig. 1*h*). In contrast, microinjection of IRS-1 IgG completely blocked the ability of these hormones to stimulate DNA synthesis. Interestingly, the growth stimulatory effects of serum were not inhibited by the IRS-1 IgG. Results of several such experiments are summarized in graphic form in Fig. 2. Insulin increased BrdUrd incorporation from a basal level of 19% ± 1% in quiescent cells to 55% ± 5% in cells that were either uninjected or injected with control preimmune rabbit IgG (Fig. 2). Microinjection of IRS-1 IgG before insulin stimulation completely prevented this increase in cell cycle progression. IGF-I increased BrdUrd incorporation to 60% ± 2% of cells and, as was the case with insulin, this stimulatory effect was unchanged by control IgG injection and completely inhibited by microinjection of IRS-1 IgG (Fig. 2). In contrast, microinjection of IRS-1 IgG had no effect on serum-stimulated BrdUrd incorporation. Comparable results were obtained with microinjection of IRS-1 IgG into a rat fibroblast cell line expressing 3 × 10^5 human insulin receptors (n = 5; data not shown). These observations indicate that insulin and IGF-I stimulation of DNA synthesis and cell cycle progression does not occur when the availability/activity of IRS-1 is altered by microinjection of IRS-1 IgG.

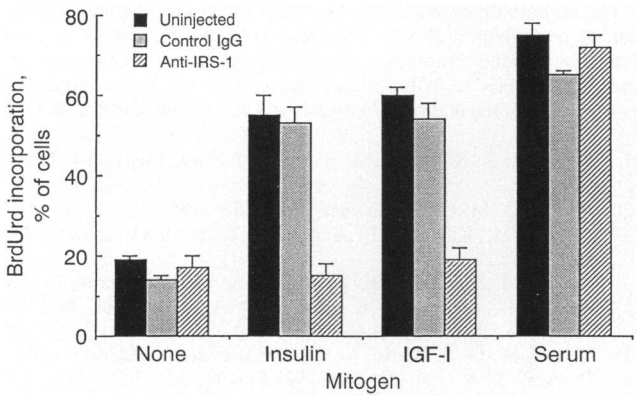

FIG. 2. Inhibition of DNA synthesis in HIRcB cells by IRS-1 antibody. Cumulative data from microinjection experiments in which purified IRS-1 antibodies or preimmune rabbit IgGs were microinjected into quiescent HIRcB cells are shown. Cells were subsequently treated with insulin (100 ng/ml), IGF-I (100 ng/ml), 10% fetal bovine serum, or with no additions. Results presented are the average of three experiments. Microinjection, labeling, staining, and analysis were all carried out as described in Fig. 1.

Because insulin-mediated mitogenic signaling has been shown to require the activity of the $p21^{ras}$ protooncogene product (15, 19), we also sought to establish the relationship between IRS-1 and $p21^{ras}$ by using an approach similar to that described above. If the activity of $p21^{ras}$ lies downstream of IRS-1 in the insulin signaling pathway that leads to DNA synthesis, it should be possible to reconstitute the signal in the presence of IRS-1 antibody by introducing constitutively active ras protein into the cells at the same time. Microinjection of purified oncogenic $p21^{ras}$ protein into quiescent cells has been shown to result in changes in cell morphology (20), induction of c-*fos* protooncogene expression (21), and initiation of DNA synthesis (18, 22). We microinjected oncogenic ras protein purified to homogeneity from a bacterial expression system (23) into quiescent HIRcB cells and found that 66% of the injected cells incorporated BrdUrd into their nuclei (compared to 19% of cells injected with carrier IgG alone). When the ras protein was coinjected with IRS-1 antibody, 59% of the injected cells were BrdUrd positive. Therefore, despite the presence of an IRS-1 blockade introduced by the IRS-1 antibody, oncogenic ras still fully stimulates DNA synthesis. This indicates either that $p21^{ras}$ lies downstream of IRS-1 in the insulin signaling pathway or that IRS-1 and $p21^{ras}$ are components of distinct pathways that converge to cause DNA synthesis.

