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Enhancement or Inhibition of Insulin Signaling by Insulin Receptor Substrate 1 Is Cell Context Dependent

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Insulin treatment of Chinese hamster ovary (CHO) cells expressing high levels of the insulin receptor (CHO/IR cells) activates both c-*fos* serum response element and activator protein 1 (AP-1) reporter genes approximately 10-fold. In contrast, parental CHO cells display only two- to threefold insulin stimulation of reporter gene activity. Transient transfection of parental CHO cells with an insulin receptor substrate 1 (IRS1) expression plasmid enhanced insulin downstream signaling in a biphasic manner, whereas IRS1 transfection of CHO/IR cells inhibited insulin signaling in a dose-dependent fashion. Further, expression of Grb2 in parental CHO cells had no effect on insulin signaling, whereas Grb2 increased insulin activation of reporter gene expression in CHO/IR cells. These data suggest that the expression levels of various effector molecules can either enhance or inhibit insulin downstream signaling events. To assess the relative effects of various insulin receptor, IRS1, and Grb2 levels on insulin signaling, parental CHO cells were transiently transfected with various combinations of expression plasmids encoding these proteins. Although expression of IRS1 resulted in a biphasic increase of insulin signaling in parental CHO cells, coexpression of IRS1 with the insulin receptor resulted in inhibition of signaling. This inhibition of insulin signaling directly correlated with an increased association of Grb2 with IRS1 and a concomitant sequestration of Grb2 away from Shc. Consistent with the Shc-Grb2 pathway as the major route for insulin-stimulated c-Fos and AP-1 transcriptional activation, the IRS1-mediated inhibition was reversed by transfection with an expression plasmid for Grb2. These data demonstrate that the extent of insulin-stimulated downstream signaling was dependent not only on the levels of individual signaling molecules but also on the formation of multiprotein complexes with specific stoichiometries.

The two major proximal intracellular targets for the kinase activity of the insulin and insulin-like growth factor 1 (IGF-1) receptors have been identified as a 185 kDa-protein, termed IRS1 (for insulin receptor substrate 1) (13, 36, 48), and proteins of 46 and 52 kDa, termed Shc (for Src homology 2/α-collagen related) (26, 32). These molecules contain insulin receptor-specific tyrosine phosphorylation sites responsible for their association with various downstream effector molecules (23, 47). For example, the tyrosine phosphorylation motifs in IRS1 provide recognition sites for the specific binding of SH2 (Src homology 2) domain-containing proteins, including the p85 subunit of the phosphatidylinositol 3-kinase, the small adaptor protein Grb2, and the SH2 domain-containing protein tyrosine-specific phosphatase SHPTP2 (34).

The central role of IRS1 as a downstream mediator of insulin action has been demonstrated by several laboratories. Unprimed *Xenopus* oocytes which are not responsive to insulin or IGF-1 become highly sensitive following injection of IRS1 (7, 8). Similarly, a myeloid cell line found to be deficient in an apparently related form of IRS1 (4PS) was unresponsive to insulin and interleukin 4 (44, 45). However, transfection with a gene encoding IRS1 restored both insulin- and interleukin 4-mediated mitogenesis (45). In addition, inhibition of IRS1 expression by use of antisense RNA and by microinjection of IRS1 antibodies also demonstrated an important role of IRS1 in insulin-stimulated DNA synthesis (28, 46).

Taken together, these observations suggest that IRS1 functions as a central protein in a multisubunit signaling complex necessary for insulin-mediated mitogenic responsiveness. As a consequence, this model predicts that the specific ratio of IRS1 associated with downstream effector molecules is critical for the physiological signaling through this complex. That is, high levels of IRS1 expression could result in deficient signaling due to sequestration of downstream targets such that each IRS1 associates with only one signaling effector molecule rather than forming a multisubunit complex. Alternatively, if additional signaling pathways are also required, then increased expression of IRS1 may compete for a limited pool of effector proteins. To test these models, we have examined the effect of insulin downstream signaling in Chinese hamster ovary (CHO) cells containing low levels of the insulin receptor, IRS1, and Grb2 in comparison with CHO cells transiently expressing high levels of these effector proteins in various combinations. The data presented in this study demonstrate that IRS1 can function as either an activator or an inhibitor of insulin-mediated signaling, depending on the expression levels of other target proteins.

MATERIALS AND METHODS

Plasmid constructs. The mammalian expression plasmid CLDN was obtained from Smith-Kline Beecham and utilizes the cytomegalovirus promoter to drive cDNA expression. The human insulin receptor containing exon 11 and the rat IRS1 cDNAs were cloned into CLDN, using convenient restrictions sites. Human Grb2 was obtained by PCR cloning from human brain poly(A)$^+$ RNA, and the resultant cDNA was verified by DNA sequence analysis prior to cloning into CLDN. The activator protein 1 (AP-1)–chloramphenicol acetyltransferase (CAT) reporter gene construct containing five copies of the collagen 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element cloned upstream of the thymidine kinase

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promoter was kindly provided by Michael Karin (University of California, San Diego). Preparation of the reporter gene constructs SRE-Luc and RSV-βGal has been previously described (49).

