

The Adipsin-Acylation Stimulating Protein System and Regulation of Intracellular Triglyceride Synthesis

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Abstract

We have previously characterized an activity from human plasma that markedly stimulates triglyceride synthesis in cultured human skin fibroblasts and human adipocytes. Based on its in vitro activity we named the active component acylation stimulating protein (ASP). The molecular identity of the active serum component has now been determined. NH₂-terminal sequence analysis, ion spray ionization mass spectroscopy, and amino acid composition analysis all indicate that the active purified protein is a fragment of the third component of plasma complement, C3a-desArg. As well, reconstitution experiments with complement factors B, D, and complement C3, the components necessary to generate C3a, have confirmed the identity of ASP as C3a. ASP appears to be the final effector molecule generated by a novel regulatory system that modulates the rate of triglyceride synthesis in adipocytes. (*J. Clin. Invest.* 1993; 92:1543-1547.) Key words: alternate complement pathway • hyperapobetalipoproteinemia • fibroblast

Introduction

In the course of studies to determine the metabolic defect in patients with hyperapobetalipoproteinemia (HyperapoB)¹ (1), we observed that adipocytes from affected individuals synthesized triglycerides less rapidly than did adipocytes obtained from normals (2). When triglyceride synthesis was measured in skin fibroblasts cultured from individuals in both groups, these differences were confirmed and shown to be due to a difference in response to a serum protein. Purification of the protein was undertaken and a single band on SDS gel electrophoresis obtained. The protein had an apparent molecular

weight of 14,000, a pI of 9.0, and, based on its in vitro activity, was named acylation stimulating protein (ASP) (3).

ASP is much more potent than insulin in stimulating the esterification of fatty acids into intracellular triglyceride in human fibroblasts and adipocytes (3). The increase in triglyceride synthesis induced by ASP is achieved by a dual effect. That is, ASP causes translocation of glucose transporters from intracellular vesicles to the cell surface, thereby increasing specific membrane glucose transport (4). By contrast, membrane transport of fatty acids is not directly affected by ASP. However, net fatty acid uptake does increase secondary to stimulation of the enzyme, diacylglycerol acyltransferase, which controls the rate limiting step in the synthesis of a triglyceride molecule (5, 6). Moreover, in vitro studies demonstrated that reduced cell responsiveness to ASP was often present in cultured human skin fibroblasts obtained from patients with HyperapoB (7), findings that have been confirmed by other investigators (8). Obviously, it was essential to determine the precise identity of ASP and this has now been done. This paper will present evidence that ASP is, in fact, identical to a fragment of the third component of complement known as C3a-desArg. This finding, when taken with the work of Spiegelman and his colleagues (9-13), points to the presence of a novel system by which intracellular triglyceride synthesis may be regulated.

Methods

S-Sepharose Fast Flow, Sephadex G-75, Mono-S HR 5/5, and columns used for standard chromatography were obtained from Pharmacia AB (Uppsala, Sweden). μ Bondapak C18 reverse phase column was from Waters Instruments, Inc. (Milford, MA) and Vydac Protein C4 was from the Separations Group (Hesperia, CA). Trifluoroacetic acid (TFA) was obtained from Chromatographic Specialties Inc. (Brockville, Ont., Canada). HPLC grade acetonitrile (ACN), hexanes, ethyl ether, and acetic acid were obtained from Fisher Scientific (Montreal, P.Q., Canada). [9,10-³H(N)]-labeled oleic acid (10.0 Ci/mmol) was obtained from Dupont-New England Nuclear (Mississauga, Ont., Canada). Oleic acid (sodium salt) and essentially fatty acid-free BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Human factor D, B, and complement C3 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Dulbecco's MEM/F12 (D-MEM/F12), Dulbecco's PBS (D-PBS), and all other tissue culture supplies were from Gibco Laboratories (Burlington, Ont., Canada). Aqueous samples were concentrated with a stirred cell concentrator equipped with a YM-3 membrane from Amicon/W.R. Grace & Co., (Beverly, MA). Organic samples were evaporated in a centrifugal vacuum concentrator by Jouan/Canberra-Packard Industries (Toronto, Ont., Canada).

