MOLECULAR AND CELLULAR BIOLOGY, July 2010, p. 3480–3492 Vol. 30, No. 14
0270-7306/10/$12.00 doi:10.1128/MCB.00057-10

Pref-1 Interacts with Fibronectin To Inhibit Adipocyte Differentiation▽

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Received 16 January 2010/Returned for modification 23 February 2010/Accepted 2 May 2010

Pref-1/Dlk1 is made as an epidermal growth factor (EGF) repeat-containing transmembrane protein but is cleaved by tumor necrosis factor alpha converting enzyme (TACE) to generate a biologically active soluble form. Soluble Pref-1 inhibits adipocyte differentiation through the activation of extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and the subsequent upregulation of Sox9 expression. However, others have implicated Notch in Pref-1 signaling and function. Here, we show that Pref-1 does not interact with, or require, Notch for its function. Instead, we show a direct interaction of Pref-1 and fibronectin via the Pref-1 juxtamembrane domain and fibronectin C-terminal domain. We also show that fibronectin is required for the Pref-1-mediated inhibition of adipocyte differentiation, the activation of ERK/MAPK, and the upregulation of Sox9. Furthermore, disrupting fibronectin binding to integrin by the addition of RGD peptides or by the knockdown of α5 integrin prevents the Pref-1 inhibition of adipocyte differentiation. Pref-1 activates the integrin downstream signaling molecules, FAK and Rac, and ERK activation by Pref-1 is blunted by the knockdown of Rac or by the forced expression of dominant-negative Rac. We conclude that, by interacting with fibronectin, Pref-1 activates integrin downstream signaling to activate MEK/ERK and to inhibit adipocyte differentiation.

Pref-1 (also called Dlk1) is synthesized as an epidermal growth factor (EGF) repeat-containing transmembrane protein, and ADAM17/tumor necrosis factor alpha converting enzyme (TACE)-mediated cleavage generates a soluble form of Pref-1 corresponding to its extracellular domain (34). Pref-1 is highly expressed in preadipocytes, but its expression is abolished during differentiation into adipocytes. The inhibitory role of Pref-1 in adipogenesis has been well documented *in vitro* (15, 17, 18, 25, 28, 30). The overexpression of Pref-1 or treatment with soluble Pref-1 in preadipocytes results in the inhibition of adipocyte differentiation (25, 27, 29, 30). Conversely, decreasing Pref-1 levels by the transfection of Pref-1 antisense sequences greatly enhances adipocyte differentiation (26). We also found that only the large soluble form of Pref-1, but not the membrane form, inhibits adipocyte differentiation (17). In this regard, the soluble form of Pref-1 in humans was identified from maternal and fetal circulation and is called fetal antigen 1 (FA1) (10). Pref-1 function in adipogenesis also has been demonstrated *in vivo*. The deletion of the Pref-1 gene in mice causes increased adiposity with a higher degree of the differentiation of adipocytes with elevated adipocyte marker expression (18). In contrast, transgenic mice overexpressing soluble Pref-1 in adipose tissue or liver show a marked decrease in adiposity, with reduced adipogenesis and adipocyte marker expression (15, 33).

Although the inhibitory role of Pref-1 in adipocyte differentiation has been well established, until recently, the signaling pathway for Pref-1 had not been elucidated. By using soluble Pref-1 in mouse embryonic fibroblasts (MEFs) from Pref-1 null embryos, we found that Pref-1 activates the MEK/ERK pathway to inhibit adipocyte differentiation (12). We also demonstrated that, in inhibiting adipocyte differentiation, MEK/ERK activation by Pref-1 upregulates Sox9, a high-mobility-group (HMG) box DNA binding transcription factor known to be involved in chondrogenesis and osteogenesis (35). We established that Sox9 directly binds the promoter regions of C/EBPβ and C/EBPδ to suppress their transcription, resulting in the inhibition of adipocyte differentiation. Others have implicated Notch in Pref-1 function, either antagonizing or enhancing Notch (3, 13). However, these contradictory phenotypic observations on the potential involvement of Notch in Pref-1 function have not been pursued by molecular or biochemical interaction studies. Regardless, in addition to adipogenesis (25, 27, 30), Pref-1 may affect differentiation processes in many tissues, including chondrogenesis, osteogenesis (32, 35), and neuroendocrine differentiation. Unlike in adults, where Pref-1 is expressed mainly in preadipocytes and certain neuroendocrine types of cells, Pref-1 is found in multiple tissues during embryonic development. In fact, we have shown that Pref-1 directs mesenchymal cells into the chondrogenic lineage but inhibits differentiation into mature chondrocytes as well as osteoblast differentiation (35). In this regard, Pref-1 is encoded by a paternally expressed gene located in an imprinted region in mouse chromosome 12. Pref-1 null and Pref-1-overexpressing transgenic mice show defects similar to those of maternal uniparental disomy 12 (UPD12) and paternal UPD12 in mice, respectively, and some of the syntenic maternal and paternal UPD14 syndromes in humans. With the known roles of imprinted genes, Pref-1 is predicted to be of critical importance during embryonic growth and development.

Fibronectin is a major component of the extracellular matrix

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▽ Published ahead of print on 10 May 2010.

(ECM). Fibronectin forms a dimer by C-terminal disulfide bonding and also can undergo fibrillogenesis. Fibronectin contains three types of repeating modules, types 1, 2, and 3. Fibronectin can bind other ECM components, such as collagen, fibrinogen, and decorin, via interaction with its various N-terminal binding domains (36). Thus, fibronectin is considered a master organizer for the biogenesis of the ECM. Fibronectin interacts with the cell surface receptor integrin, which mediates inside-out and outside-in signaling, organizing the cytoskeleton and transducing signaling in response to ECM attachment. Different types of integrins function as receptors for fibronectin as well as other ECM components, such as laminin. The activation of the classic fibronectin receptor, α5β1 integrin, causes the tyrosine phosphorylation of a number of kinases, including FAK/Src early in the cascade as well as the activation of Rho GTPases, and MAPK further downstream, to affect cell shape, migration, growth, and differentiation (9, 24, 38). In addition to its role in signaling by interaction with integrin, fibronectin plays an important role in growth factor signaling by binding specific growth factors to present them to cells and to integrate signaling along with their cognate growth factor receptors. During the differentiation of 3T3-L1 cells into adipocytes, fibronectin levels decrease, while laminin levels increase (1, 24). There is also a switch in the expression of integrin types, from fibronectin binding α5β1 integrin to laminin binding α6β1 integrin (16). In this regard, maintaining preadipocytes on fibronectin-coated dishes has been shown to prevent adipocyte differentiation, whereas the addition of a truncated fibronectin fragment enhanced adipocyte differentiation (11). The ablation of fibronectin in mice causes severe mesodermal, vascular, and neural tube defects leading to lethality early during embryogenesis, and effects on adipogenesis *in vivo* or in mouse embryonic fibroblasts (MEFs) that can undergo *in vitro* adipocyte differentiation could not be studied (6). Therefore, fibronectin-integrin signaling that regulates adipocyte differentiation has not been investigated well.

Here, we identify fibronectin as a Pref-1 interacting protein. We show that the inhibitory role of Pref-1 is, at least in part, through Pref-1 binding to the C-terminal region of fibronectin, which, in turn, activates integrin signaling to result in MEK/ERK activation and the inhibition of adipocyte differentiation.

MATERIALS AND METHODS

Plasmid construction and protein purification. The bait plasmid for yeast two-hybrid screening was generated by subcloning the Pref-1 juxtamembrane domain (amino acid [aa] 246 to 311) into NdeI and BamHI sites of the yeast pAS2 vector (Clontech) downstream of the Gal4 DNA binding domain, resulting in the pAS2-Pref-1JM construct, which did not display the autologous activation of the reporter gene *lacZ* or *his3* upon the transformation of the yeast L40 strain. Expression vectors for Pref-1EC, Pref-1EC-HA, Pref-1-hFc, and Pref-1JM-HA were generated by subcloning the Pref-1 extracellular domain and Pref-1 juxtamembrane domain fused to hemagglutinin (HA) or human Fc (hFc) into pcDNA3.1. The secreted 52-kDa Fn fragment (52Fn) was generated by inserting sequence of the Fn fragment into HindIII and DraIII sites of Myc-tagged pSecTag2 vector (Invitrogen) in frame with the N-terminal signal sequence in the vector. FnI and FnII, containing the N-terminal half of 52Fn to the end of the type II repeat 15 and the C-terminal half of 52Fn, respectively, were subcloned into HindIII and DraIII sites of the pSecTag2 vector. Expression plasmids for Notch as well as Jagged1 and Dll 1 were from Raphael Kopan (Washington University School of Medicine) and Gerry Weinmaster (UCLA). The expression plasmid for dominant-negative Rac was from Addgen. The mammalian expression and purification of Pref-1-hFc have been described previously (12).

