Repression of Adipogenesis via Syk*

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Hsien-yu Wang‡§ and Craig C. Malbon¶

From the Departments of ‡Physiology and Biophysics and ¶Molecular Pharmacology, Diabetes and Metabolic Diseases Research Program, University Medical Center, State University of New York, Stony Brook, New York 11794-8661

Gα regulates the differentiation of 3T3-L1 mouse embryonic fibroblasts to adipocytes, a process termed adipogenesis. Inducers of adipogenesis lead to a loss of Gα and derepress differentiation to adipocytes. The broad spectrum tyrosine kinase inhibitor genistein is shown to block induction of adipogenesis, suggesting an early role of tyrosine phosphorylation in adipogenesis. Staining of phosphotyrosine identified prominent staining of a 70-kDa protein, hypothesized to be the tyrosine kinase Syk. Reverse transcription and polymerase chain reaction amplification established the expression of Syk mRNA in these embryonic fibroblasts. Immuno precipitations with Syk-specific antibodies demonstrated the presence of Syk in fibroblasts and a rapid increase in the amount of phospho-Syk, peaking at 24 h post induction. Clones constitutively expressing Gα, which can no longer be induced to differentiate, no longer display increased phospho-Syk levels in response to inducers. The linkage between Gα and Syk was probed by immunoprecipitations revealing association of Syk with Gα in the absence of induction. Upon induction of adipogenesis, Gα levels decline and phospho-Syk levels as well as Syk kinase activity increase. Expression of wild-type Syk both potentiates the ability of inducers to act as well as induces adipogenesis itself. Expression of the kinase-deficient Syk had no such effects on adipogenesis. These data provide a new insight into the control of adipogenesis via Syk. Treatment with the inducers promotes a decline in Gα, increases in levels of phospho-Syk, and adipogenesis.

Green and co-workers isolated a clone of Swiss mouse 3T3-L1 cells that provides a unique model for insulin-sensitive primary fat cells (1–4). The 3T3-L1 cells differentiate over 7–10 days from an embryonic fibroblast-like state to an adipocyte phenotype when treated with inducers, such as insulin (5) or dexamethasone and methylisobutylxanthine in combination (see Ref. 6 and references therein). Heterotrimeric G-proteins participate in cell signaling via effectors that include adenylylcy clases, phospholipase C, and various ion channels (7–9) as well as in more complex biological responses, including oncogenesis (9), early (10) and neonatal (11) mouse development, as well as differentiation (6, 12).

Gα plays a key role in regulating differentiation of 3T3-L1 cells, as evidenced by the following data: Gα expression declines dramatically within 24 h of induction of differentiation; constitutive elevation of Gα in 3T3-L1 cells blocks induction of cell differentiation by known inducers; reduction of Gα levels by antisense oligodeoxynucleotides both mimics the inducer-driven decline in Gα and accelerated the cell differentiation from a 10-day process to a 3-day event in the presence of inducers, and oligodeoxynucleotides antisense to Gα alone provoke adipogenesis in the absence of the classical inducers (1, 6–15). Overexpression of the Gα2, the G-protein that antagonizes many Gα effects, stimulates adipogenesis in either the absence or the presence of the inducers (16).

How does Gα control differentiation of 3T3-L1 cells? Changes in adenylylcyclase activity and/or cyclic AMP levels do not appear to play a role in the ability of Gα to repress adipogenesis based upon the following observations: direct addition of dibutyryl cAMP itself to the cultures does not alter differentiation; elevation of intracellular cAMP concentrations by either the diterpene forskolin or pertussis toxin does not affect the differentiation process; and challenging cells with 2,5'-dideoxyadenosine to reduce intracellular cyclic AMP concentrations likewise does not alter differentiation. Cholera toxin does block adipogenesis through activation of Gα, much like expression of the constitutively active mutant form of Gα (G225T), yet both cholera and pertussis toxins elevate intracellular cAMP (6). Expression of the chimeric G-protein in which the sequence 145–235 of Gα is substituted within Gα2 at the corresponding region of this homologous protein (Gα2 1–122/ Gα1 145–235/Gα2 215–355) blocks cell differentiation as effectively as wild-type Gα (1). The domain of Gα including residues 146–235, which displays several contact regions with adenylate cyclase (17–19), is critical in controlling cell differentiation. This region of Gα includes Switch I and Switch II (20), which participate in contact with βγ complex, binding of guanine nucleotides, and adenylylcyclases (17–23). The repressor domain of Gα has been subjected to further analysis by first being trisected into smaller sequences that were substituted with the corresponding domains of Gα2 and the chimeras stably expressed in 3T3-L1 cells (24). Sequences 147–171 and 200–235, but not 172–199, of Gα are critical in control of cell differentiation. Alamine scanning mutagenesis of sequences 147–171 and 200–235 identified four amino acids (Asn167, Cys200, Val214, and Lys216) and one cluster (Leu203 and Ser205) that are critical to the ability of Gα to repress differentiation of 3T3-L1 cells (24).