Purification of ASP from human plasma. ASP was purified from human plasma in five sequential chromatographic steps that were per-

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1. Abbreviations used in this paper: ACN, acetonitrile; ASP, acylation stimulating protein; D-MEM, Dulbecco's MEM; D-PBS, Dulbecco's PBS; HyperapoB, hyperapobetalipoproteinemia; TFA, trifluoroacetic acid.

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formed in the following order: Cation exchange on S-Sepharose Fast Flow, gel filtration on Sephadex G-75, reverse phase on a semipreparative Vydac Protein C4 (1.0 × 25 cm), cation exchange on Mono-S HR 5/5, and reverse phase on μ Bondapak C18 (3.9 × 300 mm). For each chromatographic step, the activity of the column fractions were tested for their ability to stimulate triglyceride synthesis in cultured human skin fibroblasts. The S-Sepharose and Sephadex G-75 steps were performed as previously described (7). The active fractions from S-Sepharose, which correspond to the second half of the elution peak monitored by absorbance at 280 nm, were pooled (fraction A, 99 ± 30 mg of protein, 274 ± 60 ml, $n = 8$), concentrated and fractionated on G-75. Those tubes with activity were pooled and concentrated to yield fraction B (7.4 ± 3.4 mg of protein, 13 ± 1.6 ml, $n = 8$) and stored at -80°C.

Vydac protein C4. Prior to reverse phase chromatography several batches of fraction B were prepared and pooled (10–50 mg total). The pooled material was loaded on a Vydac Protein C4 column, which was eluted with a linear gradient of 25–65% solvent A (80% ACN) containing 0.1% TFA throughout over 60 min at a flow rate of 3 ml/min and collected as 1-min fractions with protein elution monitored at 214 nm. The biologic activity eluted from this column between 37 to 42% solvent A (fraction C) and the pool was stored at -80°C.

Mono-S HR 5/5. Fraction C from the Vydac C4 was loaded directly on a Mono-S HR 5/5 FPLC column equilibrated in solvent B (10 mM Tris, 10 mM NaCl, pH 7.1, sterile) and eluted from the Mono-S column with a 35-min linear 0 to 1 M NaCl gradient in solvent B at a flow of 1 ml/min and fractionated as 0.5 min per tube. Protein elution was monitored at 214 nm and activity for each fraction tested. The activity that eluted from this column between 280 to 400 mM NaCl (fraction D) was pooled and stored at -80°C.

μ Bondapak C18. A μ Bondapak C18 column was used as a final purification step and was run using the same solvent system as for the Vydac Protein C4 column. After loading Fraction D on the column, bound proteins were eluted over 60 min with a linear gradient from 0 to 60% solvent A at a flow rate of 1 ml/min and collected as 1-min fractions. The biologic activity was found to elute at 38% solvent A (fraction E).

Amino acid analysis and protein quantification. Amino acid hydrolysis was used for characterization of the final purified material. Samples were hydrolyzed in 6N HCl vapor in vacuo at 150°C for 2 h using a Pico-Tag workstation (Waters Instruments, Inc.) and analyzed for amino acid composition on a model 6300A autoanalyzer (Beckman Instruments, Inc., Fullerton, CA).

Ion spray ionization mass spectroscopy. Ion spray mass spectra of ASP were obtained using an API III triple stage mass spectrometer with ion spray interface (SCIEX, Thornhill, Ont., Canada) located at the National Research Council of Canada—Montreal Biotechnology Research Institute (14).

Edman sequencing. The NH₂-terminal amino acid sequence of ASP was determined by subjecting the samples to gas-phase micro-sequencing on a gas phase sequencer (Porton Instruments, Inc., Tarzana, CA) located at the Sheldon Biotechnology Centre of McGill University.

Culture of human skin fibroblasts. Fibroblasts were obtained from forearm skin biopsies of normolipidemic subjects. Primary cultures were established from explants and maintained in MEM with 10% FCS supplemented with penicillin/streptomycin (100 IU/ml). Fibroblasts were subcultured every 7–10 d with a split ratio of 1:2 after a 10-min incubation with 0.25% trypsin in Mg²⁺ and Ca²⁺-free D-PBS to detach the cells from the flask. Cells were used for experiments between passages 5 and 15 plated out at a concentration of 1×10^4 cells/cm² in 24-well dishes in 1 ml of medium. At or near confluency, the day before experimentation, cells were changed to serum-free D-MEM/F12.

