Transcriptional Regulation of Insulin Receptor Substrate 1 by Protein Kinase C*

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Insulin receptor substrate-1 (IRS-1) is involved in insulin signal transduction distal to receptor occupation. Targeted disruption of IRS-1 leads to insulin resistance and hyperglycemia in mice, which suggests that altered IRS-1 expression could contribute to the insulin resistance seen in non-insulin-dependent diabetes mellitus. In vitro studies using phorbol ester analogues have implicated the protein kinase C (PKC) pathway as being involved in the pathogenesis of insulin resistance. Using the MCF-7 breast cancer cell line, a role for PKC in regulating IRS-1 expression was examined. In an MCF-7 cell line (MCF-7-PKC-α) that exhibits multiple alterations in PKC isoform expression, IRS-1 content was reduced to negligible levels relative to parental MCF-7 cells. This decrease in IRS-1 content was associated with a 30-fold reduction in IRS-1 transcription. In parental MCF-7 cells, PKC inhibitors (GF109203X, bisindolylmaleimide I and staurosporine) reduced IRS-1 content. Chronic exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA; >8 h) reduced IRS-1 content and down-regulated the novel PKC-δ isoform. Bryostatin 1 inhibited TPA-induced depletion of both IRS-1 and PKC-δ expression in MCF-7 cells. Associated with TPA-induced reduction in IRS-1 content was a reduction in IRS-1 transcription. These data demonstrate that PKC can modulate IRS-1 content and suggest a potential role for PKC-δ in positively regulating IRS-1 expression.

Insulin receptor substrate-1 (IRS-1) is a cytoplasmic protein phosphorylated on tyrosine residues by ligand-induced activation of the insulin receptor (1). Insulin receptor-induced tyrosine phosphorylation of IRS-1 stimulates its binding to the 85-kDa subunit of phosphatidylinositol-3-kinase and other Src homology 2 domain-containing proteins (2–4). These phosphorylation-dependent interactions involving the IRS-1 molecule are essential in mediating signal transduction distal to occupation of the insulin receptor. Evidence of the importance of IRS-1 in insulin signal transduction is the insulin resistance, hyperinsulinemia, and mild hyperglycemia seen in mice harboring a targeted disruption of the IRS-1 gene (5, 6). Insulin resistance is a phenomenon associated with and believed to be involved in the pathogenesis and/or maintenance of noninsulin-dependent diabetes mellitus (NIDDM). Studies have demonstrated reductions of IRS-1 in muscle and liver of ob/ob mice that could potentially contribute to insulin resistance occurring in this model (7). Patients with morbid obesity, hyperinsulinemia, and NIDDM display a reduction in the skeletal muscle content of IRS-1 relative to levels seen in obese nondiabetic patients (8). Derangements in skeletal muscle insulin signal transduction are thought to contribute to the insulin resistance seen in NIDDM. Thus, decreased expression of IRS-1 could, in part, be involved in mediating certain aspects of the insulin-resistant phenotype seen in NIDDM.

Protein kinase C (PKC), which lies on signal transduction pathways modulating a diverse array of metabolic and growth-signaling cascades, has been implicated in inducing insulin resistance (9–11). In vitro studies have demonstrated that phorbol ester-stimulated activation and/or down-regulation of PKC induce insulin resistance (12, 13). PKC is a gene family consisting of at least 12 isoforms (14, 15). The existence of this gene family has led to the hypothesis that activation of individual isoforms may preferentially evoke specific cellular responses. Thus, individual PKCs may preferentially affect insulin signal transduction to either induce or attenuate resistance to this hormone.

In this report, we examined whether alterations in the PKC pathway could modulate IRS-1 expression. We found that an MCF-7 breast cancer cell line exhibiting multiple alterations in conventional and novel PKC expression (MCF-7-PKC-α cells) displayed a marked decrease in IRS-1 content relative to parental MCF-7 cells. In parental MCF-7 cells, PKC inhibitors reduced IRS-1 expression. Phorbol ester treatment of MCF-7 cells also decreased IRS-1 levels, and this effect correlated with loss of the novel PKC-δ isoform. Phorbol ester-induced decreases IRS-1 expression were mediated, at least in part, via a transcriptional mechanism. These findings indicate a possible mechanism by which alterations in the PKC pathway can negatively modulate insulin signal transduction to potentially produce an insulin-resistant state.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All reagents were obtained from Sigma unless otherwise noted. GF109203X was obtained from Calbiochem (La Jolla, CA) and prepared in Me2SO. Bryostatin 1 was isolated from *Bugula neritina* as described previously (16).