Yeast two-hybrid screening. The two-hybrid screening in yeast was carried out using an 11-day-old mouse embryo cDNA library in pGSD10 vector (Stratagene). Yeast strain L14 was sequentially transformed with pAS2-Pref-1JM and then with 2 μg of DNA from the mouse embryo cDNA library. Double transformants were selected by growth on the appropriate yeast minimal medium SD/Trp/Leu/His plates containing 3 amino-1,24-trialzole (3-AT) and by filter lift assay for β-galactosidase activity. Bait and prey constructs were introduced by electroporation into *Escherichia coli* that were grown on LB-ampicillin plates. Plasmid DNA were sequenced for verification.

***In vitro* transcription-translation and *in vitro* binding.** HA-tagged Pref-1JM (Pref-1JM-HA) and 52Fn prey expression plasmids were constructed by subcloning the fragments into pcDNA3.1(+). These constructs were used for *in vitro* transcription and translation in the presence of [^{35}S]methionine and cysteine (>1,000 μCi/nmol) (Amersham) using the T7-TnT Quick coupled system (Promega). For *in vitro* binding experiments, 10 μl of ^{35}S-labeled 52Fn proteins and Pref-1JM-HA was incubated with Pref-1JM-HA overnight at 4°C in buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20. The protein G beads preassembled with anti-HA antibody for 3 h were added to the incubation for the additional 2 h. The beads were washed four times in the same buffer followed by resuspension in 2× SDS sample buffer. The proteins in the supernatant separated by SDS-PAGE were visualized by fluorography after treatment with Enhancer (Amersham).

Far-Western analysis. Human 225-kDa Fn (225Fn) (Sigma), 110-kDa Fn (110Fn) (Chemicon), and 40-kDa Fn (40Fn) (Upstate) fragments were resolved on 7% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with Tris-buffered saline-Tween 20 (TBST) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. The membranes were incubated with serum-free conditioned medium collected from COS-7 cells transfected with either Pref-1JM-AP (Pref-1 juxtamembrane domain fused to human placenta alkaline phosphatase at the C terminus inserted into the AP-4 vector) or Pref-1EC (Pref-1 extracellular domain) for 2 h at room temperature. After being washed three times with TBST for 5 min each, the membranes were incubated with Pref-1 polyclonal antibody (1:5,000) for 1 h at room temperature, followed by being washed with TBST three times and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or with anti-AP antibody conjugated with HRP. The signals were detected by an enhanced chemiluminescence detection system (Perkin-Elmer).

Transfection of expression plasmids and siRNAs. Cells at 70 to 80% confluence were transiently transfected using Lipofectamine 2000. Seventy-two hours after transfection, the conditioned media or the cells were collected. The 3T3-L1 cells stably expressing the dominant-negative Rac fused to green fluorescent protein (GFP) and control GFP were generated by transfecting individual plasmids and treating the transfected cells with 200 μg/ml of G418 for 10 days. Pools of transfectants were used for experiments. The 3T3-L1 cells at 90% confluence were transfected with fibronectin small interfering RNA (siRNA), Notch1 siRNA, or control siRNA at 20 μM in siRNA transfection medium (Santa Cruz). Transfected cells were maintained in fresh medium containing 10% fetal bovine serum (FBS) from 6 h posttransfection.

Immunoprecipitation and Western blotting. Aliquots of 100 μl of purified Pref-1EC-HA (0.5 μg) were incubated with 0.3 or 1.0 μg of fibronectin or other fibronectin fragments for 3 h at 4°C in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and 10 μM phenylmethylsulfonyl fluoride (PMSF). After being precleared with normal rabbit IgG and protein G beads, soluble Pref-1EC-HA protein was immunoprecipitated with rabbit Pref-1 polyclonal antibody and protein G plus beads. The beads then were washed three times with the same buffer and resuspended in 2× SDS sample buffer. After being boiled for 5 min, the supernatant was separated onto 7% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). After being blocked with milk, the membranes were incubated with rabbit polyclonal Fn antibody (Santa Cruz) or anti-HA antibody (Covance) for 2 h. The membranes were washed with TBS and TBST followed by incubation with secondary antibody, goat anti-rabbit or anti-mouse IgG conjugated with HRP. The signals were detected by enhanced chemiluminescence.

3T3-L1 cell culture and adipocyte differentiation. The confluent 3T3-L1 cells were treated with 1 μM dexamethasone (Dex) and 0.5 mM methyisobutylxanthane (MIX) and 5 μg/ml insulin in Dulbecco's modified essential medium (DMEM) containing 10% FBS for 3 days. The medium contained 75% conditioned medium collected from COS-7 cells transfected with the indicated expression vectors. After 3 days, the cells were maintained in the same medium without the drugs for an additional 3 days before photography or harvesting for RNA extraction for the expression analysis of adipocyte markers. Oil red O staining and its quantification have been described previously (12).

RT-PCR and RT-qPCR. Total RNA was isolated using TRIzol reagent (Gibco-BRL). Reverse transcription (RT) was performed with 1 μg of total RNA, and the resultant cDNA populations were amplified by semiquantitative PCR for Pref-1, CCAAT/enhancer binding protein α (C/EBPα), peroxisome proliferator-activated receptor γ2 (PPARγ2), fatty acid synthase (FAS), adipocyte fatty acid binding protein (aP2/FABP4), Sox9, and adipocyte-specific secretory factor (ADSF)/resistin. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The primer pairs used in RT-PCR were described previously. Products were subjected to electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining. RT-quantitative PCR (RT-qPCR) was performed with an ABI PRISM 7900 sequence detection system (PE Applied Biosystems) to quantify the relative mRNA levels for various genes with GAPDH as the internal control. cDNAs for various genes were amplified using TaqMan gene expression assays consisting of a premade unlabeled PCR primer and TaqMan MGB probe labeled with 6-carboxyfluorescein (FAM) dye (PE Applied Biosystems). Various primers and probes were described previously (35). The statistical analysis of the qPCR was obtained using the $2^{-\Delta\Delta CT}$ method, which calculated the relative changes in the gene expression of the target normalized to an endogenous reference (GAPDH) and relative to a calibrator that serves as the control group.

Statistical analysis. Data are expressed as means ± standard errors of the means (SEM). The statistical differences in mean values were assessed by Student's *t* test. All experiments were performed at least twice, and representative data are shown. All scanned images were quantified by NIH image software.

RESULTS

Pref-1 neither interacts with nor requires Notch for its signaling. Structurally, the EGF-like repeats of Pref-1 do not contain the conserved amino acid residues that are required for EGF receptor binding but do share similarities with the Notch/Delta/Serrate (Jagged) family of EGF-like repeat-containing proteins involved in cell signaling and cell fate determination and differentiation. However, Pref-1 lacks the DSL (for Delta Serrate Lin12) domain that is conserved in all Notch ligands for receptor-ligand interaction (14). However, Pref-1 has been linked to Notch signaling with contradictory effects: Pref-1 has been reported either to enhance or to antagonize Notch in *Drosophila melanogaster* or *Candida elegans*, respectively (3, 13). Furthermore, Notch also was reported to mediate Pref-1 function in adipocyte differentiation (2). To understand whether Notch is involved in Pref-1 function, we first tested a possible Pref-1-Notch interaction *in vitro*. We cotransfected expression vectors for Notch1 with HA-tagged Delta1 (Dll-HA) as a positive control, an unrelated HA-tagged desnutrin (desnutrin-HA) as a negative control, or biologically active HA-tagged Pref-1 extracellular domain (Pref-1EC-HA) (Fig. 1A, left). Immunoprecipitation with HA-agarose and subsequent immunoblotting with anti-Notch antibodies clearly detected a strong interaction between Notch and Dll. In contrast, similarly to the unrelated protein desnutrin, the cotransfection of Pref-1 with Notch did not show an interaction (Fig. 1A, right).

We next tested whether Notch is required for Pref-1 signaling. We employed the γ-secretase inhibitor L-685458, which inhibits Notch signaling by preventing the intramembrane proteolysis of Notch and thus the release of the Notch intracellular domain. As expected, treating 3T3-L1 cells with soluble Pref-1 at 50 nM activated ERK effectively (Fig. 1B, left). Even when 3T3-L1 cells were pretreated with L-685458 for 24 h, Pref-1-mediated ERK activation was not diminished, clearly showing that Notch activation is not required for Pref-1 signaling. We next performed the siRNA-mediated knockdown of Notch. The transfection of Notch siRNA decreased Notch protein levels by 75%. In these Notch knockdown cells, similarly to control siRNA-transfected cells, the time-dependent phosphorylation of ERK1/2 clearly was observed upon the addition of soluble Pref-1 (Fig. 1B, right). These results demonstrate that Pref-1-mediated ERK/MAPK activation is independent of Notch. To further examine the potential involvement of Notch in Pref-1 function, after blocking Notch signaling by treatment with L-685458 or by the transfection of Notch siRNA, we tested the effects of Pref-1 on adipocyte differentiation. As shown by the lipid-filled, rounded adipocyte morphology in Fig. 1C, approximately 50% of the control 3T3-L1 cells differentiated into adipocytes upon treatment with the adipogenic agents Dex/MIX/insulin. As we reported previously, less than 20% of the cells treated with soluble Pref-1 differentiated into adipocytes. On the other hand, treatment with L-685458 or transfection with Notch siRNA enhanced adipocyte differentiation to 90 and 85%, respectively, compared to that of control cells. In these cells, Pref-1 still inhibited adipocyte differentiation by 55%. The quantification of lipid accumulation by Oil red O staining and by the measurement of expression levels of the adipogenic transcription factors C/EBPα and PPARγ reflected similar changes in the degree of adipocyte differentiation. These results indicate that the inhibitory effect of Pref-1 on adipocyte differentiation is independent of Notch signaling.

