Fyn-Dependent Regulation of Energy Expenditure and Body Weight Is Mediated by Tyrosine Phosphorylation of LKB1

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SUMMARY

Fyn null mice display reduced adiposity associated with increased fatty acid oxidation, energy expenditure, and activation of the AMP-dependent protein kinase (AMPK) in skeletal muscle and adipose tissue. The acute pharmacological inhibition of Fyn kinase activity with SU6656 in wild-type mice reproduces these metabolic effects and induced a specific reduction in fat mass with no change in lean mass. LKB1, the main upstream AMPK kinase (AMPKK) in peripheral tissues, was redistributed from the nucleus into the cytoplasm of cells treated with SU6656 and in cells expressing a kinase-deficient, but not a constitutively kinase-active, Fyn mutant. Moreover, Fyn kinase directly phosphorylated LKB1 on tyrosine 261 and 365 residues, and mutations of these sites resulted in LKB1 export into the cytoplasm and increased AMPK phosphorylation. These data demonstrate a crosstalk between Fyn tyrosine kinase and the AMPK energy-sensing pathway, through Fyn-dependent regulation of the AMPK upstream activator LKB1.

INTRODUCTION

Fyn is a member of the large Src family of nonreceptor tyrosine kinases that share conserved structural domains. The Src homology 1 (SH1) domain contains the catalytic tyrosine kinase activity, and the SH2 domain binds tyrosine-phosphorylated substrates. In particular, Fyn SH2 domain binds the tyrosine 528 residue in the carboxy-terminal tail of the protein, stabilizing the structure into an inactive conformation, thereby inhibiting the tyrosine kinase SH1 domain (Sicheri and Kuriyan, 1997; Sicheri et al., 1997; Songyang et al., 1995). The dephosphorylation of this site is required to release the SH2 domain and to activate the tyrosine kinase activity of Fyn.

Several studies have implicated Src kinase family members in mediating a subset of insulin signaling events. For example, Fyn was reported to directly associates with insulin-stimulated tyrosine-phosphorylated IRS and c-Cbl proteins (Myers et al., 1996; Ribon et al., 1998; Sun et al., 1996). In addition, Src family kinases have been found to activate the phosphatidylinositol (PI) 3-kinase signaling pathway, an established link to the stimulation of glucose transport in skeletal muscle and adipocytes (Choudhary et al., 2006). Upon posttranslational modifications such as palmitoylation and/or N-myristoylation, the Fyn kinase dynamically and reversibly redistributes between the cell interior and the plasma membrane (Alland et al., 1994; Filipp et al., 2003; Shenoy-Scaria et al., 1994). Several studies have also implicated Fyn in the regulation of insulin signaling through lipid raft microdomains. For example, Fyn was reported to be the kinase responsible for 3T3L1 adipocyte insulin-stimulated caveolin tyrosine phosphorylation and to associate with lipid raft proteins flotillin and CD36 (Bull et al., 1994; Huang et al., 1991; Mastick and Saltiel, 1997). In this regard, CD36, also known as fatty acid translocase (FAT), facilitates long-chain fatty acid uptake in skeletal muscle and adipose tissue and is linked to phenotypic features of the metabolic syndrome, including insulin resistance and dyslipidemia (Drover and Abumrad, 2003; Drover et al., 2005; Meex et al., 2005; Pravenec et al., 2003). Thus, the physical association of Fyn with CD36 further suggests a functional coupling between lipid raft organization and the regulation of fatty acid translocation and potentially fatty acid metabolism. More recently, we found that Fyn null mice display markedly improved insulin sensitivity and improved plasma and tissue triglyceride/nonesterified fatty acid levels coupled with higher rates of energy expenditure and fatty acid oxidation in the fasted state (Bastie et al., 2007). This was directly correlated with increased AMPK-dependent protein kinase (AMPK) T172 α subunit phosphorylation, increased AMPK activity, and inhibition of acetyl-CoA carboxylase (ACC) function.

AMPK is a heterotrimeric complex composed of one catalytic α plus two regulatory subunits, β and γ. Each functional AMPK complex is composed of multiple isoforms with overlapping tissue distributions (Cheung et al., 2000; Daval et al., 2006). Skeletal muscle primarily expresses the α2 subunit as well as both β and all three γ isoforms, whereas adipose tissue primarily expresses the α1 subunit with both β and γ1 and γ2 isoforms (Daval et al., 2006; Towler and Hardie, 2007). The regulation of AMPK activity depends on the type of subunits assembled and cellular energy status, being activated when the AMP/ATP ratio increases, which occurs in states of cellular nutritional deficiency. Binding of AMP to the γ subunit results in a conformational change that may decrease AMPK as a substrate for the PP2C phosphatase (Steinberg, 2007). Alternatively, it was reported that AMP binding increases the ability of upstream kinases (AMPK kinases) to phosphorylate the activating...
threonine residue (T172) in the α subunit (Towler and Hardie, 2007). In neurons, the calcium-stimulated activation of AMPK is dependent upon the Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaMKK) family that phosphorylates the α subunit T172 residue (Hawley et al., 2005). Although CaMKKs have also been shown to activate AMPK in the skeletal muscle under mild tetanic contraction, CaMKK expression is very low in peripheral tissues and is primarily restricted to brain, testis, thymus, and T cells (Jensen et al., 2007; Anderson et al., 1998). In contrast, LKB1 is expressed in insulin-responsive tissues, and muscle-specific LKB1 knockout mice are unable to activate AMPK (Alessi et al., 2006; Sakamoto et al., 2005).

