Biochemical and Biophysical Research Communications **272,** 293–297 (2000)
doi:10.1006/bbrc.2000.2759, available online at http://www.idealibrary.com on IDEAL®

Identification of Novel Membrane and Secreted Proteins Upregulated during Adipocyte Differentiation

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Received March 22, 2000

Adipose tissue is the largest organ in the body that secretes soluble proteins such as cytokines. A preadipocyte cell line 3T3-L1 has been widely used for investigations of mechanisms of adipocyte differentiation. 3T3-L1 cells convert to adipocytes in the presence of 1-methyl-3-isobutylxanthine, dexamethasone, and insulin. We screened a cDNA library derived from differentiated 3T3-L1 cells, using the SST-REX method (signal sequence trap by retrovirus-mediated expression screening method). Screening of 4×10^5 clones gave rise to 63 known and 8 novel clones. The known clones represented 28 independent proteins, 21 of which were secreted proteins and 7 were membrane proteins. The novel clones represented 7 independent proteins, 5 of which had no similarity to known proteins. Interestingly, most of these novel genes showed differentiation- and tissue-specific expression. The present results indicate that adipocytes specific genes or adipocyte differentiation-related genes encoding membrane and secreted proteins can be readily identified if signal sequence trap screening of differentiated adipocyte-derived cDNAs is done.

Key Words: **adipocyte; 3T3-L1; differentiation; signal sequence trap; retrovirus cDNA library.**

Adipose tissue was thought to be a storage place of fat until this tissue was shown to produce a cytokine called leptin, the mutation of which resulted in hereditical obesity in mice (1). Adipose tissue is now recognized to be the largest secretory organ. Other secretory proteins produced from adipocytes include TNF-α and plasminogen activator inhibitor-1 (PAI-1). TNF-α affects lipid and glucose metabolism, and is overexpressed in adipose tissues of genetically obese rodents (2), and this leads to insulin resistance (3). PAI-1 is highly expressed in visceral adipocytes and is involved in development of vascular disease in case of visceral obesity (4). Only a few cytokines have been identified in adipocytes, however it seems reasonable to consider that adipose tissue may secrete other soluble factors which regulate lipid and glucose metabolism. We screened adipocyte cDNA libraries to search for secreted molecules which have a short, amino-terminal hydrophobic peptide called a signal peptide (5). We used a 3T3-L1 cell line as the source of cDNA libraries, as it is a well-characterized model for adipocyte differentiation (6, 7).

For this we made use of an efficient signal sequence trap method which we recently developed using retrovirus-mediated gene transfer (8). We screened a total of 4×10^5 clones, and identified 63 cDNAs coding for known membrane or soluble proteins, and 8 novel cDNAs which likely code membrane or soluble proteins.

MATERIALS AND METHODS

Cell culture. 3T3-L1 cells were cultured in 5% CO_2 at 37°C in DMEM supplemented with 10% FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin (DMEM-FCS). Cells were grown to confluence and maintained for two days. For induction of differentiation, the medium was changed to DMEM-FCS containing 0.5 mM 1-methyl-3-isobutylxanthine, 0.25 μM dexamethasone, and 10 μg/ml insulin. Two days later, the medium was changed to DMEM-FCS, and the cells were allowed to differentiate. Ba/F3, a murine IL-3-dependent pro-B cell line was cultured in RPMI 1640 medium containing 10% FCS and 2 ng/ml murine IL-3 (R&D Systems).

RNA isolation. Poly(A)$^+$ RNAs were extracted from 3T3-L1 cells under three different conditions; in the log phase, at confluency, and 6 days after differentiation induction, using Fast Track 2.0 mRNA Isolation Kits (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Construction and screening of the cDNA library for SST. To identify cDNAs encoding secreted and membrane proteins from adipocytes, we constructed and screened an SST library from differentiated 3T3-L1 cells, as described (8). Briefly, complementary DNA (cDNA) was synthesized from the poly(A)$^+$ RNA by random hexamers, using the SuperScript Choice System (GIBCO-BRL, Rockville,

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0006-291X/00 $35.00

MD, USA), and was then inserted into *Bst*XI sites of the pMX-SST vector, using *Bst*XI adapters (Invitrogen, Carlsbad, CA). The ligated DNA was amplified in DH10B cells (Electromax, GIBCO-BRL) to construct an SST-REX library and the library DNA was prepared using QIAGEN Plasmid Kits (QIAGEN Inc., Valencia, CA).

