Functional Expression of Insulin Receptor Substrate-1 Is Required for Insulin-stimulated Mitogenic Signaling*

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To examine the role of the insulin receptor substrate-1 (IRS-1) in mediating insulin biological responsiveness, we generated Chinese hamster ovary cell lines expressing antisense IRS-1 RNA. These cells displayed morphological alterations as well as markedly reduced growth rates compared to the parental cells. Furthermore, the antisense IRS-1-expressing cell lines had decreased insulin-stimulated IRS-1 tyrosine phosphorylation, reduced phosphatidylinositol 3-kinase activation, and decreased thymidine incorporation relative to the parental cell line. To assess the role of IRS-1 in mediating insulin signaling, we have established two clonal CHO cell lines expressing antisense IRS-1 RNA. These data demonstrate that functional expression of the IRS-1 protein is essential for insulin signaling and the mitogenic potential of these cells.

EXPERIMENTAL PROCEDURES

Isolation of Antisense IRS-1-expressing Cells—The mammalian expression vector (CLDN) used in these studies was provided by Smith-Kline Beecham (King of Prussia, PA). It is a high copy number plasmid (pUC19) containing the dihydrofolate reductase gene for methotrexate selection and the neomycin gene for Geneticin (geneticin analog) selection. The 5′ end (~1 kbpase pair) of IRS-1 was cloned in the negative orientation into CLDN and transfected into DG44 cells (a CHO cell line derivative) using Lipofectin (Life Technologies, Inc.). Stably cell lines resistant to 500 μg/ml Geneticin were selected, and the antisense IRS-1 RNA was amplified once with 20 μg methotrexate.

Cell Culture—The CHO cell line used in these studies (DG44.neo) and the two IRS-1 anti-sense clonal lines SRI-5 and SRI-9 were maintained in α-minimal essential medium (Diabetes and Endocrinology Research Center, University of Iowa) supplemented with 10% fetal bovine serum and 500 μg/ml Geneticin. Prior to stimulation with insulin, the cells were arrested at 80% confluence by overnight incubation with serum-free α-minimal essential medium containing 0.5% bovine serum albumin. Cells were incubated in the presence or absence of 100 nM insulin for 5 min at 37 °C, followed by ice-cold washes in phosphate buffered saline, and then frozen in liquid N2. Whole cell lysates were prepared by thawing the cells in 1 ml of 1% Triton X-100 lysis buffer (50 mM Hepes, pH 7.8, 1 mM Trition X-100, 2.5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, 0.5 trypsin inhibitory units of aprotinin, and 10 μM leupeptin). Triton X-100-insoluble material was removed by centrifugation at 100,000 × g for 60 min, and total supernatant protein was determined by the method of Bradford.

Phosphatidylinositol 3-Kinase Assay—Cell lysates were immunoprecipitated overnight at 4 °C with either an αIRS-1 antibody (kindly provided by Dr. Alan Saltiel, Parke Davis) or with α85 (purchased from Upstate Biotechnology Inc.). The amount of primary antibody required to maximally immunoprecipitate the antigen from a given amount of total protein was determined in separate experiments (data not shown). Immune complexes were bound by adding 50 μl of a protein A-Sepharose suspension (1:1 with phosphate-buffered saline) for 2 h and centrifuged at 10,000 × g for 1 min. The immunoprecipitates were assayed for PI 3-kinase activity essentially as previously described (17).

Phosphotyrosine Blotting—The whole cell lysates (400 μg of protein) were separated on reducing SDS-polyacrylamide gels (7.5%) and transferred to nitrocellulose membranes (1 A, 90 min, 4 °C) using Towbin's transfer buffer (18) containing 0.075% SDS and 0.5 μl sodium vanadate. The membranes were incubated for 1 h at 23 °C with 0.3 μg/ml αPY (phosphotyrosine antibody provided by Dr. Peter Wilden, University of Missouri). Following extensive washing the nitrocellulose membranes were incubated 30 min at 23 °C with 125I-protein A, washed

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The abbreviations used are: IRS-1, insulin receptor substrate-1; SH2, src homology 2; PI 3-kinase, phosphatidylinositol 3-kinase; SRE, serum response element; Luc, luciferase; CHO, Chinese hamster ovary cells; CHO/HR, CHO cells expressing human insulin receptor; DG44, CHO derivative; RSV-LaCZ, Rous sarcoma virus promoter/β-galactosidase gene.