Cell culture and $CaPO_4$ transient transfection. Parental CHO cells expressing ~5 × 10^3 insulin receptors per cell and stably transfected CHO cells expressing ~3 × 10^6 human insulin receptors per cell (CHO/IR cells) were obtained as previously described (12). These cells were maintained in minimal Eagle's medium containing nucleotides plus 10% fetal bovine serum. Cells were transiently transfected by the calcium phosphate coprecipitation method with CsCl double-banded DNA as previously described (49). Briefly, 16 h prior to use, the cells were plated at 2 × 10^6 cells per 100-mm-diameter dish and transfected with various plasmid DNAs totaling 23 μg as indicated in the figure legends. Twelve hours following transfection, the cells were glycerol shocked and placed into serum-free Ham's F12 medium for 12 h and treated with and without 100 nM insulin. Whole cell extracts were prepared 5 to 6 h later for determination of luciferase, CAT, and β-galactosidase activities. All transfections were performed at least two times, in triplicate, using at least two different preparations of plasmid DNA. To correct for differences in transfection efficiencies between plates within an experiment, the luciferase and CAT activities in each extract were normalized to β-galactosidase activity. Statistical analysis was performed by using Student's *t* test, with $P < 0.05$ considered statistically significant.

Transient transfection by electroporation. To obtain a high degree of transfection efficiency necessary for immunoprecipitation and Western blotting (immunoblotting) of whole cell extracts, parental CHO and CHO/IR cells were electroporated with a total of 40 μg of plasmid DNA at 340 V and 960 μF. Under these conditions, approximately 25% of the cells remained viable, and approximately 80 to 90% of the surviving cells were transfected as determined by parallel transfection with CMV-βGal (data not shown). Thirty-six hours following transfection, the cells were serum starved for 6 h and either untreated or incubated for 5 min in the presence of 100 nM insulin.

Western blotting and immunoprecipitation. Whole cell extracts were prepared by detergent solubilization in a lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 1% Triton X-100, 3 mM $MgCl_2$, 2 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μg of aprotinin per ml, 1.5 μM pepstatin) for 1 h at 4°C. The resultant cell extracts were subjected to Western blotting with a phosphotyrosine antibody, kindly provided by Peter A. Wilden, University of Missouri. Shc and IRS1 immunoprecipitations were performed by a 10-fold dilution of the detergent-solubilized cell extracts and incubation with 4 μg of a Shc rabbit polyclonal antibody (Upstate Biotechnology, Inc.) or IRS1 rabbit polyclonal antibody (Upstate Biotechnology, Inc.) for 2 h at 4°C. The samples were then incubated with protein A-Sepharose for 1 h at 4°C. The resulting immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with a Grb2 monoclonal antibody (Transduction Laboratories).

RESULTS

It has been previously established that the c-*fos* serum response element and the TPA response element (AP-1 binding site) of the collagen promoter are highly sensitive to transcriptional activation by insulin (33, 41, 42, 49). We therefore used both SRE-Luc and AP-1–CAT reporter genes as a convenient downstream assay for insulin signaling. Transient transfection of parental CHO cells with the SRE-Luc reporter construct demonstrated an approximate threefold insulin stimulation of luciferase activity compared with untreated cells (Fig. 1A). Cotransfection of the reporter plasmid with increasing amounts of an expression plasmid for IRS1 resulted in a biphasic insulin stimulation of luciferase activity. Maximal insulin stimulation (~7-fold) occurred when the cells were cotransfected with 2 μg of IRS1. As the concentration of IRS1 plasmid increased, the extent of insulin-stimulated luciferase activity decreased and by 18 μg approached the levels observed in the untransfected CHO cells. This effect was not due to squelching, as the total amount of plasmid DNA used in all the transfections was maintained at a constant level. In contrast to parental CHO cells, insulin stimulated the SRE-Luc reporter gene approximately ninefold in CHO cells stably expressing high levels of the insulin receptor (Fig. 1B). Under these conditions, cotransfection of the luciferase reporter gene with various amounts of the IRS1 expression plasmid resulted in a continual decline in insulin-stimulated luciferase activity. At the highest amount of IRS1 expression plasmid tested, the magnitude of insulin-stimulated SRE-Luc reporter activity was also similar to that observed in the parental CHO cells.

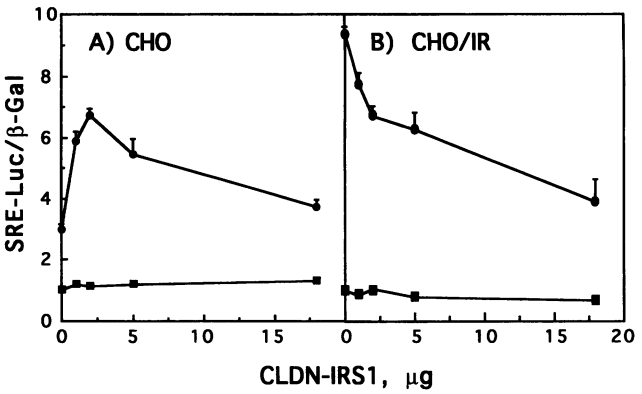

FIG. 1. Expression of IRS1 can enhance or inhibit SRE-Luc reporter gene activity. Parental CHO (A) and CHO/IR (B) cells were transfected by the $CaPO_4$ method with 1 μg of SRE-Luc, 2 μg of AP-1–CAT, 2 μg of RSV-βGal, and various amounts of the IRS1 expression plasmid CLDN-IRS1 as described in Materials and Methods. The total amount of DNA used in the transfections was maintained at 23 μg by adjustment with the empty CLDN vector. Twelve hours following transfection, the cells were serum starved for 12 h and either untreated (■) or incubated with 100 nM insulin for 6 h (●). Cell extracts were prepared and assayed for luciferase and β-galactosidase activities. The data are presented as the amount of SRE-Luc activity divided by the amount of β-galactosidase (β-Gal) activity present in the extract. The data presented are averages and standard errors of the means from four independent experiments, each performed in triplicate.

