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Posttranscriptional Control of Adipocyte Differentiation through Activation of Phosphoinositide 3-Kinase*

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Adipocytes are essential for regulation of energy homeostasis, not only by serving as a massive energy reserve by secreting hormones and cytokines that affect food intake or metabolic regulation (1, 2). Because mature adipocytes do not undergo cell division, the number of these cells is thought to increase as a result of differentiation of preadipocytes into mature adipocytes (1, 2). Studies with cultured cell lines have shed light on the regulation, especially at the transcriptional level, of adipocyte differentiation (1–3).

Trans-activation of adipocyte-specific genes is thought to be induced by a cascade of key transcriptional factors including PPARg, C/EBP families (5–7), and ADD1/SREBP1 (8), which combine to regulate each other (9–12). Thus, these factors coordinate the expression of genes, such as those encoding insulin-sensitive glucose transporter GLUT4, the fatty acid-binding protein aP2, and adipisin, which contribute to the creation and maintenance of the adipocyte phenotype (13). In addition to regulation at the transcriptional level, posttranscriptional mechanisms also may be important in determining the amounts of various proteins during adipocyte differentiation. For example, activation of PPARg by a thiazolidinedioline derivative has been shown to result in a decrease in the amount of P2Ac protein without an effect on the abundance of its mRNA (14), suggesting that PPARg regulates not only transcriptional but also posttranscriptional mechanisms. However, the regulatory events that occur subsequent to sequential gene transcription during adipogenesis remain largely unknown.

Phosphoinositide (PI)3-kinase, a lipid kinase composed of an Src homology 2 domain-containing regulatory subunit (p85) and a 110-kDa catalytic subunit (p110), catalyzes phosphorylation of the D3 position of phosphoinositides. This enzyme is important in a wide variety of cellular processes, including intracellular trafficking, organization of the cytoskeleton, cell growth and transformation, and prevention of apoptosis (15, 16). This enzyme has also been suggested to play a role in the differentiation of several cell lines (17, 18). We have now investigated the role of PI 3-kinase in adipocyte differentiation and, more specifically, at which level in the differentiation process it might act.

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents—Antibodies to phosphotyrosine (PY20 and RC20) were obtained from Transduction Laboratories. Antibodies to p85 (F12) (19), to IRS1 (1D6 and pep80) (20), to PPARg (PY20) to GLUT4 (20), to IRS2, and to the insulin-like growth factor (IGF) 1 receptor (aIR3) were kindly provided by D. Bernlohr (University of Minnesota, St. Paul, MN), M. F. White (Joslin Diabetes Center, Boston, MA), and R. A. Roth (Stanford University, Stanford, CA), respectively. A monoclonal antibody to the insulin receptor (29B4) was obtained from Santa Cruz Biotechnology. Probes for Northern blot analysis were as described (9, 22, 23). Genestin, rapamycin, and 5,8,11,14-eicosatetraynoic acid (ETYA) were obtained from Sigma. Triglazozone was kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan).

Cell Culture—3T3-F442A or 3T3-L1 preadipocytes were maintained under an atmosphere of 7.5% CO2 in Dulbecco’s minimum essential medium containing 25 mm glucose and supplemented with 10% calf serum, 2 mm l-glutamine, 500 ml penicillin, and 50 µg/ml streptomycin. Adipocyte differentiation was induced by treating confluent 3T3-F442A cells for 2 days with insulin (5 µg/ml) and ETYA (100 µM) in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, and then for 2 days with insulin (5 µg/ml) dissolved in the same medium. Alternative

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* The abbreviations used are: PI, phosphoinositide; IRS, insulin receptor substrate; ETYA, 5,8,11,14-eicosatetraynoic acid; IBMX, isobutylmethylxanthine; b-gal, b-galactosidase; m.o.i., multiplicity of infection; pfu, plaque-forming unit(s); IGF, insulin-like growth factor.
tively, the cells were treated with troglitazone (30 μg/ml) in the same medium for only 2 days. Differentiation of 3T3-L1 preadipocytes was induced by exposing confluent cells to insulin (5 μg/ml), isobutylmethylxanthine (IBMX) (0.5 mM), and dexamethasone (0.25 μM) for 2 days, and then to insulin (5 μg/ml) alone for an additional 2 days. After incubation with these reagents, the basal medium was replenished every other day. Staining of cells with oil red O was performed as described (22).

