Regulation of Insulin Receptor, Insulin Receptor Substrate-1 and Phosphatidylinositol 3-Kinase in 3T3-F442A Adipocytes. Effects of Differentiation, Insulin, and Dexamethasone

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Insulin rapidly stimulates tyrosine kinase activity of its receptor resulting in phosphorylation of its cytosolic substrate insulin receptor substrate 1 (IRS-1), which in turn associates with and activates the enzyme phosphatidylinositol 3-kinase (PI 3-kinase). In the present study we have examined these three initial steps in insulin action during the differentiation of 3T3-F442A adipocytes and after treatment with dexamethasone or insulin.

The differentiation of 3T3-F442A cells was characterized by a 13-fold increase in insulin receptor protein, a 9-fold increase in IRS-1, and a 10- and 4.5-fold increase in their insulin-stimulated phosphorylation, respectively. The mRNA expression of these two proteins showed a similar 8-fold increase during differentiation. In addition there was a 3.5-fold increase in PI 3-kinase protein [85 kilodalton (kDa) subunit] and a 16-fold increase in IRS-1-associated PI 3-kinase activity between day 0 and day 8 of differentiation.

Dexamethasone (1 μM) treatment of differentiated cells induced a further 48% ($P < 0.05$) increase in insulin receptor level, but the autophosphorylation of the receptor was decreased by 31 ± 1% ($P < 0.02$). At the same time there was a decrease by 56 ± 4% ($P < 0.005$) in IRS-1 protein and by 31 ± 1% ($P < 0.001$) in IRS-1 phosphorylation. The expression of insulin receptor mRNA was unchanged, but the expression of IRS-1 mRNA was decreased by ~75% after dexamethasone. By contrast, dexamethasone induced a 69% increase in the level of PI 3-kinase as determined by immunoblotting. The combined effect of decreased IRS-1 phosphorylation and increased PI 3-kinase protein was a minimal change (15% decrease) in the association/activation between IRS-1 and PI 3-kinase.

Chronic treatment with 100 nM insulin induced a time- and dose-dependent decrease in insulin receptor and IRS-1 protein levels reaching a nadir of 34 ± 5% ($P < 0.005$) and 39 ± 5% ($P < 0.01$) of control levels after 24 h, respectively. There was an even more marked decrease in the phosphorylation level of these proteins. Chronic insulin treatment also produced a 30% decrease in PI 3-kinase protein levels and a ~50% decrease in the association/activation between IRS-1/PI 3-kinase. The expression of insulin receptor and IRS-1 mRNA was unchanged during chronic insulin treatment. Thus three of the early steps in insulin action may have an important role in the process of adipocyte differentiation and represent points of regulation in hormone-induced insulin resistance in 3T3-F442A adipocytes. (Molecular Endocrinology 8: 545–557, 1994)

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INTRODUCTION

Certain clones of 3T3 cells (3T3-L1 and 3T3-F442A) can differentiate into adipocytes with high frequency in cell culture in a process closely resembling the differentiation of adipocytes in early life (1). The development of the fatty phenotype is accompanied by a marked increase in triglyceride content, as well as increased activity of lipogenic and lipolytic enzymes (2). Sensitivity to hormones known to affect adipocyte metabolism develops concomitantly with the expression of the fatty phenotype. With differentiation, the cells show an increase in insulin binding (3–5) and in insulin receptor mRNA (6, 7) and a parallel increase in insulin-stimulated glucose uptake and oxidation (5, 7). These character-

istics make the 3T3-F442A cells an attractive model for studies of insulin action at the postreceptor level.

Over the past decade, there has been a dramatic increase in our understanding of the early postreceptor events in insulin action. After insulin binding to the α-subunit of its receptor, there is an activation of the kinase in the β-subunit (8, 9). This catalyzes the intramolecular autophosphorylation of specific tyrosine residues of the β-subunit, further enhancing the tyrosine kinase of the receptor toward other protein substrates (10). In most cells, this leads to the subsequent tyrosyl phosphorylation of a cytoplasmic protein with an apparent mol wt between 160 and 185 K, called insulin receptor substrate 1 (IRS-1) (10–12). Considerable evidence indicates that insulin receptor tyrosine kinase and associated IRS-1 phosphorylation are essential for many, if not all, of the biological effects of insulin (8, 13–15). In studies performed using *in vivo* models of insulin action, cells in culture, as well as *in vitro* reconstitution systems, phosphorylated IRS-1 associates with the lipid metabolizing enzyme phosphatidylinositol 3-kinase (PI 3-kinase), thus activating the enzyme (16, 17). Thus, the insulin receptor, IRS-1, and PI 3-kinase represent three of the earliest steps in insulin action. In addition, recent studies have indicated that in states of insulin resistance, alterations of these steps may contribute to the altered insulin action profile (18–22).

In the present study we have examined the phosphorylation state of the insulin receptor and IRS-1 and PI 3-kinase activity after insulin stimulation *in vivo*, as well as the levels of these three proteins and their mRNAs during differentiation of 3T3-F442A adipocytes. We also examined the regulation of these early steps of insulin action in mature 3T3-F442A cells by chronic treatment with two hormones that can induce insulin resistance, *i.e.* insulin or dexamethasone (18–22).

RESULTS

Expression of Insulin Receptor and IRS-1 during the Differentiation of 3T3-F442A Adipocytes

Differentiation of 3T3-F442A cells was enhanced by the addition of 5 μg/ml insulin for the first 6 days followed by culture for 48 h in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum without insulin. By the end of this period 90–95% of the cells acquired the characteristic adipocyte morphology accumulating a large number of lipid droplets. As previously reported (4, 5), differentiation is characterized by a marked increase in insulin binding, which increased 13-fold between day 0 and day 8 (Fig. 1A). This increase in insulin binding is primarily accounted for by an increase in receptor number with no significant change in receptor affinity (4, 5). The increase in receptor level was further confirmed by immunoblotting with a C terminus antibody against insulin receptor (Fig. 2C). Insulin-stimulated receptor tyrosine phosphorylation also increased 10-fold as cells progressed from nondifferentiated to completely differentiated 3T3-F442A adipocytes (Figs. 1A and 2A).

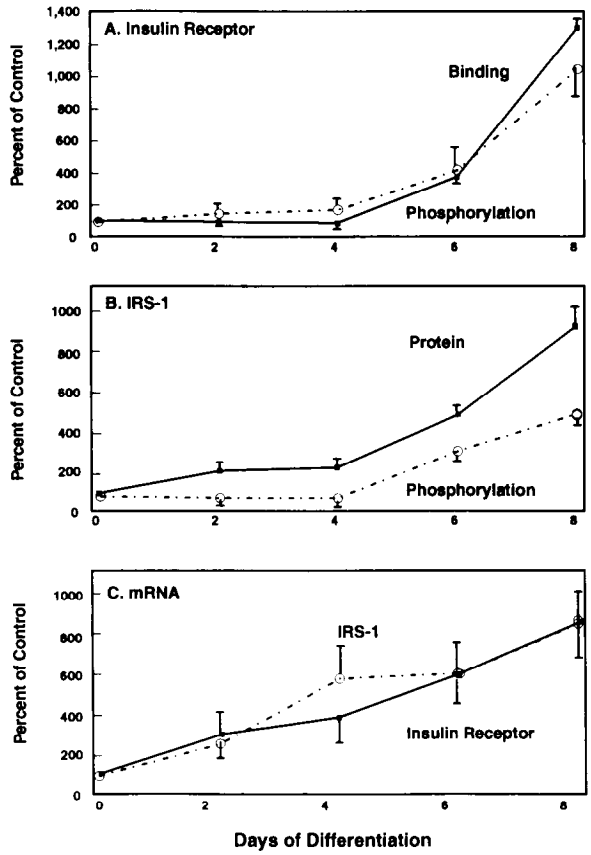

Fig. 1. Effect of Differentiation on Insulin Receptor and IRS-1 in 3T3-F442A Cells

A, Insulin binding and insulin receptor phosphorylation during the differentiation of 3T3-F442A cells. Insulin binding in cells on different days of differentiation was determined as described in *Materials and Methods*, and receptor tyrosine phosphorylation levels were determined by scanning densitometry of autoradiograms of antiphosphotyrosine antibody. The results represent the mean ± SEM of four separate experiments. B, IRS-1 protein and phosphorylation levels during differentiation of 3T3-F442A cells. Scanning densitometry of autoradiograms of anti-IRS-1 and anti-phosphotyrosine antibody were used to determine IRS-1 protein and phosphorylation levels. The results represent the mean ± SEM of four separate experiments and are expressed as percent of control (day 0). C, Effect of differentiation on expression of insulin receptor and IRS-1 mRNA in 3T3-F442A cells. One microgram of total RNA prepared from cells in different days of differentiation was reverse transcribed into cDNA in the presence of the three 3′-primers (1 pmol of each primer/reaction). One twentieth of total cDNA was subjected to PCR using end-labeled primers for insulin receptor (IR), IRS-1, or β_2-microglobulin (β2 M). Negative and positive controls were used as described in *Methods* (data not shown).