High-titer retroviruses representing the SST-REX library were produced using a packaging cell-line BOSC23 (9), and infected to Ba/F3 cells as described (10). After a 1-day infection period, selection of factor-independent Ba/F3 cells was started in the absence of IL-3, using 96-well multititer plates. The integrated cDNAs were isolated from the factor-independent Ba/F3 cells by genomic PCR and then sequenced, as described (8).

Northern blot analysis. One microgram of poly(A)$^+$ RNAs was electrophoresed on a 1.0% agarose gel containing 2% formaldehyde, and then transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech Ltd.). The membrane was probed with ^{32}P-labeled DNA fragments derived from factor-independent clones in hybridization buffer (50% formamide, 10× Denhardt's reagent, 5× SSC, 0.1% SDS, 200 μg/ml denatured salmon sperm DNA) at 42°C. To assess the amount of RNA in each lane, one of the membranes was probed by a cDNA for murine glyceraldehyde phosphate dehydrogenase (GAPDH), as a loading control. After hybridization, the filter was washed in 0.1× SSC, 0.1% SDS at 42°C, and autoradiographed. A mouse multiple tissue Northern blot was purchased from Clontech (Palo Alto, CA), and was hibridized with the probes, as described above.

TABLE 1

Clones from Adipocyte Libraries Identical to Previously Identified Proteins

No.	Identity	Number of clones
Secretion protein		(Total) 50
Extracellular matrix and related protein		(Total) 28
1	Procollagen alpha-2(I)	5
2	Collagen alpha-1 (III)	2
3	Collagen alpha-1 (VI)	2
4	Collagen alpha-2 (IV)	2
5	Collagen alpha-1 (IV)	1
6	Collagen alpha-1 (XV)	1
7	Procollagen C-proteinase enhancer protein	3
8	Cysteine-rich glycoprotein SPARC	4
9	Extracellular matrix associated protein (Sc1)	3
10	Entactin (ENT)	2
11	Fibulin-2	1
12	Lysyl oxidase	1
13	Dystroglycan (DAG1)	1
Other secretion protein		(Total) 22
14	Adipocyte complement-related protein of 30 kDa (Acrp30)	10
15	Sulfated glycoprotein (Sgp1)	4
16	Lipoprotein lipase	2
17	Cystatin C	2
18	FK506-binding protein (FKBP23)	1
19	Epithelin	1
20	Disulfide isomerase-related protein (ERp72)	1
21	Interferon receptor soluble isoform (IFNAR2)	1
Membrane protein		(Total) 13
22	Amyloid precursor-like protein 2 (APLP2)	4
23	Amyloid beta protein precursor	1
24	Syndecan-1	3
25	Lysosomal membrane glycoprotein-typeA	2
26	Rat ribophorin I homologue	1
27	Tissue factor Cf-3	1
28	Putative transmembrane receptor (frizzled 7)	1
		(Total) 63

TABLE 2

Novel Clones from Adipocyte Libraries

Clone	Similarity	Number of clones
101	No similarity to database sequences	1
102	No similarity to database sequences	1
103	No similarity to database sequences	1
104	No similarity to database sequences	1
105	Cell surface protein MCAR	1
106	Collagen alpha 1(XI)	2
107	No similarity to database sequences	1

RESULTS

Analysis of isolated cDNA clones. Using SST-REX, we screened 4×10^5 clones from differentiated 3T3-L1 cells, and isolated 100 factor-independent Ba/F3 clones, 71 of which gave a single PCR band. Sixty-three out of the 71 integrations contained the 5′ sequence of cDNAs for 28 known proteins, and all of which harbored the signal sequence (Table 1). Of the 63 cDNAs identified, 50 (79%) coded for secreted proteins, including 28 extracellular matrix (ECM) proteins or related proteins, and 13 (21%) coded for membrane proteins. The remaining 8 clones represented 7 independent novel proteins (listed in Table 2). Five proteins had no similarity with known proteins, one was similar to the cell surface protein MCAR (murine coxsackievirus and adenovirus receptor) (11), and the other showed similarity to collagen alpha 1(XI).

