Requirement of Src-Family Tyrosine Kinases in Fat Accumulation†

Yutong Sun,‡ Yong-Chao Ma,‡,§ Jianyun Huang, Krystina Y. Chen, Deirdre K. McGarrigle, and Xin-Yun Huang*

Department of Physiology, Weill Medical College, Cornell University, New York, New York 10021

Received May 16, 2005; Revised Manuscript Received August 18, 2005

ABSTRACT: Src-family tyrosine kinases mediate many receptor signals to various biological responses. Here we investigate the requirement of Src-family tyrosine kinases in adipogenesis. The biochemical mechanism by which insulin induces adipogenesis, converting fibroblast cells to adipocytes, is not clear. We show that fibroblast cells deficient of three ubiquitously expressed Src-family members (Src, Yes, and Fyn), SYF cells, are refractory to hormonally induced fat accumulation. The defect is rescued by reintroduction of c-Src into SYF cells. Furthermore, Src-family tyrosine kinases are required in the early steps of insulin signaling; it is responsible for the tyrosine phosphorylation of adaptor protein c-Cbl. Deficiency of c-Cbl blocked adipogenesis. These genetic and biochemical data clearly demonstrate that Src-family tyrosine kinases serve as a critical signal relay, via phosphorylation of c-Cbl, for fat accumulation, and provide potential new strategies for treating obesity.

Src-family tyrosine kinases are one major group of cellular signal transducers (*1*, *2*). These tyrosine kinases can be activated by various extracellular signals through receptor tyrosine kinases, integrins, G protein-coupled receptors, antigen and Fc receptors, and cytokine receptors. They can modulate a variety of cellular functions, including proliferation, differentiation, survival, adhesion, morphological changes, and migration (*2*). Studies on Src-family tyrosine kinases have led to insights into the role of protein tyrosine phosphorylation in cell physiology, the signaling pathways by which cell surface receptors regulate cell growth and differentiation, and the function of modular domains [such as Src-homology 2 (SH2)[1] and SH3] that mediate protein−protein interactions in forming signaling complexes. The crystal structures of Src-family tyrosine kinases reveal that the kinase activity is regulated by the formation and disruption of intramolecular interactions involving the SH2 and SH3 domains (*3*, *4*). Activation of Src-family tyrosine kinases allows the SH2 and SH3 domains to participate in intermolecular interactions that target Src-family tyrosine kinases to their substrates. Phosphorylation of these substrate proteins either directly modifies their functions or leads to the assembly of signaling complexes that initiate signaling cascades. Furthermore, malfunction of Src-family tyrosine kinases leads to human diseases; for example, a subset of advanced human colon cancers contained an activating mutation in c-Src (*5*). The physiological functions of Src-family tyrosine kinases continue to be revealed.

The global prevalence of obesity, leading to insulin-resistant type II diabetes, is on the rise (*6*). An understanding of fat cell formation is essential in combatting this undesirable trend. In addition to their critical role in energy balance, adipocytes also secrete hormones and cytokines such as leptin which play essential roles in integrating the body's metabolic program. Conversion of fibroblast cells to adipocytes has been extensively studied from the point of view of transcriptional regulation by the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) and the CCAAT/enhancer binding proteins C/EBPα (*7*−*9*). These studies took advantage of the model preadipocyte cell lines such as 3T3-L1 cells (*10*). These cells differentiate into adipocytes after hormonal treatment with insulin (or insulin-like growth factor-1) (*10*). The peptide hormone insulin plays central roles in obesity and diabetes. Activation of insulin receptors on preadipocytes stimulates adipogenesis. The model cell adipogenesis recapitulates most of the key features of adipogenesis in animals. Differentiated 3T3-L1 adipocytes deposit triglyceride in cytoplasmic lipid droplets and express genes that are also expressed in primary adipocytes (*11*). Studies of this process have led to the identification of several key transcriptional factors that orchestrate adipogenesis, including C/EBPα, β, and δ, and PPARγ (*7*). Indeed, overexpression of these transcriptional factors such as PPARγ in primary fibroblasts as well as established fibroblast cell lines permits these fibroblast cells to be induced to differentiate into adipocytes by hormonal treatment (*7*). Furthermore, PPARγ ligands, most notably the thiazolidinedione (TZD) class, ameliorate insulin resistance and represent exciting new therapies for type II diabetes.

In this report, we have investigated the role of Src-family tyrosine kinases in adipogenesis as monitored by fat accumulation. We found that deficiency of Src-family tyrosine kinases blocked hormonally induced fat accumulation.

† Supported by grants from the National Institutes of Health. J.H. was supported by a postdoctoral fellowship from the Charles H. Revson Foundation.

* To whom correspondence should be addressed. Telephone: (212) 746-6362. Fax: (212) 746-8690. E-mail: xyhuang@med.cornell.edu.

‡ These authors contributed equally to this work.

§ Present address: Division of Neuroscience, Children's Hospital, Boston, MA 02115.

[1] Abbreviations: SH2, Src-homology-2; SH3, Src-homology-3; PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT/enhancer binding proteins; TZD, thiazolidinedione; DMEM, Dulbecco's modified Eagle's medium; IBMX, 3-isobutyl-1-methylxanthine.

10.1021/bi0509090 CCC: $30.25
Published on Web 10/13/2005

Furthermore, we have provided a biochemical explanation for this genetic requirement of Src-family tyrosine kinases in this adipogenesis process. We have shown that Src-family tyrosine kinases are required for insulin-induced tyrosine phosphorylation of the adaptor c-Cbl. Moreover, fibroblast cells deficient in c-Cbl failed to accumulate fat after hormonal treatment. Hence, we have demonstrated that Src-family tyrosine kinases play a critical role in fat accumulation.