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Recently, a major proximal intracellular target for the insulin receptor tyrosine-specific protein kinase has been identified as a 185-kDa protein (1), termed IRS-1, for insulin receptor substrate-1 (2-6). This molecule contains numerous potential tyrosine phosphorylation sites, six of which are found within a YXXM motif (Tyr-Met-Xaa-Met) and three in the related motif YXXM (2). It is generally thought that tyrosine phosphorylation of IRS-1 provides a multimeric docking substrate for specific SH2 (src homology-2) domain-containing proteins. In the case of IRS-1, this signaling complex has been suggested to include the phosphatidylinositol 3-kinase (PI 3-kinase) (7), the SH2 domain-containing tyrosine-specific phosphatase (8), and the small adaptor molecule Grb2 (9, 10). Although Grb2 has been reported to mediate the coupling of tyrosine-phosphorylated receptors with guanylnucleotide exchange factors (11-14), a functional role for the PI 3-kinase or SH2 domain-containing tyrosine-specific phosphatase in mediating insulin signaling has not been established. In addition, the specific role of IRS-1 in mediating insulin signaling has also been difficult to address experimentally due to its ubiquitous tissue and cell type distribution (5, 15). Although expression of mammalian IRS-1 in unprimed oocytes has been reported to augment insulin stimulation of germinal vesicle breakdown (16), expression of IRS-1 in cells containing high levels of insulin receptors has also been observed to attenuate the insulin-stimulated increases in thymidine incorporation (15). In order to further address the role of IRS-1 in mediating insulin signaling, we have established two clonal CHO cell lines expressing antisense IRS-1 RNA. These data demonstrate that functional expression of the IRS-1 protein is essential for insulin signaling and the mitogenic potential of these cells.
again, and exposed to Kodak XAR film at -80°C.

Cell Growth and [3H]Thymidine Incorporation—Cells were plated at 1 x 10^5 cells/35-mm well, and 24 h later the growth media was replaced with serum-free a-minimal essential medium containing 0.5% bovine serum albumin. Cells were incubated with various concentrations of insulin for 18 h at 37°C and pulse-labeled with 0.5 pCi/ml [3H]thymidine for an additional 3 h. The labeled cells were washed, solubilized in 1 ml of phosphate-buffered saline, 0.1% SDS, and trichloroacetic acid was added to a final concentration of 10%. The trichloroacetic acid pellet was solubilized with 1 N NaOH, neutralized with HCl and the amount of incorporated [3H]thymidine determined by liquid scintillation counting. In parallel, cell number was determined at different times after plating by trypsinization and counting the released cells with a hemocytometer.

SRE-Luc Activity—The insulin stimulation of SRE-Luc transcription was determined as previously described (19). The plasmids used for these experiments were SRE-Luc, RSV-LacZ, and the entire rat IRS-1 cDNA (kindly provided by Dr. Morris White, Joslin Diabetes Center) subcloned into CLDN to generate the mammalian expression plasmid CLDN/IRS-1. Briefly, cells were cotransfected with 1 µg of the SRE-Luc reporter gene and 2 µg of RSV-LacZ, either with or without 20 µg of the rat IRS-1 expression plasmid (CLDN/IRS-1). Twelve h later the cells were glycerol-shocked, serum-starved for an additional 12 h, and then incubated with or without 100 nM insulin for 6 h. Cell extracts were prepared and assayed for both luciferase and β-galactosidase activity.