Measurement of biologic activity. The ability of column fractions to stimulate triglyceride synthesis was determined in cultured human skin fibroblasts as described previously (7). Aliquots of column fractions from reverse phase chromatography were dried in a centrifuge evaporator and reconstituted in D-PBS. Following an overnight preincubation in serum-free D-MEM/F-12, the media of the cells was replaced with

400 μ l/well of D-MEM/F12 supplemented with 125 μ M of [9,10-³H(N)]-labeled oleic acid (average sp act: 40 ± 10 DPM/pmol, $n = 11$) complexed to BSA (15) in a 5:1 molar ratio and 100 μ l/well of the test fraction aliquot. At the end of the incubation period (usually 18 h), the cells were washed three times with 1 ml ice-cold D-PBS and extracted with two 1-ml vol of heptane/isopropanol (3:2). The lipids in the organic extract were separated and quantified as described previously (3). Soluble cell protein was dissolved in 0.1 N of NaOH and measured by the method of Bradford (16), using a commercial kit (Bio-Rad Laboratories, Richmond, CA). BSA was used as protein standard. Each column fraction was assayed in duplicate or triplicate. Data is expressed as mean ± SD.

Biologic activity of in vitro generated C3a. C3a was generated enzymatically in vitro by incubating 100 μ g of C3 with 10 μ g of factor D, and 100 μ g of factor B in 2 mM of MgCl₂, as previously described (17). The mixture as well as each individual component, C3, factor B, and factor D were assayed for biologic activity as above. The presence of C3a in the mixture was confirmed and quantified, using a commercial RIA kit specific for C3a-desArg (Amersham International, Oakville, Ont., Canada). SDS-PAGE was performed by the method of Laemmli (18), using precast 5–20% gradient gels (Bio-Rad Laboratories).

Results

The purification of ASP is shown in Fig. 1. Fractionated plasma containing small basic proteins isolated by ion exchange and gel filtration (as previously described [7]) was further purified on a reverse phase HPLC Vydac C4 column, and bioactivity was measured as the capacity of each fraction to stimulate fatty acid esterification to form triglyceride in normal human skin fibroblasts (see Methods). The column profile is shown in Fig. 1 A, and the activity eluted between 37 and 42% of solvent A. Overall, recovery of activity averaged 60%. The active fractions (as highlighted in Fig. 1 A) were loaded onto an ion exchange Mono S column and fractionated with a salt gradient. Activity was recovered between 280 and 400 mM NaCl (Fig. 1 B). Finally, the active fraction of the Mono S column was chromatographed on a C18 column and the activity in

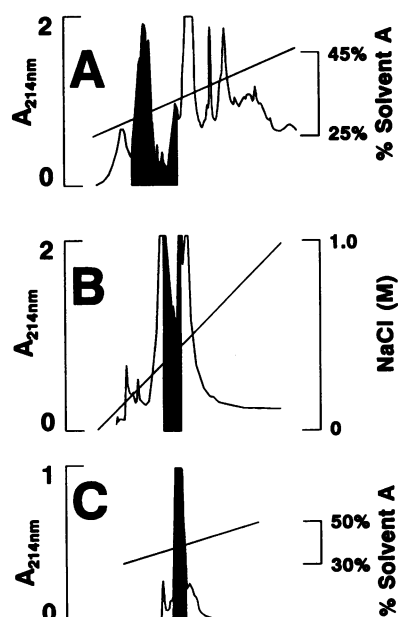


Figure 1. Purification of ASP. ASP was purified and assayed for biologic activity as described in Methods. Plasma was fractionated by cation exchange on S-Sepharose and gel filtration on Sephadex G-75, as previously described (7). Fraction B was resolved sequentially by reverse phase on Vydac Protein C4 (A), cation exchange on Mono-S HR 5/5 (B), and reverse phase on μ Bondapak C18 (C). Fractions were assayed for stimulation of incorporation of [³H]-oleate into intracellular triglycerides. Darkened areas indicate the fractions that contained biologic activity and that were pooled for the subsequent step. Normal fibroblasts were used for the bioassay. Protein elution was monitored at 214 nm. Solvent A is 80% ACN with 0.1% TFA.

