

Proteomic analysis of adipocyte differentiation: Evidence that $\alpha 2$ macroglobulin is involved in the adipose conversion of 3T3 L1 preadipocytes

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Adipogenesis is an important aspect of energy homeostasis. Here we have used a differential proteome mapping strategy to identify intracellular proteins that are differentially expressed during adipose conversion of 3T3 L1 preadipocytes. Two-dimensional gel electrophoresis analysis identified 8 proteins that are induced following hormone-evoked differentiation. In addition, we found that a $\alpha 2$ macroglobulin fragment was abundantly present in 3T3 L1 preadipocytes, but was virtually undetectable in fully differentiated adipocytes. Metabolic radiolabeling with (³⁵S)methionine and Northern blot analysis indicated that the intracellular $\alpha 2$ macroglobulin fragment in preadipocytes was derived from the extracellular culture medium, not *de novo* synthesis. Incubation of preadipocytes with an anti- $\alpha 2$ macroglobulin polyclonal antibody caused depletion of the intracellular $\alpha 2$ macroglobulin fragments, and also enhanced spontaneous adipose conversion. These results suggest that intracellular $\alpha 2$ macroglobulin fragment inhibits adipocyte differentiation, and that hormone treatment induces differentiation at least in part by suppression of intracellular $\alpha 2$ macroglobulin activity in 3T3 L1 preadipocytes.

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1 Introduction

The prevalence of obesity has risen dramatically over the past decade in many industrialized countries. Obesity is a major hazard primarily because it significantly increases the risk of type 2 diabetes and cardiovascular disease [1]. The major mechanism for this linkage is the ability of obesity to engender insulin resistance, which is characterized by diminished insulin sensitivity of target tissues including liver, skeletal muscle and adipocytes. Insulin resistance leads not only to type 2 diabetes, but also to a cluster of metabolic syndromes, including hypertension, dyslipidemia and atherosclerosis [2, 3]. Adipose tissue is an im-

portant endocrine organ that secretes a variety of biologically active molecules (adipokines), such as leptin, adiponectin, resistin and tumour necrosis factor α [4, 5]. A recent proteomics-based study for secreted proteins from adipocytes has also identified many previously uncharacterized adipokines produced from this cell line [6]. These adipokines play an important role in regulating energy metabolism, cardiovascular tone and immune responses. Dysregulated production of these adipokines from adipocytes is now proposed to be one of the major factors that link obesity with its related metabolic syndromes [7, 8].

Obesity is characterized by excess intra-abdominal adipose tissue, which is now recognized to develop through a combination of increases in both cell number (hyperplasia) and cell size (hypertrophy) [9]. Although the emphasis of earlier obesity research has been on the regulation of intracellular triacylglycerol stores in pre-existing adipocytes, it is now becoming clear that new adipocyte formation (adipogenesis) is also a component of adipose tissue enlargement [10]. The mechanisms of adipogenesis have

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Abbreviations: C/EBP, CCAAT/enhancer binding protein; PPAR γ , peroxisome proliferator-activated receptor γ

been extensively studied in preadipocyte culture systems including 3T3 L1 cells [11]. When cultured in defined media, 3T3 L1 cells deposit triglyceride in cytoplasmic lipid droplets and express genes that are also expressed in adipocytes *in vivo*. Genetically based approaches have led to the identification of key transcription factors in the complex transcriptional cascade that is activated during this process. These factors include peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer binding protein (C/EBP), and adipocyte differentiation and determination factor 1-sterol regulatory element binding protein 1c [12, 13]. Sequential activation of these transcription factors induces expression of adipocyte-specific genes, including enzymes, structural proteins, hormone receptors and a variety of secretory factors involved in the regulation of energy homeostasis.

Despite this promising progress, the detailed molecular events that occur during adipogenesis remain to be fully understood. A recent microarray-based study [11] has identified thousands of genes that are differentially expressed at different stages of adipogenesis, suggesting that the cellular programs associated with adipocyte differentiation are considerably more complex than previously thought, and that a great number of uncharacterized regulatory events are involved in this process. In particular, there is also evidence indicating that post-transcriptional events might also play an essential role in this process [14]. In the present study, we have used a proteomics-based approach to identify cellular proteins specifically involved in the conversion of 3T3 L1 preadipocytes into adipocytes. We identified eight differentially expressed/modified proteins, of which some had not previously been found to be associated with adipogenesis. Notably, we found that a fragment of α 2 macroglobulin, a general protease inhibitor predominantly secreted from liver tissue [15], accumulated with high abundance in 3T3 L1 preadipocytes as well as in preadipocytes isolated from mouse peri-uterine fat pads. The accumulation of this α 2 macroglobulin fragment was dramatically decreased to a virtually undetectable level at the early stage (day 2) of differentiation. Incubation of 3T3 L1 preadipocytes with an anti- α 2 macroglobulin polyclonal antibody induced the spontaneous differentiation of the cells, suggesting that this α 2 macroglobulin fragment plays an inhibitory role during adipogenesis.