To determine if this cell context difference in IRS1-mediated insulin signaling was unique to the SRE-Luc reporter gene system, we also examined the effect of IRS1 on expression from the AP-1–CAT reporter (Fig. 2). As observed in Fig. 1 for the SRE-Luc reporter gene, insulin stimulated AP-1–CAT reporter gene activity approximately twofold in parental CHO cells, and this level increased to approximately fivefold when cells were cotransfected with 2 μg of the IRS1 expression plasmid (Fig. 2A). Cotransfection with higher concentrations of the IRS1 expression plasmid resulted in a decrease in insulin-stimulated CAT reporter activity without any signifi-

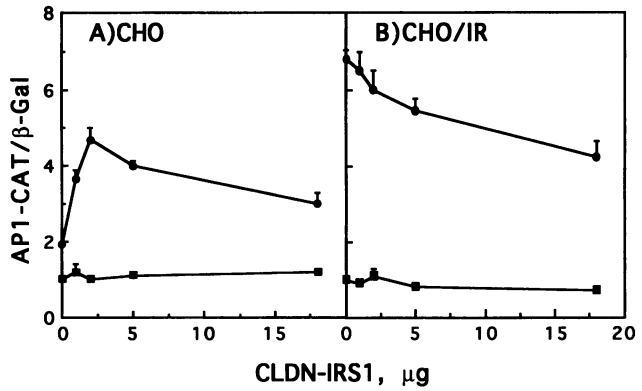

FIG. 2. Effect of IRS1 expression on AP-1–CAT reporter gene activity in CHO and CHO/IR cells. Parental CHO (A) and CHO/IR (B) cells were transfected with the reporter gene constructs as described for Fig. 1. Cell extracts were assayed for CAT and β-galactosidase activities in extracts prepared from either untreated (■) or 100 nM insulin-treated (●) cells. The data are presented as the amount of AP-1–CAT activity divided by the amount of β-galactosidase (β-Gal) activity present in the extract. The data presented are averages and standard errors of the means from three independent experiments, each performed in triplicate.

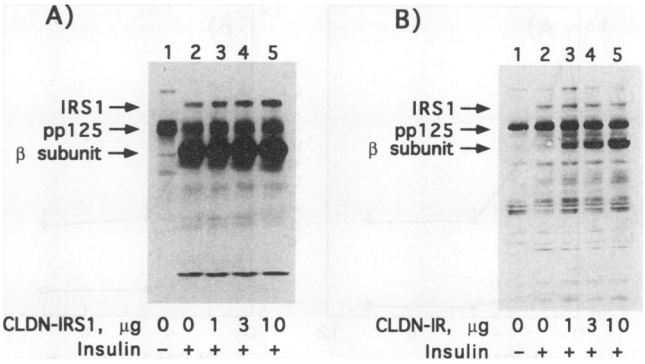

FIG. 3. Transfection of parental CHO and CHO/IR cells with expression plasmids for the insulin receptor or IRS1 resulted in increased tyrosine phosphorylation. (A) CHO/IR cells were either mock transfected (lanes 1 and 2) or transfected with increasing amounts of the IRS1 expression plasmid CLDN-IRS1 (lanes 3 to 5) by the $CaPO_4$ method as described in Materials and Methods. (B) Parental CHO cells were either mock transfected (lanes 1 and 2) or transfected with increasing amounts of the insulin receptor expression plasmid CLDN-IR (lanes 3 to 5). The cells were either left untreated (lane 1) or stimulated with 100 nM insulin for 5 min (lanes 2 to 5) prior to the preparation of detergent-soluble total cell extracts. The samples were then subjected to Western blotting with a phosphotyrosine antibody. The blots shown are representative phosphotyrosine blots independently performed two times for insulin receptor expression and three times for IRS1 expression.

cant effect on basal activity. In cells expressing high levels of the insulin receptor (CHO/IR cells), insulin increased AP-1–CAT activity approximately sevenfold (Fig. 2B), which progressively decreased in an IRS1 plasmid concentration-dependent manner.

These data demonstrate that the ability of insulin to increase reporter activity decreased in direct proportion to the amount of transfected IRS1 expression plasmid. To confirm that this effect was associated with increased levels of tyrosine-phosphorylated IRS1, CHO/IR cells were transiently transfected with various amounts of the IRS1 expression plasmid and subjected to phosphotyrosine Western blotting (Fig. 3A). In the absence of insulin, there was essentially no detectable tyrosine phosphorylation of IRS1 or the insulin receptor β subunit (Fig. 3A, lane 1). In contrast, insulin stimulation resulted in a marked increase in both IRS1 and insulin receptor β-subunit tyrosine phosphorylation (Fig. 3A, lane 2). Further, transfection with increasing amounts of the IRS1 expression plasmid resulted in a dose-dependent increase in the extent of insulin-stimulated IRS1 tyrosine phosphorylation without any significant effect on the insulin receptor β subunit (Fig. 3A, lanes 3 to 5). Under these conditions, the efficiency of $CaPO_4$ transfection remained constant (1.8 to 2.2%), as determined by parallel transfections with CMV-βGal (data not shown). Since only a small proportion of the cells were transfected under these conditions, the modest increase in IRS1 phosphorylation in the total cell population reflected substantial amounts of functionally expressed IRS1 protein in the fraction of cells that were transfected.

