Only the large soluble form of preadipocyte factor-1 (Pref-1), but not the small soluble and membrane forms, inhibits adipocyte differentiation: role of alternative splicing

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We originally identified preadipocyte factor-1 (Pref-1) as an inhibitor of adipogenesis by the fact that constitutive expression of full-length Pref-1A inhibits differentiation of 3T3-L1 cells into adipocytes. Subsequently, we found that the membrane form of Pref-1 is proteolytically processed at two sites in the extracellular domain, resulting in the larger (50 kDa) and smaller (25 kDa) soluble forms. A specific form(s) of Pref-1, which is active in inhibiting adipocyte differentiation, has not been elucidated. Here, various artificial constructs and alternative-splicing variants of Pref-1 were stably transfected into 3T3-L1 cells, or conditioned media from COS cells transfected with the various forms were added into differentiating 3T3-L1 cells. Judging by Oil Red O staining for lipid accumulation and expression of adipocyte markers, we determined that, unlike the full-length Pref-1A and the constructed large soluble form, the artificial membrane form of Pref-1 lacking the processing site proximal to the membrane was not effective in inhibiting adipogenesis. Furthermore, conditioned media from COS cells transfected with the construct containing only the first three epidermal growth factor repeats, corresponding to the small soluble form, was not effective in inhibiting adipocyte differentiation. Of the four alternative-splicing products, Pref-1A and Pref-1B, which generate both large and small soluble forms, inhibited adipogenesis, whereas Pref-1C and Pref-1D, which lack the processing site proximal to the membrane and therefore generate only the smaller soluble form, did not show any effect. We conclude that only the large soluble form, and not the transmembrane or the small soluble form, of Pref-1 is biologically active and that alternative splicing therefore determines Pref-1 function in adipocyte differentiation.

Key words: EGF repeats, secreted protein, 3T3-L1 cell, transmembrane.

INTRODUCTION

We originally identified preadipocyte factor-1 (Pref-1) by differential screening of a 3T3-L1 preadipocyte cDNA library [1]. Pref-1 is highly expressed in preadipocytes and its expression is abolished during 3T3-L1 adipocyte differentiation. Pref-1 can be found only in the stromal/vascular fraction, but not the adipocyte fraction in adipose tissue. We have shown that constitutive expression of Pref-1A, one of the four major alternative splicing products (Pref-1A–Pref-1D), in 3T3-L1 cells inhibits adipocyte differentiation; conversely, transfection of antisense Pref-1 enhances differentiation [1–3]. This indicates that Pref-1 is an inhibitor of adipogenesis [4,5]. Recently, Pref-1 has been shown to increase upon leptin treatment, accompanying potential 'de-differentiation' of adipocytes to preadipocytes [6]. Pref-1 has also been shown to increase in lipodystrophic transgenic mice over-expressing sterol-regulated-element-binding protein 1c (SREBP-1c) in adipose tissue [7]. Furthermore, the *Pref-1* gene has been reported recently to be imprinted and paternally expressed. Given the role of imprinted genes in fetal growth and development in general, and the expression of Pref-1 that we observed during the embryonic stages in a variety of tissues, Pref-1 may function beyond the regulation of adipogenesis [1,8–12].

Pref-1 is a transmembrane protein containing six epidermal growth factor (EGF)-like repeats in its extracellular domain, a juxtamembrane region, a single transmembrane domain and a short cytoplasmic tail. We found that Pref-1 contains two proteolytic processing sites in the extracellular domain, one located near the fourth EGF repeat and the other in the region proximal to the transmembrane domain [13]. Upon cleavage, Pref-1 produces a 50 kDa soluble fragment corresponding to the full ectodomain in addition to a smaller soluble fragment of 25 kDa. We have also found that the four major alternative-splicing products (Pref-1A–Pref-1D) result from an in-frame deletion of the juxtamembrane region and the sixth EGF repeat. Pref-1A and Pref-1B contain both proteolytic processing sites and generate the 50 kDa form, corresponding to the full ectodomain, and smaller soluble fragments of 25 kDa. On the other hand, due to the deletion of the cleavage site proximal to the membrane, Pref-1C and Pref-1D generate only the smaller soluble fragments. This suggests that alternative splicing may be functionally significant if the membrane and the soluble forms have differing biological effects. In this regard, alternative splicing, in general, has been shown to be important in regulating gene expression and function [14,15]. However, it is not known whether these alternative-splicing variants of Pref-1 have different functions, if any.

Pref-1 shares structural similarity with other EGF-repeat-containing proteins, such as EGF and transforming growth factor α, which are growth factors, and Notch and Delta, which

Abbreviations used: Pref-1, preadipocyte factor-1; Pref-1EC, Pref-1 extracellular domain; Pref-1Δ21, Pref-1 with a 21 amino acid deletion in its juxtamembrane region; EGF, epidermal growth factor; FBS, fetal bovine serum; Dex, dexamethasone; MIX, methylisobutylxanthine; FAS, fatty acid synthase; SCD, stearoyl CoA desaturase; HA, haemagglutinin; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcriptase PCR; C/EBPα, CCAAT-enhancer-binding protein α.

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are crucial for controlling cell fate during embryonic development. Unlike growth factors, Pref-1 does not appear to have mitogenic effects (L. Chen, B. Mei and H. S. Sul, unpublished work). Structurally, Pref-1 is most closely related to Delta by its number of EGF repeats and by other conserved amino acid residues. However, Pref-1 does not contain the so-called DSL domain (Delta, Serrate and LAG2; conserved in all Notch ligands) which is the functional binding domain mediating Notch–Delta interaction. Recently, it has been found that both Delta and Notch are proteolytically processed, as is Pref-1. Delta is cleaved at the cell surface to release a soluble extracellular fragment, and this soluble form is biologically active [16]. Notch has to be processed to generate a functional receptor and upon binding of a ligand such as Delta, the cytoplasmic region of Notch is further cleaved and released for downstream signalling [17].