Pref-1 interacts with fibronectin. Since the results described above indicate that Notch is not involved in Pref-1 signaling, a yet-to-be-identified Pref-1 partner(s) or receptor(s) that mediates signaling by soluble Pref-1 should be present at the extracellular or plasma membrane compartment. To identify interacting partners of Pref-1, we performed yeast two-hybrid screening. As bait, we employed the Pref-1 juxtamembrane domain (aa 246 to 311), which, unlike full-length Pref-1EC, did not show nonspecific binding. We screened a total of 4.5×10^6 tryptophan and leucine auxotrophic transformants of the 11-day mouse embryo cDNA library. One interacting clone (1106A) was identified as bait specific. As shown in Fig. 2A, left, yeast that was double transformed with the candidate prey clone 1106A and bait Pref-1JM grown on minimal medium plates showed a robust β-galactosidase activity. In contrast, yeast double transformed with empty vector and bait Pref-1JM did not show β-galactosidase activity. This prey clone contained a partial sequence of fibronectin, corresponding to type III repeat 14, the adjacent type III connecting segment (IIICS), type III repeat 15, and type I repeats 1 to 3 (Fig. 2A, right). This 52-kDa fibronectin (Fn) fragment is designated 52Fn.

First, we tested whether the bait Pref-1EC-HA and the prey 52Fn that we identified by yeast two-hybrid screening can interact in mammalian cells. We cotransfected 52Fn-Myc and Pref-1EC-HA mammalian expression plasmids into COS-7 cells. Coimmunoprecipitation with either Myc or HA beads followed by Western blotting with HA or Myc antibodies, respectively, showed a clear interaction between Pref-1-EC and 52Fn (Fig. 2B) in mammalian cells. We next used lysates from COS-7 cells overexpressing Myc-tagged 52Fn in a pulldown assay with preassembled Pref-1EC-HA on protein G agarose beads. An unrelated protein, desnutrin, was used as a control in place of 52Fn for interaction with Pref-1. Interaction with 52Fn was detected upon incubation (but not upon incubation with the unrelated protein desnutrin) only with Pref-1EC-HA preassembled to anti-HA beads, but not with the beads coated

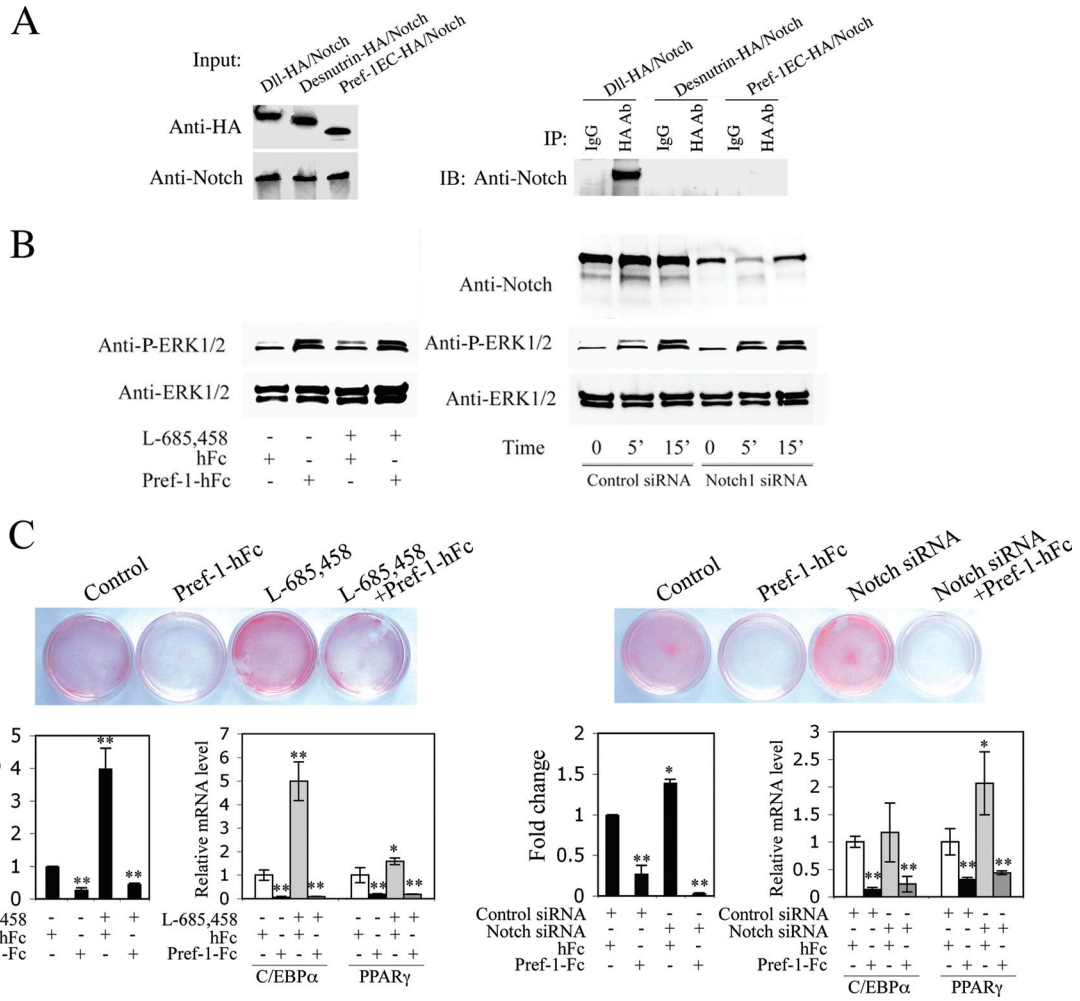

FIG. 1. Pref-1 does not interact with Notch or require Notch for its signaling. (A) COS-7 cells were cotransfected with Notch together with Dll-HA, desnutrin-HA, and Pref-1 EC-HA. Cell lysates were subjected to immunoprecipitation (IP) with control IgG or anti-HA agarose beads followed by Western blotting with anti-Notch antibody. (B) 3T3-L1 cells were treated with 1 μM γ-secretase inhibitor L-685458 (Tocris Bioscience) for 24 h, serum starved for 4 h, and then treated with 50 nM hFc or Pref-1-hFc for 15 min. Western blotting using anti-phosphorylated ERK1/2 and total ERK1/2 antibodies are shown. 3T3-L1 cells were transfected with either control or Notch siRNA. After 48 h of transfection, cells were treated with hFc or Pref-1-hFc. Western blotting was performed using anti-Notch, phosphorylated ERK1/2, and total ERK1/2 antibodies. (C) 3T3-L1 cells were subjected to adipocyte differentiation in the presence of 1 μM γ-secretase inhibitor L-685458 or after Notch siRNA transfection in the presence of Pref-1-hFc. Oil red O staining (upper), quantification of lipid staining (lower left), and adipocyte marker expression levels by RT-qPCR (lower right) are shown.

with the unrelated protein AdPLA-HA (Fig. 2C). To examine if Pref-1JM directly interacts with 52Fn, we performed an *in vitro* binding assay using *in vitro*-transcribed and -translated products labeled with [^{35}S]methionine and cysteine. Labeled 52Fn was incubated with Pref-1JM-HA and immunoprecipitated with an anti-HA antibody. 52Fn was detected only when Pref-1JM-HA was present during immunoprecipitation (Fig. 2D, left). The use of an anti-Pref-1 antibody for immunoprecipitation produced identical results (data not shown). We next tested *in vitro* interaction using the purified Pref-1EC and purified full-length 225-kDa fibronectin (225Fn) as well as a shorter 110-kDa fibronectin fragment (110Fn), which corresponds to the central domain containing type III repeats 1 to 11 of the cell binding domain including the RGD motif but lacking the C-terminal region. Pref-1EC was incubated with 225Fn or 110Fn. Immunoprecipitation with Pref-1 antibody followed by Western blotting showed that, whereas Pref-1EC was present in all samples, only 225Fn, but not 110Fn, was detected in the precipitates. These results indicate not only the interaction of Pref-1EC with full-length fibronectin but also a requirement of the C-terminal region of fibronectin for interaction with Pref-1 (Fig. 2E). For far-Western analysis, we used 225Fn, 110Fn, and 40Fn, which corresponds to the shorter C-terminal region of type III repeats 12 to 15 and IIICS and thus overlaps with 52Fn. Fibronectin and its fragments separated by SDS-PAGE and transferred onto nitrocellulose membranes were incubated with Pref-1JM containing an alkaline phosphatase (AP) tag. Using an anti-Pref-1 antibody, we detected the dose-dependent binding of Pref-1JM-AP to 225Fn and 40Fn but not to 110Fn (Fig. 2F, left). Similar results were obtained when we used purified Pref-1EC (Fig. 2F, right), clearly showing an interaction of biologically active Pref-1EC