LKB1 is a serine/threonine kinase originally identified as a tumor suppressor protein mutated in Peutz-Jeghers syndrome that controls diverse cellular processes, including cellular polarity, cancer, and metabolism (Hemminki et al., 1997; Jenne et al., 1998). Regulation of LKB1 appears to be a complex process that involves phosphorylation on diverse residues (S31, S325, T366, and S431) and association into a ternary complex with MO25 and STRADα or STRADβ. Serine 431 in LKB1 is highly conserved in all organisms except Caenorhabditis elegans and is phosphorylated by p90 ribosomal S6 protein kinase (RSK) and protein kinase A (PKA). Although the phosphorylation of S431 was initially described as critical for LKB1 activity, more recent studies have suggested that it might not be necessary and that other activation mechanisms might exist (Fogarty and Hardie, 2009). In particular, LKB1 subcellular localization is an important event regulating LKB1 activity, as LKB1 functions as a tumor suppressor only when it localizes in the cytoplasm and appears to be inactive when restricted to the nucleus of cells (Alessi et al., 2006). Recent studies have demonstrated that MO25 stabilizes the interactions between LKB1 and STRADα and that the ternary complex is cytoplasmically localized, whereas the monomeric LKB1 protein and/or the dimeric LKB1/MO25 complex are primarily nuclear localized (Boudeau et al., 2003). In addition, LKB1 was reported to undergo sirtuin-mediated deacetylation with the acetylated form restricted to the nucleus and was redistributed to the cytoplasm following deacetylation (Lan et al., 2008).

We recently reported that conventional Fyn knockout mice displayed increased energy expenditure and fatty acid oxidation due to increased activation of adipose tissue and skeletal muscle AMPK activity, resulting in reduced adipose tissue mass and enhanced insulin sensitivity (Bastie et al., 2007). Importantly, the enhanced catabolism occurred predominantly in the fasted state, and the Fyn null mice were able to convert to a normal anabolic state during the feeding cycle. These data suggested that pharmacological inhibition of Fyn function could induce selective weight loss by decreasing fat mass and simultaneously increasing insulin sensitivity. In addition, this approach would identify the acute role of the Fyn tyrosine kinase activity in adult animals without regard to putative developmental/adaptive changes present in the conventional Fyn null mice. In this study, we examined the physiologic and molecular signaling events regulating energy metabolism using the selective Src family kinase inhibitor SU6656 (Blake et al., 2000). We observed that acute treatment with SU6656 rapidly increases energy expenditure, fatty acid oxidation, and AMPK α subunit T172 phosphorylation in wild-type mice, mimicking the metabolic phenotype of the Fyn null mice. Mechanistically, AMPK activation resulted from a Fyn-dependent tyrosine phosphorylation of LKB1 that regulates the subcellular localization of LKB1 and activation of AMPK independent of STRADα association. Together, these data describe a signaling pathway accounting for the control of energy expenditure by Fyn, and support the therapeutic potential of acute Fyn inhibition in vivo.

**RESULTS**

**Fyn Activity Inhibition Increases Energy Expenditure and Lipid Utilization**

Loss of Fyn kinase protein in the conventional Fyn knockout mice leads to increased lipid utilization and energy expenditure, particularly in the fasting/resting state (Bastie et al., 2007). To examine the effects of acute Fyn inhibition, we investigated the in vivo effects of the pharmacological selective Src family kinase inhibitor SU6656. Wild-type mice were placed into metabolic chambers and allowed to acclimatize for 48 hr. Intrapertorineal injection of SU6656 or vehicle was performed at the beginning of the light cycle (fasting/resting state), and the respiratory quotient (RQ), oxygen consumption (VO₂), energy expenditure (EE), and physical activity were monitored for the following 12 hr. Both animal groups displayed identical carbohydrate utilization during the dark cycle preceding the injection, as shown by similarly high respiratory quotients (Figure 1A). As expected, the RQ gradually decreased in the vehicle-treated mice during the light cycle as the mice normally switched to lipid utilization. However, the decrease of RQ was more pronounced in SU6656-treated mice and was particularly more effective during the first 3 hr following the injection (Figure 1A). Oxygen consumption and energy expenditure were similar in both groups before the injection and were decreased during the light period as the animals were resting with reduced basal metabolism. However, both oxygen consumption and energy expenditure remained 14% more elevated in the SU6656-injected group (Figures 1B and 1C) without any alteration in physical activity in either the dark or light cycle (Figure 1D).