2 Materials and methods

2.1 Reagents

Goat antihuman α 2 macroglobulin antiserum, goat non-immune serum, dexamethasone and isobutylmethylxanthine (IBMX) were purchased from Sigma (St. Louise,

MO, USA). Insulin, high glucose DMEM and fetal calf serum were from Invitrogen (Carlsbad, CA, USA). All the consumables for 2-DE were products of Amersham Biosciences (Uppsala, Sweden). Type I collagenase was from Worthington (Freehold, NJ, USA). (35 S)Trans-label (85% methionine and 15% cysteine) was from ICN (Irvine, CA, USA). Trizol reagent and random labeling kit were purchased from Invitrogen.

2.2 3T3 L1 cell culture, differentiation induction and metabolic radiolabeling

3T3 L1 cells were maintained in subconfluent cultures in high glucose DMEM supplemented with 10% fetal calf serum. Differentiation of postconfluent cells was initiated by incubation with 0.25 μ M dexamethasone, 0.5 mM IBMX and 10 μ g/mL insulin for 48 h, as previously described [16]. This was followed by incubation with 10 μ g/mL insulin for 48 h. The cells were then maintained in the complete medium for another 96 h. Oil red O staining for lipid droplets demonstrated that over 90% of cells exhibited typical morphology of adipocytes. For metabolic radiolabeling, 3T3 L1 preadipocytes or adipocytes were starved in DMEM without methionine and cysteine for 1 h, and then incubated with 50 μ Ci of (35 S)labeled methionine and cysteine *per* mL for a further 8 h. The radiolabeled cells were then harvested for further 2-DE analysis.

2.3 Fractionation of adipose tissue

Adipose tissue was separated into stromal and adipocyte fractions as previously described [11]. Briefly, freshly excised peri-uterine fat pads from 8-week old, female FVB/N mice were rinsed in saline, minced, and digested for 1 h at 37°C in Krebs-Ringer bicarbonate (pH 7.4) with 4% w/v BSA and 1.5 mg/mL type I collagenase. The digested tissue was filtered through a 200 μ m nylon mesh to remove undigested tissue and centrifuged at 500 \times g for 5 min. The floating adipocyte fraction was removed, washed in buffer, and recentrifuged to isolate free adipocytes. The stromal-vascular pellet was resuspended in erythrocyte lysis buffer (154 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA), filtered through a 30 μ m nylon mesh to remove endothelial cells, and centrifuged at 500 \times g for 5 min. The adipocytes and stromal-vascular cells were then used for 2-DE analysis.

2.4 2-DE

Preparation of cell lysates, protein quantification and 2-DE analysis were performed as described previously [17, 18], with the following alterations. To improve sample

solubility and avoid protein precipitation, 2 M thiourea was included in the lysis buffer and samples were loaded by cup into the first dimensional strip during first dimensional electrophoresis. Immobiline Drystrip 4–7 linear gels were used throughout. After electrophoresis, proteins were visualized by silver or CBB R250 staining, or by autoradiography. To identify differentially expressed proteins during adipose conversion, four pieces of silver-stained 2-D gels from either 3T3 L1 preadipocytes or adipocytes were digitized at 300 × 300 pixels/cm using a sharp JX-325 scanner (Sharp Electronics, Osaka, Japan) and a green/blue filter. The gel features were detected, quantified and matched using PDQUEST software (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The proteins whose expression levels were altered by at least 3-fold were chosen for further analysis.

2.5 RP-HPLC and N-terminal amino acid sequencing

To characterize proteins of interest, the relevant spots were excised from CBB-stained gels. Gel pieces were pooled and subjected to in-gel trypsin digestion. The tryptic peptide mixtures were separated by RP HPLC on a Jupiter 5 μ C₁₈ column (Phenomenex, Torrance, CA, USA), as described previously [19]. Only well resolved peaks from each sample were subjected to N-terminal amino acid sequencing using the Edman degradation method with a Perkin Elmer (Procise, Model 492) protein sequencer (Perkin Elmer, Norwalk, CT, USA).

2.6 MALDI-TOF MS analysis

MALDI-TOF analysis was conducted as previously described [16]. Briefly, 0.5 μ L of tryptic peptide mixtures were mixed with an equal amount of CHCA matrix (10 mg/mL in 60% ACN/0.3%TFA), spotted onto the sample plates and air-dried. Reflectron mass spectrometric analyses were performed on a Voyager DE PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) using a pulsed laser beam (nitrogen laser, $\lambda = 337$ nm). All ion spectra were recorded in the positive mode with an accelerating voltage of 20 kV. The spectrometer was externally calibrated using Cal Mix 2 standard mixture. Acquired spectra were searched against databases such as SWISS-PROT, TrEmbl or EST databases.