Adenovirus Vectors—An adenovirus vector encoding Δp85, bovine p85α lacking the binding site for the catalytic subunit of PI 3-kinase, was as described previously (20). Complementary DNA encoding p110N, a COOH-terminal deletion mutant of bovine p110 regulatory subunits of the enzyme2. An adenovirus vector containing the lacZ gene (AdCAcLacZ) (25) was kindly provided by I. Saito (Tokyo University, Japan). For adenovirus-mediated gene transfer, cells cultured to subconfluence were infected with various adenovirus vectors at the indicated multiplicity of infection (m.o.i.). Two days after infection, differentiation was induced as described above.

PI 3-Kinase Assay, Northern Blot, and Immunoblot Analysis—3T3-F442A or 3T3-L1 preadipocytes were induced to differentiate as described above. At the indicated times, the cells were lysed as described (20), and PI 3-kinase activity in immunoprecipitates prepared with antibodies to phosphotyrosine or to IRS1 was assayed as described (20). For Northern blot analysis, cells were harvested at the indicated time and total RNA was extracted with the use of an RNeasy kit (Qiagen). Twenty micrograms of total RNA were then subjected to Northern analysis with various probes as described previously (9, 22, 23). Lysates containing ~100 μg of protein were subjected to immunoblot analysis.

RESULTS

Activation of PI 3-Kinase during Adipocyte Differentiation—Differentiation of 3T3-F442A preadipocytes was induced with insulin and the arachidonic acid analog ETYA, and, at various times, the cells were lysed and PI 3-kinase activity was assayed in immunoprecipitates prepared with antibodies to phosphotyrosine. PI 3-kinase activity was markedly increased 4 days after induction of differentiation, but it had returned to basal values by 8 days (Fig. 1A). A similar activation of PI 3-kinase was observed when differentiation was induced by troglitazone (Fig. 1A), a direct activator of PPARγ (26); however, the peak of PI 3-kinase activity was delayed by 2 days compared with that in cells treated with insulin and ETYA, consistent with the 2-day delay in lipid accumulation apparent in troglitazone-treated cells (data not shown). Furthermore, differentiation of 3T3-L1 preadipocytes induced by a combination of insulin, IBMX, and dexamethasone was accompanied by a transient activation of PI 3-kinase at a similar stage (Fig. 1B). These data suggested that activation of PI 3-kinase is not a direct effect of the reagents used to induce differentiation, but is rather a characteristic event associated with adipocyte differentiation.

Role of Tyrosine Phosphorylation of IRS1 in PI 3-Kinase Activation during Adipocyte Differentiation—We next investigated which tyrosine-phosphorylated protein associates with PI 3-kinase during adipocyte differentiation. Specific antibodies to the p85 regulatory subunit of PI 3-kinase precipitated an ~180-kDa tyrosine-phosphorylated protein from 3T3-F442A cells induced to differentiate with insulin and ETYA or with troglitazone (Fig. 2A). The time course of the association of this phosphorylated protein with p85 was similar to that of PI 3-kinase activation during differentiation. Four days after induction of differentiation with insulin and ETYA, a 180-kDa tyrosine-phosphorylated protein was also detected in immunoprecipitates prepared with antibodies to p110 catalytic subunit of PI 3-kinase; furthermore, immunoprecipitates prepared with antibodies to p85 reacted with antibodies to IRS1 (Fig. 2B). Immunoprecipitates prepared with antibodies to IRS1 also reacted with antibodies to phosphotyrosine (Fig. 2B). Moreover, PI 3-kinase activity in immunoprecipitates prepared with antibodies to IRS1 increased during adipocyte differentiation with a time course similar to that observed for PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates (Figs. 1A and 2C). These data suggested that tyrosine phosphorylation of IRS1 contributes to the activation of PI 3-kinase during adipogenesis.