MATERIALS AND METHODS

3T3-L1 Cell Adipogenesis. 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in 10 cm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Life Technologies) supplemented with 10% calf serum (Invitrogen-Life Technologies). Confluent cultures were induced to differentiate by incubation of cells with DMEM containing 10% fetal bovine serum (Invitrogen-Life Technologies), 1 μg/mL insulin (Sigma), 0.25 μM dexamethasone (Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma), and 0.5 μM synthetic PPARγ ligand rosiglitazone. After 3 days, the medium was changed to DMEM, 10% fetal bovine serum, 1 μg/mL insulin, and 0.5 μM rosiglitazone for an additional 2 days. The medium was then changed to DMEM containing 10% fetal bovine serum. Under these conditions, more than 90% of the cells were morphologically differentiated into adipocytes after 8 days. Adipogenesis was verified via staining of accumulated lipid droplets with Oil Red O (Sigma). Differentiated adipocytes were fixed with 3.7% formaldehyde in 1× PBS for 5 min and then washed three times with 1× PBS. Cells were stained for 10 min with Oil Red O (0.5% in 2-propanol) diluted with 1.5 volumes of 1× PBS. Giemsa (1%) (Sigma) was used to stain nuclei and cytosol. To test the effect of PP2 and PP3 on adipogenesis, 5 μM PP2 or 10 μM PP3 (Calbiochem) was added to the differentiation medium starting from day 1.

Differentiation of Mouse Embryonic Fibroblast Cells. MEF/3T3, SYF, SYF/c-Src (SYF cells with stable reintroduction of c-Src), Abl$^{-/-}$/Arg$^{-/-}$, c-Cbl$^{-/-}$, and c-Cbl$^{-/-}$/c-Cbl (Cbl$^{-/-}$ cells with stable reintroduction of c-Cbl) cells have been previously described (*12*−*14*). These cells were infected with retroviruses expressing PPARγ2 in pMSCVpuro (*15*). Preparation of retroviral stocks and the infection conditions that were used have previously been described (*16*). Puromycin was used for the selection of stable cell lines. The c-Cbl$^{-/-}$/c-Cbl+PPARγ2 cells were selected at a higher concentration of puromycin (8 μg/mL). These cells were then differentiated as 3T3-L1 cells as described above.

c-Src Immunocomplex Kinase Assay. The Src immunocomplex kinase assay was conducted as previously described (*13*, *17*). Whole cell extracts were made from proliferating 3T3-L1 cells (~80% confluent) and from cells 2 days after differentiation. Src immunoprecipitation was done with a monoclonal antibody to Src (Oncogene Science). The Src kinase assay was performed using 5 μg of a purified fusion protein of glutathione *S*-transferase and the cytoplasmic domain of human erythrocyte band 3 (GST−CDB3) as a substrate (*17*).

Cbl Tyrosine Phosphorylation Assay. Whole cell lysates were prepared from MEF/3T3, SYF, or SYF/Src fibroblast cells with stable expression of PPARγ2. Cells were either treated or not treated with insulin (100 nM) or with insulin-like growth factor-1 (IGF-1) (10 nM) for the indicated time. c-Cbl was immunoprecipitated from cell lysates with polyclonal anti-c-Cbl antibody (Santa Cruz Biotechnology). After SDS−PAGE, the tyrosine phosphorylation of c-Cbl was detected with a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology).

ERK Kinase Assay. The p44/42 MAP kinase assay was performed with a kit from Cell Signaling Technology as previously described (*17*). Whole cell lysates were prepared from MEF/3T3, SYF, or SYF/Src fibroblast cells stably expressing PPARγ2. Cells were either treated or not treated with insulin (100 nM) for 5 min. A monoclonal antibody for phospho-p44/42 ERK MAPK (cross-linked to agarose beads) was added to immunoprecipitate active ERK from cell lysates. Substrates (200 μM ATP and 2 μg of Elk-1−GST fusion protein) were added, and the reaction was allowed to proceed at 30 °C for 30 min. After SDS−PAGE, the ERK activity (the phosphorylation of Elk-1 by ERK) was assessed by Western blotting with an anti-phospho-Elk-1 antibody.

Akt Kinase Assay. The Akt kinase assay was performed with a kit from Cell Signaling Technology. Akt was immunoprecipitated with a monoclonal anti-Akt antibody cross-linked to agarose beads from cell lysates prepared from MEF/3T3, SYF, or SYF/Src cells stably expressing PPARγ2. Cells were treated or not treated with insulin (100 nM) for 5 min. ATP (200 μM) and 1 μg of GSK−3α/β fusion protein were added to the immunocomplex as substrates. The phosphorylation reaction was allowed to proceed for 30 min at 30 °C. After SDS−PAGE, the activity of Akt (the phosphorylation of GSK−3α/β by Akt) was analyzed by Western blotting with an anti-phospho-GSK−3α/β antibody.

Detection of Adipsin and C/EBPα *Proteins*. Whole cell lysates were prepared from MEF/3T3, SYF, or Cbl$^{-/-}$ cells stably expressing PPARγ2 at the indicated days during differentiation. After SDS−PAGE, expression of adipsin and C/EBPα proteins was monitored by Western blots with anti-adipsin and C/EBPα antibodies (Santa Cruz Biotechnology).

RESULTS

Sensitivity of Adipogenesis of 3T3-L1 Cells to Src-Family Tyrosine Kinase Inhibitors. Insulin induces adipogenesis of 3T3-L1 preadipocytes, as assayed by histological Oil Red O staining for the accumulation of triglyceride as cytoplasmic lipid droplets in differentiated cells (*10*) (Figure 1A,B). Large lipid droplets (stained red) are seen in adipocytes (Figure 1B). For comparison, no lipid droplet staining was seen in 3T3-L1 cells grown to confluence prior to insulin treatment (Figure 1A). To investigate the possible involvement of Src-family tyrosine kinases in this insulin-induced adipogenesis, we first examined the effect of the Src-family tyrosine kinase inhibitor PP2 (*18*). As shown in Figure 1C, in the presence of PP2, insulin-induced differentiation of 3T3-L1 cells was blocked, while a negative control compound PP3 had no effect (Figure 1D). Treatment with another Src-family kinase inhibitor PP1 also inhibited fat accumulation (data not shown). Furthermore, during adipogenesis of 3T3-L1 cells, the kinase activity of endogenous c-Src was increased (Figure 1E). These data indicate that Src-family tyrosine kinase activity is necessary for insulin-induced adipogenesis.