In parallel there was a 9-fold increase in IRS-1 protein levels from preadipocytes to differentiated adipocytes (Figs. 1B and 2B) and a 4.5-fold increase in insulin-stimulated IRS-1 phosphorylation (Figs. 1B and 2A). The lesser increase in IRS-1 phosphorylation, as com-

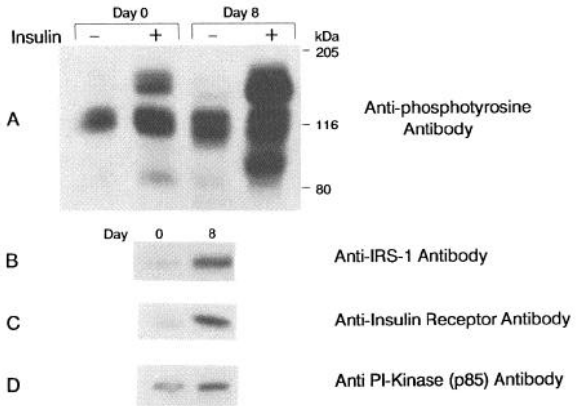

Fig. 2. Effect of Differentiation of 3T3-F442A Fibroblasts (Day 0) in Adipocytes (Day 8) on Insulin-Stimulated Tyrosine Phosphorylation

The cells in different phases of differentiation were treated with 100 nM insulin for 1 min (lanes 2 and 4), extracted, and homogenized in extraction buffer A as described in *Methods*. After centrifugation aliquots corresponding to the same number of cells were resolved on 6% SDS-polyacrylamide gel and transferred to nitrocellulose, and the proteins were detected with antiphosphotyrosine antibody (A), anti-IRS-1 antibody (B), antiinsulin receptor antibody (C), anti-PI 3-kinase antibody (D) and [^{125}I]protein A and subjected to autoradiography.

pared to IRS-1 protein and insulin receptor phosphorylation, suggested the possibility of an increase in cytosolic protein tyrosine phosphatase (PTPase) which might act preferentially on IRS-1 *vs*. insulin receptor. Indeed, there was a 50% increase in cytosolic PTPase activity from day 0 to day 8 differentiation (data not shown), which may contribute to the differential increase in tyrosine phosphorylation of these proteins.

To determine whether the changes in insulin receptor and IRS-1 protein levels were regulated at the mRNA level, we determined the expression of mRNA in these two proteins using quantitative polymerase chain reaction (PCR). During differentiation there was a progressive and gradual increase in mRNA level of both insulin receptor and IRS-1 reaching an 8-fold increase by day 8 when 90–95% of the cells have the adipocyte morphology (Fig. 1C). These results are in agreement with previous reports on the expression of insulin receptor mRNA during the differentiation of 3T3-L1 preadipocytes (6, 7) and suggest that the increase in gene transcription of insulin receptor and IRS-1 play a key role in the increased expression of these proteins during differentiation. β_2-Microglobulin, which was used as a control, also increased during differentiation, but to a much lesser extent (data not shown).

Effect of Differentiation on PI 3-Kinase in 3T3-F442A Cells

To determine the impact of changes in IRS-1 expression and phosphorylation on further downstream effects, PI 3-kinase activity was measured in nondifferentiated and completely differentiated cells (Fig. 3B). A very low level of basal PI 3-kinase activity was present in anti-IRS-1 immunoprecipitates from 3T3-F442A fibroblasts, which rapidly increased about 6-fold following acute insulin stimulation (Fig. 3B). After differentiation into adipocytes, the basal PI 3-kinase activity in anti-IRS-1 immunoprecipitates was 10 times higher than in 3T3-F442A fibroblasts, and insulin stimulation produced a further 8-fold increase over basal levels. In other words, there was a 16-fold increase in IRS-1-associated PI 3-kinase activity after acute insulin stimulation in 3T3-F442A adipocytes (day 8) as compared to fibroblasts (day 0). Based on immunoblotting the total cell extracts with an antibody to the 85 kDa of PI 3-kinase, the levels of this enzyme protein increased only ~3-fold during differentiation of the 3T3-F442A cells (Fig. 3A). Thus, the increase in the amount of the enzyme can account for only a part of the increase in PI 3-kinase activity associated with IRS-1 after differentiation and suggests that other regulatory events or factors might play a role in the control of this enzyme.

Effect of Dexamethasone on Insulin Receptor and IRS-1 Phosphorylation in 3T3-F442A Adipocytes

When fully differentiated 3T3-F442A adipocytes were treated with dexamethasone (1 μM) for 6 h there was a 42 ± 16% increase in insulin receptor levels as detected by immunoblotting with antireceptor antibody ($P < 0.05$). After 24 h of dexamethasone, insulin receptor levels increased only slightly to 48 ± 21% ($P < 0.05$) (Figs. 4D and 5A). By contrast, insulin stimulation of insulin receptor autophosphorylation as detected by immunoblotting with antiphosphotyrosine antibodies decreased in 3T3-F442A with dexamethasone reaching a nadir of 69 ± 1% of control levels after 24 h of treatment ($P < 0.02$) (Figs. 4A and 5B). When the change in phosphorylation was normalized for the increase in receptor content, there was a ~50% decrease in receptor autophosphorylation per receptor, after 12 h and 24 h of dexamethasone treatment. During the same time course, there was a progressive and significant decrease in IRS-1 protein levels, reaching 44 ± 4% of control levels ($P < 0.005$) after 24 h (Figs. 4B and 5A). The level of insulin-stimulated tyrosine phosphorylation of IRS-1 showed a parallel decrease reaching 73 ± 1% ($P < 0.01$) and 69 ± 1% ($P < 0.01$) of control values at 12 h and 24 h of dexamethasone treatment, respectively (Figs. 4A and 5B). However when the latter data were corrected for the level of IRS-1, there was actually a slight, although not significant increase in the apparent stoichiometry of IRS-1 phosphorylation.

To test the hypothesis that the changes in insulin receptor (increase) and IRS-1 (decrease) induced by dexamethasone might be regulated at the mRNA levels, insulin receptor and IRS-1 mRNA were estimated by quantitative PCR in 3T3-F442A adipocytes before and after dexamethasone (1 μM) treatment. Insulin receptor mRNA did not change during glucocorticoid treatment (Fig. 6, A and D). However, IRS-1 mRNA was signifi-

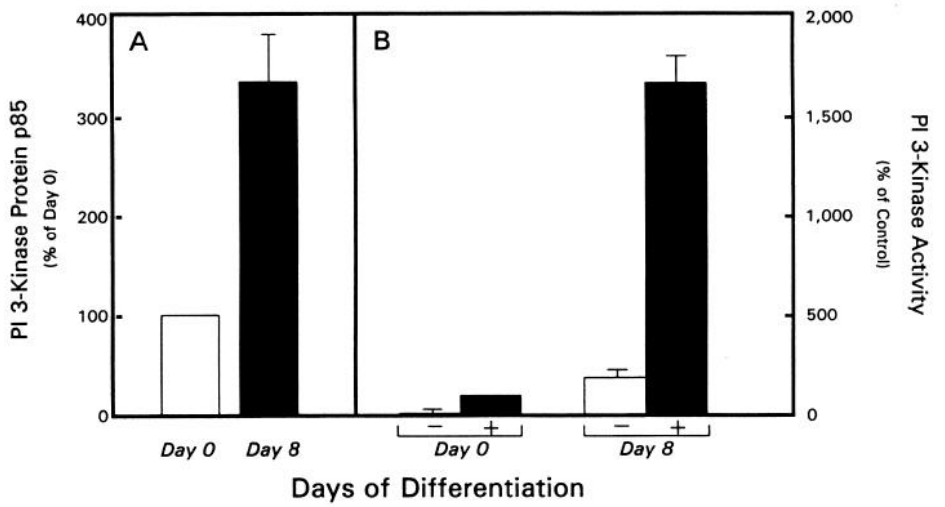

Fig. 3. Effect of Differentiation of PI 3-Kinase in 3t3-F442A Cells

A, PI 3-kinase protein levels during the differentiation of 3T3-F442A cells. The data are expressed as mean + SEM of scanning densitometry of autoradiograms on four experiments and are normalized per cell number. B, Insulin stimulation of IRS-1-associated PI 3-kinase during the differentiation of 3T3-F442A cells. The proteins were extracted and processed as described in *Methods* and incubated at 4 C with anti-IRS-1 antibodies and protein-A sepharose. PI 3-kinase in the washed immunoprecipitates was assayed as described in *Methods*. The resulting labeled lipids were extracted, separated by TLC, and visualized by autoradiography. [^{32}P] phosphate incorporated into PI 3-phosphate was quantitated by Cerenkov counting. The *right panel* shows PI 3-kinase activity in anti-IRS-1 immunoprecipitates from cells on day 0 (fibroblasts) and day 8 (adipocytes) before (−) and after (+) insulin stimulation. Quantification was done by Cerenkov counting of the [^{32}P]phosphate incorporated into PI(3)P in immunoprecipitates from cells in different days of differentiation and represented as percent of basal (day 0). The results are expressed as mean ± SEM of three separate experiments.