Immunoprecipitates prepared with antibodies to IRS2, a protein closely related to IRS1, also reacted with antibodies to phosphotyrosine (Fig. 2B); the corresponding tyrosine-phosphorylated protein migrated slightly more slowly than that precipitated with antibodies to IRS1 (Fig. 2B), consistent with the slightly larger molecular size of IRS2 relative to that of IRS1 (27). Moreover, immunoprecipitates prepared with antibodies to p85 reacted with antibodies to IRS2, suggesting that IRS2 also undergoes tyrosine phosphorylation and associates with PI 3-kinase during adipocyte differentiation (Fig. 2B). However, the mobilities of the tyrosine-phosphorylated proteins detected in the immunoprecipitates prepared with antibodies to p110 or to p85 were similar to that of the phosphoprotein precipitated with antibodies to IRS1; they were slightly greater than that of IRS2. It is thus likely that the major tyrosine-phosphorylated protein associated with PI 3-kinase is IRS1, although IRS2 is also phosphorylated and may, at least in part, contribute to the activation of PI 3-kinase during adipocyte differentiation.

Because IRS proteins are well characterized substrates of the insulin receptor family of tyrosine kinases, we examined whether the insulin receptor or the IGF-1 receptor is activated during adipocyte differentiation. The amount of the insulin receptor protein was markedly increased during differentiation.
Role of PI 3-Kinase in Adipocyte Differentiation

Tyrosine phosphorylation of IRS1 and its association with PI 3-kinase during differentiation of 3T3-F442A cells. A, association of tyrosine-phosphorylated proteins with PI 3-kinase during differentiation of 3T3-F442A cells induced with insulin and ETYA (Fig. 2D), as consistent with a previous report (28). However, tyrosine phosphorylation of ~100 kDa proteins, of which molecular size correspond to those of the insulin and IGF-1 receptors, was barely detectable in the immunoprecipitates prepared with antibodies to the insulin receptor (Fig. 2D) or to the IGF1 receptor (data not shown). When the membrane was exposed to a film for a long time, faint tyrosine-phosphorylated bands were evident in the immunoprecipitates with antibodies to the insulin receptor at 2–4 days after the induction of differentiation with insulin and ETYA. However, the extent of tyrosine phosphorylation during adipogenesis was much smaller than that in differentiated 3T3-F442A cells that were treated with 100 nM insulin for 10 min (Fig. 2D). At 4 days after induction, an ~180-kDa and an ~190-kDa tyrosine phosphorylated proteins, which are likely to be IRS1 and IRS2, were apparent in anti-phosphotyrosine immunoprecipitates (Fig. 2E); the extent of tyrosine phosphorylation of these proteins was similar to those of a 180-kDa and a 190-kDa protein in differentiated 3T3-F442A cells that were treated with insulin. A 100-kDa protein, of which molecular size corresponds to those of the insulin and the IGF-1 receptors, also became tyrosine-phosphorylated at 4 days after induction; however, the extent of tyrosine phosphorylation of the 100-kDa protein was, again, much less than that of a 100-kDa protein in differentiated 3T3-F442A cells that were treated with insulin. A 100-kDa protein, of which molecular size corresponds to those of the insulin and the IGF-1 receptors, also became tyrosine-phosphorylated at 4 days after induction; however, the extent of tyrosine phosphorylation of the 100-kDa protein was, again, much less than that of a 100-kDa protein in differentiated 3T3-F442A cells that were treated with insulin. A 100-kDa protein, of which molecular size corresponds to those of the insulin and the IGF-1 receptors, also became tyrosine-phosphorylated at 4 days after induction; however, the extent of tyrosine phosphorylation of the 100-kDa protein was, again, much less than that of a 100-kDa protein in differentiated 3T3-F442A cells that were treated with insulin. 

