Expression of the insulin receptor substrate-1 (IRS1) or Shc cDNA resulted in both increased protein and insulin-stimulated tyrosine phosphorylation of IRS1 and Shc proteins, respectively. Although expression of Shc had no significant effect on insulin-stimulated mitogen-activated protein (MAP) kinase gel shift or c-fos transcriptional activation, expression of IRS1 inhibited these responses. The effect of IRS1 expression on the formation of multisubunit signaling complexes was determined by a series of indirect co-immunoprecipitations. Grb2 immunoprecipitation from IRS1-transfected and insulin-treated cells demonstrated an increased co-immunoprecipitation of Sy6 and the p85 regulatory subunit of the phosphatidylinositol 3-kinase. Similarly, cell extracts immunoprecipitated with a p85 antibody displayed an increased co-immunoprecipitation of Sy6 and Grb2. However, expression of IRS1 increased the extent of Grb2 associated with IRS1 with a concomitant reduction in the amount of Grb2 associated with Shc. Furthermore, increased expression of Shc reduced the amount of Grb2 bound to IRS1 with a concomitant increase in Grb2 associated with Shc. Together, these data demonstrated that IRS1 and Shc compete for a limited cellular pool of Grb2, and insulin activation of MAP kinase and c-fos transcription predominantly occur through the Shc-Grb2 signaling pathway.

Growth factor binding to receptor tyrosine kinases results in autophosphorylation and generation of recognition sites for various Src homology 2 (SH2) domain containing effector proteins (1-4). The association of tyrosine-phosphorylated receptors with these effector molecules form distinct receptor signaling complexes responsible for downstream biological responsiveness. However, unlike most receptor tyrosine kinases, the insulin receptor itself does not significantly associate with these effector molecules, but instead tyrosine-phosphorylates intermediate proteins responsible for the formation of multisubunit signaling complexes (5, 6). The first proximal intracellular target for the kinase-activated insulin receptor has been identified as a 185-kDa protein, termed IRS1 for insulin receptor substrate-1 (7-9).

More recently a second target, composed of two related proteins (46 and 52 kDa), termed Shc for Src homology 2a-collagen-related has been identified (10, 11). These proteins contain insulin receptor-specific tyrosine phosphorylation sites responsible for their association with various downstream effector molecules. In the case of IRS1, these include binding sites for the SH2 domains of the phosphatidylinositol 3-kinase, protein tyrosine-specific phosphatase Sy6, and the small adapter proteins Grb2 and Nck (12-15). In contrast, tyrosine phosphorylation of the Shc proteins has only been shown to directly induce the association with Grb2 (12, 16).

In addition to SH2 domains, many proteins involved in intracellular signaling events also contain Src homology 3 (SH3) domains which are responsible for the binding to various proline-rich motifs (17). For example, the SH3 domains of Grb2 bind to proline-rich sequences found in the microtubule-associated protein dynamin and the guanylnucleotide exchange factor for Ras, termed Son of Sevenless or SOS (18-21). The association of the Grb2-SOS complex with tyrosine-phosphorylated receptors and/or Shc have been directly implicated in the activation of the Ras signaling pathway (22-26). However, the relative insulin signaling properties between Grb2 association with IRS1 or with Shc is highly controversial. Studies examining the interactions of SOS with IRS1 and Shc were unable to detect any significant insulin-stimulated association of SOS with IRS1, although under identical conditions SOS was co-immunoprecipitated with Shc (27). In addition, the majority of Ras guanylnucleotide exchange activity was found in Shc immunoprecipitates with only a relatively small fraction associated with IRS1 immunoprecipitates (28). These data would support a major role for the Shc-Grb2-SOS complex in mediating insulin activation of the Ras pathway. However, several studies have clearly demonstrated the presence of SOS in IRS1 immunoprecipitates (29). Furthermore, expression of insulin receptor mutants that poorly tyrosine-phosphorylate Shc but normally tyrosine-phosphorylate IRS1 also display complete activation of Ras (30). More recently, expression of IRS1 has been shown to either activate or inhibit insulin signaling dependent upon other cell context factors (31, 32). Thus at present, the relative role of IRS1 and Shc in mediating insulin stimulation of downstream signaling is not at all clear and conflicting as well as overlapping evidence exists for both pathways.