RESULTS AND DISCUSSION

Several studies have demonstrated that IRS-1 is a proximal target for the insulin receptor kinase, which upon tyrosine phosphorylation associates with multiple intracellular signaling molecules including the PI 3-kinase (20-22), Grb2 (9, 10), and SH2 domain-containing tyrosine-specific phosphatase (8). In order to assess the role of IRS-1 in mediating downstream signaling, we transfected DG44 cells (CHO-derived cell line lacking dihydrofolate reductase) with the antisense IRS-1/CLDN construct. Following selection with 500 µg/ml Genetecin and a single round of amplification with 20 nm methotrexate, we isolated 11 individual clones. Of these, two clones had a substantially reduced rate of growth compared to DG44/neo cells (CLDN vector without insert, Fig. 1A). These two clonal cell lines were termed SRI-5 and SRI-9. Any further attempts to amplify the antisense RNA by increased methotrexate selection resulted in a complete cessation of cell growth.

Since one of the cellular effects of insulin is the stimulation of DNA synthesis, we also examined the effect of insulin on DNA synthesis ([3H]thymidine incorporation) in the DG44/neo, SRI-5, and SRI-9 cell lines (Fig. 1B). As typically observed, stimulation of the DG44/neo cells with saturating insulin concentrations stimulated a 19-fold increase in [3H]thymidine incorporation with an EC50 of approximately 10 nM. In contrast, maximal insulin stimulation of [3H]thymidine incorporation was greatly inhibited in both the SRI-5 and SRI-9 antisense cell lines. Despite the large decrease in maximal responsiveness, insulin sensitivity was apparently unchanged between the control and antisense SRI-5 and SRI-9 cell lines. The inhibition of insulin-stimulated [3H]thymidine incorporation observed in the antisense cell lines (Fig. 1B) was much greater than the inhibition of growth rate (Fig. 1A). However, it should be noted that the growth rate experiments were performed in the presence of serum, whereas the thymidine incorporation experiments were done in the absence of serum. Thus, the relatively smaller inhibition of growth probably reflects the presence of other growth factors in serum that are mediated by intracellular pathways not utilizing IRS-1. Despite the differing magnitudes of inhibition, these data demonstrate that expression of antisense IRS-1 RNA in DG44 cells resulted in a marked reduction in insulin-stimulated DNA synthesis and cell growth.

In addition to alterations in growth properties, these clonal cell lines also displayed several morphological differences (Fig. 2). DG44/neon cells had a normal fibroblastic appearance, whereas the SRI-5 and SRI-9 cells were substantially more refractile with a more rounded shape. These differences were more apparent at lower cell densities and tended to disappear as the cells approached confluence (data not shown).

To determine whether these growth and morphological changes were a consequence of decreased IRS-1 tyrosine phosphorylation, we next performed phosphotyrosine Western blot analysis (Fig. 3, A and B). In the absence of insulin, one major tyrosine-phosphorylated band occurred at approximately 120
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Fig. 3. Relative levels of IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity in CHO/hIR, DG44/neo, SRI-5, and SRI-9 cell lines. A, CHO/hIR (lanes 1 and 2), DG44/neo (lanes 3 and 4), and IRS-1 antisense RNA cell lines (lanes 5–8) were incubated in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 100 nM insulin for 5 min at 37 °C. Cell lysates containing 400 μg of total protein were separated on SDS-polyacrylamide gels (7.5%), transferred to nitrocellulose, and immunoblotted with αPY. B, quantitation of IRS-1 tyrosine phosphorylation by densitometric analysis from four independent experiments. C, comparison of insulin-stimulated PI 3-kinase activity in control DG44/neo, SRI-5, and SRI-9 cell lines. Control and IRS-1 antisense RNA cell lines were incubated with 100 nM insulin for 5 min, and cell lysates were prepared and immunoprecipitated with αIRS-1 and αp85. PI 3-kinase enzyme activity in the αIRS-1 immunoprecipitates was normalized with respect to total cellular PI 3-kinase activity determined in the αp85 immunoprecipitates. The relative PI 3-kinase activity was expressed as a percentage of that found in the DG44/neo cells. These data were from three independent experiments.

