Pref-1 is a highly glycosylated Delta-like transmembrane protein containing six epidermal growth factor-like repeats in the extracellular domain. Pref-1 is abundantly expressed in preadipocytes, but expression is down-regulated during adipocyte differentiation. Forced expression of Pref-1 in 3T3-L1 cells was reported to inhibit adipocyte differentiation. Here we show that efficient and regulated processing of Pref-1 occurs in 3T3-L1 preadipocytes releasing most of the extracellular domain as a 50-kDa heterogeneous protein, previously isolated and characterized as FA1. Unexpectedly, we found that forced expression of the soluble form, FA1, or full-length Pref-1 did not inhibit adipocyte differentiation of 3T3-L1 cells when differentiation was induced by standard treatment with methylisobutylxanthine, dexamethasone, and high concentrations of insulin. However, forced expression of either form of Pref-1/FA1 in 3T3-L1 or 3T3-F442A cells inhibited adipocyte differentiation when insulin or insulin-like growth factor-1 (IGF-1) was omitted from the differentiation mixture. We demonstrate that the level of the mature form of the IGF-1 receptor is reduced and that IGF-1-dependent activation of p42/p44 mitogen-activated protein kinases (MAPKs) is compromised in preadipocytes with forced expression of Pref-1. This is accompanied by suppression of clonal expansion and terminal differentiation. Accordingly, supplementation with insulin or IGF-1 reduced p42/p44 MAPK activation, clonal expansion, and adipocyte differentiation in a dose-dependent manner.

Adipocyte differentiation is a complex process that involves dramatic changes of cell morphology and gene expression (for review, see Refs. 1–5). Primary mouse embryonic fibroblasts and several established preadipocyte cell lines can be induced to differentiate into adipocytes when treated with an appropriate combination of adipogenic factors, including methylisobutylxanthine (Mix),1 dexamethasone (Dex), and insulin or IGF-1. Growth factors like EGF and platelet-derived growth factor (PDGF) or cytokines, such as interleukin-11 (IL-11), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β), can inhibit adipogenesis and cause reversal of the adipocyte phenotype. In addition, growth hormone has been shown to inhibit differentiation of primary preadipocytes (6), but it exerts a proadipogenic action on several preadipocyte cell lines, including 3T3-L1 (14–16).

Pref-1 cDNA was cloned from a 3T3-L1 cDNA library in a screening for genes that were differentially expressed during adipocyte differentiation (17). Pref-1 is abundantly expressed in preadipocytes, whereas expression in mature adipocyte is undetectable (17). In 3T3-L1 cells, pref-1 expression has been demonstrated to be down-regulated by a change from growth in normal calf serum to growth in fetal calf serum, and an element in the pref-1 promoter involved in this down-regulation has been identified (18). In addition, the proadipogenic action of dexamethasone has been related to a dexamethasone-mediated acute down-regulation of pref-1 transcription (19). Forced expression of Pref-1 was reported to inhibit adipogenesis, and accordingly, Pref-1 was suggested to play an important role in the maintenance of the preadipose state (17). In accordance with this hypothesis, a reduction in the level of pref-1 expression in 3T3-L1 cells by expression of pref-1 antisense RNA decreased the requirements for dexamethasone to support adipocyte differentiation (19), and a reduction of pref-1 expression in Balb/c 3T3 cells dramatically increased their ability to undergo adipose conversion in response to supraphysiological levels of insulin (20, 21). Finally, the generation of Pref-1-null mice demonstrated that lack of Pref-1 expression is associated with obesity and that pref-1 in mice, as in humans, is a paternaly expressed imprinted gene (22).

Pref-1 belongs to the epidermal growth factor (EGF)-like protein family, the members of which are characterized by the presence of EGF-like motifs in their extracellular domains. Members of the EGF-like protein family can function as soluble or transmembrane proteins that exert their effects through protein-protein interaction and play important roles in cellular growth and differentiation. One well-characterized subclass includes EGF and transforming growth factor-α. These proteins...
teins arise by ectodomain processing and release from transmembrane precursors and exert their action upon binding to the EGF receptor. Interestingly, processing and release are not prerequisites for biological activity (for review, see Ref. 23). One of the best-characterized examples involving lateral protein-protein interaction of transmembrane proteins is provided by the Notch-Delta or Notch-Serrate/Jagged systems that play pivotal roles in cell fate determination during neurogenesis (24). Recently, Notch-1 was also shown to be implicated in adipocyte differentiation (25). The organization of the pref-1 gene and the structure of the individual EGF-like repeats strongly suggest that Pref-1 is most closely related to the Delta family (26).