Expression of novel adipocyte genes during differentiation. We then used Northern blot to examine expression patterns of the 7 novel genes (Nos. 101–107), during differentiation (Fig. 1).

Expression of mRNA changed along with differentiation, in all but clone 102. The mRNA of clone 101, 104, 106, and 107 increased after differentiation. Clone 105 had two bands, and the intensity of the lower band increased, when the cells differentiated to adipocytes. Expression of clone 103 increased when 3T3-L1 cells were grown to confluence.

Northern blot analysis of multiple mouse tissues. We also investigated expression profiles of the novel adipocyte-derived genes by Northern blot analysis on mRNA derived from various mouse tissues (Fig. 2). We selected 6 genes, the expression of which increased significantly in the adipocytes. Expression of clone 101 was remarkably high in testis, and mRNA was weak in heart, spleen, and liver. Clone 103 was specifically expressed in heart and brain and the lower-molecular band was weak in testis. Expression of clone 104 was remarkably high in heart and liver, and weak in brain, lung, and kidney. Clone 105 was transcribed only in

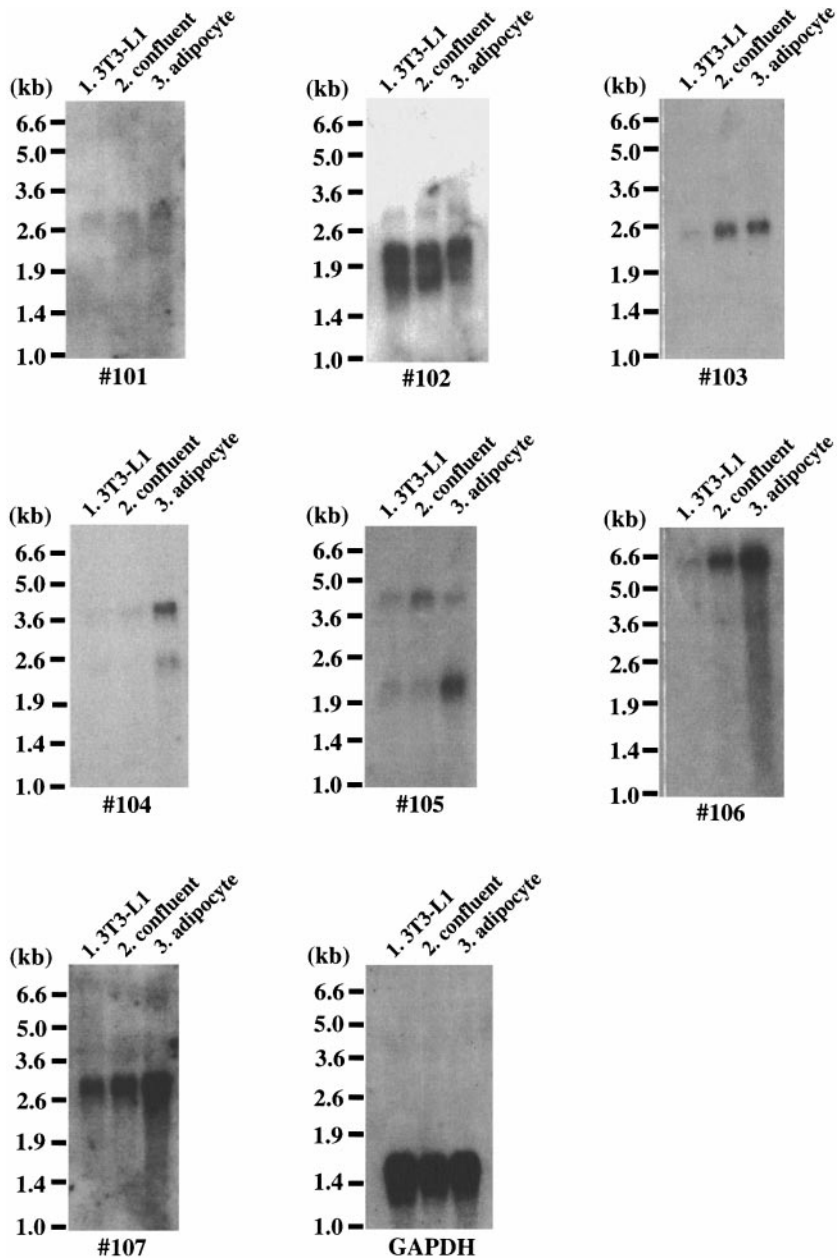

FIG. 1. Expression of novel adipocyte genes during differentiation. Expression patterns of 7 novel genes including clones 101–107 during differentiation were investigated by Northern blot analysis. Poly(A)$^+$ RNAs were prepared from growing 3T3-L1 cells (lane 1), confluent 3T3-L1 cells (lane 2), and 3T3-L1 cells that were induced to differentiate into adipocytes (lane 3). To assess the amount of RNA in each lane, one of the membranes was probed using a cDNA for murine glyceraldehyde phosphate dehydrogenase (GAPDH) as a loading control.

heart and brain. The higher-molecular band was detected only in the brain and expression of clone 106 was detected only in the heart. Clone 107 mRNA levels in heart, liver, kidney, and testis were higher than in other organs.

DISCUSSION

We searched for adipocyte-derived cDNA encoding secreted and membrane proteins using SST-REX, a novel signal sequence trap method which we recently established, and obtained 63 known cDNAs and 8 novel ones. All of the sixty-three cDNAs contained the 5′ sequence of cDNAs for 28 known proteins, and all of which were membrane or secreted proteins; there was no background clone in this screening. Fifty of 63 clones (79%) coded for secreted proteins. In particular, 28 out of 63 clones (44%) were extracellular matrix (ECM) proteins or related proteins such as collagens, suggesting that the potential for fibroblasts to secrete