**FIG. 2.** Tyrosine phosphorylation of IRS1 and its association with PI 3-kinase during differentiation of 3T3-F442A cells. A, association of tyrosine-phosphorylated proteins with PI 3-kinase during differentiation. At the indicated times after induction of differentiation with insulin plus ETYA (upper panel) or with troglitazone alone (lower panel), cells were lysed and subjected to immunoprecipitation (IP) with antibodies to p85 (αp85). The immunoprecipitates were then subjected to immunoblot analysis with antibodies to phosphotyrosine (αPY). The positions of molecular size standards are indicated in kilodaltons. B, differentiation-dependent tyrosine phosphorylation of IRS1 and IRS2. Before or 4 days after induction of differentiation with insulin plus ETYA, cells were subjected to immunoprecipitation with antibodies to p110, to p85, to IRS1 (1D6), or to IRS2. The resulting immunoprecipitates were subjected to immunoblot analysis with antibodies to phosphotyrosine (RC20), to IRS1 (pep80), or to IRS2. C, activation of PI 3-kinase during differentiation. PI 3-kinase activity was precipitated with antibodies to IRS1 and assayed at the indicated times after induction of differentiation with insulin plus ETYA (open columns) or with troglitazone alone (closed columns). D, the insulin receptor does not undergo significant tyrosine phosphorylation during adipogenesis. 3T3-F442A cells induced to differentiate with insulin plus ETYA were lysed at the indicated times after induction; alternatively, 8 days after induction, the cells were serum-deprived for 16 h, treated with 100 nM insulin for 10 min, and then lysed. The lysates were subjected to immunoprecipitation (IP) with antibodies to the β subunit of the insulin receptor, then to immunoblot analysis with antibodies to the β subunit of the insulin receptor (upper panel) or to phosphotyrosine (middle and lower panels). The immunoblot probed with antibodies to phosphotyrosine, visualized using a chemiluminescent detection kit, was exposed to a film for 30 s (middle panel) or for 5 min (lower panel). E, tyrosine phosphorylation of various proteins during differentiation of 3T3 F442A cells. 3T3 F442A cells were lysed before or 4 days after induction of differentiation with insulin plus ETYA or, at 8 days after induction, the cells were serum-deprived for 16 h, treated with 100 nM insulin for 10 min, and then lysed. The lysates were subjected to immunoprecipitation (IP) with antibodies to phosphotyrosine and then to immunoblot analysis with antibodies to phosphotyrosine. Data are representative of three (A and B) or two (D and E) experiments. Quantitative data in C were means ± S.E. of three experiments.
Role of PI 3-Kinase in Adipocyte Differentiation

We finally examined the effect of rapamycin on the expression of GLUT4 protein because this reagent partially inhibits mitogen-stimulated bulk protein synthesis at posttranscriptional level.

We examined the effects of genistein, a broad spectrum pharmacological inhibitor of tyrosine kinases (29). Addition of genistein to 3T3-F442A cells 3 days after the induction of differentiation with insulin plus ETYA resulted in marked inhibition of the increase in GLUT4 mRNA, as well as similar increases in the amounts of adipin and ap2 proteins, was markedly inhibited by either αp85 or p110N at an m.o.i. (30 pfu/cell) sufficient to prevent lipid accumulation, these adipocyte marker proteins were undetectable by immunoblot analysis. These data suggested that activation of PI 3-kinase is required for expression of adipocyte-specific proteins at a posttranscriptional step.

Fig. 3. Effects of dominant negative mutants of PI 3-kinase on enzyme activity during differentiation. 3T3-F442A cells were infected with adenoviruses encoding β-gal (AxCA LacZ), αp85 (AxCAΔp85), or p110N (AxCAp110N) at an m.o.i. of 30 pfu/cell. After 48 h, the cells were induced to differentiate with insulin and ETYA (A) or with troglitazone (B). PI 3-kinase activity precipitated with antibodies to phosphotyrosine was assayed at the indicated times. Data are means ± S.E. of three independent experiments.