**Cell Culture**—The MCF-7 human breast cancer cell line was purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mm glutamine, 10 mm Hepes, 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in 5% carbon dioxide. Viability was assessed by trypsin blue exclusion.

**Western Blot Analysis**—Hot Laemmli lysis buffer was added directly to cells pelleted by centrifugation as we have previously described (17). After heating at 100 °C for 5 min, the cells were sheared by 10 passages through a 25-gauge needle, and 105 cells in 10 μl of buffer were loaded per lane. Western blotting was done as described elsewhere (18). IRS-1 and antiphosphotyrosine antisera were purchased from Upstate Bio-

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¶ The abbreviations used are: IRS, insulin receptor substrate; NIDDM, non-insulin-dependent diabetes mellitus; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; GF109203X, bisindolylmaleimide I.
been previously described (17, 19). Antisera to the α, β, and γ PKC isoforms have been previously described (17, 19). Antisera to the δ, θ, and μ isoforms were purchased from Transduction Laboratories. Antisera to the ε and η isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sample buffer for Western analysis using the IRS-1 antibody contained 1 mM sodium fluoride and 1 mM sodium ortho-vanadate in addition to the other components.

Immunoprecipitation—To immunoprecipitate IRS-1, cells were rinsed with ice-cold phosphate buffered saline and resuspended in immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 1 mM EDTA, 1 mM EGTA, and 0.1% SDS) in the presence of both protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium ortho-vanadate, 1 mM sodium fluoride, and 1 μM aprotinin). Five hundred μl of immunoprecipitation buffer was used per 1 × 10^7 cells. The samples were incubated on ice for 30 min and then spun in a microcentrifuge for 5 min at 4°C. Supernatants were collected and centrifuged at 123,000 × g for 1 h at 4°C. Supernatants from this centrifugation were collected and precleared of nonspecific absorbing material with 50 μl of normal rabbit serum/ml of lysate. Preclearing rabbit serum antibodies were removed by adding 50 μl of 50% protein A/G-agarose (Santa Cruz Biotechnology)/ml of supernatant, incubated for 30 min at 4°C, and spun in a microcentrifuge for 5 min. Supernatants from this centrifugation were collected, and protein concentration was determined. Five μg of IRS-1 antibody was added to 500 μg of protein and incubated at 4°C overnight. Twenty μl of protein A/G-agarose was added and incubated for 2 h at 4°C with agitation. Samples were microcentrifuged for 2 min at 4°C, and the pellet was washed twice with immunoprecipitation buffer, once with TSA solution (10 mM Tris, pH 7.4, and 0.5 mM sodium chloride) and once with 20 mM Tris, pH 7.4. After the final wash, the pellet was resuspended in 40 μl of 2 × immunoprecipitation sample buffer (120 mM Tris, pH 6.8, 200 mM dithiothreitol, 15% glycerol, and 4% SDS) and boiled for 5 min at 100°C. The samples were cooled and microcentrifuged for 1 min, supernatants were removed, and 20 μl of each sample was subjected to SDS-polyacrylamide gel electrophoresis and Western analysis with an antiphosphotyrosine antibody as described above.

Northern Blot Analysis—Total RNA was isolated via a single step guanidium thiocyanate-phenol extraction technique using TRIzol Reagent (Life Technologies, Inc.). Poly(A)^+ RNA was further isolated using the PolyATtract mRNA isolation system obtained from Promega (Madison, WI). The RNA was size fractionated on a 1.5% agarose/formaldehyde gel. Ethidium bromide staining of gels was performed to confirm the integrity of RNA and to assess the equivalency of loading. The RNA was transferred to nylon membranes (Gibco-BRL, Gaithersburg, CA) via capillary action and cross-linked with UV transillumination. Dr. Charles Garner (Department of Biochemistry and Molecular Biology, Texas Tech University Health Science Center, Lubbock TX) generously provided the IRS-1 cDNA.

Nuclear Run-on Analysis—Nuclei were isolated, and transcriptional run-on analysis was performed as described previously (20). Hybridization of nascent RNA to the IRS-1 cDNA was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with Image Quant software. The IRS-1 signal was normalized to the genomic signal.

