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Cleavage of Membrane-Associated pref-1 Generates a Soluble Inhibitor of Adipocyte Differentiation

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pref-1 is an epidermal growth factor-like repeat protein present on the surface of preadipocytes that functions in the maintenance of the preadipose state. pref-1 expression is completely abolished during 3T3-L1 adipocyte differentiation. Bypassing this downregulation by constitutive expression of full-length transmembrane pref-1 in preadipocytes drastically inhibits differentiation. For the first time, we show processing of cell-associated pref-1 to generate both a soluble pref-1 protein of approximately 50 kDa that corresponds to the ectodomain and also smaller products of 24 to 25 kDa and 31 kDa. Furthermore, while all four of the alternately spliced forms of pref-1 produce cell-associated protein, only the two largest of the four alternately spliced isoforms undergo cleavage in the juxtamembrane region to release the soluble 50-kDa ectodomain. We demonstrate that addition of *Escherichia coli*-expressed pref-1 ectodomain to 3T3-L1 preadipocytes blocks differentiation, thus overriding the adipogenic actions of dexamethasone and methylisobutylxanthine. The inhibitory effects of the pref-1 ectodomain are blocked by preincubation of the protein with pref-1 antibody. That the ectodomain alone is sufficient for inhibition demonstrates that transmembrane pref-1 can be processed to generate an inhibitory soluble form, thereby greatly extending its range of action. Furthermore, we present evidence that alternate splicing is the mechanism that governs the production of transmembrane versus soluble pref-1, thereby determining the mode of action, juxtacrine or paracrine, of the pref-1 protein.

Adipose tissue is central to the maintenance of energy balance, and caloric intake in excess of energy utilization leads to obesity. Obesity may arise from increased size of individual adipose cells due to lipid accumulation or increased number of adipocytes arising from differentiation of adipose precursor cells to mature adipocytes. Overfeeding studies in rodents indicate a lifelong ability to make new fat cells in response to a high-fat diet and reveal that preadipocytes continue to undergo differentiation under the appropriate nutritional and hormonal cues throughout adulthood (10, 25, 26). Recent work reveals that the *ob* gene product, leptin, is an adipocyte-produced hormone involved in the regulation of appetite (17, 36, 51). Preadipocyte cell lines, such as 3T3-L1, differentiate in vitro in a process that biochemically and morphologically resembles in vivo adipocyte differentiation (14, 15). This spontaneous differentiation is accelerated by treatment of confluent preadipocytes with dexamethasone and methylisobutylxanthine (39). During differentiation, the fibroblastic preadipocyte becomes spherical and accumulates lipid; this is accompanied by dramatic alterations in the synthesis of cytoskeletal, extracellular matrix (ECM) proteins and those required for lipid metabolism, nutrient transport, and hormone responsiveness (reviewed in reference 42). Studies of genes expressed during adipocyte that differentiation, such as fatty acid binding protein (aP2), have demonstrated transactivation by C/EBPα (8, 19) and PPARγ (47), whose ligand has recently been identified as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (13, 24). Use of exogenous factors with positive or negative effects on adipogenesis have indicated that differentiation requires the appropriate combinatorial action of hormones, growth factors, and ECM (reviewed in reference 42). Preadipocytes, therefore, must integrate signals from the extracellular environment for differentiation to ensue. For example, while expression of C/EBPα and PPARγ2 promotes adipose-specific gene expression in cells not committed to the adipocyte lineage (20, 50), these effects generally require the addition of dexamethasone.

We originally identified preadipocyte factor 1 (pref-1) during a differential screening of a 3T3-L1 preadipocyte cDNA library designed to isolate genes that regulate adipogenesis (41). pref-1 is a transmembrane protein with epidermal growth factor (EGF)-like repeats in the extracellular domain, a juxtamembrane region, a single transmembrane domain, and a short cytoplasmic tail. The pref-1 transcript undergoes alternate splicing, with four major forms of the transcript detected in 3T3-L1 preadipocytes. The longest form, pref-1A, is most abundant; however, in-frame juxtamembrane deletions result in three additional transcripts. pref-1 is a unique regulatory molecule; it is expressed in preadipocytes, in contrast to the transcription factors, C/EBP and PPARγ, that are only detected in conditions permissive for differentiation. pref-1 is readily detected in preadipocytes but is totally absent in mature fat cells, indicating complete downregulation of *pref-1* during adipocyte differentiation. pref-1 mRNA levels are lower in 3T3-L1 preadipocytes than in the closely related but differentiation-defective 3T3-C2 cells. The level of pref-1 mRNA is decreased by treatment with a combination of the adipogenic inducing agents dexamethasone and methylisobutylxanthine (44) and by fetal calf serum, a component usually required for differentiation. Moreover, constitutive expression of pref-1 in 3T3-L1 preadipocytes inhibits their conversion to adipocytes as determined by cell morphology, level of adipocyte-expressed mRNAs, and degree of lipid accumulation. These observations indicate that pref-1 could maintain the preadipose phenotype and that pref-1 downregulation is integral to adipocyte differentiation.

The most striking feature of pref-1 is the presence of six tandem EGF-like repeats in the extracellular domain. The EGF-like repeat, first identified in epidermal growth factor, is a 35- to 40-amino-acid domain with conserved spacing of six cysteine residues. EGF-like domains are present in a number of molecules where they mediate protein-protein interaction to

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control cell growth and differentiation (1). A single EGF-like domain is the functional unit of EGF, transforming growth factor α (TGFα), and other growth factors that interact with the EGF receptor (32). Cleavage of a precursor transmembrane form at the amino and carboxyl termini of the EGF-like unit(s) releases the soluble growth factor (5). The extent of processing varies with the site of synthesis and is not requisite for biological activity (3, 33, 35, 46, 49). The importance of EGF-like domains in development is clearly illustrated by the *Drosophila* cell-fate determination proteins Notch (48) and Delta (28), transmembrane proteins that contain 36 and 9 EGF-like repeats, respectively. In contrast to the EGF-like growth factors, Notch and Delta function as transmembrane proteins; no processing of the ectodomain and/or release of EGF-like repeats occurs. Notch is a multifunctional receptor with pleiotropic effects. For example, interaction of the EGF-like repeats of Delta with those of Notch on adjacent cells transduces a lateral inhibitory signal during development of the neurogenic ectoderm (11). These studies suggest that EGF-like repeats function either as soluble ligands or as juxtacrine membrane-bound signalling or transducing molecules. Notably, the tandem arrangement of the pref-1 EGF-like repeats, the amino acid sequence within individual EGF-like repeats, as well as the interruption of its EGF-like repeats by introns (40), indicate that overall, *pref-1* structure resembles that of *Drosophila delta*. pref-1 has been independently cloned as delta-like protein dlk (29) on the basis of its expression in several types of tumors. This observation, together with the function of pref-1 in adipocyte differentiation, has led us to hypothesize that in addition to the specific role of pref-1 in adipocyte differentiation, pref-1 could have a general role in maintaining the undifferentiated state. Indeed, pref-1 mRNA is detected in several embryonic tissues but not in their adult counterparts (41). The downregulation of pref-1 expression in differentiation, the inhibitory effects of its forced expression, and its EGF-like structural motif all predict that pref-1 may function in a manner analogous to that of Notch and Delta by interacting with EGF-like repeat proteins on adjacent cells or in the ECM to actively maintain the preadipose state.

In this report, we address whether transmembrane pref-1 undergoes processing to release a soluble factor and if the pref-1 ectodomain alone can generate the adipogenic inhibitory signal. By individual transfection of alternately spliced pref-1 cDNAs we show that membrane-associated pref-1 undergoes processing to produce several small soluble forms and a soluble product of 50 kDa that corresponds to the complete ectodomain. We find that two of the four alternately spliced pref-1 cDNAs do not generate this largest soluble form. When the pref-1 ectodomain produced as pref-1/glutathione *S*-transferase (GST) fusion protein is added to 3T3-L1 cells, their differentiation is drastically inhibited. Therefore, pref-1 not only functions as a transmembrane protein to affect adjacent cells but can act as a soluble inhibitor of adipocyte differentiation, with its mode of action, juxtacrine or paracrine, determined by alternate splicing.

MATERIALS AND METHODS

Cell culture and transfection. 3T3-L1 cells and COS cells were maintained in Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum. For transfection, either COS-7 or COS-CMT cells were utilized as noted and seeded at 10^6 cells per 100-mm-diameter dish the day prior to transfection. Two micrograms of supercoiled DNA was transfected per dish utilizing DEAE-dextran (Stratagene). The pref-1 expression constructs utilized encompassed the open reading frame of pref-1 subcloned into either pcDNA1 or pcDNA1AMP (Invitrogen). For transfection of COS-7 cells, cells and DNA were kept in contact for 45 min, rinsed with phosphate-buffered saline (PBS), and incubated for 4 h in DMEM containing 10% fetal calf serum and 100 μM chloroquine; the medium was then changed to DMEM with 10% fetal calf serum. Unless otherwise stated, cells and medium were harvested at 72 h posttransfection. Transfection of COS-CMT cells was performed as described above except that cells were maintained in DMEM with 10% serum plus (JRH Biosciences, Lenexa, Kans.) for 24 h following the onset of transfection and growth medium was supplemented with 100 μM $ZnCl_2$ beginning at 24 h posttransfection.

