A Proteomic Approach for Identification of Secreted Proteins during the Differentiation of 3T3-L1 Preadipocytes to Adipocytes*

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We have undertaken a systematic proteomic approach to purify and identify secreted factors that are differentially expressed in preadipocytes versus adipocytes. Using one-dimensional gel electrophoresis combined with nanoelectrospray tandem mass spectrometry, proteins that were specifically secreted by 3T3-L1 preadipocytes or adipocytes were identified. In addition to a number of previously reported molecules that are up- or down-regulated during this differentiation process (adipsin, adipocyte complement-related protein 30 kDa, complement C3, and fibronectin), we identified four secreted molecules that have not been shown previously to be expressed differentially during the process of adipogenesis. Pigment epithelium-derived factor, a soluble molecule with potent angiogenic properties, was found to be highly secreted by preadipocytes but not adipocytes. Conversely, we found hippocampal cholinergic neurostimulating peptide, neutrophil gelatinase-associated lipocalin, and haptoglobin to be expressed highly by mature adipocytes. We also used liquid chromatography-based separation followed by automated tandem mass spectrometry to identify proteins secreted by mature adipocytes. Several additional secreted proteins including resistin, secreted acidic cysteine-rich glycoprotein/osteonectin, stromal cell-derived factor-1, cystatin C, gelsolin, and matrix metalloprotease-2 were identified by this method. To our knowledge, this is the first study to identify several novel secreted proteins by adipocytes by a proteomic approach using mass spectrometry. Molecular & Cellular Proteomics 1:213–222, 2002.

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Obesity is now increasingly recognized as a major health concern especially in the affluent world (1). Obesity predisposes a variety of other illnesses ranging from hypertension and coronary heart disease to type II diabetes mellitus. Until recently, the adipose tissue was considered to play a passive role in the body by merely acting as a storage depot for fat. However, adipocytes are involved actively in maintaining the energy balance in the body (2–4). Adipocytes have an important role in processes such as satiety, bone function, and reproduction, and most of these functions are carried out via proteins secreted by adipocytes capable of acting locally or at distant sites (5). One such example is leptin that is mainly secreted by the adipose tissue and acts on the hypothalamus to regulate food intake (6). In addition to leptin, other factors such as adipin, tumor necrosis factor-α, insulin-like growth factor-1, vascular endothelial growth factor, and Acrp30/AdipoQ that are secreted by adipose tissue have been identified (7–11). Injection of the globular domain of Acrp30 was recently shown to cause weight loss in mice when they were maintained on a high fat and high sucrose diet again demonstrating the importance of adipocyte-specific proteins in metabolism (12). 3T3-L1 cells are an excellent model system for studying the behavior of fibroblasts as they differentiate into adipocytes when subjected to a differentiation regimen consisting of insulin, dexamethasone, and methylisobutylxanthine (mix) (13). We therefore chose preadipocytes and day 9 adipocytes to examine the profile of secreted proteins in greater detail. Several secreted proteins that were reported previously to be up- or down-regulated during the differentiation process were found in this study including several collagens, adipin, Acrp30, complement C3, entactin/nidogen, and fibronectin. In addition, we found several molecules that were not described previously to be secreted by adipocytes or to be expressed differentially during the process of adipogenesis. For instance, PEDF,1 a serpin inhibitor with potent angiogenic

1 The abbreviations used are: PEDF, pigment epithelium-derived factor; Acrp30, adipocyte complement-related protein 30; HCNP, hippocampal cholinergic neurostimulating peptide; LC, liquid chromatography; MS/MS, tandem spectrometry; NGAL, neutrophil gelatinase-associated lipocalin precursor; SPARC, secreted acidic cysteine-rich glycoprotein; DMEM, Dulbecco’s modified Eagle’s medium; MOPS, 4-morpholinepropanesulfonic acid; SDF, stromal cell-derived factor.
activity (14), was detected as a protein secreted by preadipocytes but not by adipocytes. Conversely, we found an acute phase reactant, haptoglobin, and two smaller polypeptides, hippocampal cholinergic neurostimulating peptide (HCNP) and neutrophil gelatinase-associated lipocalin (NGAL), that were produced by adipocytes (15, 16). We showed that whereas haptoglobin was correspondingly up-regulated at the mRNA level, NGAL and HCNP showed no change in mRNA expression levels suggesting that the regulation of these protein levels occurs post-transcriptionally.