To explore the specificity of the previously observed inhibitory phenotype associated with microinjection of IRS-1 IgG, we studied the effect of microinjection on the mitogenic activities of several other growth factors. The experiments were conducted in a manner analogous to those described above, except that after microinjection of IRS-1 IgG, cells were treated with epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), or 10% serum. The HIRcB cells responded to these stimulatory agents by increasing BrdUrd uptake from basal levels (≈20%) to 50% ± 3%, 44% ± 4%, and 75% ± 3%, respectively. Microinjection of IRS-1 IgG or preimmune IgG before stimulation with these agents was without effect on BrdUrd incorporation in all cases (Table 1). Thus, the inhibition of insulin and IGF-I signal transduction shown in Figs. 1 and 2 is due to specific perturbation of the signaling pathways downstream of the receptors for these two ligands.

To further establish that the observed inhibitory phenotype was the direct result of antibody interaction with IRS-1 within the cell, the interfering IRS-1 IgG was pretreated with an immobilized IRS-1–glutathione *S*-transferase fusion protein before microinjection. This pretreatment depleted the IRS-1 antibodies from the polyclonal IgG preparation and the affinity-depleted IgG preparation was no longer able to recognize IRS-1 protein on Western blots when compared at the same dilution as untreated antibodies (data not shown). No significant alteration of total IgG concentration occurred as a result of this pretreatment. When the preadsorbed IgG preparation was tested for its ability to inhibit insulin action by microinjection studies, all inhibitory activity had been removed and the microinjected cells incorporated BrdUrd and progressed through the cell cycle to the same extent as uninjected or control injected cells (Table 1). These results establish that the inhibitory effect of the IRS-1 IgG observed in Figs. 1 and 2 is due to the anti-IRS-1 antibodies within the polyclonal IRS-1 IgG preparation.

Our results demonstrate that not only does microinjection of IRS-1 IgG inhibit insulin action, it also inhibits IGF-I

Table 1. Specificity of the inhibitory effect of IRS-1 IgG

Mitogen	Uninjected	IRS-1 antibody injected	Preadsorbed IRS-1 antibody
		HIRcB cells	
None	19 ± 1	17 ± 3	—
Insulin	55 ± 5	15 ± 3	56 ± 5
IGF-I	60 ± 2	19 ± 3	51 ± 2
EGF	50 ± 3	47 ± 1	—
bFGF	44 ± 4	39 ± 4	—
Serum	75 ± 3	72 ± 3	—
		Rat-1 cells	
None	25 ± 1	—	
IGF-I	55 ± 3	24 ± 4	

Quiescent cells were stimulated after microinjection with the indicated hormones or growth factors at the following concentrations: insulin, 100 ng/ml; IGF-I, 100 ng/ml; EGF, 1 μg/ml; bFGF, 1 ng/ml. BrdUrd was simultaneously added to the medium and incubation was carried out for 16 h as described (18). The percentage of cells that stained positive for BrdUrd is presented as the mean of three separate experiments ± SEM in which at least 200 cells were injected per coverslip. Comparable results were obtained in a rat fibroblast cell line expressing 3×10^5 insulin receptors ($n = 5$).

action. However, since the HIRcB cell line overexpresses insulin receptors, leading to the formation of insulin–IGF-I receptor hybrids, it seemed important to determine whether the inhibition of IGF-I action in these cells was due to specific blockade of signals originating from homodimeric IGF-I receptors. To assess this issue, we conducted experiments in untransfected, parental Rat-1 fibroblasts. As previously reported (24), the parental cells express $\approx 10^5$ IGF-I receptors and respond to this growth factor with stimulation of thymidine uptake, BrdUrd incorporation, and cell growth. In the current studies (Table 1), IGF-I treatment increased BrdUrd incorporation from basal values of 26% ± 1% to a stimulated value of 55% ± 3%. Microinjection of IRS-1 IgG, before IGF-I stimulation, reduced the percentage of Rat-1 fibroblasts that responded to IGF-I to basal levels. When these observations are taken together with the data in Figs. 1 and 2, one can conclude that transmembrane signaling of DNA synthesis via the IGF-I receptor requires IRS-1.