Lack of Effect of Inhibition of PI 3-Kinase Activation on Expression of PPARγ on Transcriptional Regulation of Genes Encoding Adipocyte Marker Proteins—We next examined the effects of the dominant negative mutants of PI 3-kinase on the expression of PPARγ, a key transcriptional regulator during adipocyte differentiation. Both PPARγ mRNA and protein were apparent within 2 days after induction of differentiation of 3T3-F442A cells with insulin plus ETYA, with the abundance of both being maximal at 4 days (Fig. 5). The increases in PPARγ mRNA and protein during adipocyte differentiation were not affected by infection of the cells with adenoviruses encoding either αp85 or p110N even at an m.o.i. of 30 pfu/cell (Fig. 5), a virus concentration sufficient for complete inhibition of lipid accumulation (Fig. 4). Furthermore, the differentiation-associated increase in the abundance of mRNAs encoding various adipocyte marker proteins, including GLUT4, adipin, and ap2, was not affected by either of the dominant negative mutants of PI 3-kinase (Fig. 6A). Moreover, the time course of the increase in GLUT4 mRNA did not differ between cells infected with viruses encoding αp85 or p110N and control cells (Fig. 6B). These data suggested that PI 3-kinase activity is not required for the transcriptional control of genes that encode adipocyte-specific proteins.

Requirement of PI 3-Kinase for the Expression of Adipocyte-Specific Proteins—We also investigated the effects of the dominant negative mutants of PI 3-kinase on the abundance of GLUT4, adipin, and ap2 proteins during adipocyte differentiation. The amount of GLUT4 was markedly increased at 4 days and maximal at 6 days after the induction of differentiation of 3T3-F442A cells with insulin plus ETYA (Fig. 7A). The increase in the amount of GLUT4, as well as similar increases in the amounts of adipin and ap2 proteins, was markedly inhibited by either αp85 or p110N at an m.o.i.-dependent manner (Fig. 7B); at an m.o.i. (30 pfu/cell) sufficient to prevent lipid accumulation, these adipocyte marker proteins were undetectable by immunoblot analysis. These data suggested that activation of PI 3-kinase is required for expression of adipocyte-specific proteins at a posttranscriptional step.

Effects of Genistein and Rapamycin on Adipocyte-Specific Protein Expression—To determine whether activation of PI 3-kinase at a specific stage is important for adipocyte differentiation, we examined the effects of genistein, a broad spectrum pharmacological inhibitor of tyrosine kinases (29). Addition of genistein to 3T3-F442A cells 3 days after the induction of differentiation with insulin plus ETYA resulted in marked inhibition of the increase in GLUT4 mRNA, as well as the activation of PI 3-kinase in anti-phosphotyrosine immunoprecipitates, normally apparent at 4 days (Fig. 8A). Genistein added at this time also inhibited the differentiation-associated increase in the amount of GLUT4 protein, without affecting the increase in GLUT4 mRNA (Fig. 8B). However, addition of genistein 6 days after induction of differentiation, at which time the activity of PI 3-kinase has already begun to decrease (Fig. 1A), did not inhibit expression of GLUT4 protein (Fig. 8B). Moreover, 8 days after induction of differentiation by insulin plus ETYA, at which time the activity of PI 3-kinase has already returned to basal values (Fig. 1A), infection of cells with viruses encoding either αp85 or p110N did not affect the amount of GLUT4 protein (data not shown). These data suggest that the transient increase in PI 3-kinase activity that occurs 4–6 days after induction of differentiation with insulin and ETYA is required for expression of adipocyte-specific proteins at a posttranscriptional level.

We finally examined the effect of rapamycin on the expression of GLUT4 protein because this reagent partially inhibits mitogen-stimulated bulk protein synthesis at posttranscriptional level (30, 31). Addition of rapamycin to the cells 3 days after the induction of differentiation did not inhibit the differentiation-associated increase in the amount of GLUT4 protein, GLUT4 mRNA (Fig. 9), or lipid accumulation normally appa-
ent at 8 days (data not shown), suggesting that rapamycin-sensitive pathways are not involved in posttranscriptional control at this stage of adipocyte differentiation.