Apart from its function in adipocyte differentiation, Pref-1 has been shown to be involved in the interaction between stromal cells and hematopoietic cells, in which dlk expression in stromal cells is directly correlated with their capability to provide a microenvironment allowing co-cultured hematopoietic stem cells to give rise to highly proliferative myeloid-erythroid progenitors (20, 27). Of particular interest is a recent report describing a role for Pref-1 in thymocyte development (28). Surprisingly, a dimeric form of Pref-1 was shown to increase thymocyte cellularity in an HES-1-dependent manner even though Pref-1 is devoid of the DSL motif generally thought to be necessary for Delta-Notch-type interactions (20).

Prior to the cloning of pref-1, a soluble form of the human homolog of Pref-1, named fetal antigen 1 (FA1), was isolated from amniotic fluid (29–31), and subsequently, mouse FA1 was isolated from mouse amniotic fluid (32). Sequence comparison revealed that FA1, Pref-1, pg2 (a human adenarc specific mRNA) (33), and dlk (delta-like protein) (34) represent polymorphic variants of the same gene (30, 35). The presence of high levels of FA1 in amniotic fluid immediately suggested that Pref-1 might also function as a soluble molecule. Accordingly, it has been shown that a bacterially expressed GST-Pref-1 fusion protein could inhibit adipocyte differentiation of 3T3-L1 cells in a dose-dependent manner (36), and purified human FA1 was reported to inhibit differentiation of primary rat preadipocytes (37). It was furthermore reported that conditioned medium from COS-7 or Balb/c cells transfected with pref-1 could prevent 3T3-L1 cells from undergoing adipocyte differentiation (21, 36), and recently it was reported that only the large soluble form of Pref-1 was able to inhibit adipocyte differentiation (38). It is generally assumed that Pref-1 is processed and released from 3T3-L1 preadipocytes, but this in fact has not been directly demonstrated.

In this report, we show that mouse Pref-1 is released from 3T3-L1 preadipocytes as a 50-kDa heterogeneous protein that is indistinguishable from mouse FA1 purified from amniotic fluid. We show that processing is regulated in a growth/differentiation-dependent manner and that a significant fraction of the synthesized Pref-1 is processed and released from 3T3-L1 cells. We confirm previous findings that pref-1 expression is down-regulated during adipocyte differentiation and show that the ratio between membrane-bound and soluble Pref-1/F1A1 is maintained during differentiation. Surprisingly, we found that forced expression of full-length Pref-1 or the soluble form of Pref-1/F1A1 did not inhibit adipocyte differentiation of 3T3-L1 cells using the standard MDI protocol that includes treatment with Mix, Dex, and supraphysiological concentrations of insulin for induction of differentiation. However, in the absence of insulin or IGF-1, forced Pref-1 expression inhibited adipocyte differentiation. These results suggested that IGF-1/IGF-1 receptor (IGF-1R) signaling was compromised in the cells expressing Pref-1, thereby rendering the cells unresponsive to the low concentration of IGF-1 in the fetal calf serum. In keeping with this, we show that IGF-1 or insulin in a dose-dependent manner bypassed the inhibitory effect of Pref-1. Finally, we present evidence that forced expression of Pref-1 reduced the level of the mature form of the IGF-1R, inhibited clonal expansion, and severely curtailed IGF-1-dependent activation of p42/p44 MAPKs.

EXPERIMENTAL PROCEDURES

Plasmin and Cloning—The retroviral vector Akvibe2 was generated by inserting retroviral sequences into the EcoRl/Sall site of pUC19. The retroviral sequences contain the long-terminal repeats and packaging sequence derived from retrovirus A-MLV (39), and the envelope glycoproteins virus IRS (internal ribosomal entry site) (40) between the two long-terminal repeats. Mouse pref-1 cDNA was generated by RT-PCR from total RNA of 3T3-L1 preadipocytes using Expand™ reverse transcriptase (Roche Applied Science). Different regions of rat pref-1 (41) and mouse pref-1 were generated by PCR and cloned into the polylinker site of Akvibe2 to generate plasmids AkvPrefPL, AkvPrefSF, AkvPrefET, AkvPrefIC, AKvPrefEF, AkvPrefSF, AkvmPrefET, and AkvmPrefIC (Fig. 2A). The sequences of the plasmids were verified by sequencing.