To address this issue, we have developed a rapid and highly efficient transient transfection protocol in which both IRS1 and Shc can be routinely expressed in 80-95% of the total cell population. This method has allowed us to examine the relationship between MAP kinase and c-fos transcription, as convenient readouts for insulin action, in comparison with the formation of IRS1 and Shc signaling complexes. The data presented in this manuscript demonstrate that IRS1 and Shc compete for a limiting pool of Grb2 and that the extent of Grb2 bound to Shc correlates with downstream insulin signaling. Thus, although IRS1 and Shc can serve as mediators of insulin biological responsiveness, the specific signaling events ob-

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The abbreviations used are: SH2, Src homology 2 domain; IRS1, insulin receptor substrate-1; SSE, serum response element; Luc, luciferase; RSV, Rous sarcoma virus promoter; CHO, Chinese hamster ovary cells; IR, insulin receptor; PI 3-kinase, phosphatidylinositol 3-kinase; β-gal, β-galactosidase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase.
observed are dependent upon the relative concentrations and stoichiometries of these multisubunit signaling complexes.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The mammalian expression plasmid, CLDN, was purchased from Stratagene and utilizes the cytomegalovirus promoter to drive cDNA expression. The Shc cDNA was kindly provided by Dr. Alan Saltiel (Parke-Davis/Warner-Lambert) and was cloned into the CLDN expression vector. The preparation of CLDN-IRS1 and the reporter gene constructs SRE-Luc and RSV-βGal have been described previously (33, 34).

Cell Culture and Transient Transfection—CHO cells expressing 3 × 10^5 human insulin receptor/cell (CHO/IR) were obtained as described previously (34). These cells were maintained in minimal Eagle’s medium containing nucleotides plus 10% fetal bovine serum. Cells were transiently transfected using the calcium phosphate co-precipitation method with CaCl2 double-doped DNA as described previously (33). Briefly, 16 h prior to use, the cells were plated at 2 × 10^5 cells/100-mm dish and transfected with various plasmid DNAs totaling 23 μg. Twelve hours following transfection, the cells were glycerol shocked and placed into serum-free Ham’s F-12 medium for 12 h.

In order to obtain a high degree of transfection efficiency necessary for immunoprecipitation and Western blotting of whole cell extracts, CHO/IR cells were electroporated with a total of 40 μg of plasmid DNA at 540 volts and 960 microfarads. Under these conditions approximately 70–80% of the electroporated CHO/IR cells were lysed; however, the surviving 20–30% of the cell population were fully viable. Within this cell population, approximately 95% of the cells were functionally transfected as determined by in situ staining for the expression of β-galactosidase activity (see Fig. 2). Thirty-six h following transfection the cells were serum-starved for 8 h and either untreated or incubated for 5 min in the presence of 100 nM insulin prior to the preparation of whole cell lysates.

In situ β-Galactosidase Staining—CHO/IR cells were washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 2.6 mM KH2PO4, pH 7.4) 24 h following transfection and fixed with 1% glutaraldehyde for 15 min at room temperature. The cells were then rinsed and incubated with 0.2% 5-bromo-4-chloro-3-indolyl Pp-galactosidase (X-Gal) solution for 4 h at 30 °C. The resulting immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting using a polyclonal phosphotyrosine antibody (aPY), kindly provided by Dr. Peter A. Wilden, University of Missouri, a MAP kinase monoclonal antibody (Zymed), a Shc monoclonal antibody (Transduction Laboratories), and a carboxyl-terminal IRS1 polyclonal antibody (Upstate Biotechnology, Inc.).