2.7 Northern and Western blot analysis

Total RNA from 3T3-L1 cells was purified using Trizol reagent. Total RNA from each sample (20 μ g) was separated on 1.2% denaturing gels, transferred onto nylon

membrane, and probed with (³²P)-labeled cDNA fragments encoding C/EBP α , PPAR γ , adiponectin or GLUT4 [4, 12, 13, 16]. The relative mRNA abundance of each gene was quantified using phosphorimaging. For Western blot analysis, 20 μ g of cellular proteins from 3T3 L1 fibroblasts or 15 μ L of culture media were separated by 7.5% or 15% SDS-PAGE, transferred to nitrocellulose membrane, and probed with rabbit anti α 2 macroglobulin antibody at 10 μ g/mL. The proteins immuno-reactive to the primary antibody were visualized by ECL detection, and quantified using ImageMaster software (Amersham Pharmacia).

3 Results

3.1 Identification of differentially expressed proteins during adipocyte conversion of 3T3 L1 cells

Figure 1 shows a representative 2-D pattern of 3T3 L1 preadipocytes and adipocytes (day 8 after differentiation). This analysis identified eight proteins (spots 1 to 8) that are reproducibly up-regulated by at least five-fold following differentiation. Three of them (spots 1 to 3) are exclusively expressed in adipocytes and are virtually undetectable in preadipocytes. Seven of the up-regulated proteins were unequivocally identified on the basis of amino acid sequence data obtained from tryptic peptides eluted from the relevant gel spots (Table 1). These include secretory proteins, protease inhibitors and proteins involved in fatty acid metabolism. Some of these proteins, such as fatty acid binding protein, malate dehydrogenase and adiponectin have previously been linked with either adipogenesis or adipocyte metabolism [10, 11]. We also found one protein (spot 9, *M*_r 20 kDa and *pI* 5.4) that was abundantly

Table 1. Identification of differentially expressed cellular proteins during adipose conversion of 3T3-L1 cells

Spot no. ^{a)}	Amino acid sequence	Accession no.	Protein no. ^{b)}
1	FDETTAD	Swissprot: p55053	Fatty acid binding protein
2	ASGEPWL	Swissprot: p08228	Superoxide dismutase
3	TWILHH	Swissprot: p56395	Mouse cytochrome B5
4	LSLDDFK	Swissnew: p20108	Mitochondrial periredoxin 3
5	FVEGLPIN	Swissnew: p40925	Malate dehydrogenase
6	DPEGQPG	Swissnew: p18242	Cathepsin D
7	AVLFTYDQ	Swissnew: p60994	Adiponectin

- a) Numbers refer to the protein spots identified in Fig. 1
 b) Amino acid sequences were matched to entries (<http://www.expasy.hcuge.ch>)

