Proprotein convertases are important mediators of the adipocyte differentiation of mouse 3T3-L1 cells

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Summary
Mouse 3T3-L1 cells are widely used to study adipocyte differentiation in vitro. When treated with insulin, dexamethasone and isobutylmethylxanthine these fibroblastic cells differentiate into round triglyceride-rich adipocytes. Because several proteins implicated in adipocyte differentiation (e.g. type 1 IGF receptors) are proteolytically activated by endoproteinases of the proprotein convertase family, we sought to determine whether these endoproteinases are crucial for adipocyte conversion. In this study, we show that expression of the proprotein convertases PACE4, PC7 and furin increases when 3T3-L1 cells are induced to differentiate into adipocytes. The differentiation was blocked in transfected cells expressing α1-antitrypsin Portland or in normal cells pre-treated with the synthetic inhibitor decanoyl-RVKR-chloromethylketone. Both inhibitors are known to specifically inactivate proprotein convertases. The block was associated with impaired proteolytic activation of proIGF-1 receptor, absence of induction of the adipogenic transcriptional factor PPARγ and marked reduction of the nuclear translocation of the C/EBPβ factor. Taken together, these data constitute evidence that proprotein convertases are crucial mediators of adipogenesis.

Key words: Gene expression regulation, Serine proteinases, Protease inhibitors, Transcription factors

Introduction
3T3-L1 cells were selected from Swiss 3T3 mouse fibroblasts for their ability to differentiate into adipocytes. Upon growth arrest in the presence of fetal bovine serum (FBS), they become rounded and accumulate cytoplasmic lipid vesicles made of newly biosynthesized triglycerides (Green and Kehinde, 1975). This differentiation is accelerated when the cells are transiently exposed to a cocktail of inducing agents including dexamethasone (Dex), isobutylmethylxanthine (IBMX) and insulin (Ins) (Rubin et al., 1978).

The determining role of transcriptional regulation in this conversion has been extensively studied. Two families of transcription factors, the CCAAT/enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptors (PPARs), play particularly important roles as mediators of adipogenic signals (Gregoire et al., 1998; Hwang et al., 1997). Among members of the C/EBP family, C/EBPβ and C/EBPδ regulate the early response to these signals. Their rapid and transient induction relays the effects of IBMX and Dex, and catalyzes the transactivation of C/EBPα and PPARγ genes (Cao et al., 1991; Yeh et al., 1995; Zhu et al., 1995). The latter factors, in turn, activate a variety of adipocyte-specific genes. Transduction of either factor in multipotent NIH-3T3 fibroblasts has been shown to promote their conversion into adipocytes (Fretyag et al., 1994; Tontonoz et al., 1994).

Adipocyte differentiation may also be determined by post-translational modifications of adipogenic proteins. Proteolytic activation of latent precursors is one such modification. Sterol regulatory element binding protein 1 (SREBP-1), otherwise known as adipocyte determination and differentiation factor 1 (ADD1), represents an example of an adipogenic protein that needs activation by proteases. This transcriptional factor is released into the nucleus after an endoplasmic-bound precursor located in the endoplasmic reticulum following two successive cleavages at distinct sites, the first by a pyrolysin-like convertase called site-1 protease (Sakai et al., 1998) or SKI-1 (Seidah et al., 1999), the second by a metalloproteinase known as site-2 protease (Brown and Goldstein, 1999). SREBP-1 regulates genes involved in the biosynthesis of cholesterol and fatty acids (Brown and Goldstein, 1999). Its downregulation has been shown to inhibit the differentiation of 3T3-L1 cells into adipocytes (Brun et al., 1996).

Receptors for insulin (InsR) and insulin-like growth factor 1 (IGF-1R) are other important mediators of adipocyte differentiation (Accili and Taylor, 1991; Smith et al., 1988) that are activated by limited proteolysis. These receptors are biosynthesized as inactive precursors and are rendered functional by a single cleavage into two chains (α and β) linked by disulphide bonds. Furin, the enzyme mediating this processing (Bravo et al., 1994; Lehmann et al., 1998) belongs to a family of serine proteinases known as proprotein convertases (PCs) (Seidah and Chretien, 1999; Zhou et al., 1999).