Western blot analysis. Cell monolayers were rinsed twice with PBS and scraped into PBS containing 2 mM phenylmethylsulfonyl fluoride (PMSF). The cell suspension was subjected to three freeze-thaw cycles, and the crude membrane fraction was recovered by centrifugation at 13,000 × *g* for 25 min at 4°C. The pellet was dissolved in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40 [NP-40], 1 mM EDTA, 2 mM PMSF) on ice for 30 min and clarified by brief spinning in a microcentrifuge, and the protein content was determined (Bio-Rad). The indicated amount of protein was loaded per lane in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore) with 10 mM 3-cyclohexylamino-1-propanesulfonic acid–10% methanol transfer buffer. The pref-1 antibody was raised against a pref-1/TrpE fusion protein (41). For immunodetection of proteins, membranes were blocked for 1 h at room temperature in 5% nonfat dry milk–0.5% Tween 20 in PBS. Subsequent incubations and washes were conducted with 1× NET (145 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100, and 50 mM Tris-HCl [pH 7.4]). Detection of the antigen-antibody complexes was accomplished via goat anti-rabbit immunoglobulin G-horseradish peroxidase (HRP) conjugate (Bio-Rad), and signals were visualized by enhanced chemiluminescence (ECL) (Amersham) per manufacturer's instructions.

Metabolic labelling and immunoprecipitation. Seventy-two to ninety hours posttransfection, cell monolayers were rinsed with PBS and incubated in methionine and cysteine-free DMEM with 10% dialyzed fetal calf serum for 20 min. Following this, 200 μCi of ^{35}S TransExpress labelling mix (NEN) per ml was added. After the indicated labelling periods, medium was collected and monolayers were rinsed with PBS. Cells were either harvested or refed with DMEM with 10% fetal calf serum for the chase periods. Following the chase period, medium was collected by sequential centrifugation at 1,100 and 17,000 × *g*. For use in immunoprecipitation cell monolayers were harvested in 1× immunoprecipitation (IP) buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 1 mM EDTA, and 2 mM PMSF), and medium samples were adjusted to 1× IP buffer.

For immunoprecipitation, equal amounts of trichloroacetic acid-precipitable counts of ^{35}S-labelled cell lysates were brought to a volume of 125 μl in lysis buffer and incubated with 10 μl of antisera for 2 h on ice. Immune complexes were collected by incubation at 4°C with either fixed *Staphylococcus aureus* (Pansorbin; Calbiochem) for 15 min or with protein A-Sepharose for 1 h, and pellets were washed three times in radioimmunoprecipitation assay buffer (1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM HEPES [pH 7.4], and 0.15 M NaCl). Samples were boiled in the presence of 2% (vol/vol) β-mercaptoethanol and fractionated on SDS-PAGE gels. For ^{35}S-labelled samples, gels were subjected to fluorography (Entensify; NEN) and exposed to Fuji RX film. For ^{32}P-labelled samples, gels were exposed to Fuji RX film with an intensifying screen.

In vitro transcription and translation. Full-length pref-1 cDNA in the *Eco*RI/*Xho*I site of the plasmid pcDNA1 was linearized at the unique 3′ *Xho*I site. Capped, full-length pref-1 sense RNA was synthesized utilizing T3 polymerase (Stratagene). One microgram of synthesized transcript was used for in vitro translation with the incorporation of ^{35}S cysteine (NEN). Products were analyzed by SDS–10% PAGE and fluorography of dried gels (Entensify).

Posttranslational modification of pref-1 protein. For tunicamycin treatment of COS cells, cells were incubated at 72 h posttransfection with 10 μg of tunicamycin/ml or vehicle control during a 6-h metabolic labelling period. For enzymatic removal of N-linked carbohydrate and neuraminic acid, crude membrane fraction pellets of 3T3-L1 cells were dissolved in digest buffer (100 mM phosphate buffer [pH 7.0], 1% NP-40, and 200 μM PMSF). Prior to digestion, 100 μg of protein was denatured by boiling for 5 min in a final concentration of 0.5% SDS and 0.1 M β-mercaptoethanol in a total volume of 50 μl. An additional 50 μl of digest buffer was added per sample, samples were adjusted to 1 mM $CaCl_2$ for neuraminidase digestion and 2 U of *N*-glycanase (peptide *N*-glycosidase Fe Boehringer Mannheim) and 10 mU of *Vibrio cholerae* neuraminidase (Boehringer Mannheim) were added as indicated for 3.5 h. Samples were analyzed by SDS–10% PAGE, and pref-1 protein was visualized by Western analysis.

Construction of c-Myc epitope-tagged pref-1 constructs. To tag the C terminus of the pref-1 protein with the human c-Myc epitope, two oligonucleotides (coding strand, 5′ GATCGAGCAGAAGCTGATCTCCGAGGAGGACCTCTA ATG 3′; noncoding strand, 5′ GATCCATTAGAGGTCCTCCTCGGAGATCA GCTTCTGCTC 3′) were designed to encode the 10-amino-acid human c-Myc epitope recognized by the monoclonal antibody 9E10 (9) followed by an in-frame stop codon. The oligonucleotides were annealed by heating for 10 min at 70°C in 25 mM Tris (pH 7.6)–5 mM $MgCl_2$–25 mM NaCl; this was followed by a slow cooling to room temperature. The Myc tag was ligated into the pref-1/pcDNA1AMP expression constructs pref-1A and pref-1B at the C terminus of the pref-1 protein, and the reading frame was confirmed by sequencing.

Construction of P-tagged pref-1 and in vitro phosphorylation. A consensus phosphorylation site for the catalytic subunit of cAMP-dependent protein kinase,

encoding amino acids RRASV (termed herein P-tag), was inserted into the *Nco*I site that occurs at nucleotide 370 in the pref-1 cDNA sequence. This site was chosen because it occurs in EGF-like repeat two between the third and fourth cysteines, an area where spacing between cysteine residues is quite variable. Two oligonucleotides representing the coding (5′CATGGGCGTCGCGCGTCTG TTG 3′) and noncoding (5′CATGCAACAGACGCGCGCGACGC 3′) strands with *Nco*I-compatible ends were annealed as described for the c-Myc oligonucleotides. The double-stranded product (P-tag) was ligated into the various c-Myc-tagged pref-1 expression constructs at the *Nco*I site.

Seventy-two hours posttransfection of COS-CMT cells, medium was collected by sequential centrifugation at 1,100 and 17,000 × *g*, and the supernatant was acetone precipitated. Protein pellets corresponding to 2 ml of medium were collected by centrifugation, dried, and resuspended in bovine heart kinase phosphorylation buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, and 12 mM $MgCl_2$). A total of 125 μl of this was used in the phosphorylation reaction that included 5 μl of γATP (3,000 Ci/mmol) and 50 U of heart muscle kinase (Sigma Chemical) in a final volume of 150 μl. Following incubation at 4°C for 30 min, 850 μl of stop solution (10 mM sodium phosphate [pH 8.0], 10 mM sodium pyrophosphate, 10 mM EDTA, and 1-mg/ml bovine serum albumin) was added, along with 110 μl of 10× IP buffer (0.2 M Tris-HCl [pH 7.4], 1.5 M NaCl, 5% sodium deoxycholate, 10% NP-40, 10 mM EDTA, and 20 mM PMSF), and samples were divided equally for immunoprecipitation with 10 μl of the indicated antisera.

pref-1/GST fusion protein production and 3T3-L1 cell differentiation. *Eco*RI/*Bam*HI-digested pGEX2TK (Pharmacia Biotech) and PCR-amplified fragments of pref-1 were used to generate expression vectors for pref-1/GST fusion proteins. GST and pref-1/GST, corresponding to the full pref-1 extracellular domain minus the signal sequence (amino acids 8 through 299) were expressed in BL-21 *Escherichia coli* and purified by affinity binding to glutathione agarose beads (Pharmacia Biotech). The proteins eluted by 5 mM reduced glutathione were dialyzed against 1× PBS, mixed with DMEM containing 0.5% FBS, and filter sterilized through a Millex 10-μm-pore-size filter (Millipore). At confluence, 3T3-L1 preadipocytes were treated for 48 h with 1 μM dexamethasone and 0.5 mM methylisobutylxanthine (dex/mix). Control GST or pref-1/GST proteins were added at the start of the differentiation protocol at a concentration of 50 nM. This concentration was maintained by the addition of proteins at subsequent medium changes. The concentration of purified proteins was determined by multiplying the purity of the protein determined by Coomassie blue staining of SDS-PAGE gels by the total protein concentration. Antisera were inoculated with equal volumes of the fusion protein before being added to the medium at a final dilution of 1:100. The effect of pref-1 inhibition could first be observed 2 days after the start of the differentiation protocol. At 5 days postinitiation of differentiation, cells were stained for lipid with Oil Red O and photographed, and RNA was extracted from parallel cultures and subjected to Northern analysis as previously described (41) utilizing ^{32}P-labelled cDNA for fatty acid synthetase, C/EBPα, stearoyl coenzyme A desaturase, and fatty acid binding protein and a labelled PPARγ1 cDNA that detects both the PPARγ1 and PPARγ2 transcripts.