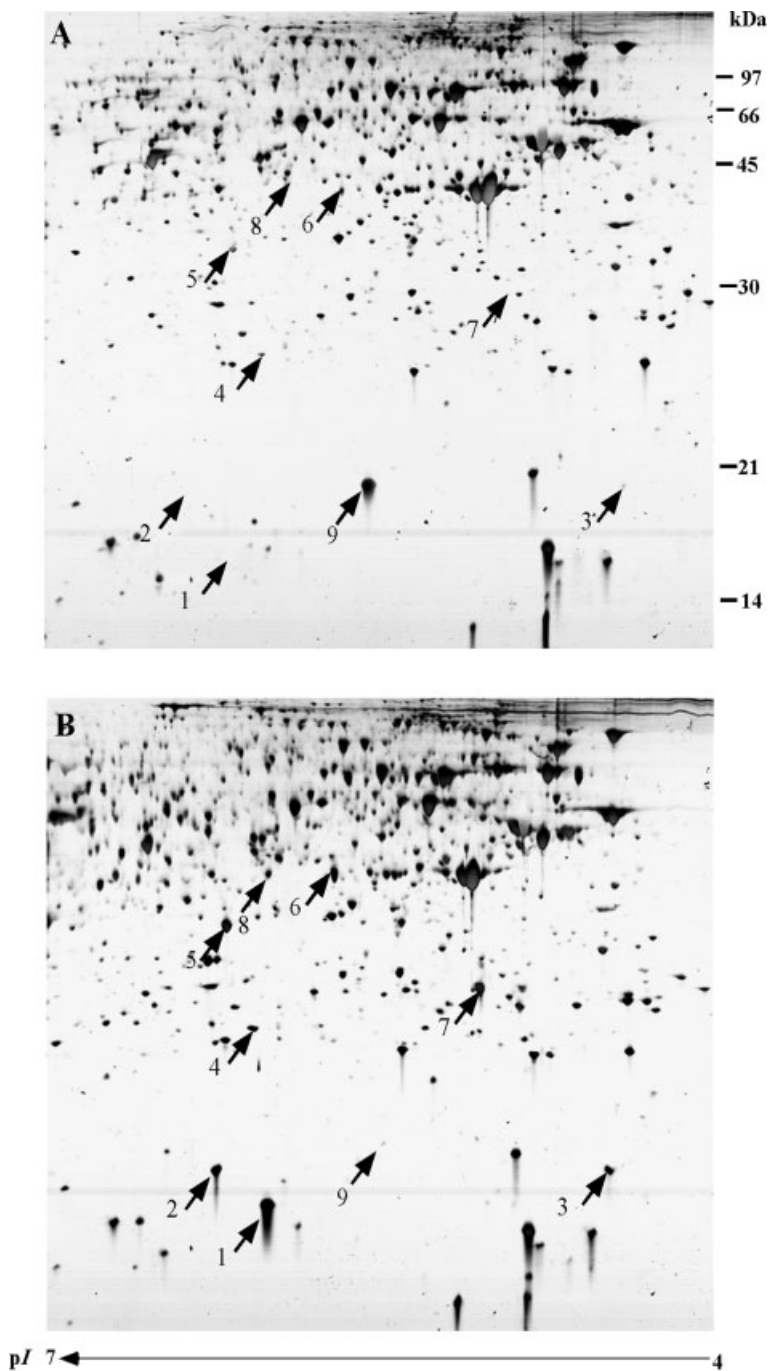


Figure 1. Representative 2-D protein profiles of 3T3 L1 preadipocytes and adipocytes. Proteins (60 μ g) from subconfluent 3T3 L1 preadipocytes (A) or adipocytes 8 days after differentiation (B) were separated by 2-DE and stained with silver. The positions of up-regulated or down-regulated proteins are denoted by numbered arrows. The gels shown are typical of three independent experiments.

expressed in preadipocytes, but was decreased dramatically to an undetectable level following differentiation (Fig. 1). Amino acid sequencing of several tryptic peptides (Table 2) revealed that this protein shares high sequence homology with human α 2 macroglobulin. Among 110 amino acid residues sequenced (Table 2), 87 were identical to those of human α 2 macroglobulin and 63 were identical to those of mouse α 2 macroglobulin.

3.2 α 2 Macroglobulin in preadipocytes was derived from extracellular culture medium, not from *de novo* synthesis

In order to ascertain the origin of this α 2 macroglobulin fragment, 3T3 L1 preadipocytes were radiolabeled with (35 S)methionine and cysteine, and the radiolabeled proteins were separated by 2-DE. Although the amino acid

Table 2. Amino acid sequences of the tryptic peptides derived from protein spot 9

RP-HPLC peptide no. ^{a)}	Amino acid sequence
1	VVQK
2	VTMNMERR
3	FEVQVR
4	NEESLVFV
5	IAQWQNLEVE
6	IAQWQNLEVENGLQQLTFPLSSEP
7	IITILEEVQVSVEGLYTYGKP
8	IVLLDESFHPLNELVPLVYVEDDL
NH ₂ -terminus	NEESLVFVQTD

a) Numbers refer to RP-HPLC fractions collected sequentially for internal amino acid sequencing

sequence of this protein contains at least two methionine residues (Table 2), autoradiographic analysis (Fig. 2) revealed that the spot corresponding to the $\alpha 2$ macroglobulin fragment was not labeled with ³⁵S even after extensive exposure (4 weeks). Given that $\alpha 2$ macroglobulin is a highly abundant circulating protein, we speculate that the intracellular $\alpha 2$ macroglobulin might be of bovine origin derived from the cell culture medium that includes 10% fetal bovine serum. This hypothesis was further confirmed by the fact that the protein in 3T3 L1 preadipocytes decreased dramatically following serum starvation for 3 days (data not shown). To investigate whether decreased levels of the $\alpha 2$ macroglobulin fragments also occur *in vivo* during adipocyte conversion, mouse preadipocytes (stromal vascular cells) and adipocytes were isolated from peri-uterine fat pads, and equal amount of proteins from these cells were separated by 2-DE. This analysis detected a protein of ~ 20 kDa which was present exclusively in stromal vascular cells, but was absent from mature adipocytes (Fig. 3). *N*-terminal sequencing of this protein spot yielded the sequence NKESVVFVQTDK, which exactly matches amino acid residues 135 and 146 of mouse $\alpha 2$ macroglobulin (SWISS-PROT: P28665). Northern blot and RT PCR analysis revealed that the gene encoding mouse $\alpha 2$ macroglobulin was not expressed in stromal vascular cells, although it can easily be detected in mouse liver tissue (data not shown). This result indicates that stromal vascular cells can take up extracellular $\alpha 2$ macroglobulin fragment probably from plasma, and that this uptake event was blocked during their conversion to adipocytes *in vivo*.