Immunoprecipitations were performed by a 10-fold dilution of the detergent solubilized cell extracts (lysis buffer without Triton X-100) and incubation with 4 μg of a Shc polyclonal antibody (aShc, Upstate Biotechnology, Inc.), Grb2 polyclonal antibody (aGrb2, Santa Cruz), or p85 polyclonal antibody (Upstate Biotechnology, Inc.) antibody for 2 h at 4 °C. Since the carboxyl-terminal IRS1 antibody used for Western blotting was not effective for immunoprecipitation, an IRS1 rabbit polyclonal antibody (aIRS1, Upstate Biotechnology, Inc.) antibody was used (aIRS1, Upstate Biotechnology, Inc.). The samples were then incubated with protein A-Sepharose for 1 h at 4 °C. The resulting immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and Western blotted (enhanced chemiluminescence detection kit, Amersham Corp.) using a Grb2 monoclonal antibody (Transduction Laboratories), IRS1 polyclonal antibody (Transduction Laboratories), and p85 polyclonal antibody (Upstate Biotechnology, Inc.), as indicated in the individual figure legends.

RESULTS

It has previously been observed that expression of IRS1 can lead to either an enhancement or inhibition of insulin signaling, depending upon the amount of IRS1 present as well as other cell context factors (31, 32). For example, expression of IRS1 in parental CHO cells was observed to increase insulin stimulation of DNA synthesis, whereas expression of IRS1 in CHO/IR cells reduced this response (31). To further characterize this phenomenon, CHO/IR cells were co-transfection with an insulin-responsive c-fos reporter gene construct (SRE-Luc) and the RSV-βGal reference gene using the CaPO4 method as described previously (32, 33). In these transiently transfected cells, insulin treatment typically resulted in an approximate 10-fold specific increase in SRE-Luc reporter gene activity (Fig. 1). However, co-transfection with the expression plasmid for IRS1 (CLDN-IRS1) had no effect on basal reporter gene expression, but attenuated the extent of insulin-stimulated SRE-Luc activity to approximately 40% of control cells. In contrast, expression of Shc had no significant effect on either basal or insulin-stimulated SRE-Luc activity.

Typically, to investigate the molecular basis for this IRS1-dependent inhibition of insulin signaling stable cell lines expressing various CDNs would be prepared and subsequently analyzed. In order to develop a rapid transient transfection method suitable for this type of cell biological analysis, we have adapted electroporation conditions to obtain 80–95% transfection efficiencies in CHO/IR cells (Fig. 2). Electroporation of CHO/IR cells at 960 microfarads and 340 V resulted in a loss of viability in approximately 70–80% of the cells (data not shown). The remaining 20–30% of the surviving cells, however, displayed normal growth characteristics. CHO/IR cells mock-transfected with 40 μg of the empty CLDN expression vector did not stain positive for β-galactosidase activity (Fig. 2A). In contrast, cells transfected with 40 μg of a β-galactosidase reporter plasmid (CLDN-βGal) demonstrated positive in situ staining in 80–95% of cells, depending upon the particular experiment (Fig. 2B). As controls, CHO/IR cells were also transfected with the empty CLDN vector (Fig. 2C) and CLDN-βGal (Fig. 2D) using the standard CaPO4 method. Under these conditions, only a small percentage of the cells (~1–3%) stained positive for β-galactosidase expression. These data demonstrated that electroporation under relatively high voltage and capacitance conditions resulted in a highly efficient transient transfection of CHO/IR cells.
Fig. 2. Comparison of transfection efficiency between the CaPO₄ and electroporation methods in CHO/IR cells. CHO/IR cells were either mock-transfected by electroporation with 40 µg of the empty CLDN vector (A) or 40 µg of CLDN-βGal (B) as described under “Experimental Procedures.” In parallel, CHO/IR cells were either mock-transfected by the CaPO₄ method with either 20 µg of the empty CLDN vector (C) or 20 µg of CLDN-βGal (D). Twenty-four hours later, the cells were fixed with paraformaldehyde and stained for β-galactosidase expression by incubation with the 5-bromo-4-chloro-3-indolyl β-D-galactoside reagent for 1 h as described under “Experimental Procedures.” The arrows in D depict two cells within that stained positive for the expression of the LacZ gene.