To examine whether the increased energy expenditure resulted in weight loss in the SU6656-injected mice, total body mass was evaluated before (T = 0 hr) and after (T = 12 hr) vehicle and SU6656 injections (Figure 2A, left and right panels, respectively). As mice generally consume 80% of their calories during the dark cycle and have a limited food intake during the light cycle, they undergo a diurnal pattern of weight gain and loss as shown by the decreased body mass at T = 12 hr in the vehicle-treated mice. The SU6656-treated animals also displayed decreased body weight 12 hr after the injection (Figure 2A, right panel), and the total weight loss was 40% greater than that of the vehicle-treated group (Figure 2B). Lean mass was slightly reduced during the light cycle, but there was no significant difference between vehicle and SU6656-injected mice (Figure 2C). In contrast, fat mass was significantly reduced in the SU6656-treated mice compared to vehicle-injected control animals (Figure 2D). To determine whether the lowered RQ in the mice treated with SU6656 was directly due to increased skeletal muscle fatty acid oxidation, red soleus and white gastrocnemius muscles were isolated 3 hr following the
vehicle or SU6656 injection and incubated with \(^{14}\text{C}\)-palmitate. The oxidation of \(^{14}\text{C}\)-palmitate to \(^{14}\text{CO}_2\) was increased 25% and 33%, respectively, in the soleus and the gastrocnemius of the SU6656-treated mice relative to vehicle treated controls (Figures 3A and 3B). The SU6656-stimulated increase in fatty acid oxidation occurred concomitantly, with an approximate 3-fold increased AMPK \(\alpha\) subunit T172 phosphorylation and increased ACC S79 (ACC1) and S221 (ACC2) phosphorylation levels (Figures 3C and 3D).

Although SU6656 is a highly selective Src family kinase inhibitor, it is not specific for Fyn. Thus, these data do not exclude a role for other Src family kinases in mediating these metabolic effects. To address this issue, we examined the metabolic effect of SU6656 on wild-type versus Fyn knockout mice. As previously reported (Bastie et al., 2007), Fyn null mice display a marked reduction in RQ compared to wild-type mice (Figure 3E). Similarly to Figure 1, SU6656 treatment of wild-type mice resulted in an enhanced conversion to fatty acid utilization characterized by a lower RQ (Figure 3E, filled versus open circles). In contrast, SU6656 treatment had no significant effect on the rate of change or the RQ level in the Fyn knockout mice (Figure 3E, filled versus open diamonds). In addition, we did not observe any alterations of body mass or physical activity after SU6656 treatment in the Fyn null mice (see Figure S1 available online). As the two most closely related kinases to Fyn are Src and Lyn, we examined the protein expression levels of Src and Lyn proteins in the Fyn null mice. As shown in Figure 3F, the levels of Src were unchanged in adipose tissue, liver, or skeletal muscle of the Fyn null mice. Although Lyn is not expressed in skeletal muscle, adiposity, and promotes weight loss most likely through a Fyn kinase-dependent mechanism.

**Fyn Regulates LKB1 Subcellular Localization**

Previous studies have demonstrated that LKB1 is predominantly nuclear localized in cultured cells, whereas its substrate target, AMPK, is primarily localized to the cytoplasm (Alessi et al., 2006). To determine if Fyn regulates LKB1 localization, we transfected the C2C12 muscle cell line with GFP-LKB1 and examined the effect of acute SU6656 treatment on subcellular LKB1 localization (Figure 4). As previously reported (Nezu et al., 1999; Tiainen et al., 2002), in vehicle-treated cells the majority of the LKB1 was nuclear localized (Figures 4A and 4B). However, treatment with SU6656 resulted in the redistribution of LKB1 into the cytoplasm in approximately 65% of the cells. Figure S2 shows representative images of multiple C2C12 cells treated with Vehicle (Figure S2A) or SU6656 (Figure S2B). Since the concentration of SU6656 (10 \(\mu\text{M}\)) is sufficient to inactivate Fyn kinase activity (Blake et al., 2000), this suggests that LKB1 subcellular localization was controlled by the catalytic activity of Fyn. We therefore transfected C2C12 and 3T3L1 adipocytes with a constitutively active Fyn mutant (Fyn-CA) in which the negative regulatory tyrosine (Y528) site was mutated to phenylalanine and a kinase-defective mutant (Fyn-KD) in which the catalytic lysine (K299) residue was mutated to methionine. Expression of Fyn-CA in C2C12 appeared to alter the morphology to a more rounded phenotype, but there was no significant effect on the nuclear localization of LKB1, whereas expression of Fyn-KD had no morphology effect but resulted in a redistribution of more than
50% of the LKB1 out of the nucleus and into the cytoplasm (Figures 4C and 4D). Figure S3 shows representative images of multiple C2C12 cells transfected with constitutively active Fyn (Fyn-CA, Figure S3A) or kinase-defective Fyn (Fyn-KD, Figure S3B).

In contrast to C2C12 cells, subcellular localization is more readily visualized in adipocytes due to the presence of large lipid droplets. Expression of Fyn-CA or Fyn-KD has no significant effect on the morphology of the adipocytes (Figure 4E). LKB1 remained nuclear localized in the presence of Fyn-CA, whereas expression of Fyn-KD resulted in decreased nuclear LKB1 with an increased cytosolic localization (Figures 4E and 4F). Figure S4 shows representative images of multiple 3T3L1 adipocytes transfected with constitutive active Fyn (Fyn-CA, Figure S4A) or kinase-defective Fyn (Fyn-KD, Figure S4B).

Since STRADα has been reported to regulate intracellular LKB1 distribution (Boudeau et al., 2003, 2004), we cotransfected 3T3L1 adipocytes with STRADα and either Fyn-CA or Fyn-KD and examined subcellular localization of tagged LKB1. As previously observed, tagged LKB1 alone was localized to nuclei of adipocytes (Figures S5Aa–S5Ac), but following coexpression with STRADα, there was an efficient redistribution of LKB1 into the cytoplasm (Figures S5Ad–S5Ag). However, LKB1 remained nuclear localized when coexpressed with the constitutively active form of Fyn kinase, even in the presence of STRADα (Figures S5Ba–S5Bd). As a control, the expression of the kinase-deficient Fyn (Fyn-KD) with STRADα had no additional effect on the cytoplasmic localization of LKB1 (Figures S2Bf–S2Bj). The quantification of these data is presented in Figure S5C. Due to the high expression levels of endogenous MO25 in adipocytes, identical results were also obtained when STRADα and MO25 were coexpressed with LKB1 (data not shown).