Thus, the relative IRS1-dependent changes in insulin-stimulated reporter gene activity most likely reflected differences in cell context rather than differences in the amount of functional IRS1 expression. This change could have arisen either from clonal selection of the CHO/IR cells versus the parental CHO cells or specifically as a result of the increased expression of insulin receptor. To distinguish between these two possibilities, parental CHO cells were transfected with various amounts of an insulin receptor expression plasmid, and then SRE-Luc (Fig. 4A) and AP-1–CAT (Fig. 4B) reporter gene activities were determined. In these experiments, levels of insulin stimulation of SRE-Luc and AP-1–CAT reporter activities were approximately three- and twofold, respectively, in parental CHO cells. Cotransfection with increasing amounts of the insulin receptor expression plasmid resulted in a dose-dependent increase in reporter gene activity to levels significantly greater than that observed in the stably transfected CHO/IR

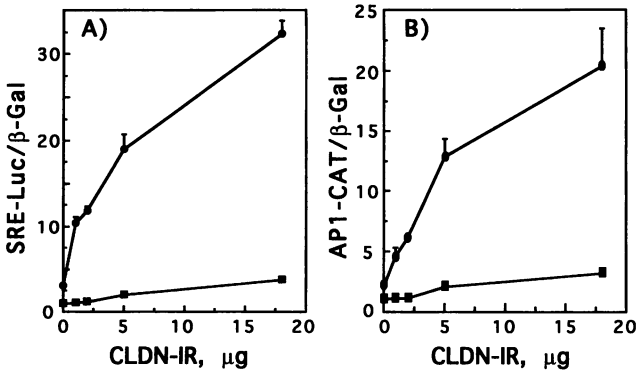

FIG. 4. Expression of the insulin receptor in parental CHO cells augments insulin stimulation of SRE-Luc and AP-1–CAT reporter gene activities. Parental CHO were transfected by the $CaPO_4$ method with 1 μg of SRE-Luc, 2 μg of AP-1–CAT, 2 μg of RSV-βGal, and various amounts of the insulin receptor expression plasmid CLDN-IR as described for Fig. 1. Twelve hours following transfection, the cells were serum starved for 12 h and either untreated (■) or incubated with 100 nM insulin for 6 h (●). Cell extracts were prepared and assayed for luciferase and CAT activities. The data are presented as the amount of SRE-Luc (A) or AP-1–CAT (B) activity divided by the amount of β-galactosidase (β-Gal) activity present in the extract. The data presented are averages and standard errors of the means from three independent experiments, each performed in triplicate.

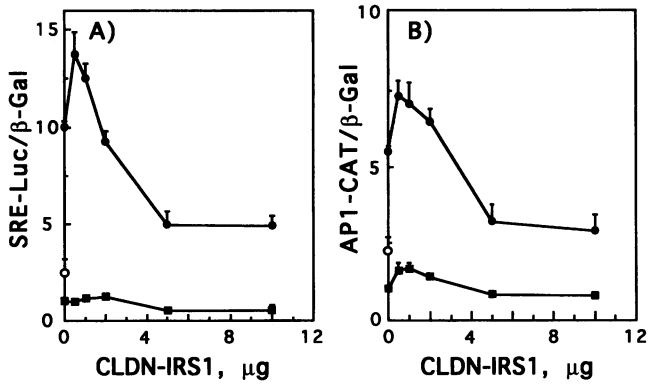

FIG. 5. IRS1 inhibition of reporter gene activities in CHO cells transfected with the insulin receptor. Parental CHO were transfected with 1 μg of SRE-Luc, 2 μg of AP-1–CAT, 2 μg of RSV-βGal, 1 μg of CLDN-IR and various amounts of the IRS1 expression plasmid CLDN-IRS1 as described for Fig. 1. Twelve hours following transfection, the cells were serum starved for 12 h and either untreated (■) or incubated with 100 nM insulin for 6 h (●). Cell extracts were prepared and assayed for luciferase and CAT activities. The data are presented as the amount of SRE-Luc (A) or AP-1–CAT (B) activity divided by the amount of β-galactosidase (β-Gal) activity present in the extract. In parallel, parental CHO cells were transfected as described above in the absence of CLDN-IR, and the amount of insulin-stimulated reporter gene activity was determined (○). The data presented are averages and standard errors of the means from three independent experiments, each performed in triplicate.