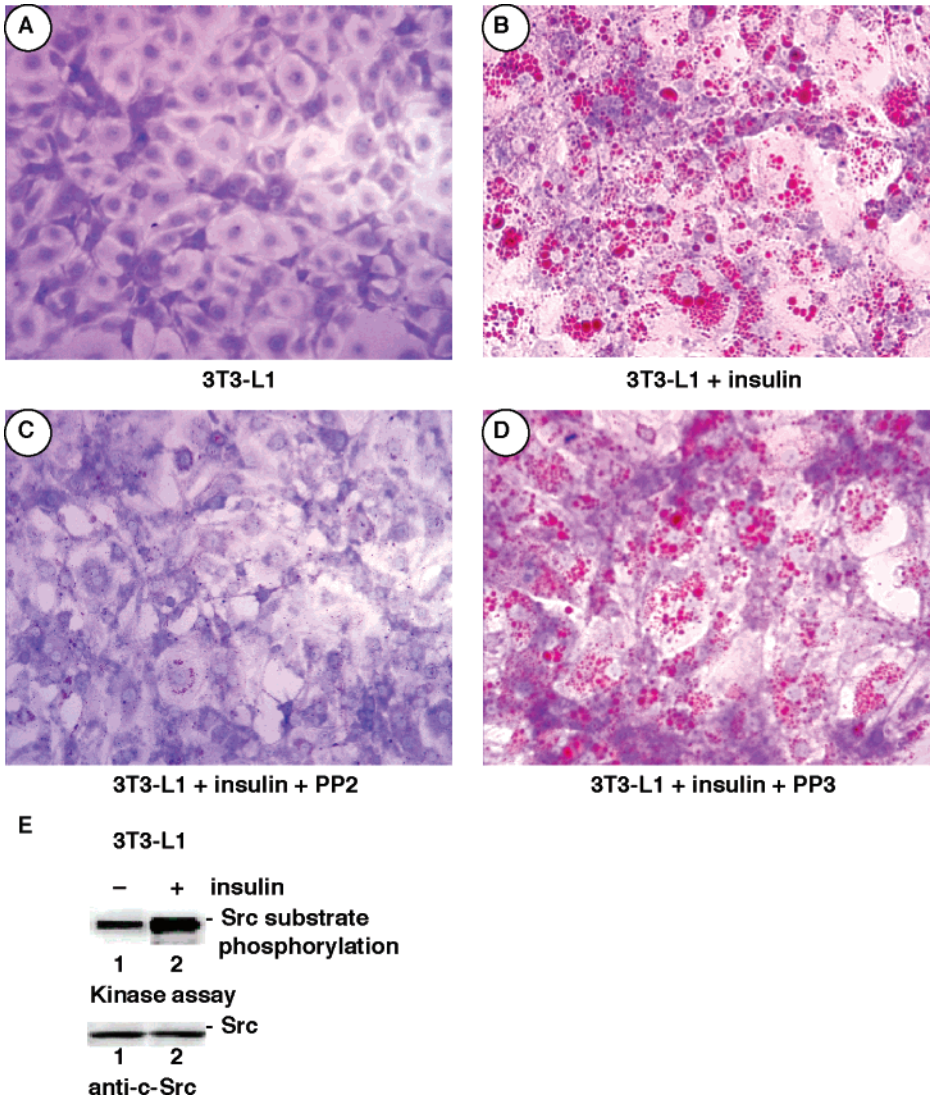

FIGURE 1: Insulin-induced differentiation of 3T3-L1 cells into adipocytes was sensitive to Src-family tyrosine kinase inhibitors. (A) Oil Red O staining of 3T3-L1 cells grown to confluence before differentiation. (B) Oil Red O staining of lipid droplets in 3T3-L1 cells 10 days after the start of the differentiation protocol. For easy labeling, "insulin" was used to represent the whole differentiation protocol; >90% of the cells displaced lipid droplets. (C) Oil Red O staining of 3T3-L1 fibroblasts 10 days after the start of the differentiation protocol with PP2 (5 μM); <30% of the cells had small lipid droplets. (D) Oil Red O staining of 3T3-L1 cells after 10 days with PP3 (10 μM), insulin, DEX, and IBMX; >90% of the cells had lipid droplets. (E) During adipogenesis of 3T3-L1 cells, the kinase activity of endogenous c-Src increased. Endogenous c-Src proteins were immunoprecipitated from undifferentiated and differentiated (for 24 h) 3T3-L1 cells. A Src substrate (GST−CDB3 fusion protein) was added to monitor the kinase activity of c-Src. The bottom panel shows that similar amounts of Src proteins were immunoprecipitated. Data are representative of three sets of experiments.

Deficiency of Src-Family Tyrosine Kinases Blocks Insulin-Induced Fat Accumulation. To genetically confirm the necessity of Src-family tyrosine kinases for insulin-induced adipogenesis, we examined insulin-induced adipogenesis using MEF/3T3 fibroblast cells (which have endogenous Src-family tyrosine kinases) and fibroblast cells without Src-family tyrosine kinases, SYF cells (Figure 2). The SYF cells were derived from Src, Yes, Fyn triple-knockout mouse embryos, and these cells lack Src-family tyrosine kinase activity (*12*, *13*). To precondition these fibroblast cells and induce insulin sensitivity, we used retroviral infection to express nuclear hormone receptor PPARγ2 (*7*). This method allows insulin to induce differentiation of mouse embryonic fibroblast cells into adipocytes (*7*). As shown in panels A and B of Figure 2, after stable expression of PPARγ2 in MEF/3T3 fibroblast cells, insulin induced fat accumulation in these cells, confirming the utility of this approach. In contrast, even after stable expression of PPARγ2 in SYF fibroblast cells, insulin failed to induce fat accumulation in these cells (Figure 2C,D). Reintroduction of c-Src back into SYF cells rescued the ability of insulin to induce adipogenesis in the presence of PPARγ2 (Figure 2E,F). Furthermore, as additional controls, we tried other tyrosine kinase-deficient fibroblast cells. For example, Abl$^{-/-}$/Arg$^{-/-}$ fibroblast cells [derived from Abl$^{-/-}$/Arg$^{-/-}$ double-knockout mouse embryos (*14*)], stably expressing PPARγ2, were still induced by insulin to accumulate fat (Figure 2G,H). These genetic data confirm that insulin requires Src-family tyrosine kinases to induce adipogenesis.