RESULTS

Cleavage yields a residual C-terminal 25-kDa cell-associated pref-1. To begin characterizing the pref-1 protein, we generated antibodies against an *E. coli*-expressed TrpE/pref-1 fusion protein. In 3T3-L1 cell lysates, a minimum of seven discrete protein bands of approximately 45 to 55 kDa are detected by the pref-1 antibody. These are abolished by preincubation of the pref-1 antisera with TrpE/pref-1 fusion protein but not with TrpE protein alone (43). Given the complex pattern of pref-1 protein in preadipocytes, partly due to expression of at least four alternate transcripts, detailed analysis of specific pref-1 protein isoforms is inherently difficult. To overcome these limitations we expressed pref-1 in COS cells, a cell type that lacks endogenous pref-1 and that has been extensively utilized to address protein structure and function. When we transfected full-length pref-1 we observed that, in addition to full-length transmembrane pref-1, a 25-kDa cell-associated protein was specifically detected by pref-1 antibody. Since pref-1 was previously known to exist only in transmembrane form, the appearance of this 25-kDa membrane-associated protein was the first indication we had that pref-1, in addition to its transmembrane location, might exist in a soluble form; the 25-kDa protein could correspond to residual pref-1 after cleavage and release of some region of the ectodomain and would thus be predicted to contain the pref-1 cytoplasmic domain.

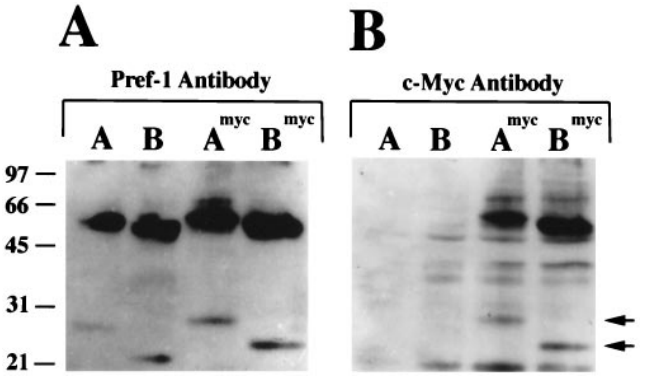

FIG. 1. Western analysis of c-Myc-tagged pref-1. Twenty-five micrograms of protein from COS-7 cells expressing pref-1A (lanes A), pref-1B (lanes B), or forms tagged with the c-Myc epitope (lanes A^{myc} and B^{myc}) were fractionated on SDS–10% PAGE. (A) Western analysis utilizing a 1:15,000 dilution of pref-1 primary antibody and a 1:2,000 dilution of goat anti-rabbit secondary antibody followed by ECL detection. (B) The same blot shown in panel A stripped and reprobed with a 1:20,000 dilution of the 9E10 primary antibody and a 1:2,000 dilution of goat anti-mouse secondary antibody followed by ECL detection. Molecular mass markers in kilodaltons are on the left, and the arrows at the right indicate the positions of the 25-kDa pref-1A and 21-kDa pref-1B protein bands.

To determine if the 25-kDa cell-associated protein corresponds to the C terminal cytoplasmic domain, we added a 10-amino-acid human c-Myc epitope tag to the extreme C terminus of cDNA expression constructs for the two longest forms of pref-1, pref-1A and pref-1B. Myc-tagged and unmodified versions of pref-1A and pref-1B were transfected into COS cells, and crude membrane fraction proteins were analyzed by Western blotting. The pref-1 antibody detects the full-length 55-kDa pref-1A and the full-length 51-kDa pref-1B in the membrane fraction. In addition, a 25-kDa protein results upon pref-1A expression, and a 21-kDa protein results by pref-1B expression (Fig. 1A). This 4-kDa size difference of the pref-1A and pref-1B proteins reflects the membrane-proximal 153-base deletion in pref-1B arising by alternate splicing. The Myc-tagged versions of pref-1A and pref-1B are also recognized. In each case the addition of the Myc tag increases the molecular mass of the pref-1 bands by 1 kDa, i.e., the size of tag. This is most apparent for the 25-kDa pref-1A and the 21-kDa pref-1B proteins. Their size increases upon addition of the Myc tag indicate that they contain the C terminus of the pref-1 protein. Reprobing of the same membrane with the 9E10 antibody specific for the Myc epitope (9) shows that only the Myc-tagged, and not the native forms, of pref-1A and pref-1B are specifically recognized (Fig. 1B). Furthermore, the identical 25-kDa pref-1A and 21-kDa pref-1B proteins are recognized by the pref-1 and the 9E10 antibodies (Fig. 1B). Given the 1-kDa size increase that occurs with the addition of the Myc tag, and the recognition of these bands by both antibodies, we conclude that full-length membrane pref-1 is probably cleaved to a residual membrane-associated protein of 25 kDa for pref-1A and 21 kDa for pref-1B and that this protein contains the pref-1 cytoplasmic domain.

The pref-1 ectodomain is cleaved to a soluble factor. Our Myc tag studies indicate that membrane pref-1 undergoes cleavage. To detect the pref-1 cleavage product in the medium and address pref-1 processing in more detail we expressed pref-1A in COS cells and performed pulse-chase analyses. At the end of the 30-min pulse period, a pref-1 protein of 55 kDa is detected by immunoprecipitation of the cell lysate with pref-1 antibody (Fig. 2A). By 7 h postsynthesis, the majority of membrane-associated pref-1A has been turned over, and it is