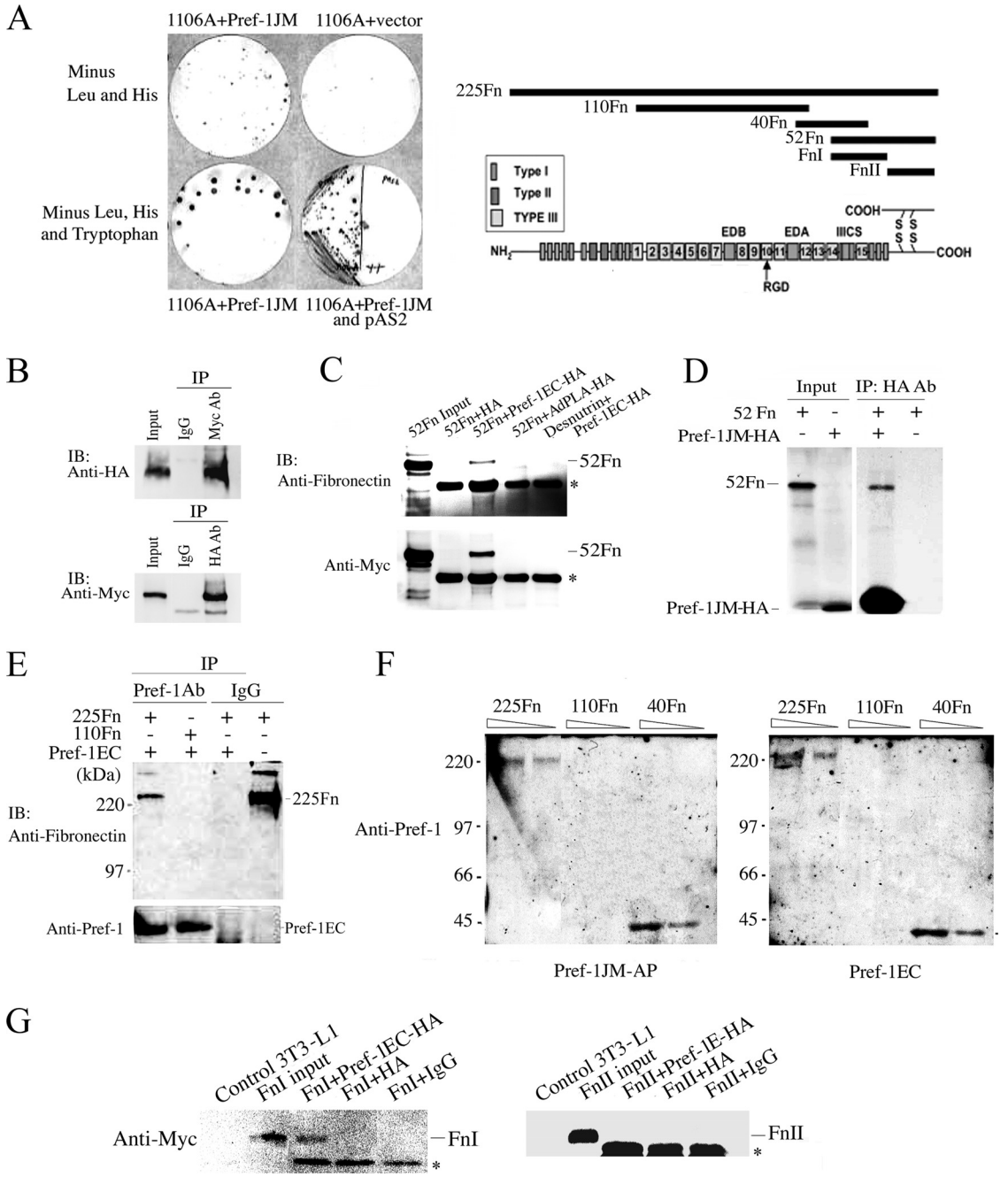

FIG. 2. Pref-1 interacts with fibronectin. (A) Pref-1JM interacts with C-terminal fibronectin in yeast-two hybrid assay (left). Staining of β-galactosidase activity of filter replicas of yeast plates double transformed with indicated plasmids. 1106A, positive clone; Pas2, empty vector. Schematic illustration of fibronectin structure and fibronectin fragments used (right panel). (B) COS-7 cells were cotransfected with Pref-1-HA and 52Fn-Myc, and lysates were subjected to IP followed by Western blotting. (C) Pulldown of 52Fn-Myc by Pref-1-HA beads. Lysates from COS-7 cells transfected with 52Fn-Myc were used for pulldown using Pref-1-HA beads and were detected by Western blotting (IB). The asterisk represents the IgG band. (D) ^{35}S-labeled Pref-1JM-HA and 52Fn were incubated, followed by IP with HA-antibody before SDS-PAGE and autoradiography. (E) Purified Pref-1EC was incubated with purified fibronectin fragments before IP with anti-Pref-1 antibody or normal IgG followed by Western blotting using anti-fibronectin antibody. (F) Fibronectin fragments separated by SDS-PAGE were transferred to nitrocellulose before incubation with Pref-1EC or Pref-1JM-AP in far-Western analysis. (G) Myc-tagged FnI or FnII was transfected into COS-7 cells, and lysates were used for pull-down assay with Pref-1EC-HA beads. The asterisk represents the IgG band.

with the fibronectin C-terminal domain. To further define the fibronectin domain that interacts with Pref-1, we generated and used two expression constructs: FnI, containing partial type III repeat 14 to partial IIICS overlapping with 40Fn, and FnII, containing the rest of the C-terminal region of 52Fn. Upon cotransfection and coimmunoprecipitation, we detected the interaction of Pref-1EC with FnI but not with FnII (Fig. 2G). Taken together, these results show that Pref-1, via its

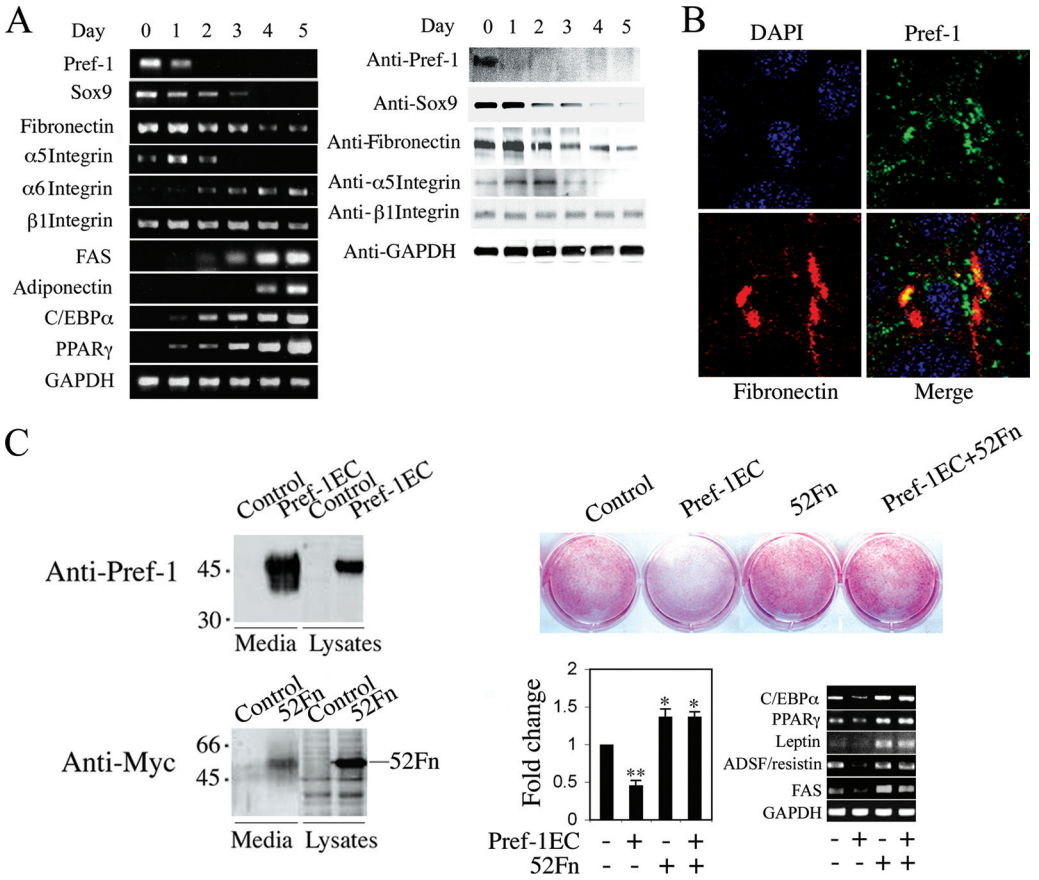

FIG. 3. Relationship between Pref-1 and fibronectin in the regulation of adipocyte differentiation. (A) Semiquantitative RT-PCR and Western blotting for various genes and proteins, respectively, during 3T3-L1 adipocyte differentiation. (B) Immunofluorescence micrograph for localization of Pref-1 and fibronectin. 3T3-L1 cells were immunostained with goat anti-Pref-1 antibody as the primary antibody and labeled with anti-goat IgG–Alexa fluor 488 (green). The cells also were stained with mouse antifibronectin antibody and labeled with anti-mouse IgG–Alexa fluor 594 (red). DAPI staining (blue) also is shown. (C) Pref-1EC and 52Fn-Myc were transfected into COS-7 cells (left), and the collected conditioned media were added to 3T3-L1 cells during differentiation. Oil red O staining (right upper panel) and its quantification (right lower left panel) are shown. Results are means ± SEM. The intensity of control cells after differentiation was defined as 1. $P < 0.05$ (*) and $P < 0.01$ (**) compared to control cells. Semiquantitative RT-PCR for the expression of adipocyte genes is shown.