The PC family also includes PACE4, PC1/3, PC2, PC4, PC5/6 and PC7/8. Furin, PACE4, PC5 and PC7 are widely expressed. PC1 and PC2 are primarily found in endocrine and neuroendocrine cells. PC4 expression is mostly confined to the testis. PCs act in the secretory pathways where they cleave precursor proteins after selected pairs of basic residues. Their...
substances include precursors to hormones and neuropeptides, viral
glycoproteins and bacterial toxins (Seidah and Chretien, 1999; Zhou et al., 1999).

Of all the PCs, furin has the widest variety of proteins among
its substrates. This type I-membrane-bound enzyme cycles between the TGN and the surface of all cells (Molloy et al., 1994). It cleaves its substrates after an R-X-K/R-R (X represents any amino acid) motif. This motif is found at the processing site of proInsR and proIGF-IR as well as that of precursors to extracellular matrix components such as stromelysin-3, fibrillin, and membrane type I matrix metalloproteinase (Lönnqvist et al., 1998; Santavicca et al., 1996; Yana and Weiss, 2000). Furin cleavage of these precursors could thus be integral to mitogenic cell signaling and to plasma membrane remodeling. In this context, furin has been implicated in the growth and differentiation of gastric surface mucous cells and of cardiocytes (Konda et al., 1997; Sawada et al., 1997).

The importance of PCs in adipogenesis has not been examined before. In this report, we describe the regulation of several PC genes during adipocyte differentiation of 3T3-L1 cells. We also show that PC-specific inhibitors block this differentiation at an early stage, confirming the involvement of these proteinases for the process.

Materials and Methods
Cell culture and differentiation
3T3-L1 preadipocytes were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS. Two days after they reached confluence (experimental day 0), they were induced to differentiate with 10 μg/ml of Ins, 0.5 mM IBMX and 1 μM Dex. On day 2 and every other day afterwards, fresh medium was substituted until day 7. Alternatively, post-confluent cells were incubated in medium containing varying concentrations of the synthetic inhibitor decanoyl-RVKR-chloromethylketone (dec-RVKR-CMK), for 24 hours before and for the 48 hours of adipogenic stimulation. The N-α-p-Tosyl-L-Phe-CMK, H-Glu-Gly-Arg-CMK peptides (Bachem), and for the 48 hours of adipogenic stimulation. The N-RVKR-chloromethylketone (dec-RVKR-CMK), for 24 hours before.

Table 1. Primers used for RT-PCR amplification

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Strand*</th>
<th>Sequence (5′→3′)</th>
<th>PCR fragment length (bp)</th>
</tr>
</thead>
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<tr>
<td>PC1</td>
<td>S</td>
<td>GGATCGCTTGCAATGATCCAAATGTCG</td>
<td>398</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTCATGCAGCTCGTACATGACAA</td>
<td></td>
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<tr>
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<td>GAGACCCGCTTCCCAAGAATC</td>
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<td>S</td>
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<tr>
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<tr>
<td>PC7</td>
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<tr>
<td></td>
<td>AS</td>
<td>AAGGAGACTCCTCCCTCTCACA</td>
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</tr>
<tr>
<td>PACE4</td>
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<td>GCATAGAAGGAAATCACCACAG</td>
<td>462</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>TGGATCCCCATACAGGAGGACAG</td>
<td></td>
</tr>
<tr>
<td>Furin</td>
<td>S</td>
<td>TGGACCCATGGTATGGCTACAG</td>
<td>576</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GGACAGACTGTTTTCGTTTTGA</td>
<td></td>
</tr>
<tr>
<td>Adipsin</td>
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<td>170</td>
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<tr>
<td></td>
<td>AS</td>
<td>GGTTCACCTTCATTTTCTGTTCCG</td>
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<tr>
<td>PPARγ</td>
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<tr>
<td></td>
<td>AS</td>
<td>CACCCGCTTGTTGGCCGATG</td>
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</table>

*S, sense; AS, antisense.

Establishment of clonal lines of transfectant 3T3-L1 cells
The pcDNA3 expression vector (Invitrogen) and a derivative carrying a full-length cDNA for the PC inhibitor α1-antitrypsin Portland (α1-PDX) (Anderson et al., 1993; Benjannet et al., 1997; Dufour et al., 1998; Jean et al., 1998; Munzer et al., 1997; Tsuji et al., 1999a) were transfected into 3T3-L1 preadipocyte cells using the DOSPER Liposomal Transfection Reagent (Boehringer Mannheim). Independent G418-resistant clonal cell lines were established from clones picked from different transfection culture dishes.