This study demonstrates that several secreted factors that differ between any two states can be identified in a single experiment using one-dimensional electrophoresis and tandem mass spectrometry. We also used an alternative method that avoids the gel electrophoresis step altogether. For this experiment, supernatants from mature adipocytes were digested with trypsin in solution, and the tryptic peptides were separated by liquid chromatography. The peptides were eluted and subjected to automated fragmentation and sequencing (LC-MS/MS). This procedure resulted in the identification of 12 additional molecules, five of which have not been described previously to be secreted by adipocytes. Our proteomic approach is complementary to microarray experiments using oligonucleotide or cDNA arrays, because proteins that are not differentially expressed at the mRNA levels cannot be identified using microarrays. More importantly, our proteomic analysis allowed us to enrich for and directly examine only one set of cellular proteins (secreted proteins in detail). Identification of novel secreted molecules using this proteomic methodology will allow further detailed experiments to dissect the roles of such proteins in adipose biology and in various metabolic pathways in general.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Growth Factors, and Antibodies—**3T3-L1 preadipocytes were grown in DMEM with 10% calf serum plus antibiotics in 10% CO₂ at 37 °C. Mouse 3T3-L1 preadipocytes were differentiated essentially as described previously (13). Briefly, cells were grown to confluence in DMEM with 10% calf serum plus antibiotics in 10% CO₂. Two days after the cells reached confluence (day 0), they were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine (mix; Sigma), 1 μM dexamethasone (Sigma), and 167 μM insulin (Novo-Nordisk). After 48 h (day 2), the medium was replaced with DMEM supplemented with 10% fetal bovine serum and 167 μM insulin. After an additional 48 h (day 4), insulin was withdrawn, and the medium was changed every second day.

**Harvesting of Supernatants from Preadipocytes and Adipocytes—**3T3-L1 cells were grown to confluence. They were then started on a differentiation protocol as described above. For metabolic labeling, the cells were washed twice using 30 ml of serum-free medium each time and left in 12 ml of serum-free medium for an additional 18 h. Extreme care was taken not to disrupt the cells during this washing step. The supernatants were then harvested, centrifuged once, and filtered using a 0.2-μm filter. The samples were dialyzed against water (molecular mass cutoff — 3500 Da; Pierce) and dried in a vacuum centrifuge. 50 μg of the protein sample (derived from 4 × 10⁶ cells) was loaded onto an SDS-PAGE gel that was subsequently silver-stained as described previously (17). For LC-MS/MS experiments, ~250 μg of protein sample (obtained from 2 × 10⁶ cells) was digested by trypsin and analyzed.

