Communication

A Novel Serum Protein Similar to C1q, Produced Exclusively in Adipocytes*

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We describe a novel 30-kDa secretory protein, Acrp30 (adipocyte complement-related protein of 30 kDa), that is made exclusively in adipocytes and whose mRNA is induced over 100-fold during adipocyte differentiation. Acrp30 is structurally similar to complement factor C1q a hibernation-specific protein isolated from the plasma of Siberian chipmunks; it forms large homo-oligomers that undergo a series of post-translational modifications. Like adipsin, secretion of Acrp30 is enhanced by insulin, and Acrp30 is an abundant serum protein. Acrp30 may be a factor that participates in the delicately balanced system of energy homeostasis involving food intake and carbohydrate and lipid catabolism. Our experiments also further corroborate the existence of an insulin-regulated secretory pathway in adipocytes.

Insulin-induced glucose transport occurs in heart, striated muscle, and fat tissue. In adipocytes, glucose uptake increases 20- to 30-fold in the presence of insulin. Glucose transport is mediated by the sodium-independent facilitative glucose transporters GLUT1 and GLUT4, which, in response to insulin, translocate from an intracellular compartment to the plasma membrane (1, 2). GLUT4, which is expressed only in fat and skeletal and cardiac muscle, is the primary transporter involved in this process and is the predominant transporter expressed in these tissues (3, 4). Insulin also causes translocation of several receptor proteins from intracellular membranes to the plasma membrane (5-7). Adipocytes are a principal storage depot for triglycerides and express a specific transport protein allowing them to import free fatty acids (8).

Adipocytes also secrete several proteins potentially important in homeostatic control of glucose and lipid metabolism. Adipsin, equivalent to Factor D of the alternative complement pathway (9), is synthesized exclusively in adipocytes, and its secretion is enhanced severalfold by insulin (10). The function of adipsin in fat cell biology is not known, nor are the roles of complement factors C3 and B that are also secreted by adipocytes (11). Tumor necrosis factor α, also secreted by adipocytes, is a key mediator of insulin resistance in animal models of non-insulin-dependent diabetes mellitus. Tumor necrosis factor α directly interferes with the signaling of insulin through its receptor and consequently blocks biological actions of insulin including insulin-stimulated glucose uptake (reviewed in Ref. 12). Adipocytes are the only cell type known to secrete the ob gene product (13). In the absence of db (db/db) or ob (ob/ob) mice, its presumed receptor, the ob gene product (db/ob) mice the mice overeat and become obese and diabetic (14). Here we describe another novel protein, Acrp30,1 that is exclusively synthesized in adipose tissue and whose synthesis in a serum. Like adipsin, secretion of Acrp30 is enhanced severalfold by insulin. While we do not know the function of this protein, its sequence and structural resemblance to complement factor C1q is intriguing. Importantly, our experiments confirm the existence of an insulin-regulated secretory pathway in adipocytes.

EXPERIMENTAL PROCEDURES

Cloning of Acrp30 and DNA Analysis—A full-length cDNA library templated by mRNA from 3T3-L1 adipocytes at day 8 of differentiation (15) was screened with a digoxigenin-labeled cDNA fragment obtained from the random sequencing screen. Labeling, hybridization, and detection were performed according to the manufacturer’s instructions (Boehringer Mannheim). One of the positive clones obtained was subjected to automated sequencing on a Applied Biosystems 373-A Sequencer. The entire 1.3-kb insert was sequenced at least 2 independent times on one and once on the complementary strand. Sequence analysis was performed with the DNASTAR package and showed an open reading frame of 741 bp encoding a protein of 28 kDa. The sequence has been submitted to GenBank™ and has the accession number U37222. Homology searches were performed at NCBI using the BLAST network service, and alignments were performed with the Megalign program from DNASTar using the Clustal algorithm. Only the globular domain for the type X collagen was used for the alignment (residues 562-680).

Generation of Specific Antibodies—A peptide corresponding to the sequence, EDDVTTTEELAPALV (residues 18-32), was used to generate specific anti-Acrp30 antibodies in rabbits (multiple antigen peptide technology, Research Genetics).

mRNA Isolation and Analysis—Isolation of mRNA from tissues and from 3T3-L1 cells at various stages of differentiation was as described in Ref. 13, as was 32P labeling of DNA, agarose gel electrophoresis of mRNA, and its transfer to nylon membranes.

Pulse-Chase Experiments and Immunoprecipitations—3T3-L1 adipocytes were starved for 30 min in Dulbecco’s modified Eagle’s medium (ICN) lacking cysteine and methionine and then labeled for 10 min in the same medium containing 0.5 μCi/ml Express Protein Labeling Reagent (1000 Ci/mmol) (DuPont NEN). The cells were then washed twice with Dulbecco’s modified Eagle’s medium supplemented with unlabeled cysteine and methionine, and then fresh growth medium containing 300 μM cycloheximide was added. At the indicated time points, the medium was collected. Insoluble material from the medium was removed by centrifugation (15,000 × g for 10 min); the supernatant was then precipitated with 50 μl of Protein A-Sepharose for 10 min at 4°C and then immunoprecipitated with 50 μl of affinity-purified anti-Acrp30 antibody for 2 h at 4°C. Immunoprecipitates were washed 4 times in lysis buffer (1% Triton X-100, 60 mM octyl glucoside, 80 mM NaCl, 20 mM Tris, pH 8.0, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis).