The present study was designed to examine whether both soluble and membrane forms of Pref-1 regulate adipocyte differentiation and the possible role of alternative splicing in producing a biologically active form of Pref-1. Here, we clearly show that only the larger soluble form is active and sufficient to inhibit adipocyte differentiation, and that neither the smaller soluble form nor the membrane form of Pref-1 affects adipogenesis. We also found that alternative splicing determines Pref-1 function by controlling production of the larger biologically active soluble form.

MATERIALS AND METHODS

Construction of plasmids

Pref-1A, Pref-1B, Pref-1C and Pref-1D expression vectors were constructed by subcloning the open reading frames into pcDNA3.1 (Invitrogen) for transient and stable transfections [13,18]. The Pref-1 extracellular domain (Pref-1EC) was amplified by PCR using primers 5′-TTTTTTAAGCTTGCCGCCATGATCGCGACCGGAGCCCTC-3′ (nucleotides 1–21 of the Pref-1A coding sequence are underlined) and 5′-TTTAAGCTTACAAGCTGTAATCTGGAACATCGTATGGGTATCCCTCGGTGAGGAGAGGG-3′ (nucleotides 894–912 of the Pref-1A coding sequence are underlined) with a haemagglutinin (HA) tag at its C-terminus and then subcloned into pcDNA3.1 vector. To construct the expression vector for Pref-1A deleted of 21 amino acids in its juxtamembrane region (Pref-1Δ21), two PCR fragments generated by primer pair I (5′-TTTTTTAAGCTTGCCGCCATGATCGCGACCGGAGCCCTC-3′, with nucleotides 1–21 of Pref-1A coding sequence underlined, and 5′-TTTTTTATCGATGGGCTGCTGAACAGGCAGCTC-3′, with nucleotides 826–846 of Pref-1A underlined) and primer pair II (5′-TTTTTTATCGATGGACAGGCCATCTGCTTCACC-3′, nucleotides 910–930 of Pref-1A underlined, and 5′-TTGGATCCTACAAGCTGTAATCTGGAACATCGTATGGGTAGATCTCCTCATCACCAGCC-3′, with nucleotides 1137–1155 of Pref-1A underlined) were ligated at a *Cla*I site and subcloned into pcDNA3.1. The deleted region of 21 amino acids (EQHILKVSMKELNKSTPLLTE) contains the proteolytic processing site proximal to the transmembrane region. An HA tag was added at the C-terminus of Pref-1Δ21.

Pref-1-EGF repeats 1–3 were amplified by PCR with primer 5′-TTTTTTAAGCTTGCCGCCATGATCGCGACCGGAGAGCCCTC-3′ (nucleotides 1–21 of the Pref-1A coding sequence are underlined) and primer 5′-TTTGGATCCTTACAAGCTGTAATCTGGAACATCGTATGGGTAGGGCCCAGCCTTGTGCTGGC-3′ (nucleotides 371–390 of Pref-1A are underlined) and subcloned into pcDNA3.1 with an HA tag at the C-terminus.

Construction of the P-tagged expression vector for Pref-1A and Pref-1D and *in vitro* phosphorylation of the conditioned media were carried out as described previously [13]. Briefly, a consensus phosphorylation site for the catalytic subunit of cAMP-dependent protein kinase, RRASV (hereon referred to as the P-tag), was inserted into the *Nco*I site that occurs in the second EGF repeat of Pref-1. The conditioned media from COS cells transfected with Pref-1A P-tag and Pref-1D P-tag were collected 72 h after transfection. After sequential centrifugation at 1100 ***g*** and 17000 ***g***, both for 10 min, the supernatant was precipitated with acetone, the pellet resuspended and the phosphorylation reaction carried out using [γ-^{32}P]ATP. Following immunoprecipitation with anti-Pref-1 antibody, the products were analysed by SDS/PAGE (12 % gels) and autoradiography.

Transient transfection

Various forms of Pref-1 in pcDNA3.1 expression vectors were transiently transfected into COS cells using DEAE–dextran in Dulbecco's modified Eagle's medium (DMEM) with 10 % Serum Plus (JRH Biosciences) as described in [19]. Then, 24 h after transfection, the media were changed to DMEM supplemented with 10 % fetal bovine serum (FBS; Life Technologies). The conditioned media were collected 72 h after transfection, centrifuged at 500 ***g*** for 5 min, and stored at 4 °C for less than a week before use. The transfected COS cells were maintained in serum-free or serum-containing DMEM as indicated.

Stable transfection and adipocyte differentiation

Exponentially growing 3T3-L1 cells were transfected with 20 μg of plasmid DNA by the calcium phosphate co-precipitation method [20]. Drug-resistant colonies were selected in 400 μg/ml G418. For each stable transfection, 24 individual clones were first isolated and the remaining cells were used as pools of transfectants. Individual clones were screened for the expression of transfected Pref-1 by reverse transcriptase PCR (RT-PCR) using the pcDNA3.1 vector reverse primer (5′-CCTCGACTGTGCCTTCTA-3′), which is located 110 bp downstream of the stop codon for Pref-1, and the two Pref-1-specific primers. One of the Pref-1-specific primers (5′-CTGGATTCGTCGACAAGACCTGCA-3′, with nucleotides 599–622 of the Pref-1A coding sequence underlined) was used for screening cells expressing Pref-1A, Pref-1EC and Pref-1D. Cells transfected with Pref-1A generate a PCR product of 669 bp, whereas cells expressing Pref-1EC and Pref-1D generate PCR products of 443 and 390 bp, respectively. The last 18 nucleotides at the 3′-end of the other Pref-1-specific primer (5′-AGCTGCCTGTTCAGCAGCCCATCGAT-3′, with nucleotides 826–845 of the Pref-1A coding sequence underlined) are Pref-1Δ21-specific, and cells expressing Pref-1Δ21 generate a PCR product of 415 bp.