To assess whether this efficient transient transfection procedure was amenable to the expression of various intracellular effector proteins mediating insulin receptor signaling, we next determined the relative extent of IRS1 and Shc protein expression using this method. CHO/IR cells were electroporated with cDNAs encoding for IRS1 and Shc, and whole cell extracts were then immunoblotted with specific antibodies for IRS1 and Shc (Fig. 3). Mock-transfected CHO/IR cells displayed weak immunoblotting with the IRS1-specific antibody which was not readily visualized at this exposure level (Fig. 3A, lanes 1 and 2). Prolonged exposure of the autoradiogram indicated identical amounts of immunoblotted IRS1 in the basal and insulin-stimulated states (data not shown). However, following electroporation with the IRS1 expression plasmid, there was a large increase in the amount of immunoblotted IRS1 protein (Fig. 3A, lanes 3 and 4). Similar to the control cells, identical amounts of IRS1 protein were observed in both the basal and insulin-stimulated states. In addition, insulin treatment resulted in a somewhat decreased mobility of IRS1, suggesting covalent modification by phosphorylation.

As observed for IRS1, there was also a relatively low level of immunoblotted Shc proteins in control cells which did not significantly change following insulin-stimulation (Fig. 3B, lanes 1 and 2). Again, following electroporation with the Shc expression plasmid (CLDN-Shc), there was a large increase in the amount of immunoblotted Shc proteins of 52 and 46 kDa (Fig. 3B, lanes 3 and 4). Similar to the control (mock-transfected) cells, identical amounts of the Shc proteins were observed in both the basal and insulin-stimulated states.

Having established an increase in protein expression, we next determined whether these proteins would function as accessible substrate for insulin-stimulated tyrosine phosphorylation (Fig. 4). Phosphotyrosine immunoblotting of control (mock-transfected) CHO/IR cells demonstrated an insulin-dependent increase in insulin receptor β subunit and IRS1 tyrosine phosphorylation compared with nonstimulated cells (Fig. 4A, lanes 1 and 2). Expression of IRS1 had no significant effect on the basal state tyrosine phosphorylation of the whole cell extracts (Fig. 4A, lane 3). However, insulin stimulation resulted in an increase in the extent of IRS1 tyrosine phosphorylation without any significant effect on insulin receptor β subunit tyrosine phosphorylation (Fig. 4A, lane 4). Similarly, transfection of CHO/IR cells with the Shc expression plasmid did not affect the basal state tyrosine phosphorylation in whole cell extracts (Fig. 4B, compare lanes 1 and 3). Although the insulin-stimulated tyrosine phosphorylation of Shc was poorly detected in control cells (Fig. 4B, lane 2), following transfection with the Shc expression plasmid there was an increase in the tyrosine phosphorylation of the 52-kDa Shc protein (Fig. 4B, lane 4). As expected, insulin-stimulated tyrosine phosphorylation of IRS1 and the insulin receptor β subunit were also unaffected by the increased expression of Shc.

Since these data demonstrated that transient electroporation of CHO/IR cells resulted in the efficient functional expression of both IRS1 and Shc, we next determined whether the IRS1-dependent inhibition of insulin downstream signaling was recapitulated under these electroporation conditions (Fig. 5). Insulin treatment of CHO/IR cells electroporated with the
IRS1-Grb2 and Shc-Grb2 Signaling Pathways

