A 12.5-kDa cysteine-rich adipose tissue-specific secretory factor (ADSF/resistin) is a novel secreted protein rich in serine and cysteine residues with a unique cysteine repeat motif of CX_12CXC_6CXX_8CXC_6CXX_6CC. A single 0.8-kilobase mRNA coding for this protein was found in various murine white adipose tissues including inguinal and epididymal fats and also in brown adipose tissue but not in any other tissues examined. Two species of mRNAs with sizes of 1.4 and 0.8 kilobases were found in rat adipose tissue. Sequence analysis indicates that this is because of two polyadenylation signals, the proximal one with the sequence AATAAA and the distal consensus sequence AATAAA. The mRNA level was markedly increased during 3T3-L1 and primary preadipocyte differentiation into adipocytes. Its expression in adipose tissue is under tight nutritional and hormonal regulation; the mRNA level was very low during fasting and increased 25-fold when fasted mice were refed a high carbohydrate diet. It was also very low in adipose tissue of streptozotocin-diabetes and increased 25-fold upon insulin administration. Upon treatment with the conditioned medium from COS cells transfected with the expression vector, conversion of 3T3-L1 cells to adipocytes was inhibited by 80%. The regulated expression pattern suggesting this factor as an adipose sensor for the nutritional state of the animals and the inhibitory effect on adipocyte differentiation implicate its function as a feedback regulator of adipogenesis.

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Adipose tissue is the major energy reservoir in higher eukaryotes; storing triacylglycerol in periods of energy excess and its mobilization during energy shortage are its primary purposes. During adipose tissue development, genes that code for the lipid transport and lipogenic and lipolytic enzymes are induced to carry out the adipocyte function of triacylglycerol synthesis, storage, and mobilization. For the past decades, in vitro systems including preadipocyte cell lines such as 3T3-L1 cells as well as primary preadipocytes in culture have been extensively used (1–3). Transcriptional activation of adipocyte genes has been the focus of much research. CCAAT enhancer-binding protein (C/EBPα) and PPARγ have been shown to be critical in directing adipocyte-specific gene expression and adipogenesis (4–7). In animals, the activities of critical enzymes in triacylglycerol biosynthesis and lipolysis are tightly controlled by nutritional and hormonal conditions (8). For example, feeding causes an induction whereas fasting causes suppression of lipogenic enzymes. Elevated insulin over a high carbohydrate diet feeding is thought to induce these enzymes in lipogenesis. The role of insulin can also be demonstrated by administration of insulin to diabetic animals. These enzymes have also been shown to be expressed at a high level in the adipose tissue of animal obesity models including ob/ob and db/db mice as well as Zucker rats (9). Hyperinsulinemia may be responsible for elevated levels of the enzymes.

The role of adipose tissue mainly as an organ for energy storage and mobilization has recently been expanded by the discovery of leptin (10, 11). Leptin is primarily made and secreted by mature adipocytes. It binds to its receptor in the hypothalamus and may function in regulating body fat mass (12, 13). Other immune system-related proteins such as TNF-α, adipin, and ACRP30/AdipoQ along with vascular function-related molecules such as angiotensinogen and plasminogen activator inhibitor type I have been shown to be secreted by adipose tissue (14–16). In addition, adipocytes also secrete factors such as Pref-1, which inhibits adipocyte differentiation (17–19). Although the precise functions of these molecules are not clear, adipose tissue as a secretory organ to regulate other physiological processes as well as energy balance and homeostasis is now well established. Adipose tissue must secrete factors reflecting the nutritional status and regulating adipose tissue mass.

We report here the identification and function of a serine/cysteine-rich adipocyte-specific secretory factor (ADSF) that does not belong to known classes of cysteine-rich proteins. Its mRNA is expressed only in adipose tissue. The mRNA is induced markedly during differentiation of 3T3-L1 and primary preadipocytes. In fasted or diabetic animals, its expression is very low or non-detectable in adipose tissue but increases markedly upon feeding or insulin administration. Furthermore, when treated with the conditioned medium from COS cells transfected with the expression vector, adipose conversion of 3T3-L1 cells was inhibited, indicating its potential role as a feedback regulator of adipogenesis. During the submission of this manuscript, the Lazar laboratory (20) reported this protein as a TZD down-regulated factor contributing to insulin resistance.

EXPERIMENTAL PROCEDURES

GeneFilter Microarray Analysis—Adipose tissue-specific genes were examined by microarray analysis using rat GeneFilter membranes (R-protein; SCD, stearoyl CoA desaturase; DMEM, Dulbecco’s modified Eagle’s medium; TZD, thiazolidinediones; bp, base pairs; nt, nucleotide; HA, hemagglutinin; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; EST, expressed sequence tag.
search Genetics). Filters were hybridized with α-32P-labeled cDNAs synthesized with 5 μg of total RNA. Spots exclusively hybridized with cDNA probes prepared from adipose tissue RNA were used for sequence analysis.