Requirement of Src-Family Tyrosine Kinases for Tyrosine Phosphorylation of Cbl. The data given above pharmacologically and genetically demonstrate that Src-family tyrosine kinases are essential for insulin-induced adipogenesis. To investigate the possible biochemical mechanism for this requirement for Src-family tyrosine kinases in insulin signaling, we examined the early signaling pathways initiated by

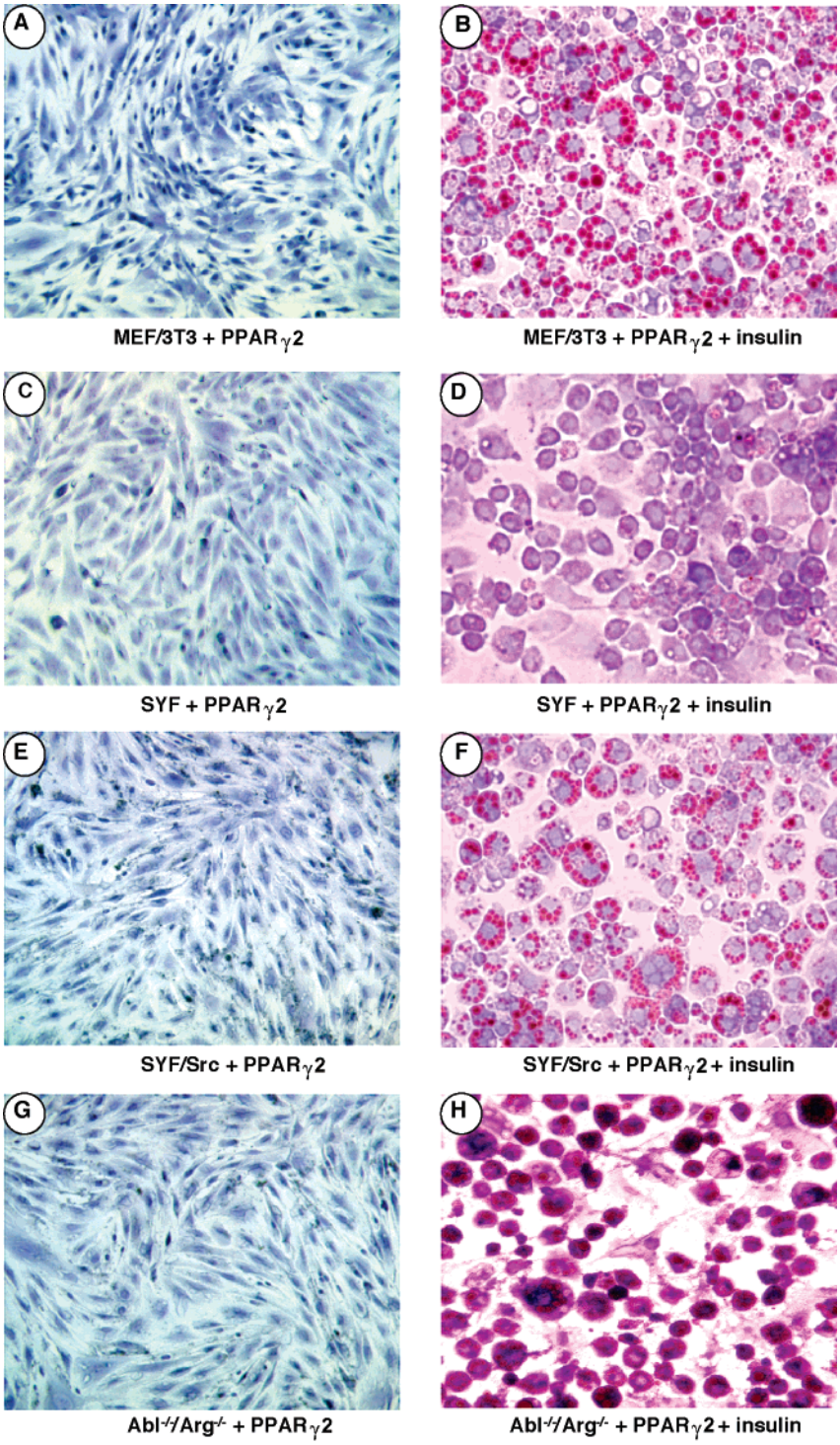

FIGURE 2: Insulin failed to induce adipogenesis of Src-family tyrosine kinase deficient fibroblast cells. (A) Oil Red O staining of MEF/3T3 cells after stable expression of PPARγ2, grown to confluence before insulin induction. MEF/3T3 cell lines were established from wild-type mouse embryonic fibroblast cells using the 3T3 protocol. (B) Oil Red O staining of MEF/3T3 cells after stable expression of PPARγ2, and insulin induction for 10 days. For better visualization of the red staining of lipid droplets, the photograph was adjusted to be brighter and was taken with higher magnification than in A; >90% of the cells had lipid droplets. (C) Oil Red O staining of SYF cells after stable expression of PPARγ2, grown to confluence before insulin induction. SYF cell lines were established from mouse embryos deficient in c-Src, Yes, and Fyn tyrosine kinases. (D) Oil Red O staining of SYF cells after stable expression of PPARγ2, and insulin induction for 12 days. No lipid droplets were observed. (E) Oil Red O staining of SYF/Src cells after stable expression of PPARγ2, grown to confluence before insulin induction. SYF/Src cells are SYF cells with stable re-expression of c-Src. (F) Oil Red O staining of SYF/Src cells after stable expression of PPARγ2, and insulin induction for 10 days; >90% of the cells had lipid droplets. (G) Oil Red O staining of $Abl^{-/-}/Arg^{-/-}$ cells after stable expression of PPARγ2, grown to confluence before insulin induction. $Abl^{-/-}/Arg^{-/-}$ cell lines were established from mouse embryos deficient in the Abl-family tyrosine kinases (Abl and Arg). (H) Oil Red O staining of $Abl^{-/-}/Arg^{-/-}$ cells after stable expression of PPARγ2, and insulin induction for 10 days; >90% of the cells had lipid droplets. Data are representative of three sets of experiments.

insulin. Insulin can activate the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase/Akt (protein kinase B) pathway, and the Cbl/Crk/C3G/TC10 pathway (*19*). Early studies have shown that, in adipocytes,

tyrosine phosphorylation of the adaptor protein c-Cbl recruits other signaling components, including the adaptor protein CrkII, exchange factor C3G, and small G protein TC10, for insulin-induced glucose transporter GLUT4 translocation to the plasma membrane, which facilitates glucose uptake (*20*, *21*). To examine whether Src-family tyrosine kinases are required for insulin-induced tyrosine phosphorylation of Cbl during adipogenesis, we investigated the tyrosine phosphorylation status of Cbl in MEF/3T3, SYF, and SYF/Src cells (all stably expressing PPARγ2) with or without insulin stimulation. As shown in Figure 3A, we found that, while insulin induced tyrosine phosphorylation of Cbl in MEF/3T3+PPARγ2 cells, deficiency of Src-family tyrosine kinases prevented the tyrosine phosphorylation of Cbl. Reexpression of Src in SYF+PPARγ2 cells rescued the insulin-induced tyrosine phosphorylation of Cbl (Figure 3A). Consistent with an essential role for Src-family tyrosine kinases in Cbl phosphorylation, treatment of MEF/3T3+PPARγ2 cells with PP2 blocked insulin-induced tyrosine phosphorylation of Cbl (data not shown). We also investigated the two other early signaling pathways initiated by insulin. We found that insulin-induced activation of Akt and MAPK pathways was not affected by the deficiency of Src-family tyrosine kinases (Figure 3B,C). Our biochemical data demonstrate that Src-family tyrosine kinases are required for insulin-induced tyrosine phosphorylation of Cbl.