juxtamembrane region, specifically interacts with fibronectin through a C-terminal domain corresponding to the type III repeat 14 to the partial IIICS region.

Fibronectin is required for Pref-1 inhibition of adipocyte differentiation. We compared the expression pattern of Pref-1 with fibronectin and integrins during the differentiation of 3T3-L1 cells into adipocytes. As predicted, the expression of FAS, adiponectin, and the adipogenic transcription factors C/EBPα and PPARγ were markedly increased during differentiation. On the other hand, Pref-1, as well as its downstream target Sox9, were drastically downregulated and were absent upon differentiation. Fibronectin expression also was decreased during differentiation, although it could be detected at a very low level upon differentiation (Fig. 3A, left). The expression of the α5 integrin subunit was decreased during adipocyte differentiation to an undetectable level. In contrast, the expression of the α6 integrin subunit was increased during differentiation, while the β1 integrin subunit showed no significant change. Western blotting for Pref-1, Sox9, fibronectin, and the α5 integrin subunit showed similar changes at the protein levels to those at the mRNA levels during differentiation (Fig. 3A, right). Immunostaining for Pref-1 and fibronectin in 3T3-L1 cells indicated the colocalization of these two proteins in the extracellular compartment (Fig. 3B). Overall, changes in the expression of fibronectin and the α5 integrin subunit followed Pref-1 expression during adipocyte differentiation.

To start testing the effect of Pref-1 interaction with fibronectin on adipocyte differentiation, we subjected 3T3-L1 cells to adipocyte differentiation using conditioned media from cells transfected with 52Fn or Pref-1EC (Fig. 3C, left). Compared to control cells, cells treated with 52Fn alone differentiated into adipocytes to a somewhat higher degree (by ~35%), as judged by the quantification of Oil red O staining (Fig. 3C). On the other hand, cells treated with Pref-1EC showed the inhibition of differentiation, with a 60% lower differentiation than that of control cells. In contrast, cells treated with both Pref-1EC and 52Fn showed 35% higher differentiation than that of control cells, similarly to cells treated with 52Fn alone. The expression of the adipogenic transcription factors C/EBPα and PPARγ, as well as the other adipocyte markers, leptin and FAS, also indicated similar differences in adipocyte differentiation. Adi-

pocyte marker levels were lower in cells treated with Pref-1EC compared to those in control cells but were somewhat higher in cells treated with 52Fn alone or in cells treated with both 52Fn and Pref-1EC. These results show the blunting of the inhibitory effect of soluble Pref-1 on adipocyte differentiation by the presence of 52Fn. An excess of 52Fn containing the fibronectin dimerization domain as well as the Pref-1 interacting domain enhanced adipocyte differentiation and, more importantly, prevented the inhibitory function of Pref-1, probably by acting in a dominant-negative fashion.

To further examine the functional relationship between Pref-1 and fibronectin, we performed the siRNA-mediated knockdown of fibronectin in 3T3-L1 cells. Fibronectin expression levels were decreased by approximately 75% 48 h after transfection with fibronectin siRNA (Fig. 4A, middle). Fibronectin siRNA-transfected cells subjected to an adipogenic procedure differentiated to adipocytes at a 45% higher level than that of control siRNA-transfected cells, as judged by lipid staining and its quantification. Pref-1-hFc treatment caused the inhibition of differentiation by 50% in control siRNA-transfected cells. In contrast, Pref-1-hFc treatment did not significantly affect adipocyte differentiation in fibronectin knockdown cells (Fig. 4A, middle). Expression levels of adipocyte markers showed similar differences in differentiation to those judged by Oil red O staining. Expression levels of adiponectin, leptin, ADSF, aP2, and FAS, as well as the adipogenic transcription factors C/EBPα and PPARγ, were greatly increased in fibronectin knockdown cells compared to that in control cells (Fig. 4A, bottom). Pref-1 treatment caused a decrease in adipocyte marker expression in control cells but not in fibronectin knockdown cells. In contrast, expression levels of endogenous Pref-1 as well as the Pref-1 target gene, Sox9, were low in control cells differentiated into adipocytes but were greatly increased upon the addition of soluble Pref-1, indicating the inhibition of adipocyte differentiation. Fibronectin knockdown cells subjected to adipocyte differentiation showed lower Pref-1 and Sox9 levels compared to those of control cells, and treatment with soluble Pref-1 during differentiation did not lead to any increase in their expression levels. Similar results also were obtained from 3T3-F442A cells. Overall, these results demonstrate that the inhibitory effect of Pref-1 on adipocyte differentiation is blocked by the knockdown of fibronectin, an indication of the requirement of fibronectin for Pref-1 function.

Previously, we have shown that Pref-1 inhibits adipocyte differentiation by upregulating Sox9 expression through the activation of ERK1/2. We therefore examined in these fibronectin siRNA-transfected preadipocytes the immediate effect of Pref-1 signaling, ERK1/2 phosphorylation, and the expression of Sox9. As predicted, Pref-1 rapidly increased ERK1/2 phosphorylation within 5 min in cells transfected with control siRNA, and ERK1/2 activation remained high up to 15 min. In contrast, in fibronectin siRNA-transfected cells, basal levels of ERK1/2 phosphorylation were lower than those in control cells. More importantly, the increase in ERK1/2 activation was not significant in fibronectin knockdown cells and was barely detectable at either 5 or 15 min after the addition of soluble Pref-1 (Fig. 4B, left). Furthermore, we detected a 50% decrease in Sox9 levels in fibronectin knockdown cells compared to that in control siRNA-transfected cells. In addition, Pref-1 treatment upregulated Sox9 expression in control cells but not in fibronectin knockdown cells (Fig. 4B, right). These results clearly show the requirement of fibronectin for Pref-1 downstream signaling and the inhibition of adipocyte differentiation, occurring via the direct interaction between Pref-1 and fibronectin. Previously we reported that, by inducing Sox9, Pref-1 suppresses the promoter activities of C/EBPβ and C/EBPδ, transcription factors that are induced and are critical early during adipocyte differentiation. We therefore examined whether Pref-1 interaction with fibronectin affects the Pref-1-mediated suppression of C/EBPβ and C/EBPδ promoter activities. As shown in Fig. 4C, promoter activities of C/EBPβ and C/EBPδ were higher in fibronectin siRNA-transfected cells than that of control siRNA-transfected cells. However, the suppression of C/EBPβ and C/EBPδ promoter activities by Pref-1 observed in control cells was no longer detected in fibronectin knockdown cells. These results indicate that fibronectin has its own suppressive effects on C/EBPβ or C/EBPδ promoter activity, but more importantly, the Pref-1 suppression of C/EBPβ or C/EBPδ promoter activities is dependent on its interaction with fibronectin.