LKB1 Is a Substrate for Fyn Tyrosine Kinase

Since Fyn-KD, but not Fyn-CA, induced the redistribution of LKB1 into the cytoplasm, we next examined whether LKB1 can interact and/or is a substrate for the Fyn tyrosine kinase catalytic activity. First, we observed that endogenous LKB1 coimmunoprecipitated with Fyn kinase in skeletal muscle and in 3T3L1 adipocytes (Figures 5A and 5B). We next coexpressed Fyn with LKB1 in 3T3L1 adipocytes. Immunoblotting of LKB1 immunoprecipitates with the PY100 phosphotyrosine antibody demonstrated Fyn-induced tyrosine phosphorylation of LKB1 (Figures 5C and 5D). LKB1 is a direct substrate target of Fyn, as purified His-LKB1-tagged fusion protein was tyrosine phosphorylated by purified recombinant Fyn kinase in vitro (Figure 5E). To identify the LKB1 tyrosine sites’ phosphorylated by Fyn, we utilized Phosphosite Detector from JPT peptide technology. As shown in Figure S6, 141 overlapping peptides (12–15 residues in length) corresponding to the LKB1 sequence were subjected to in vitro phosphorylation utilizing purified recombinant Fyn tyrosine kinase. This analysis identified five tyrosines (Y60, Y156, Y166, Y261, and Y365) as potential LKB1 phosphorylation acceptor sites for the Fyn kinase. To identify the phosphorylation sites in vivo, we next generated single point mutants where each tyrosine site was substituted by a phenylalanine residue. Coexpression of Fyn-CA with wild-type LKB1 and each individual mutant demonstrated equal protein expression levels for all LKB1 mutants (Figure 5F). Tyrosine phosphorylation levels were decreased with LKB1-Y261F mutant, and a substantially greater reduction was obtained with LKB1-Y365F (Figure 5G). In addition, while both single mutants (Y261F and Y365F) partially reduced LKB1 phosphorylation, the double mutation Y261/365F had a more pronounced decrease in LKB1 tyrosine phosphorylation (Figures 5H and 5I).

Figure 2. SU6656-Induced Fyn Inhibition Promotes Fat Mass Loss

(A) Body weight distribution of vehicle and SU6656-injected mice before (T = 0) and after (T = 12 hr) the injection.

(B) Total weight loss 12 hr after the injection of vehicle (open bar) and SU6656 (dark bar) treated animals.

(C) Fat mass before (T = 0) and after (T = 12 hr) vehicle or SU6656 injection.

(D) Lean mass before (T = 0) and after (T = 12 hr) vehicle or SU6656 injection (see also Figure S1).

Results are expressed as mean ± SEM.
LKB1 Subcellular Distribution Is Regulated by Tyrosine Phosphorylation

Since inhibition of Fyn kinase activity by SU6656 treatment or expression of a Fyn kinase-deficient mutant (dominant negative) resulted in redistribution of LKB1 out of the nucleus, we examined the subcellular localization of the phosphorylation-defective mutants of LKB1 (Figure 6). As previously observed, in adipocytes wild-type LKB1 was predominantly nuclear localized as well as the LKB1-Y60F mutant (Figures 6Aa–6Af). Similarly, both LKB1 mutants Y156F and Y166F were also nuclear localized (data not shown). In contrast, both the LKB1 Y261F and Y365F mutants displayed a greater cytosolic distribution, similar to that observed for the SU6656-treated and Fyn-KD-transfected cells (Figures 6Ba–6Bf). Similarly, the LKB1 double mutation Y261/365F displayed a predominant cytoplasmic distribution (Figures 6Ca–6Cc). To determine if the Fyn-dependent tyrosine phosphorylation at Y261 and Y365 was directly responsible for LKB1 nuclear localization, we coexpressed constitutively active Fyn (Fyn-CA) with the LKB1-Y261/365F double mutant. In this case, LKB1-Y261/365F remained cytosolic, and Fyn-CA was ineffective in redistributing this mutant into the nucleus (Figures 6Da–6Dd). Quantification of these data is presented in Figure 6E with approximately 20% of LKB1-WT and LKB1-Y60F displaying a cytoplasmic localization, whereas 55%–60% of LKB1-Y261F and LKB1-Y365F were in the cytoplasm. Moreover, nearly 90% of the LKB1-Y261/365F double mutant was found in the cytoplasm, and coexpression of Fyn-CA was ineffective in altering the subcellular localization of the LKB1-Y261/365F double mutant.