Transfection and Differentiation—BOSC23 packaging cells (42) were grown in 9-cm dishes in DMEM (Invitrogen, high glucose) containing 10% fetal calf serum (Sigma) until they reached 80% confluence and then transfected with 7.5 μg of Akvibe2 and 7.5 μg of pUC19. The retroviral sequences contained the long-terminal repeats and mouse pref-1 cDNA were inserted into the respective EcoRI cloning vectors using the 3β[3(O,N',N'-dimethylaminoethane)carbamoyl]-cholesterol procedure as described previously (43). Six hours after transfection, the medium was replaced with fresh medium, and after another 48 h of incubation, the virus-containing medium was harvested and either frozen down at –80 °C or used immediately for transduction. Before transduction, the viral suspension was mixed with 1 volume of fresh DMEM and 6 μg/ml hexadimethrine bromide (Sigma). The 3T3-L1 cells were transduced at 50% confluence, and the following day, the cells were split 1:6 and replated under selection of 600 μg/ml G418 (Sigma). Seven days later, the selected clones were pooled and replated for differentiation experiments.

Culture and Differentiation—Following selection, the transduced 3T3-L1 cells were grown in DMEM containing 10% calf serum (CS) (Sigma) and 400 μg/ml G418 (Sigma) until they reached confluence, at which time G418 was removed. At 2 days postconfluence (defined as day 0), the cells were induced to differentiate as described earlier (44). Briefly, the medium was changed to DMEM containing 10% fetal calf serum (FCS), 1 μM dexamethasone (Dex, Sigma), 0.5 mM isobutylmethylxanthine (Mix, Aldrich). Insulin or IGF-1 (Roche Molecular Biochemicals) was added as indicated. Dex and Mix were maintained until day 2, and insulin/IGF-1 when added was maintained until day 4. At the time points indicated, media were collected, and the cells were either harvested for analysis of protein by Western blotting and ELISA, or stained with Oil Red O or used immediately for transfection, and 3T3-C2 cells were cultured and treated according to the standard MDI differentiation protocol (DMEM, 10% FCS, 1 μM Dex, 0.5 mM Mix, and 1 μg/ml insulin). 3T3-F442A cells were grown in DMEM containing 10% CS until confluence and then induced to differentiate by switching to DMEM containing 10% FCS, 10 mM insulin, and 2 mM T3. Medium was renewed every second day for both cell types. For the analysis of MAPK and Akt activation, the transduced 3T3-L1 cells were maintained in DMEM containing 10% CS until 2 days post confluence and then switched to differentiation medium (DMEM, 10% FCS, 1 μM Dex, and 0.5 mM Mix with or without 10 nM IGF-1) and cultured until the indicated time points when cell lysates were prepared for Western blotting.

Purification of Mouse Pref-1/FA1—Pref-1/FA1 was purified from medium conditioned by 3T3-L1 preadipocytes or from murine amniotic fluid by immunospecific affinity chromatography as described previously (32).

ELISA—ELISA was performed as described (32) with affinity-purified rabbit anti-mFA1 as the capture antibody. The plates were incubated overnight with test samples and calibrator (mouse amniotic fluid in 2-fold dilution series). Biotinylated affinity-purified rabbit anti-mFA1 was used as the detector antibody, and peroxidase-conjugated streptavidin, H₂O₂, and 0-phenylenediamine were used to develop the reaction.

Triglyceride Measurement—mPref-1-transduced 3T3-L1 cells were lysed in Tris-C1 buffer by sonication. The triglyceride content was measured using the GPO-Kit as recommended by the manufacturer (Sigma).

Multiplex RT-PCR—RNA purification, reverse transcription, and
multiplex RT-PCR were performed as previously described (45). For PCR, 20 cycles were performed. The primer sets used were: Mouse TBP upstream: 5′-ACC CTT CAC CAA TGA CTC CTA TG; downstream: 5′-ATG ATG ACT GCA GAA AAT CGC. Mouse IGF-1R upstream: 5′-TCA GCC TTC ATC CGC AAG AC; downstream: 5′-CAA GGA GAC CAA GGC ATG AG.

Western Blotting—Whole cell extracts and conditioned medium from 3T3-L1-vector, 3T3-L1-mPrefSF, and 3T3-L1-mPrefSF cells were subjected to Western blotting. For cell extracts, 100 µg of total protein was loaded; for conditioned medium, 10 µl was loaded. The following antibodies were used: rabbit anti-mouse FA1 and anti-rat FA1 antibody (32), anti-mouse aP2 antibody (kindly provided by David A. Bernlohr), anti-mouse PPARγ2 antibody (Santa Cruz Biotechnology, sc-7273), anti-IGF-1R β-subunit antibody (Santa Cruz Biotechnology, sc-718-G), anti-TBP antibody (Santa Cruz Biotechnology, sc-275), anti-MAPK antibodies (Cell Signaling Technology, #9102 for total MAPK p42/p44 and #9106L for phosphorylated MAPK p42/44), and anti-Akt antibodies (Cell Signaling Technology, #9272 for total Akt and #9271L for phosphorylated Akt). Visualization was achieved by enhanced chemiluminescence.