Pools of drug-resistant cells and isolated individual clones were used for the differentiation assay. Cells at confluence were treated with 1 μM dexamethasone (Dex) and 0.5 mM methylisobutylxanthine (MIX) in DMEM containing 10 % FBS for 3 days. Cells were then maintained in the media without Dex/MIX for an additional 3 days before being stained with Oil Red O or harvested for total RNA extraction [21]. In experiments where conditioned media were used, cells were cultured in media containing 75 % conditioned media and 25 % DMEM with 10 % FBS plus 1 μM Dex and 0.5 mM MIX. After 3 days, the cells were maintained in the same media without the drugs for an additional 3 days.

Northern-blot analysis

After differentition, 3T3-L1 cells were harvested in Trizol reagent (Life Technologies) to extract total RNA. Total RNA (20 μg) was separated on formaldehyde-denatured agarose gels and transferred to Hybond-N nylon membranes (Amersham Bioscience). The membranes were UV cross-linked to immobilize the RNA before being hybridized in ExpressHyb hybridization solution (Clontech). Probes were generated by labelling with [α-^{32}P]dCTP using the Primer-It II random-primer-labelling kit (Stratagene). The membranes were washed with 2 × SSC/0.05 % SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) followed by 0.1 × SSC/0.1 % SDS before being autoradiographed with Kodak BioMax films at −80 °C.

Western-blot analysis

Transfected COS cells were lysed in buffer containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1 % SDS. Protein concentration was determined by the Bradford method using BSA as a standard (Bio-Rad). Cell lysate and conditioned media were fractionated by SDS/PAGE (12 % gels) and transferred to Protran nitrocellulose membranes (Schleicher & Schuell) in transfer buffer containing 25 mM Tris, 192 mM glycine and 20 % methanol. After being blocked with 1 % BSA, the membranes were incubated with anti-Pref-1 antibody, from rabbit with Pref-1/TrpE fusion protein produced from *Escherichia coli* [13], or anti-HA antibodies (Covance), followed by horseradish peroxidase conjugates (Bio-Rad) as secondary antibodies. Signals were visualized by enhanced chemiluminescence (Amersham Bioscience).

RT-PCR

RT-PCR was conducted with the Access RT-PCR system (Promega) using 1 μg of total RNA. The RT-PCR products were analysed on agarose gels with different thermal cycles to ensure that PCR was within the linear range. Primers used in RT-PCR were for CCAAT-enhancer-binding protein α (C/EBPα) (5′-AAGCCAAGAAGTCGGTGGA-3′ and 5′-CAGTTCACGGCTCAGCTGTTC-3′), fatty acid synthase (FAS; 5′-AGGGGTCGACCTGGTCCTCA-3′ and 5′-GGGTGGTTGTTAGAAAGAT-3′), stearoyl CoA desaturase (SCD; 5′-TGGGCAAGTGCTAATGGACCTC-3′ and 5′-GGGACAAGAGATGGTTGATGGTTC-3′) and β-actin (5′-TCCTATGTGGGTGACGAGGC-3′ and 5′-CATGGCTGGGGTGTTGAAGG-3′). The relative intensity of the PCR products on the gel was analysed with Molecular Analyst (Bio-Rad).

RESULTS

The larger soluble form of Pref-1, but not the membrane form, inhibits adipocyte differentiation

In order to examine the biological effectiveness of the membrane form of Pref-1, and to distinguish the roles of the soluble and membrane forms, we constructed expression vectors for Pref-1A (the largest full-length Pref-1) and Pref-1EC and examined their effectiveness in inhibiting adipocyte differentiation of 3T3-L1 cells. We also used an artificial form of Pref-1, Pref-1Δ21, constructed by deleting the cleavage site proximal to the membrane, which therefore could not be processed to generate the large soluble form (Figure 1). We stably transfected Pref-1A, Pref-1EC and Pref-1Δ21 into 3T3-L1 cells. After drug selection, pools of drug-resistant transfected cells were subjected to the differentiation assay. The expression of various forms of transfected Pref-1 was verified by RT-PCR using specific primers, as

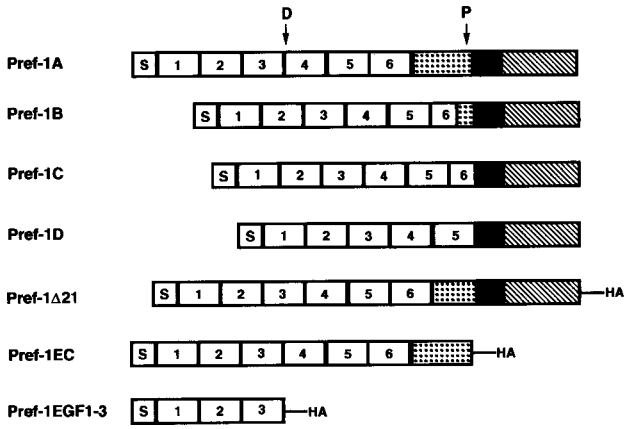

Figure 1 Pref-1 and Pref-1 constructs

Pref-1A has a secretary signal sequence (S), six EGF repeats (boxes labelled 1–6), a juxtamembrane region (dotted box), a transmembrane domain (black box) and a cytoplasmic domain (hatched box). Pref-1A has proximal (P) and distal (D) proteolytic processing sites, as indicated. The major alternatively spliced forms, Pref-1B–Pref-1D, have an in-frame deletion at the juxtamembrane region and EGF repeat 6. Pref-1Δ21 is an artificial Pref-1 with a 21 amino acid deletion corresponding to the processing site proximal to the transmembrane region (P-site). Pref-1EC only includes the extracellular domain of Pref-1, representing the large soluble form. Pref-1 EGF1-3 only includes the N-terminal three EGF-like repeats, representing the small soluble form. HA, HA epitope.