In the present study we show the ability of a tyrosine kinase inhibitor to block adipogenesis in response to dexamethasone and methylisobutylxanthine and provide compelling evidence for a central role of the tyrosine kinase Syk in the Gα-mediated regulation of adipogenesis. The data suggest that Syk associates with Gα and that inducers of adipogenesis promote Syk phosphorylation/activation. Blockade of the decline in Gα or of tyrosine kinase activity with genistein blocks the adipogenic response to inducers, whereas overexpression of Syk itself promotes adipogenesis.
Stable Expression of Syk, Syk mutants, and Gα chimera in 3T3-L1 Cells—Mouse embryo fibroblast 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA). Plasmids pRc/Syk and pRc/Syk (K−), harboring the cDNA for the kinase-dead version of Syk, were kindly provided by Dr. Anthony L. DeFranco (Department of Microbiology and Immunology, University of California, San Francisco, CA). The PCR expression vector for Gα (pCW1 Gα) and its empty counterpart (pCW1) were kindly provided from Dr. Gary L. Johnson (Basic Sciences, National Jewish Center for Immunology, Denver, CO). Cells were maintained in culture in 100-mm Petri dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The protocols for stable transfection of 3T3-L1 cells employed in these studies were described previously (6, 16). Stably transfected clones were selected (400 μg/ml) and then maintained (100 μg/ml) in the presence of the active form of the gentamicin analogue, G418 sulfate (Life Technologies, Inc.).

Immunoprecipitations and Immunoblotting—Whole cell lysates were prepared in a buffer composed of 130 mM NaCl, 5 mM EDTA, 10 mM HEPES, 6 mM dithiothreitol, 1% Triton X-100, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 5 mg/ml aprotinin, 5 mg/ml leupeptin, and 0.2 mM freshly prepared phenylmethylsulfonlfyl fluoride. Washed cells were lysed directly in the Petri dishes, and the mixture was collected by aspiration and subjected to centrifugation, with the resultant supernatant referred to as the whole cell lysate. Aliquots of whole cell lysates (1–3 mg protein from each subclone) were subjected to immunoprecipitation with antibodies to Syk, to PY20, or to Gα. The antibodies were coupled to protein A/G-agarose (Santa Cruz Biotechnology). To create the proper control protein A/G-agarose preparations, normal mouse/rabbit serum was coupled to the matrix, under the same conditions following the protocol of the commercial supplier. Immunoprecipitates and aliquots of whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (1). The separated proteins were transferred to nitrocellulose, and the blots were stained with polyclonal antibodies described previously (6). Stably transfected clones were selected (400 μg/ml) and then maintained (100 μg/ml) in the presence of the active form of the gentamicin analogue, G418 sulfate (Life Technologies, Inc.).

RESULTS AND DISCUSSION

Analysis of the role of protein kinase action in the regulation of adipogenesis revealed a key transient role for calcium, calmodulin-dependent protein kinase II but little role for either cyclic AMP-dependent protein kinase (protein kinase A) or protein kinase C (25). To probe a possible role for tyrosine kinase(s) in adipogenic conversion of 3T3-L1 cells, we established a protocol (panel d). The presence of the dimethyl sulfoxide has no effect on the growth of the culture or their ability to undergo adipogenic conversion in response to D/M. At day 10, cells were fixed by D/M-induced adipogenesis was explored (Fig. 1).