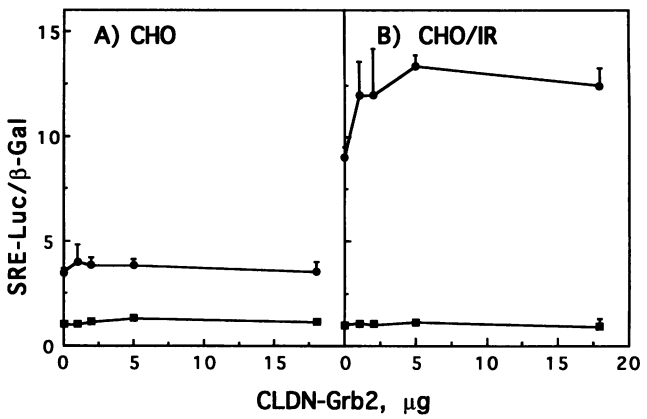

FIG. 6. Expression of Grb2 specifically enhances insulin stimulation of SRE-Luc reporter gene activity in CHO/IR cells. Parental CHO (A) and CHO/IR (B) cells were transfected with the reporter gene constructs and various amounts of the Grb2 expression plasmid CLDN-Grb2 as described for Fig. 1. Cell extracts were prepared from either untreated cells (■) or cells incubated with 100 nM insulin for 6 h (●). The cell extracts were assayed for luciferase and β-galactosidase activities. The data are presented as the amount of SRE-Luc activity divided by the amount of β-galactosidase (β-Gal) activity present in the extract. The data presented are averages and standard errors of the means from four independent experiments, each performed in triplicate.

cell line (compare Fig. 1B with Fig. 4A and Fig. 2B with Fig. 4B). It should be noted that expression of the insulin receptor had a relatively small effect on SRE-Luc and AP-1–CAT activities in the basal state (Fig. 4). However, the small enhancement of basal reporter gene activities (~2 to 4-fold) was substantially less than the large increase in insulin-stimulated activities (~20 to 30-fold). In any case, these data confirm that the greater degree of insulin-stimulated reporter gene activity in CHO/IR cells than in parental CHO cells was not due to clonal variation but was a result of increased insulin receptor expression.

To demonstrate that transfection with the insulin receptor expression plasmid resulted in increased functional levels of insulin receptor kinase activity, parental CHO cells were subjected to phosphotyrosine Western blotting (Fig. 3B). In the absence or presence of insulin, there was no detectable insulin receptor β-subunit tyrosine phosphorylation (Fig. 3B, lanes 1 and 2). However, following transfection with increasing amounts of the insulin receptor expression plasmid, there was a dose-dependent increase in the extent of insulin-stimulated β-subunit tyrosine phosphorylation (Fig. 3B, lanes 3 to 5). It should be noted that since only a small percentage of the cell population were transfected under these conditions (~2.0%), there was no detectable change in the extent of tyrosine-phosphoryled IRS1 in the total cell extracts. Nevertheless, these data directly demonstrate that transfection with the insulin receptor expression plasmid resulted in a functional increase in the insulin receptor protein kinase.

This being the case, cotransfection of the parental CHO cells with a fixed amount of the insulin receptor expression plasmid and various amounts of the IRS1 expression plasmid should result in a marked inhibition of insulin-stimulated reporter gene activity (Fig. 5). As expected, parental CHO cells displayed an approximate 3-fold insulin-stimulated increase in SRE-Luc (Fig. 5A) and AP-1–CAT (Fig. 5B) reporter activities, whereas cotransfection with 1 μg of the insulin receptor expression plasmid resulted in 12- and 6-fold increases, respectively. Coexpression of the insulin receptor with 1 μg of the IRS1 expression plasmid resulted in small increase in SRE-Luc (30%) and AP-1–CAT (50%), respectively (Fig. 5). However, as the concentration of the IRS1 expression plasmid increased, there was a marked decrease in insulin-stimulated reporter gene activity. Under these conditions, transfection with 5 μg of the IRS1 expression plasmid reduced the extent of insulin stimulation to a value similar to that observed for the parental CHO cells in the absence of the insulin receptor. These data demonstrate that appropriate relative levels of the insulin receptor and IRS1 are essential for productive downstream insulin signaling.

Previous studies have reported that increased expression of the small adaptor protein Grb2 augments insulin stimulation of mitogen-activated protein (MAP) kinase activity (31). Since tyrosine-phosphorylated IRS1 has been observed to associate with Grb2 (19, 32, 39), we next investigated the effect of Grb2 expression on SRE-Luc and AP-1–CAT reporter gene activity (Fig. 6 and 7). Transfection of various amounts of the Grb2 expression plasmid had no significant effect on insulin stimulation of SRE-Luc activity in parental CHO cells (Fig. 6A). However, Grb2 was consistently observed to increase insulin-stimulated SRE-Luc reporter activity approximately 1.4-fold in CHO/IR cells (Fig. 6B). Essentially identical results were obtained for the insulin stimulation of AP-1–CAT reporter gene activity in parental CHO cells (Fig. 7A) and CHO/IR cells (Fig. 7B).