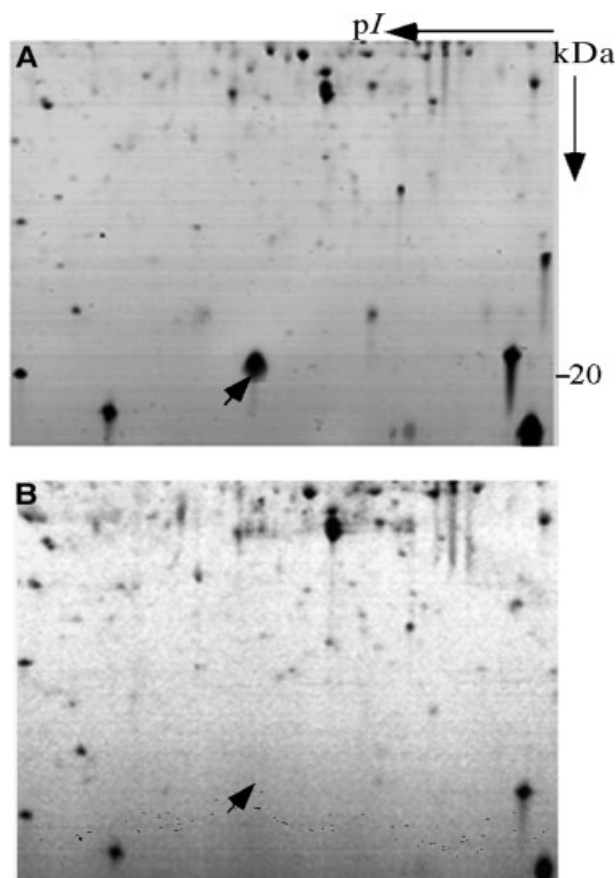


Figure 2. 2-DE protein pattern illustrating metabolically labeled 3T3 L1 preadipocytes. Subconfluent 3T3 L1 preadipocytes were radiolabeled with (³⁵S)methionine and cysteine as described in Section 2.2. The labelled proteins were separated by 2-DE. The proteins were visualized by either silver-staining (A) or autoradiography (B). Note that the spot indicated with an arrow was not labelled with (³⁵S)methionine, although the adjacent spots were.

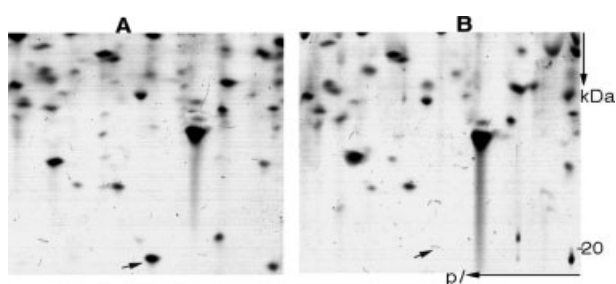


Figure 3. Murine $\alpha 2$ macroglobulin fragment is present in mouse preadipocytes but not in adipocytes. Mouse preadipocytes (stromal vascular cells) and adipocytes were isolated from peri-uterine fat pads as described in Section 2.3. Proteins (60 μ g) from either preadipocytes (A) or adipocytes (B) were subjected to 2-DE analysis as in Fig. 1. Note that the arrow-denoted protein was confirmed to be murine $\alpha 2$ macroglobulin fragments by NH₂-terminal protein sequencing.

3.3 Incubation of 3T3-L1 cells with a $\alpha 2$ macroglobulin antibody induces spontaneous differentiation

Time course analysis revealed that the levels of $\alpha 2$ macroglobulin fragment in 3T3 L1 cells decreased significantly within a 24 h period following hormonal induction of differentiation, and dropped to an undetectable level after 48 h (Fig. 4). This change is followed by expression of adipocyte specific genes such as adiponectin and fatty acid binding proteins. This result indicates that decreased accumulation of $\alpha 2$ macroglobulin occurs at the early stage of differentiation. To explore the potential role of $\alpha 2$ macroglobulin in adipogenesis, we treated 3T3 L1 preadipocytes with a polyclonal antibody against $\alpha 2$ macroglobulin. Silver-staining analysis of 2-D gels revealed that incubation of subconfluent 3T3 L1 preadipocytes with the antibody for 72 h led to the depletion of intracellularly accumulated $\alpha 2$ macroglobulin fragments to a undetectable level, whereas nonimmune serum did not cause this effect (Fig. 5). Western blot analysis showed that this treatment drastically decreased both full-length $\alpha 2$ macroglobulin and its low M_r fragments in 3T3 L1 preadipocytes (Fig. 6), indicating that the decreased intracellular accumulation of the low M_r fragments is not due to reduced degradation of full-length $\alpha 2$ macroglobulin. Furthermore, the 20 kDa $\alpha 2$ macroglobulin fragments are virtually undetectable in the culture medium of 3T3 L1 preadipocytes treated without or with anti $\alpha 2$ macroglobulin polyclonal antibody, thus excluding the possibility that the low M_r fragments are released into the culture medium following antibody treatment. These results collectively suggest that cleavage of $\alpha 2$ macroglobulin into its low M_r

fragments occur within 3T3 L1 preadipocytes, and that the antibody-mediated depletion of the intracellular low M_r $\alpha 2$ macroglobulin fragments is perhaps due to its ability to block $\alpha 2$ macroglobulin uptake from the culture medium.

Morphological analysis of cells treated with anti $\alpha 2$ macroglobulin antibody revealed that, 4 days after confluence, occasional cells start to accumulate lipid droplets (data not shown). By 6 days after confluence, widespread accumulation of lipid droplets was easily observed (Fig. 7A). In sharp contrast, only sporadic occurrence of lipid laden cells (less than 1 in 100) was observed in cells treated with nonimmune serum (Fig. 7B). Northern blot analysis demonstrated that mRNA expression of several adipocyte-specific markers, including the transcription factors C/EBP α and PPAR γ , adiponectin and GLUT4 [4, 5, 12, 13], is significantly induced following treatment with the anti $\alpha 2$ macroglobulin antibody (Fig. 8). These results indicate that depletion of intracellularly accumulated $\alpha 2$ macroglobulin fragments enhances spontaneous adipose conversion of 3T3 L1 cells.