The tyrosine kinase inhibitor genistein blocks the ability of dexamethasone and methylisobutylxanthine to induce adipogenesis in 3T3-L1 embryonic fibroblasts: dose-response relationship to genistein. A, at confluence (day 0), cultures of mouse 3T3-L1 embryonic fibroblasts were treated without (panel a) or with (panel b) D/M. The D/M inducers were removed after incubation for 2 days, and the cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum for 10 days. A second replicate set of cells was maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in the presence of D/M but exposed to 100 μM genistein (panel c), a broad spectrum tyrosine kinase inhibitor for 2 h prior to and during the addition of the inducers. The genistein was dissolved in dimethyl sulfoxide (0.5%), and this vehicle was added to all of the cultures lacking the genistein, as a control (panel d). The presence of the dimethyl sulfoxide has no effect on the growth of the culture or their ability to undergo adipogenic conversion in response to D/M. At day 10, cells were fixed by D/M-induced adipogenesis was explored (Fig. 1).

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Tyrosine phosphorylation of cellular proteins of mouse 3T3-L1 clones during early adipogenesis: analysis by immuno- 
blotting (IB). Crude cell lysates were prepared from wild-type 3T3-L1 clones challenged with dexamethasone and methylisobutylxanthine to 
induce adipogenesis over 48 h post-confluence. The samples (3 mg of 
cellular protein of each whole cell extract) were subjected to immuno-
precipitation reactions employing antibodies to phosphotyrosine (PY20) 
that were first chemically coupled to protein A/G-agarose and the im-
munoprecipitates (IP) subjected to SDS-polyacrylamide gel electro-
phoresis. Protein A/G-agarose prepared in the absence of the PY20 
antibodies was coupled to normal rabbit serum to provide a control for 
the immune precipitations. The resolved proteins from the immunocom-
exes were transferred to nitrocellulose blots. Then the blots were 
stained with the PY20 antibody. Immune complexes were made visible 
by chemiluminescence and the use of a second goat anti-mouse IgG to 
which horseradish peroxidase was coupled. The phosphotyrosine con-
tent of three prominent proteins (p120, p84, and p70) increased within 
48 h of treatment with the inducers D/M. The data shown are repre-
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sentative of more than three independent experiments performed on as 
many separate cultures of 3T3-L1 cells.

To probe further tyrosine kinase action in adipogenesis, whole cell lysates of 3T3-L1 cells treated for varying times with 
and without the inducers D/M were prepared and subjected to 
immunoprecipitation with either PY20 antibody coupled to pro-
tein A/G-agarose or the protein A/G-agarose coupled to normal 
rabbit serum as a control. The immunoprecipitates were sub-
jected to SDS-PAGE, transferred to nitrocellulose blots, and 
stained for phosphotyrosine content using the PY20 antibody 
with a Syk-specific antibody (BR13). The p70 phosphotyrosine-contain-
ing phosphoprotein was established as authentic Syk by staining with 
three different polyclonal antibodies and one monoclonal antibody, ob-
tained from independent sources. Immune complexes were made visible 
by chemiluminescence and the use of a second goat anti-rabbit IgG to 
which horseradish peroxidase was coupled. The phosphotyrosine content of 
the ~70-kDa band was observed to increase within 24 h and 
thereafter declined to the base-line value. The p120 and p84 
species displayed a temporal pattern of tyrosyl phosphorylation 
differing from that of p70.

Based upon its electrophoretic mobility and phosphotyrosine 
content, the p70 species was suspected to be Syk, or a homolog 
of Syk. The Syk/ZAP-70 nonreceptor protein-tyrosine kinases 
are major elements in signal transduction of T- and B-cells, 
regulating cellular processes that include antibody production, 
lymphokine production, proliferation, differentiation, and ap-
optosis (26–29). A role for a Syk-like tyrosine kinase has not 
been proposed in other cell lines, so it was important to deter-
mine whether Syk was expressed in the 3T3-L1 cells and 
whether its phosphorylation/activation was altered by adipo-
gensis. Reverse transcription and polymerase chain reaction 
(RT-PCR) amplification with Syk-specific primers reveal the 
presence of Syk mRNA in the 3T3-L1 cells (Fig. 3A) with the
same mobility as the signal obtained from pRc/Syk plasmid positive control (P). RT-PCR amplification with primers specific for glyceraldehyde-phosphate dehydrogenase establishes equivalent sample loadings. PCR performed using 3T3-L1 cell RNA as template was devoid of signal, demonstrating the absence of contamination of the RNA samples with DNA.