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each fraction monitored. The activity is indicated in the profile in Fig. 1 C, and ASP constituted the active fraction isolated, which eluted at 38% solvent A.

ASP was characterized by amino acid terminal sequencing. The first 10 amino terminal amino acids yielded a sequence that exactly matched the sequence previously published for a fragment of complement C3, complement C3a (NH₂-Ser-Val-Gin-Leu-Thr-Glu-Lys-Arg-Met-Asp) (19). No other sequence was present in the active material. CNBr treatment, which cleaves at methionine, followed by Edman degradation amino terminal sequencing, did not reveal any unexpected sequences (i.e., any sequences other than those that are predicted from cleavage of C3a). Moreover, as shown in Table I, ASP amino acid composition matches that of C3a very closely. Of interest, ASP purified from plasma contains 10 Arg, as does C3a-desArg, whereas C3a itself contains 11. These results were confirmed when protein mass analysis was performed by ion spray ionization. This demonstrated that the mass of the protein was 8,933±0.3 mass units, a mass that corresponds not to C3a (9,088.7) but to C3a-desArg (8,932.5) (19).

C3a is generated through the concerted actions of complement B, C3, and D (20). We therefore obtained purified complement C3, B, and D and incubated them together in 2 mM MgCl₂ under conditions reported to generate C3a in vitro (17). This material was then tested for its ability to stimulate triglyceride synthesis in human skin fibroblasts. The results are shown in Fig. 2. The basal level of triglyceride synthesis in the fibroblasts was 29±3.3 nmol/mg cell protein. Addition of a partially purified preparation of ASP increased triglyceride synthesis to 55±6.6 nmol/mg cell protein, a 87% increase above basal. Addition of a mixture of B/C3/D resulted in a similar stimulation of triglyceride synthesis as seen with ASP. In contrast, addition of the individual components, complement C3, complement factor B, and factor D did not increase triglyceride synthesis when added at the same concentrations as in the

Table I. Amino Acid Composition

Amino Acid	ASP*	C3a [†]	C3a-desArg [‡]
Asx	4.9	5	5
Thr	2.8	3	3
Ser	3.4	4	4
Glx	9.0	9	9
Pro	2.1	2	2
Gly	4.1	4	4
Ala	3.9	4	4
Cys	6.1	6	6
Val	3.5	3	3
Met	2.7	3	3
Ile	2.1	2	2
Leu	6.9	7	7
Tyr	1.8	2	2
Phe	2.9	3	3
His	2.2	2	2
Lys	7.8	7	7
Arg	9.9	11	10

* The amino acid composition of ASP, determined as described in Methods, was compared to published compositions of C3a and C3a-desArg (18).

[†] Amino acid composition of C3a, as reported by T. Hugli (18).

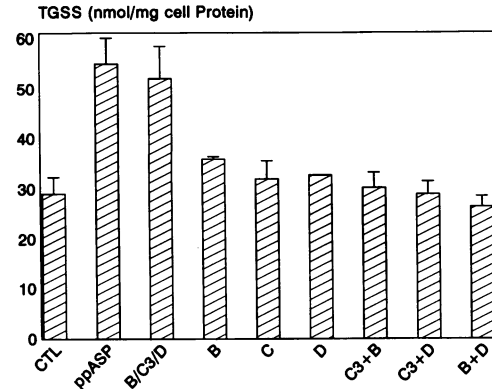


Figure 2. Biologic activity of in vitro generated C3a. C3a was generated in vitro by incubating C3 with factor B and factor D as described in Methods. The ability of the mixture, as well as each of the components: factor B and factor C3 [8 µg/ml], factor D [0.8 µg/ml], and partially purified plasma ASP [20 µg/ml] to stimulate triglyceride synthesis was determined as described in Methods. Results are expressed as nmol triglyceride synthesized per mg cell protein±SD for an average of two experiments.