**DISCUSSION**

We have now shown that PI 3-kinase activity precipitated with antibodies to phosphotyrosine was transiently increased during adipocyte differentiation. This increase in PI 3-kinase activity does not appear to be attributable to a direct effect of insulin used for induction of differentiation, because insulin was present in the induction medium for the first 4 days whereas PI 3-kinase activation was apparent only 4–6 days after induction in 3T3-F442A cells treated with insulin plus ETYA. Eight days later, cells were stained with oil red O. Original magnification, ×200. Data are representative of three experiments.

**FIG. 4.** Effects of dominant negative mutants of PI 3-kinase on adipocyte differentiation of 3T3-F442A cells. Cells were infected or not (A and B) with adenovirus vectors encoding either β-gal at an m.o.i. of 30 pfu/cell (C), Δp85 at a respective m.o.i. of 3, 10, or 30 pfu/cell (D–F), or p110N at a respective m.o.i. of 3, 10, or 30 pfu/cell (G–I). After 48 h, cells were induced (B–I) or not (A) to differentiate with insulin plus ETYA. Eight days later, cells were stained with oil red O. Original magnification, ×200. Data are representative of three experiments.

**FIG. 5.** Effects of dominant negative mutants of PI 3-kinase on the abundance of PPARγ mRNA and protein during differentiation of 3T3-F442A cells. Cells were infected or not (control) with adenovirus vectors encoding Δp85 or p110N at an m.o.i. of 30 pfu/cell and subsequently induced to differentiate with insulin plus ETYA. At the indicated times, PPARγ mRNA (A) and protein (B) were assayed by Northern blot and immunoblot analysis, respectively. Data are representative of three experiments.

**FIG. 6.** Effects of dominant negative mutants of PI 3-kinase on the abundance of adipocyte-specific marker (GLUT4, adipsin, and aP2) mRNAs during differentiation of 3T3-F442A cells. A, cells were infected or not (control) with adenovirus vectors encoding β-gal, Δp85, or p110N at the indicated m.o.i. and subsequently induced (or not) to differentiate with insulin plus ETYA. Eight days after induction, mRNAs encoding GLUT4, adipsin, and aP2 were assayed by Northern blot analysis. B, cells were infected or not (control) with adenovirus vectors encoding Δp85 or p110N at an m.o.i. of 30 pfu/cell and subsequently induced to differentiate with insulin plus ETYA. At the indicated times, GLUT4 mRNA was assayed by Northern analysis. Data are representative of three experiments.
and aP2) proteins during differentiation of 3T3-F442A cells. The abundance of adipocyte-specific marker (GLUT4, adipsin, and aP2) proteins during differentiation of 3T3-F442A cells. A, effects of genistein on PI 3-kinase activity and tyrosine phosphorylation of IRS1. Cells were induced to differentiate with insulin plus ETYA and, after 3 or 6 days, 100 μM genistein was added; the medium containing genistein was replenished every other day. GLUT4 mRNA (upper panel) and protein (lower panel) were assayed before and 8 days after induction of differentiation. Data are representative of three experiments.

ETYA or in 3T3-L1 cells treated with insulin, IBMX, and dexamethasone. Indeed, little increase in tyrosine phosphorylation of the insulin receptor or the IGF1 receptor was observed in 3T3-F442A cells induced to differentiate with insulin plus ETYA. These data indicate that the transient increase in PI 3-kinase is due not to activation of the insulin or the IGF1 receptor by insulin, which was added to the induction media, but instead to a programmed event during adipocyte differentiation. A similar activation of PI 3-kinase observed during the differentiation of 3T3-F442A cells induced by troglitazone alone also supports this conclusion.