Fig. 3. Electroporation of CHO/IR cells with expression plasmids for IRS1 and Shc increased the relative amount of protein expression. A, CHO/IR cells were electroporated with 40 µg of the empty CLDN vector (lanes 1 and 2) or 40 µg of CLDN-IRS1 (lanes 3 and 4). Thirty-six hours later, the cells were serum-starved for 6 h and subsequently untreated (lanes 1 and 3) or incubated with 100 nM insulin (lanes 2 and 4) for 5 min prior to detergent solubilization. The whole cell extracts were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting with an IRS1-specific antibody (αIRS1) as described under “Experimental Procedures.” B, CHO/IR cells were electroporated with 40 µg of the empty CLDN vector (lanes 1 and 2) or 40 µg of CLDN-Shc (lanes 3 and 4). The cells were then serum-starved and either left untreated (lanes 1 and 3) or stimulated for 5 min with 100 nM insulin (lanes 2 and 4) followed by Shc Western blotting (αShc) as indicated in A. These were representative Western blots independently performed four times for IRS1 transfected cells and three times for Shc-transfected cells.

Fig. 4. Expression of IRS1 and Shc proteins in CHO/IR cells resulted in increased insulin-stimulated tyrosine phosphorylation of IRS1 and Shc. A, CHO/IR cells were electroporated with 40 µg of the empty CLDN vector (lanes 1 and 2) or 40 µg of CLDN-IRS1 (lanes 3 and 4). Thirty-six hours later, the cells were serum-starved for 6 h and subsequently untreated (lanes 1 and 3) or incubated with 100 nM insulin (lanes 2 and 4) for 5 min prior to detergent solubilization. The whole cell extracts were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting with a phosphotyrosine-specific antibody (αPY) as described under “Experimental Procedures.” B, CHO/IR cells were electroporated with 40 µg of the empty CLDN vector (lanes 1 and 2) or 40 µg of CLDN-Shc (lanes 3 and 4). The cells were then serum-starved and either left untreated (lanes 1 and 3) or stimulated for 5 min with 100 nM insulin (lanes 2 and 4) followed by phosphotyrosine Western blotting as indicated in A. These were representative Western blots independently performed four times for IRS1-transfected cells and three times for Shc-transfected cells.

SRE-Luc reporter plasmid demonstrated an approximate 8-fold specific increase in luciferase activity compared with unstimulated cells. As previously observed for CaP04 transfected cells (Fig. 1), expression of IRS1 inhibited the extent of insulin-stimulated SRE-Luc reporter activity without any effect on basal activity. In addition, expression of Shc again had no significant effect on either basal or insulin-stimulated SRE-Luc activity. These data demonstrate that transfection of these cDNAs by electroporation mirrors the insulin stimulation of SRE-Luc activity determined by the standard CaP04 transfection procedure (Fig. 1). Furthermore, the data presented in Figs. 2–4 demonstrate that the rapid transient transfection obtained by electroporation was essentially equivalent to the preparation of stable cell lines.

Recently, one complete pathway linking the insulin receptor to the transcriptional activation of the c-fos SRE has been identified (35–38). This pathway requires Ras activation as an upstream mediator of the Raf/MEK/MAP kinase pathway, resulting in the phosphorylation and activation of several transcription factors (TCF and SRF) necessary for SRE responsiveness. To determine if the IRS1-mediated inhibition of insulin-stimulated SRE-Luc activity resulted from a blockade of MAP kinase activation, MAP kinase gel shift assays were performed (Fig. 6). The 44- and 42-kDa forms of MAP kinase (ERK1 and ERK2, respectively) have been established to require both tyrosine and threonine phosphorylation for activation (39, 40). These phosphorylation events result in a characteristic decreased mobility of MAP kinase on SDS-polyacrylamide gel electrophoresis (41, 42). As can be seen in Fig. 6A, insulin treatment of control cells induced a nearly complete shift in the mobility of the 42-kDa (ERK2) MAP kinase (Fig. 6A, lanes 1 and 2). In contrast, CHO/IR cells transfected with IRS1 displayed a reduction in the proportion of gel shifted MAP kinase (Fig. 6A, lanes 3 and 4). Consistent with the expression of Shc having no effect on insulin stimulation of SRE-Luc reporter gene activity (Figs. 1 and 4), Shc expression did not alter the ability of insulin to induce the mobility shift of MAP kinase (Fig. 5B, lanes 1–4).