To confirm these results in vivo, we took advantage of skeletal muscle transfection by electroporation as described previously (Prud’homme et al., 2006). The expressed Flag-LKB1-WT is predominantly detected in the muscle syncytia that parallel the sarcolemma (Figures 7Aa–7Ac). In contrast, expression of the Flag-LKB1-Y261/365F double mutant resulted in a more cytoplasmic localization (Figures 7Ad–7Af). The low level of nonspecific background labeling is shown in Figures 7Ag–7Ai, and a larger magnification of LKB1 localization is provided in Figure 7B. Although it is very difficult to detect the endogenous LKB1 in vivo by immunofluorescence due to the quality of the currently available antibodies, we also observed an increased LKB1 signal in the cytoplasm of muscle cells of wild-type mice treated with SU6656 (data not shown).

To determine if Fyn kinase also induces the tyrosine phosphorylation of LKB1 in vivo, tibialis anterior muscles were transfected with Fyn-CA, and endogenous LKB1 was immunoprecipitated and immunoblotted with PY100 (Figure S7A and S7B). In the absence of Fyn-CA, there was a relatively low level of LKB1 tyrosine phosphorylation that was significantly increased by the
expression of Fyn-CA. To demonstrate the functional consequence of LKB1 distribution, we first assessed the effect of the LKB1-Y261/365F double mutant on AMPK phosphorylation by coexpression with the GST-AMPKα subunit in HeLa cells. There was a low level of AMPKα subunit T172 phosphorylation when coexpressed with LKB1-WT that was increased in the presence of the LKB1-Y261/365F double mutant (Figure S7C). Moreover, there was a marked increase in endogenous AMPKα subunit T172 phosphorylation in tibialis anterior muscle transfected with the LKB1-Y261/365F double mutant compared to LKB1-WT (Figure S7D).

**DISCUSSION**

AMP-activated protein kinase (AMPK) is considered a cellular “energy sensor” directly regulated by alterations of the intracellular AMP/ATP ratio that occur during prolonged fasting and refeeding (Hardie, 2008a; Hardie et al., 2006; Hue and Rider, 2007; Schimmack et al., 2006). During states of low energy, activation of AMPK results in the phosphorylation and inhibition of ACC activity, thereby lowering malonyl-CoA levels, leading to increased fatty acid oxidation and a reduction in fatty acid synthesis (Brownsey et al., 2006). In contrast, during states of caloric excess AMPK is inactive, resulting in increased ACC activity and inhibition of fatty acid oxidation with a concomitant increase in fatty acid storage. The critical role of AMPK in determining energy balance has been clearly demonstrated in both AMPK knockout and overexpression of dominant-interfering AMPK mutants that display insulin resistance, reduced energy expenditure, and inability to undergo normal metabolic switching between carbohydrate and fatty acid fuels (Hardie, 2008a, 2008b; Viollet et al., 2003). Thus, mechanisms and factors controlling AMPK activity are key issues in the balance of lipid and glucose metabolism that may provide novel therapeutic opportunities.

Previously, we demonstrated that Fyn functions as a negative regulator of fatty acid oxidation through the inhibition of AMPK in skeletal muscle and adipose tissue (Bastie et al., 2007). This was based upon the observation that conventional Fyn null mice displayed enhanced fatty acid oxidation in adipose tissue and skeletal muscle, increased AMPK activity, increased energy expenditure, and insulin sensitivity. However, due to the constitutive loss of Fyn expression, these data could neither address potential developmental tissue adaptations that could be responsible for these metabolic alterations nor distinguish whether this resulted from a loss of Fyn kinase activity or protein interaction functions. Therefore, the mechanism responsible for AMPK activation in the Fyn null mice remained enigmatic.

To address these issues, we first took advantage of a selective pharmacological approach to acutely inhibit Src family kinase activity that recapitulated the metabolic phenotype observed in...
the Fyn null mice, including increased fatty acid oxidation, energy expenditure, and AMPK active site phosphorylation. The metabolic effect of the SU6656 inhibitor was likely specific for Fyn, as this agent had no effect in Fyn null mice. These changes in whole-body metabolism occurred relatively rapidly (2–3 hr) upon acute treatment with SU6656, consistent with inhibition of Fyn kinase activity rather than developmental compensation or other Fyn-protein interactions being responsible for the observed phenotype. Moreover, the phenotypic characteristics of Fyn inhibition are essentially the same as those reported for other animal models with increased AMPK activity (Viollet et al., 2009). Taken together, these data strongly indicate that Fyn kinase activity per se serves to functionally inhibit AMPK activity. Importantly, the acute inhibition of Fyn resulted in significant weight loss due to decreased adipose tissue mass without any significant change in lean mass, supporting a potential therapeutic approach for modulating Fyn function.

In this regard, the primary upstream kinase activator of AMPK in peripheral tissues is LKB1, and we speculated that this was a potential Fyn target responsible for the regulation of AMPK. Several studies have demonstrated that LKB1 activity depends on its subcellular localization, LKB1 being active in the cytoplasm and inactive when restricted to the nucleus of cultured cells (Alessi et al., 2006; Baas et al., 2003). Since AMPK is predominantly localized in the cytoplasm, nuclear export of LKB1 would be required for LKB1-dependent phosphorylation of AMPK. Our data demonstrate that activated Fyn kinase activity maintains LKB1 predominantly in the nucleus and that inhibition of Fyn kinase activity results in LKB1 redistribution into the cytoplasm of cultured cells. Moreover, skeletal muscle nuclei are peripherally organized adjacent to the sarcolemma, and LKB1 is also peripherally localized in skeletal muscle in vivo. Although there appears to be partial overlap with nuclei, inhibition of Fyn kinase activity also results in LKB1 redistribution into the cytoplasm in a manner similar to the cultured cells.