Immunoblots of proteins from whole cell lysates of 3T3-L1 cultures subjected first to SDS-PAGE and then stained with Syk-specific antibodies make visible a prominent 70-kDa species (Fig. 3B). This same 70-kDa species was stained with antibodies from several independent sources, including a mouse monoclonal and several rabbit polyclonal antibodies. Treatment of the cultures with the inducers D/M for up to 48 h does not appear to influence the steady-state level of expression of Syk in the 3T3-L1 cells. The level of G<sub>s</sub> subunit of heterotrimeric G-proteins does not change during adipogenesis. Re-probing the blots with antibodies to G<sub>q</sub> established the equivalence of sample loading of SDS-PAGE gels (Fig. 3C). The immunoblotting data are in agreement with the analysis of Syk mRNA by RT-PCR. Taken together, the immunoblotting and RT-PCR results establish the presence of the tyrosine kinase Syk and its mRNA in the mouse 3T3-L1 embryonic fibroblasts.

The initial observation of a p70 phosphoprotein whose phosphotyrosine content increased upon adipogenic induction by D/M (Fig. 2) was explored by use of antibodies capable of immunoprecipitating Syk from whole cell lysates. 3T3-L1 cells were challenged with inducers for 30 min to 48 h, and immunoprecipitation reactions were performed using the cell lysates followed by SDS-PAGE of the immunoprecipitates (Fig. 4A). Immunoblots of the immunoprecipitates of anti-Syk antibodies were stained with antibodies to phosphotyrosine (PY20) as well as with the anti-Syk antibodies itself. The phosphotyrosine content of Syk increased ~2-fold from 12 to 24 h post-induction. Within the 24–48-h period post-induction, phosphotyrosine content of Syk returns to the initial, base-line value, in good agreement with the earlier observations (Fig. 2). The levels of phosphotyrosine were found to decline by 50–60% within 30 min of induction (not shown) but then increased reproducibly within 24 h to ~2-fold over the zero time values.

The staining of the blots with anti-Syk antibodies revealed some variation in the amount of Syk immunoprecipitated over the time course of induction (Fig. 4A). Stripping and then re-staining of the PY20 blots with anti-Syk antibodies demonstrates a modest decrease in the amount of immunoprecipitable Syk when measured at 12 h. The ratio of PY20 (phosphotyrosine content) signal to Syk signal ranged from 0.5 (at 30 min post-induction) to >2 (at 24 h post-induction) when compared with those for clones not treated with inducers. Control studies performed with increasing amounts of the cell lysate for immune precipitations from the untreated clones and the clones treated with inducer further establish, once corrected for the amount of immunoprecipitable Syk, that the phosphotyrosine content of Syk increases ~2-fold at 24 h post-induction of adipogenesis (Fig. 4B). The conditions employed for immunoprecipitation reactions performed with 1–4 mg of cell lysate protein demonstrate a linear relationship up to and including 3 mg of protein (Fig. 4B). The ratio of PY20 (phosphotyrosine content) to immunoreactive Syk ranged from 2.5 to 3.1 for the immunoprecipitation reactions performed with 1–3 mg of lysate protein/immunoprecipitation reaction for the clones stimulated with the inducers for 24 h. The results displayed earlier (Figs. 2 and 3) and all other immunoprecipitations were performed at a protein content within this linear range of the assay.

The central role of G<sub>α</sub> in adipogenesis and increases in phosphotyrosine content of Syk during adipogenesis provoked a test of a possible linkage between G<sub>α</sub> and Syk in this unique system in which G<sub>α</sub> represses adipogenesis and inducers such as D/M act to derepress the actions of G<sub>α</sub> (1, 6, 24, 25). This possible involvement of a nonreceptor tyrosine kinase with the α-subunit of a heterotrimeric G-protein was strengthened further by the recent discovery of the association of nonreceptor tyrosine kinase Btk with the α-subunit of the heterotrimeric G-proteins G<sub>q</sub> (30, 31) and G12 (32). Immunoprecipitations of Syk were performed using whole cell lysates from 3T3-L1 sta-
Gα Repression of Adipogenesis via Syk

![Image](52x630 to 294x729)