These results demonstrate that microinjection of anti-IRS-1 antibodies into living cells abrogates the ability of these cells to subsequently respond to insulin's stimulatory effects on DNA synthesis. Interestingly, the IRS-1 IgG was equally potent at blocking IGF-I action, consistent with earlier findings that stimulation of various cell types with IGF-I leads to tyrosine phosphorylation of IRS-1 (24, 25). This inhibitory effect is specific for the signaling pathways used by insulin and IGF-I receptors, since the stimulatory effects of EGF, FGF, and serum were not inhibited by microinjection of the IRS-1 IgG. As such, these data also indicate that either the signaling pathways for insulin and IGF-I are distinct from those used by EGF and FGF, or that they converge distal to the position of IRS-1 in the insulin/IGF-I signaling cascade. Microinjection studies recently completed with inhibitory antibodies to Raf-1 kinase in an approach identical to that described in this report indicate that the latter possibility is most likely to be correct (D.W.R., unpublished data). These formulations are consistent with the fact that neither EGF nor FGF induces IRS-1 phosphorylation. The inhibition caused by the polyclonal IRS-1 IgG was not due to nonspecific toxic effects of the microinjection process, since microinjection of control IgG was not inhibitory, and IRS-1 IgG did not block EGF or FGF action. Furthermore, preadsorption of the IRS-1 IgG against an IRS-1–glutathione *S*-transferase fusion protein effectively depleted the inhibitory effect of the IgG preparation. This latter result strongly indicates that it is the IRS-1 antibodies within the polyclonal IRS-1 IgG that are responsible for the inhibitory effect. However, the precise mechanism whereby microinjection of an antibody directed against the N terminus of IRS-1 interferes with IRS-1 function remains to be defined.

Presumably, IRS-1 acts as a signaling molecule by binding to SH2-containing proteins through its YMXM motifs and other phosphorylated tyrosine residues. In this way, IRS-1 serves as a docking molecule bringing SH2-containing proteins into proximity with the insulin or IGF-I receptor where they can be phosphorylated (5, 8). This, then, would serve to propagate the insulin signal downstream. Perhaps the most well studied example of this docking protein function relates to the PI 3-kinase. The p85 subunit of PI 3-kinase binds, through its SH2 domain, to specific YMXM motifs of IRS-1 both *in vitro* and *in vivo*, and insulin stimulation leads to tyrosine phosphorylation and activation of PI 3-kinase (5, 8). In recent studies, we have examined the function of this interaction *in vivo* by using the microinjection technique. Thus, we have microinjected a glutathione *S*-transferase fusion protein containing the N-terminal SH2 domain of p85 and found that this markedly inhibits insulin and IGF-I stimulation of gene transcription and BrdUrd uptake (B. Jhun, A.R.S., D.W.R., and J.M.O., unpublished data).

Microinjection of specific antibodies into mammalian cells has been used previously to establish that critical signal transduction molecules, such as the protooncogene products ras (26), fos (27), and jun (28) are necessary for cell growth. Although it is well known that IRS-1 is a direct substrate of both the insulin and IGF-I receptors, a functional role for IRS-1 in biologic signaling has not been directly shown to date. The current studies demonstrating that microinjection of anti-IRS-1 antibodies into living cells blocks insulin and IGF-I action would appear to establish this fact. Thus, we conclude that IRS-1 is a critical coupling molecule connecting activated insulin and IGF-I receptors to downstream signaling pathways. In interpreting these results, it should be cautioned that we have assessed only the mitogenic effects of insulin and IGF-I. Evidence exists indicating that insulin exerts its metabolic and mitogenic actions through divergent signaling pathways, but the point of divergence is unknown. Whether microinjection of IRS-1 IgG would inhibit the metabolic actions of these hormones remains to be determined. Until such information is available, the potential role of IRS-1 in the biologic actions of insulin and IGF-I, apart from mitogenic signaling, is an open question.