Mechanistically, our data indicate that Fyn-dependent regulation of LKB1 localization occurs through the tyrosine phosphorylation of two critical sites on LKB1 (Y261 and Y365). Taken together, these data strongly indicate that Fyn kinase activity per se serves to functionally inhibit AMPK activity. Importantly, the acute inhibition of Fyn resulted in significant weight loss due to decreased adipose tissue mass without any significant change in lean mass, supporting a potential therapeutic approach for modulating Fyn function.

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localization also results in increased AMPK phosphorylation on T172 both in skeletal muscle in vivo and in cultured cells.

We therefore propose a working hypothesis in which, similar to other regulated proteins that undergo nuclear import/export (i.e., Foxo1), LKB1 continually undergoes nuclear import/export such that, under basal steady-state levels, the equilibrium favors nuclear localization. However, this equilibrium shifts to greater cytoplasmic localization by either increasing nuclear export and/or reducing nuclear import through various regulatory events. For example, previous studies have shown that these include binding to STRADα/MO25 and LKB1 deacetylation (Boudeau et al., 2003; Lan et al., 2008). Since Fyn is predominantly a nonnuclear protein being distributed throughout the cell including the plasma membrane, endomembranes, and the cytoplasm (Alland et al., 1994; Davy et al., 1999; Filipp and Julius, 2004; Filipp et al., 2003; Parravicini et al., 2002), we hypothesize that Fyn interacts with and tyrosine phosphorylates the nuclear exported LKB1 that, in turn, increases its rate of nuclear import, resulting in a high steady-state level of nuclear LKB1. Inhibition of Fyn kinase prevents LKB1 tyrosine phosphorylation on Y261 and Y365, reducing the rate of nuclear import that now results in a greater steady-state level of LKB1 in the cytoplasm. One appealing model that can account for these observations is that Fyn-dependent LKB1 tyrosine phosphorylation prevents the assembly of LKB1 into the LKB1/STRADα/MO25 ternary complex, thereby increasing LKB1 nuclear localization. Tyrosine dephosphorylation would then allow for the formation of the ternary complex and promote cytosolic LKB1 localization and kinase activation.

In summary, we have demonstrated that LKB1 is a direct substrate for Fyn tyrosine kinase, that LKB1 subcellular distribution is regulated by tyrosine phosphorylation on Y261 and Y365, and that Fyn-dependent redistribution of LKB1 into the cytoplasm results in increased phosphorylation/activation of AMPK. Importantly, the positive metabolic effects observed in Fyn null mice (decreased adiposity and increased energy expenditure) are reproduced by the acute pharmacological inhibition of Fyn activity, resulting in weight loss via decreased adiposity without affecting lean mass. Since the deregulation of the whole-body energy homeostasis is one of the main events leading to the development of obesity, insulin resistance, and diabetes, these data highlight the therapeutic potential of inhibiting Fyn kinase signaling.

**EXPERIMENTAL PROCEDURES**

**Animals**

Eight- to ten-week-old male C57BL6/J, pp59fyn null mice and their controls were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in a facility equipped with a 12 hr light/dark cycle. Animals were fed ad libitum a standard chow diet (Research Diets, New Brunswick, NJ) containing 75.9% (Kcal) carbohydrates, 14.7% protein, and 9.4% fat. All studies were conducted in accordance with the Animal Care and Use Committee at the University of British Columbia.
Total body fat and lean mass were measured before (T = 0) and after (T = 12 hr) the injection.

Western Blot Analysis

Animals were injected with vehicle or SU6656 (4 mg/kg) at the beginning of the light cycle. They were anesthetized and sacrificed by cervical dislocation 3 hr after the injection. Tissues were rapidly freeze clamped in liquid nitrogen and stored at −80°C. Protein preparation and blotting were performed as described below. Membranes were blocked with 5% milk and 0.1% BSA in Tris-buffered saline for 60 min at room temperature and incubated overnight at 4°C with the indicated antibodies (Cell Signaling, Upstate, and Santa Cruz Biotechnology, Lincoln, NE). Alternatively, immunoblots were incubated with IRDye800CW goat anti-mouse (H+L) or IRDye680 goat anti-rabbit (H+L) secondary antibodies, and signal was detected with the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE).

Fatty Acid Oxidation in Isolated Muscles

Mice were sacrificed by cervical dislocation at the end of the light cycle. They were anesthetized and sacrificed by cervical dislocation 3 hr after the injection. Skeletal muscles (red soleus and white gastrocnemius) were rapidly removed from the animals in a cold room (4°C) with a pair of scissors, and tissues were rapidly freeze clamped in liquid nitrogen and stored at −80°C. Protein preparation and blotting were performed as described below. Membranes were blocked with 5% milk and 0.1% BSA in Tris-buffered saline for 60 min at room temperature and incubated overnight at 4°C with the indicated antibodies (Cell Signaling, Upstate, and Santa Cruz Biotechnology, Lincoln, NE). Alternatively, immunoblots were incubated with IRDye800CW goat anti-mouse (H+L) or IRDye680 goat anti-rabbit (H+L) secondary antibodies, and signal was detected with the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE).