4 Discussion

$\alpha 2$ macroglobulin is an acute phase glycoprotein mainly expressed and secreted from liver tissue [15, 20]. The protein is a general protease inhibitor that has been implicated in various functions, such as increasing expression of the platelet-derived growth factor receptor [21] and regulating the effects of transforming growth factor- β [22–24]. There is also a study indicating that $\alpha 2$ macroglobulin is genetically and functionally linked with Alz-

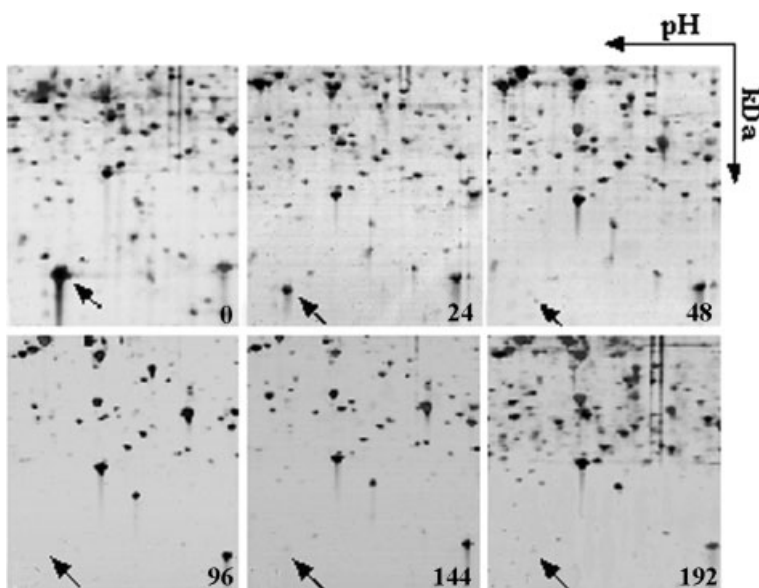


Figure 4. Time dependent decrease of intracellularly accumulated $\alpha 2$ macroglobulin fragments during differentiation of 3T3 L1 cells. Proteins (60 μ g) from 3T3 L1 cells harvested at different time intervals (0, 24, 48, 96 and 192 h) after differentiation induction were separated by 2-DE and stained with silver. The arrows denote $\alpha 2$ macroglobulin fragments.

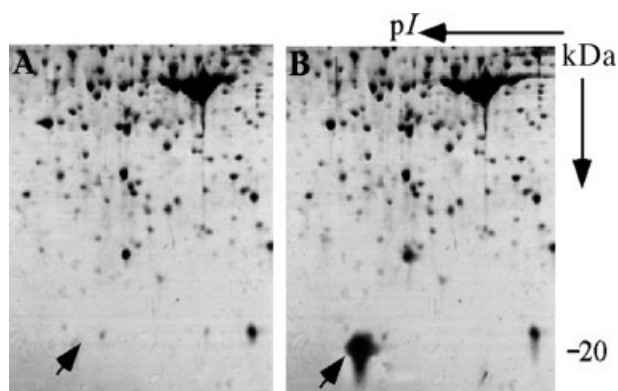


Figure 5. Depletion of intracellularly accumulated $\alpha 2$ macroglobulin fragment in 3T3 L1 preadipocytes following treatment with a polyclonal antibody against $\alpha 2$ macroglobulin. Subconfluent cells were treated with anti- $\alpha 2$ macroglobulin antibody at a concentration of 100 $\mu\text{g}/\text{mL}$ (A), or with the same concentration of nonimmune serum (B). Three days after treatment, cells were harvested, and proteins from the cells analyzed by 2-DE. Note that the arrow-denoted protein spot corresponding to $\alpha 2$ macroglobulin fragments is absent in the antibody-treated cells.