There are several possible mechanisms that could potentially account for inhibition of insulin signaling by IRS1 overexpression, but not Shc. Since tyrosine-phosphorylated IRS1 simultaneously associates with multiple effector proteins, high intracellular levels of tyrosine-phosphorylated IRS1 could effectively dilute out these proteins such that only a single SH2 domain containing target protein was bound to a given IRS1 molecule. To examine this possibility, we performed a series of co-immunoprecipitations between Grb2, Syp, and the p85 regulatory subunit of the phosphatidylinositol (P1)-3-kinase which have been established to form a multisubunit signaling complex with IRS1 (43). As expected, immunoprecipitation of Grb2 from control (mock-transfected) cells demonstrated an insulin-dependent co-immunoprecipitation of Syp (Fig. 7A, lanes 1 and 2) and the p85 regulatory subunit of the PI 3-kinase (Fig. 7B, lanes 1–4).
A. Experimental Procedures.

stimulated mobility shift of were serum-starved for 6 h and subsequently untreated MAP or incubated with 100 nM insulin electroporated with 40 pg of the empty CLDN vector untreated two times each for the IRSl and the Shc-transfected cells.

lanes 1 and 2). Expression of IRS1 resulted in a substantial increase in the amount of Syp (Fig. 7A, lanes 3 and 4) and p85 (Fig. 7B, lanes 3 and 4) that was co-immunoprecipitated following insulin treatment.

In a complementary study, we next co-immunoprecipitated Grb2 and Syp using an antibody directed against the p85 regulatory subunit of the PI 3-kinase (Fig. 8). Immunoprecipitation with the p85 antibody from control cells treated with insulin demonstrated the co-immunoprecipitation of Syp (Fig. 8A, lanes 1 and 2) and Grb2 (Fig. 8B, lanes 1 and 2). Similar to Fig. 7, expression of IRS1 increased the amount of co-immunoprecipitated Syp (Fig. 8A, lanes 3 and 4) and Grb2 (Fig. 8B, lanes 3 and 4) in the p85 immunoprecipitate from insulin-stimulated cells. Taken together, these data demonstrate that a sufficient accessible cellular pool of Syp, Grb2, and p85 effector proteins were present to form a multisubunit complex even in the presence of increased IRS1 expression. Thus, high levels of IRS1 did not inhibit insulin-dependent signaling by preventing the formation of a multisubunit IRS1 signaling complex.

In addition to IRS1, Grb2 has also been shown to directly associates with tyrosine-phosphorylated Shc (12, 16). Previous studies have also suggested that Grb2 may be rate-limiting for the activation of the Ras pathway (27, 32, 44). Thus, increased IRS1 expression could potentially compete for Grb2 binding to Shc. To further test this hypothesis, we next determined the amount of Grb2 associated with IRS1 and Shc (Fig. 9). In the absence of insulin there was no specific Grb2 protein detected in the IRS1 immunoprecipitate (Fig. 9A, lanes 1 and 3), whereas following insulin stimulation, immunoprecipitation of IRS1 resulted in the specific association with Grb2 (Fig. 9A, lane 2). As expected, increased expression of IRS1 resulted in an increase in the amount of Grb2 bound to IRS1 following insulin stimulation (Fig. 9A, compare lanes 2 and 4). Similarly, Shc immunoprecipitation demonstrated the presence of co-immunoprecipitated Grb2 from insulin-stimulated cells (Fig. 9B, lanes 1 and 2). In contrast, increased expression of IRS1 reduced the extent of insulin-stimulated Grb2 associated with Shc (Fig. 9B, lanes 3 and 4). Quantitation of these data indicated that increased expressed of IRS1 reduced the amount of Grb2 associated with Shc by 67 ± 5% relative to the untransfected control cells. Thus, these data demonstrate that high levels of IRS1 expression compete with Shc for a limited pool of Grb2 molecules.