Pref-1 signaling and function in adipocyte differentiation is through fibronectin-integrin signaling. Since we found that Pref-1-fibronectin interaction is required for the Pref-1 inhibition of adipocyte differentiation and that the fibronectin receptor α5β1 integrin is detected as the major integrin in preadipocytes, we next examined if integrin is involved in Pref-1 function during adipocyte differentiation. To disrupt fibronectin-integrin interaction, we employed RGD peptides corresponding to the cell binding sequence of fibronectin. When we tested various concentrations of RGD peptides (0.10 to 1.00 μM) in 3T3-L1 cells, RGD peptides at >0.25 μM resulted in cell detachment during adipocyte differentiation (data now shown). We therefore used RGD peptides at 0.25 μM to examine the requirement of the fibronectin-integrin interaction for the Pref-1 effect on adipocyte differentiation. Upon treatment with differentiation agents, 50% of control cells treated with hFc and DGR peptides differentiated into adipocytes. Cells treated with RGD peptides displayed enhanced differentiation of 85%, as judged by microscopic morphology and lipid accumulation (Fig. 5A). Pref-1-hFc treatment decreased the degree of differentiation to 20 to 25% in control cells treated with DGR peptides. This inhibitory effect of Pref-1 on differentiation was blocked in cells treated with RGD peptides, with cells displaying 80% differentiation. Expression levels of adipocyte markers similarly reflected changes in adipocyte differentiation. Levels of adiponectin, leptin, ADSF/resistin, aP2/FABP4, FAS, C/EBPα, and PPARγ were higher, whereas endogenous Pref-1 levels were lower in cells treated with RGD peptides than in control cells treated with DGR peptides. More importantly, an increase in endogenous Pref-1 levels observed in control DGR-treated cells by soluble Pref-1 was absent in RGD peptide-treated cells (Fig. 5A). These data suggest that RGD peptides that can competitively bind the cell-binding domain of the integrin receptor prevent the Pref-1 inhibition of adipocyte differentiation. We next transfected the α5 integrin subunit siRNA into 3T3-L1 cells that showed an 80% decrease in α5 integrin expression levels. These cells were subjected to differentiation in the presence of soluble Pref-1. Cells transfected with the α5 integrin subunit siRNA showed

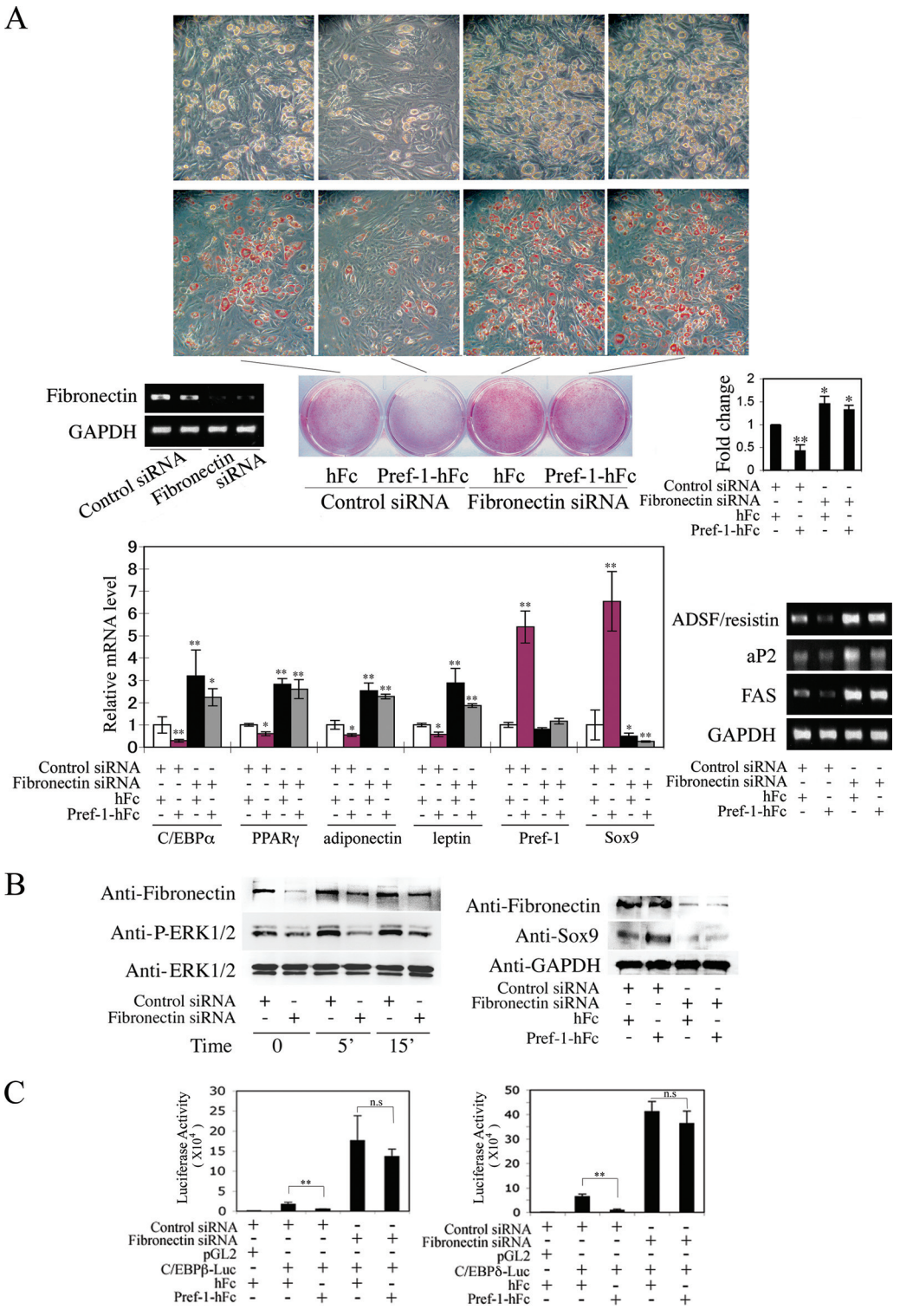

FIG. 4. Fibronectin is required for Pref-1 inhibition of adipocyte differentiation. (A) The 3T3-L1 cells transfected with fibronectin or control siRNA were subjected to adipocyte differentiation in the presence of hFc or Pref-1-hFc. Microscopic morphology (upper), Oil red O staining, and the quantification of lipid accumulation (middle) are shown. RT-qPCR and semiquantitative RT-PCR are shown as well (bottom). The value of control siRNA-transfected cells treated with hFc after differentiation was defined as 1. $P < 0.05$ (*) and $P < 0.01$ (**) compared to control cells. (B) Pref-1 null MEFs were transfected with control or fibronectin siRNA and treated with Pref-1-hFc. Western blotting was performed with antifibronectin, phosphorylated ERK1/2, and total ERK1/2 antibodies (left). Control or fibronectin siRNA-transfected cells were treated with hFc or Pref-1-hFc for 8 h before being harvested for Sox9 protein levels via Western blotting (right). (C) 3T3-L1 cells were transfected with fibronectin siRNA along with 1.0 kb C/EBPβ promoter-luciferase construct (left) or 2.0 kb C/EBPδ-promoter-luciferase construct (right). After 48 h, cells were treated with control hFc or Pref-1-hFc for an additional 16 h. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega). pRL-SV40 was used as internal control. Results are means ± SEM. **, $P < 0.01$. n.s, no significant difference.