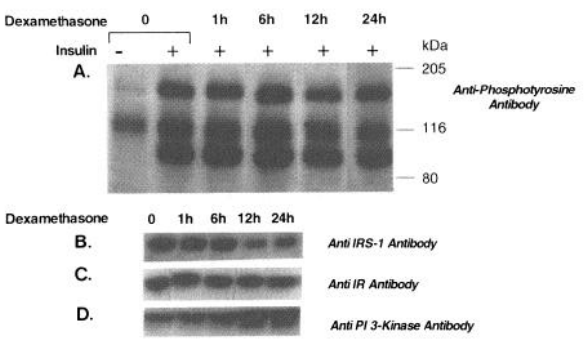

Fig. 4. Time Course of Dexamethasone on Insulin-Stimulated Tyrosine Phosphorylation in 3T3-F442A Adipocyte Cells

Cells were treated with or without 1000 nM dexamethasone for 1, 6, 12, and 24 h, and were stimulated with normal saline (lane 1) or 10^{-7} M insulin (lanes 2, 3, 4, 5, and 6) for 1 min and then extracted and homogenized in extraction buffer A at 4 C as described in *Methods*. After centrifugation, aliquots with the same amount of protein were resolved on 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with antiphosphotyrosine antibody (A), anti-IRS-1 antibody (B),antiinsulin receptor antibody (C), or anti-PI 3-kinase antibody (D), and [^{125}I]protein A and subjected to autoradiography.

cantly decreased to 67 ± 1% ($P < 0.01$), 22 ± 13% ($P < 0.01$), and 24 ± 13% ($P < 0.01$) of control cells after 1 h, 6 h, and 24 h of dexamethasone treatment, respectively (Fig. 6, B and D). β_2-Macroglobulin mRNA, used as control, did not change during dexamethasone treatment (Fig. 6C). These results suggest that the decrease in IRS-1 protein level is secondary to a decrease in IRS-1 mRNA. In on-going studies we have found that this is due to a change in IRS-1 mRNA half-life rather than transcriptional regulation of the gene (E. Araki and C. R. Kahn, submitted for publication).

To determine whether the reductions in insulin receptor and IRS-1 phosphorylation were accompanied by a reduction in the association/activation between IRS-1/PI 3-kinase, 3T3-F442A adipocytes were treated with dexamethasone (1 μM) for 24 h, stimulated with insulin for 1 min, and then extracted as described in *Materials and Methods* for PI 3-kinase activity. A low level of basal PI 3-kinase activity was found in anti-IRS-1 immunoprecipitates from control cells and cells treated with dexamethasone. After insulin stimulation, there was 10-fold increase in IRS-1-associated PI 3-kinase activity in control cells and an 8.5-fold increase in cells treated with dexamethasone (Fig. 7). This modest 15% reduction in IRS-1-associated PI 3-kinase activity is much less than the reduction in IRS-1 protein and/or phosphorylation levels. Immunoblotting the cell extracts with an antibody to the 85-kDa subunit of PI 3-kinase revealed a progressive increase in PI 3-kinase protein levels following dexamethasone treatment reaching a maximum of 169 ± 22% of control levels after 24 h (Fig. 8A). Thus, an increase in PI 3-kinase enzyme level may prevent a major decline in IRS-1-associated PI 3-kinase activity as might have been expected by the decrease in IRS-1 phosphorylation levels.

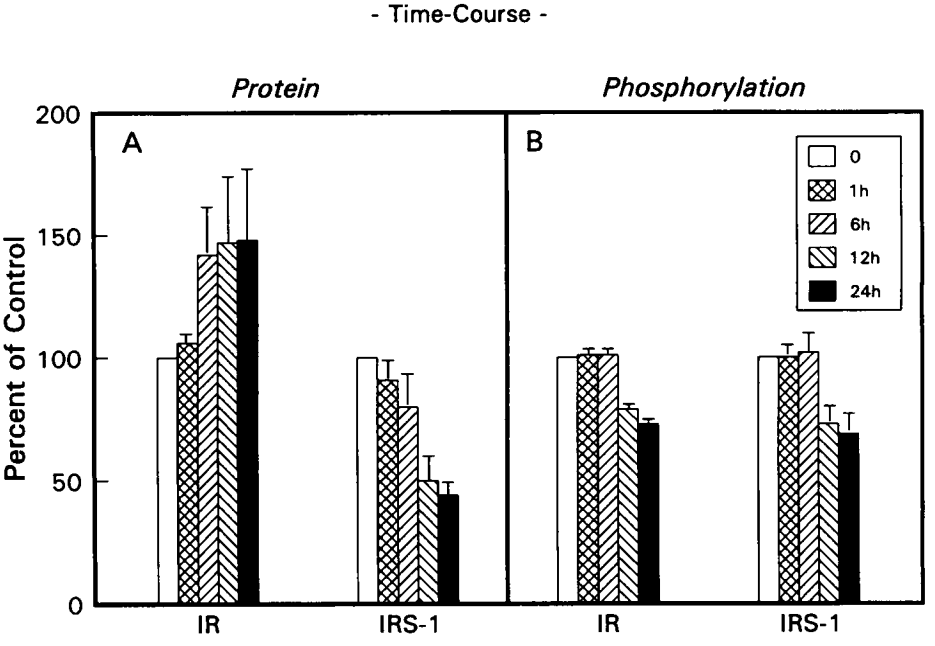

Fig. 5. Insulin Receptor and IRS-1 Protein (A) and Tyrosine Phosphorylation Levels (B) during the Time-Course of Dexamethasone in 3T3-F442A Adipocytes Cells

Cells were treated for 1, 6, 12, and 24 h with or without 1000 nM dexamethasone or 100 nM insulin, extracted, and homogenized in extraction buffer A at 4 C as described in *Methods*. After centrifugation, aliquots with the same amount of protein were resolved on 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with antireceptor and anti-IRS-1 antibodies and [^{125}I] protein A and subjected to autoradiography.The data are expressed as mean ± SEM of scanning densitometry of autoradiograms performed on five experiments for insulin receptor and IRS-1 concentration and four experiments for tyrosine phosphorylation levels of both and are normalized per protein.

Time Course of Insulin Treatment on Insulin Receptor and IRS-1 Phosphorylation in 3T3-F442A Adipocyte Cells

To evaluate the effect of chronic insulin exposure on insulin receptor and IRS-1 phosphorylation, differentiated 3T3-F442A adipocytes cells were treated with 100 nM insulin for periods up to 24 h. Between 1 and 24 h, there was a progressive decrease in insulin receptor level, as detected by immunoblotting with receptor antibodies, reaching a nadir of 34 ± 5% of control levels ($P < 0.005$) after 24 h of insulin treatment (Figs. 9C and 10A). Chronic exposure of differentiated 3T3-F442A cells to 100 nM insulin also resulted in a decrease in IRS-1 levels reaching 39 ± 5% ($P < 0.01$) of basal levels at 24 h (Figs. 9B and 10A). In addition, following acute insulin stimulation and up to 6 h of insulin treatment, there was a decrease in mobility reflecting an increase in the degree of phosphorylation of IRS-1 resulting from insulin stimulation. This was confirmed by direct immunoblotting with antiphosphotyrosine antibodies (Fig. 9A). Thus, in the basal state there was a constitutively phosphorylated protein, in 3T3-F442A adipocytes, with molecular mass of 120 kilodaltons (kDa). After insulin stimulation for 1 min the insulin receptor β-subunit (95 kDa) and IRS-1 (broad band between 165–185 kDa) appeared and became prominently phosphorylated in the phosphotyrosine immunoblotting. Following chronic insulin treatment there was an increase in basal insulin receptor and IRS-1 phosphorylation. The increase in basal insulin receptor and IRS-1 phosphorylation were maximal at 1 h, decreasing progressively until 24 h. Acute insulin stimulation did not change the pattern of the chronic insulin effect on phosphorylation of both proteins during the time course of 24 h. By comparison with control cells, acute insulin-induced insulin receptor phosphorylation was decreased to 68 ± 1% ($P < 0.05$), 16 ± 2% ($P < 0.005$), and 16 ± 4% ($P < 0.005$) after 6 h, 12 h, and 24 h of chronic insulin treatment, respectively, with similar changes in IRS-1 phosphorylation (Figs. 9A and 10B). The reduction in insulin receptor phosphorylation was greater than the decrease in insulin receptor protein levels, suggesting a modest decrease in the stoichiometry of insulin receptor autophosphorylation during chronic insulin treatment. The change in IRS-1 phosphorylation was parallel to the change in IRS-1 protein levels during the first 6 h of insulin treatment. The decrease in IRS-1 phosphorylation levels became more marked after 12 h (15 ± 2%, $P < 0.005$) and 24 h (9 ± 1%, $P < 0.002$) of chronic insulin treatment (Figs. 9A and 10B) also suggesting a reduction in IRS-1 phosphorylation when normalized per IRS-1 molecule.