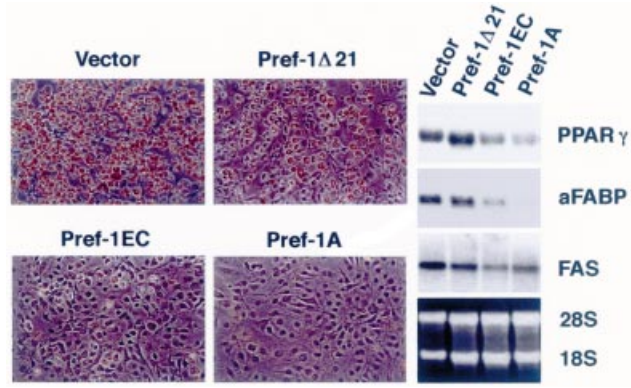

Figure 2 Stably transfected Pref-1A and Pref-1EC, but not the membrane form of Pref-1, inhibit adipocyte differentiation of 3T3-L1 cells

3T3-L1 preadipocytes were stably transfected with pcDNA3.1 control, expression constructs for full-length Pref-1A, Pref-1EC corresponding to the large soluble form of Pref-1A or Pref-1Δ21 lacking the processing site proximal to the membrane. Stable pools of cells were subjected to a differentiation assay as described in the Materials and methods section. Transfected cells were replated and subjected to the differentiation procedure. Cells were stained with Oil Red O for lipid accumulation, and total RNA was extracted for Northern-blot analysis of adipocyte markers. PPARγ, peroxisome proliferator-activated receptor γ; aFABP, adipocyte fatty acid-binding protein.

described in the Materials and methods section. The confluent cells were treated with Dex and MIX for 3 days and maintained in drug-free media for an additional 3 days. Cells were then either stained with Oil Red O for lipid accumulation or harvested for RNA preparation. As shown in Figure 2, the percentage of cells differentiated into adipocytes, as judged by lipid staining of a pool of 3T3-L1 cells transfected with Pref-1A, was dramatically decreased in comparison with that of cells transfected with vector alone. As we reported previously, the expression levels of adipocyte differentiation markers, FAS, peroxisome proliferator-

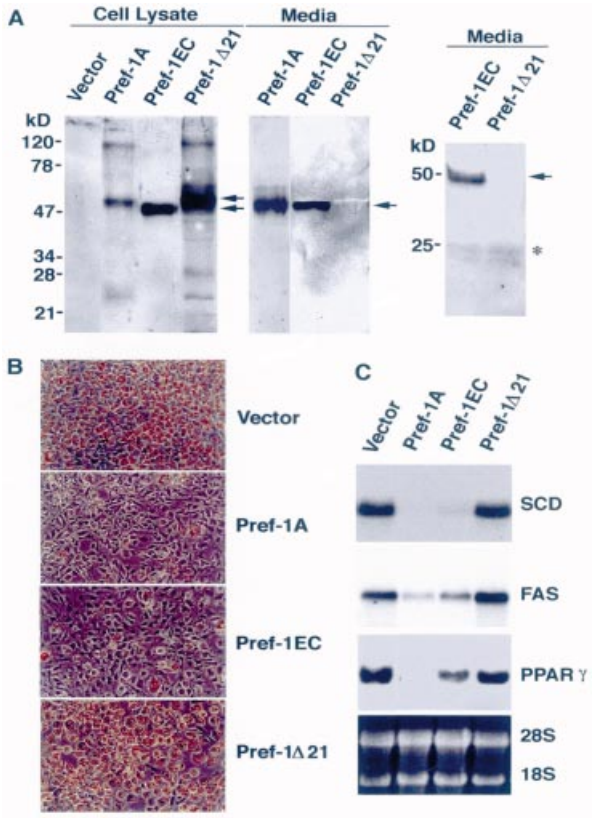

Figure 3 Effects of conditioned media from COS cells transfected with Pref-1A, Pref-1EC and Pref-1Δ21 on 3T3-L1 adipocyte differentiation

3T3-L1 cells were differentiated in conditioned media from COS cells transfected with pcDNA3.1 control, Pref-1A, Pref-1EC or Pref-1Δ21 expression vectors. (**A**) Western-blot analysis of Pref-1 expression in transfected COS cell lysates and conditioned media. In the left-hand and middle panels, COS cells were cultured in serum-free DMEM. In the right-hand panel, COS cells were cultured in DMEM with 10% FBS. The arrows indicate Pref-1 in cell lysate and the larger soluble fragment in the conditioned media. The asterisk indicates the smaller soluble fragments in conditioned media. (**B**) Oil Red O staining of 3T3-L1 cells differentiated in the conditioned media with 10% FBS. (**C**) Northern-blot analysis for adipocyte marker expression in differentiated cells. PPARγ, peroxisome proliferator-activated receptor γ.