Bromodeoxyuridine Labeling—3T3-L1-vector, 3T3-L1-mPrefSF, and 3T3-L1-mPrefFL cells were grown to 2-day post confluency in DMEM plus 10% CS and then treated with differentiation medium containing DMEM, 10% FCS, 1 µM DEX, 0.5 mM Mix, and different concentrations of IGF-1 as indicated. The cells were labeled with BrdUrd between the period from 12 to 24 h after the start of the treatment, and BrdUrd incorporations were carried out using the 5-Bromo-2′-deoxyuridine Labeling and Detection Kit I according to the instructions of the manufacturer (Roche Applied Science).
confirm this observation. On the contrary, the maximal levels of Pref-1/FA1 in medium conditioned by confluent 3T3-C2 and in lysates were only ~30% of those determined for 3T3-L1 cells (Fig. 1, compare e and g with a and c).

When 3T3-C2 cells were treated according to the MDI protocol for induction of adipogenesis, a moderate decline in the levels of Pref-1/FA1 in both conditioned medium and lysates were observed. A nadir in the level of Pref-1/FA1 in lysates was observed around day 3, but the magnitude of changes was minimal compared with 3T3-L1 cells (Fig. 1, f and h). Without MDI treatment the level of Pref-1/FA1 expression remained essentially constant in the period from day 1 to day 4 and then declined.

**Forced Expression of Pref-1/FA1 Does Not Prevent MDI-induced Differentiation of 3T3-L1 Cells**—It was originally reported that forced expression of Pref-1 in 3T3-L1 inhibited differentiation (17), and this observation has been confirmed in several subsequent studies (17, 19, 21). Furthermore, it was reported that expression of the Pref-1 ectodomain or peptides corresponding to the individual EGF-like repeats 1, 4, and 5 inhibited adipocyte differentiation (21), and recently, it was reported that only the large soluble form of Pref-1 inhibited adipocyte differentiation (38).

We amplified different regions of the rat Pref-1 cDNA by PCR and inserted the fragments into the Akvibe2 retroviral vector (Fig. 2A). After transduction with the relevant retroviral vector, the cells were selected with G418, pooled, and subjected to treatment with differentiation inducers according to the MDI protocol (44). On day 10, the cells were either Oil Red O-stained or harvested. ELISA quantification confirmed that the cells lysates and conditioned medium from Pref-1-expressing 3T3-L1 cells contained Pref-1/FA1 levels 2- to 13-fold higher than the vector control cells (results not shown). Surprisingly, the differentiation potential was maintained in all the pools of cells transduced with the different constructs as determined by cell morphology and intercellular lipid accumulation (Fig. 2B). These results indicated that forced expression of rat Pref-1 in 3T3-L1 cells did not interfere overtly with the MDI-induced adipocyte differentiation.