Fig. 5. Adipogenesis and the increase in the immunoprecipitable, tyrosine-phosphorylated Syk observed within 24 h of the induction of adipogenesis by dexamethasone and methylisobutyl xanthine in mouse 3T3-L1 embryonic fibroblasts are both blocked by constitutive expression of Gα. At confluence (day 0), cultures of mouse 3T3-L1 embryonic fibroblasts stably transfected with either empty vector alone (pCW1(EV)) or the expression vector for Gα (pCW1(Gα)) were treated without (0 time) or with D/M for 0.5, 12, 24, and 48 h. Immunoprecipitation (IP) of Syk was performed with whole cell lysates (1.2 mg of total cellular protein) and anti-Syk antibody. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to nitrocellulose blots. The immunoblots (IB) were stained with the PY20 antibody that specifically recognizes phosphotyrosine as well as the anti-Syk antibody. Immune complexes were made visible by chemiluminescence and the use of a second either goat anti-rabbit IgG or anti-mouse IgG to which horseradish peroxidase was coupled. The data shown are representative of more than three independent experiments performed on as many separate cultures of 3T3-L1 cells.

Fig. 6. Co-immunoprecipitation of Gα and Syk in whole cell lysates from mouse 3T3-L1 embryonic fibroblasts: immunoprecipitation analysis with anti-Syk antibodies. At confluence (day 0), cultures of mouse 3T3-L1 embryonic fibroblasts stably transfected with either empty vector alone (pCW1(EV)) or the expression vector for Gα (pCW1(Gα)) were treated without (0 time) or with D/M for 24 h. Immunoprecipitation (IP) of Syk was performed with whole cell lysates and anti-Syk antibodies coupled to protein A/G-agarose and protein A/G-agarose coupled to normal rabbit serum as a control. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to nitrocellulose blots (IB). The duplicate blots were stained with antibodies either to Gα or to Syk. Immune complexes were made visible by a second goat anti-rabbit IgG to which calf alkaline phosphatase was coupled. The data shown are representative of more than three independent experiments performed on as many separate cultures of 3T3-L1 cells.

| IP: Ab to Syk | +D/M | control |
|---------------|------|---------|
| **pCW1(EV)**  |      |         |
| 0.5 | 0.6 | 0.7 | 1.6 | 0.9 | 0.6 | 0.7 | 0.5 | 0.5 | Ratio (PY20/Syk) |
| 0.5 | 12  | 24  |     |     |     |     |     |     |                 |
| +D/M |      |      |     |     |     |     |     |     |                 |
| IB: Gα      |      |      |     |     |     |     |     |     |                 |
| 24  | 0   | 24  |     |     |     |     |     |     |                 |

The data shown are representative of more than three independent experiments performed on as many separate cultures of 3T3-L1 cells.

Staining of anti-Syk immunoprecipitations with antibodies to Gα blocked by constitutive expression of Gα (Fig. 6) or those from wild-type 3T3-L1 cells (not shown), stain the clones stably transfected either with an empty expression vector (pCW1) or with the same vector constitutively expressing Gα (pCW1-Gα). 3T3-L1 clones that constitutively express Gα fail to undergo adipogenesis in response to inducers by virtue of their maintenance of Gα levels (1). Much like wild-type 3T3-L1 cells, the clones stably transfected with the empty vector alone display increased levels of phosphorylated Syk (Fig. 5) and robust adipogenesis in response to D/M (0 time) or with D/M for 0.5, 12, 24, and 48 h. Immunoprecipitation (IP) of Syk was performed with whole cell lysates (1.2 mg of total cellular protein) and anti-Syk antibody. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to nitrocellulose blots. The immunoblots (IB) were stained with the PY20 antibody that specifically recognizes phosphotyrosine as well as the anti-Syk antibody. Immune complexes were made visible by chemiluminescence and the use of a second either goat anti-rabbit IgG or anti-mouse IgG to which horseradish peroxidase was coupled. The data shown are representative of more than three independent experiments performed on as many separate cultures of 3T3-L1 cells.

Upon induction of adipogenesis, the amount of Syk available for Syk association. Immunoprecipitations performed with antibodies to Gα constitutively express exogenous Gα display, in contrast, persistent levels of Gα content in the anti-Syk immunoprecipitations in the presence of inducers (Fig. 6). In keeping with the inability of the Gα-expressing clones to undergo adipogenic conversion in response to the inducers D/M, the amount of Syk available for immunoprecipitation remained unchanged in response to inducers and equivalent to untreated Gα-expressing clones and untreated wild-type 3T3-L1 clones.