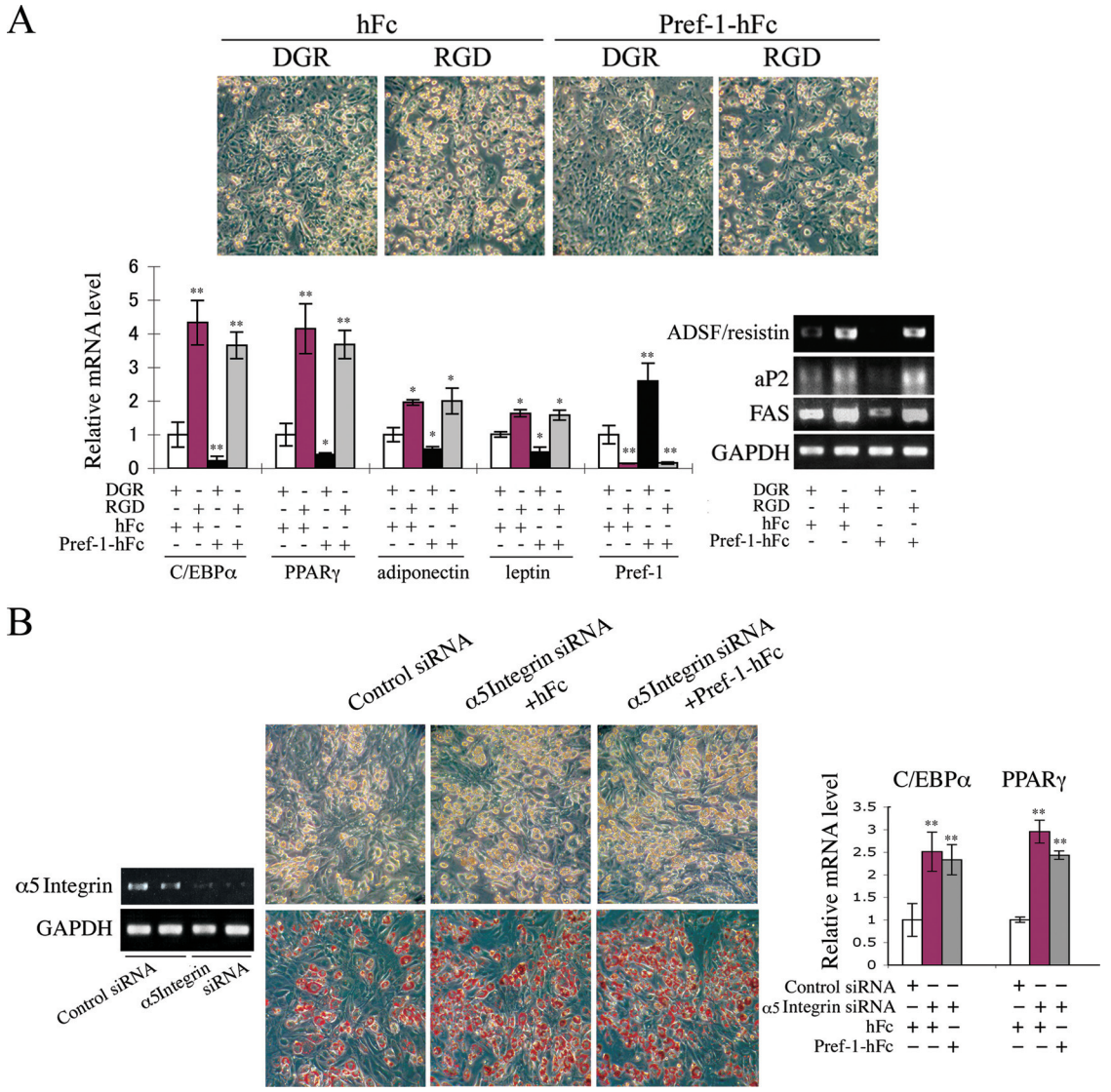

FIG. 5. α5 integrin is required for Pref-1 inhibition of adipocyte differentiation. (A) 3T3-L1 cells were differentiated in the presence of control DGR or RGD peptides and in the presence of hFc or Pref-1-hFc. Morphology and adipocyte marker expression levels by RT-qPCR and RT-PCR are shown. The value from cells treated with DGR peptides and hFc after differentiation was defined as 1. $P < 0.05$ (*) and $P < 0.01$ (**) compared to control cells. (B) Knockdown of α5 integrin in 3T3-L1 cells was verified by RT-PCR (left). Cells transfected with control or α5 integrin siRNA were subjected to adipocyte differentiation in the presence of hFc or Pref-1-hFc. Microscopic morphology, Oil red O staining (middle), and expression levels by RT-qPCR (right) are shown. **, $P < 0.01$ compared to control cells.

greater lipid droplet accumulation accompanied by higher levels of C/EBPα and PPARγ expression compared to cells transfected with control siRNA (Fig. 5B). Pref-1 treatment did not block this increase in adipocyte differentiation of α5 integrin subunit siRNA-transfected cells. These results indicate that Pref-1 cannot inhibit adipocyte differentiation in the absence of integrin, providing further evidence of the requirement of integrin for Pref-1 function.

Upon binding by its ligand, integrin activates various downstream signaling events, including the phosphorylation of focal adhesion kinase (FAK) and Src, as well as the activation of the Rho-like GTPase family of proteins, including Rac, RhoA, and Cdc42, that cycle between inactive GDP-bound forms and active GTP-bound forms. When we examined the effect of Pref-1 on integrin downstream signaling, we found that the phosphorylation of FAK was increased by 2.5-fold upon Pref-1 treatment, whereas Src phosphorylation did not show significant changes (Fig. 6A, left). By using Rhotekin and p21-activated protein kinase (Pak1) agarose beads, GTP-bound Rac1 as well as RhoA and Cdc42, respectively, were isolated from cell lysates. Western blotting showed that the active form of Rac1 increased approximately 2-fold upon Pref-1 treatment, whereas the active form of RhoA was barely detectable and the active form of Cdc42 showed no significant changes (Fig. 6A, right). Since Rac is known to be activated downstream of FAK, we next tested the function of Rac in Pref-1-mediated MEK/ERK activation. 3T3-L1 cells were stably transfected with dominant-negative Rac fused to GFP. The high-level expression of the dominant-

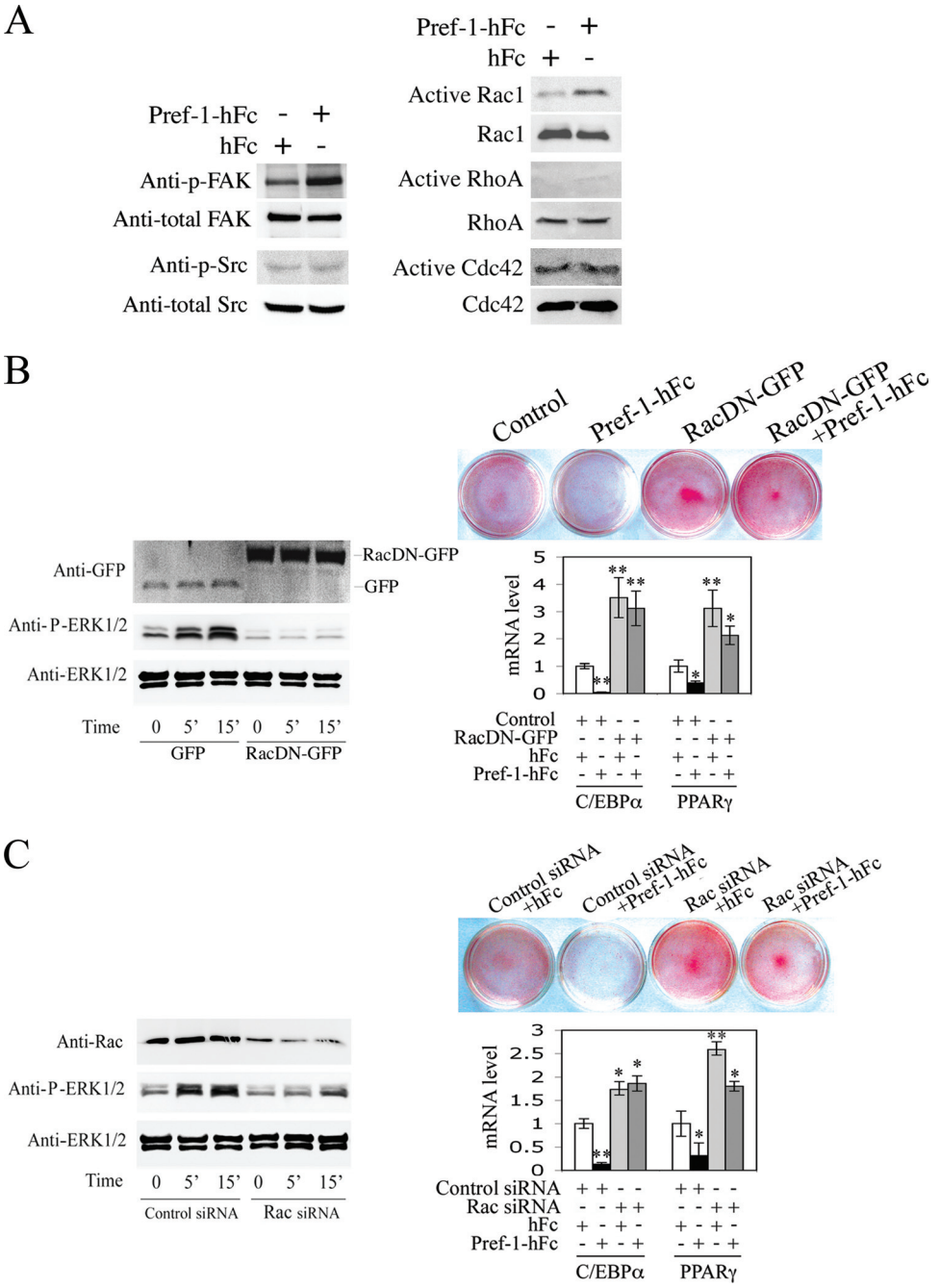

FIG. 6. Downstream signaling pathway involved in the activation of ERK by Pref-1. (A) 3T3-L1 cells were treated with hFc or Pref-1-hFc for 1 h. Western blotting is shown. (B) Cells stably expressing control GFP or dominant-negative Rac (RacDN-GFP) were treated with Pref-1-hFc. Western blotting was performed with anti-GFP, phosphorylated ERK1/2, and total ERK1/2 antibodies (left). Oil red O staining and adipocyte marker expression levels determined by RT-qPCR are shown. The values from cells that first were transfected with control GFP vector or cells treated with control hFc after differentiation was defined as 1. $P < 0.05$ (*) and $P < 0.01$ (**) compared to control cells. (C) Western blotting of lysates from 3T3-L1 cells transfected with either control or Rac siRNA, treated with hFc or Pref-1-hFc, using the indicated antibodies. Oil red O staining and adipocyte marker expression levels by RT-qPCR are shown. The values from cells transfected with control siRNA or treated with control hFc after differentiation was defined as 1. $P < 0.05$ (*) and $P < 0.01$ (**) compared to control cells.

negative Rac was detected by Western blotting using a GFP antibody. As expected, Pref-1 treatment caused a robust time-dependent activation of ERK1/2 in control cells, whereas Pref-1 did not cause changes in ERK1/2 phosphorylation in cells stably expressing a dominant-negative Rac, clearly showing a blunting of the Pref-1 effect (Fig. 6B, left). We also observed a significant decrease in ERK1/2 activation upon the siRNA-mediated knockdown of Rac (Fig. 6C, left). Furthermore, as judged by Oil red O staining and expression levels of C/EBPα and PPARγ, adipocyte differentiation was enhanced in cells transfected with dominant-

negative Rac-GFP or Rac siRNA. More importantly, Pref-1 could no longer prevent adipocyte differentiation in these cells (Fig. 6B and C, right), demonstrating a requirement for Rac in the Pref-1 inhibition of adipocyte differentiation. Overall, these results demonstrate that the integrin signaling pathway involving FAK-Rac mediates the Pref-1 activation of ERK1/2 and the subsequent inhibition of adipocyte differentiation.