Because our results clearly contrasted previously published results, we speculated whether this was a result of species differences between rat and mouse Pref-1. Consequently, we cloned the mouse pref-1 cDNA and repeated the experiments using retroviral vectors that harbored the full-length mouse pref-1 cDNA (mPrefFL) or a cDNA encoding the soluble form of mouse Pref-1/FA1 (mPrefSF). As shown in Fig. 3A, 3T3-L1 cells expressing the full-length or the soluble form both achieved more than 80% differentiation, comparable to the vector cells. Western blotting (Fig. 3B) detected a high intensity band of 50 kDa in the conditioned medium from mPrefSF and mPrefFL cells on both day 0 and day 10, indicating high levels of ectopic Pref-1 expression. These bands co-migrated with FA1 purified from mouse amniotic fluid (results not shown), indicating that the transduced full-length Pref-1 was correctly processed and released into the medium. In the cell lysates, a broad band around 50 kDa, representing the full-length Pref-1, was detected in 3T3-L1-mPrefFL and 3T3-L1-mPrefSF cells on both day 0 and day 10, indicating high levels of ectopic Pref-1 expression. These bands co-migrated with FA1 purified from mouse amniotic fluid (results not shown), indicating that the transduced full-length Pref-1 was correctly processed and released into the medium. In the cell lysates, a broad band around 50 kDa, representing the full-length Pref-1, was detected in 3T3-L1-mPrefFL and 3T3-L1-mPrefSF cells on both day 0 and day 10, indicating high levels of ectopic Pref-1 expression. These bands co-migrated with FA1 purified from mouse amniotic fluid (results not shown), indicating that the transduced full-length Pref-1 was correctly processed and released into the medium. In the cell lysates, a broad band around 50 kDa, representing the full-length Pref-1, was detected in 3T3-L1-mPrefFL cells on both day 0 and day 10. The heterogeneous nature of the bands particularly in the cell lysates is likely to reflect the different degree of glycosylation (46) and/or alternative splicing of pref-1 gene transcripts (26). In our Western analysis, we did not detect bands between 21 and 31 kDa in the conditioned medium of Pref-1-transduced 3T3-L1 cells as reported previously for Pref-1-transfected COS-7 cells (36). To exclude the possibility that our deviating results were related
Insulin or IGF-1 Treatment Bypasses Pref-1-mediated Inhibition of Adipocyte Differentiation—Our results demonstrating that adipocyte conversion of 3T3-L1 preadipocytes proceeded in the presence of forced expression of Pref-1 contradicted previous observations and indicated that Pref-1 down-regulation, contrary to the general notion, is not a prerequisite for adipocyte differentiation. The MDI differentiation protocol involves combined treatment with Mix, Dex, and insulin, whereas the inhibitory action of forced expression of Pref-1 was observed in cultures not treated with insulin (17). In the MDI procedure, insulin is used at supraphysiological concentrations, and hence, insulin is able to initiate signaling also through the IGF-1R. Thus, one interpretation of our experiments would be that the level of IGF-1 normally present in fetal calf serum became limiting for adipocyte differentiation of the Pref-1 over-expressing 3T3-L1 preadipocytes. Consequently, we analyzed whether the requirements for exogenous insulin or IGF-1 to induce differentiation were increased in 3T3-L1 preadipocytes that overexpressed the full-length or the soluble form of Pref-1 in comparison with 3T3-L1 cells transduced with the empty retroviral vector.

Fig. 4A shows that a significant proportion of the 3T3-L1 cells transduced with the empty retroviral vector differentiated into fat-laden adipocytes even in the absence of added insulin or IGF-1. In contrast, 3T3-L1 cells expressing the full-length or the soluble form of Pref-1 differentiated very poorly in the absence of exogenously added insulin or IGF-1. Addition of insulin or particularly IGF-1 to the vector-transduced cells increased differentiation in a dose-dependent manner. In 3T3-L1 cells expressing the soluble form of Pref-1/FA1, addition of IGF-1 to a concentration of 100 nM almost completely restored adipocyte differentiation, whereas the same concentration only partially restored differentiation of 3T3-L1 cells expressing the full-length Pref-1. Insulin at 100 nM was less efficient in restoring adipocyte differentiation of cells expressing either the full-length or the secreted form of Pref-1, with adipocyte differentiation being most efficiently suppressed in the cells expressing full-length Pref-1. It should be noted that insulin was used at a concentration of 167 nM in the standard MDI protocol. The same pattern was observed when differentiation was estimated from quantification of lipid accumulation using the GPO kit (Fig. 4B). Accumulation of cellular lipid was severely reduced in the 3T3-L1 cells that expressed the soluble form of Pref-1; in cells expressing full-length Pref-1, the reduction in lipid accumulation was even more pronounced. In parallel with the results obtained by Oil Red O staining, the use of the GPO-kit also revealed that accumulation of lipid was most effectively restored by the addition of IGF-1. Part of this increased accumulation can be ascribed to the greater number of differentiated cells in the IGF-1-treated cells. It is noteworthy that the percentage of differentiated 3T3-L1-mPrefSF cells was higher than that of 3T3-L1-mPrefFL cells at the same concentration of insulin or IGF-1 (Fig. 4A), even though Western blotting indicated that the level of Pref-1 in the medium of 3T3-L1-mPrefSF cells was higher than that in the medium of 3T3-L1-mPrefFL cells (Fig. 4C). This finding was corroborated by ELISA quantification, which demonstrated that the concentration of Pref-1/FA1 in the medium conditioned by cells expressing the full-length form of Pref-1 was 2 μg/ml, whereas medium conditioned by cells expressing the soluble form of Pref-1 contained 3 μg/ml (results not shown). Western blotting revealed that expression of PPARγ and the adipocyte marker gene aP2 was decreased in the 3T3-L1-mPrefSF and 3T3-L1-mPrefFL cells. Addition of IGF-1 or insulin restored expression of the marker genes in a dose-dependent manner paralleling the IGF-1/insulin-dependent rescue of adipocyte differentiation (Fig. 4C).