This being the case, then increased expression of Shc should also reduce the amount of Grb2 associated with IRS1. To confirm this prediction, immunoprecipitation of IRS1 from control cells demonstrated the co-immunoprecipitation of Grb2 in the insulin-stimulated state (Fig. 10A, lanes 1 and 2), whereas increased expression of Shc reduced the amount of Grb2 co-immunoprecipitated with IRS1 (Fig. 10A, lanes 3 and 4). Similarly, Shc immunoprecipitation demonstrated the insulin-dependent association with Grb2 (Fig. 10B, lanes 1 and 2). As expected, increased expression of Shc resulted in an increase in the extent of insulin-stimulated Grb2 bound to Shc (Fig. 10B, lanes 3 and 4). Thus, these data directly demonstrate that the formation of both IRS1-Grb2 and Shc-Grb2 signaling complexes were in direct competition with each other.
**DISCUSSION**

Expression of various wild type and mutant cDNAs in tissue culture cells has provided a very powerful tool to dissect the signaling pathways linking receptor tyrosine kinases to downstream biological responses. In the vast majority of cases, this has required the preparation and isolation of stable cell lines expressing cDNAs of interest. In addition to the time required to generate these stable cell lines, there are several concerns over clonal selection and compensatory cell context changes that may occur during isolation. To circumvent these difficulties, we examined the transfection efficiency of electroporation under high voltage and capacitance conditions in CHO/IR cells. Electroporation with an expression vector for the Luc2 gene resulted in the expression of β-galactosidase activity in no less than 80%, and in most cases greater than 95%, of the viable cell population. This method was applicable to several other proteins as electroporation of expression plasmids for IRS1 and Shc also markedly increased their protein levels.

It is also important to recognize that following this procedure only approximately 25% of the total cell population remained viable. However, this surviving cell population displayed the identical characteristics of the total initial cell population, in terms of c-fos transcriptional activation, cell growth, and IRS1- but not Shc-dependent inhibition of insulin signaling. This suggests that there was no selection pressure induced by the electroporation procedure itself or via the introduction of these expression plasmids which would result in a nonrepresentative cell population. Thus, these data presented in this manuscript demonstrated that electroporation can be used as an effective procedure to generate a large population of cells expressing a particular gene of interest similar to the preparation of stable cell lines.

It is interesting to note that electroporation with the expression plasmid for Shc resulted in high levels of both the 52- and 46-kDa isoforms of Shc. The presence of these two protein products from this cDNA was consistent with the alternative translation start site usage from a single Shc transcript (10). In addition, previous studies have demonstrated that platelet-derived growth factor and epidermal growth factor stimulation resulted in the tyrosine phosphorylation of both Shc species, presumably at the carboxy-terminal Tyr317 residue (12). However, we have observed that only the 52-kDa Shc protein was tyrosine-phosphorylated in response to insulin in both CHO/IR and CHO/IR cells transfected with the expression plasmid for Shc. Since the amino-terminal 58 amino acids that were deleted in the 46-kDa Shc protein do not contain any tyrosine residues, this differential insulin-dependent tyrosine phosphorylation most likely reflects a regulatory role for this domain in mediating substrate specificity. Alternatively, this domain may be responsible for differential targeting of the 52- and 46-kDa isoforms to distinct subcellular locations. The possibility that this distinctive pattern of Shc tyrosine phosphorylation may underlie tyrosine kinase receptor signaling specificity will require further investigation.

In any case, having demonstrated the utility of electroporation to express these proteins, we wished to examine the relative signaling role of IRS1 and Shc in mediating biological actions of insulin. It has been well documented that increased expression of IRS1 in several cell types increased insulin sensitivity and responsiveness of DNA synthesis (31, 45, 46). In addition, expression of antisense IRS1 RNA or microinjection of IRS1 antibodies also inhibited insulin-stimulated DNA synthesis and growth (47, 48). Surprisingly, however, increased expression of IRS1 in a cell line expressing very high levels of the insulin receptor (CHO/IR) resulted in an attenuation of insulin-stimulated DNA synthesis as well as in c-fos transcription (31, 32). In the present study, we have confirmed that high levels of IRS1 expression inhibited insulin stimulation of SRE-Luc activity which correlated with a blockade of MAP kinase gel shift and, thus, kinase activation.