**RT-PCR Analysis—**Total RNA was prepared as described previously (17). Reverse transcription reactions were performed in a 25-μl volume containing 1 μg of total RNA, 3 μg of random hexamers (Amersham Biosciences), 50 μM Tris-Cl, pH 8.3, 75 μM KCl, 3 mM MgCl₂, 10 μM dithiothreitol, 40 units of RNA-guard (Amersham Biosciences), 0.9 μM dNTPs, and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Reactions were left for 10 min at room temperature, followed by incubation at 37 °C for 1 h. After cDNA synthesis, the reaction mix was diluted with 50 μl of water. Multiplex reverse transcription-polymerase chain reaction (RT-PCR) was performed essentially as described (18). Briefly, the PCR reaction was performed in a 25-μl volume containing 1.5 μl of diluted cDNA, 50 μM KCl, 10 mM Tris-Cl, pH 9.0, 1.5 mM MgCl₂, 0.1% Triton X-100, 40 μM dATP, dTTP, and dGTP, 20 μM dCTP, 5 pmol of each primer, 1.25 units of Taq polymerase, and 1.25 μM of α-32P-dCTP (6000 Ci/mmole) (PerkinElmer Life Sciences). The reaction mix was denatured by heating at 94 °C for 1 min. Denaturation was followed by 15, 20, or 25 cycles (depending on the set of primers used) of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 40 s. All reactions contained the TATA-binding protein primer set as an internal standard. Reactions amplifying NGAL were performed with 25 cycles, adipin and Acpr30 with 15 cycles, and PEDF, HCNP, and haptoglobin with 20 cycles. Ten micrometers of each reaction were dried down and resuspended in formamide dye mix (98% deionized formamide, 10 mM EDTA, pH 8.0, 0.2% bromphenol blue, 0.2% xylene cyan) and loaded onto 0.4 mm, 8 μm urea, 1x TBE (TBE borate/EDTA), 6% polyacrylamide gels. Electrophoresis was performed for 3 h at 50 watts. The gels were dried and exposed overnight on a PhosphorImage storage screen and subsequently scanned on a PhosphorImager plate (Molecular Dynamics, Sunnyvale, CA).

Primers used for multiplex RT-PCR were as follows (upstream and downstream, respectively): PEDF, CCAGACTTACAACTCCTTCGG and GGTCAGGATTCTGCTTATGA; HCNP, TGGACGCAGGCAGGCC- AAATGCG and CTCCTGCTGACACCAGACCGA; NGAL, CTACGAA- CTGATCCCTGCCC and CCAGCCCTGGAGCTTGGAACA; adipin, TGGACAGTGAAGTGCTAAGC and GCAGGTTGCCGTTCGAT- AT; haptoglobin, TGGGTCACCTCTCGT and CCAGCGACTGTTGTC- ACCAT; Acrp30, TATCGCTACGTTCTCAAGG and TGCGCTTGG- CCCAATCTTTTG; TATA-binding protein, ACCCTCAACAATGACT- CCTATG and ATGATAGCTGCAACGAAACTTG.

**Northern Blot Analysis—**20 μg of total RNA was resolved on a denaturing gel containing 1.2% agarose, 20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, transferred to a Hybond membrane (Amersham Biosciences), and immobilized by UV cross-linking. Probe fragments corresponding to PEDF and haptoglobin were labeled with a Prime-It RmT Random primer labeling kit (Stratagene) using [α-32P]dCTP (6000 Ci/mmole) (PerkinElmer Life Sciences), and hybridization was performed overnight at 42 °C in a buffer containing 50% deionized formamide, 2.5× Denhardt’s solution, 0.38% SDS, 50% dextran sulfate, 2.5× saline/sodium phosphate/EDTA, and 0.1 mg/ml salmon sperm DNA.

**Mass Spectrometric Analysis of Secreted Proteins—**The bands indicated in Fig. 2 were excised from one-dimensional silver-stained polyacrylamide gel and processed as described (19, 20). After reduction and alkylation of bands, proteins were in-gel digested with an excess of modified, sequencing grade trypsin (Promega, Madison,
RESULTS AND DISCUSSION