RESULTS

Decreased IRS-1 Transcription in MCF-7-PKC-α Cells That Display Altered PKC Isoform Content—In examining the role of PKC in mediating breast cancer progression, PKC-α was stably overexpressed in MCF-7 breast cancer cells (MCF-7-PKC-α cells) (18). In addition to overexpressing the transfectected PKC-α isoform, the MCF-7-PKC-α cells also display the de novo induction of the PKC-β and PKC-δ isoforms, decreases in the novel PKC-ε, PKC-η, and PKC-μ isoforms, and a slight reduction in the atypical PKC-i isoform (Fig. 1).

The MCF-7-PKC-α cells display several characteristics consistent with progression to a more biologically aggressive breast cancer phenotype. Specifically, these cells lack the estrogen receptor, display enhanced vimentin expression, grow quite efficiently in soft agar, and are highly tumorigenic with metastatic potential when inoculated in nude mice (18). Given the possible role of tyrosine kinase-signaling pathways in the malignant progression of breast cancer (21), we examined certain aspects of these signal transduction cascades in an attempt to better understand the molecular mechanisms underlying the phenotypic changes displayed by MCF-7-PKC-α cells. Since tyrosine phosphorylation of IRS-1 is involved in transmitting signals distal to occupation of insulin-like growth factor 1 and insulin receptors, the phosphorylation status of IRS-1 was examined. Extracts from parental MCF-7 and MCF-7-PKC-α cells grown in 10% fetal calf serum were subjected to immunoprecipitation with IRS-1 antisera and Western blot analysis with antiphosphotyrosine antiserum (Fig. 2A). Tyrosine phosphorylation of IRS-1 was detected in parental MCF-7 cells grown in 10% fetal calf serum (Fig. 2A). Interestingly, MCF-7-PKC-α cells did not contain tyrosine-phosphorylated IRS-1 (Fig. 2A). To examine whether the difference in phosphorylated IRS-1 levels between these cells was secondary to a decrease of IRS-1 content in MCF-7-PKC-α cells, Western blot analysis of IRS-1 was done (Fig. 2B). IRS-1 content was dramatically reduced in MCF-7-PKC-α cells.

To determine the level at which the reduction in IRS-1 content occurred in MCF-7-PKC-α cells, IRS-1 mRNA expression was analyzed (Fig. 3). Northern blot analysis using poly(A)^+ RNA demonstrated a reduction of the 6.9-kb IRS-1 mRNA transcript in MCF-7-PKC-α cells relative to that detected in parental and vector-transfected MCF-7 cells (Fig. 3). To examine the mechanism by which this reduction in IRS-1 mRNA was mediated, nuclear run-on analysis was performed (Fig. 4A). Transcription of the IRS-1 gene was reduced by 30-fold in MCF-7-PKC-α cells relative to that seen in parental MCF-7 cells (Fig. 4B). Given this marked reduction in IRS-1 transcription, these data suggest that reduced IRS-1 expression in MCF-7-PKC-α cells may, at least in part, be mediated at a transcriptional level.

Due to the multiple phenotypic changes exhibited by the MCF-7-PKC-α cells, in addition to altered PKC isoform expression, it was impossible to ascertain whether these alterations in PKC isoform expression were directly involved in mediating the reduction in IRS-1 expression. Thus, to more closely examine the role of the PKC signal pathway in regulating IRS-1...
expression, studies manipulating PKC activation status were performed in parental MCF-7 cells.

**PKC Inhibitors and TPA-induced Down-regulation of PKCs Reduced IRS-1 Content in Parental MCF-7 Cells**—MCF-7 cells contain conventional (α), novel (ε, δ, γ, and μ), and atypical (ζ and ι) PKC isoforms (Fig. 1). Using PKC inhibitors GF109203X and staurosporine, we examined whether PKC inhibition would decrease IRS-1 levels. GF109203X inhibits conventional and novel PKCs, with a higher degree of sensitivity exhibited against the novel PKC subfamily (22, 23). Staurosporine is a potent inhibitor of all PKCs but lacks the high degree of PKC specificity as that exhibited by GF109203X (22, 23). After a 24-h exposure, both inhibitors significantly reduced IRS-1 levels in MCF-7 cells (Fig. 5). These treatments did not reduce MCF-7 cell viability (data not shown). These results suggested that maintenance of the PKC pathway was, at least, partially necessary to maintain the IRS-1 levels contained in parental MCF-7 cells.