Dose-Response of Chronic Insulin Treatment on Insulin Receptor and IRS-1 Phosphorylation Levels in 3T3-F442A Adipocytes Cells

Figures 11 and 12 show the concentration dependence of insulin on insulin receptor and IRS-1 phosphorylation

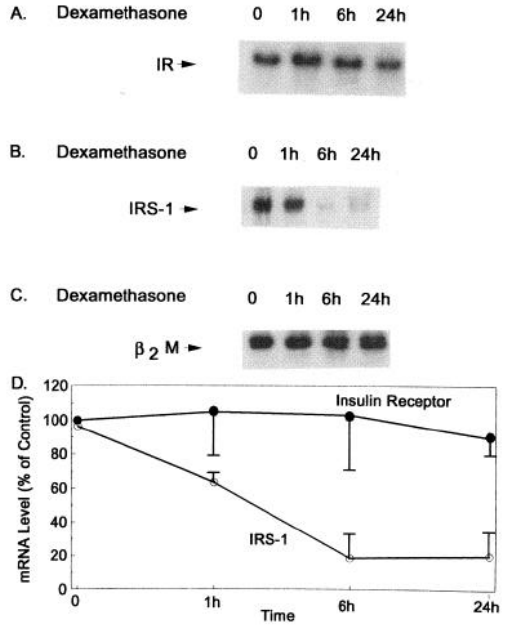

Fig. 6. Time-Course of Dexamethasone on Expression of Insulin Receptor and IRS-1 mRNA in 3T3-F442A Adipocytes

One microgram of total RNA prepared from control cells (0) or cells treated with 1000 nM dexamethasone for 1 h, 6 h, or 24 h was reverse transcribed into cDNA in the presence of the three 3′-primers (1 pmol of each primer/reaction) and subjected to PCR using end-labeled primers for insulin receptor (IR) (panel A), IRS-1 (panel B), or β_2-microglobulin (β2 M (panel C). Panel D, Effect of dexamethasone on the expression of insulin receptor and IRS-1 mRNA in 3T3-F442A adipocytes. Scanning densitometry of autoradiograms was performed on four experiments similar to panel A. The data are represented as percent of control (day 0) and are expressed as mean ± SEM.

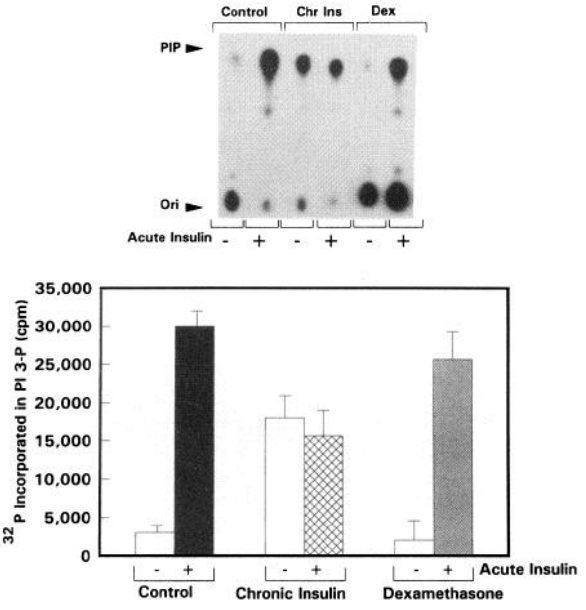

Fig. 7. Insulin Stimulation of IRS-1-Associated PI 3-Kinase in Control Cells and Cells Chronically Treated with Insulin or Dexamethasone

The proteins were extracted and processed as described in *Methods*, and incubated at 4 C with anti-IRS-1 antibodies and protein-A Sepharose. PI 3-kinase in the washed immunoprecipitates was assayed as described in *Methods*. The resulting labeled lipids were extracted, separated by TLC, and visualized by autoradiography. [^{32}P]phosphate incorporated into PI-3P was quantitated by Cerenkov counting. The *upper panel* shows PI 3-kinase activity in anti-IRS-1 immunoprecipitates from cells treated with 100 nM insulin or 1000 nM dexamethasone for 24 h or without these hormones (control) before (−) and after (+) acute insulin stimulation. Ori indicates the origin; PIP indicates the position of migration of PI(4)P standard. *Lower panel*, Quantification by Cerenkov counting of the [^{32}P] phosphate incorporated into PI(3)P in immunoprecipitates from control and cells chronically treated with insulin or dexamethasone. The results are expressed as mean ± SEM of three separate experiments.

in 3T3-F442A adipocytes. The level of insulin receptor detected by immunoblotting with antiinsulin receptor antibody did not change after treatment with 0.01 nM insulin, but decreased significantly with 1 nM and reached 14 ± 9% ($P < 0.01$) of control level with 100 nM insulin (Fig. 12A). At the same time the levels of IRS-1 protein did not change with 0.01 nM and 1 nM insulin, and were reduced to 43 ± 8% of control levels after 24 h of 100 nM insulin ($P < 0.02$) (Figs. 11B and 12A).

On phosphotyrosine immunoblots there was a progressive decrease in acute insulin-induced insulin receptor phosphorylation levels reaching a nadir of 29 ± 4% ($P < 0.01$) after 24 h of exposure of the cells to 100 nM insulin (Figs. 11A and 12A). At all concentrations of chronic insulin there was no difference between insulin receptor protein levels and insulin receptor phosphorylation levels suggesting that the autophosphorylation of insulin receptor did not change on a per receptor basis. The decrease observed with IRS-1 phosphorylation was more marked after 0.01 nM insulin for 24 h, but very similar, and paralleled the decrease in receptor phosphorylation with the two highest doses (Figs. 11A and 12B). The decrease in IRS-1 phosphorylation level at all concentrations of insulin was also more marked than the decrease in IRS-1 protein levels suggesting a reduction in IRS-1 phosphorylation per molecule and a reduction in insulin receptor tyrosine kinase activity toward its endogenous substrate IRS-1. No consistent change in insulin receptor and IRS-1 mRNA levels were observed in the time course of insulin treatment (data not shown), indicating that the decrease in the level of these two proteins induced by insulin is not regulated at mRNA levels.

Characteristics of Insulin-Stimulated PI 3-Kinase Activity in 3T3-F442A Adipocytes Cells Treated with Insulin

To determine the effect of reduction in IRS-1 expression and phosphorylation induced by insulin on further downstream effects, control cells and cells chronically treated with insulin were acutely stimulated with 100 nM insulin

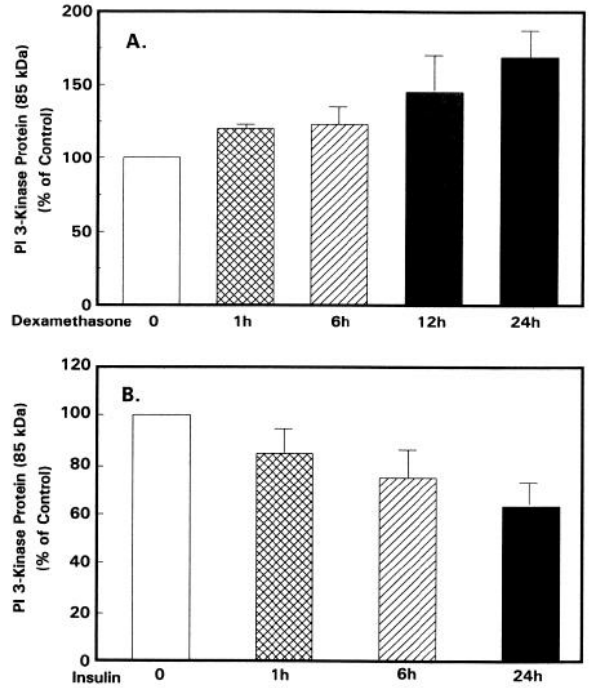

Fig. 8. Effect of Dexamethasone and Insulin on PI 3-Kinase Levels in Differentiated 3T3-F442A Cells

A, PI 3-kinase protein levels during the time course of dexamethasone in 3T3-F442A adipocytes cells; B, PI 3-kinase protein levels during the time course of insulin in 3T3-F442A adipocytes cells. Cells were treated for 1 h, 6 h, 12 h, and 24 h with 1000 nM dexamethasone or 100 nM insulin, extracted, and homogenized in extraction buffer A at 4 C as described in *Methods*. After centrifugation aliquots with the same amount of protein were resolved on 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with anti-PI 3-kinase antibodies and [^{125}I]protein A and subjected to autoradiography. Scanning densitometry of autoradiograms was performed on four experiments. The data are expressed as mean ± SEM and are normalized per protein.

and extracted, and immunoprecipitates with antibodies to IRS-1 were assayed *in vitro* for PI 3-kinase activity. A low level of basal PI 3-kinase activity was present in the anti-IRS-1 immunoprecipitates of control 3T3-F442A adipocytes cells before insulin stimulation, and after 1 min of insulin stimulation there was a 10-fold increase in IRS-1-associated PI 3-kinase activity (Fig. 7). Chronic insulin (100 nM) treatment for 24 h produced a sustained increase in basal IRS-1-associated PI 3-kinase activity of ~6-fold (Fig. 7). During the period of chronic insulin treatment, IRS-1-associated PI 3-kinase activity did not change over basal with further acute insulin stimulation. Indeed, by comparison with acute insulin stimulated in control cells there was a 53% decrease after 24 h of 100 nM insulin (Fig. 7). This decrease in PI 3-kinase activity in immunoprecipitates of IRS-1 was accompanied by a reduction in PI 3-kinase protein levels of 27 ± 11% ($P < 0.05$) and 38 ± 8% ($P < 0.02$) at 6 h and 24 h, respectively, as determined by immunoblotting with antibodies to the 85 kDa subunit of PI 3-kinase (Fig. 8B).