Deficiency of c-Cbl Blocks Insulin-Induced Fat Accumulation. Since Src-family tyrosine kinases are required for the tyrosine phosphorylation of Cbl initiated by insulin, this might provide a biochemical mechanism for the dependency of insulin-initiated adipogenesis on Src-family tyrosine kinases that we observed in Figures 1 and 2. If indeed Src-family tyrosine kinases and Cbl function in this insulin-induced adipogenesis, a cellular physiological prediction is that deficiency of c-Cbl in fibroblast cells should block insulin-induced fat accumulation. Thus, we studied the ability of insulin to induce adipogenesis in c-Cbl$^{-/-}$ fibroblast cells (from c-Cbl knockout mice) (*22*). We generated c-Cbl$^{-/-}$ fibroblast cells stably expressing PPARγ2, and attempted to differentiate these cells. As shown in panels A and B of Figure 4, deficiency of c-Cbl blocked insulin-induced fat accumulation in c-Cbl$^{-/-}$ cells stably expressing PPARγ2. This blockage is due to the lack of c-Cbl since reintroduction of c-Cbl into c-Cbl$^{-/-}$ fibroblast cells restored the ability of insulin to induce adipogenesis (Figure 4C,D). These data provide cell physiological evidence that c-Cbl is essential for insulin-induced fat accumulation. Hence, both Src-family tyrosine kinases and Cbl are required for fat accumulation. Src-family tyrosine kinases are essential for tyrosine phosphorylation of Cbl. Thus, phosphorylation of Cbl by Src-family tyrosine kinases is the likely biochemical basis for the participation of Src-family tyrosine kinases in fat accumulation.

To further confirm the defects of adipogenesis caused by the absence of c-Cbl and Src-family tyrosine kinases, we examined the protein levels of some adipose-specific genes at various times after addition of the differentiation mixture (Figure 5A). Adipsin is a fat-cell-specific protein induced during adipogenesis (*23*). Although adipsin proteins were detected in MEF/3T3+PPARγ2 cells during differentiation, these proteins were undetectable in SYF+PPARγ2 or Cbl$^{-/-}$+PPARγ2 cells (Figure 5A). The lack of adipsin

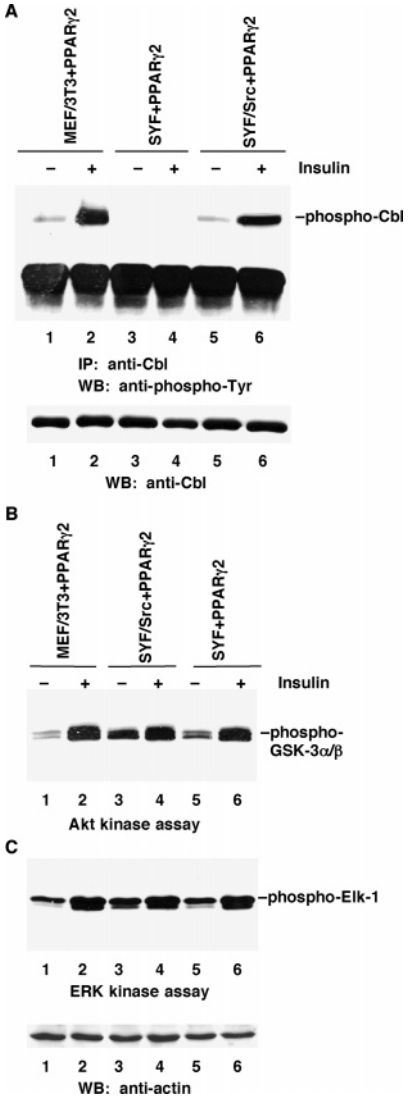

FIGURE 3: Src-family tyrosine kinases are required for insulin-induced tyrosine phosphorylation of adaptor protein c-Cbl. (A) Whole cell extracts prepared from MEF/3T3(PPARγ2), SYF-(PPARγ2), and SYF/Src(PPARγ2) cells, treated or not treated with insulin (100 nM for 30 min), were subjected to immunoprecipitation with an anti-Cbl antibody. After SDS−PAGE, the tyrosine phosphorylation of c-Cbl was detected with a monoclonal anti-phosphotyrosine antibody (top). At the bottom is a Western blot with anti-c-Cbl antibody. (B) In the Akt kinase assay, Akt was immunoprecipitated by a monoclonal anti-Akt antibody cross-linked to agarose beads from cell lysates prepared from MEF/3T3, SYF/Src, or SYF cells with stable expression of PPARγ2. Cells were either treated or not treated with insulin (100 nM) for 5 min. ATP (200 μM) and 1 μg of GSK−3α/β fusion protein were added to the immunocomplex as substrates. The phosphorylation reaction was allowed to proceed for 30 min at 30 °C. After SDS−PAGE, the activity of Akt (the phosphorylation of GSK−3α/β by Akt) was analyzed by Western blotting with anti-phospho-GSK−3α/β antibody. (C) In the ERK kinase assay, whole cell lysates were prepared from MEF/3T3, SYF/Src, or SYF fibroblast cells with stable expression of PPARγ2. Cells were treated or not treated with insulin (100 nM) for 5 min. A monoclonal antibody for phospho-p44/42 ERK MAPK (cross-linked to agarose beads) was added to immunoprecipitate active ERK from cell lysates. Substrates (200 μM ATP and 2 μg of Elk-1−GST fusion protein) were added, and the reaction was allowed to proceed at 30 °C for 30 min. After SDS−PAGE, the ERK activity (the phosphorylation of Elk-1 by ERK) was measured by Western blotting with an anti-phospho-Elk-1 antibody. The bottom panel shows a Western blot with anti-actin antibody for normalization of the protein samples used in panels B and C. Data are representative of three to five experiments.