Animal Treatments—Mice were fasted for 48 h, or fasted mice were refed a 58% carbohydrate fat-free diet (21). Induction of diabetes and insulin treatments were carried out as described previously (21).

RESULTS AND DISCUSSION

Identification of cDNA Sequence—To identify novel genes that are only expressed in adipocytes and are induced during adipocyte differentiation, we compared expression levels of rat expressed sequence tag (EST) sequences by cDNA microarray. RNA samples were prepared from white adipose tissue as well as brown adipose tissue, liver, muscle, and brain. The RNAs were used to synthesize cDNAs for hybridization, and those sequences that were expressed only in white and brown adipose tissues were identified. Candidate EST clones were sequenced, and one such sequence was identified as that coding for a novel adipose-tissue-specific, serine- and cysteine-rich secreted protein. This gene was originally named as FIZZ3, which belongs to a gene family whose founding member, FIZZ1, is implicated as a possible mediator of neuronal function and airway hyperactivity (25). While this manuscript was being reviewed, the protein was also identified as a TGR down-regulated adipocyte protein, resistin, which may contribute to insulin resistance (20). From now on, we refer to the novel serine/cysteine-rich adipocyte-specific secretory factor as ADSF/resistin.

The cDNA sequence of rat ADSF/resistin is shown in Fig. 1A. It reveals a 1174-bp cDNA with two potential polyadenylation signal sequences, the proximal one at 524 nt and the distal one at 1149 nt. Northern blot analysis revealed two mRNAs with sizes of 1.4 and 0.8 kb found in rat adipose tissue with the differences in size attributed to the 3′-untranslated region. In murine fat tissues, on the other hand, we detected only one mRNA, which corresponds to the shorter rat mRNA. When we sequenced murine cDNA, we found that unlike the rat cDNA, the mouse homolog was shorter in the 3′-untranslated region with only one polyadenylation signal sequence corresponding to the rat proximal sequence. The mRNA species detected in Northern blot analysis and sequence analysis indicate that the difference in the size of rat and murine mRNAs is in the 3′-untranslated region. Interestingly, mouse cDNA sequence contains a consensus polyadenylation signal AATAAA. In contrast, rat cDNA sequence reveals the proximal polyadenylation signal AATACAA, with a single base mismatch, which probably is a weak signal and therefore causes the generation of the longer mRNA using the distal polyadenylation signal of AATAAA. Overall between rat and murine sequences there is 68% homology in nucleotide sequence (85 and 43% in coding and noncoding regions, respectively). The open reading frame encodes a 114 amino acid protein with a calculated molecular mass of 12.5 kDa and 75% homology between the rat and mouse proteins. The human homolog was shorter in the 3′-untranslated region with only one polyadenylation signal sequence corresponding to the rat proximal sequence. The mRNA species detected in Northern blot analysis and sequence analysis indicate that the difference in the size of rat and murine mRNAs is in the 3′-untranslated region.

Preparation of the Stromal Vascular Fraction from Rat Adipose Tissue and Primary Cell Culture—The adipose-derived stromal vascular fractions from rats were prepared as has been described previously (23). Briefly, the subcutaneous inguinal fat deposits from female Zucker rats were dissected and the lymph nodes were removed. The stromal vascular cells were obtained by collagenase (Sigma, 540 units/mg) digestion at 1 mg/ml at 37 °C for 45 min in Hepes-phosphate buffer (10 mM HEPES, pH 7.4, 135 mM NaCl, 2.2 mM CaCl2, 1.25 mM MgSO4, 0.45 mM KH2PO4, 2.17 mM Na2HPO4·5H2O, and 2% w/v bovine serum albumin). The cell suspension was filtered through a 10μm nylon filter and centrifuged at 400 × g for 10 min. The pellets were washed, filtered through a 25 μm nylon filter, and plated at a density of 2.5 × 104 cells/cm2 in DMEM, 10% FBS plus Dex and MIX. After 72 h, the medium was replaced by DMEM, 10% FBS plus insulin only.

Transient Transfection in COS cells—The pcDNA3.1 expression vector was transiently transfected into COS cells using DEAE-dextran in DMEM with 10% serum plus (JRH Biosciences) as described previously (24). Twenty-four hours after transfection, the medium was changed to DMEM supplemented with 10% FBS. The conditioned medium was collected 72 h after transfection, centrifuged at 500 × g for 5 min, and stored at 4 °C for less than a week before use.