A Proteomic Strategy to Identify Molecules Secreted during the Process of Adipocyte Differentiation—Several molecules that are expressed differentially during the process of adipocyte differentiation have been identified over the course of the last decade or so. A key family of nuclear hormone receptor regulatory element-binding protein families have been identified, and finally digested with trypsin. The tryptic peptides were eluted with 95% methanol/5% formic acid directly into a nanoelectrospray needle (MDS-Proteomics, Odense, Denmark). Nanoelectrospray tandem mass spectrometry analysis was performed either on a Q-TOF mass spectrometer (Micromass, Manchester, United Kingdom) or on a QSTAR Pulsar (PE Sciex, Toronto, Canada) equipped with a nanoelectrospray source (MDS-Proteomics), and fragmentation spectra were obtained. The resulting "peptide sequence tags" (22) were used to search the nrdb database (EBI) using the PepSea program (MDS-Proteomics). When a peptide match was found in the database, the retrieved peptide sequence was verified against the MS/MS spectrum. LC-MS/MS analysis was performed on an Agilent Capillary LC system coupled to a quadrupole time-of-flight mass spectrometer (QSTAR Pulsar; PE Sciex, Toronto, Canada). The sample was loaded off-line onto a column packed with a 5-μm Zorbax C18 resin. Peptides were eluted using a 7-40% gradient of organic phase in 150 min. Buffer A was 0.1% acetic acid, 0.005% HFBA (heptafluorobutyric acid), and Buffer B was 90% acetonitrile, 0.4% acetic acid, 0.005% HFBA. The MS data was obtained in pulsing mode using information-dependent acquisition based on a 1-s MS survey scan followed by up to three MS/MS scans of 2 s each. The resulting data was searched against a non-redundant protein database by using MASCOT (www.matrixscience.com) and SEQUEST (ThermoFinnigan, Foster City, CA). The typical search parameters were 2-Da mass accuracy for parent ions and 50-ppm accuracy for MS/MS data. One missed cleavage was allowed. Proteins were identified based on multiple matches to peptides from the same protein, either by MASCOT score for each peptide ion or by SEQUEST Xcorr and DelCn coefficient values. For MASCOT searches, a positive score was defined to be greater than 30 for each peptide ion. Positive assignment using SEQUEST was based on Xcorr scores greater than 1.5 for singly charged peptide ions and 2.5 and 3 for doubly and triply charged ions, respectively. If only a single peptide was identified, the assignment was confirmed by manual interpretation of the MS/MS spectrum by applying a sequence tag algorithm using the PepSea database search engine.

Identification of Secreted Molecules That Are Differentially Expressed by Preadipocytes and Adipocytes by Nanoelectrospray Tandem Mass Spectrometry—The bands that were expressed differentially between preadipocytes and adipocytes compared with each other were excised, reduced and alkylated, and finally digested with trypsin. The tryptic peptides from each band were analyzed by nanoelectrospray tandem mass spectrometry. Table I shows a list of all of the proteins identified in this study, and Fig. 3 shows the spectra that were obtained by tandem mass spectrometric analysis. We identi-
Fied fibronectin as a down-regulated protein (band 1) that has been shown previously to be down-regulated at the mRNA level (28, 29). Down-regulation of fibronectin at the protein level was demonstrated recently and shown to be critical for adipocyte differentiation (30). We also found procollagen type I α2 (band 9) to be down-regulated during the adipocyte differentiation process. Again, it has been shown to be regu-
A list of secreted proteins that are up-regulated during adipogenesis

| Upregulated secreted proteins | Band number | Database accession No. |
|------------------------------|-------------|-----------------------|
| Type VI collagen α 3         | 2, 3, 4     | AAC23667              |
| Complement factor C3         | 5, 7, 10, 11| AAC42013              |
| Entactin/nidogen             | 6           | NP_05047              |
| Haptoglobin                  | 12, 13, 14  | NP_059066             |
| Adipsin                      | 12, 14      | NP_038487             |
| Acrp30                       | 15          | AAA80543              |
| HCNP                         | 16          | BAA03276              |
| NGAL                         | 16          | CAA32762              |