activated receptor γ and adipocyte fatty acid-binding protein, were decreased by 70–90%, as shown by the Northern-blot analysis (Figure 2). Pref-1EC contains the full ectodomain with the signal sequence and therefore would be secreted into the media. It also contains the distal processing site and it can be cleaved further to generate the small soluble form. However, differentiation of Pref-1EC-transfected cells was impaired by 90% compared with vector-transfected control cells, as judged by Oil Red O staining. Northern-blot analysis showed that, although at slightly higher levels than those observed in cells transfected with Pref-1A, the cells transfected with Pref-1EC had decreased expression of adipocyte markers. Pref-1Δ21 was constructed by deleting 21 amino acids (EQHILKVSMKELNK-STPLLTE) where the proximal processing site is located (Figure 1). Cells transfected with Pref-1Δ21 express a membrane-associated form containing all six EGF repeats and most of the juxtamembrane region at its extracellular domain. These cells generated a smaller soluble form resulting from the cleavage at the distal processing site but not the larger soluble form. Oil Red O staining and Northern-blot analysis for adipocyte markers showed that a pool of 3T3-L1 cells stably transfected with Pref-1Δ21 had differentiation capability similar to that of control vector-transfected cells, indicating that Pref-1Δ21 did not affect adipocyte differentiation (Figure 2). We also observed essentially the same results when individual stable clones expressing Pref-1A, Pref-1EC and Pref-1Δ21 at high levels were isolated and compared for differentiation (results not shown). These stable transfection experiments demonstrate that the large soluble form of Pref-1 inhibits adipogenesis, but that neither the membrane form of Pref-1 nor the smaller soluble form can regulate adipocyte differentiation.

Since stable transfection may cause changes in the differentiation capability of cells, we employed conditioned media from COS cells transfected with Pref-1 to further demonstrate that the large soluble form is sufficient for inhibiting adipocyte differentiation. We first documented the levels of various ectopically expressed forms of Pref-1 in COS cells transfected with Pref-1A, Pref-1EC or Pref-1Δ21. As shown in Figure 3(A), left-hand panel, Western-blot analysis of cell lysates using anti-Pref-1 antibodies demonstrated that, as expected, Pref-1A-transfected cells had membrane-associated full-length Pref-1A, shown as a diffuse band of 55 kDa due to glycosylation. Pref-1Δ21-transfected cells also showed a band of a similar size due to its small deletion, which does not make it possible to distinguish it from full-length Pref-1A under the conditions employed. Pref-1EC-transfected cells showed the full-length ectodomain form of approx. 50 kDa. Vector-transfected control COS cells did not express endogenous Pref-1. As we have reported previously, conditioned media from COS cells transfected with Pref-1A maintained in serum-free media secreted the large soluble form of 50 kDa. Although the size difference between the intact membrane form of Pref-1A (55 kDa) and the large soluble form (50 kDa) is not obvious in this figure, we demonstrated the difference previously by subjecting the samples to longer periods of electrophoresis [13]. Similar to the cells transfected with Pref-1A, those transfected with Pref-1EC showed the large soluble form of 50 kDa, whereas those transfected with Pref-1Δ21 did not secrete the large soluble form. In this experiment, where serum-free media were used to eliminate the strong BSA band, we did not detect the predicted small soluble forms. We therefore carried out Western-blot analysis for the media from the COS cells maintained in 10% FBS, a condition which increases the expression levels and which we employed in generating conditioned media for the differentiation assay. As shown in Figure 3(A), right-hand panel, we could then detect the small soluble forms in conditioned media from COS cells transfected with Pref-1EC or Pref-1Δ21. The signal from the small soluble forms was not as strong as that from the large soluble form on the Western blot in Figure 3(A). This may be due to the fact that the anti-Pref-1 polyclonal antibody that we generated using Pref-1 expressed as a fusion protein in *E. coli* may be directed to the more C-terminal domain and therefore shows only a weak signal towards the small soluble form. Previously we have demonstrated the generation of approximately equimolar concentrations of these two forms by transfection of a P-tagged expression vector [13]. Therefore, we conclude that, whereas the conditioned media from Pref-1EC-transfected cells contained both large and small soluble forms, the media from cells transfected with Pref-1Δ21 contained only the small soluble form. We compared the efficacy of these conditioned media for inhibiting adipogenesis (Figures 3B and 3C). When conditioned media from COS cells transfected with Pref-1A and Pref-1EC were added to differentiating 3T3-L1 cells, the number of cells converted to adipocytes decreased to a similar extent of approx. 60%, as judged by Oil Red O staining and the expression levels of SCD, FAS and peroxisome proliferator-activated receptor γ, which were reduced markedly. In

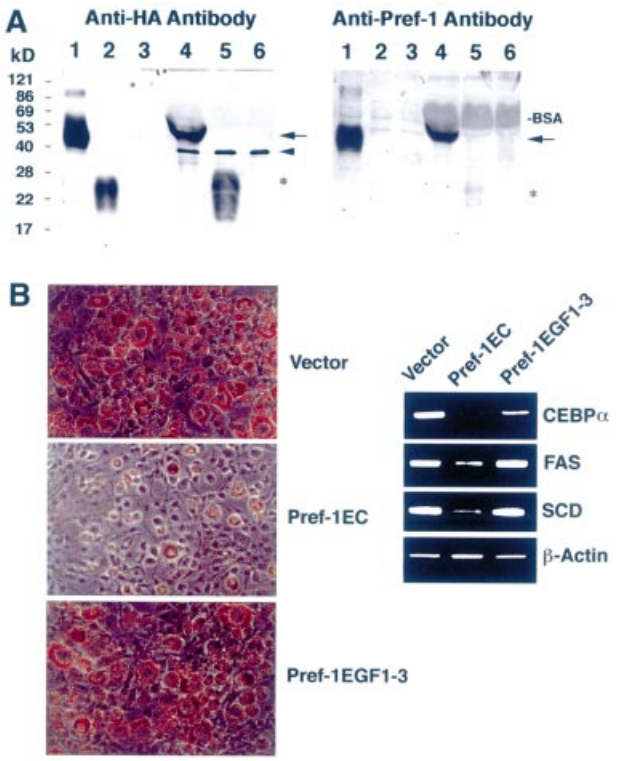

Figure 4 Conditioned media from COS cells transfected with Pref-1 EGF1-3, corresponding to the small soluble form, are not effective in inhibiting 3T3-L1 differentiation