The ability of Grb2 to augment insulin signaling in CHO/IR cells but not in parental CHO cells suggested that Grb2 function was limiting in the CHO/IR cells. We therefore determined the amount of IRS1-associated Grb2 in CHO/IR and CHO/IR cells transfected with the IRS1 expression plasmid (Fig. 8A). As expected, in the absence of insulin, there was essentially no IRS1-immunoprecipitated Grb2 protein in ei-

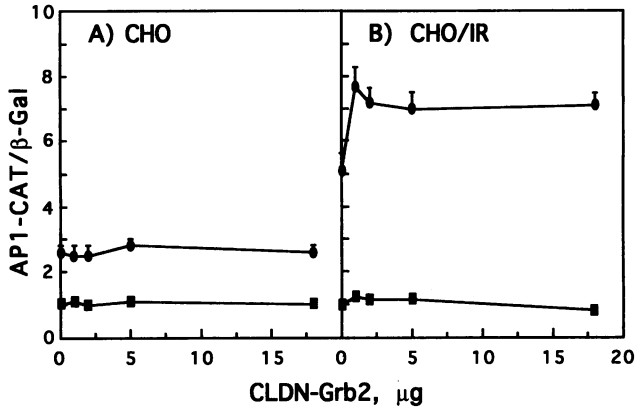

FIG. 7. Effect of Grb2 expression on insulin-stimulated AP-1–CAT reporter gene activity in CHO and CHO/IR cells. Parental CHO (A) and CHO/IR (B) cells were transfected with the reporter gene constructs and various amounts of the Grb2 expression plasmid CLDN-Grb2 as described for Fig. 1. Cell extracts were prepared from either untreated cells (■) or cells incubated with 100 nM insulin for 6 h (●). The cell extracts were assayed for CAT and β-galactosidase activities. The data are presented as the amount of AP-1–CAT activity divided by the amount of β-galactosidase (β-Gal) activity present in the extract. The data presented are averages and standard errors of the means from three independent experiments, each performed in triplicate.

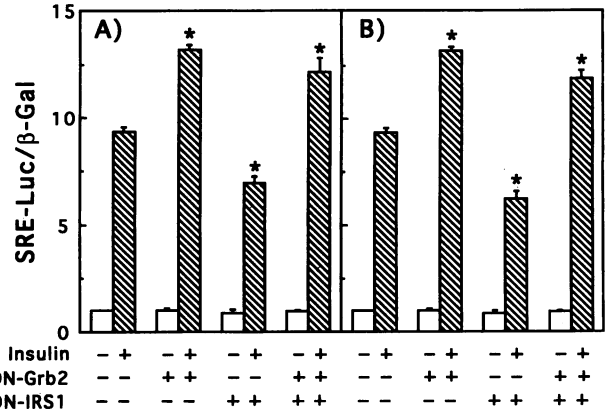

FIG. 9. Restoration of IRS1 inhibited insulin-stimulated SRE-Luc reporter activity by coexpression with Grb2 in CHO/IR cells. (A) CHO/IR cells were transfected with and without various combinations of the Grb2 expression plasmid CLDN-Grb2 (10 μg) and the IRS1 expression plasmid CLDN-IRS1 (3.0 μg) as indicated. (B) CHO/IR cells were transfected with and without various combinations of the Grb2 expression plasmid CLDN-Grb2 (10 μg) and the IRS1 expression plasmid CLDN-IRS1 (5.0 μg) as indicated. Sixteen hours following transfection, the cells were serum starved for 12 h and either untreated (open boxes) or incubated with 100 nM insulin for 6 h (hatched boxes). Cell extracts were prepared and assayed for luciferase and β-galactosidase activities. The data are presented as the amount of SRE-Luc activity divided by the amount of β-galactosidase (β-Gal) activity present in the extract. The data presented are averages and standard errors of the means from three independent experiments, each performed in triplicate. *, $P < 0.01$ compared with insulin-treated control cells.

ther control (Fig. 8A, lane 1) or IRS1-transfected (Fig. 8A, lane 3) CHO/IR cells. In contrast, insulin stimulation resulted in the association of Grb2 with tyrosine-phosphorylated IRS1 (Fig. 8A, lane 2). Further, following expression of IRS1, there was a substantial increase in the amount of IRS1-associated Grb2 in the insulin-stimulated state (Fig. 8A, lane 4). Thus, the inhibition of insulin signaling in CHO/IR cells overexpressing

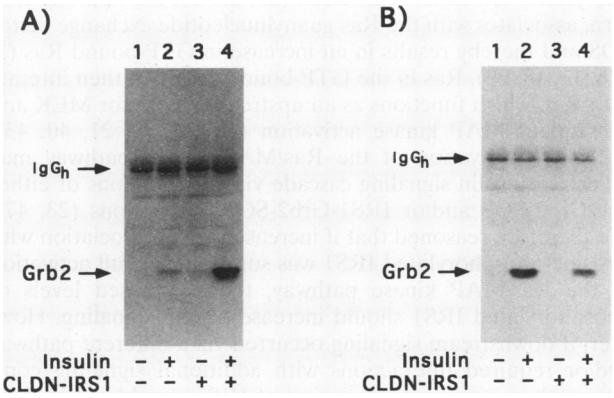

FIG. 8. Expression of IRS1 decreased the amount of Shc-associated Grb2. CHO/IR cells were either mock transfected (lanes 1 and 2) or transfected with 40 μg of the expression plasmid for IRS1 (lanes 3 and 4) by electroporation as described in Materials and Methods. Subsequently, the cells were either left untreated (lanes 1 and 3) or stimulated with 100 nM insulin for 5 min (lanes 2 and 4) and detergent solubilized in lysis buffer. The cell extracts were then subjected to immunoprecipitation with an IRS1 antibody (A) or a Shc antibody (B). The immunoprecipitates were then subjected to Western blotting with a Grb2 antibody. The blots are from a representative experiment independently performed three times. IgG_h, immunoglobulin G heavy chain.

IRS1 probably did not result from insufficient levels of IRS1-associated Grb2.