There are several general mechanisms that can be envisioned to account for this apparent paradoxical effect of IRS1 expression on insulin signaling. First, since IRS1 functions as a multisite docking protein for several SH2 domain containing effector proteins, an over abundance of tyrosine-phosphorylated IRS1 could titrate out these proteins. Under such a cir-
currence, only a single SH2 domain target protein would be bound to any given IRS1 molecule, thereby preventing the formation of a multisubunit signaling complex. This possibility was examined by co-immunoprecipitation of the p85 regulatory subunit of the PI 3-kinase, Syk, and Grb2, all of which have been previously demonstrated to indirectly associate via binding to tyrosine-phosphorylated IRS1 (5, 6). However, the data presented in this manuscript demonstrated that, under identical conditions which inhibited the insulin stimulation of MAP kinase phosphorylation and SRE-Luc expression, there was sufficient amounts of p85, Syk, and Grb2 to generate a multisubunit complex with IRS1 as the core molecule.

Having excluded this possible mechanism, it was also possible that increased expression of IRS1 resulted in a sequestration of protein components away from other necessary signaling pathways. In this regard, both tyrosine-phosphorylated IRS1 and Shc bind to the SH2 domain of the small adapter protein Grb2 (12, 16). Grb2 has also been shown to directly associate with the Ras guanyl-nucleotide exchange factor SOS, thereby linking Ras activation via the stimulation and/or appropriate targeting of SOS (20–26). Consequently, the SOS-mediated increase in GTP-bound Ras provides a physical interaction with Raf initiating downstream activation of the Raf/MEK/MAP kinase cascade (49–56). Thus, the insulin stimulation of MAP kinase and SRE-Luc transcriptional activation reflect insulin signaling via combinations of either Shc-Grb2-SOS and/or IRS1-Grb2-SOS interactions.

Since the involvement of Grb2 has been established in the MAP kinase pathway leading to SRE-Luc activation, we reasoned that relatively high levels of IRS1 expression could have recruited or sequestered Grb2 away from Shc. Alternatively, it was possible that increased IRS1 expression could have engaged and/or activated an inhibitory protein to the Grb2-SOS signaling pathway. However, the data presented in this manuscript demonstrate that the amount of Grb2 associated with Shc was decreased in IRS1 over expressing cells, whereas the amount of Grb2 bound to IRS1 was increased. In addition, increased expression of Shc resulted in a loss of IRS1 bound Grb2 with a concomitant increase in Shc associated Grb2. These data demonstrate that Shc and IRS1 compete for a limited pool of Grb2. Furthermore, since increased expression of IRS1 inhibited insulin responsiveness, where Shc had no effect, strongly suggests that the Shc-Grb2 pathway was predominant over the IRS1-Grb2 pathway in mediating insulin signaling for MAP kinase and c-fos transcriptional activation.

In summary, the data present in this manuscript demonstrate the use of electroporation as a highly efficient method to introduce and functionally express various proteins involved in insulin receptor tyrosine kinase signaling. This rapid method for protein expression has several advantages over the preparation of stable cell lines most notably the absence of clonal selection which may introduce significant changes in cell properties. Utilizing this method, we have determined the basis for IRS1-mediated inhibition of insulin downstream signaling which only occurs in cell expressing high levels of both the insulin receptor and IRS1. The inhibition of insulin signaling under these conditions resulted from a sequestration of a limited pool of Grb2 away from Shc due to the high level of tyrosine-phosphorylated IRS1 relative to Shc. Furthermore, these data demonstrate that the formation of the Shc-Grb2 complex rather than an IRS1-Grb2 complex was the major pathway for insulin-stimulated MAP kinase and c-fos transcriptional activation.

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