Immune precipitations performed with antibodies to Gα (Fig. 7A) confirm the observations obtained with anti-Syk immunoprecipitations, i.e. Gα associates with Syk, and induction of adipogenesis results in loss of Syk associated with Gα. Expression of Gα blocks adipogenesis and the decline in Gα-Syk association. Immune precipitations performed with anti-Gα2 antibodies, in contrast, reveal no association with Syk (not shown). Analysis of the activation of Syk under these same conditions using the phosphotyrosine-specific antibody PY20 in cell lysates first depleted of Gα similarly reveals Syk activation only in the cells capable of adipogenic conversion in response to D/M (Fig. 7B). Measurement of Syk kinase activity was performed directly in the anti-Syk immunoprecipitates from clones expressing the empty vector (pCW1(EV)) or pCW1 (Gα), using the band 3 GST fusion protein (GST-CDB3) as a substrate. A sharp increase in Syk activity was observed at 24 h of adipogenic induction (Fig. 7C). No such activation of Syk was observed in anti-Syk immunoprecipitations from the clones constitutively expressing Gα (pCW1(Gα)) (Fig. 7C). The activity of Syk increased 3-fold at 24 h post-treatment with the inducers only for the clones expressing the empty vector (Fig. 7D). The clones stably expressing Gα, in contrast, fail to display any increase in Syk activity following the challenge with inducers (Fig. 7D).

Immune precipitations performed with protein A/G-agarose coupled with normal rabbit serum, in contrast, displayed no Syk kinase activity (Fig. 7C).
Co-immunoprecipitation of Gαs and Syk in whole cell lysates from mouse 3T3-L1 embryonic fibroblasts: immunoprecipitation analysis with anti-Gα antibodies and Syk kinase determination. At confluence (day 0), cultures of mouse 3T3-L1 embryonic fibroblasts stably transfected with either empty vector alone (pCW1(EV)) or the expression vector for Gαs (pCW1(Gαs)) were treated without (0 time) or with D/M for 24 h. A, immunoprecipitation of Gαs was performed in whole cell lysates with anti-Gα antibodies coupled to protein A/G-agarose. The immunoprecipitates (IP) were subjected to SDS-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to nitrocellulose blots. The blots were stained with antibodies either to Syk or to Gαs. B, the whole cell lysate depleted with anti-Gα antibodies was then treated with antibodies to Syk, and the immunoprecipitates were subjected to SDS-PAGE. The resolved proteins were subjected to immunoblotting (IB) and stained with antibodies either to Syk or to PY20. Note that in the wild-type cells, the loss of Gαs induced by adipogenesis leads to the appearance of phosphorylated Syk, whereas the cells constitutively expressing Gαs fail to display activated Syk. Immune complexes were made visible by a second goat anti-rabbit IgG to which horseradish peroxidase is coupled or a goat anti-mouse IgG to which horseradish peroxidase is coupled. The data shown are representative of more than three independent experiments performed in as many separate cultures of 3T3-L1 cells. C, Syk kinase activation occurs in response to induction of adipogenesis but not in 3T3-L1 clones in which the induction of adipogenesis and decline in Gαs is prevented by constitutive expression of Gαs. Whole cell lysates were prepared from clones constitutively expressing Gαs and those harboring the empty vector, both treated with or without dexamethasone and methylsulfonylbutyloxanthine for 24 h. Immunoprecipitations were performed with antibodies to Syk coupled to protein A/G-agarose or with the protein A/G-agarose coupled to normal rabbit serum as a control. The Syk kinase activity was measured in the immunoprecipitates using a GST-band 3 fusion protein as the substrate. D, Syk kinase activity assayed as described in C. The data displayed are the mean values of replicate experiments with deviation from the mean of less than 10% (n = 2).

If Syk acts as a mediator for some feature of Gα action in the derepression of adipogenic conversion by D/M, expression of the Syk itself might influence adipogenesis in these cells. Syk action has been studied largely in T- and B-cells and their signal transduction. A possible role for Syk in other differentiation pathways, such as adipogenesis, would be rather novel. The presence of Syk in immunoprecipitations of Gαs as well as the presence of Gαs in Syk immunoprecipitations are important observations, but they do not establish a biological role for the interaction in adipogenesis. The increase in both the amount of phosphorylation and kinase activity of Syk accompanying adipogenesis is, however, quite revealing. The inhibition of adipogenesis in response to D/M by the tyrosine kinase inhibitor genistein and the block of Syk activation and adipogenesis by the constitutive expression of Gαs also are quite compelling and lend greater weight to the immunoprecipitation data.