Our analysis identified several up-regulated secreted proteins. Adipocyte complement-related protein 30 kDa (Acrp30), complement factor C3 precursor, and adipsin were found to be produced mainly by adipocytes. Acrp30 is a protein known to be secreted exclusively by adipocytes, and its mRNA is induced 100-fold during the process of adipocyte differentiation (10). It was also cloned in an independent study and designated as AdipoQ (11). Acrp30 has four domains; its C-terminal globular domain was shown recently to increase fatty acid oxidation in muscle and to cause weight loss in mice when they were put on a regimen of a high fat and high sucrose diet (12). Acrp30 presumably undergoes proteolytic cleavage in vivo to produce a C-terminal fragment containing the globular domain alone, which migrates at 16 kDa (12). In this study, we have identified Acrp30 from a band that migrates at 30 kDa indicating that it is the uncleaved version of Acrp30. Complement factor C3 was identified from bands 5, 7, 10, and 11. Its mRNA and protein levels have been shown previously to increase dramatically as adipocytes differentiate into adipocytes (32, 33). Activation of C3 is a central step in the alternative complement pathway. The complement factor C3 precursor, which is ~200 kDa, is composed of α and β chains that are linked by a disulfide bond (34). The form of C3 migrating at 110 kDa that we have identified is the α chain whereas the form migrating at 70 kDa is the β chain. C3a and C3b are derived by proteolytic cleavage of the complement C3 precursor and correspond to its N and C terminus, respectively. Cleavage of C3a to C3adesArg makes it capable of inducing triglyceride synthesis and glucose transport indicating its intimate involvement in energy metabolism adipocytes (35–37). Adipsin was identified from bands 12 and 14 as an up-regulated protein. It was originally isolated as an mRNA species that was up-regulated over 200-fold during the adipocyte conversion process (38). It was also shown subsequently to be up-regulated at the protein level (39) and is secreted in two forms that differ in their glycosylation patterns, 37 and 44 kDa (40); both of these alternative forms of adipsin were identified in our study. Entactin/nidogen was another protein that we identified as an up-regulated protein. It was identified by Tsuruga et al. (27) as a differentially expressed mRNA using a signal sequence trap method and was shown to be up-regulated 30-fold at the protein level during adipocyte differentiation using immunoprecipitating antibodies (41). Entactin can form a ternary complex with type IV collagen and laminin thereby helping in the formation of the basement membrane (41). We found collagen type VI α3 to be secreted mainly by adipocytes confirming the results of a recent study that found this collagen expressed mainly in adipocytes using a cDNA-based subtraction strategy (42). We had also identified the α3 subunit of type VI collagen as a protein up-regulated in adipogenesis by our subtractive antibody-screening method (26).

Characterization of Four Previously Undescribed Secreted Molecules That Are Expressed Differentially—In addition to the secreted molecules described above that have been reported previously to undergo up-regulation when preadipocytes differentiate into adipocytes, we found four secreted molecules that have not been reported previously to be expressed differentially. Fig. 2B shows a band around 50 kDa that is seen in supernatants from preadipocytes but not adipocytes. Sequencing revealed this band (band 8) to be a PEDF or stromal cell-derived factor-3 (SDF-3) (43, 44) (Fig. 3A). It belongs to the serine protease inhibitor family and induces differentiation of cultured human retinoblastoma cells into neurons (45). It has been also been shown recently to act as a potent angiogenesis inhibitor (14). Daily administration of recombinant PEDF conferred protection from ischemia-induced retinopathy in a mouse model of retinopathy (46). Multiplex RT-PCR analysis showed that PEDF mRNA transcript is expressed in preadipocytes but not in mature adipocytes (Fig. 4A). To examine the time course of down-regulation of PEDF transcript, we performed a Northern blot analysis. As shown in Fig. 4B, PEDF transcript is abundant in preadipocytes but is hardly detectable by day 3. This pattern is similar to another molecule, Pref-1, that is expressed by preadipocytes but not by adipocytes (47).