Cell Culture

C2C12 myoblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum. Differentiation into myotubes was initiated by switching the myoblasts to DMEM supplemented with 2% horse serum for 4–6 days as described previously (Yaffe and Saxel, 1977a, 1977b). 3T3L1 preadipocytes were cultured in DMEM supplemented with 2% horse serum for 4–6 days as described previously (Yaffe and Saxel, 1977a, 1977b). 3T3L1 preadipocytes were cultured in DMEM supplemented with 10% calf serum at 37°C. Confluent cultures were induced to differentiate into adipocytes as described previously (Min et al., 1999).

cDNA Constructs

cDNA constructs were generated by RT-PCR performed on spleen total RNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with a pair of oligonucleotides: 5’-CACACATTCCACTCAAGAG-3’ and 5’-CAAGCAGGCTGCTGAATGTCAAGG-3’. The PCR product was separated on 2% agarose gel, and the specific single band was extracted using the QIAquick PCR purification kit (QIAGEN). The purified PCR product was cloned into the pcDNA3.1D/V5-His-TOPO using the pcDNA3.1 Directional TOPO Expression Kit (Invitrogen, Carlsbad, CA). pcDNA3.1-Fyn-V5 was obtained using the oligonucleotides 5’-CACACATTCCACTCAAGAG-3’ and 5’-CAAGCAGGCTGCTGAATGTCAAGG-3’ and following the same protocol. pcDNA3.1-Fyn-KD(K299M)-V5 was constructed approved by and performed in compliance with the guidelines of the Yeshiva University Institutional Animal Care and Use Committee (IACUC).

Oxygen and carbon dioxide consumption were simultaneously determined by Oxymax open-circuit indirect calorimetry system (eight-cage system) (Columbus Instruments). Animals were allowed to acclimatize for two complete light and dark cycles (48 hr), and SU6656 injections were performed at the beginning of the light cycle the following day. Measurements were subsequently taken 12 hr following the injection. Data were analyzed as the average of 1 hr measurements for each mouse. Instrument settings were as follows: gas flow rate, 0.6 l/min; sample flow rate, 0.5 l/min; settle time, 120 s; measure time, 60 s.

Total body mass (g) was recorded before (T = 0) and 12 hr (T = 12 hr) after the injection.

**Figure 7. Subcellular Localization of LKB1 in Skeletal Muscle In Vivo Is Regulated by Tyrosine Phosphorylation**

(A) Tibialis anterior was transfected with pcDNA-Flag-LKB1-WT (Ad–Ac), or the pcDNA empty vector (Ag–Al) cDNAs. Immunofluorescence was performed on 10 μm frozen sections for the localization of LKB1 (Flag antibody) and nuclei (DAPI). (B) Magnified images of muscles transfected with pcDNA-Flag-LKB1-WT (Ba–Bc) or the pcDNA-Flag-LKB1-Y261/365F double mutant (Bd–Bf) is shown to more easily visualize the change in LKB1 localization (see also Figure S7).

**Cell Metabolism**

**Fyn Regulation of Energy Expenditure**

Total body mass and magnetic resonance imaging were performed as described below. Membranes were blocked with 5% milk and 0.1% BSA in Tris-buffered saline for 60 min at room temperature and incubated overnight at 4°C with the indicated antibodies (Cell Signaling, Upstate, and Alpha Diagnostic International) in Tris-buffered saline and 0.05% Tween 20 (TBST) containing 1% BSA. Blots were washed in TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:30,000) for 30 min at room temperature. Membranes were washed in TBST, and antigen-antibody complexes were visualized by chemiluminescence using an ECL kit (Pierce). Alternatively, immunoblots were incubated with IRDye800CW goat anti-mouse (H+L) or IRDye680 goat anti-rabbit (H+L) secondary antibodies, and signal was detected with the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE).
Cell Metabolism

Fyn Regulation of Energy Expenditure

by overlapping extension PCR. The gene encoding Fyn was amplified with the pair of oligonucleotides 5’-CACATGCTGTTGCTGAAATGAAG-3’ and 5’-GCATGAGTTAGGTCCACTTCCTTTT-3’ and the pair of oligonucleotides 5’-AAGGACTCGTTGCGAGGCTC-3’ and 5’-TGAAGCTGGAGGTTTA-3’. Product was mixed, and a second PCR was performed using the oligonucleotides 5’-CACATGCTGTTGCTGAAATGAAG-3’ and 5’-CAGGTTTCGCGTAT-3’. PCR products were extracted and purified. Each product was mixed, and a second PCR was performed using the oligonucleotides 5’-CACATGCTGTTGCTGAAATGAAG-3’ and 5’-CAGGTTTCGCGTAT-3’. PCR products were cloned into the pcDNA3.10/V5-His-TOPO. The pYX-LKB1 construct was obtained from Open biosystems (Rockford, IL) and used to generate the pcDNA3.1-Flag-LKB1 construct. The gene encoding LKB1 was amplified with the oligonucleotides 5’-ATGGAGTACCAAGGAGATGACGACAGAAAATGAGCTGCGGAGGCCCTC-3’ and 5’-TCACTCTGGCTGCTGAGACGCAGAGACGTGGTAGCAG-3’. LKB1-Y166F, 5’-GGCGCTGGAATTCCTACACAGC-3; LKB1-Y166F, 5’-GGCGCTGGAATTCCTACACAGC-3’. Sections were washed three times under 0.22 kV, 950 mC/mg for 30–48 hr.