Semi-quantitative RT-PCR
Total RNA was extracted using a guanidine isothiocyanate method previously described (Day et al., 1992). The RNA was treated with RNase-free DNasel (Life Technologies) and 2 μg were reverse-transcribed into cDNA using the SuperScript II Reverse Transcriptase (Life Technologies). Three microliters of the reverse transcriptase reaction were used for PCR amplification of cDNA fragments for PC1, PC2, PC5, PC7, PACE4, furin, adipsin, PPARγ and γ2 (collectively called PPARγ) α1-PDX or the ribosomal protein L30 as internal standard. The sequence of the PCR primers is shown in Table 1. The PCR reaction mixes contained 0.5 units of rTaq DNA polymerase (Life Technologies), 1× PCR buffer, 0.2 mM dNTP, 0.5 μM sense and antisense primers for a specific cDNA, and L30 in a 50 μl volume. PCR was performed with PC or α1-PDX primers for a total of 32-37 cycles, together with L30 primers for the last 20 cycles. For PPARγ and adipsin semi-quantification, the reaction was conducted for 25 cycles with their respective primers and L30 primers. The number of cycles was pre-determined to fall within the linear range of amplification of each PCR product. Each cycle involved a 94°C/1 minute denaturation step, a 60°C/1 minute annealing step, and a 72°C/1 minute polymerization step. The PCR products were electrophoresed on agarose gels, stained with ethidium bromide, revealed by UV irradiation and analyzed by densitometry using the National Institutes of Health Image software. The authenticity of the amplified sequences was verified by restriction enzyme mapping.

Western blot analysis
Cells were washed with PBS and scraped off dishes in 1 ml of PBS. They were sedimented by centrifugation, lysed by the addition of 100 μl of SDS-gel buffer, and heated to 100°C for 5 minutes (Cao et al., 1991). Extracted proteins were fractionated by 12% SDS-PAGE and electrophoresed on agarose gels, stained with ethidium bromide, revealed by UV irradiation and analyzed by densitometry using the National Institutes of Health Image software. The authenticity of the amplified sequences was verified by restriction enzyme mapping.
proteins were treated or not with 50 units of calf intestine alkaline phosphatase (CIAP) (Boehringer) for 1 hour at 37°C; they were then diluted in SDS-gel buffer, boiled for 5 minutes and analyzed by Western blotting for C/EBPβ proteins.

To examine proIGF-1R proteolytic processing, we used transfected control and α1-PDX preadipocytes as well as normal preadipocytes treated at confluency with 0 or 100 μM dec-RVKR-CMK for 48 hours. Whole-cell extracts in 60 mM Tris-HCl buffer, pH 7.5/1% SDS were prepared; aliquots corresponding to 100 μg of proteins were fractionated by 8% SDS-PAGE and analyzed by immunoblotting as described above using an antibody against the β subunit of IGF-1R (Santa Cruz Biotechnology).

IRS-1 phosphorylation was examined by sequential probing of a blot carrying 50 μg of proteins from control and α1-PDX cells, stimulated or not with insulin (10 μg/ml). Probing was conducted subsequently with an anti-IRS-1 antibody and, after membrane stripping using Re-Blot Plus-Mild (Chemicon), with an anti-phosphotyrosine antibody. Both antibodies were obtained from Santa Cruz Biotechnology.

Electrophoretic mobility shift assay (EMSA)

A 5′-[32P]-labeled double-stranded C/EBP consensus oligonucleotide (5′-GAT CGA TTT CGC AAT C-3′) (Osada et al., 1996) was used as probe. The binding mixture contained 10 μg of nuclear extract, 2 μg of poly dI-dC, 20 mM Hepes, pH 7.9, 4% Ficoll, 0.5 mM DTT, 1 mM MgCl2, 50 mM KCl and 20,000 cpm of labeled probe. It was incubated for 40 minutes at 4°C. In competition assays, the mixture was supplemented with a 100-fold molar excess of unlabeled oligonucleotide prior to adding the labeled probe. In supershift assays, nuclear extracts were incubated for 90 minutes with 1 μg of an antibody directed against the C-terminus of C/EBPβ prior to adding the labeled probe. The binding mixtures were electrophoresed at 100 V at room temperature in a 6% nondenaturing polyacrylamide gel containing 10% glycerol. The gel was then dried and subjected to autoradiography.