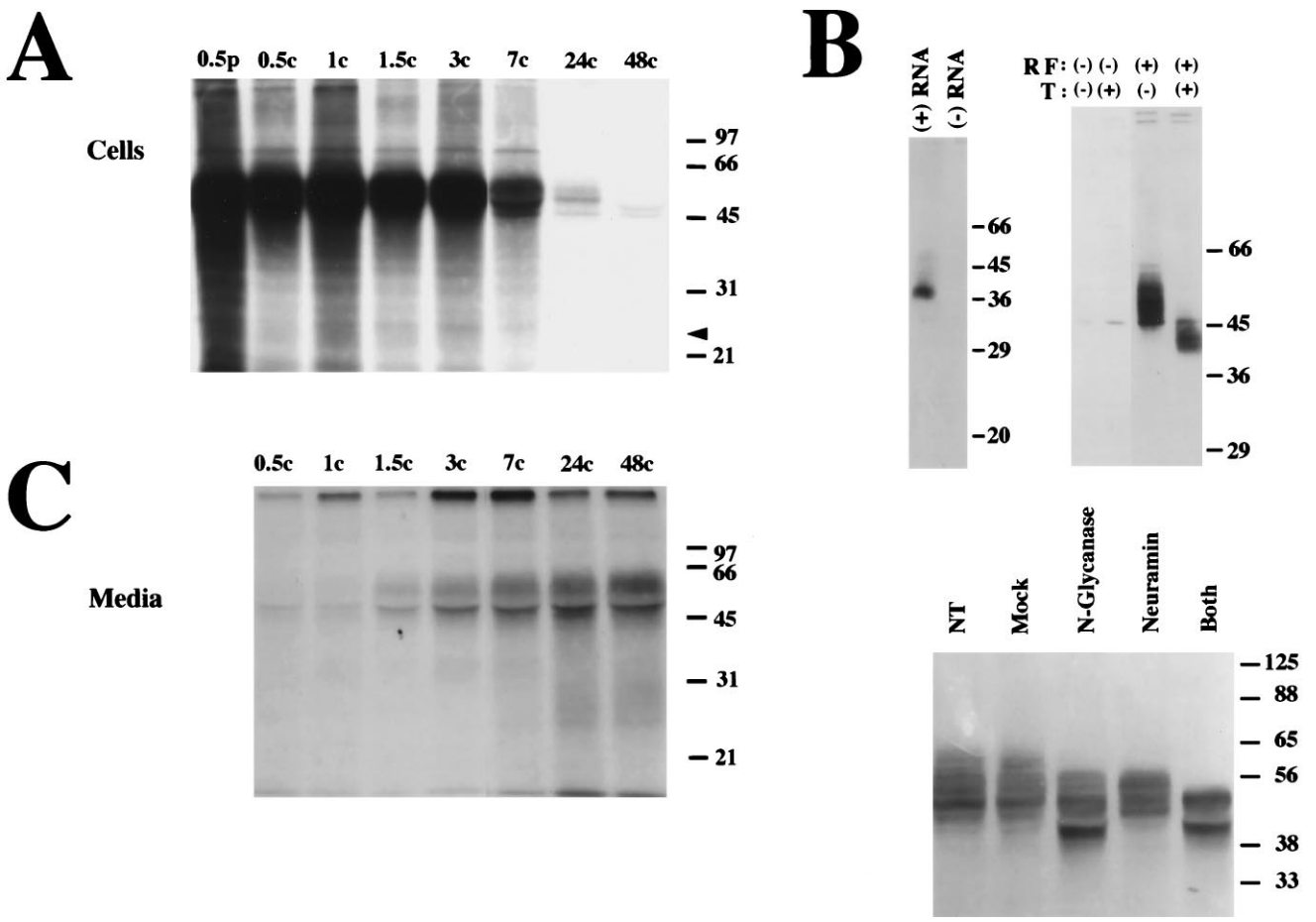

FIG. 2. Analysis of pref-1 processing. (A) Pulse-chase analysis of cellular pref-1. pref-1A-expressing COS-CMT cells were pulse-labelled with [^{35}S]cysteine and methionine for 30 min (0.5p) and subjected to the indicated chase periods (c) in hours followed by immunoprecipitation of cell lysates with pref-1 antibody and SDS-PAGE. Cells were harvested at indicated time points. Normal sera controls indicate that the approximately 50-kDa doublet band seen at 24 and 48 h is nonspecific (43). The exposure time for cell-associated pref-1 is approximately one-fifth that for soluble pref-1 shown in panel C. The arrowhead indicates the position of the 25-kDa product. (B) Posttranslational modification of pref-1. The left part of the panel shows results of in vitro translation of in vitro-transcribed pref-1 RNA (+), or a no RNA control (−), in the presence of [^{35}S]cysteine. The right part of the panel shows results from COS cells transfected with the correct (RF+) or reverse (RF−) orientation of the pref-1 open reading frame and which were subjected to metabolic labelling with [^{35}S]cysteine and methionine in the presence (T+) or absence (T−) of tunicamycin, immunoprecipitated with pref-1 antibody, and analyzed by SDS-PAGE. The lower part of the panel shows results from denatured crude membrane fraction protein from 3T3-L1 cells which was either not treated (NT), incubated without addition of enzyme (mock), or treated with *N*-glycanase, neuraminidase, or both *N*-glycanase and neuraminidase for 3.5 h. Following digestion, 50-μg samples were fractionated on SDS–10% PAGE gels and subjected to Western analysis using pref-1 antisera at a dilution of 1:800 and a 1:2,000 dilution of goat anti-rabbit HRP secondary antibody. (C) Pulse-chase analysis of soluble pref-1. pref-1A-expressing COS-CMT cells were pulse-labelled with [^{35}S]cysteine and methionine for 30 min and subjected to the indicated chase periods (c) in hours followed by immunoprecipitation of medium with pref-1 antibody and SDS-PAGE. Samples of medium were collected at indicated time points. The exposure time for the samples was approximately five times longer than that for the pulse-chase analysis of cell-associated pref-1 shown in panel A. Molecular mass markers in kilodaltons are shown on the right.

undetectable at 48 h. At the same time a 25-kDa product (Fig. 2A), which is the size of the residual membrane and cytoplasmic domain of pref-1 detected by Western blotting shown in Fig. 1, is in the cell lysate. However, the prominence and relative ratio of the 25-kDa form to that of full-length membrane-associated pref-1 differs in our Western blot versus pulse-chase analyses. This apparent discrepancy may be because whereas Western analysis likely reflects steady-state actual molar ratios, the signals of the metabolically labelled proteins are based on their content of methionine and cysteine and reflect a single time point of synthesis. Since the majority of the pref-1 extracellular domain consists of six tandem EGF-like repeats, each containing six cysteine residues, the intensity of bands detected by pulse-chase studies would be skewed toward full-length pref-1 in contrast to the residual membrane-associated 25-kDa form.

In these analyses, the cell-associated 55-kDa pref-1 appears as a broader signal than that shown in Fig. 1. Although this could be attributable to the detection technique used, immunoprecipitation versus Western analysis and gel resolution, the diffuse nature of the 55-kDa cell-associated pref-1 shown in Fig. 2A suggests posttranslational modification of the protein. There are three consensus sites for N-linked glycosylation in the pref-1 extracellular domain. We determined the size of the pref-1 primary translation product and utilized tunicamycin, an inhibitor of N-linked glycosylation to assess whether pref-1 protein in transfected COS cells contains *N*-glycan (Fig. 2B). In vitro translation results in an approximately 39-kDa protein, which is in agreement with the predicted size of the pref-1 primary translation product (Fig. 2B, left panel). Metabolic labelling and immunoprecipitation of pref-1-expressing COS cells reveals that cell-associated pref-1 protein is reduced to a

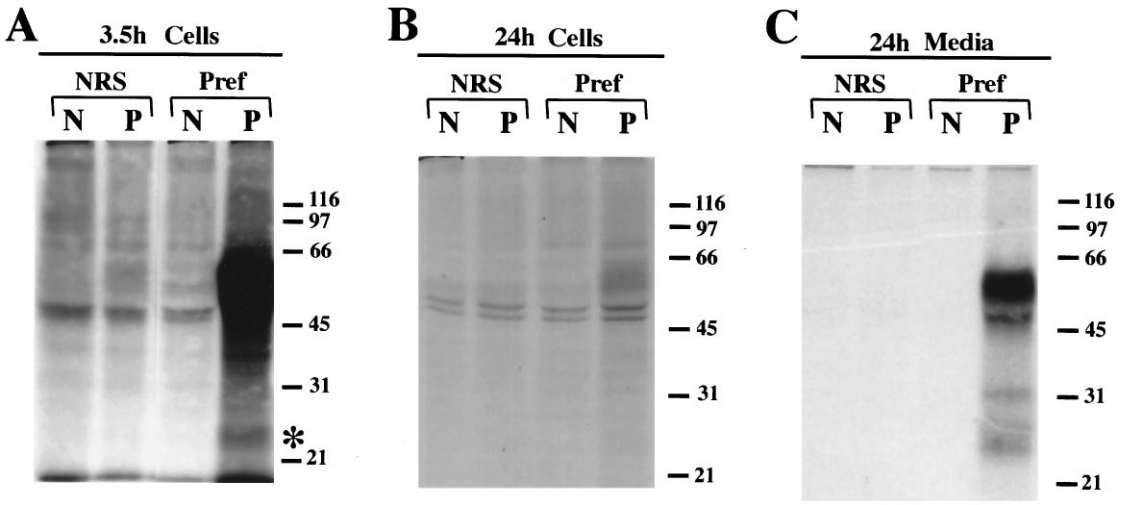

FIG. 3. Detection of soluble forms of pref-1 in conditioned medium. Nontransfected (N) or COS-CMT cells transfected with pref-1A (P) were metabolically labelled with ^{35}S for 3.5 h, cells and medium were subjected to immunoprecipitation with normal rabbit sera (NRS) or pref-1 antisera (Pref), and products were fractionated on SDS–10% PAGE gels. (A) Cells harvested following the 3.5-h labelling period. The asterisk indicates a band that may correspond to a residual 25-kDa pref-1 protein associated with the cytoplasmic membrane (Fig. 1 and 2A). (B) Cells harvested 24 h after the onset of the 3.5-h labelling period. (C) Medium harvested 24 h after the onset of the 3.5-h labelling period. Molecular mass markers in kilodaltons are on the right.

more discrete band of 45 kDa in the presence of tunicamycin. These bands are not present when an expression construct containing the opposite orientation of the pref-1 reading frame is employed (Fig. 2B, right panel). This indicates that all of the heterogenous cell-associated proteins we detect correspond to various forms of pref-1. To further address this, crude membrane preparations of 3T3-L1 preadipocytes were treated with *N*-glycanase and neuraminidase, followed by Western analysis. No treatment and mock treatment show multiple discrete pref-1 protein bands. Digestion with *N*-glycanase, neuraminidase, or a combination confirms pref-1 is a glycoprotein that contains N-linked oligosaccharide and sialic acid (Fig. 2B, lower panel). The presence of sialic acid in pref-1 may therefore explain the 6-kDa size difference between the in vitro-translated product and pref-1 protein present in tunicamycin-treated cells. We conclude that the heterogenous nature of pref-1 protein is due to posttranslational modifications that occur within 30 min of synthesis.

Pulse-chase analysis of the medium (Fig. 2C) demonstrates that a soluble 50-kDa form of pref-1 appears 1.5 h postsynthesis and accumulates thereafter. In addition, a diffuse signal between 21 and 31 kDa is present in the medium at 24 h. The increase in soluble pref-1 in the medium with a concomitant decrease in the membrane-associated form (Fig. 2A) is consistent with a precursor-product relationship and indicates cell-associated pref-1 is processed to release soluble products. This does not necessarily indicate that all of membrane-associated pref-1 undergoes processing; the decrease in the 55-kDa cell-associated form over time is likely due to the combined effects of cleavage to soluble forms and recycling and/or turnover of membrane pref-1. To further address the nature of these smaller proteins in the medium, a longer labelling period was used. COS cells were transfected with pref-1A, and cells were harvested 3.5 h after labelling or cells and medium were harvested 24 h after the onset of labelling. After the 3.5-h labelling period a pref-1 band of approximately 55 kDa, corresponding to full-length pref-1A, is detected in pref-1-transfected cells (Fig. 3A). It is not present in nontransfected controls nor is it detected with normal rabbit sera. An additional band (Fig. 3A) may correspond to the residual cytoplasmic membrane-associated 25-kDa pref-1 protein noted in Fig. 1 and 2A. As in the pulse-chase analysis, cellular pref-1 protein is barely detectable 24 h postlabelling (Fig. 3B). However, this longer labelling identified, in addition to the prominent 50-kDa soluble form, 24- to 25-kDa and 31-kDa proteins in the medium (Fig. 3C). The diffuse nature of the 24- to 25-kDa doublet suggests it could arise by differential posttranslational modification of the same polypeptide backbone. The low amounts of these smaller soluble forms may indicate a slow cleavage event due to a limiting proteolysis system. As the immunoprecipitation analysis revealed the cell-associated and soluble pref-1 to be close in size, 55 kDa versus 50 kDa, and since these analyses were performed on separate SDS-PAGE gels, to confirm this size difference we directly compared the size of cell-associated and soluble pref-1 by resolving them in adjacent lanes of an SDS-PAGE gel. Figure 4 shows the result of this size comparison analyzed by Western blotting pref-1 protein is not detected in nontransfected COS cells whereas transfection of pref-1A results in an approximately 55-kDa cell-associated pref-1 protein and an approximately 50-kDa form in the medium. These findings are in agreement with the metabolic labelling results shown in Fig. 2 and 3 and taken together indicate that full-length cell-associated pref-1 can undergo processing to release a 50-kDa soluble form.