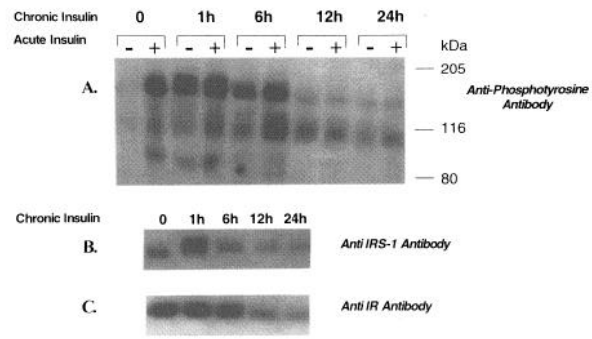

Fig. 9. Time Course of Chronic Insulin on Insulin-Stimulated Tyrosine Phosphorylation in 3T3-F442A Adipocytes Treated with Insulin

The cells were chronically treated with or without 100 nM insulin for 1, 6, 12, and 24 h (0), and were acutely stimulated with normal saline (lanes 1, 3, 5, 7, and 9) or 10^{-7} M insulin (lanes 2, 4, 6, 8, and 10) for 1 min, then extracted and homogenized in extraction buffer A at 4 C as described in *Methods*. After centrifugation, aliquots with the same amount of protein were resolved on 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with antiphosphotyrosine antibody (A), anti-IRS-1 antibody (B), antiinsulin receptor antibody (C) and [^{125}I]protein A and subjected to autoradiography.

DISCUSSION

Although the exact molecular events linking the insulin receptor tyrosine kinase to its final cellular actions remain poorly understood, several of the early steps in the insulin action cascade have been defined at a molecular level. Following insulin binding, the activated insulin receptor kinase catalyzes tyrosine phosphorylation of a cytoplasmic protein of relative molecular mass between 165 and 185 kDa termed IRS-1 (10–12). IRS-1 is a substrate of insulin, insulin-like growth factor-1, and interleukin-4 receptors (10–12, 23, 24) and following its phosphorylation can associate with proteins containing Src homology 2 (SH2) domains through specific tyrosyl phosphorylation sites (16, 25–28). This association leads to an activation of the enzyme PI 3-kinase (16, 17). In the present study we have evaluated the regulation of expression and function of the proteins involved in these early steps in insulin action during the differentiation of 3T3-F442A cells, as well as the effect of chronic treatment with dexamethasone or insulin on fully differentiated adipocytes.

Our results show that the differentiation process is accompanied by a marked increase in insulin receptor and IRS-1 levels. These results are in accordance with previous studies using 3T3-L1 cells (3–5, 29). In addition, we find that the increase in insulin receptor and IRS-1 levels parallel an increase in mRNA of these proteins suggesting that these changes are regulated at the transcriptional level. Interestingly, the increase in insulin- stimulated receptor and IRS-1 phosphorylation was less dramatic than the increase in the level of these two proteins. This may be due to an increase in the cytoplasmic phosphotyrosine phosphatase activity

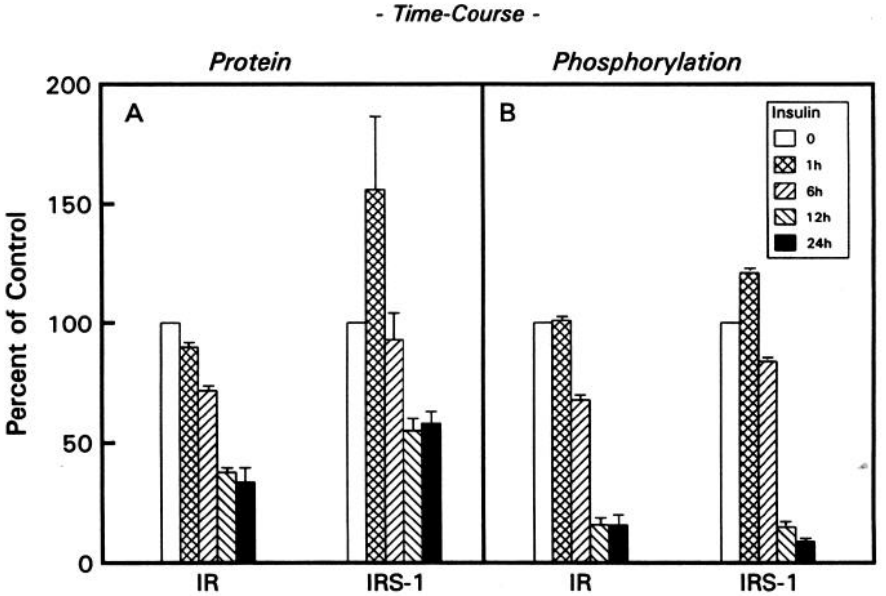

Fig. 10. Insulin Receptor and IRS-1 Protein (A) and Tyrosine Phosphorylation Levels (B) during the Time Course of Chronic Insulin in 3T3-F442A Adipocytes

Scanning densitometry of autoradiograms was performed on five experiments for insulin receptor and IRS-1 concentration and four experiments for tyrosine phosphorylation levels of both. The data are expressed as mean ± SEM and are normalized per protein.

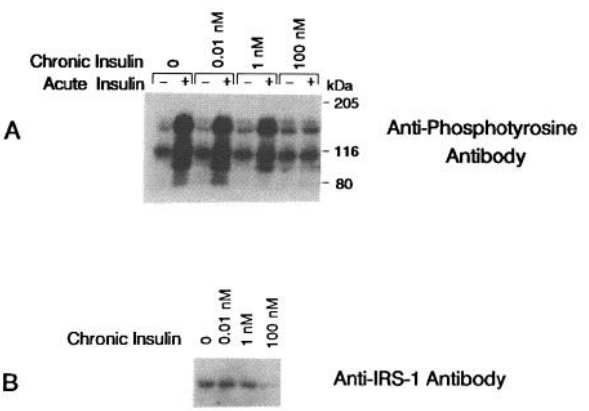

Fig. 11. Dose-Response of Insulin-Stimulated Tyrosine Phosphorylation in 3T3-F442A Adipocytes Chronically Treated with Insulin

Cells were treated for 24 h with or without 0.01 nM, 1 nM, and 100 nM insulin for 24 h and then were acutely stimulated with 10^{-7} M insulin for 1 min. The cells were extracted and homogenized in extraction buffer A at 4 C as described in *Methods*. After centrifugation, aliquots with the same amount of protein were resolved on 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with antiphosphotyrosine antibody (A), anti-IRS-1 antibody (B) and [^{125}I]protein A and subjected to autoradiography.

which increases 50% as the cells differentiate from fibroblast to adipocyte. The differences between the increase in IRS-1 protein and phosphorylation levels in 3T3-F442A adipocytes were more marked then those for the insulin receptor and suggest that IRS-1 may be more sensitive to dephosphorylation and/or subject to other regulatory influences. This is in accordance with the hypothesis of Mooney and Bordwell (30) who found that IRS-1 is dephosphorylated at a rate 4 times faster than the receptor in permeabilized adipocytes. During differentiation there is also an increase in PI 3-kinase levels and together with the increase in IRS-1 phosphorylation level this results in a large increase in association/activation between IRS-1/PI 3-kinase. These results suggest that an increase at all three early post-binding steps in insulin action, *i.e.* insulin-induced insulin receptor and IRS-1 phosphorylation and activation of PI 3-kinase, play a role in the increased insulin responsiveness observed in the adipocyte phenotype.

We studied the effect of chronic treatment with insulin and dexamethasone on these early steps in insulin action in the fully differentiated adipocytes, since these two hormones are known to induce insulin resistance in a variety of physiological and pathological states (31, 32). Chronic insulin treatment induced a time- and dose-dependent decrease in insulin receptor and IRS-1 protein and phosphorylation level. Reduction of insulin receptor levels and kinase activity by chronic insulin treatment has previously been demonstrated in other intact cell systems (32), although this has not been uniformly observed (33). The decrease in IRS-1 levels paralleled the decrease in protein levels in the first 6 h, but after this there was a reduction in IRS-1 phosphorylation greater than could be accounted for by the decrease in IRS-1 protein. In this context, the changes in insulin receptor kinase in the same direction may have at least a permissive role, since the IRS-1 protein

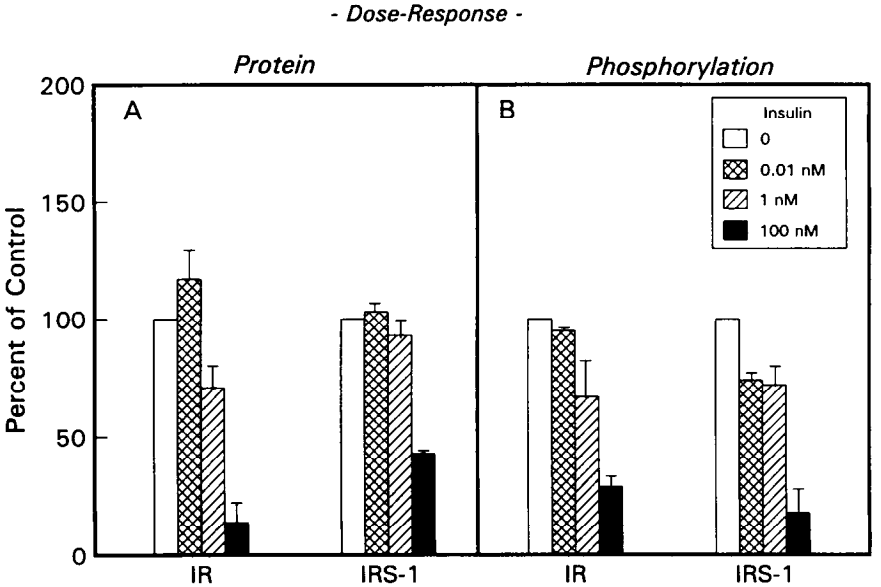

Fig. 12. Insulin Receptor and IRS-1 Protein (A) and Tyrosine Phosphorylation Levels (B) during the Dose-Response of Chronic Insulin in 3T3-F442A Adipocytes

Scanning densitometry was performed on autoradiograms from four experiments for insulin receptor concentration, five experiments for IRS-1 concentration, and four experiments for tyrosine phosphorylation. The data are expressed as mean ± SEM and are normalized per protein.

levels are not the only determinant of IRS-1 phosphorylation levels (19). Whether these regulatory defects induced by chronic hyperinsulinemia reflect alterations in receptor and IRS-1 serine phosphorylation or some other regulatory event is unknown. Recently, Rice *et al.* (29, 34) demonstrated that chronic exposure of 3T3-L1 adipocytes to insulin induced a decrease in IRS-1 protein levels in accordance with our results. We also showed similar results using insulin-sensitive rat hepatoma cells (M. J. A. Saad, F. Folli, and C. R. Kahn, submitted). The present study demonstrates that the reduction in insulin receptor and IRS-1 protein levels in 3T3-F442A adipocytes is not due to a decrease in the mRNA of these two proteins, suggesting that this desensitization process may be related to an increase in the rate of degradation of these proteins. Indeed, pulse-chase labeling studies in 3T3-L1 adipocytes demonstrate that the rate of degradation of IRS-1 protein is about 10 times faster in insulin-treated cells than in control cells (34).