Several of the up-regulated bands (bands 12, 13, and 14) (Fig. 2B) were found to correspond to haptoglobin (Fig. 3B), which is a dimer of two subunits, α and β, that are derived from processing of a single polypeptide chain. We found the partially and fully glycosylated form of prohaptoglobin migrating at 45 and 45 kDa, as well as the core glycosylated β subunit migrating at ~38 kDa (48). Haptoglobin is an acute phase protein and is mainly synthesized by the liver and is the major hemoglobin-binding protein. Its level in plasma is sharply up-regulated during inflammation, infection, pregnancy, trauma, and malignancy. The expression of haptoglobin has been studied in some detail in hepatocytes and demonstrated to be regulated by a variety of cytokines and drugs including interleukin-1, interleukin-6, transforming growth fac-
tor-β, dexamethasone, and forskolin (49–51). The cAMP-dependent regulation of the haptoglobin gene presumably occurs via CCAAT/enhancer-binding proteins. Up-regulation of haptoglobin production during the process of adipogenesis has not been shown previously. To test whether the up-regulation of haptoglobin was at the mRNA level, we performed a multiplex RT-PCR analysis. As shown in Fig. 4A, haptoglobin mRNA is detected in adipocytes but not in preadipocytes. A time course analysis carried out using mRNA isolated from various stages of adipocyte differentiation clearly shows the up-regulation of haptoglobin mRNA beginning at day 3 of adipogenesis (Fig. 4B).

We also found two other factors, NGAL or 24p3 and HCNP to be up-regulated in adipocytes (Fig. 3, C and D). NGAL was described originally as an oncogene whose expression increases dramatically after infection with polyoma or SV40 virus (16, 52). It belongs to a family of fatty acid-binding proteins called lipocalins (53). HCNP was isolated from hip-

**Fig. 3.** Nanoelectrospray tandem mass spectrometric identification of four newly identified secreted molecules that are expressed differentially. Proteins separated by SDS-PAGE in Fig. 2 were subjected to in-gel digestion by trypsin and analyzed by tandem mass spectrometry. A, spectrum from MS/MS analysis of protein band 8 corresponding to PEDF. The spectrum shows the fragmentation pattern of a doubly charged parent ion. B, spectrum from MS/MS analysis of protein band 12 corresponding to haptoglobin. C, spectrum from MS/MS analysis of protein band 16 corresponding to NGAL. D, spectrum from MS/MS analysis of protein band 16 corresponding to HCNP. The y series of ions (C-terminal fragments), as well as those from the b series (N-terminal fragments), are shown. The sequence of the peptides as deduced from the spectrum and database search are given at the top of each panel.
Proteomic Analysis of Proteins Secreted by Adipocytes

**Fig. 4.** mRNA analysis of differentially expressed secreted proteins. A, RT-PCR results of PEDF, haptoglobin, NGAL, and HCNP. Primers specific for PEDF, haptoglobin, NGAL, HCNP, adipin, and Acrp30 were used as indicated to amplify the transcripts from total RNA isolated from preadipocytes and day 9 adipocytes. Total RNA was isolated from day 9 adipocytes as in panel A, and −20 μg was resolved by agarose gel electrophoresis and transferred to nitrocellulose. 32P-labeled probes specific for PEDF and haptoglobin were used as described under “Experimental Procedures.”

In the case of adipocytes, multiplex RT-PCR showed that there is no alteration in the mRNA levels of these two secreted molecules suggesting that the difference in protein level may be expected to post-transcriptional effects (Fig. 4A). Because of lack of commercially available good immunoprecipitating or Western blotting antibodies against these two proteins, we are unable to measure quantitatively the changes in their expression levels. Using quantitative mass spectrometry techniques that employ incorporation of a deuterium- or hydrogen-labeled affinity tag onto cysteine residues, it may be possible to quantify the expression level changes at the protein level (57).