complete mixture. Moreover, addition of any two of the factors together: B + D, B + C3, and C3 + D also did not result in stimulation of triglyceride synthesis as shown in Fig. 2. That C3a/ASP is indeed generated when B, C3, and D are incubated together under appropriate conditions is shown in Fig. 3. Both factor B and factor D demonstrate single bands on SDS gel electrophoresis (lanes 1 and 3) (21). Complement C3 is composed of 2 chains, C3α (110 kD) and C3β (70 kD) attached by disulfide bridges that demonstrate two bands on gel electrophoresis (lane 2) (21). Incubation of factors B, C3, and D produces several changes. Factor D enzymatically cleaves factor B to generate Ba and Bb of apparent molecular masses 30 and 60 kD, as indicated by arrows on Fig. 3 (lane 4). In addition, the binding of factor Bb to C3 will form the C3 convertase that

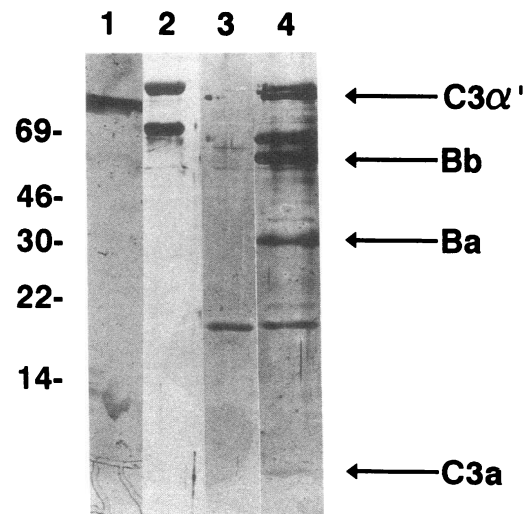


Figure 3. Gel Electrophoresis of individual components B, C3, and D and in vitro generated C3a/ASP. 0.5 µg of complement factor B, C3, and D were applied per lane. For the mixture, 1.5 µg of a 10:10:1 wt/wt mixture of factors B, C3, and D were applied after incubation to generate C3a/ASP as described in Methods.

generates C3 α' (101 kD) and C3a (9 kD). All of the generated products of the reaction: C3 α' , Bb, Ba, and C3a can be visualized on the gel as indicated by arrows.

The capacity of in vitro-generated C3a/ASP to stimulate triglyceride synthesis as compared to partially purified plasma ASP is shown in Fig. 4. The amount of C3a mass present in both the purified complement component mixture and in the partially purified ASP derived from a small basic fraction of serum was measured by commercial RIA. Increasing concentrations of ASP/C3a-desArg (as measured by RIA) were added to cells and triglyceride synthetic activity assessed. As shown in Fig. 4, the curves closely parallel each other. Therefore, C3a generated in vitro is capable of stimulating triglyceride synthesis and the ASP triglyceride synthetic activity of the small basic partially purified serum fraction can be attributed principally to the C3a-desArg component.

Discussion

The data presented indicate that ASP is identical in terms of amino acid composition, molecular mass, and NH₂-terminal amino sequence to a component of human complement, C3a-desArg. This result was not anticipated, and, at first glance, appears physiologically implausible, given that the precursor of C3a-desArg, C3a, is generally considered to be an anaphylatoxin generated through complement activation. In vertebrates, the complement system plays a key role in a successful antimicrobial response. Organisms may be lysed following activation of either the classical complement pathway, which is antibody-dependent, or the alternate complement pathway, which is antibody-independent, and which is the pathway of present interest.

Until the work of Spiegelman and his colleagues (9–13), activation of this alternate complement pathway was not known to have any effect on lipid metabolism in vivo. How-

ever, their studies do indicate that components of complement may indeed play important roles in adipose tissue metabolism. They first demonstrated that murine 3T3 adipocytes on differentiation express large amounts of a message for a protein, which they named adipsin (9). Adipsin is secreted from such cells and plasma levels of adipsin and adipose tissue adipsin mRNA are both markedly suppressed in many, although not all, models of experimental obesity (10). From this and other data, they concluded that adipsin might play a critical role in regulating the rate of lipolysis in adipocytes (10).