Immunohistochemistry

Confluent preadipocytes were induced to differentiate for 24 hours; they were then fixed with 50% ethanol/1% H2O2 and incubated for 90 minutes with the anti-C/EBPβ antibody (diluted 1:250). The immunoreaction was amplified using a horseradish peroxidase-based Tyramine Signal Amplification (TSA) Plus DNP kit (NEN Life Science Products), and revealed using the chromogenic substrate 3,3′ dianinobenzidine.

Results

Regulation of furin, PC7 and PACE4 during adipocyte differentiation

We first determined by semi-quantitative RT-PCR the relative levels of PC mRNAs in 3T3-L1 cells before and after their differentiation into adipocytes. This technique was chosen for its sensitivity, considering the low levels of some PCs in these cells. In both cell types, transcripts for PC7 were the most abundant, followed by those for furin and PACE4 (Fig. 1A). PC1 and PC2 transcripts were present at very low levels (not shown). PC5 mRNA transcripts were not detected. Expression of the testis-restricted PC4 was not examined. There were more transcripts for PC7, furin and PACE4 in adipocytes than in preadipocytes (Fig. 1A,B). The kinetics of this increase is shown in Fig. 1B. The starting level of PACE4 transcripts was very low, but it increased from day 3 to about tenfold the starting level on day 7 (P<0.001). PC7 mRNA increased gradually to threefold the starting level by day 7 (P<0.005). Furin transcripts, in contrast, sharply increased to 2.5-fold the starting level (P<0.005) during the 2-day inducing treatment, sharply diminished on day 3 and gradually increased again to twice the starting level by day 7 (P<0.005). The changes in the mRNA levels of the three PCs during adipocyte differentiation suggested that these enzymes play a role in this process.

For comparison, we also examined the relative levels of these transcripts in epididymal white adipose tissue (WAT) from C57Bl/6 mice. Compared with 3T3-L1 adipocytes, WAT contained more PACE4 transcripts, less PC7 transcripts and markedly less furin transcripts (Fig. 1A). These differences may be due to the fact that, unlike 3T3-L1 adipocytes, the WAT is made of a heterogeneous populations of cells (Smas and Sul, 1995) that may have distinct patterns of PC transcripts.

PC inhibitors block 3T3-L1 adipogenesis

To assess the importance of these PCs for adipogenic conversion, we produced clonal lines of 3T3-L1 transfectants carrying either the pcDNA3 vector (control cell lines) or a pcDNA3/α1-PDX for expression of the PC-specific inhibitor α1-PDX (α1-PDX cell lines). Because 3T3-L1 cells are notorious for spontaneously giving rise to differentiation-resistant cells, 3 control and 16 α1-PDX transfectant cell lines were established. These cell lines were induced to differentiate and stained with Oil Red O. A typical staining before and after adipogenic treatment is illustrated in Fig. 2A. All 3 control cell lines stained very strongly with Oil Red O, an indication that they accumulated substantial amounts of cytoplasmic

![Fig. 1. Levels of PACE4, PC7 and furin transcripts in 3T3-L1 cells during adipocyte conversion. (A) Total RNA extracted from 3T3-L1 preadipocytes and adipocytes was analyzed by semi-quantitative RT-PCR as described in Materials and Methods. RNA from epididymal WAT was also examined for comparison. (B) Ratios between PC and L30 densitometric values obtained by semi-quantitative RT-PCR were determined. They are presented as percent of maximal expression of each PC. The values represent means±s.e.m. of five independent experiments.](image-url)
triglycerides. By contrast, 14 out of the 16 cell lines derived from transfection with the \( \alpha_1 \)-PDX expression vector exhibited noticeably reduced staining with Oil Red O (Fig. 2A), indicative of low triglyceride content. By light microscopy, only 2-5\% of cells in these lines contained lipid vesicles typical of fully differentiated adipocytes (not shown). Transcripts for \( \alpha_1 \)-PDX were detectable by RT-PCR in the 14 lines. They were absent in control lines. A representative analysis of the PCR product is shown in Fig. 2B. All subsequent experiments involving transfected cells were conducted with the 3 control cell lines and 3 randomly selected \( \alpha_1 \)-PDX-positive cell lines. Transcripts were observed in \( \alpha_1 \)-PDX cells and not in control cells.