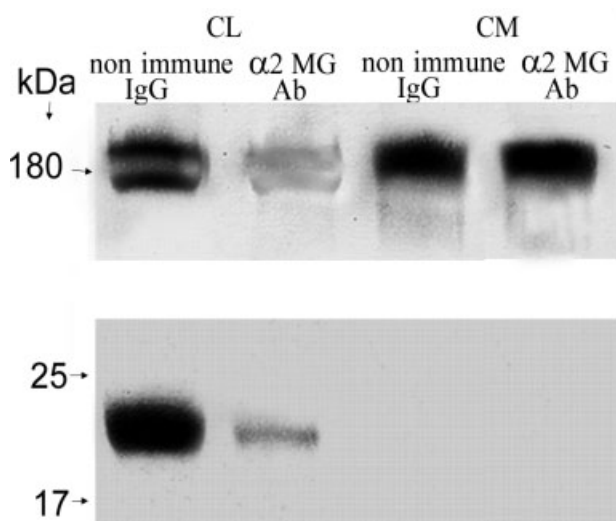


Figure 6. Effect of anti- $\alpha 2$ macroglobulin polyclonal antibody on intracellular accumulation of full-length $\alpha 2$ macroglobulin and its low M_r fragments. Subconfluent cells were treated with anti- $\alpha 2$ macroglobulin antibody ($\alpha 2$ MG Ab) at a concentration of 50 $\mu\text{g}/\text{mL}$, or with the same concentration of nonimmune IgG as a control. Three days after treatment, cells and culture medium were harvested respectively. Cell lysates (CL; 20 μg) or culture medium (CM, 15 μL) from each sample was incubated with SDS-PAGE loading buffer at 37°C for 45 min, separated by either 7.5% (top panel) or 15% (low panel) SDS-PAGE, transferred onto nitrocellulose membrane, and then probed with anti- $\alpha 2$ macroglobulin polyclonal antibody as described in Section 2.7.

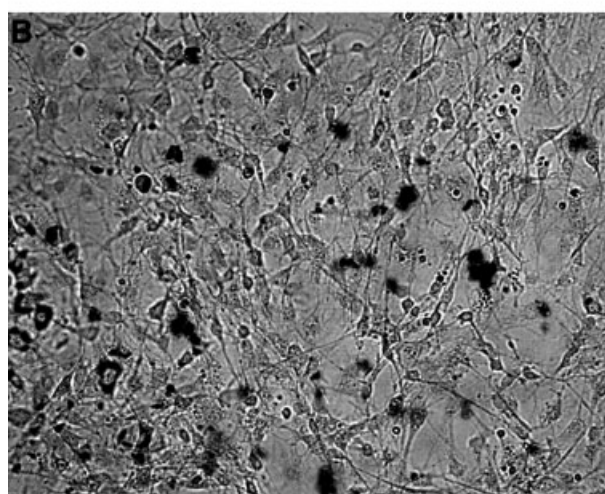
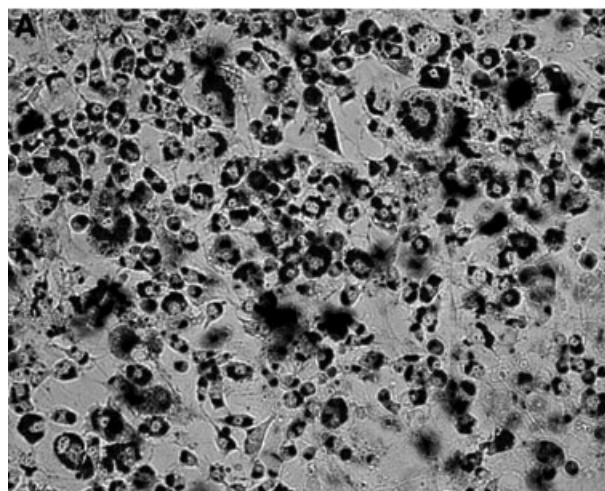


Figure 7. Treatment with a polyclonal antibody against $\alpha 2$ macroglobulin enhances the spontaneous differentiation of 3T3 L1 preadipocytes. Subconfluent 3T3 L1 preadipocytes were treated with the anti- $\alpha 2$ macroglobulin antibody (A) or nonimmune IgG (B) for 3 days as in Fig. 6. Once confluent, the cells were maintained in the same medium for another 6 days, then stained with oil red O and visualized with a Zeiss (Göttingen, Germany).

heimer's disease [25]. 2-DE patterns of intracellular $\alpha 2$ macroglobulin and its fragments have previously been reported [26], and those of serum alpha 2 macroglobulin have been examined [27]. In the present study, we have demonstrated that a $\alpha 2$ macroglobulin fragment is present in 3T3-L1 preadipocytes maintained in DMEM culture medium containing 10% fetal bovine serum, and its intracellular concentrations decline with the progression of differentiation (Figs. 1 and 4). Although the amino acid sequence of bovine $\alpha 2$ macroglobulin is currently unavailable, several lines of evidence demonstrate that intra-

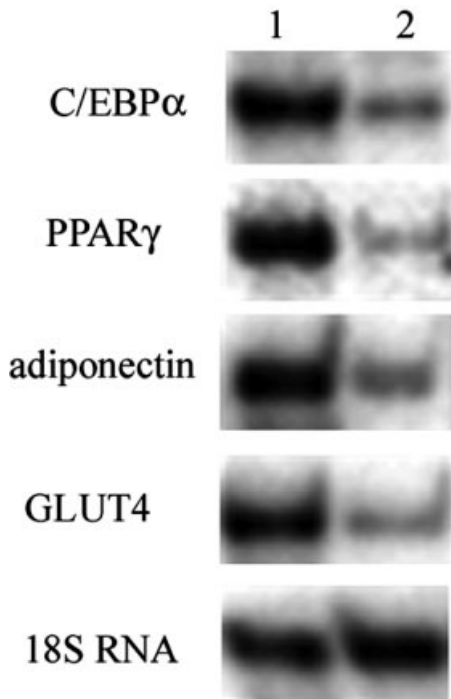


Figure 8. Incubation with anti $\alpha 2$ macroglobulin antibody induces expression of adipocyte-specific genes. 3T3 L1 preadipocytes were incubated with the anti $\alpha 2$ macroglobulin antibody ($\alpha 2$ MG Ab) or nonimmune IgG as in Fig. 7. Total RNA from each sample (20 μ g) was subjected to Northern blot analyses for adiponectin, GLUT₄, PPAR γ , C/EBP α and 18S RNA as described in Section 2.7.