Localization of cleavage and regulation of soluble pref-1 production by alternate splicing. Identification of multiple soluble forms of pref-1 and a 25-kDa cell-associated form indicates that membrane pref-1 is subject to two cleavage events. Based on the 50-kDa molecular mass for the large soluble form, this cleavage event would occur near the cell membrane. The cleavage event that generates smaller soluble pref-1 would be predicted to occur at a more membrane-distal site. To study the generation of the soluble pref-1 in more detail, we used two approaches: (i) addition of a phosphorylation site tag (P-tag) to the pref-1 extracellular domain and (ii) determination of the effect of various juxtamembrane deletions on the appearance of soluble pref-1. We hypothesized that processing from the N terminus may generate the smaller soluble forms of pref-1 detected by metabolic labelling. To determine which portion of the extracellular domain of pref-1 is released as the soluble

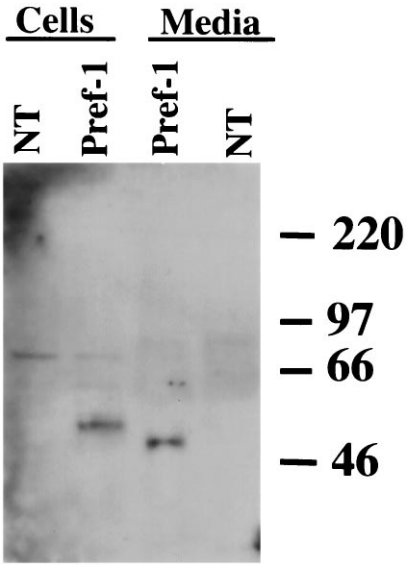

FIG. 4. Western analysis of cell-associated and soluble pref-1. Cells and conditioned medium were harvested from COS-CMT cells transfected with pref-1A (Pref-1) or nontransfected (NT) controls. Fifteen micrograms of the cell lysate and 5 μl of conditioned medium were fractionated on SDS–10% PAGE gels, subjected to Western analysis using a 1:15,000 dilution of pref-1 primary antibody and a 1:5,000 dilution of goat anti-rabbit–HRP secondary antibody, and products were visualized by ECL.

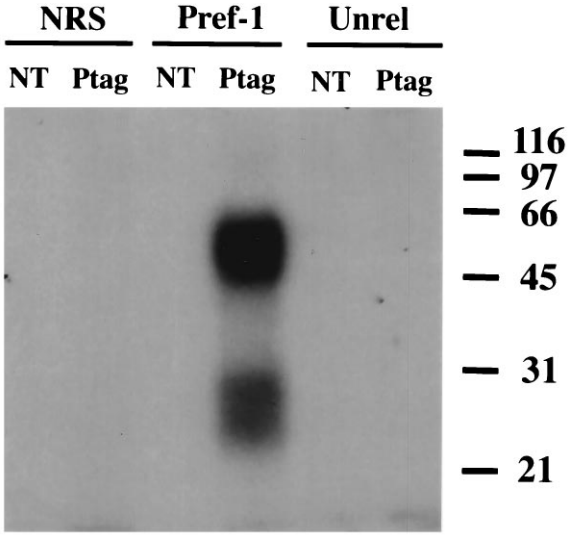

FIG. 5. Analysis of P-tagged pref-1 in medium. Conditioned medium collected from nontransfected (NT) COS-CMT cells or COS-CMT cells transfected with the P-tagged version of pref-1A (Ptag) was in vitro phosphorylated with ^{32}P and immunoprecipitated with either normal rabbit sera (NRS), pref-1 antisera (Pref-1), or antisera raised against an unrelated TrpE fusion protein (Unrel). Immunoprecipitates were fractionated by SDS–10% PAGE and subject to autoradiography. Molecular mass markers in kilodaltons are on the right.

form(s), a consensus phosphorylation site (P-tag) for cAMP-dependent protein kinase was added near the N terminus of the pref-1 extracellular domain. To minimize the effects of the addition of six amino acids on overall structure, the P-tag was inserted in the second EGF-like repeat between the third and fourth cysteines, an area with variable cysteine spacing. P-tagged pref-1A was expressed in COS cells and the medium was in vitro phosphorylated and immunoprecipitated (Fig. 5). We detected a phosphorylated protein of 50 kDa, the same soluble product noted by metabolic labelling; given its size this protein likely corresponds to the full ectodomain. The doublet of 24 to 25 kDa is also observed by use of the P-tag. These proteins therefore contain the second EGF repeat and thus probably the N-terminal region of pref-1. They are not detected in nontransfected COS cells nor when normal sera or an unrelated antisera was used in immunoprecipitation. We therefore predict that a pref-1 processing event occurs at a site C terminal to the P-tag to generate the N-terminal, P-tagged 24- to 25-kDa doublet. This membrane-distal event would also explain our detection of the 25-kDa residual cell-associated pref-1 which is apparent upon expression of pref-1A as shown in Fig. 1. The sizes of the soluble forms detected by metabolic labelling and P-tag are identical. The differences observed in the relative ratio of the 50-kDa to the 24- to 25-kDa soluble form may be attributed to inherent differences in the two detection methods. While metabolic labelling at cysteine residues, abundant in the EGF-like repeat motif, follows a population of pref-1 synthesized during a specific period at 72 h posttransfection, by in vitro phosphorylation each pref-1 molecule is labelled at a single P-tag site. The signals of the various soluble forms determined by in vitro phosphorylation likely reflect a steady-state level of their molar ratios accumulated from 24 to 72 h posttransfection.

The four alternately spliced forms of pref-1 that we previously identified in 3T3-L1 preadipocytes, and which have various in-frame extracellular juxtamembrane deletions, provided a system in which to test our hypothesis that the 50-kDa soluble pref-1 derives from an extracellular membrane-proximal cleavage. The structures of these alternate forms are depicted in Fig. 6B. Transfection of each of the four major alternate forms of the pref-1 cDNA results in membrane-associated pref-1 proteins whose molecular masses decrease in correspondence to their respective deletions (43). To address soluble pref-1 production, the four P-tagged alternately spliced forms were expressed in COS cells. The medium was subject to in vitro phosphorylation at the P-tag site, immunoprecipitation, and SDS-PAGE analysis. Strikingly, whereas each isoform expresses the 24- to 25-kDa doublet in the medium, the large soluble form is produced only by pref-1A and pref-1B; little if any large soluble pref-1 is generated by the two alternately spliced isoforms with larger juxtamembrane deletions, pref-1C and pref-1D (Fig. 6A). These data reveal that the cleavage that generates large soluble pref-1 occurs within a sequence common to pref-1A and pref-1B. Furthermore, the observation that pref-1B results in the large soluble form and pref-1C does not indicates that the sequence present in pref-1B, but deleted in pref-1C, contains the membrane-proximal processing site for the generation of the large soluble pref-1. This localizes the cleavage event to within the 22-amino-acid juxtamembrane sequence PEQHILKVSMKELNKSTPLLTE (Fig. 6C). Interestingly, our localization of the membrane-proximal cleavage to within the sequence PEQHILKVSMKELNKSTPLLTE agrees with the protein sequence of fetal antigen 1 (FA1), reported during the course of our experiments. FA1 is a circulating fetal protein with undetermined function that likely corresponds to the complete extracellular domain of human pref-1 (23). The N terminus of FA1 begins after the pref-1 signal sequence, and although the extreme C terminus of FA1 has not been unambiguously assigned, it falls within the 22-amino-acid sequence that we determined contains the membrane-proximal cleavage site for the release of the 50-kDa soluble pref-1. Although no consensus processing sites are present in this 22-amino-acid sequence, the sequence is nota-