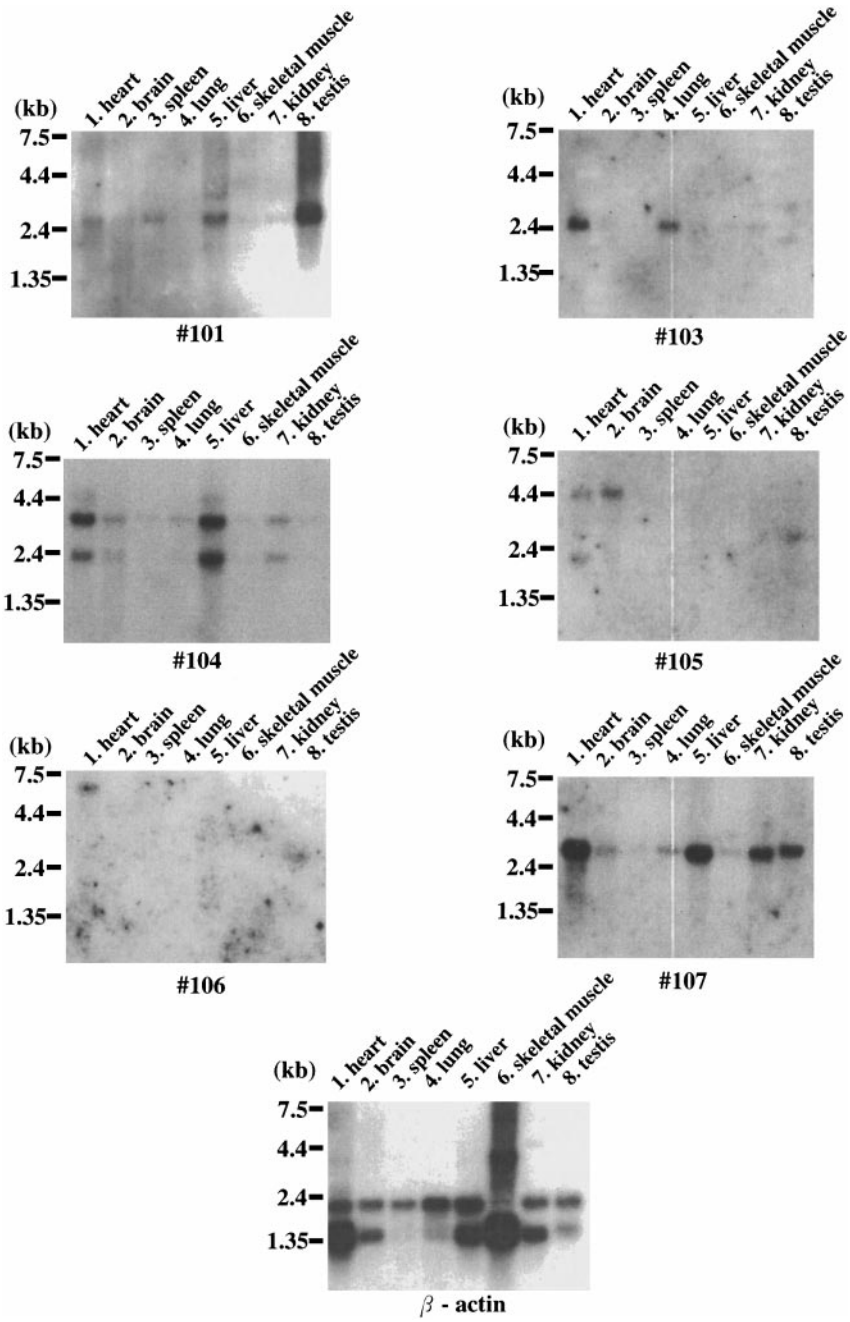

FIG. 2. Tissue distribution of novel adipocyte-derived genes. Expression profiles of the novel adipocyte-derived genes (except for clone 102) were investigated using Northern blot analysis on mRNA derived from various mouse tissues. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. The membrane was probed using cDNA for murine β-actin as a loading control, to assess the amount of RNA in each lane.

ECM proteins was enhanced during differentiation into adipocytes. It was reported that synthesis and secretion of collagen alpha-1 (IV) and alpha-2 (IV) are negligible in fibroblasts, yet remarkably enhanced in adipocytes. Enhanced secretion of entactin was also observed (12). The present results are consistent with these findings and suggest that synthesis of ECM proteins has a role in differentiation of adipose tissue.

Among the known cDNAs we obtained, some encoded for adipocyte-specific proteins, including adipocyte complement-related protein of 30 kDa (Acrp30) which harbors collagen repeats (13) (cloned 10 times)

and lipoprotein lipase (LPL) (cloned twice). Acrp 30 mRNA is induced over 100-fold during adipocyte differentiation (13), and LPL is the adipocyte-specific gene involved in the accumulation of fat, the expression of which is enhanced by PPARγ in adipocytes. Proteins such as syndecan-1 and lysosomal membrane glycoprotein-typeA are the housekeeping genes.