To further confirm the importance of PCs for adipocyte differentiation, we incubated untransfected post-confluent 3T3-L1 cells, for 24 hours before and for the 48 hours of adipogenic stimulation, in medium containing \( \alpha_1 \)-AT, \( \alpha_1 \)-PDX or dec-RVKR-CMK. They were then cultured for 5 days in normal medium and stained with Oil Red O on day 7 to assess conversion. (A) Staining was reduced in cells treated with 8 \( \mu \)M \( \alpha_1 \)-PDX, but not in those treated with \( \alpha_1 \)-AT. (B) Treatment with dec-RVKR-CMK reduced the staining in a concentration-dependent manner. The same results were obtained in four separate experiments. (C) By general cellular morphology, blockage of adipocyte conversion was partial at 20 \( \mu \)M dec-RVKR-CMK and complete at 100 \( \mu \)M.

Fig. 2. \( \alpha_1 \)-PDX expression inhibits 3T3-L1 adipocyte differentiation. (A) Three control and 16 \( \alpha_1 \)-PDX 3T3-L1 independent transfectant cell lines at confluence were treated (or not) with adipogenesis-inducing agents for 2 days. They were subsequently cultured for 5 days, fixed and stained with Oil Red O on day 7. A typical staining of differentiated and undifferentiated cells is shown. Unlike control cells, \( \alpha_1 \)-PDX transfectant cell lines failed to respond to the adipogenic treatment. (B) Expression of \( \alpha_1 \)-PDX transgene by RT-PCR in control and \( \alpha_1 \)-PDX transfectants cell lines. Transcripts were observed in \( \alpha_1 \)-PDX cells and not in control cells.

Fig. 3. Effect of exogenously added serine protease inhibitors on adipocyte conversion. Post-confluent 3T3-L1 cells were incubated for 24 hours before and for 48 hours during the adipogenic induction in medium containing \( \alpha_1 \)-AT, \( \alpha_1 \)-PDX or dec-RVKR-CMK. They were then cultured for 5 days in normal medium and stained with Oil Red O on day 7 to assess conversion. (A) Staining was reduced in cells treated with 8 \( \mu \)M \( \alpha_1 \)-PDX, but not in those treated with \( \alpha_1 \)-AT. (B) Treatment with dec-RVKR-CMK reduced the staining in a concentration-dependent manner. The same results were obtained in four separate experiments. (C) By general cellular morphology, blockage of adipocyte conversion was partial at 20 \( \mu \)M dec-RVKR-CMK and complete at 100 \( \mu \)M.
Altered expression of adipins, PPARγ and C/EBPβ in α1-PDX-expressing cells

To further characterize the phenotype of the α1-PDX-transduced cells, we examined by semi-quantitative RT-PCR the relative levels of PPARγ and adipins. The former is an early marker of adipogenic conversion and the latter a late marker of the adipocyte phenotype. Representative results are illustrated in Fig. 4. Expectedly, the levels of PPARγ and adipin mRNA transcripts markedly increased in control cells after adipogenic induction. By contrast, in α1-PDX-expressing cells, a similar treatment produced only a slight and belated induction in the level of adipin transcripts and none at all for PPARγ transcripts.

The gene for PPARγ is one of those activated by C/EBPβ in the cascade of transcriptional events leading to the adipogenic conversion of 3T3-L1 cells (Zhu et al., 1995). We therefore examined how C/EBPβ expression was affected by α1-PDX transduction. By western blot analysis, total extracts from control and α1-PDX-expressing cells contained comparable levels of the LIP (liver-enriched inhibitory protein) and LAP (liver-enriched activating protein) C/EBPβ isoforms (Fig. 5A). These levels transiently increased during the 2-day treatment with inducing agents, as expected (Cao et al., 1991). However, when nuclear extracts from these transfectant lines were analyzed by C/EBPβ-specific EMSA, the complexes observed with nuclear extracts from α1-PDX-expressing cells were of lesser intensity and of faster electrophoretic mobility than those observed with nuclear extracts from control cells (Fig. 5B, lane 1 versus lane 4). In both cases, the oligonucleotide electrophoretic shift was specifically due to C/EBPβ binding since the complexes could be retarded in the presence of an anti-C/EBPβ antibody (Fig. 5B, lanes 2,5) and abrogated in the presence of unlabeled competing oligonucleotide (Fig. 5B, lanes 3,6). These results suggested that, compared with control cells, α1-PDX-expressing cells contained less or less active C/EBPβ in their nuclei. We conducted a comparative western blot analysis of the nuclear extracts to verify these possibilities. Nuclear extracts from control cells contained two LAP immunoreactive bands of nearly equal intensities; those from α1-PDX-expressing cells contained noticeably less of both isoforms, and even less of the slower-migrating one (Fig. 5C, lanes 1,2). The latter represented a phosphorylated form of the faster-migrating isof orm, as shown by its disappearance in CIAP-treated nuclear extracts (Fig. 5C, lanes 3,4).