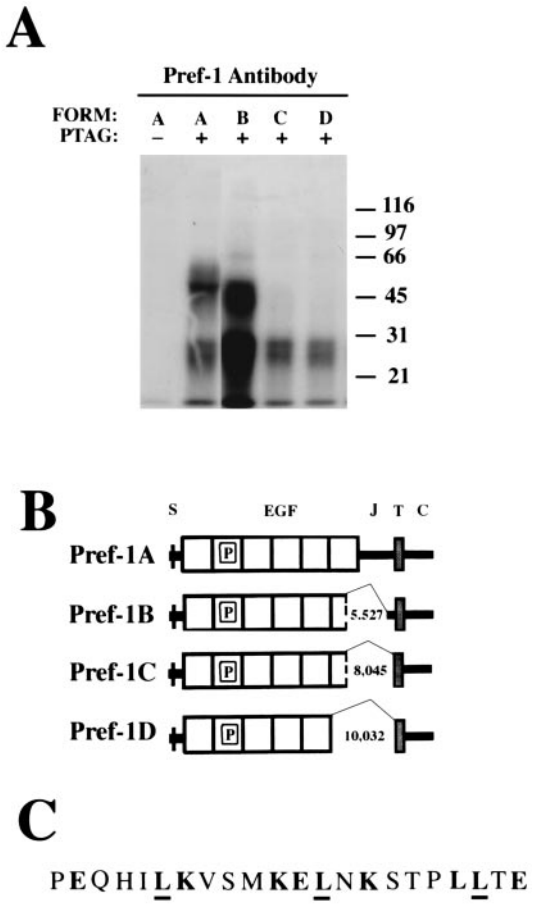

FIG. 6. Effect of alternate splicing on appearance of soluble pref-1. (A) The various alternately spliced (A, B, C, D) and P-tagged (PTAG) forms of pref-1 were expressed in COS-CMT cells. The presence (+) or absence (−) of the P-tag is indicated. Conditioned medium was subjected to in vitro phosphorylation with ^{32}P, and following immunoprecipitation with pref-1 antibody, products were analyzed by SDS–12.5% PAGE and autoradiography. Molecular mass markers in kilodaltons are on the right. (B) The predicted structures of the four alternately spliced forms of the pref-1 cDNA are shown. S, signal sequence; EGF, EGF-like repeat; J, juxtamembrane; T, transmembrane; C, cytoplasmic domain P, location of the P-tag in the second EGF-like repeat. The thin connecting line represents the area deleted in each of the forms of the protein and the number shown indicates the calculated molecular weight of the primary amino acid sequence deleted. (C) The 22-amino-acid juxtamembrane sequence, present in pref-1B but absent in pref-1C, predicted to be involved in release of the 50-kDa soluble pref-1 is shown. The glutamic acid, lysine, and leucine residues are shown in bold. The leucines spaced every seventh amino acid, as in leucine zipper motifs, are underlined.

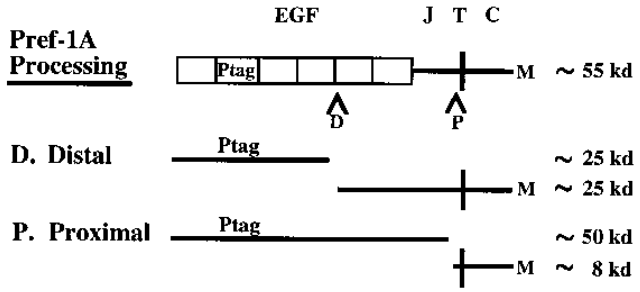

FIG. 7. Proposed model for processing of membrane-associated pref-1. The structure of full-length pref-1A is shown at top. EGF, EGF-like repeats; J, juxtamembrane; T, transmembrane; C, cytoplasmic; Ptag, location of the P-tag in the second EGF-like repeat; M, location of the C-terminal Myc-epitope tag. The predicted processing events are shown by arrowheads and are designated D for the membrane-distal event and P for the membrane-proximal event. The corresponding cleavage products are outlined below, and approximate molecular masses in kilodaltons are on the right. The model incorporates data from Western blot, pulse chase, and P-tag studies with the proposed cleavage sites assigned based on the sizes of membrane-bound and soluble pref-1. The membrane-proximal site can be assigned to be within 22 amino acids of the transmembrane domain based on the differential effects of alternate splicing on the generation of the 50-kDa soluble pref-1. This cleavage event would also predict the generation of a residual membrane-associated protein of approximately 8 kDa. The membrane-distal site is putatively placed between the fourth and fifth EGF-like repeat. Cleavage at this location predicts the generation of proteins corresponding to the small soluble form of approximately 25 kDa and the residual cell-associated 25-kDa pref-1. Furthermore, this is the location of the alanine- and valine-rich sequence (see Discussion) that is similar to sites involved in the processing of several other transmembrane proteins. The percentage of membrane-expressed pref-1 that is subject to each processing event and whether the two cleavages occur independently or sequentially remain to be established. The slight differences in the observed and predicted sizes of the products of pref-1 cleavage may arise from as yet unidentified cleavage events. The origin of the minor 31-kDa product observed via metabolic labelling has not been determined and for this reason is not included in this model.

ble for the distinct spacing of lysine, glutamic acid, and leucine (Fig. 6C). The glutamic acids occur every tenth residue and the lysines every fourth residue. Most interestingly, the leucines are spaced every seventh residue, reminiscent of the leucine zipper motif for protein-protein interaction. However, the presence of proline, which disrupts alpha-helical structures, argues against a typical leucine zipper motif. Together the above-described results indicate that the 55-kDa membrane-associated pref-1 can undergo two cleavage events. These are depicted in Fig. 7. A membrane-distal event generates the P-tagged approximately 24- to 25-kDa soluble pref-1 and the residual 25-kDa membrane-associated protein containing the pref-1 cytoplasmic domain. A membrane-proximal event within the sequence PEQHILKVSMKELNKSTPLLTE generates the 50-kDa soluble form of pref-1. Cleavage at this membrane-proximal site would be predicted to result in a residual cell-associated pref-1 with a calculated molecular mass of 8 kDa that was perhaps too small to be detected in our experiments. Furthermore, the differential effects of alternate splicing on the production of soluble pref-1 demonstrate that this is a mechanism for determining the type(s) of soluble and/or transmembrane pref-1 produced. Our findings therefore indicate that pref-1 has the potential to function not only in a juxtacrine fashion as a transmembrane protein but as a soluble protein with paracrine actions.

Soluble pref-1 acts to inhibit adipocyte differentiation. Although our studies have not defined the exact area of cleavage in vivo, results of pulse-chase analyses and the transfection of alternately spliced isoforms of pref-1 indicate that the full pref-1 ectodomain is present in culture medium as the result of a membrane-proximal cleavage event. We have previously shown that constitutive expression of full-length pref-1 drastically inhibits 3T3-L1 adipocyte differentiation. Whereas all four alternate forms express the 24- to 25-kDa soluble product, only the largest soluble form is differentially generated; it is derived from the pref-1A and pref-1B isoforms but not the pref-1C and pref-1D isoforms. While we have employed COS cells to address processing of specific pref-1 isoforms, the existence of the pref-1 ectodomain (FA1) in fetal circulation is definitive evidence that the pref-1 processing we detect occurs in vivo and strongly indicates an in vivo function for soluble pref-1. Since the only model system for pref-1 action described to date is the inhibition of adipocyte differentiation, we therefore addressed the bioactivity of soluble pref-1 in adipocyte differentiation. The entire pref-1 extracellular domain, corresponding to the 50-kDa soluble pref-1, was produced as a GST