In addition to IRS1, Grb2 has been shown to associate with another insulin receptor tyrosine-phosphorylated substrate, Shc (25, 26, 29, 30, 32). To determine if IRS1 expression had any effect on the Shc-Grb2 complex, we next performed a Shc immunoprecipitation from control and IRS1-transfected CHO/IR cells (Fig. 8B). As in Fig. 8A, in the absence of insulin, there was no specific Grb2 protein in the Shc immunoprecipitate (Fig. 8B, lanes 1 and 3), whereas following insulin stimulation, the immunoprecipitation of Shc resulted in the specific association of Grb2 (Fig. 8B, lane 2). However, in CHO/IR cells that were transfected with IRS1, there was a substantial decrease in the amount of Grb2 protein associated with Shc (Fig. 8B, lane 4). These data demonstrate that IRS1 and Shc were competing for a limited pool of Grb2 and that high levels of tyrosine-phosphorylated IRS1 can sequester Grb2 away from Shc.

Since the data presented in Fig. 8 indicated that the amount of Grb2 associated with Shc may be limiting, we next examined the ability of Grb2 to restore the IRS1 inhibition of insulin-stimulated reporter gene activity (Fig. 9). As observed in Fig. 6, insulin increased SRE-Luc reporter activity approximately 10-fold in CHO/IR cells; this level was further enhanced when the cells were cotransfected with 10 μg of the Grb2 expression plasmid (Fig. 9A). To partially inhibit insulin stimulation of SRE-Luc activity (Fig. 1), CHO/IR cells were transfected with 3.0 μg (Fig. 9A) or 5.0 μg (Fig. 9B) of the IRS1 expression plasmid. In the presence of 3.0 μg of IRS1, insulin stimulation of SRE-Luc activity was reduced from 10-fold to 7-fold. In contrast, cotransfection with 3.0 μg of the IRS1 expression plasmid and 10 μg of the Grb2 expression plasmid resulted in

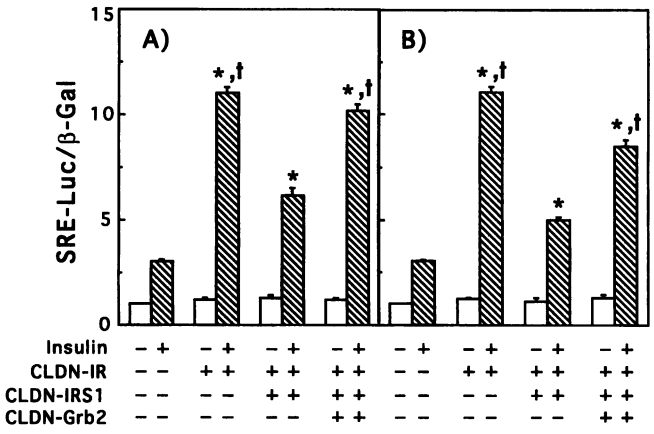

FIG. 10. Recovery of IRS1 inhibited insulin-stimulated SRE-Luc reporter activity in parental CHO cells by coexpression with Grb2. (A) Parental CHO cells were transfected with and without various combinations of the insulin receptor expression plasmid CLDN-IR (1 μg), the IRS1 expression plasmid CLDN-IRS1 (3.0 μg), and the Grb2 expression plasmid CLDN-Grb2 (10 μg) as indicated. (B) Parental CHO cells were transfected with and without various combinations of the insulin receptor expression plasmid CLDN-IR (1 μg), the IRS1 expression plasmid CLDN-IRS1 (5.0 μg), and the Grb2 expression plasmid CLDN-Grb2 (10 μg) as indicated. Sixteen hours following transfection, the cells were serum starved for 12 h and either untreated (open boxes) or incubated with 100 nM insulin for 6 h (hatched boxes). Cell extracts were prepared and assayed for luciferase and β-galactosidase activities. The data are presented as the amount of SRE-Luc activity divided by the amount of β-galactosidase (β-Gal) activity present in the extract. The data presented are averages and standard errors of the means from three independent experiments, each performed in triplicate. *, $P < 0.01$ compared with insulin-treated control cells; †, $P < 0.01$ compared with insulin-treated cells transfected with the expression vector for IRS1 (CLDN-IRS1).

an 11-fold insulin stimulation of SRE-Luc activity. Similarly, transfection of the CHO/IR cells with 5.0 μg of the IRS1 expression plasmid inhibited insulin stimulation of SRE-Luc activity from 10-fold to approximately 6-fold (Fig. 9B). The IRS1-mediated reduction in insulin-stimulated SRE-Luc activity was again fully restored upon cotransfection with 10 μg of the Grb2 expression plasmid. Essentially identical results were also obtained for insulin stimulation of AP-1–CAT reporter gene activity (data not shown).