Identification of mRNA and Northern Blot Analysis—The total RNA from the rat tissues was prepared by guanidine isothiocyanate/cesium chloride centrifugation. The total RNA from the cells prepared using Trizol reagent (Life Technologies, Inc.). RNA was electrophoresed in 1% formaldehyde-agarose gel in 2.2M formaldehyde, 20 m M MOPS, 1 mM EDTA, and transferred to Hybond N (Amerham Pharmacia Biotech). After UV cross-linking, the membranes were hybridized with the α-32P-labeled cDNA probes in ExpressHyb solution (CLONTECH). The membranes were exposed to x-ray film with an intensifying screen, and the signals were scanned using the Molecular Analyst (Bio-Rad). Western Blot Analysis—Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride for 30 min on ice. The protein content was determined by Bradford assay (Bio-Rad). Thirty μg of protein were subjected to SDS-polyacrylamide gel electrophoresis, electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore), and immunodetected using mouse anti-HA antiseraum (Covance) and goat anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad) by using an enhanced chemiluminescence detection kit (Bio-Rad).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)—Total RNA was reverse transcribed at 37 °C for 60 min, and the products were used as the template for two PCR reactions in tube containing target genes with actin as an internal control. The primers were used PPARα (PCR product of 375 bp in length), 5′-ATGCCATTCTGGCCCACCAAAC-3′ and 5′-CTTGTCACTTCTCCACAGCAT-3′; aFABP (PCR product of 310 bp in length) 5′-CTTGTTCCTCGAGAATACT-3′ and 5′-ACCT-CTCTGGTTTTCTCTAT-3′; FAS (PCR product of 120 bp in length), 5′-AAGGCGGTCGGACTTCATGAAAGAT-3′ and actin (PCR product of 282 bp in length), 5′-TCTCTAGTTGTTGATATGGAAG-3′.
FIG. 1. Comparison of structure between mouse and rat ADSF/ Resistin and its expression. A, coding sequences are capitalized. Lines indicate identical residues, and the possible polyadenylation signals are boxed. Full and partial corresponding nucleotide sequences are in the TIGR database under the following tentative consensus sequence numbers; mouse DNA sequence, TC148909 and rat DNA sequence, TC119941, respectively. B, an alignment of the predicted amino acids from mouse and rat using the ClustalW program is shown. C, Northern blot analysis of rat and mouse mRNAs in adult white adipose tissues. Positions of 28 S and 18 S ribosomal RNA are shown. Cell lysates and conditioned medium from COS cells transiently transfected with HA-tagged expression vector, lysate from control vector transfected cells, were markedly increased during differentiation into adipocytes. As anticipated, expression of aFABP (aP2) was induced during adipocyte conversion, whereas actin expression was decreased by ~50%. We also carried out in vitro differentiation of stromal vascular cells isolated from rat adipose tissue. Differentiation of adipocyte precursor cells isolated from the epididymal fat pads may better mimic in vivo processes of adipogenesis. Two species (1.4 and 0.8 kb) of rat mRNAs were undetectable in freshly plated preadipocytes of stromal vascular fractions from fat pads were subjected to in vitro adipocyte differentiation. RNA prepared from cells at the indicated time points of 0 (at confluence), day 1, day 3, day 4, and day 6 were subjected to Northern blot analysis for ADSF/resistin, aFABP, SCAR and PPARγ. C, Two different preparations of stromal vascular fractions from fat pads were subjected to in vitro adipocyte differentiation. RNA prepared from cells at the indicated time points of 0 (at confluence), day 0.5, day 2.5, and day 9 were subjected to Northern blot analysis for ADSF/resistin, aFABP, SCAR and PPARγ.

Inclusion of brain, heart, small intestine, kidney, liver, lung, and skeletal muscle. A single 0.8-kb mRNA was detected only in murine adipose tissue (data not shown). We also detected the two mRNAs at similar levels in all regions of rat white adipose tissues including epididymal and inguinal fat pads. Interestingly, these mRNAs were also found in brown adipose tissue although in lower amounts than in white adipose tissue. We next fractionated adipose tissue into stromal vascular fractions, which mainly contain preadipocytes and adipocyte fractions. We found that unlike in adipocyte fractions, these mRNAs were not detectable in stromal vascular fractions suggesting that ADSF/resistin expression may be induced during adipogenesis.