3T3-L1 cells were differentiated in conditioned media from COS cells transfected with pcDNA3.1 control, Pref-1EC or Pref-1 EGF1-3 expression vectors. (**A**) Western-blot analysis for Pref-1 expression in transfected COS cell lysates and conditioned media. COS cells were cultured in DMEM with 10% FBS. Transfectants in the left-hand panel were detected with anti-HA antibody and those in the right-hand panel were detected with anti-Pref-1 polyclonal antibody. Lanes 1–3, lysates of COS cells transfected with Pref-1EC, Pref-1 EGF1-3 and empty vector, respectively; lanes 4–6, conditioned media from COS cells transfected with Pref-1EC, Pref-1 EGF1-3 and empty vector, respectively. The arrows indicate Pref-1EC in cell lysates and the larger soluble fragment in the conditioned media. The asterisks indicate the smaller soluble fragments in conditioned media. The arrowhead indicates a non-specific band recognized by the anti-HA antibody. (**B**) Oil Red O staining of 3T3-L1 cells differentiated in the conditioned media with 10% FBS (left-hand panel) and RT-PCR analysis for expression of adipocyte markers in differentiated cells (right-hand panel). β-Actin was used as a control.

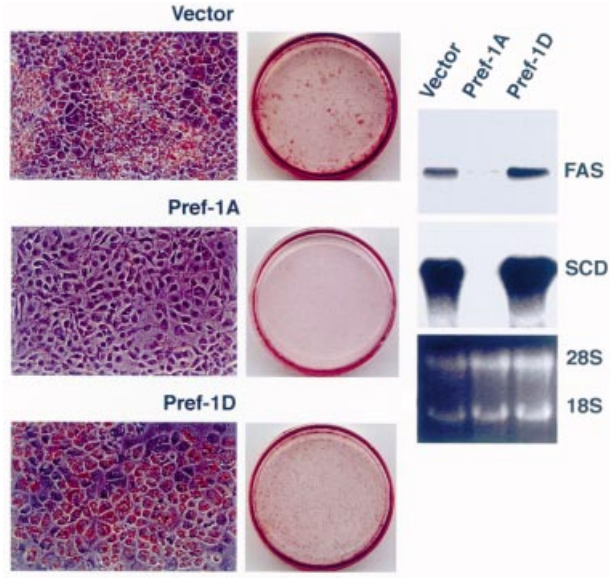

Figure 5 Stable transfection of Pref-1D does not affect 3T3-L1 adipocyte differentiation

3T3-L1 preadipocytes were stably transfected with pcDNA3.1 control, Pref-1A or Pref-1D expression vectors and subjected to the differentiation assay. The differentiated individual stable clones were stained with Oil Red O and total RNA was prepared for Northern blotting of adipocyte-marker expression.

contrast, conditioned media from COS cells transfected with Pref-1Δ21 did not affect adipocyte differentiation (Figure 3). These results demonstrate further that the large soluble form of Pref-1 is responsible for inhibiting adipocyte differentiation. Furthermore, the small soluble form may not be effective in inhibiting adipogenesis.

To further confirm that the small soluble form is not biologically active in inhibiting adipogenesis, we attempted to make an expression construct containing only the smaller soluble form. We determined previously that the processing site proximal to the N-terminus is present between EGF repeats 3 and 4. We constructed an expression vector corresponding to Pref-1 EGF repeats 1–3, which represents the small soluble form. Pref-1 EGF1-3 and Pref-1EC, both HA-tagged at the C-terminus, were transfected into COS cells (Figure 4A). The cell lysates and the conditioned media were fractionated in two identical SDS/PAGE gels and subjected to Western-blot analysis with anti-HA and anti-Pref-1 antibodies (Figure 4A). With anti-HA antibody (Figure 4A, left-hand panel), Pref-1 EGF1-3 was detected in both cell lysates and media as diffused bands at about 25 kDa, which were similar in size and pattern to the small soluble form generated from Pref-1EC and Pref-1Δ21 (Figure 3A, right-hand panel). The diffuse nature of the bands was probably the result of glycosylation, as we determined previously. Pref-1EC was detected as a diffuse band at approx. 50 kDa at a similar molar concentration to that of Pref-1 EGF1-3. The small soluble form was not detected by the anti-HA antibody when conditioned media from cells transfected with Pref-1EC were used because Pref-1EC was HA-tagged at the C-terminus. Anti-Pref-1 antibody on an identical Western blot (Figure 4A, right-hand panel) detected a strong band corresponding to Pref-1EC, whereas that for Pref-1 EGF1-3 was much weaker. This confirmed further that the anti-Pref-1 antibody is not sensitive to the small soluble form, as seen in Figure 3. As expected, when conditioned media from COS cells transfected with Pref-1 EGF1-3 were employed, adipocyte differentiation of 3T3-L1 cells was not inhibited, as judged by Oil Red O staining and by C/EBPα, FAS and SCD mRNA levels determined by RT-PCR (Figure 4B). These data further confirm our observation that only the large soluble form, and not the small soluble form, is biologically active.