To recapitulate the ability of Grb2 to ameliorate the IRS1 inhibition of insulin-stimulated SRE-Luc activity in parental CHO cells, these cells were transfected with the insulin receptor to increase insulin signaling to an extent similar to that in the CHO/IR cells (Fig. 10). Under these conditions, expression of the insulin receptor (1 μg of plasmid) increased insulin stimulation of SRE-Luc reporter activity (~11-fold) compared with the parental CHO cells (~3.5-fold). At this concentration of transfected insulin receptor, there was no significant change in basal SRE-Luc reporter activity. Coexpression of the insulin receptor with 3.0 μg (Fig. 10A) or 5.0 μg (Fig. 10B) of the IRS1 expression plasmid substantially reduced insulin-stimulated SRE-Luc activity to approximately 7- or 5-fold, respectively. However, inclusion of 10 μg of the Grb2 expression plasmid completely restored the insulin stimulation of reporter gene activity upon inhibition with 3.0 μg of IRS1 (Fig. 10A). Similarly, the inhibition of insulin-stimulated SRE-Luc reporter activity with 5.0 μg of the IRS1 expression plasmid was partially restored (~8-fold) by coexpression with 10 μg of the Grb2 expression plasmid (Fig. 10B).

DISCUSSION

Although various studies have demonstrated an important role for IRS1 in mediating insulin and IGF-1 receptor-dependent downstream signaling events, in some cases IRS1 expression has paradoxically been observed to inhibit insulin signaling. For example, several studies have demonstrated that various cell lines expressing high levels of the insulin receptor display increased levels of insulin-stimulated tyrosine phosphorylation of IRS1 and enhanced insulin signaling (for reviews, see references 14, 23, 37, 38, and 47). Similarly, a cell line that has low levels of endogenous insulin receptors when stably selected for high expression of IRS1 displayed high levels of IRS1 tyrosine phosphorylation and enhanced insulin mitogenic responsiveness (35). In contrast, expression of both the insulin receptor and IRS1 at high levels resulted in a marked reduction in insulin-stimulated DNA synthesis (35). Thus, the biological signaling role of IRS1 appears to differ depending on cell context factors.

There are two possible mechanisms which could account for the cell context-dependent effects of IRS1 expression. First, several studies have demonstrated that tyrosine-phosphorylated IRS1 can bind to several different effector proteins, including the phosphatidylinositol 3-kinase, the small adaptor protein Grb2, and the tyrosine-specific protein phosphatase SHPTP2, via their respective SH2 domains (1, 2, 13, 16, 17, 19, 22, 31, 32, 39, 50). These observations have led to the concept of an insulin-induced assembly of a multisubunit signaling complex with IRS1 as the core molecule (23, 47). One question raised as a consequence of this model is whether a single effector protein bound to IRS1 is sufficient for the activation of a particular downstream signaling event or whether the interaction of multiple effectors on IRS1 is required. High levels of IRS1 expression could potentially dilute out the effector proteins such that only a single individual SH2 domain target protein can be bound to any given IRS1 molecule, thereby preventing the formation of a multisubunit signaling complex.

Alternatively, it has been recently reported that another insulin receptor substrate, termed Shc, when tyrosine phosphorylated associates with Grb2 (25, 26, 29, 30, 32). Grb2, in turn, associates with the Ras guanylnucleotide exchange factor SOS and thereby results in an increase in GTP-bound Ras (3, 5, 6, 11, 18, 29). Ras in the GTP-bound state can then interact with Raf, which functions as an upstream kinase for MEK and subsequent MAP kinase activation (10, 15, 20, 21, 40, 43). Thus, the activation of the Ras/MAP kinase pathway may reflect an insulin signaling cascade via combinations of either Shc-Grb2-SOS and/or IRS1-Grb2-SOS interactions (23, 47). We therefore reasoned that if increased Grb2 association with tyrosine-phosphorylated IRS1 was sufficient for full activation of the Ras/MAP kinase pathway, then increased levels of phosphorylated IRS1 should increase insulin signaling. However, if downstream signaling occurred via a different pathway and/or required interactions with additional signaling complexes, then increased expression of IRS1 should result in an inhibition of insulin signaling due to sequestration of protein components away from these other pathways.

To distinguish between these possibilities, we have taken advantage of the established downstream targets of insulin action (SRE-Luc and AP-1–CAT reporter gene activities) that are mediated through the MAP kinase pathway (for recent reviews, see references 4 and 9). The data obtained with use of these reporters directly demonstrate that the increased expression of IRS1 can either enhance or inhibit insulin signaling, depending on the relative levels of IRS1 and other cell context factors. The inhibition of insulin signaling in cells expressing

both high levels of IRS1 and insulin receptors was consistent with a recruitment of Grb2 into an IRS1 complex with the concomitant reduction in amount of Grb2 associated with Shc. Thus, these data would indicate that the Shc-Grb2-SOS complex is a major route for the insulin activation of the Ras/MAP kinase pathway. This conclusion is consistent with a recent study in PC12 cells which suggested that only tyrosine kinase receptors that signal through the Shc-Grb2-SOS pathway couple to MAP kinase activation (24). In contrast, cells containing low levels of either insulin receptors or IRS1, and hence relatively low levels of tyrosine-phosphorylated IRS1, would not be able to efficiently compete for the available pool of Grb2.

Taken together, these data suggest that only a fraction of the cellular content of IRS1 at any given time functions as an accessible substrate for the insulin receptor kinase. Thus, increased expression of the insulin receptor and/or IRS1 will result in a net increase in tyrosine-phosphorylated IRS1 molecules capable of participating in a multisubunit signaling complex as well as in sequestering effector molecules from other tyrosine-phosphorylated substrates such as Shc. Thus, the downstream biological responses observed in any given cell type will reflect not only the properties of particular signaling molecules in a functional complex but also the relative proportions of signaling complexes generated on the basis of the amount of available effector proteins.

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