Antibodies to either p110 or to p85 coprecipitated a tyrosine-phosphorylated protein of 180 kDa during differentiation of 3T3-F442A cells, and the time course of the association of this protein with p85 is similar to that of PI 3-kinase activation in anti-phosphotyrosine immunoprecipitates. This 180-kDa protein reacted with antibodies to IRS1 on immunoblot analysis. Moreover, the time courses of PI 3-kinase activity precipitated with antibodies to IRS1 were similar to that of PI 3-kinase activity precipitated with antibodies to IRS2, suggesting that IRS1 contributes to the activation of PI 3-kinase during adipocyte differentiation. We also showed that IRS2 undergoes tyrosine phosphorylation and associates with PI 3-kinase during adipocyte differentiation. However, the mobility of the tyrosine-phosphorylated protein observed in the immunoprecipitates prepared with antibodies to PI 3-kinase more closely resembles that of IRS1 than that of IRS2. These data suggest that the major tyrosine-phosphorylated protein that associates with PI 3-kinase during differentiation of 3T3-F442A cells is IRS1, although IRS2 also may contribute to PI 3-kinase activation during adipocyte differentiation. Sun et al. (32) have shown that the amount of IRS2 in 3T3-L1 cells and isolated rat adipocytes is less than that of IRS1. Our data may reflect the relative abundance of these two proteins in 3T3-F442A cells. IRS1 was originally identified as a substrate of the insulin receptor family of tyrosine kinases and was subsequently shown to become phosphorylated in response to ligands, such as growth hormone as well as various interferons and interleukins (27), whose receptors are thought to be coupled with the JAK family of tyrosine kinases (33). However, we could not detect tyrosine phosphorylation of Jak1, Jak2, Jak3, or Tyk2 in 3T3-F442A cells 4 days after induction of differentiation with insulin and ETYA (data not shown), suggesting that none of these tyrosine kinases is responsible for PI 3-kinase activation during adipocyte differentiation. Immune precipitation and im-

![Fig. 7](image_url)

**Fig. 7.** Effects of dominant negative mutants of PI 3-kinase on the abundance of adipocyte-specific marker (GLUT4, adipsin, and aP2) proteins during differentiation of 3T3-F442A cells. A, effects of genistein on PI 3-kinase activity and tyrosine phosphorylation of IRS1. Cells were induced to differentiate with insulin plus ETYA and, after 3 or 6 days, 100 μM genistein was added; the medium containing genistein was replenished every other day. GLUT4 mRNA (upper panel) and protein (lower panel) were assayed before and 8 days after induction of differentiation. Data are representative of three experiments.

![Fig. 8](image_url)

**Fig. 8.** Effects of genistein on activation of PI 3-kinase, tyrosine phosphorylation of IRS1, and the abundance of GLUT4 mRNA and protein during the differentiation of 3T3-F442A cells. A, effects of genistein on PI 3-kinase activity and tyrosine phosphorylation of IRS1. Cells were induced to differentiate with insulin plus ETYA and, after 3 days, incubated in the absence or presence of 100 μM genistein. PI 3-kinase activity precipitated with antibodies to phosphotyrosine (upper panel) and tyrosine phosphorylation of IRS1 (lower panel) were examined before (day 0) and 4 days after induction. Data are representative of two experiments, and quantitative data are means ± S.E. of three independent experiments. B, effects of genistein on the abundance of GLUT4 mRNA and protein. Cells were induced to differentiate with insulin plus ETYA and, after 3 or 6 days, 100 μM genistein was added; the medium containing genistein was replenished every other day. GLUT4 mRNA (upper panel) and protein (lower panel) were assayed before and 8 days after induction of differentiation. Data are representative of three experiments.

![Fig. 9](image_url)

**Fig. 9.** Effects of rapamycin on the abundance of GLUT4 mRNA and protein. 3T3-F442A cells were induced to differentiate with insulin plus ETYA and, after 3 days, 20 ng/ml of rapamycin was added; the medium containing rapamycin was replenished every other day. GLUT4 mRNA (upper panel) and protein (lower panel) were assayed 8 days after induction of differentiation. Data are representative of two experiments.
munoblot analysis with antibodies to phosphorytrosine revealed that several proteins other than IRS1 became tyrosine-phosphorylated during differentiation of 3T3-F442A cells. Thus, one of these tyrosine-phosphorylated proteins may be a tyrosine kinase that phosphorylates IRS1.