Taken together, these results suggest that hyperinsulinemia by itself can induce alterations in three of the earliest steps in insulin action which may account, in part, for the phenomenon of insulin-induced desensitization. Altered autophosphorylation of the insulin receptor and kinase activity of the receptor toward endogenous substrate, as indicated by the phosphorylation of IRS-1 and activation of PI 3-kinase, also appear to be important in the altered signal transduction observed in cultured cells expressing mutant insulin receptors. Indeed, direct evidence that this pathway is required for insulin action is most clearly derived from experiments in which kinase-deficient receptors are overexpressed in various cell types (35, 46). In these cells, the absence of biological activity correlates specifically with the inability of the mutant receptor to stimulate the tyrosyl phosphorylation of IRS-1 and consequently PI 3-kinase. Thus, reduced insulin receptor and IRS-1 phosphorylation and decreased PI 3-kinase activity may have an important role in reduced insulin action induced by chronic insulin treatment.

Chronic dexamethasone treatment induces a slight increase in insulin receptor levels in 3T3-F442A adipocytes. A similar increase is observed in Fao cells (M. J. A. Saad, F. Folli, and C. R. Kahn, 1993; submitted), as well as several other cell types (36, 37). This effect of dexamethasone occurs with no change in the level of insulin receptor mRNA, suggesting that a decrease in receptor degradation may account for the increase in receptor protein levels. Despite the increase in receptor protein, insulin-induced receptor autophosphorylation is decreased after dexamethasone treatment. The mechanisms for the decrease is unclear but might involve, for example, increased tyrosine phosphatase activity or increased serine-threonine phosphorylation of the receptor.

IRS-1 protein levels were decreased after dexamethasone treatment in accordance with previous data (29) and could be accounted for by a decrease in the level of mRNA of IRS-1. In parallel there is a decrease in IRS-1 phosphorylation, reflecting the decrease in protein levels as well as a decrease in the stoichiometry of phosphorylation level. Recently we demonstrated an opposite effect of dexamethasone on IRS-1 protein levels and phosphorylation in another cell line, the Fao hepatoma cell, *i.e.* both IRS-1 protein and phosphoryl-

ation increased after dexamethasone treatment. This confirms our previous data in animal tissues that IRS-1 expression has a tissue-specific regulation (18, 19).

Chronic corticosteroid treatment caused a mild and nonsignificant decrease in IRS-1-associated PI 3-kinase activity. This phenomenon is in contrast to the decrease in IRS-1 protein and phosphorylation level and is due, at least in part, to an increase in the levels of PI 3-kinase protein. The increase in insulin-induced activation of PI 3-kinase activity during the differentiation process in these cells, as well as its differential regulation by insulin and dexamethasone in the differentiated state, parallels the changes observed in insulin-stimulated glucose transport (5, 38). *In vivo* data seem to corroborate these findings. Glucose transport, as well as IRS-1 phosphorylation and PI 3-kinase activation after insulin stimulation, are severely impaired in hyperinsulinemic ob/ob and goldthioglucose obese mice (18, 20, 21, 39). Furthermore, a specific inhibitor of PI 3-kinase (R. Cheatham, C. Vlahos, and C. R. Kahn, manuscript in preparation) is able to selectively and efficiently inhibit glucose transport in these cells, without affecting other postreceptor insulin signaling pathways such as the microtubule-associated protein kinase. Thus, it is reasonable to speculate that the IRS-1/PI 3-kinase pathway may be linked to activation of glucose transport and this cell type may help us to elucidate the regulatory mechanisms involved in this process.

It has been recently shown that specific phosphorylation sequences in the IRS-1 molecule are involved in the interaction with specific SH2 domains containing proteins. More specifically, phosphorylated YVNI and YIDL motifs bind GRB-2 and SHPTP-2, respectively (28), whereas phosphorylated YMXM or YXXM motifs bind PI 3-kinase (16). GRB-2 is the mammalian homolog of the *Caenorhabditis elegans* protein *sem*-5, a critical component in the signal transduction pathway that links receptor tyrosine kinases to *ras* activation (27, 28, 40). SHPTP2/syp appears to be the mammalian homologue of the *Drosophila* gene *corkscrew* (*csw*) (41), which, in combination with the polehole gene product, belong to a terminal transduction pathway of the signals generated by torso, a receptor protein tyrosine kinase (42). Thus IRS-1 appears to be a multisite docking protein, capable of activating or inhibiting different signal generating pathways. Based on this and other studies, it seems likely that the final metabolic or growth-promoting effects of insulin may be dictated by tissue-specific expression and regulation of IRS-1 and interacting proteins, as well as other components of the insulin signaling pathway. Changes in the stoichiometry of phosphorylation could be associated with changes in the sites phosphorylated and thus serve as a point for divergence of insulin resistance as well as insulin signaling.

In conclusion, during differentiation of 3T3-F442A fibroblast to adipocytes there is a marked increase in insulin receptor, IRS-1 protein, phosphorylation and PI 3-kinase levels and in the association/activation between IRS-1/PI 3-kinase. Chronic insulin treatment induces a decrease in insulin receptor and IRS-1 level and phosphorylation and also in the association/activation of IRS-1 with PI 3-kinase. Chronic dexamethasone treatment induces an increase in insulin receptor and PI 3-kinase protein levels and a decrease in IRS-1 protein and phosphorylation levels. This results in a mild decrease in the association/activation of IRS-1/PI 3-kinase. Some of these effects are at the transcriptional level; others are not. This study underscores the importance of the complex regulation of different components of the insulin signal transduction pathway and further emphasize the concept of a tissue-, as well as hormone-specific, insulin resistance.

MATERIALS AND METHODS

Materials

Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad Laboratories (Richmond, CA). HEPES, phenylmethylsulfonylfluoride (PMSF), antipain, aprotinin, leupeptin, peptstatin, benzamidine hydrochloride, dithiothreitol (DTT), ATP, phosphatidylinositol-4-monophosphate, Triton X-100, Tween 20, glycerol, and BSA (fraction V) were from Sigma Chemical Co. (St. Louis, MO). Nonidet NP-40 was from Calbiochem (La Jolla, CA), phosphatidylinositol from Avanti (Alabaster, AL), silica gel TLC plates from Merck (Gibbstown, NJ), protein A-sepharose 6 MB from Pharmacia (Upsala, Sweden), [^{125}I] protein A from ICN Biomedicals (Costa Mesa, CA), [γ-^{32}P]ATP from New England Nuclear-Dupont (Wilmington, DE), and nitrocellullose paper (BA85, 0.2 μm) was from Schleicher & Schuell (Keene, NH). Polyclonal antiphosphotyrosine (αpY) antibodies were raised in rabbits and affinity purified on phosphotyramine columns (43). Anti-IRS-1 antibodies were raised in rabbits using a synthetic peptide (Pep 80) derived from the amino acid sequence (YIPGATMGTSPALTGDEAA) corresponding to residues 489–507 of the protein and affinity purified on a column prepared by coupling the synthetic peptide to Affi-Gel 10 (Bio-Rad) as previously described (11). Anti-rat-PI 3-kinase (p85 subunit) antiserum was from UBI (Lake Placid, NY). Antiinsulin receptor antibody was raised in rabbits using a synthetic peptide derived from the amino acid sequence (KKNGRILTLPRSNPS) corresponding to the C terminus of the β-subunit of the protein.

Cell Culture

3T3-F442A cells were grown in DMEM medium with 10% calf serum in 5% humidified CO_2 atmosphere. Cells were differentiated in 10% CO_2 in DMEM medium supplemented with 10% fetal calf serum for 8 days. The differentiation was enhanced by the addition of 5 μg/ml insulin for the first 6 days. Cells were then withdrawn from insulin and maintained in DMEM plus 10% fetal calf serum for 2 days. By the end of this period 90–95% of the cells acquired the characteristic adipocyte morphology.