Alternative splicing determines generation of biologically active Pref-1

To address whether the alternatively spliced forms of Pref-1 have differing effects on adipocyte differentiation, we first compared the biological effectiveness of Pref-1A and Pref-1D. Because Pref-1D contains the largest in-frame deletion, of all the Pref-1 alternative-splicing variants it is the smallest and the most different from Pref-1A in structure. Stable transfectants of Pref-1A and Pref-1D were generated and the degree of differentiation was assessed. As shown in Figure 5, the stable clonal cells

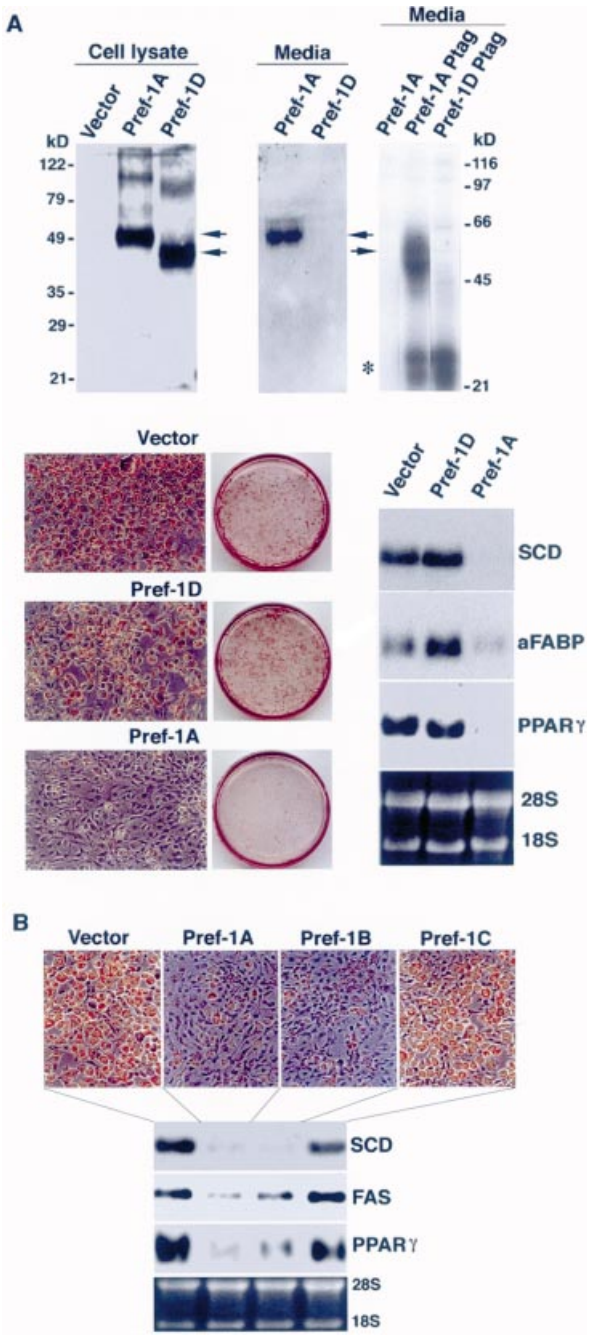

Figure 6 Conditioned media from COS cells transfected with Pref-1C and Pref-1D do not inhibit 3T3-L1 adipocyte differentiation

3T3-L1 cells were differentiated in conditioned media from COS cells transfected with pcDNA3.1 control, Pref-1A, Pref-1B, Pref-1C or Pref-1D expression vectors. (**A**) The top-left two panels show immunoblot analysis for Pref-1 expression in cell lysates and serum-free conditioned media from COS cells transfected with Pref-1A, Pref-1D or empty vector. The top-right panel shows the phosphorylated secreted Pref-1 in conditioned media from COS cells transfected with the P-tagged expression vector for Pref-1A and Pref-1D, as described in the Materials and methods section. The arrows indicate Pref-1 in cell lysates and the larger soluble fragment in the conditioned media. The asterisk indicates the smaller soluble fragments in the conditioned media. Lower left panel: Oil Red O staining of 3T3-L1 cells differentiated in serum-containing conditioned media from COS cells transfected with Pref-1A, Pref-1D or empty vector. Lower right panel: Northern-blot analysis of adipocyte-marker expression after differentiation. (**B**) Oil Red O staining of 3T3-L1 cells differentiated in serum-containing conditioned media from COS cells transfected with Pref-1A, Pref-1B, Pref-1C or vector alone and Northern-blot analysis of adipocyte markers after differentiation. PPARγ, peroxisome proliferator-activated receptor γ; aFABP, adipocyte fatty acid-binding protein.

expressing Pref-1D were converted to adipocytes to a similar extent to the vector-transfected control cells, as judged by lipid accumulation and the high expression of adipocyte markers, FAS and SCD. As expected, Pref-1A-transfected cells did not undergo adipose conversion. Pools of cells stably transfected with Pref-1D showed similar results (not shown).