**Forced Expression of Pref-1/FA1 Diminishes the Level of the Mature Form of the IGF-1 Receptor and Attenuates IGF-1-dependent Signaling—**IGF-1 is essential for adipocyte differentiation (47). Given our observation that IGF-1 overcomes Pref-1-dependent inhibition of adipocyte differentiation, we asked whether Pref-1 expression altered IGF-1R expression. By using RT-PCR we detected no significant differences in the expression of IGF-1R mRNA in 3T3-L1-vector, 3T3-L1-mPrefSF, and 3T3-L1-mPrefFL cells (Fig. 5A). However, when protein extracts from 3T3-L1-vector, 3T3-L1-mPrefSF, and 3T3-L1-mPrefFL preadipocyte cells were analyzed by Western blotting using an antibody specific for the IGF-1R β-subunit, we found that the level of the mature IGF-1R β-subunit was markedly decreased in the 3T3-L1 cells overexpressing the full-length Pref-1.

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reduced in cells expressing full-length Pref-1, whereas a significant, but less pronounced repression was observed in 3T3-L1-mPrefSF cells (Fig. 5B). The reduction in IGF-1R β-subunit levels was about 60 and 40% in cells expressing the full-length and the soluble form of Pref-1, respectively (Fig. 5C).

The discrepancy between IGF-1R mRNA and protein indicates posttranscriptional regulation of expression possibly at the level of protein processing. Identical results were obtained in 3T3-F442A cells overexpressing the soluble form of Pref-1 or full-length Pref-1 (results not shown). In conclusion, our observation that Pref-1/FA1 overexpression down-regulates the number of mature IGF-1R molecules in preadipocytes suggests that forced expression of Pref-1/FA1, at least in part by reducing the number of functional receptors, might interfere with IGF-1-dependent signaling.

Because forced expression of Pref-1 led to a reduction in the number of mature IGF-1 receptors, we hypothesized that this would impair IGF-1-dependent downstream signaling. IGF-1-dependent signaling plays an important role in triggering the phase of mitotic clonal expansion during adipocyte differentiation. Consequently, we examined whether forced expression of either the soluble form of Pref-1 or full-length Pref-1 affected mitotic clonal expansion of 3T3-L1 cells. Indeed, forced expression of either the soluble form of Pref-1 or full-length Pref-1 reduced BrdUrd incorporation substantially in the absence of added IGF-1 indicating that clonal expansion was impaired, and addition of IGF-1 restored BrdUrd incorporation (Fig. 6).

IGF-1R-dependent signaling leads to activation of Akt/PKB and p42/p44 MAPK. Activation of Akt/PKB promotes adipocyte differentiation (48), whereas conflicting data on the role of p42/p44 MAPK in adipocyte differentiation have been presented. However, it appears that the initial phase of adipocyte differentiation, including clonal expansion, requires MAPK activity, whereas sustained MAPK activity blocks terminal differentiation (49–52). In addition, it was recently shown that transient early activation of the MEK/MAPK pathway promotes adipogenesis by increasing expression of PPARγ and C/EBPα (53). To examine how ectopic expression of Pref-1/FA1 affected IGF-1R downstream signaling, we compared activation of MAPK and Akt/PKB in 3T3-L1 cells with forced expression of either the soluble form or full-length Pref-1/FA1 in response to treatment with standard differentiation medium (DMEM, 10% FCS, Mix, and Dex) with or without 10 nM IGF-1 (Fig. 7). Treatment with medium containing IGF-1 led to a robust transient activation of p42/p44 MAPK and a more sustained activation of Akt/PKB in vector-transduced cells. In cells expressing either the soluble form or full-length Pref-1 activation of Akt/PKB proceeded unabated, whereas activation of p42/p44 MAPK was clearly diminished, but still significant. In cells not receiving IGF-1 no activation of Akt/PKB was observed at the time points examined. In vector-transduced cells, activation of p42/p44 MAPK still occurred, but this activation was almost completely abolished in cells expressing either the soluble form or full-length Pref-1. Of note, not only the levels of activated p42/p44 MAPK were reduced in Pref-1-overexpressing cells, also the duration of the activation period was reduced. We conclude that the level of IGF-1 (insulin) in FCS is insufficient to activate p42/p44 MAPK in cells overexpressing either the soluble form or full-length Pref-1. Supplementation with supraphysiological concentrations of insulin or IGF-1 overcomes this deficiency, allowing a transient activation of p42/p44 MAPKs restoring clonal expansion and differentiation.