Although phorbol esters activate conventional and novel PKCs, chronic exposure to TPA induces the preferential down-regulation of specific PKC isoforms. Exposure of parental MCF-7 cells to 10 nM TPA for 24 h only minimally down-regulates the PKC-α, -ε, and -μ isoforms, substantially reduces the novel PKC-δ isoform, and slightly increases PKC-γ (Fig. 6A). Under these same conditions, IRS-1 content decreased (Fig. 6B). Combined with the ability of PKC antagonists to decrease IRS-1 levels, these results from chronic TPA treatment experiments suggested that down-regulation of a PKC isoform could be involved in mediating the TPA-induced reduction in IRS-1 expression. Given that PKC-δ was the isoform most down-regulated by TPA treatment, and given that the MCF-7-PKC-α cells display marked reduction in both PKC-δ and IRS-1 (Figs. 1 and Fig. 2B), these results suggest potential importance of PKC-δ in maintaining IRS-1 expression.

**Correlation between PKC-δ Down-regulation and Reduced IRS-1 Expression in MCF-7 Cells**—To assess the extent of the correlation between the TPA-induced reduction in IRS-1 expression and PKC-δ down-regulation, a TPA time course and TPA dose-response curve for both PKC-δ and IRS-1 were established (Fig. 7). After exposure to 10 nM TPA, IRS-1 levels decreased between the 8- and 24-h treatment points (Fig. 7A). Under identical conditions, down-regulation of PKC-δ preceded the decrease in IRS-1 and was apparent within a 4-h exposure (Fig. 7A). After a 24-h exposure to varying concentrations of TPA, decreases in IRS-1 were observed at a dose of 1 nM (Fig. 7B). PKC-δ down-regulation was also apparent after a 24-h exposure to 1 nM TPA and was maximal at a TPA concentration of 10 nM (Fig. 7B). These data indicate that TPA-induced decreases in IRS-1 expression are preceded by down-regulation of PKC-δ and that these events are induced by similar TPA concentrations. These findings further suggest a role for PKC-δ in maintaining IRS-1 levels in MCF-7 cells.

**Bryostatin 1 Blocks Both TPA-induced Decreases in IRS-1**
Expression and PKC-δ Down-regulation—Bryostatin 1, a macrocyclic lactone, interacts with the phorbol ester binding site to activate the phorbol ester-responsive PKCs (24). Although bryostatin 1 activates PKCs, it displays the unusual property of inhibiting TPA-induced responses in certain cells (24–27). Although the molecular mechanisms for this inhibitory response have yet to be fully elucidated, it has been proposed that TPA down-regulates PKC-δ and reduces IRS-1 levels in MCF-7 cells. A, Western blot analysis using isoform-specific PKC antisera was done on extracts derived from MCF-7 cells treated with vehicle or 10 nM TPA for 24 h. B, IRS-1 content determined by Western blot analysis using extracts prepared from the same cells used in A. These experiments were repeated with similar results.
pression. In addition, studies in other insulin-responsive cell lines will need to be done to determine whether the effects of PKC-δ on IRS-1 expression represent a generalized phenomenon or are restricted to only specific cell types.

The decreases in IRS-1 observed in MCF-7-PKC-α cells and TPA-treated parental MCF-7 cells were regulated, at least in part, at a transcriptional level. In MCF-7-PKC-α cells, the reduction in IRS-1 transcription was accompanied by a reduction in IRS-1 mRNA levels. This correlation suggested that transcriptional alterations may contribute to regulating the expression of IRS-1. Unlike insulin- and glucocorticoid-induced modulation of IRS-1 expression, which occur at the protein and mRNA stability levels, respectively (28–31), PKC-mediated regulation of IRS-1 expression appears to involve a transcriptional mechanism. However, because this work was focused on addressing the transcriptional regulation of IRS-1, the possibility that the PKC pathway may also affect these other levels of regulation still exists. Thus, in this work we provide evidence for another means and level at which IRS-1 expression can be regulated.

With respect to insulin signaling, chronic exposure to phorbol esters has been shown to induce insulin resistance in a variety of cultured cell models (12, 13). Although our work does not exclude other possibilities, it does indicate that a reduction in IRS-1 expression may be one potential mechanism by which phorbol esters negatively modulate insulin signal transduction. Indeed, further work directly comparing insulin sensitivity and IRS-1 levels after manipulations of the PKC pathway in insulin-responsive cell types will be necessary to confirm the significance of PKC-mediated regulation of IRS-1 expression in altering insulin sensitivity. Through such studies, a better understanding of both the mechanisms by which the PKC pathway affects insulin signaling and the putative role of PKC in mediating insulin resistance in NIDDM may be achieved.

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