We also examined biological activity of Pref-1D using conditioned media from COS cells transiently transfected with Pref-1D. The lysates of the Pref-1D-transfected COS cells showed a 50 kDa protein, the predicted size of the membrane form of Pref-1D, which is smaller than the 55 kDa Pref-1A due to the in-frame deletion (Figure 6A). Conditioned media from Pref-1A-transfected cells contained the large soluble form of 50 kDa, whereas those from Pref-1D-transfected cells did not show any secreted protein. Although the smaller soluble form is present in media collected from Pref-1A-transfected cells, as we reported previously [13], it was difficult to detect it by the Western blot with anti-Pref-1 polyclonal antibody. However, when we used expression vectors containing a phosphorylation site on the second EGF repeat of Pref-1A and Pref-1D, the smaller soluble phosphorylated forms were detected in approximately equal molar concentrations in conditioned media from cells transfected with Pref-1A or Pref-1D. As shown in Figure 6(A), although Pref-1D produces the small soluble form, it cannot generate the larger fragment because of a deletion in the region containing the proteolytic processing site proximal to the membrane. The conditioned media from the transfected cells were tested for the effects on 3T3-L1 adipocyte differentiation by Oil Red O staining and by adipocyte-marker expression. Unlike the conditioned media from Pref-1A-transfected COS cells that effectively inhibited adipogenesis, those from Pref-1D-transfected COS cells did not affect adipocyte differentiation at all. Our results demonstrate clearly that the smaller soluble form generated by Pref-1D cannot inhibit adipocyte differentiation (Figure 6). Similarly, conditioned media from cells transfected with Pref-1B, which contained both larger and smaller soluble forms, inhibited preadipocyte differentiation whereas conditioned media from cells transfected with Pref-1C, which only generated the small soluble form [13], did not (Figure 6B). To this point, our results showed that both the artificial membrane form Pref-1Δ21, which has a small deletion of 21 amino acids including the proximal processing site, and the physiological alternative-splicing forms of Pref-1C and Pref-1D, which have a deletion at the sixth EGF repeat and the juxtamembrane region containing the proximal processing site, did not inhibit adipocyte differentiation. Only those forms that generate the larger soluble fragment, Pref-1A, Pref-1B and Pref-1EC, were capable of inhibiting 3T3-L1 cell differentiation. The results presented here, therefore, show clearly that alternative-splicing variants of Pref-1 have different functions in regulating adipocyte differentiation. In addition to preadipocytes, Pref-1 has been detected in adult adrenal glands, pituitary gland, β-cells in the islets of Langerhans, placenta, thymus and tumours of neuroendocrine origin [1,10–12]. The expression pattern of those alternatively spliced forms was found to be different among those tissues (B. Mei and H. S. Sul, unpublished work), suggesting that these different forms may play distinct physiological roles in those different tissues. However, no information is available now about the physiological significance of these alternative-splicing products.

DISCUSSION

We originally cloned Pref-1 from a 3T3-L1 preadipocyte cDNA library as an inhibitor of adipogenesis and found four major alternative-splicing products for Pref-1. Pref-1 is proteolytically

processed at the extracellular domain, and releases large (50 kDa) and small (25 kDa) soluble fragments [13]. By using conditioned media collected from COS cells transfected with the various forms of Pref-1 we found that only the larger soluble fragment, corresponding to the full ectodomain, which is produced by Pref-1A and Pref-1B, inhibits adipocyte differentiation. The smaller soluble forms, which are generated by all four alternative-splicing variants, are not functional. Pref-1 may function in autocrine and/or paracrine fashions. Delta, an EGF-repeat-containing protein that interacts with cell-surface receptor Notch and controls cell-fate determination, has been thought to be a transmembrane ligand expressed on adjacent cells. Recently, however, Delta has also been demonstrated to release a soluble extracellular domain capable of binding to and acting through Notch. Delta is processed by the metalloprotease Kuzbanian at the cell surface and the loss of Kuzbanian function results in phenotypes that are similar to the loss of Notch signalling, suggesting that proteolytic cleavage of Delta may be essential for its function [16,17].

In contrast to the larger soluble form, the membrane form of Pref-1 does not affect adipogenesis. We demonstrated this by transfecting both Pref-1Δ21, an artificial Pref-1A which has a deletion of the 21 amino acids at the juxtamembrane region including the proximal processing site, and Pref-1D, a native splicing variant which contains five of the six EGF repeats but lacks the proximal processing site. These two forms are expressed as membrane-associated Pref-1 but are not capable of releasing the functional large soluble form. We found that neither of these forms affects adipocyte differentiation. Garces et al. [22] hypothesized that the membrane form of Pref-1 might play a role opposite to the soluble form in controlling adipogenesis. However, our observation indicates strongly that the membrane form of Pref-1 is not functional in regulating adipocyte differentiation, and the large soluble fragment is the only biologically active form. This suggests that Pref-1 does not regulate adipogenesis in a juxtacrine fashion. We do not know at present if the membrane form of Pref-1 has a distinct unidentified function. In this regard, both soluble and membrane forms of the EGF-repeat-containing growth factors are believed to function in cell growth [23]. However, recent studies have shown that the membrane-bound forms may have a different function or potency from the soluble forms; the membrane-anchored heparin-binding EGF induces growth inhibition and apoptosis rather than stimulating proliferation, as soluble heparin-binding EGF does in a haematopoietic cell line [24–26].

Alternative splicing is a mechanism for generating functionally distinct proteins from the same gene and is employed to regulate expression and function of genes involved in virtually every aspect of cell function, including cell growth, cell proliferation and cell death [14,15]. Alternative splicing is also used as a means to generate both membrane-associated and soluble forms of a protein from a single gene. The results we present here demonstrate clearly that four alternatively spliced Pref-1 forms have a drastically different effect on adipocyte differentiation. Both Pref-1A and Pref-1B dramatically inhibit adipogenesis but Pref-1C and Pref-1D have no effect. It is interesting to note that although Pref-1C is the dominant form in 3T3-L1 cells (B. Mei and H. S. Sul, unpublished work), Pref-1C, as in the case of Pref-1D, appears not to be biologically active in regulating adipocyte differentiation because it does not generate the large soluble fragment. In agreement, constitutive expression of Pref-1D did not affect adipocyte differentiation. Whether the membrane-associated variants of Pref-1 have physiological function in other tissues is not clear. To this end, an interesting example of potential differential function of membrane-associated and soluble forms produced by alternative splicing was presented recently by attractin/mahogany, which also contains EGF-like repeats [27,28]. Alternative-splicing variants may have their own unique tissue and subcellular distributions to meet specific needs [16,29]. Alternative splicing of several integrin subunits leads to variations in the sequence of both extracellular and cytoplasmic domains with specific expression patterns, which may provide mechanisms to regulate subtly the ligand binding and signalling activity [30]. Disruption of the ratios of alternatively spliced Wilms' tumour gene (*WT1*) affects urogenital development causing Denys–Drash syndrome [31]. The physiological significance of the alternatively spliced products of Pref-1 and their subtle functional differences remain to be elucidated.

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