Transfection of C2C12 Myotubes and 3T3L1 Adipocytes

Transfection of C2C12 myotubes and 3T3L1 adipocytes were electroporated as previously described (Waters et al., 1999). A suspension of 3T3L1 adipocytes was electroporated with 500 μg of plasmid under low-voltage condition (0.16 kV, 950 μF). C2C12 myotubes were electroporated with a total of 250 μg of plasmid under 0.22 kV, 950 mC/mg for 5 days later. Animals were sacrificed 5 days after the second set of electroporation. The bilateral anterior muscles were rapidly removed and immediately embedded into optimal cutting temperature (O.C.T.) compound (Sakura Fine-tex USA, Inc., Torrance, CA). Tissues were frozen in liquid nitrogen. Frozen sections (10 μm) were prepared and subjected to immunofluorescence labeling as previously described, using anti-Flag mouse mAb antibody and Alexa Fluor 488 anti-mouse secondary antibody. Sections were washed three times with PBS and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) and were imaged as described above. Setting (iris pinhole), laser intensity, gain, and offset) were fixed and identical for all samples. Muscle extracts were also used for immunoprecipitation and immunoblotting as described below.

Signal Quantification

The ratio of cytosolic and nuclear LKB1 was quantified using the Image J software (National Institutes of Health). Images of 15 representative cells were processed, and results represent mean ± SE from three independent experiments.

Immunoprecipitation

Cells were homogenized in a NP-40 lysis buffer containing 25 mM HEPES (pH 7.4), 10% glycerol, 50 mM sodium fluoride, 10 mM sodium phosphate, 137 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM PMFS, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 μg/ml leupeptin and rocked for 10 min at 4°C. Muscle extracts (100 mg) were homogenized in the Bullet Blender (Next Advance, Inc., Avon, MA). Cells were homogenized using zirconium silicate beads (speed 8 for 3 min) in the buffer described above. Homogenates were centrifuged for 10–30 min at 13,000 g at 4°C, and supernatants were collected. Protein concentration was determined using the BCA protein assay. Cell lysates (3–4 mg) were incubated with 10 μg of antibody for 2 hr at 4°C. TrueBlot anti-rabbit Ig (IP Beads (50 μl) (eBioscience, Inc., San Diego, CA)) were added, and samples were rocked for 60 min at 4°C. Samples were washed three times with NP-40 lysis buffer and were resuspended in 100 μl of Laemmli buffer containing 50 mM DTT. Samples were heated at 90–100°C for 10 min and centrifuged at 10,000 x g for 3 min. Supernatants were collected and loaded on 10% SDS-polyacrylamide gels.

Immunofluorescence

In Vitro LKB1 Phosphorylation Assay

His-LKB1 fusion protein was purified using HisPur Purification kit and Slide-A-Lyzer Dialysis Cassette (Pierce, Rockford, IL). His-LKB1 protein (1 μg) was incubated with the recombinant His-FynT kinase (1 μl) (Calbiochem, Gibbstown, NJ) in the presence of Srrg ATP cocktail (Millipore, Billerica, MA), and kinase reaction was performed for 1 hr at 35°C. Samples were separated on 10% SDS-polyacrylamide gels, and immunoblotting was performed with PY100 monoclonal antibody and LKB1 polyclonal antibody. Signals were detected with the Odyssey Infrared Imaging System (Li-COR Biotechnology, Lincoln, NE).

Transfection of C2C12 Myotubes and 3T3L1 Adipocytes

Transfection of C2C12 myotubes and 3T3L1 adipocytes were electroporated as previously described (Waters et al., 1999). A suspension of 3T3L1 adipocytes was electroporated with 500 μg of plasmid under low-voltage condition (0.16 kV, 950 μF). C2C12 myotubes were electroporated with a total of 250 μg of plasmid under 0.22 kV, 950 μC/mg. Adipocytes and myotubes were allowed to adhere onto collagen-coated tissue culture dishes for 30–48 hr.

Culture Metabolism

In Vivo LKB1 Phosphorylation Assay

His-LKB1 fusion protein was purified using HisPur Purification kit and Slide-A-Lyzer Dialysis Cassette (Pierce, Rockford, IL). His-LKB1 protein (1 μg) was incubated with the recombinant His-FynT kinase (1 μl) (Calbiochem, Gibbstown, NJ) in the presence of Srrg ATP cocktail (Millipore, Billerica, MA), and kinase reaction was performed for 1 hr at 35°C. Samples were separated on 10% SDS-polyacrylamide gels, and immunoblotting was performed with PY100 monoclonal antibody and LKB1 polyclonal antibody. Signals were detected with the Odyssey Infrared Imaging System (Li-COR Biotechnology, Lincoln, NE).

Transfection of Skeletal Muscle In Vivo

Three-month-old wild-type mice were anesthetized with isoflurane, and the right tibialis anterior was injected with 125 μg of pcDNA-Flag-LKB1 WT or pcDNA-Flag-LKB1 (−Y261F) (Invitrogen, Carlsbad, CA) and were imaged as described above. Setting (iris pinhole), laser intensity, gain, and offset) were fixed and identical for all samples. Muscle extracts were also used for immunoprecipitation and immunoblotting as described below.

Statistics

Results are expressed as mean ± standard error of the mean (SEM). Differences between animals and/or treatments were tested for statistical significance (p < 0.05) using Student’s unpaired t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.cmet.2009.12.010.

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