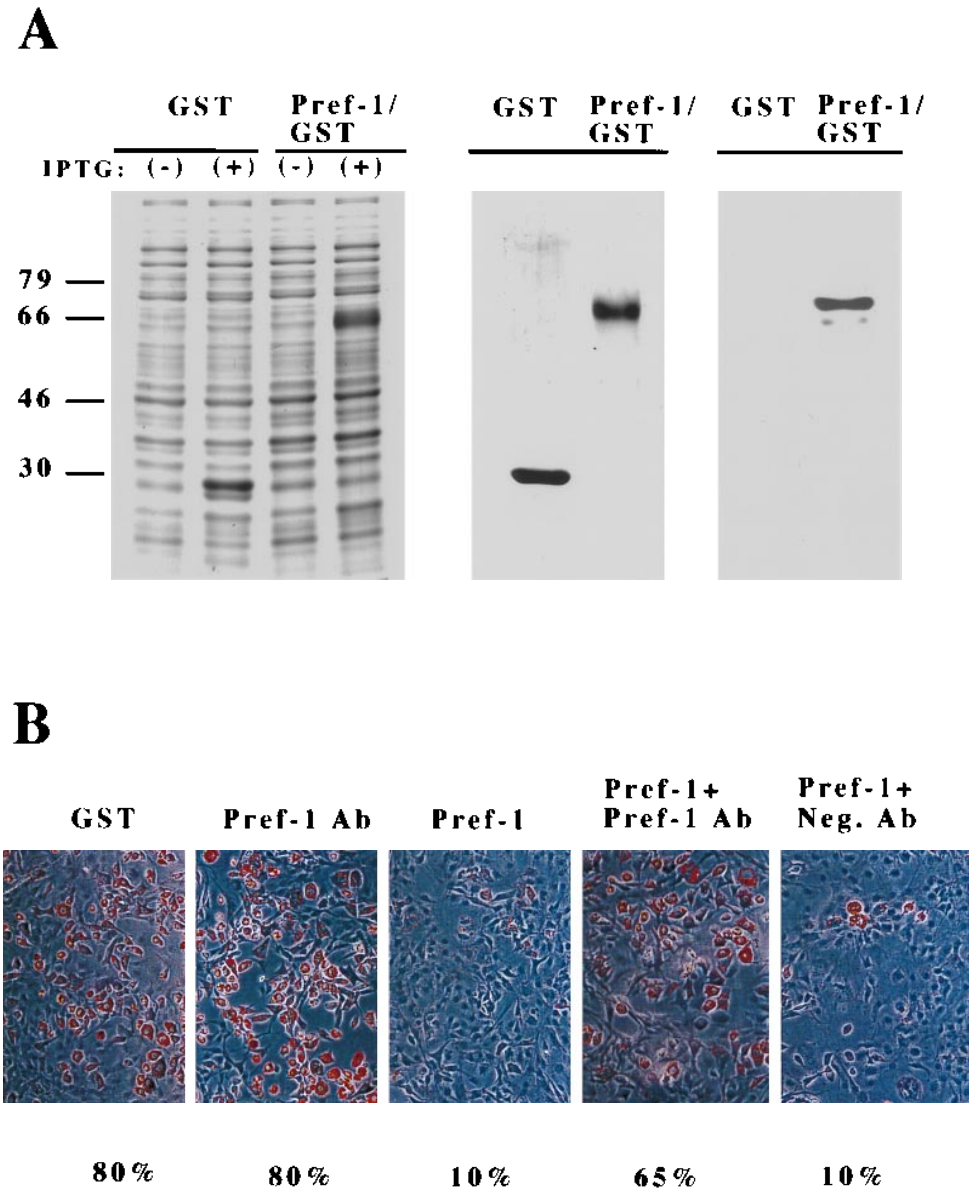

FIG. 8. Production and activity of pref-1/GST fusion protein. (A) Expression, purification, and Western analysis of pref-1/GST fusion protein. Left, Coomassie blue-stained SDS-PAGE gel of total protein from uninduced (−) and IPTG-induced (+) BL-21 *E. coli* harboring either the GST or pref-1/GST expression constructs; middle, affinity-purified 29-kDa GST and the 63-kDa pref-1/GST fusion protein on a Coomassie blue-stained SDS-PAGE gel; right, Western analysis of purified GST and pref-1/GST using pref-1 antibody. Molecular mass markers in kilodaltons are on the left. (B) The inhibitory effect of pref-1/GST on 3T3-L1 differentiation assessed by Oil Red O staining of cellular lipid. Additions to the standard dex/mix differentiation treatment are noted above photomicrographs. GST, GST protein; Pref-1 Ab, antibody directed against a pref-1/TrpE fusion protein; Pref-1, pref-1/GST fusion protein; Neg. Ab, antibody directed against an unrelated TrpE fusion protein. The percentages of lipid-containing cells are indicated below the photomicrographs.

fusion protein in *E. coli*. Cells harboring the GST or pref-1/GST expression construct show an identical pattern of proteins upon Coomassie blue staining of SDS-PAGE gels. Induction of protein expression with isopropyl-β-D-thiogalactopyranoside (IPTG) results in proteins of the size predicted for GST alone (29 kDa) or pref-1/GST (63 kDa); these are the most abundant proteins detected (Fig. 8A, left panel). Coomassie blue staining of soluble fusion proteins after affinity binding to glutathione agarose beads shows a single band, indicating purification to near homogeneity (Fig. 8A, middle panel). Western analysis reveals that the pref-1/GST fusion protein but not GST alone is specifically detected by pref-1 antibody (Fig. 8A, right panel). To test the effect of soluble pref-1 on adipocyte differentiation, confluent 3T3-L1 preadipocytes were treated with dex/mix to initiate differentiation. The medium was supplemented with either the GST protein or the pref-1/GST fusion protein. Additionally, to address the specificity of the effects of pref-1/GST, pref-1 antibody or an antibody against an unrelated TrpE fusion protein was utilized. After 5 days, cells were fixed and stained with Oil Red O, and the degree of adipocyte differentiation was judged by cell morphology and the percentage of lipid-containing cells (Fig. 8B). Addition of either the GST protein or pref-1 antibody had no discernable effects; 80% of cells differentiated to adipocytes as indicated by high lipid content and rounded appearance. Addition of pref-1/GST fusion protein markedly inhibited differentiation, and these cells

TABLE 1. Concentration-dependent inhibition of 3T3-L1 differentiation by GST-pref-1 fusion protein[a]

Protein concentration (nM)	% Adipocyte conversion	
	Expt 1	Expt 2
0	>70	>70
5	>70	>70
10	20–50	30–60
25	<20	20–50
50	<20	<20
100	<20	<20

[a] Confluent 3T3-L1 cells in quadruplicate dishes were subject to dex/mix treatment in the presence of the indicated concentrations of fusion protein. Six days after induction cells were stained for lipids with Oil Red O and examined microscopically for percentage of adipocyte conversion. The average of four dishes is indicated.

had very little lipid accumulation and maintained fibroblast morphology with only 10% of the cells differentiating. Furthermore, the inhibitory effects of the fusion protein on adipocyte differentiation are attenuated by preincubation of pref-1/GST with antiserum against pref-1. These cultures show 65% differentiation, whereas preincubation with an unrelated control serum does not affect the inhibitory action of pref-1/GST as evidenced by only 10% differentiation. This indicates that the inhibitory effects of the pref-1/GST fusion protein are specifically due to the pref-1 ectodomain. To address whether there is a dose-response effect of pref-1 action, we tested the inhibitory action of pref-1 at protein concentrations of 0, 5, 10, 25, 50, and 100 nM. As is shown in Table 1, the inhibitory effects of pref-1 are first noted at 10 nM, and maximum inhibition is observed at 50 nM. These data, as well as the blocking effects of pref-1 antibody shown in Fig. 8B, are consistent with the existence of a specific pref-1 receptor. However, the biological nature of the assay system, namely the inhibition of adipocyte differentiation, limits more detailed determination of the kinetics of pref-1 interaction with its predicted receptor. Such analyses await the identification and isolation of the pref-1 receptor by interaction cloning or other methods.

We next addressed the inhibition of adipocyte differentiation in detail at the molecular level. The effects of soluble pref-1 on the level of adipocyte-expressed RNAs was determined and correlated with morphological evidence of adipocyte differentiation. Cells were treated with dex/mix alone or supplemented with GST or pref-1/GST and stained for lipid with Oil Red O 5 days after initiation of differentiation (Fig. 9A); we observed the same inhibitory effects for pref-1 which are shown in Fig. 8B. Northern analysis for five adipocyte-expressed mRNAs reveal that compared to cells differentiated with the standard dex/mix treatment or with the addition of GST protein, pref-1-treated cells have only 20% of the levels of the terminal marker mRNAs for fatty acid synthase, stearoyl coenzyme A desaturase, and fatty acid binding protein (Fig. 9B). Moreover, the levels of mRNA for C/EBPα and PPARγ are similarly decreased, indicating the inability of cells to express these transcription factors in the presence of soluble pref-1. This suggests that the inhibitory effects of soluble pref-1 are exerted early in differentiation. The results indicate that

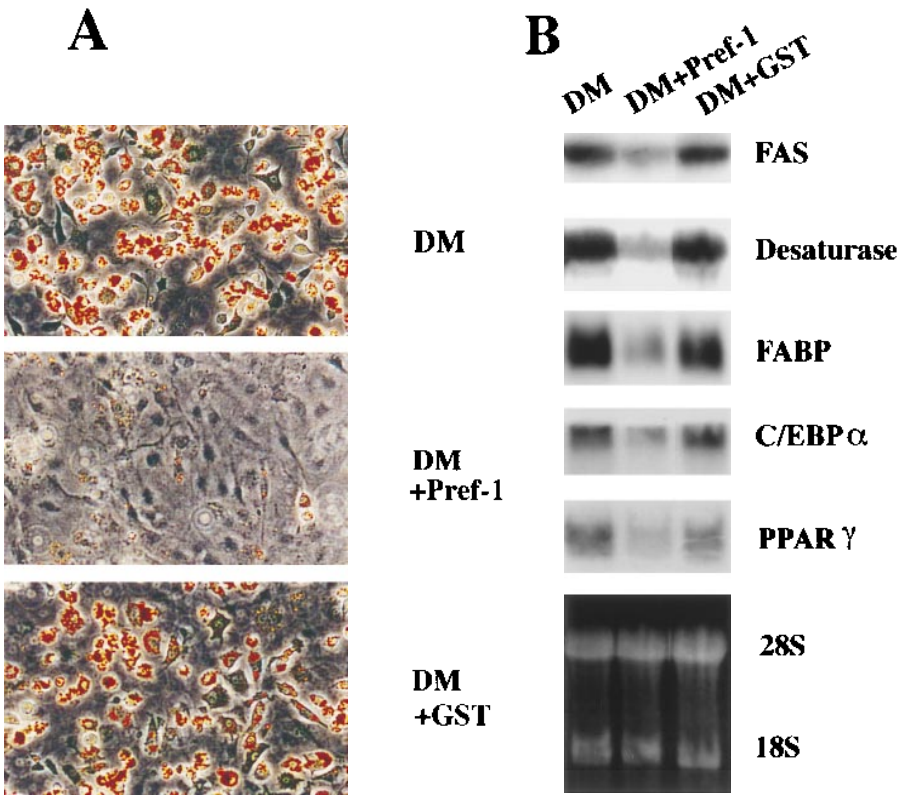

FIG. 9. Soluble pref-1 inhibits adipocyte differentiation. (A) Confluent 3T3-L1 preadipocytes were subject to standard in vitro differentiation conditions (DM) or supplemented with either the pref-1/GST fusion protein (DM+Pref-1) or GST control (DM+GST) throughout the course of differentiation. At 5 days after initiation of differentiation, cultures were stained with Oil Red O and photographed. Typical microscopic fields are shown. (B) Ten micrograms of total RNA from parallel cultures were subject to Northern blot analysis using the indicated ^{32}P-labelled cDNA probes. The PPARγ signal appears as a doublet since both the γ1 and γ2 isoforms are detected. Representative ethidium bromide staining of the Northern gel is shown at the bottom.

the pref-1 ectodomain alone, corresponding to the soluble form we detect in conditioned medium, is sufficient for the inhibitory effect of pref-1 in adipocyte differentiation. This further suggests that the pref-1 molecule, in either transmembrane or soluble form, probably functions as a ligand to initiate and/or maintain signals inhibitory to adipogenesis.