The differentiated adipocytes (after day 8 and before day 11) were chronically treated with insulin or dexamethasone with a time course and dose-response as described in *Results*. Cells not treated with these hormones were used as controls. In all experiments the cells were also acutely stimulated without or with 100 nM porcine insulin for 1 min at 37 C. The incubations were stopped by quickly removing the medium and freezing the cell monolayers with liquid nitrogen. Cells were homogenized immediately in 1 ml 50 mM HEPES (pH

7.4), containing 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM EDTA, 2 mM sodium vanadate, 1% Triton X-100, 2 mM PMSF, and 0.1 mg/ml aprotinin (buffer A). The monolayers were scraped from the dishes, and the insoluble material was sedimented by centrifugation at 55,000 rpm at 4 C in a Beckman 70.1 Ti rotor for 60 min (Beckman Instruments, Palo Alto, CA). The supernatant was used as a sample or immunoprecipitated with anti-IRS-1 antibody.

Protein Analysis by Immunoblotting

The samples were treated with Laemmli sample buffer (44) with 100 mM DTT and heated in a boiling water bath for 4 min. For total extracts, similar size aliquots of sample (150 μg protein) were subjected to SDS-PAGE (6% tris acrylamide) in a Bio-Rad miniature slab gel apparatus (45). Electrotransfer of proteins from the gel to nitrocellulose was performed for 1 h at 90 V (constant) in the Bio-Rad miniature transfer apparatus (Mini-Protean) as described (30), but with 0.02% SDS added to the transfer buffer to enhance elution of high molecular mass proteins. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4 C in blocking buffer (3% BSA, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). Prestained mol wt standards were myosin (205 K), β-galactosidase (116 K), BSA (80 K), and ovalbumin (49.5 K).

The nitrocellulose blot was incubated with antiinsulin receptor antibody (0.3 μg/ml), antiphosphotyrosine antibodies (αpY, 0.3 μg/ml), anti-IRS-1 antibodies (αPep 80, 0.3 μg/ml), or with anti-PI 3-kinase antibody (αp85, 1:500 dilution) diluted in blocking buffer for 4 h at 22 C and washed for 60 min in the blocking buffer without BSA. The blots were then incubated with 2 μCi [^{125}I]protein A (30 μCi/μg) in 10 ml blocking buffer for 1 h at 22 C and washed again as described above for 2 h. [^{125}I] protein A bound to the antiphosphotyrosine and anti-IRS-1 antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −70 C for 12–48 h. Band intensities were quantitated by optical densitometry (Molecular Dynamics, Sunnyvale, CA) of the developed autoradiogram.

PI 3-Kinase Activity

PI 3-kinase activity was measured by *in vitro* phosphorylation of phosphatidylinositol (17, 46). The cells were incubated in the absence or presence of insulin (100 nM) for 1 min and washed once with ice-cold PBS and twice with buffer B [20 mM Tris (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4]. The cells were solubilized on ice with ice-cold buffer B containing 1% NP-40, 10% glycerol, aprotinin (2 μg/ml), antipain (10 μg/ml), leupeptin (5 μg/ml), pepstatin (0.5 μg/ml), benzamidine (1.5 mg/ml), and PMSF (34 μg/ml), and insoluble material was removed by centrifugation at 15,000 rpm in a 70Ti rotor (Beckman) for 50 min. IRS-1 was immunoprecipitated from aliquots of the supernatant containing 1 mg protein with anti-IRS-1 (αPep80, 0.3 μg/ml) or preimmune rabbit immunoglobulin G (0.3 μg/ml) followed by protein-A-sepharose 6 MB. The immunoprecipitates were washed successively in PBS containing 1% NP-40 and 100 μM Na_3VO_4 (3 times), 100 mM Tris (pH 7.5) containing 500 mM $LiCl_2$ and 100 μM Na_3VO_4 (3 times), and 10 mM Tris (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4 (2 times). The pellets were resuspended in 50 μl 10 mM Tris (pH 7.5) containing 100 mM NaCl and 1 mM EDTA. To each pellet was added 10 μl 100 mM $MgCl_2$ and 10 μl phosphatidylinositol (2 μg/μl) sonicated in 10 mM Tris (pH 7.5) with 1 mM EGTA.

The PI 3-kinase reaction was started by the addition of 10 μl 440 μM ATP containing 30 μCi [^{32}P]ATP. After 10 min at room temperature with constant shaking, the reaction was stopped by the addition of 20 μl 8 N HCl and 160 μl $CHCl_3$-methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate (Merck) coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$-CH_3OH-H_2O-NH_4OH (60:47:11.3:2), dried, and visualized by autoradiography. The radioactivity in spots that comigrated with a PI-4 standard was measured by Cerenkov counting.

Quantitative PCR

Quantitative PCR was performed as previously described (47). For IRS-1, the 5′ primer was AGTGGCCATGGCTCCAC, and the 3′-primer was TTGCCACCCATGCAGAT; for insulin receptor, the 5′-primer was TTCCGAGACCTCAGTTTC, and the 3′-primer was TGTGACTTACAGATGGT; for β_2- microglobulin, the 5′-primer was TTCAGCAAGGACTGGTCT, and the 3′-primer was CTGCTTACATGTCTCGAT. Primers for the insulin receptor and β_2- microglobulin spanned at least one intron to avoid contamination of the PCR product by amplification of genomic DNA. RNA was extracted by RNAzol method (Cinna/Biotecx Laboratories, Houston, TX). Total RNA (1 μg) from 3T3-F442A cells obtained during differentiation or after treatment with insulin or dexamethasone were reverse transcribed into cDNA using all three 3′- primers (1 pmol primer/reaction). After [^{32}P] end-labeling of the PCR primers, 5% of the total cDNA was used as a template for the PCR reaction. The final PCR conditions were 10 pmol of each primer/reaction, denaturing at 94 C for 30 sec, annealing at 57 C for 30 sec, and extension at 72 C for 1 min. Based on pilot tests, linear amplification of IRS-1 mRNA was achieved using 50 ng total RNA/reaction and 21–27 cycles (47). The final PCR products were separated on a 5% acrylamide gel in 0.5 × Tris/borate/EDTA buffer. Gels were dried and subjected to autoradiography, and the radioactivity in the bands was determined by Cerenkov counting. As a negative control, the reverse transcription reaction followed by PCR was performed without addition of RNA. As positive controls, 10 ng human IRS-1 or insulin receptor cDNA was used as a PCR template. To determine the amount of IRS-1 PCR product derived from any contaminating genomic DNA, the reverse transcription and PCR reactions were also performed by omitting the reverse transcriptase.

Assay of PTPase Activity

The assay of PTPase activity was performed using a synthetic peptide derived from the autophosphorylated regulatory region of the insulin receptor β-subunit (residues 1154–1165) as a phosphatase substrate (48). The peptide was labeled with insulin receptors that were solubilized from a membrane fraction of Chinese hamster ovary cells transfected with the cloned human insulin receptor and partially purified by wheat-germ-agglutinin-agarose chromatography. Aliquots of the lectin column eluate were incubated with 1 μM insulin for 30 min at 4 C, after which 500 μCi [^{32}P]ATP were added at a final concentration of 25 μM, followed by $MnCl_2$ to 5 mM and 1 mg of the 1154–1165 peptide in a volume of 350 μl. After 16 h at 4 C, 1 ml of 5% (wt/vol) trichloroacetic acid was added, and the ^{32}P-labeled peptide in the supernatant was purified on AG1-X2 acetate resin followed by passage through a C18 Sep-Pak cartridge (Waters Associates, Milford, MA) and lyophilized. When prepared in this way, the peptide is monophosphorylated on tyrosine-1162 residue. PTPase activity was assayed by incubating portions of the cell extracts with labeled substrate in a reaction buffer containing 1 mM DTT and 2 mM EDTA in 50 mM HEPES, pH 7.0, at 30 C. Reactions were terminated by the addition of 1 mM H_2SO_4 and 5 mM silicotungstic acid, and [^{32}P]phosphate released from the labeled peptide was measured by organic extraction of the supernatant.

Other

For insulin binding, cells grown in six-well plates were washed twice with PBS plus 1% BSA and incubated in the same

medium supplemented with protease inhibitors and 90 pM [^{125}I] insulin (1.4 cpm/fmol; ICN Radiochemicals) for 2 h at 4 C. The cells were washed three times with cold PBS and solubilized in 1% Triton X-100. The nonspecific binding, determined in the presence of 400 nM nonradioactive insulin, was subtracted. Assays were performed in triplicate. Protein determination was performed by the Bradford dye method (49) using the Bio-Rad reagent and BSA as the standard.

Experiments were always performed studying samples from the chronically dexamethasone or insulin-treated cells in parallel with a control cells. During the differentiation the control is 3T3-F442A fibroblasts on day 0. Comparisons were made using paired and unpaired *t* test as appropriate. The minimal level of significance used was $P < 0.05$.

Experimental Animals

The animals used for the required experiments in this report were treated in accord with the "Guidelines for Care and Use of Experimental Animals."