**High Throughput Automated LC-MS/MS Identification of Several Previously Undescribed Proteins Secreted by Adipocytes—** Because the analysis of bands excised from one-dimensional gels is still cumbersome, we decided to test the feasibility of avoiding gel electrophoresis altogether. To this end, we subjected the proteins isolated from supernatants of adipocytes to trypsin digestion in solution. The tryptic peptides were loaded onto a nano-LC column, eluted sequentially from the column, and the eluting peptides were fragmented on-line by the mass spectrometer (58, 59). However, because this method analyzes all of the proteins secreted by mature adipocytes and not merely the differentially expressed secreted proteins, it provides a larger catalog of proteins secreted by adipocytes. It can be quite difficult to compare two different runs in a typical LC experiment. All the molecules identified by analysis of individual bands from a one-dimension gel (with the exception of NGAL and HCNP) were again identified by LC-MS/MS. Table II lists the additional molecules that we identified by this approach. Twelve additional secreted factors were found by the LC-MS/MS approach. Three of these factors, resistin, SPARC/osteonectin, and matrix metalloproteinase-2/gelatinase A, have already been implicated in the adipose tissue metabolism or obesity. Resistin was identified recently as a secreted molecule that is down-regulated (at the mRNA and protein levels) by administration of thiazolidinediones, which function as insulin sensitizers (60, 61). The same molecule was obtained by homology searching by a different group and designated earlier as FIZZ3 (62). Administration of recombinant resistin induces an insulin-resistant state in mice, and resistin levels are higher in obese versus normal mice leading to the suggestion that it is involved in the pathogenesis of type II diabetes (60). SPARC/osteonectin was similarly found recently to be increased markedly in several models of obesity in mice, and its expression in adipose tissue was induced by insulin (63). Our finding of SPARC/osteonectin as a secreted adipocyte protein by LC-MS/MS confirmed our previous observations by subtractive antibody screening (26). Another molecule that we identified, matrix metalloproteinase-2 or gelatinase A, was recently reported to be expressed at a higher level in gonadal fat pad of mice on a high fat diet compared with normally fed mice (64). We also identified SDF-1 or pre-B cell growth stimulating factor from adipocytes supernatants (65, 66). This CXC chemokine, which is a ligand for CXCR4/fusin (67, 68), has not been shown previously to be secreted by adipocytes. Two calcium-binding proteins, calumenin and calvasculin, previously not identified in relation to adipocytes, were also found to be secreted by adipocytes (69, 70). Also, two protease inhibitors, cystatin C and colligin-1, were found in the LC-MS/MS approach (71, 72).

| Protein identified       | Database accession No. |
|--------------------------|------------------------|
| Resistin                 | NP_075360              |
| SPARC/osteonectin        | NP_033268              |
| Cystatin 3               | NP_034106              |
| SDF-1                    | P40224                 |
| Calumenin                | NP_031620              |
| Gelatinase               | NP_034484              |
| Colligin-1               | NP_004344              |
| Matrix-metalloproteinase 2| NP_032636             |
| Mouse placental calcium-binding protein | P07091 |
| Type III collagen α 1    | P08121                 |
| Type VI collagen α 1     | NP_034063              |
| Type VI collagen α 2     | S21369                 |

**TABLE II**

A list of additional secreted proteins identified by LC-MS/MS from adipocyte supernatants
CONCLUSIONS

Proteomic methods using mass spectrometry have now been used to characterize several protein complexes such as the spliceosome (74) or for a systematic identification of proteins from organelles such as the Golgi apparatus and mitochondria, among others (75, 76). Mass spectrometry-derived data can also be used for annotation of sequence databases to identify secreted proteins and to determine their tissue expression (77). We have shown that a simple one-dimensionalelectrophoresis can be coupled with mass spectrometric identification of secreted proteins that are up- or down-regulated during adipogenesis. Further, LC-MS/MS can be used to identify secreted proteins in a high throughput and automatable fashion. We have identified several proteins that are involved intimately in adipose tissue biology, validating our approach. In addition, we have validated the increased expression of some proteins that were known to be up-regulated at the mRNA level. Finally, we have identified several other secreted molecules whose function in adipogenesis is not yet characterized. This strategy can be adopted easily to find novel factors whose secretion from cells is regulated by cytokines, growth factors, or drugs. Quantitation of changes in protein levels will require different strategies such as addition of isotope-coded affinity tags after harvesting of proteins (57) or labeling of growing cells in culture with stable isotope-

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