Induction of ADSF/Resistin During Adipocyte Differentiation—To determine whether ADSF/resistin expression is regulated during adipocyte differentiation, mRNA levels were examined in 3T3-L1 preadipocytes during differentiation into adipocytes (Fig. 2B). The ADSF/resistin mRNA was not detectable in the preadipocyte stage (Day 0). But levels of the 0.8-kb murine mRNA, as predicted by the murine origin of 3T3-L1 cells, were markedly increased during differentiation into adipocytes. As anticipated, expression of aFABP (aP2) was induced during adipocyte conversion, whereas actin expression was decreased by ~50%. We also carried out in vitro differentiation of stromal vascular cells isolated from rat adipose tissue. Differentiation of adipocyte precursor cells isolated from the epididymal fat pads may better mimic in vivo processes of adipogenesis. Two species (1.4 and 0.8 kb) of rat mRNAs were undetectable in freshly plated preadipocytes of stromal vascular fractions, but their expression was increased dramatically during adipocyte conversion initiated by Dex and MIX treatment. The increase in mRNA levels during adipogenesis of both 3T3-L1 and primary preadipocytes was similar to or somewhat later than that of other late adipocyte markers, such as adipocyte fatty acid-binding protein and stearyl CoA reductase. The results of 3T3-L1 and primary preadipocytes in culture along with its tissue distribution clearly show that the mRNA levels increase during late stages of adipocyte tissue development, and therefore ADSF/resistin is expressed only in adipose tissue in mature rodents.

Additional figures and tables are located in the Supplemental Data.
...tion of insulin. We tested whether the observed increase in mRNA levels during fasting/refeeding is caused by an increase in insulin secretion. We utilized streptozotocin-diabetic mice. The mRNA levels were low in the adipose tissue of diabetic mice (Fig. 3B). Upon insulin administration, the mRNA level was increased 23-fold after 30 min. As predicted, FAS mRNA levels increased 15-fold after 30 min. Actin mRNA levels did not change appreciably by fasting/refeeding or by diabetes/insulin administration. These results indicate that ADSF/resistin is regulated in a fashion similar to the lipogenic enzymes, fatty acid synthase. However, unlike the lipogenic enzymes, which are induced both in liver and adipose tissue during feeding and by insulin, ADSF/resistin is expressed only in adipose tissue and induced by nutrition and insulin.

ADSF/Resistin Inhibits Adipocyte Differentiation—Given that it is expressed only in adipose tissue and highly induced when the animals are in a fed state, we predicted that ADSF/resistin might promote adipocconversion of preadipocytes. Our hypothesis is that this protein may be a signal to generate adipocytes for the increased capacity to store excess energy. We collected medium from COS cells transfected with expression vector containing HA-tagged full-length mouse cDNA. The condition medium was added to differentiating 3T3-L1 cells to test their effect on adipogenesis. 3T3-L1 cells maintained in conditioned medium collected from COS cells transiently transfected with control vector differentiated into lipid-laden adipocytes as shown in oil red O staining (Fig. 4A). Unexpectedly, those cells maintained in medium from COS cells transfected with HA-tagged expression vector did not undergo extensive adipocyte conversion as judged by lipid staining. Similarly, expression of adipocyte markers, PPARγ, aFABP, and fatty acid synthase was decreased by 80% when cells were treated with conditioned medium from COS cells transfected with HA-tagged expression vector (Fig. 4B). The actin mRNA level of these cells was somewhat higher, as expected. These results clearly demonstrate an inhibitory effect of ADSF/resistin on adipocyte conversion.

Mature adipocytes, the main cellular components of adipose tissue, are uniquely equipped to function in energy storage and balance under tight hormonal control. However, with the recent realization that adipocytes secrete factors known to play a role in appetite control, immune response, and vascular disease, a much more complex and dynamic role for adipose tissue has emerged. The best known example is leptin, a hormone that is primarily made and secreted by mature adipocytes to regulate adipose fat mass. However, several adipocyte-specific factors with unknown functions including adipin, acylation stimulation protein, and Acrp30/AdipoQ are secreted along with other well understood factors such as TNF-α, angiotensinogen, and plasminogen activator inhibitor type-I. Most of these factors appear to be related to immune or vascular functions. These examples clearly establish that the adipocytes behave as endocrine as well as paracrine/autocrine cells. The exact role of the ADSF/resistin secreted from adipose tissue is not yet known. It is composed of cysteine-rich domain with unique cysteine spacing and may potentially participate in protein-protein interaction. It is exclusively made in adipose tissue and is secreted to the medium. Its exclusive expression in adipocytes, its large increase during the late stage of adipogenesis, and its dramatic induction during fasting/refeeding and by insulin administration to streptozotocin-diabetic animals suggest that this factor may be involved in sensing the nutritional status of the animals to affect adipogenesis. Some of these properties are most similar to those observed with leptin, which is secreted only by adipocytes and is induced dramatically by fasting/refeeding and by diabetes/insulin. We speculate that this factor may be a molecule that serves as a feedback signal to restrict adipose tissue formation. It is also possible that it functions in an opposite manner to known differentiation agents such as DEX, MIX, and insulin during adipocyte differentiation. The recent report of ADSF/resistin as a TZD down-regulated adipose tissue factor, which may antagonize insulin action by linking obesity to diabetes, is also intriguing and needs further study.

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