DISCUSSION

We previously reported that only the soluble Pref-1 but not the transmembrane form is biologically active in inhibiting adipocyte differentiation (17). Our prediction is that soluble Pref-1 has to interact with either an extracellular or transmembrane protein. Here, using a yeast-two hybrid system, we identified fibronectin as a Pref-1-interacting protein. In this regard, other growth factors, such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and transforming growth factor β (TGF-β), also bind various regions of fibronectin. These growth factors function by forming a complex that contains both integrin and the cognate growth factor receptors to transduce signaling (8). We show here that Pref-1JM and Pref-1EC directly interact with the C-terminal region of fibronectin. We further defined the Pref-1-interacting domain of fibronectin to be FnI corresponding to the region containing partial FnIII14 and IIICS and found that interaction with fibronectin is necessary for the Pref-1 inhibition of adipocyte differentiation. It has been reported previously that adipocyte differentiation was inhibited when preadipocytes were grown on fibronectin-coated dishes or when fibronectin was added as a soluble form to the culture media (4, 31). Fibronectin has been shown to inhibit differentiation by preventing the cytoskeletal and morphological changes necessary for preadipocytes to become adipocytes. Although also detected in our present study, we are not addressing the role of fibronectin in adipocyte differentiation *per se*. Rather, as a Pref-1 interaction protein, we are addressing the involvement of fibronectin in the Pref-1 inhibition of adipocyte differentiation. In our present study, we show that the addition of a fibronectin fragment containing dimerization and Pref-1-interacting domains or the knockdown of fibronectin enhances adipocyte differentiation. More importantly, the addition of this fibronectin fragment or the knockdown of fibronectin prevents the Pref-1 inhibition of differentiation as well as the Pref-1 activation of MEK/ERK and Sox9 induction, indicating the requirement of fibronectin for Pref-1 function.

Previously, others reported that Pref-1 affected adipocyte differentiation through Notch, although the interaction of full-length Pref-1 and Notch has never been reported (2, 14). In *Drosophila* wing development, only the membrane form, but not the soluble form, of Pref-1 was reported to antagonize Notch (3). On the contrary, it has been reported that Pref-1 acted synergistically with a Notch ligand in activating Notch in *C. elegans* (13). There are additional conflicting reports on the effect of Notch on adipocyte differentiation. In the presence of the Notch ligand, Jagged1, Notch signaling was activated, causing the inhibition of adipocyte differentiation. Contrary to this inhibitory effect of Notch on adipocyte differentiation, the knockdown of the downstream molecule of Notch, Hes-1, did not enhance, but rather inhibited, adipocyte differentiation (22). Notch also has been reported to be required for adipocyte differentiation (5). However, Notch1 knockout cells could efficiently differentiate into adipocytes, showing the dispensable role of Notch in adipocyte differentiation (19). We also found in the present study that Notch is not required, but in fact it suppresses adipocyte differentiation. More importantly, we have shown here that Pref-1 does not interact with Notch, nor is it required for Pref-1 signaling and function. Our results also exclude the possibility that Pref-1 interacts with classic DSL domain-containing Notch ligands to affect Notch signaling. This is in agreement with the fact that Notch itself is not known to affect MEK/ERK, although Notch can either enhance or antagonize growth factor-mediated MEK/ERK activation depending on the context. Since the phenotypic relationship others observed between Pref-1 and Notch is not supported by biochemical interaction studies we present here, we conclude that the phenotypic link is indirect at best.

It is well established that fibronectin binds integrin to affect cell proliferation, cell adhesion, and differentiation (23). In addition, many growth factors can bind to various regions of fibronectin. These growth factors function by forming a complex containing the fibronectin receptor, integrin, and respective cognate growth factor receptors to transduce signaling (8). For example, vascular endothelial growth factor (VEGF) binds fibronectin through the C-terminal domain of Fn (type III repeats 13 and 14) to bind integrin along with the VEGF receptor, juxtaposing the two receptors, to induce cellular responses, including ERK/MAPK activation, which promotes endothelial cell proliferation and migration (37). Fibronectin also can bind hepatocyte growth factor (HGF) and form a complex with the HGF receptor, Met, and α5β1 or αVβ3 integrin to activate ERK or phosphatidylinositol 3 (PI3) kinase, promoting proliferation and migration, respectively (21). TGF-β binds to fibronectin through forming a complex with other proteins, and its incorporation into ECM is necessary for subsequent TGF-β signaling (8). How does the binding of Pref-1 to fibronectin activate the downstream signaling of ERK/MEK to inhibit adipocyte differentiation? α5β1 integrin is the major integrin for fibronectin binding in preadipocytes. We found that treating 3T3-L1 cells with RGD peptides or the knockdown of the α5 integrin subunit enhances adipocyte differentiation, indicating that fibronectin interaction with α5 containing integrin has an inhibitory effect on adipocyte differentiation. We also found that 52Fn corresponding to the C-terminal region of FnII14 and IIICS enhances adipocyte differentiation. This observation is consistent with the previous report that FnIII14 peptides stimulated adipocyte differentiation in ST-13 cells (11). The addition of excess 52Fn containing the C-terminal dimerization domain probably prevented the dimerization of endogenous fibronectin and assembly and, thus, interaction with integrin. More importantly, we found that blocking fibronectin binding to integrin by RGD peptides or by the knockdown of α5 integrin prevents the inhibitory effect of Pref-1 on adipocyte differentiation, indicating the requirement of the fibronectin-integrin interaction for Pref-1 function. We also found that the addition of 52Fn blocks the Pref-1-mediated inhibition of adipocyte differentiation. Since 52Fn contains the Pref-1-interacting domain, the loss of the Pref-1 inhibition of differentiation must be through the prevention of Pref-1 binding to endogenous fibronectin. Overall,

our results indicate the requirement of a Pref-1-fibronectin-integrin interaction for the Pref-1 inhibition of adipocyte differentiation.

Intracellular signaling for fibronectin-integrin has been well studied. Fibronectin-integrin binding activates the FAK/Src complex and Rho-like GTPase family members, including Rac, RhoA, and Cdc42, which can activate the ERK/MAPK pathway (7). Thus, the activation of the ERK/MAPK pathway by fibronectin can be blocked by anti-α4 and anti-α5 integrin antibodies or by RGD peptides. In the present study, we found that treating 3T3-L1 preadipocytes with soluble Pref-1 results in the activation of FAK and Rac. In this regard, not only α5 integrin subunit expression but also Rac activation decrease during adipocyte differentiation. Furthermore, the overexpression of the α5 integrin subunit has been reported to increase Rac activation, and a constitutively active Rac inhibited adipocyte differentiation (16), demonstrating an inhibitory effect of α5 integrin and Rac on adipocyte differentiation. More importantly, we have shown here that MEK/ERK activation by Pref-1 was blunted in cells in which fibronectin was knocked down. Furthermore, the Pref-1 activation of MEK/ERK was blunted by the knockdown of Rac or in cells with the forced expression of dominant-negative Rac, clearly linking integrin and the activation of integrin downstream of Rac to Pref-1 function. Our findings are consistent with the notion that, by directly binding to fibronectin, Pref-1 activates α5β1, the major integrin in preadipocytes, and its downstream signaling pathway, including Rac, to maintain preadipocytes in an undifferentiated state. It is likely that there is a positive feedback loop between fibronectin and ERK/MAPK activation. Fibronectin can activate ERK/MAPK to affect cell proliferation, adhesion, and differentiation. Conversely, fibronectin mRNA and protein levels are ERK/MAPK dependent in that treatment with inhibitors of ERK activation, or the knockdown of ERK, decreases fibronectin protein and expression levels as well as fibronectin assembly (20). Therefore, MEK/ERK activation by Pref-1 may increase fibronectin levels. In conclusion, our present studies clearly show that Pref-1 directly binds fibronectin and activates integrin downstream signaling to inhibit adipocyte differentiation. However, it remains to be determined whether this complex also contains a yet-to-be-identified cognate transmembrane receptor for Pref-1, as is the case for some of the growth factors that bind fibronectin. Further studies will be required to determine the existence and nature of this entity.

ACKNOWLEDGMENTS

We thank Raphael Kopan and Gerry Weinmaster for providing expression vectors for Notch, Jagged1, and Dll 1. We thank Robin Duncan and Maryam Ahmadian for the careful reading of the manuscript.

The work was supported by NIH grant DK50808 to H.S.S.

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