cellular $\alpha 2$ macroglobulin fragments in 3T3 L1 preadipocytes were of bovine origin derived from the extracellular culture medium, not from *de novo* synthesis. First, the sequence for $\alpha 2$ macroglobulin fragments is not of murine origin, but is 79% identical with human $\alpha 2$ macroglobulin (Table 2). Second, metabolic labeling experiments showed that (³⁵S)methionine was not incorporated into the protein, although it contains at least two methionine residues (Fig. 2). Third, intracellularly accumulated $\alpha 2$ macroglobulin fragment was completely depleted by incubation of 3T3 L1 cells with anti $\alpha 2$ macroglobulin polyclonal antibody (Fig. 5). Fourth, degenerate PCR analysis confirmed that the gene encoding this protein was not expressed in 3T3 L1 preadipocytes. The physiological relevance of this finding was supported by the fact that accumulation of $\alpha 2$ macroglobulin fragments also occurs in preadipocytes directly derived from mouse, but not in mature adipocytes (Fig. 3).

It is important to note that $\alpha 2$ macroglobulin was identified from spots of approximately 180 kDa in a 2-DE database [28], whereas the $\alpha 2$ macroglobulin fragment present in 3T3 L1 preadipocytes is \sim 20 kDa. Since this fragment

was not present in the fetal bovine serum used for cell culture (Fig. 6), we concluded that $\alpha 2$ macroglobulin was taken up from the culture medium and then fragmented within the cells. One of the potential mechanisms underlying the fragmentation of $\alpha 2$ macroglobulin might be due to its activity as a protease inhibitor. During the entrapment of different proteases, $\alpha 2$ macroglobulin could also be cleaved by the proteases [29, 30]. In line with our results, two recent studies by Kondo *et al.* [31, 32] have demonstrated the accumulation of a 20 kDa $\alpha 2$ macroglobulin fragment in human fibroblast cell lines (OUMS-24 and KMS-6). This accumulation decreased dramatically following immortalization [31, 32]. These studies also confirmed that the 20 kDa $\alpha 2$ macroglobulin fragments were derived from the extracellular medium. However, the functional relevance of these findings in fibroblasts is not yet clear.

Our present study indicated that accumulation of $\alpha 2$ macroglobulin in 3T3 L1 preadipocytes inhibits their conversion to adipocytes. This conclusion is supported by the observation that depletion of intracellular $\alpha 2$ macroglobulin by treatment with an anti $\alpha 2$ macroglobulin polyclonal antibody is associated with spontaneous differentiation of 3T3 L1 preadipocytes, as shown by increased intracellular lipid accumulation (Fig. 7) and elevated expression of adipocyte-specific markers (Fig. 8). The mechanisms by which $\alpha 2$ macroglobulin inhibits adipocyte conversion of 3T3 L1 cells remain to be defined. Notably, proteolytic processes have been shown to play a critical role during the early stage of adipocyte differentiation [33–35]. For instance, the calcium-activated protease, calpain, is required for activation of C/EBP α , a key transcription factor involved in adipogenesis [35, 36]. Exposure of preadipocytes to the calpain inhibitor *N*-acetyl-leu-leu-norleucinal or overexpression of calpastatin, a specific endogenous calpain inhibitor, blocks adipocyte differentiation. HIV protease inhibitors have also been found to suppress preadipocyte differentiation *in vivo* and *in vitro* [37, 38]. Given that $\alpha 2$ macroglobulin is a protease inhibitor with broad specificity, it could block adipocyte differentiation by suppressing proteolytic events required for this process. Alternatively, the $\alpha 2$ macroglobulin fragment may have activities distinct from those of full-length $\alpha 2$ macroglobulin, which may be involved in the suppression of adipocyte differentiation.

5 Concluding remarks

Adipogenesis is a complex process that involves thousands of proteins at different stages of differentiation. Expression of many genes, such as insulin-growth factor-1 receptor [39], GADD [40], inhibitor of DNA binding

protein [41], c-fos [42], and pref-1 [43], is decreased during differentiation. Down-regulation of some of these genes has been shown to play an essential role in the early stage of differentiation, such as cell growth arrest and initiation of transcriptional programs. In line with these results, our present study shows that decreased concentration of intracellular $\alpha 2$ macroglobulin fragments in 3T3 L1 preadipocytes occurs at the early stage of differentiation. This process seems to be functionally important for adipose conversion *in vitro*. However, the mechanisms by which intracellular $\alpha 2$ -macroglobulin inhibit adipogenesis remain elusive. Further characterization of the molecular events underlying this process is likely to shed new light on the molecular events of adipocyte differentiation.

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