DISCUSSION

pref-1 exists in both transmembrane and soluble forms. We demonstrate by pulse-chase analyses and in vitro phosphorylation at the P-tag site that full-length pref-1 undergoes cleavage at a membrane-proximal site to release an N-terminal soluble product of 50 kDa. This soluble form inhibits adipocyte differentiation. The differential effects of alternate splicing on the production of the 50-kDa soluble pref-1 predicts cleavage occurs extracellularly near the transmembrane domain at a membrane-proximal site within the 22-amino-acid sequence PEQHILKVSMKELNKSTPLLTE. This agrees with the protein sequence of the human fetal protein FA1, reported during the course of our studies, that corresponds to the pref-1 extracellular domain. The simplest interpretation of our data is that the spliced-out sequence removes a processing site. This has strong similarities to the effect of alternate splicing in the c-kit ligand where the KL-1 form is processed while the alternately spliced KL-2 form is not efficiently cleaved due to a juxtamembrane deletion encompassing the preferred processing site (12, 21). This 22-amino-acid sequence does not contain any recognizable motifs such as the basic residues that are processing sites for kex2/furin proteases (18) or the small apolar amino acids where cleavage of TGFα occurs (31). It is of interest to note that the splicing event removes portions of the juxtamembrane region, including sequences reminiscent of a leucine zipper.

These findings place pref-1 into that class of proteins which can act either as transmembrane or soluble molecules. Among EGF-like repeat proteins, ectodomain processing and release has been demonstrated only for those growth factors that function through the EGF receptor and related receptors. Transfection studies with EGF and TGFα have allowed detailed analysis of their processing from transmembrane precursors; however, processing is not requisite for their biological activity. Membrane-anchored forms of EGF and TGFα bind and activate the EGF receptor (2, 32). The full 160-kDa pro-EGF produced by the kidney is active (37). Ectodomain release also occurs for transmembrane molecules other than the EGF-like repeat growth factors, including the c-kit ligand (7, 21), tumor necrosis factor 1 receptor (16, 27), and the β-amyloid precursor protein (4). While our data indicate that the 50-kDa soluble form of pref-1 corresponds to the full ectodomain as the result of membrane-proximal cleavage, formulation of a complete model for the generation of the minor soluble forms of pref-1 is not yet possible. In addition to a prominent soluble form of 50 kDa, we also find that the 24- to 25-kDa form contains the P-tag placed near the pref-1 N terminus. We can speculate that subsequent cleavage of the 50-kDa form of soluble pref-1 at the membrane-distal site to generate the 24- to 25-kDa soluble pref-1 could serve as a mechanism to inactivate the larger soluble form or otherwise modulate its activity. However, as we have not yet clearly delineated which portion of the pref-1 ectodomain the smaller soluble proteins derive from, we cannot at this time address with any certainty their function. Nevertheless, a membrane-distal processing event would be predicted to occur at a site C terminal to the P-tag inserted in the second EGF-like repeat. This would release the 24- to 25-kDa soluble form. We predict that this membrane-distal event also generates the 25-kDa residual cell-associated pref-1 that we determined by Myc-epitope tagging to contain the pref-1 cytoplasmic domain. With the assumption that the size of this 25-kDa residual cell-associated pref-1 is attributable solely to primary amino acid sequence, the 25-kDa residual cell-associated protein would correspond to the extreme C terminus of pref-1 up to EGF-like repeat five. Inspection of the primary amino acid sequence of pref-1 within the region bordered by the P-tag and the transmembrane domain reveals an area of small apolar amino acids, Val-Ala-Ala, between the fourth and fifth EGF-like repeats. This is similar to the cleavage site(s) used for the release of mature soluble EGF, TGFα, and KL-1 from transmembrane precursors (30, 34). Preliminary studies indicate site-directed mutagenesis of the pref-1 Val-Ala-Ala sequence alters the amount and appearance of soluble pref-1 (43).

Functional implications of pref-1 processing. The work presented demonstrates that the pref-1 ectodomain/GST fusion protein, which corresponds to the 50-kDa soluble form, inhibits adipocyte differentiation, as we have previously shown for the membrane-associated form. Since the 50-kDa soluble form of pref-1 has inhibitory activity similar to that of the full-length membrane-associated form, release of the pref-1 ectodomain as a soluble factor allows switching between two active forms of pref-1, thereby regulating its range of action. Therefore, pref-1 not only functions in a juxtacrine manner as a transmembrane protein to affect adjacent cells but can have paracrine actions as a soluble inhibitor of adipocyte differentiation. We have confirmed the inhibitory effects of soluble pref-1 by treating confluent 3T3-L1 preadipocytes with dex/mix in the presence of conditioned medium from transfected COS cells. Following a 2-day dex/mix treatment, cells were maintained in 50% fresh growth medium-50% conditioned medium. While cells treated with conditioned medium from mock-transfected COS cells differentiated well, as judged by the number of lipid-containing cells, conditioned medium from pref-1A-transfected COS cells drastically reduced adipocyte differentiation (43). Thus, use of two different approaches, GST fusion protein and conditioned medium, demonstrates the inhibitory action of soluble pref-1 and indicates that the bioactivity of the GST fusion protein is similar to that produced by COS cells. Although both the transmembrane and soluble pref-1 are active in the inhibition of adipocyte differentiation, future studies may reveal finer distinctions in their respective functions, as demonstrated for the kit ligand where the soluble factor does not fully substitute for the actions of membrane-bound kit ligand in vivo (12). These inhibitory effects observed with pref-1A-conditioned medium are additional evidence for an in vivo role of soluble pref-1 in the regulation of adipocyte differentiation. Moreover, we have observed that treatment of 3T3-L1 preadipocytes with conditioned medium from COS cells transfected with pref-1A markedly inhibits adipocyte differentiation, while conditioned medium from COS cells transfected with the most deleted alternate form, pref-1D, does not affect adipocyte differentiation (43). We therefore hypothesize that the mode of function, juxtacrine or paracrine, depends on the alternate pref-1 transcript expressed.

The temporal expression of genes during adipocyte differentiation suggests a hierarchy of regulatory events. Based on expression pattern and transfection studies, C/EBP and PPARγ have been shown to be central to adipogenesis. However, factors such as cell confluence/growth arrest, fetal calf serum, dexamethasone, and an ECM environment conducive to adipocyte differentiation may govern expression and action of these transcription factors. The absolute downregulation of pref-1 during adipocyte conversion and the inhibitory effects of

forced pref-1 expression in preadipocytes suggest it has a unique regulatory function in this process. In conditions under which preadipocytes normally differentiate, addition of soluble pref-1 prevents expression of both PPARγ and C/EBPα, the regulatory molecules that transactivate adipocyte genes and lead to adipogenesis. This is consistent with the concept that downregulation of pref-1 is a prerequisite for C/EBPα and PPARγ induction and adipocyte differentiation. Our experiments here suggest that, via the generation of a soluble inhibitory form, pref-1 is likely to have a wider range of function than was first predicted on the basis of its synthesis as a transmembrane protein. The inhibitory effects of fibronectin (45) and collagen (22) on adipocyte differentiation indicate cytoskeletal and/or ECM remodelling is requisite for adipocyte differentiation. By analogy, pref-1, as either a transmembrane or soluble protein, may exert its inhibitory effects through interaction of its EGF-like repeats with EGF-like repeats present in cell surface or ECM components to maintain the preadipose phenotype. It is intriguing given its structural similarities to the Notch-Delta family, that pref-1 is processed to generate soluble forms. Work presented here does not rule out the possibility that transmembrane pref-1 may act as a receptor to transduce inhibitory signals. However, the fact that the pref-1 ectodomain alone inhibits adipocyte differentiation indicates that generation of the inhibitory signal does not require the pref-1 cytoplasmic region. This suggests that soluble pref-1 acts as a signalling molecule through an as yet unidentified receptor. It is highly unlikely that pref-1 acts through the EGF receptor. Not only are the spacing and conservation of amino acids required for EGF-receptor interaction (38) absent in pref-1, but we have failed to detect for pref-1 the mitogenic effect normally associated with EGF receptor function (6). We hypothesize the existence of an EGF repeat containing receptor for pref-1 that could be analogous in action to the Notch-Delta receptor-ligand pair.

Although we address here the role of soluble pref-1 in adipocyte differentiation, other findings point to a broader role for pref-1 in differentiation and development. pref-1 is detected in various tissues early in embryogenesis but not in corresponding adult tissues (41). Expression of the pref-1 homolog dlk has been linked to small cell lung carcinoma and neuroendocrine tumors (29). In the larger context we hypothesize that pref-1 may maintain undifferentiated states in a number of cell types, and its downregulation may be required for differentiation. The expression of alternately spliced forms of pref-1, each with potentially distinct functions and ranges of action, may be temporally and/or spatially restricted during development. The finding that FA1, the pref-1 extracellular domain, is present in fetal circulation supports a broader in vivo role for the processing and effects of soluble pref-1 than we have described. Along these lines it is tempting to speculate that soluble pref-1 may repress adipogenesis in vivo, a process that, depending on the species, occurs late in gestation or neonatally.

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