Reduced translocation of C/EBPβ into the nucleus of stimulated α1-PDX-expressing cells was further confirmed by immunohistochemistry. The results are shown in Fig. 5D. After a 24-hour stimulation with the adipogenic agents, C/EBPβ immunoreactivity in control cells was concentrated in the nucleus (Fig. 5Db), it was mostly perinuclear in the α1-PDX-expressing cells (Fig. 5Dd). The specificity of the immunoreaction was ascertained by omitting the anti-C/EBPβ antibody in the protocol (Fig. 5Da,c).

PC inhibitors block the processing of proIGF-1 receptors and mitotic clonal expansion

The observed blockage by PC inhibitors of the transcriptional cascade leading to adipocyte differentiation was most probably due to a failure in the signaling pathways normally induced by adipogenic treatment. The insulin and IGF-1 signaling pathway is one that is likely to be affected by the inhibitor since the latter may reduce activation of proIGF-1R by furin (Lehmann et al., 1998). To verify that the proIGF–1R processing is abolished by PC inhibition, 3T3-L1 confluent preadipocytes were treated or not with dec-RVKR-CMK and analyzed for proIGF–1R processing by immunoblotting. The results are shown in Fig. 6A. In the absence of the inhibitor, there was complete proIGF–1R processing since only the mature IGF-1Rβ chain was detected (Fig. 6A, lane 1). In dec-RVKR-CMK-treated cells, by contrast, proIGF–1R was much more abundant than the mature IGF-1Rβ chain (Fig. 6A, lanes 2,3,4). This observation confirms the role of PCs in proIGF–1R processing in 3T3-L1 cells. It also suggests that the block of adipocyte differentiation may be due to inhibition of proIGF–1R processing.

To confirm that the IGF–1R pathway was affected, we analyzed IRS–1 phosphorylation in control and α1-PDX cells (Fig. 6B). This analysis was conducted by sequential probing of a blot carrying proteins from control and α1-PDX cells, stimulated or not with insulin. The insulin concentration used was high enough to activate type 1 IGF–1R. The first probing conducted with an anti-IRS–1 antibody revealed an IRS–1 band in both control and α1-PDX cells. The second probing conducted with an anti-phosphotyrosine antibody revealed a band overlapping that of IRS–1 only in insulin-stimulated control cells.

Early following differentiation induction, 3T3-L1 preadipocytes undergo ~2 rounds of mitotic clonal expansion as they express the early adipogenic genes (Tang and Lane,
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It was recently demonstrated that mitotic clonal expansion was induced only by insulin and not by IBMX or Dex (Qiu et al., 2001). We therefore examined whether this event was altered in \( \alpha_1 \)-PDX cells. To do so, we induced post-confluent control and \( \alpha_1 \)-PDX cells and, three days later, we determined their number. For control cells, the number increased almost fourfold, whereas it did not significantly change in \( \alpha_1 \)-PDX cells (Fig. 6C), indicating that \( \alpha_1 \)-PDX expression affected the mitotic clonal expansion step.