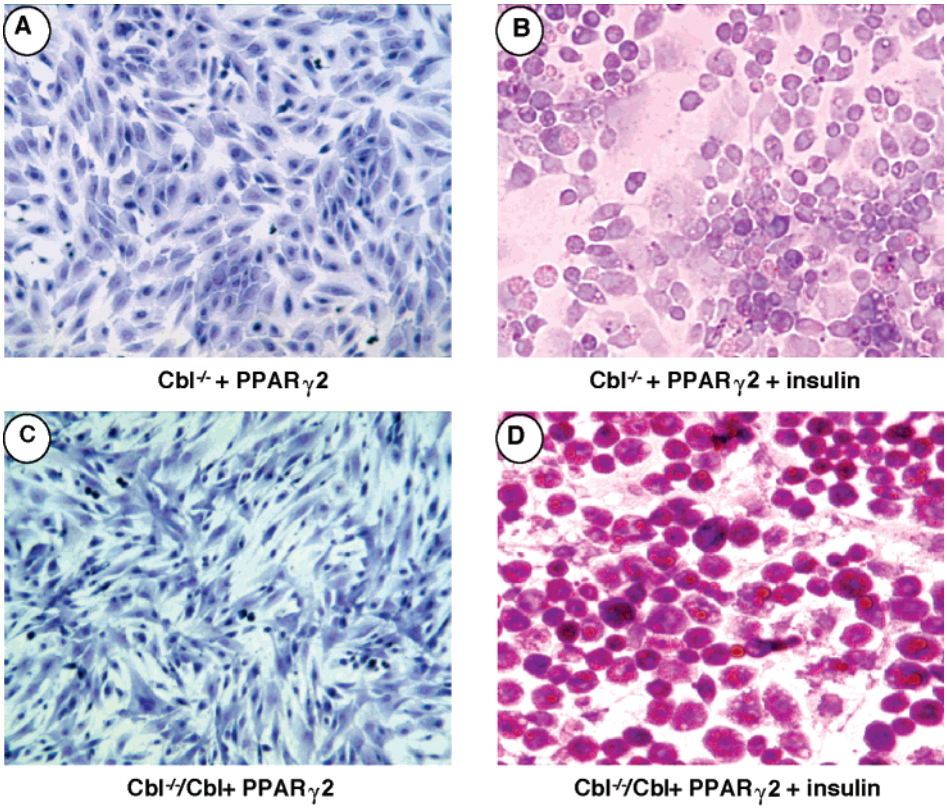

FIGURE 4: Insulin failed to induce fat accumulation in c-Cbl-deficient fibroblast cells. (A) Oil Red O staining of c-Cbl$^{-/-}$ cells after stable expression of PPARγ2, grown to confluence before insulin induction. Cbl$^{-/-}$ cell lines were established from mouse embryos deficient in c-Cbl. (B) Oil Red O staining of Cbl$^{-/-}$ cells after stable expression of PPARγ2, and insulin induction for 10 days. Other than nuclei staining, no lipid droplets were observed in any cells. (C) Oil Red O staining of Cbl$^{-/-}$/Cbl cells after stable expression of PPARγ2, grown to confluence before insulin induction. Cbl$^{-/-}$/Cbl cells are Cbl$^{-/-}$ cells with stable re-expression of c-Cbl. (D) Oil Red O staining of Cbl$^{-/-}$/Cbl cells after stable expression of PPARγ2, and insulin induction for 10 days. Lipid droplets were clearly accumulated in >90% of the cells. Data are representative of three sets of experiments.

expression in SYF+PPARγ2 or Cbl$^{-/-}$+PPARγ2 cells reflects the lack of adipogenesis in these cells. A similar pattern was observed for the differentiation-associated transcription factor C/EBPα (Figure 5A).

We also investigated the c-Cbl tyrosine phosphorylation induced by IGF-1. As shown in Figure 5B, as for insulin, IGF-1 also induced an increased level of tyrosine phosphorylation of c-Cbl in MEF/3T3+PPARγ2 cells. As previously reported (*24*), neither insulin nor IGF-1 induced tyrosine phosphorylation of c-Cbl in MEF/3T3 fibroblast cells or 3T3-L1 cells (data not shown). Although the molecular basis is not clear, expression of PPARγ2 in MEF/3T3 cells modified these cells in such a way that insulin and IGF-1 could induce sustained (at least for up to 60 min) tyrosine phosphorylation of c-Cbl in MEF/3T3+PPARγ2 cells (Figure 5B).

DISCUSSION

Here we have provided pharmacological, genetic, and biochemical data demonstrating that Src-family tyrosine kinases play a critical role in adipogenesis. To the best of our knowledge, this is the first report demonstrating a physiological role for Src-family tyrosine kinases in fat accumulation. Src-family tyrosine kinase inhibitors decreased the degree of adipogenesis of 3T3-L1 preadipocytes. Deficiency of Src-family tyrosine kinases blocked the adipogenesis of fibroblast cells. Insulin-induced tyrosine phosphorylation of the adaptor protein Cbl required Src-family tyrosine kinases. Fibroblast cells with deletion of c-Cbl were refractory to be differentiated into adipocytes. Consistent with our findings, Src-family tyrosine kinases, such as Fyn, Lyn, and Lck, have been shown to physically interact with c-Cbl in lymphocytes (*24*−*27*). Furthermore, the basal c-Src kinase activity was increased during the progression from proliferating to growth-arrested to differentiating 3T3-L1 cells (*28*). Moreover, c-Cbl knockout mice had reduced (by 50%) adipose mass and lower triglyceride levels in the liver (*29*). Interestingly, these c-Cbl$^{-/-}$ mice also exhibited improved insulin action and higher energy expenditure (*29*). The complex phenotype of these mice might reflect the fact that, in addition to its role as an adaptor protein, c-Cbl also has an intrinsic E3 ubiquitin ligase activity (*30*).