**DISCUSSION**

Efficient Processing of Membrane-bound Pref-1 in 3T3-L1 Preadipocytes Leads to Accumulation of High Levels of the 50-kDa Ectodomain in the Medium—Substantial evidence has
accumulated that the membrane-bound and the soluble form of Pref-1/FA1 play important roles in controlling cellular growth and differentiation. In this study we show that a significant proportion of the membrane-bound Pref-1 in 3T3-L1 preadipocytes undergoes proteolytic processing resulting in the release of high levels of the soluble form of Pref-1/FA1 to the medium. The released Pref-1/FA1 is immunologically indistinguishable from the form found in amniotic fluid and exhibits the same heterogeneity on Western blotting. A comparison of the amounts of Pref-1/FA1 in cell lysates and medium indicated that the rate of processing increased when cells were maintained at confluency, whereas no significant changes in processing and release took place during the ensuing period of adipocyte differentiation.

The heterogeneity of Pref-1/FA1 proteins is generated by differential glycosylation (31, 46), alternative splicing (26), and heterogeneous processing at the C terminus (31, 46). By transfection into COS-7 cells, it was shown that the six mRNA splice variants only pref-1A and pref-1B gave rise to processed and released products of 50 kDa (36). This also led to the hypothesis that differential splicing during adipocyte differentiation might perturb the ratio between mRNAs giving rise to Pref-1/FA1 (here named dlk) isoforms capable of undergoing processing and mRNAs encoding proteins that remained membrane-bound (21). In addition, it was suggested that the membrane-bound form and the soluble form of Pref-1 played opposing roles in the control of adipocyte differentiation. However, data presented in this study and by Smas and co-workers (19, 38) do not support this hypothesis. Following ectopic expression of the pref-1A cDNA in COS-7 cells, processing of the ectodomain gave rise to additional soluble products with molecular masses between 24 and 31 kDa. We have been unable to detect similar products in conditioned medium from 3T3-L1 cells. This may be related to poor recognition of these processing products (38), or, alternatively, it may indicate that processing of Pref-1/FA1 exhibits cell type specificity.

It was originally reported that the differentiation-defective 3T3-C2 cell line exhibited a 3-fold increase in Pref-1 expression in comparison to 3T3-L1 cells, and it was suggested that this increased level of Pref-1 expression was related to the inability of 3T3-C2 cells to undergo adipocyte differentiation (17). Our findings do not substantiate this notion. We found that the levels of Pref-1/FA1 in medium conditioned by and in the lysates of our line of 3T3-C2 cells were only ~30% of those seen in 3T3-L1 cells, questioning whether Pref-1 expression is a decisive factor governing the lack of adipose conversion of 3T3-C2 cells.

**Pref-1FA1-dependent Inhibition of Adipocyte Differentiation Is Bypassed by Addition of IGF-1 or Insulin**—The initial observation that forced expression of Pref-1 prevented adipocyte differentiation of 3T3-L1 cells, established a role for Pref-1 as an inhibitor of adigenesis and led to the general concept that down-regulation of Pref-1 expression is a prerequisite for adipocyte differentiation (17, 38). In our experiment utilizing the widely used differentiation protocol based on treatment with Mix, Dex, and insulin (44), we were unable to observe any inhibition of adigenesis by forced expression of Pref-1. Similarly, treatment of 3T3-L1 cells with medium from Pref-1-overexpressing cells did not prevent adipocyte differentiation in response to addition of adigenic inducers (results not shown). In addition, we showed that overexpression of Pref-1/FA1 in 3T3-F442A cells was compatible with differentiation induced by combined treatment with T3 and insulin.

We noted that in the original experiments of Smas and Sul (17) differentiation was induced without insulin. Because insulin is known to enhance differentiation of 3T3-L1 cells in part by activating IGF-1R-dependent signaling, we speculated whether Pref-1 overexpression interfered with insulin/IGF-1 signaling during initiation of adipocyte differentiation. Indeed, when insulin was omitted from the differentiation mixture leaving only the IGF-1 present in the fetal calf serum available for initiation of adipocyte differentiation, overexpression of full-length Pref-1 or the soluble form of Pref-1/FA1 almost completely inhibited differentiation. Accordingly, we demonstrated that IGF-1 and insulin in a dose-dependent manner rescued adipocyte differentiation of 3T3-L1 cells overexpressing either forms of Pref-1/FA1, with IGF-1 being the most effective. Although the level of soluble Pref-1 in the medium was significantly higher for cells overexpressing the soluble form of Pref-1/FA1 than for cells overexpressing full-length Pref-1, a stronger inhibition was observed in cells overexpressing full-length Pref-1. This seems to be at variance with the recent findings of Sul and co-workers (38) showing that only the soluble form of Pref-1/FA1 was able to inhibit adipocyte differentiation. This conclusion was based on the inability of a mutant Pref-1 lacking 21 amino acid residues of the extracellular juxtamembrane region, including the major processing site to inhibit adipocyte differentiation. In contrast, our results indicate that forced expression of full-length Pref-1 more efficiently inhibits adipocyte differentiation than forced expression of the soluble form of Pref-1/FA1 even though the level of the soluble form of Pref-1 in the medium is lower for cells with forced expression of full-length Pref-1. The apparent discrepancy between our results and those of Sul and co-workers might be...
explained if subtle differences in processing would yield a more potent inhibitor of adipocyte differentiation when full-length Pref-1 is the substrate for processing rather than the truncated soluble version. Alternatively, deletion of the 21-amino acid region might abolish the functionality of the membrane-bound form of Pref-1 in processes involving protein-protein interactions with other membrane-bound or soluble factors. In a broader sense this is reminiscent of the Pref-1-mediated up-regulation of HES-1 expression in thymocytes that apparently required either membrane-bound or dimeric Pref-1 (28).