To further test the possible role of Syk more directly in the cells, 3T3-L1 cells were stably transfected with pCDNA3 expression vector harboring either Syk or a kinase-deficient Syk (Syk(K−)), and the stable transfectants were studied. Clones were selected, and the expression of the wild-type and kinase-deficient mutants was established by immunoblotting of whole cell lysates (Fig. 8A). Expression of Syk and the kinase-deficient mutant form of Syk were approximately 2-fold greater than the level of endogenous expression of Syk. In the presence of the inducers D/M, only the clones overexpressing Syk demonstrated marked oil red O staining (45% stained positive) at day 3 post-induction (Fig. 8B and Table I). Because the adipogenic conversion typically is a 7–10-day process in response to inducers, the appearance of lipid droplets at day 3 reveals early differentiation in the clones overexpressing Syk. The wild-type 3T3-L1 clones as well as the Syk kinase-deficient clones, in contrast, displayed much less positive staining with oil red O at day 3 post-induction. At day 7 post-induction with D/M, the clones overexpressing Syk still demonstrate enhanced oil red O staining (>60% stained positive), although the wild-type clones now display a positive staining also (Table I). The clones expressing the Syk kinase-deficient mutant generally displayed much less oil red O staining than the clones overexpressing Syk, suggesting a need for kinase-active Syk in adipogenesis. Expression of kinase-deficient Syk, however, does not block the ability of the cells ultimately to differentiate. The endogenous level of wild-type Syk may well be sufficient to permit adipogenesis in the presence of the expressed levels of mutant, kinase-deficient Syk obtained in these clones. Thus, overexpression of Syk facilitates the ability of D/M to induce adipogenic conversion in 3T3-L1 cells.

The ability of Syk to influence adipogenesis in the absence of inducers was explored. At 10 days after confluence, cultures of 3T3-L1 begin to display some adipogenic conversion in the absence of inducers (Fig. 8C). The wild-type clones reveal oil red O staining (14% positive staining) by day 10 (Table I). The clones overexpressing Syk display more marked adipogenic conversion, reflecting by enhanced positive staining of lipid by oil red O (>30% positive staining; see Table I). Expression of the kinase-deficient Syk resulted in a modest but clear reduction in adipogenic conversion, displaying oil red O staining that is less than that observed in the wild-type cells. When overexpressed at levels approximately 2-fold over endogenous levels, Syk itself appears capable of promoting adipogenic conversion.
Induction of adipogenesis with D/M increased phosphotyrosine content of Syk in the wild-type cells as well as in the clones stably overexpressing Syk (Fig. 9). The amount of Syk increased 2-fold in the Syk-overexpressing clones as compared with wild-type cells, as determined by immunoblotting. Thus, Syk is phosphorylated and activated in response to induction of adipogenesis. Overexpression of Syk is adipogenic itself, enhances adipogenesis in response to D/M, and results in in-

### Table I

| Inducers (+D/M) | Day | Wild type | Syk-expressing | Syk(K-) expressing |
|----------------|-----|-----------|----------------|--------------------|
| No             | 1   | 0.2 ± 1.2 | 0              |
| No             | 10  | 14.7 ± 1.2| 33.3 ± 2.2     | 09.3 ± 2.6         |
| Yes            | 03  | 07.9 ± 1.4| 45.3 ± 2.6     | 11.7 ± 3.1         |
| Yes            | 07  | 40.0 ± 4.5| 61.2 ± 1.5     | 37.7 ± 4.2         |

*a Denotes *p < 0.05 for difference from wild-type values.

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**Fig. 8.** Overexpression of Syk, but not of the kinase-deficient mutant of Syk, enhances the adipogenic conversion of 3T3-L1 cells in response to inducers. Mouse 3T3-L1 cells were stably transfected with pCDNA3 expression vector alone (WT) or a version expressing either wild-type Syk (Syk) or the kinase-deficient (Syk(K-)) mutant form of Syk. Expression of each protein was established by immunoblotting of samples of whole cell lysates subjected to SDS-PAGE and staining with anti-Syk antibodies. Clones from all three were selected and propagated, and their adipogenic responses to D/M were examined. At confluence (day 0), cultures of mouse 3T3-L1 embryonic fibroblasts were treated without (-D/M) or with (+D/M) dexamethasone and methylisobutylxanthine. Dexamethasone and methylisobutylxanthine were removed after incubation for 2 days, and the cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for the number of days indicated. At the day of post-confluent growth indicated (day 1, 3, 7, or 10), cells were fixed by 3% paraformaldehyde for 5 min and stained for lipid accumulation with oil red O for 10 min. Hematoxylin (1%) was used to stained nuclei. Adipogenesis of four individual stably transfected clones was examined under a Zeiss Axiophot microscope. The red-stained bodies of the cytosol are oil droplets. Bar, 100 μm. Data presented are representative of experiments performed on each of the four separate clones assayed on at least two occasions, providing essentially identical data. The percentage of the cells of each clone that scored positive (oil red O staining) were quantified for each of the clones and conditions described (Table I).