We used two structurally diverse dominant negative mutants of PI 3-kinase to inhibit activation of the enzyme during adipocyte differentiation because pharmacological inhibitors have been shown to attenuate the activity of enzymes other than PI 3-kinase (15, 34). Whereas Δp85 inhibits PI 3-kinase activation by competing with the endogenous enzyme for association with tyrosine-phosphorylated proteins, p110N inhibits the enzyme by competing with endogenous p110 for association with p85.2 Overexpression of these mutants inhibited both the activation of PI 3-kinase and adipocyte differentiation induced by exposure of 3T3-F442A cells either to insulin plus ETYA or to troglitazone alone, indicating that PI 3-kinase activation is required for adipogenesis.

We showed that Δp85 or p110N did not affect expression of PPARγ at either the mRNA or protein level, possibly consistent with the observation that the increases in PPARγ mRNA and protein induced by insulin plus ETYA appeared to peak before maximal PI 3-kinase activation. Although PPARγ is an important transcriptional regulator during adipocyte differentiation, other transcription factors, such as C/EBPα, -β, -δ, and ADD-1/SREBP1 also contribute to this process, at least in 3T3-L1 cells induced with insulin, IBMX, and dexamethasone (2, 9, 12). The differentiation-associated increases in the abundance of mRNAs encoding the adipocyte marker proteins GLUT4, adipin, and aP2 in 3T3-F442A cells also were not inhibited by expression of the dominant negative mutants of PI 3-kinase. Thus, transcriptional control of the corresponding genes appears to be independent of PI 3-kinase, regardless of the transcription factors involved in this control.

Akt, also known as protein kinase B, is one of the downstream effectors of PI 3-kinase (35). It was reported that Akt fused with a viral Gag protein or tagged with a membrane-targeted signal sequence shows higher kinase activity than wild type Akt, and that overexpression of these mutant proteins into 3T3-L1 preadipocytes caused spontaneous differentiation with increased expression of mRNA of aP2 or C/EBPα (36, 37). These findings suggest that signals transmitted through PI 3-kinase are involved in transcriptional regulation of adipogenesis. However, we have shown that activation of PI 3-kinase was essentially excluded at a specific stage of adipocyte differentiation. It is thus possible that expression of a constitutively active Akt at a stage when PI 3-kinase is not normally activated modulates the transcriptional control which is not physiologically regulated by PI 3-kinase during adipocyte differentiation.

Inhibition of PI 3-kinase activation during differentiation prevented the increase in the amounts of adipocyte-specific proteins in 3T3-F442A cells, presumably by a posttranscriptional mechanism given that the corresponding mRNAs were unaffected. This effect could theoretically be mediated through control of protein translation or control of protein turnover. In this regard, PI 3-kinase is thought to contribute to ligand-stimulated protein synthesis at several steps, including activation of initiation factor eIF-2B (38), phosphorylation of PHAS-I/4E-BP1 (39), and activation of p70 S6 kinase (40). Furthermore, PI 3-kinase might contribute to the differentiation of cells in which this process is sensitive to pharmacological inhibitors of the enzyme (17, 18), possibly acting through a mechanism similar to that described here. It is known that rapamycin, a bacterial macrolide, attenuates mitogen-stimulated protein synthesis by 10–30% (30, 31) probably through interfering translational control of mRNA that possess a poly-pyrimidine tract at their transcriptional start site (40, 41). We have shown that addition of rapamycin to 3T3-F442A cells 3 days after the induction of differentiation with insulin plus ETYA had no effect on the differentiation-associated increase in the amount of GLUT4 protein or adipocyte differentiation assessed by lipid accumulation, whereas addition of genistein at the same stage of differentiation completely abolished the expression of GLUT4 at posttranscriptional step. These results suggest that rapamycin sensitive-mechanism is not required for adipogenesis at this stage of differentiation.

In conclusion, we have demonstrated a stage-specific activation of PI 3-kinase, to which tyrosine phosphorylation of IRS1 may contribute, during adipocyte differentiation. Furthermore, this activation of PI 3-kinase increases the expression of adipocyte-specific proteins at a posttranscriptional level. The tyrosine kinase responsible for the activation of PI 3-kinase and the mechanism by which this protein kinase is activated remain unknown, but their identification and elucidation, respectively, should increase further our understanding of the regulation of adipocyte differentiation.

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