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1. Kahn, C. R. & White, M. F. (1988) *J. Clin. Invest.* **82,** 1151–1156.
2. Rosen, O. M. (1987) *Science* **237,** 1452–1458.
3. Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1987) *Science* **215,** 181–187.
4. Sun, X. J., Rothenberg, P. L., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J. & White, M. F. (1991) *Nature (London)* **352,** 73–77.
5. Myers, M. G. & White, M. F. (1993) *Diabetes* **42,** 643–650.
6. Pawson, T. & Gish, D. G. (1992) *Cell* **71,** 359–362.
7. Lavan, B. E., Kuhne, M. R., Garner, C. W., Anderson, D., Reedijk, M., Pawson, T. & Lienhard, G. E. (1992) *J. Biol. Chem.* **267,** 11631–11636.
8. Backer, J. M., Myers, M. G., Jr., Shoelsen, S. E., Chin, D. J., Sun, X.-J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J. & White, M. F. (1992) *EMBO J.* **11,** 3469–3479.
9. Yonezawa, K., Ueda, H., Hara, K., Nishida, K., Ando, A., Chavanieu, A., Matsuba, H., Shii, K., Yokono, K., Fukoi, Y., Calas, B., Grigorescu, F., Dhand, R., Gout, I., Otsu, M.,

Waterfield, M. D. & Kasuga, M. (1992) *J. Biol. Chem.* **267,** 15958–15966.
10. Takata, Y., Webster, N. J. G. & Olefsky, J. M. (1991) *J. Biol. Chem.* **266,** 9135–9139.
11. Ahn, N. G., Seger, R. & Krebs, E. G. (1992) *Curr. Opin. Cell Biol.* **4,** 992–999.
12. McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ullrich, A. & Olefsky, J. M. (1987) *J. Biol. Chem.* **262,** 14663–14671.
13. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67,** 31–40.
14. Stumpo, D. J. & Blackshear, P. J. (1991) *J. Biol. Chem.* **266,** 455–460.
15. Jhun, B. H., Meinkoth, J. L., Leitner, J. W., Draznin, B. & Olefsky, J. M. (1994) *J. Biol. Chem.*, in press.
16. Takata, Y., Webster, N. J. G. & Olefsky, J. M. (1991) *J. Biol. Chem.* **266,** 9135–9139.
17. Thies, R. S., Ullrich, A. & McClain, D. A. (1989) *J. Biol. Chem.* **264,** 12820–12825.
18. Rose, D. W., McCabe, G., Feramisco, J. R. & Adler, M. J. (1992) *J. Cell Biol.* **119,** 1405–1411.
19. Medema, R. H., Wubbolts, R. & Bos, J. L. (1991) *Mol. Cell. Biol.* **11,** 5963–5967.
20. Bar-Sagi, D. & Feramisco, J. R. (1986) *Science* **233,** 1061–1068.
21. Stacey, D. W., Watson, T., Kung, H. F. & Curran, T. (1987) *Mol. Cell. Biol.* **1,** 523–527.
22. Feramisco, J. R., Gross, M., Kamata, T., Rosenberg, M. & Sweet, R. W. (1984) *Cell* **38,** 109–117.
23. Gross, M., Sweet, R. W., Sathe, G., Yokoyama, S., Fasano, O., Goldfarb, M., Wigler, M. & Rosenberg, M. (1985) *Mol. Cell. Biol.* **5,** 1015–1024.
24. McClain, D. A., Maegawa, H., Thies, R. S. & Olefsky, J. M. (1990) *J. Biol. Chem.* **265,** 1678–1682.
25. Myers, M. G., Sun, X. J. K., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M. & White, M. F. (1993) *Endocrinology* **132,** 1421–1430.
26. Feramisco, J. R., Clark, R., Wong, G., Arnheim, N., Milley, R. & McCormick, F. (1985) *Nature (London)* **314,** 639–642.
27. Riabowol, K. T., Vosatka, R. J., Ziff, E. B., Lamb, N. J. C. & Feramisco, J. R. (1988) *Mol. Cell. Biol.* **8,** 1670–1676.
28. Kovary, K. & Bravo, R. (1991) *Mol. Cell. Biol.* **11,** 4466–4472.