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REFERENCES

1. Green H, Kehinde O 1976 Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7:105–113
2. Spooner PM, Chernick SS, Garrison MM, Scow RO 1979 Development of lipoprotein lipase activity and accumulation of triacylglycerol in differentiating 3T3-L1 adipocytes: effect of prostaglandin F2α, 1-methyl-3-isobutylxanthine, prolactin and insulin. J Biol Chem 254:1305–1310
3. Rubin CS, Lai E, Rosen OM 1977 Acquisition of increased hormone sensitivity during *in vitro* adipocyte development. J Biol Chem 252:3554–3557
4. Reed BC, Kaufman SH, Mackall JC, Student AK, Lane MD 1977 Alterations in insulin binding accompanying differentiation of 3T3-L1 preadipocytes. Proc Natl Acad Sci USA 71:4876–4880
5. Karlsson FA, Grunfeld C, Kahn CR, Roth J 1979 Regulation of insulin receptor and insulin responsiveness in 3T3-L1 fatty fibroblasts. Endocrinology 104:1383–1392
6. Sibley E, Kastelic T, Kelly TJ, Lane MD 1989 Characterization of the mouse insulin receptor gene promoter. Proc Natl Acad Sci USA 86:9732–9736
7. Garcia de Herreros A, Birnbaum MJ 1989 The acquisition of increased insulin- responsive hexose transport in 3T3-L1 adipocytes correlates with expression of a novel transporter gene. J Biol Chem 264:19994–19999
8. Rosen O 1987 After insulin binds. Science 237:1452–1458
9. Kasuga M, Karlsson FA, Kahn CR 1982 Insulin stimulates the phosphorylation of the 95,000 dalton subunit of its own receptor. Science 215:185–187
10. White MF, Maron R, Kahn CR 1985 Insulin rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells. Nature 318:183–186
11. Rothenberg PL, Lane WS, Karasik A, Backer J, White MF, Kahn CR 1991 Purification and partial sequence analysis of pp185, the major cellular substrate of the insulin receptor tyrosine kinase. J Biol Chem 266:8302–8311
12. Sun XJ, Rothenberg PL, Kahn CR, Backer JM, Araki E, Wilden P, Cahill DA, Goldstein BJ, White MF 1991 Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature 352:73–77
13. Maegawa H, Olefsky JM, Thies S, Boyd D, Ullrich A, McClain DA 1988 Insulin receptors with defective tyrosine kinase inhibit normal receptor function at the level of substrate phosphorylation. J Biol Chem 263:12629–12637
14. Ebina Y, Araki E, Taira M, Shimada F, Mori M, Craik CS, Siddle K, Pierce SB, Roth RA, Rutter WJ 1987 Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin and antibody-stimulated glucose uptake and receptor kinase activity. Proc Natl Acad Sci USA 84:704–708
15. White MF, Livingston JN, Backer JM, Lauris V, Dull TJ, Ulrich A, Kahn CR 1988 Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. Cell 54:641–649
16. Backer JM, Myers Jr MG, Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skolnik EY, Schlessinger J, White MF 1992 The phosphatidylinositol 3-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J 11:3469–3479
17. Folli F, Saad MJA, Backer JM, Kahn CR 1992 Insulin stimulation of phosphatidylinositol 3-kinase activity and association with IRS-1 in liver and muscle of the intact rat. J Biol Chem 267:22171–22177
18. Saad MJA, Araki E, Miralpeix M, Rothenberg PL, White MF, Kahn CR 1992 Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. J Clin Invest 90:1839–1849
19. Saad MJA, Folli F, Kahn JA, Kahn CR 1993 Modulation of insulin receptor, insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone treated rats. J Clin Invest 92:2065–2072
20. Folli F, Saad MJA, Backer JM, Kahn CR 1993 Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin resistant and insulin-deficient diabetes mellitus. J Clin Invest 92:1787–1794
21. Heydrick SJ, Jullien D, Gautier N, Tanti JF, Giorgetti S, Van Obberghen E, Le Marchand-Brustel Y 1993 Defect in skeletal muscle phosphatidylinositol 3-kinase in obese insulin-resistant mice. J Clin Invest 91:1358–1366
22. Giorgino F, Almahfouz A, Goodyear LD, Smith RJ 1993 Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle *in vivo*. J Clin Invest 91:2020–2030
23. Myers Jr MG, Sun XJ, Cheatham B, Jachna BR, Glasheen EM, Backer JM, White MF 1993 IRS-1 is a common element of insulin and IGF-1 signaling to the phosphatidylinositol 3′-kinase. Endocrinology 132:1421–1430
24. Wang L, Keegan AD, Li W, Lienhard GE, Pacini S, Gutkind JS, Myers MG, Sun XJ, White MF, Aaronson SA, Paul WE, Pierce JH 1993 Common elements in IL-4 and insulin signaling pathways in factor dependent hematopoietic cells. Proc Natl Acad Sci USA 90:4032–4036

25. Koch CA, Anderson D, Moran MF, Ellis C, Pawson T 1991 SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 252:668–674
26. Kuhne' MR, Pawson T, Lienhard GE, Feng G-H 1993 The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp. J Biol Chem 268:11479–11481
27. Skolnik EY, Lee C-H, Batzer A, Vicentini LM, Zhou M, Myers MG, Backer JM, Ulrich A, White MF, Schlessinger J 1993 The SH2-SH3 domain-containing protein GRB-2 interacts with tyrosine-phosphorylated IRS-1 and Shc: implications for insulin control of *ras* signalling. EMBO J 12:1929–1936
28. Sun XJ, Crimmins DL, Myers MG, Miralpeix M, White MF 1993 Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. Mol Cell Biol 13:7418–7428
29. Rice KM, Lienhard GE, Garner CW 1992 Regulation of the expression of pp160, a putative insulin receptor signal protein, by insulin, dexamethasone and iso-methyl-3-isobutylxanthine in 3T3-L1 adipocytes. J Biol Chem 267:10163–10167
30. Mooney RA, Bordwell KL 1992 Differential dephosphorylation of the insulin receptor and its 160 kDa substrate (pp160) in rat adipocytes. J Biol Chem 267:14054–14060
31. Amatruda JM, Livingston JN, Lockwood DH 1985 Cellular mechanisms in selected states of insulin resistance: human obesity, glucocorticoid excess and chronic renal failure. Diab Metab Rev 3:293–317
32. Treadway JL, Whittaker J, Pessin JE 1989 Regulation of the insulin receptor kinase by hyperinsulinemia. J Biol Chem 261:15136–15143
33. Lima FB, Thies RS, Garvey WT 1991 Glucose and insulin regulate insulin sensitivity in primary cultured adipocytes without affecting insulin receptor kinase actvity. Endocrinology 128:2415–2426
34. Rice KM, Turnbow MA, Garner CW 1993 Insulin stimulates the degradation of IRS-1 in 3T3-L1 adipocytes. Biochem Biophys Res Commun 190:961–967
35. Kapeller R, Chen KS, Yoakim M, Schaffhausen BS, Backer JM, White MF, Cantley LC, Ruderman NB 1991 Mutations in the juxtamembrane region of the insulin receptor impair activation of phosphatidylinositol 3-kinase by insulin. Mol Endocrinol 5:769–77749
36. Fantus G, Saviolakis GA, Hedo JA, Gorden P 1982 Mechanisms of glucorticoid-induced increase in insulin receptors of cultured lymphocytes. J Biol Chem 257:8277–8283
37. Knutson VP 1986 The acute and chronic effects of glucocorticoids on insulin receptor and insulin responsiveness. Transient fluctuations in intracellular receptor level parallel transient fluctuations in responsiveness. J Biol Chem 261:10306–10312
38. Grunfeld C, Baird K, Van Obberghen E, Kahn CR (1981) Glucocorticoid-induced insulin resistance *in vitro*: evidence for both receptor and postreceptor defects. Endocrinology 109:1723–1730
39. Le-Marchand-Brustel Y, Jeanrenaud B, Freychet P 1978 Insulin binding and effects in isolated soleus muscle of lean and obese mice. Am J Physiol 234:E348-E358
40. Baltensperger K, Kozma LM, Cherniack AD, Klarlund JK, Chawla A, Banerjee U, Czech MP 1993 Binding of the ras activator son of sevenless to insulin receptor substrate-1 signaling complexes. Science 260:1950–1952
41. Freeman RM, Plutzky J, Neel BG 1992 Identification of a human src homology 2-containing protein-tyrosine phosphatase: a putative homolog of *Drosophila* corkscrew. Proc Natl Acad Sci USA 89:11239–11243
42. Bishop JM 1991 Molecular themes in oncogenesis. Cell 64:235–248
43. Pang DT, Sharma BR, Shafer JA 1985 Purification of the catalytically active phosphorylated form of insulin receptor kinase by affinity chromatography with O-phosphotyrosil-binding antibodies. Arch Biochem Biophys 242:176–186
44. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the bacteriophage. Nature 227:680–685
45. Towbin H, Staehlin J, Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci USA 76:4350–4354
46. Backer JM, Schroeder GG, Kahn CR, Myers Jr MG, Wilden PA, Cahill DA, White MF 1992 Insulin stimulation of phosphatidylinositol 3-kinase activity maps to insulin receptor regions required for endogenous substrate phosphorylation. J Biol Chem 267:1367–1374
47. Araki E, Sun XJ, Haag III, BL, Chuang LM, Zhang Y, Yang-Feng TL, White MF, Kahn CR 1993 Human skeletal muscle insulin receptor substrate-1: characterization of the cDNA, gene and chromosomal localization. Diabetes 42:1041–1054
48. Hashimoto N, Zhang WR, Goldstein BJ 1992 Insulin receptor and epidermal growth factor receptor dephosphorylation by three major rat liver protein-tyrosine phosphatases expressed in a recombinant bacterial system. Biochem J 284:569–576
49. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein dye binding. Anal Biochem 72:248–254