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1 The abbreviations used are: Acrp30, adipocyte complement-related protein of 30 kDa; Endo H, endoglucosidase H; BS3, bis(sulfosuccinimidyl)suberate; kb, kilobase(s); bp, base pair(s); PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
Acrp30, a Novel Serum Protein Similar to C1q

**FIG. 1.** A, predicted structure of Acrp30. The protein consists of an amino-terminal signal sequence (SS) followed by a sequence of 27 amino acids lacking significant homologies to any entries in the GenBank™ database. This region is followed by a stretch of 22 collagen repeats with 7 "perfect" Gly-X-Pro repeats (dark hatched boxes) clustered at the beginning and end of the domain interspersed with 15 "imperfect" Gly-X-Y repeats (light hatched boxes). The C-terminal 137 amino acids probably form a globular domain. B, alignment of the amino acid sequences of Acrp30, Hib27, a member of the hibernation-specific protein family; C1q-C, one of the subunits of complement C1q; Coll type X, the globular domain of the type X collagen. Conserved residues are shaded. For simplicity, other members of each family are not shown, but shaded conserved residues are in most instances conserved within each protein family. Only the globular domain for the type X collagen was used for the alignment (residues 562–693). C, Northern blot analysis of Acrp30 expression. The left panel shows poly(A) RNA isolated from various tissues probed with the full-length Acrp30 cDNA. The predominant Acrp30 mRNA is 1.4 kb and is expressed only in adipose tissue and cultured 3T3-L1 adipocytes. Overexposure of the autoradiogram does not reveal expression in any other tissue. The right panel shows induction of the Acrp30 message during differentiation of 3T3-L1 fibroblasts to adipocytes. Induction of Acrp30 occurs primarily between days 2 and 4 of differentiation, the same time as induction of the insulin receptor and the insulin-responsive glucose transporter GLUT4. Numbers on the left indicate molecular mass standards (in kb). Equal loading of RNA was documented by probing the stripped filter with a cDNA encoding the cytosolic hsp70 protein (lower panel).
intracellular and extracellular 35S-labeled Acrp30. Indeed, judged by densitometric scanning of the immunoprecipitates of Acrp30 is secreted into the medium at 2.5 to 3 h of chase as 3T3-L1 adipocytes unstimulated by insulin, 50% of newly made or Golgi compartments, by analogy to a similar modification in the structurally related mannann-binding protein (23). In 3T3-L1 adipocytes unstimulated by insulin, 50% of newly made Acrp30 is secreted into the medium at 2.5 to 3 h of chase as judged by densitometric scanning of the immunoprecipitates of intracellular and extracellular 35S-labeled Acrp30. Indeed, Acrp30 can be detected by Western blotting in normal mouse serum. A protein of the identical molecular weight can be detected by Western blot analysis of 3T3-L1 adipocytes (not shown). The anti-peptide antibody is specific for the mouse homologue, as it does not cross-react with bovine, human, or rabbit serum (Fig. 2).

To examine effects of insulin on Acrp30 secretion, we monitored the discrete population of newly made protein generated in a short pulse with labeled amino acids followed by inhibition of further protein synthesis by cycloheximide. This offers increased sensitivity compared to examining secretion of the entire cellular complement of Acrp30, particularly in light of the very long t1/2 for secretion of Acrp30. Fig. 3 shows that, during the first 60 min of chase, insulin causes a 4-fold increase in secretion of newly made Acrp30. After 60 min, the rates of Acrp30 secretion are the same in unstimulated and insulin-stimulated cells. Similarly, insulin causes a 4-fold increase in adipin secretion during the first 30 min of chase, but, afterwards, the rate of adipin secretion is the same in control and insulin-treated cells (Fig. 3 and Ref. 10). The ability of insulin to abolish the lag in adipin secretion has been seen in several separate experiments. We hypothesize that a fraction of newly made adipin and Acrp30 are sorted, probably in the trans-Golgi reticulum, into regulated secretory vesicles whose exocytosis is induced (in an unknown manner) by insulin, whereas the balance is sorted into vesicles that are constitutively exocytosed. Partial sorting of protein hormones into regulated secretory vesicles has been seen in other types of cultured cells (24, 25). We do not know how insulin causes an increase in protein secretion; insulin could cause a more efficient overall processing of secretory proteins in 3T3-L1 adipocytes. We are currently isolating other adipocyte-specific secretory proteins to study this process in detail.

Complement factor C1q consists of three related polypeptides that form heterotrimeric subunits containing a three-stranded collagen "tail" and three globular "heads"; six of these subunits generate an 18-mer complex often referred to as a "bouquet of flowers" (reviewed in Ref. 26). The experiments in contains one potential N-glycosylation site, within the collagen domain, but apparently is not glycosylated; Endo H treatment did not cause a shift in molecular mass of Acrp30 at any time during a metabolic pulse-chase experiment (not shown). Acrp30 does become modified post-translationally, since, after 20 min of chase, there was a small but reproducible reduction in gel mobility. This most likely represents hydroxylation of collagen domain proline residues in the endoplasmic reticulum or Golgi compartments, by analogy to a similar modification in the structurally related mannann-binding protein (23).