We observed no effect of the deficiency of Src-family tyrosine kinases on insulin activation of ERK MAPK in fibroblast cells. Indeed, the ERK MAPK pathway is not required for induction of adipogenesis. Sustained activation of ERK MAPK inhibits adipogenesis via phosphorylation and inhibition of PPARγ (*31*). Overexpression of a membrane-targeted constitutively active form of Akt in 3T3-L1 preadipocytes was reported to cause spontaneous differentiation (*32*, *33*). Since our data showed that deficiency of Src-family tyrosine kinases had no effect on insulin activation of Akt, insulin likely signals through two pathways involving Src-family tyrosine kinases and Akt leading to adipogenesis.

It is not currently clear at which stage of the adipogenesis process the Src-family tyrosine kinases exert their role. Adipogenesis includes initial growth arrest followed by

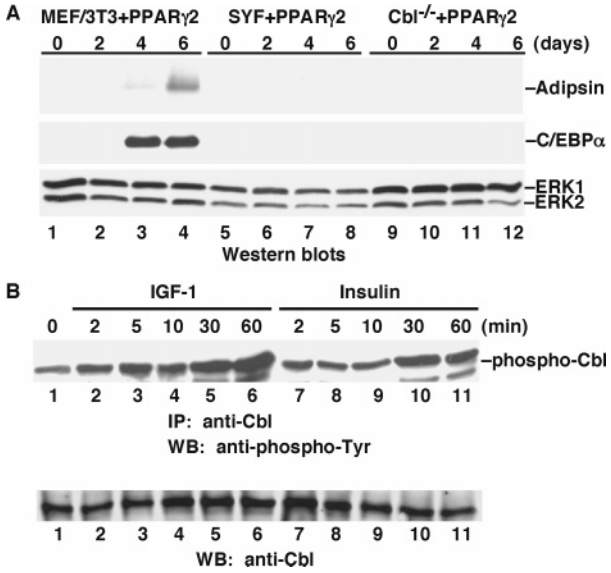

FIGURE 5: Deficiency of Src-family tyrosine kinases or c-Cbl blocked the differentiation program. (A) MEF/3T3+PPARγ2, SYF+PPARγ2, and Cbl$^{-/-}$+PPARγ2 cells were treated with the differentiation mixture for 0, 2, 4, or 6 days. At the indicated time, whole cell lysates were prepared from these cells and Western blotted with anti-adipsin (top panel) or anti-C/EBPα (middle panel) antibodies. The bottom panel was blotted with anti-ERK1/2 antibody to show similar amounts of proteins used in each lane. (B) Time course of tyrosine phosphorylation of c-Cbl in MEF/3T3+PPARγ2 cells induced by IGF-1 or insulin. Whole cell lysates were made from cells treated for 0, 2, 5, 10, 30, or 60 min. After immunoprecipitation with anti-c-Cbl antibody, the samples were separated by SDS−PAGE. The top panel was probed with anti-phosphotyrosine antibody. The bottom panel was blotted with anti-c-Cbl antibody. Data are representative of three sets of experiments.

clonal expansion. This process ceases coincident with the expression of PPARγ and C/EBPα, which lead to permanent growth arrest and the fully differentiated adipocytes. It was proposed that insulin, through insulin receptor substrate-1 and -2 (IRS-1 and IRS-2, respectively), upregulates the mRNA expression of PPARγ and C/EBPα (*34*). However, since overexpression of PPARγ in SYF cells did not lead to adipogenesis, our data point to additional roles for insulin in adipogenesis. Since at the early stage of the differentiation process dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) were also used, Src-family tyrosine kinases could play signaling roles downstream of these molecules as well, in addition to downstream of insulin. In differentiated 3T3-L1 adipocytes, phosphorylated Cbl recruits the CrkII−C3G complex to lipid rafts, where guanine nucleotide exchange factor C3G activates small GTP-binding protein TC10 (*21*). Whether this same Cbl pathway functions during adipogenesis and how the Src-Cbl signaling leads to eventual fat accumulation need further investigation.

Our data suggest that perhaps Src-family tyrosine kinase inhibitors could be explored as obesity treatments. Although no successful clinical application of Src-family kinase inhibitors has been reported, an inhibitor for a related non-receptor tyrosine kinase Abl is effective in the treatment of chronic myelogenous leukemia (*35*). This clinical reward highlights the hope for kinase inhibitors for treating other diseases. Few epidemics have affected more individuals than the current explosive rise in obesity. Obesity in turn promotes type II diabetes. Increased adipocyte differentiation and increased insulin sensitivity of existing adipocytes and muscle cells are major obstacles in the treatment of type II diabetes. A major challenge in treating type II diabetes is to increase insulin sensitivity without promoting adipocyte differentiation and triglyceride stores; these would counteract the initial benefits of such treatments because clinically more and larger adipocytes will increase insulin resistance. Our work suggests that potential new strategies could be designed to circumvent the problem.

ACKNOWLEDGMENT

We thank M. Lampson and T. McGraw for advice on the 3T3-L1 cell adipogenesis assay and for rosiglitazone, B. Spiegelman for the PPARγ2 retroviral construct, A. Koleske for the Abl$^{-/-}$/Arg$^{-/-}$ cells, M. David for the Cbl$^{-/-}$ cells, and T. McGraw, L. Levin, W. Lowry, and members of our laboratory for critical reading of the manuscript and discussion.