**Forced Expression of Pref-1/FA1 Impairs IGF-1/IGF-1R Signaling Pathways**—It is well established that IGF-1 is essential for initiation of adipocyte differentiation. The supraphysiologically concentrations of insulin used in the standard differentiation protocols mimics IGF-1 by interacting with the IGF-1R (47, 54). In preadipocytes, IGF-1 binds to the archetypical IGF-1R, whereas in adipocytes the IGF-1R/hybrid receptor prevails (55). In our experiments significant differentiation of Pref-1/FA1-expressing cells was restored using 10 nM IGF-1, prevails (55). In our experiments significant differentiation of Pref-1/FA1-expressing cells was restored using 10 nM IGF-1, and exogenous IGF-1 is required to initiate differentiation. In normal fetal calf serum used for differentiation, the concentration of IGF-1 is very low. Approximately 13,000 IGF-1 receptors are present per cell in 3T3-L1 preadipocytes (47). Interestingly, it was reported that 15,000–22,000 IGF-1 receptors per cell were needed to render mouse embryo fibroblasts responsive to IGF-1 (56). That the number of active receptors on the cell surface is critical in determining the downstream MAPK activation, and hence, the cell growth/differentiation has also been seen in other cell types (reviewed in Ref. 57). In our experiment, overexpression of Pref-1/FA1 in 3T3-L1 and 3T3-F442A cells resulted in a significant reduction in the level of the mature IGF-1R β-subunit. Thus, it is conceivable that a Pref-1/FA1-dependent reduction in the density of functional IGF-1Rs on the surface of preadipocytes would result in impaired IGF-1/IGF-1R signaling. In Balb/c 3T3 cells expressing either sense or antisense pref-1/dlk1, no effect of Pref-1/FA1 on IGF-1R levels was observed (58). The reason for this discrepancy remains to be established, but it may be cell line-dependent.

IGF-1/IGF-1R-mediated signaling regulates pathways leading to activation of the PI3K/Akt/PKB and Ras/Raf/MEK/ MAPK pathways. In 3T3-L1 cells, IGF-1/IGF-1R preferentially activates the Shc/Grb2/SOS-dependent pathway of MAPK activation, whereas insulin/IR activates the IRS/PI3K-dependent pathway leading to Akt/PKB activation (59). Our results indicate that forced expression of Pref-1 did not affect IGF-1-dependent Akt/PKB activation, whereas activation of p42/44 MAPK was strongly inhibited in the early stage of differentiation. Inclusion of IGF-1 in the differentiation mixture rescued the differentiation of Pref-1/FA1-overexpressing cells and restored p42/44 MAPK activation. These results suggest that the inhibition of differentiation by Pref-1 overexpression is associated with the down-regulation of MAPK activation. Our result complements the recent observation that down-regulation of Pref-1/dlk expression in Balb/c 3T3 cells enhanced IGF-1-dependent activation of p42/p44 MAPKs (58). The role of p42/p44 MAPKs in adipogenesis has been a matter of dispute. Anti-sense-mediated elimination of p42/p44 MAPK expression was reported to prevent insulin-induced adipocyte differentiation and DNA synthesis in 3T3-L1 cells (49), and similarly, terminal differentiation of 3T3-F442A induced by insulin/T/E 

**Acknowledgment**—We thank Dr. David A. Bernlohr for the anti-aP2 antibody.

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J. Biol. Chem. 2003, 278:20906-20914.
doi: 10.1074/jbc.M300022200 originally published online March 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300022200

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