### Discussion

The possibility of a physiological link between endoproteinases and adipogenesis has been of increased scientific interest in recent years following the observation that AIDS patients treated with aspartyl protease inhibitors develop a lipodystrophy syndrome (Carr, 2000; Dowell et al., 2000; Nguyen et al., 2000; Caron et al., 2001). Our laboratory studies the role of another class of endoproteinases, namely the PC family of serine proteases, in adipocyte differentiation. In this report, we show that expression of PACE4, PC7 and furin during adipocyte conversion of 3T3-L1 cells increases in distinctive fashions. The mRNA level of PC7 increases in a nearly linear fashion; that of PACE4 begins after the 2-day treatment with inducing agents, and that of furin transcripts was maximal during the treatment. Interestingly, the levels of furin transcripts change in parallel with that of C/EBP\( \beta \). Three different promoters, P1, P1A, and P1B, drive transcription of the furin gene (Ayoubi et al., 1994). The P1 promoter carries a C/EBP binding element that can be activated by transduced
C/EBPβ (Ayoubi et al., 1994). Activation of this promoter may explain the observed correlation in the levels of furin transcripts and immunoreactive C/EBPβ during adipogenic stimulation. The increase in PACc4 mRNA may be due to SREBP-induced activation of its gene since its promoter contains a functional E box (Tsuji et al., 1999b). SREBP-1, which is known to be induced in 3T3-L1 cells after adipogenic stimulation (Kim and Spiegelman, 1996), may activate the PACc4 gene through this element. There is a PPAR binding site in the PC7 gene promoter (F. Sirois and M. Mbikay, unpublished). Thus the increase of PC7 transcripts may result from the activation of its gene by PPAR following its induction during adipogenic conversion.

To assess the importance of PCs in the adipocyte differentiation of 3T3-L1 cells, we produced transfectant cell lines expressing the PC inhibitor α1-PDX. The majority (14/16) of these cell lines failed to convert into adipocytes when subjected to an adipogenic treatment. The two lines that were able to convert were not characterized. They may have derived either from spontaneously G418-resistant cells, from cells that had incorporated a fragment of the expression vector containing the resistance gene but not the α1-PDX transgene, or from cells that had incorporated the full vector but in a transcription-repressing genomic environment. Endogenous expression of α1-PDX did not cause any change in cell growth or in the levels of apoptosis markers (not shown). The fact that addition of recombinant α1-PDX or the synthetic PC inhibitor dec-RVKR-CMK to the culture medium of a native population of 3T3-L1 cells induced a similar differentiation block is a strong indication that the observations made with α1-PDX transfectant lines were due to the action of this inhibitor and not caused by some clonal variation among 3T3-L1 cells. Other peptidyl CMKs such as N-α-p-Tosyl-L-Phe-CMK and H-Glu-Gly-Arg-CMK had no inhibiting effect on adipocyte differentiation when added in the medium (not shown).

The differentiation block was associated with absence of PPARγ induction. These factors regulate a variety of genes for proteins involved in lipid metabolism (Tontonoz et al., 1994). PPAR gene activation is mediated by members of the C/EBP (Wu et al., 1996) and SREBP (Fajas et al., 1999) families. The PPARγ gene promoter carries two functional C/EBP binding sites (Zhu et al., 1995) and two E-boxes (Fajas et al., 1999). Adipogenic stimuli induce as much C/EBPβ in α1-PDX-expressing cells as in control cells. However, the amount of C/EBPβ LAP isoform, particularly its phosphorylated isoform, is dramatically reduced in the nucleus. Moreover, nuclear C/EBPβ from α1-PDX-transduced cells, together with a consensus C/EBP oligonucleotide, forms a complex of faster electrophoretic mobility on EMSA. The abnormal EMSA and western blot banding patterns of nuclear C/EBPβ from α1-PDX-expressing cells may be due to inefficient post-translational modification of the factor. Thus, nearly complete failure of C/EBPβ translocation into the nucleus and abnormal interactions with its binding sites in the PPARγ promoter may explain why this gene is not activated by adipogenic signals in α1-PDX-expressing cells. C/EBPβ is an early response factor in the adipogenic signaling pathways as indicated by its rapid increase following adipogenic stimulation. This regulation is reportedly mediated by cAMP in response to IBMX (Yeh et al., 1995). It has been reported that C/EBPβ can be phosphorylated at multiple sites and that, depending on the phosphorylated site, its DNA-binding activity either increases or decreases (Trautwein et al., 1993; Trautwein et al., 1994; Piwien-Pilipuk et al., 2001). Moreover, it appears that