REFERENCES

1. Hunter, T., and Cooper, J. A. (1985) Protein-tyrosine kinases, *Annu. Rev. Biochem. 54*, 897−930.
2. Thomas, S. M., and Brugge, J. S. (1997) Cellular functions regulated by Src family kinases, *Annu. Rev. Cell Dev. Biol. 13*, 513−609.
3. Xu, W., Harrison, S. C., and Eck, M. J. (1997) Three-dimensional structure of the tyrosine kinase c-Src, *Nature 385*, 595−602.
4. Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) Crystal structure of the Src family tyrosine kinase Hck, *Nature 385*, 602−9.
5. Irby, R. B., Mao, W., Coppola, D., Kang, J., Loubeau, J. M., Trudeau, W., Karl, R., Fujita, D. J., Jove, R., and Yeatman, T. J. (1999) Activating SRC mutation in a subset of advanced human colon cancers, *Nat. Genet. 21*, 187−90.
6. Friedman, J. M. (2000) Obesity in the new millennium, *Nature 404*, 632−4.
7. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor, *Cell 79*, 1147−56.
8. Lowell, B. B. (1999) PPARγ: An essential regulator of adipogenesis and modulator of fat cell function, *Cell 99*, 239−42.
9. Rosen, E. D., Walkey, C. J., Puigserver, P., and Spiegelman, B. M. (2000) Transcriptional regulation of adipogenesis, *Genes Dev. 14*, 1293−307.
10. Green, H., and Meuth, M. (1974) An established pre-adipose cell line and its differentiation in culture, *Cell 3*, 127−33.
11. Spiegelman, B. M., Choy, L., Hotamisligil, G. S., Graves, R. A., and Tontonoz, P. (1993) Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes, *J. Biol. Chem. 268*, 6823−6.
12. Klinghoffer, R. A., Sachsenmaier, C., Cooper, J. A., and Soriano, P. (1999) Src family kinases are required for integrin but not PDGFR signal transduction, *EMBO J. 18*, 2459−71.
13. Ma, Y. C., Huang, J., Ali, S., Lowry, W., and Huang, X. Y. (2000) Src tyrosine kinase is a novel direct effector of G proteins, *Cell 102*, 635−46.
14. Koleske, A. J., Gifford, A. M., Scott, M. L., Nee, M., Bronson, R. T., Miczek, K. A., and Baltimore, D. (1998) Essential roles for the Abl and Arg tyrosine kinases in neurulation, *Neuron 21*, 1259−72.
15. Rosen, E. D., Hsu, C. H., Wang, X., Sakai, S., Freeman, M. W., Gonzalez, F. J., and Spiegelman, B. M. (2002) C/EBPα induces adipogenesis through PPARγ: A unified pathway, *Genes Dev. 16*, 22−6.
16. Lowry, W. E., Huang, J., Ma, Y. C., Ali, S., Wang, D., Williams, D. M., Okada, M., Cole, P. A., and Huang, X. Y. (2002) Csk, a critical link of G protein signals to actin cytoskeletal reorganization, *Dev. Cell 2*, 733−44.
17. Wan, Y., Kurosaki, T., and Huang, X. Y. (1996) Tyrosine kinases in activation of the MAP kinase cascade by G-protein-coupled receptors, *Nature 380*, 541−4.
18. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. (1996) Discovery of a novel, potent, and Src family-selective

tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation, *J. Biol. Chem. 271*, 695−701.
19. Saltiel, A. R., and Pessin, J. E. (2002) Insulin signaling pathways in time and space, *Trends Cell Biol. 12*, 65−71.
20. Baumann, C. A., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., Bickel, P. E., Pessin, J. E., and Saltiel, A. R. (2000) CAP defines a second signalling pathway required for insulin-stimulated glucose transport, *Nature 407*, 202−7.
21. Chiang, S. H., Baumann, C. A., Kanzaki, M., Thurmond, D. C., Watson, R. T., Neudauer, C. L., Macara, I. G., Pessin, J. E., and Saltiel, A. R. (2001) Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10, *Nature 410*, 944−8.
22. Blesofsky, W. A., Mowen, K., Arduini, R. M., Baker, D. P., Murphy, M. A., Bowtell, D. D., and David, M. (2001) Regulation of STAT protein synthesis by c-Cbl, *Oncogene 20*, 7326−33.
23. Rosen, B. S., Cook, K. S., Yaglom, J., Groves, D. L., Volanakis, J. E., Damm, D., White, T., and Spiegelman, B. M. (1989) Adipsin and complement factor D activity: An immune-related defect in obesity, *Science 244*, 1483−7.
24. Ribon, V., and Saltiel, A. R. (1997) Insulin stimulates tyrosine phosphorylation of the proto-oncogene product of c-Cbl in 3T3-L1 adipocytes, *Biochem. J. 324* (Part 3), 839−45.
25. Donovan, J. A., Wange, R. L., Langdon, W. Y., and Samelson, L. E. (1994) The protein product of the c-cbl protooncogene is the 120-kDa tyrosine-phosphorylated protein in Jurkat cells activated via the T cell antigen receptor, *J. Biol. Chem. 269*, 22921−4.
26. Tezuka, T., Umemori, H., Fusaki, N., Yagi, T., Takata, M., Kurosaki, T., and Yamamoto, T. (1996) Physical and functional association of the cbl protooncogen product with an src-family protein tyrosine kinase, p53/56lyn, in the B cell antigen receptor-mediated signaling, *J. Exp. Med. 183*, 675−80.
27. Fukazawa, T., Reedquist, K. A., Trub, T., Soltoff, S., Panchamoorthy, G., Druker, B., Cantley, L., Shoelson, S. E., and Band, H. (1995) The SH3 domain-binding T cell tyrosyl phosphoprotein p120. Demonstration of its identity with the c-cbl protooncogene product and in vivo complexes with Fyn, Grb2, and phosphatidylinositol 3-kinase, *J. Biol. Chem. 270*, 19141−50.
28. Sekimoto, H., and Boney, C. M. (2003) C-Terminal Src kinase (CSK) modulates insulin-like growth factor-I signaling through Src in 3T3-L1 differentiation, *Endocrinology 144*, 2546−52.
29. Molero, J. C., Jensen, T. E., Withers, P. C., Couzens, M., Herzog, H., Thien, C. B., Langdon, W. Y., Walder, K., Murphy, M. A., Bowtell, D. D., James, D. E., and Cooney, G. J. (2004) c-Cbl-deficient mice have reduced adiposity, higher energy expenditure, and improved peripheral insulin action, *J. Clin. Invest. 114*, 1326−33.
30. Thien, C. B., and Langdon, W. Y. (2001) Cbl: Many adaptations to regulate protein tyrosine kinases, *Nat. Rev. Mol. Cell Biol. 2*, 294−307.
31. Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ, *Science 274*, 2100−3.
32. Magun, R., Burgering, B. M., Coffer, P. J., Pardasani, D., Lin, Y., Chabot, J., and Sorisky, A. (1996) Expression of a constitutively activated form of protein kinase B (c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation, *Endocrinology 137*, 3590−3.
33. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation, *J. Biol. Chem. 271*, 31372−8.
34. Miki, H., Yamauchi, T., Suzuki, R., Komeda, K., Tsuchida, A., Kubota, N., Terauchi, Y., Kamon, J., Kaburagi, Y., Matsui, J., Akanuma, Y., Nagai, R., Kimura, S., Tobe, K., and Kadowaki, T. (2001) Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation, *Mol. Cell. Biol. 21*, 2521−32.
35. Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C. L. (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia, *N. Engl. J. Med. 344*, 1031−7.

BI0509090