In Northern blot analysis, most SST clones obtained from adipocytes cDNA libraries showed adipocyte-specific expression patterns. For example, the expression of clone 103 was hardly detectable in 3T3-L1 preadipocytes but did appear in confluent cells and adipocytes. It may be an early marker of adipocyte differentiation, such as lipoprotein lipase, collagen, and FAAR (fatty acid-activated receptor) (14). The expressions of early marker genes are essential and important as a prerequisite for subsequent preadipocyte differentiation. As the level of clone 106 mRNA increased during differentiation, it is likely to have a role in adipocyte differentiation. The expression pattern of clone #105 is of interest as two transcripts were detected in Northern blot analysis, these might be derived from distinct genes. If one of the two transcripts is an alternative spliced form of the other, it would be of interest to determine how they are regulated before and after differentiation. The significance of the differential regulation of multiple mRNA bands remains to be determined.

In summary, we obtained 7 novel genes out of 71 SST clones derived from adipocytes when we screened 4×10^5 clones and most showed differentiation-specific expression without subtraction. The feasibility of SST screening to identify novel soluble and membrane proteins involved in lipid metabolism seems apparent.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan, and from the Ministry of Health and Welfare of Japan. This work was also supported by ONO Medical Research Foundation. Department of Hematopoietic Factors is supported in part by Chugai Pharmaceutical Ltd. We also thank Ms. Mariko Ohara for her providing language assistance.

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