kDa in the control cells (Fig. 3A, lane 1). This band has been observed in the phosphotyrosine blots of other cell types and was constitutively tyrosine-phosphorylated in an insulin-independent manner (1). In CHO cells overexpressing the human insulin receptor (CHO/hIR), insulin stimulation for 5 min resulted in a marked increase in tyrosine phosphorylation of the 185-kDa IRS-1 protein and the 95-kDa β-subunit of the insulin receptor (Fig. 3A, lanes 1 and 2) with no significant change in the 120-kDa protein. Insulin stimulation of the DG44/neo cells also increased tyrosine phosphorylation of IRS-1, but phosphorylation of the β-subunit was not observed (Fig. 3A, lanes 3 and 4). The inability to detect autophosphorylation of the insulin receptor β-subunit reflects the relatively low level of insulin receptors present in the DG44/neo cells. In contrast to the DG44/neo cells, insulin stimulation of the SRI-5 and SRI-9 cell lines resulted in a reduced level of IRS-1 tyrosine phosphorylation (Fig. 3A, lanes 6 and 8). Quantitation of IRS-1 tyrosine phosphorylation determined from multiple Western blots consistently demonstrated a reduction of 30 and 50% compared to control values in the SRI-5 and SRI-9 cells, respectively (Fig. 3B). Although we were unable to directly assess the relative amounts of IRS-1 protein due to the lack of an appropriate immunoblotting IRS-1 antibody, the αPY Western blots demonstrate that the IRS-1 antisense-expressing cell lines have reduced levels of tyrosine-phosphorylated IRS-1 protein in response to insulin.

It has been well established that tyrosine phosphorylation of IRS-1 results in the association of IRS-1 with PI 3-kinase (20–22). This association is dependent upon tyrosine phosphorylation of IRS-1 YMMX motifs (23, 24) and their specific recognition by SH2 domains present in the p85 subunit of PI 3-kinase (25, 26). We therefore determined the amount of IRS-1-associated PI 3-kinase activity in the DG44/neo, antisense SRI-5, and antisense SRI-9 cell lines (Fig. 3C). The amount of insulin-stimulated αIRS-1 immunoprecipitated PI 3-kinase activity in the SRI-5 cell line was reduced approximately 20% compared to DG44/neo cells, whereas the SRI-9 cell line was reduced to 40% of DG44/neo. Thus, insulin-mediated association of IRS-1 with PI 3-kinase was also impaired in the antisense IRS-1 RNA-expressing clones, which is consistent with the reduction in insulin-stimulated tyrosine phosphorylation of IRS-1 protein (Fig. 3, A and B).

Insulin has also been shown to be a potent activator of immediate early gene expression and, in particular, the c-fos promoter (19, 27). Insulin stimulation of control cells transfected with a SRE fused to the thymidine kinase-luciferase reporter gene resulted in a 4-fold increase in luciferase activity (Fig. 4A). In contrast, insulin-stimulated SRE-Luc activity approximately 2.2-fold in the SRI-5 and 2-fold in the SRI-9 cell lines. In order to demonstrate that the decrease in insulin-stimulated SRE-Luc activity was a specific consequence of the decreased phosphorylation of IRS-1, we co-transfected the cells with an expression plasmid for rat IRS-1 (Fig. 4B). As expected, co-transfection with IRS-1 had only a small effect on insulin stimulation of SRE-Luc activity in DG44/neo cells. However, in the SRI-5 and SRI-9 cell lines, expression of IRS-1 fully restored insulin-stimulated SRE-Luc activity. Thus,
the inhibition of insulin-stimulated c-fos transcription in the IRS-1 antisense RNA-expressing cell lines was a specific consequence of decreased levels of tyrosine-phosphorylated IRS-1.

In summary, these data demonstrate that in CHO cells, expression of IRS-1 was essential for cell growth, insulin-stimulated DNA synthesis, and transcriptional activation of the c-fos serum response element. Although the function of PI 3-kinase in insulin signaling has not yet been established, the reduced association/activation of PI 3-kinase with IRS-1 was also consistent with the inhibition of biological responsiveness in these cell lines. Surprisingly, however, both the decrease in IRS-1 tyrosine phosphorylation and PI 3-kinase association with IRS-1 were modest in comparison to the marked inhibition of cell growth, insulin-stimulated SRE-Luc activity in IRS-1 antisense cells transfected with the IRS-1 cDNA plasmid. Thus, the impaired biological responsiveness was a direct consequence of reduced IRS-1 levels, and transcriptional activation of the c-fos sequence of decreased levels of tyrosine-phosphorylated IRS-1.

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