**Fig. 9.** Tyrosine phosphorylation of Syk in wild-type mouse 3T3-L1 clones and clones stably overexpressing Syk: response to induction of adipogenesis. Crude cell lysates were prepared from wild-type 3T3-L1 clones (WT) and clones stably expressing Syk that were challenged with dexamethasone and methylisobutylxanthine to induce adipogenesis. **Left-hand panel,** the samples (3 mg of cellular protein of each whole cell extract) were subjected to immunoprecipitation reactions employing antibodies to phosphotyrosine (PY20) that were first chemically coupled to protein A/G-agarose and the immunoprecipitates (IP) subjected to SDS-polyacrylamide gel electrophoresis. Protein A/G-agarose coupled with normal rabbit serum provided a control for the immunoprecipitations. The resolved proteins from the immunocomplexes were transferred to nitrocellulose blots. Then the blots were stained with a second, anti-phosphotyrosine antibody (PY69). The p70 phosphotyrosine-containing phosphoprotein was established as authentic Syk by staining with three different polyclonal antibodies and one monoclonal antibody, obtained from independent sources. The data shown are representative of three independent experiments performed on as many separate cultures of 3T3-L1 cells. **Right-hand panel,** a sample of whole cell lysate was employed for SDS-PAGE, immunoblotting (IB), and staining with anti-Syk antibody to measure the level of Syk expression.

Induction of adipogenesis with D/M increased phosphotyrosine content of Syk in the wild-type cells as well as in the clones stably overexpressing Syk (Fig. 9). The amount of Syk increased 2-fold in the Syk-overexpressing clones as compared with wild-type cells, as determined by immunoblotting. Thus, Syk is phosphorylated and activated in response to induction of adipogenesis. Overexpression of Syk is adipogenic itself, enhances adipogenesis in response to D/M, and results in in-
Increased phosphorylation and activation in response to treatment with D/M.

Several observations herein support the notion that Gα represses adipogenesis in 3T3-L1 cells via the nonreceptor tyrosine kinase Syk, including: 1) the ability of the broad action tyrosine kinase inhibitor genistein to block adipogenic conversion; 2) phosphorylation/activation of Syk in 3T3-L1 cells early in adipogenesis; 3) increased levels of immunoprecipitable and tyrosine-phosphorylated Syk in response to inducers that is blocked by overexpression of Gα; 4) co-precipitation of Gα with immunoprecipitation of Syk; 5) co-precipitation of Syk with Gα in the naive but not the clones induced to adipogenic conversion; and 6) overexpression of Syk enhances adipogenic conversion in response to inducers as well as displays a modest capacity to induce adipogenic conversion itself in the absence of inducers like D/M. The association between Syk and Gα may be fortuitous, but recent discovery of the association between the nonreceptor tyrosine kinase Btk and the G-protein a subunits Gqα (30, 31) and G12α (32) provide an example in which a tyrosine kinase acts as an effector for a G-protein.

For adipogenesis, the sharp reduction in Gα expression that accompanies adipogenic conversion, the ability of oligodeoxynucleotides antisense to Gα to suppress Gα and to promote adipogenesis, and the ability of overexpression of Gα to block adipogenesis in response to inducers provide an interesting model for G-protein regulation of differentiation, not unlike the case of signaling in the T- and B-cells for Gqα via Btk (30, 31). Clearly overexpression of Syk promotes a robust, earlier adipogenic conversion. Based upon these observations by others and the data presented herein, we propose that Gα represses aspects of adipogenesis via Syk, a nonreceptor tyrosine kinase heretofore shown to participate in signaling and differentiation of T- and B-cells.

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