FIG. 2. Acrp30 is a secretory protein found in blood. Acrp30 can be detected by Western blotting in serum from mice; the antibody does not cross-react with calf, human, or rabbit serum. One microliter of fetal calf, rabbit, mouse, and human serum was boiled for 5 min in 2 × sample buffer and analyzed by SDS-PAGE and Western blotting with the anti-Acrp30 antibody according to standard protocols. Antibody was visualized with an anti-rabbit IgG antibody coupled to horseradish peroxidase using a chemiluminescence kit from DuPont NEN.

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disulfide-bonded together (Fig. 4B), similar to other proteins containing a collagen domain, including the macrophage scavenger receptor (27). Besides being a homo-oligomer, Acrp30 differs from C1q in containing an uninterrupted stretch of 22 perfect Gly-X-X repeats; this suggests that Acrp30 has a straight collagen stalk as opposed to the characteristic kinked collagen domain in C1q caused by imperfect Gly-X-X repeats in two of the three subunits (26).

**DISCUSSION**

We do not yet know the function of Acrp30. However, its expression exclusively in adipocytes, its enhanced secretion by insulin, and its presence in normal serum, suggests that it is, like the ob protein, involved in the control of the nutritional status of the organism. Acrp30 is a relatively abundant serum protein, accounting for up to 0.05% of total serum protein as judged by quantitative Western blotting using recombinant Acrp30 as a standard (data not shown). Even though we have no evidence at this stage, we cannot exclude the possibility that Acrp30, like C3-complement released by adipocytes (28), is converted proteolytically to a bioactive molecule.

Our experiments also corroborate the existence of a regulated secretory pathway in adipocytes. We do not yet know whether adipin and/or Acrp30 are in the same intracellular vesicles that contain GLUT4 and that fuse with the plasma membrane in response to insulin, or whether they are in different types of vesicles. Adipocytes express two members of the Rab3 family, Rab3A and Rab3D (29); these are found in vesicles of different density. Rab3B are small GTP-binding proteins involved in regulated exocytic events. Except for adipocytes, Rab3A is found only in neuronal and neuroendocrine cells; in neurons, Rab3A is localized to synaptic vesicles and is important for their targeting to the plasma membrane (30). An attractive hypothesis under test is that, in adipocytes, Rab3A is localized to vesicles containing Acrp30 and/or adipin, and that possibly Rab3B mediates insulin-triggered exocytosis of vesicles containing GLUT4. In any case, the mechanism of signal transduction from the insulin receptor to regulated exocytosis of intracellular vesicles remains an important unsolved problem.

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**FIG. 4A.** Incubation of 35S-labeled 3T3-L1 culture supernatant with increasing amounts of the BS3 cross-linking reagent, followed by immunoprecipitation with Acrp30-specific antibodies, reveals a set of cross-linked products whose molecular sizes are multiples of 30 kDa. Predominant species are trimers, hexamers, and a high molecular mass species (asterisk) that correspond to a nonamer or a dodecamer. In the lane Total, 1% of the amount of cell medium used for the cross-linking reactions was analyzed on the same gel; a comparison of the “Total” lane and lane 3 demonstrates the specificity of the antibody used for immunoprecipitation. Immuno precipitates were analyzed by gradient SDS-PAGE (7–12.5% acrylamide) followed by fluorography. Arrowheads (Amersham) together with a phosphorylase ladder (Sigma) were used as molecular mass markers. B, reducing and nonreducing SDS-PAGE of anti-Acrp30 immunocomplexes isolated from 35S-labeled 3T3-L1 medium. Medium from day 8 3T3-L1 adipocytes labeled overnight with [35S]methionine and cysteine were immunoprecipitated with anti-Acrp30 antibodies as described under “Experimental Procedures.” The sample was subjected to SDS-PAGE (7–12.5% acrylamide gradient) in the presence (reducing) or absence (nonreducing) of 50 mM dithiothreitol. Labeled proteins were detected by fluorography. C, velocity gradient centrifugation of mouse serum displays two discrete Acrp30-immunoreactive species. The smallest corresponds to a trimer of Acrp30 polypeptides and the larger a nonamer or dodecamer. One microliter of mouse serum was diluted with 50 μl of PBS and layered on top of a 4.5-ml linear 5–20% sucrose gradient in PBS and centrifuged for 10 h at 60,000 rpm in a SW60 rotor of a Beckman ultracentrifuge. Thirteen 340–μl fractions were collected from the top and analyzed by SDS-PAGE and Western blotting using anti-Acrp30 antibodies. An identical gradient was run in parallel with a set of molecular mass standards: cytochrome c (14 kDa), carboxy anhydrase (29 kDa), bovine serum albumin (68 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and apoferritin (443 kDa). The positions of these markers are indicated below the panel with hammer (Amersham) together with a phosphorylase ladder (Sigma).