Fig. 6. Effect of PC inhibitors on IGF-1 receptor pathway. (A) Confluent 3T3-L1 preadipocyte cells treated or not with dec-RVKR-CMK (100 μM) for 48 hours (lanes 1,2) as well as control and α1-PDX cells (lanes 3,4) were analyzed by immunoblot for proIGF-1R processing. The arrowhead points to an unknown immuno-crossreactive protein that was unaffected by the inhibitor. (B) IRS-1 phosphorylation was analyzed by sequential probing of a blot carrying proteins from control and α1-PDX cells, stimulated or not with insulin (10 μg/ml). The first probing was conducted with an anti-IRS-1 antibody and the second probing was conducted with an anti-phosphotyrosine antibody. (C) Effect on mitotic clonal expansion. Control and α1-PDX cells were induced or not to differentiate. After 3 days, cells from 6-well culture dishes were trypsinized and cell number was determined by counting. Data shown are the means±s.e. Results are representative of three independent experiments with three individual clones of each cell lines.
acquisition of binding activity during mitotic clonal expansion involves phosphorylation of C/EBPβ (Tang and Lane, 1999). Our results do not imply that lack of C/EBPβ translocation into the nucleus is due to an abnormal phosphorylation. They simply suggest that α1-PDX prevents PC activation of proproteins involved in the signaling pathways leading to C/EBP activation and nuclear translocation. The identity of these precursors remains to be determined.

A signaling pathway that is most certainly affected by this inhibitor involves the InsR/IGF-1R and the mitogen-activated protein kinase pathway (Boney et al., 1998). Both receptors are proven furin substrates (Bravo et al., 1994; Lehmann et al., 1998). Both are known to be crucial for the proliferation and adipocyte conversion of 3T3-L1 cells (Accili and Taylor, 1991; Smith et al., 1988). In this study, we have shown that α1-PDX as well as a synthetic inhibitor of PCs can block proIGF-1R proteolytic maturation to IGF-1R in preadipocyte cells. Unprocessed receptors would not efficiently bind IGF-1 or insulin and would thus be unable to initiate the downstream signaling normally observed with their processed forms. Signal transduction by these peptides leads to phosphorylation of insulin-receptor substrate 1 (IRS-1) (Myers et al., 1994). We show in this study that this phosphorylation does not occur in insulin-stimulated α1-PDX-expressing 3T3-L1 cells. We also show the mitotic clonal expansion step, which depends on insulin induction (Qiu et al., 2001), is inhibited in α1-PDX cells.

For optimal inhibitory effect, the synthetic inhibitor dec-RVKR-CMK must be added to post-confluent cells 24 hours before and 48 hours during adipogenic treatment. This timing is required presumably to reduce the amounts of pre-existing active PCs and PC-processed adipogenic products, such as IGF-1R, and to prevent activation of those induced during adipogenesis. The differentiation block is not reversed by removal of the inhibitor, suggesting that active PCs are most crucial in the early steps of adipocyte conversion.

Which, among furin, PACE4 and PC7, is the most critical enzyme for the adipocyte differentiation of 3T3-L1 cannot be determined solely from the use of the dec-RVKR-CMK synthetic inhibitor, as the latter inhibits all three convertases indiscriminately. However, there are several reasons to believe that furin is the determining enzyme in this process. First, it is the primary maturation enzyme for several adipocyte signaling molecules including IGF-1R, InsR and the low density lipoprotein receptor-related protein (Bravo et al., 1994; Ko et al., 1998; Lehmann et al., 1998). Second, its rapid induction early during adipogenic stimulation, in the same time window as when blockage of differentiation by the synthetic PC inhibitor is most effective, suggests that it is needed to promote the processing of these and other precursor proteins. Third, of the major three PCs found in 3T3-L1 preadipocytes, PACE4 is barely detectable at the crucial early steps of induction and PC7, which is more readily detected in these cells, is poorly inhibitable by α1-PDX compared with furin (Benjannet et al., 1997; Jean et al., 1998). Finally, furin has been implicated in the regulation of growth and differentiation of other cell types including gastric surface mucous cells and cardiomyocytes (Konda et al., 1997; Sawada et al., 1997). Moreover, it is interesting to note that the furin gene maps in a mouse chromosome 7 region that has been shown by quantitative trait linkage analysis to affect adiposity in a dominant fashion (Taylor and Phillips, 1996). As for PACE4 and PC7, their expression pattern would be compatible with yet unknown roles late in adipocyte differentiation.

To summarize, we have presented evidence that PCs play an important role in the adipocyte differentiation of 3T3-L1 cells. We have shown that blockage of adipose conversion with PC-specific inhibitors is associated with a dramatic reduction of the nuclear translocation of the C/EBPβ factor.

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References


PC inhibitors block 3T3-L1 adipogenesis