The link to the complement pathway began with their demonstration that there was considerable homology between the mouse adipsin cDNA sequence and the corresponding amino acid sequence of human factor D, a protein integral to the activation of the alternate complement pathway (11). They then isolated a cDNA for human adipsin and showed that it encoded for a protein sharing 98% amino acid sequence identity with the protein sequence for purified human complement D (12). Most recently, they demonstrated that differentiated murine 3T3 adipocytes under specific incubation conditions also synthesized and secreted the two other components involved in the initial steps of the alternate complement pathway, factor B and C3, and that C3a could be identified in the medium surrounding such cells (13). Importantly, there was no evidence that complement C2 or C5 were generated, indicating that the distal steps of the complement pathway that lead to cell lysis were not operational (13).

Our studies, on the other hand, have focused on the regulation of triglyceride synthesis in human adipose tissue. ASP causes triglyceride synthesis to rise in fibroblasts and HepG2 cells but has a much more pronounced impact on differentiated adipocytes (3). By contrast, there is no evidence that it has any effect on lipolysis (22). Moreover, our data and those of others indicate that the reduced rate of triglyceride synthesis in adipocytes from patients with HyperapoB might be due to reduced responsiveness to ASP (2, 7, 8). This observation generated the hypothesis that a reduced rate of fatty acid uptake in peripheral tissues, due to a reduced rate of triglyceride synthesis in adipocytes, would result in increased delivery of fatty acids to the liver (23). This would result in increased secretion of hepatic apoB particles, the metabolic hallmark of HyperapoB (24). These data and the evidence that there is a direct relation between the levels of ASP in plasma after an oral fat load and the rate of triglyceride clearance from plasma (25) suggest that this system is likely to be an important physiologic determinant of the rate of adipocyte triglyceride synthesis.

The physicochemical data in this study indicate that ASP and C3a-desArg are identical. C3a-desArg is the product that results when the terminal arginine is cleaved from C3a, and, given the large amounts of carboxypeptidase present in plasma, this would be the expected form in which C3a would be purified from this source. Until now, C3a-desArg has been considered to be a bioinactive product, as it does not possess any immunocytologic activity (26). The present data, however, establish that C3a-desArg is indeed bioactive insofar as it can increase intracellular triglyceride synthesis.

A critical sector of experimental evidence in this study is derived from the experiments in which the precursor proteins, factors B, D (adipsin), and C3, were incubated under appropriate conditions to generate C3a and then added to the medium bathing fibroblasts. The data indicate that triglyceride synthesis increased in direct proportion to the amount of C3a

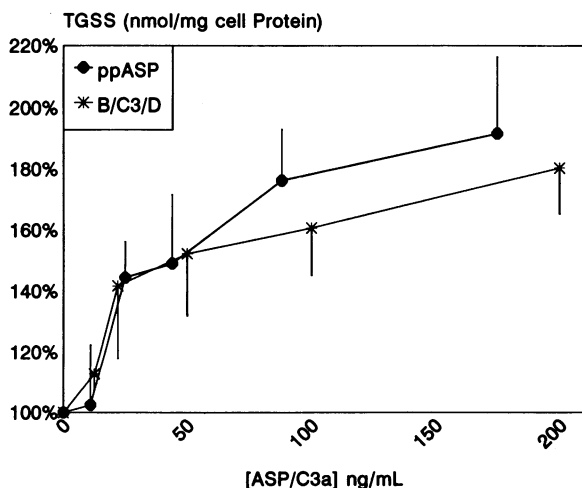


Figure 4. Concentration-dependent stimulation of triglyceride synthesis by in vitro generated C3a/ASP and serum ASP. C3a/ASP was generated in vitro, as described in Methods, and the concentration determined by RIA. The amount of C3a/ASP in a partially purified plasma preparation of ASP was also measured by RIA. The capacity to stimulate triglyceride synthesis at equivalent concentrations of ASP/C3a was determined in human skin fibroblasts. Results are expressed as percent stimulation of triglyceride synthesis \pm SD for an average of six experiments.

present. Moreover, the increase in triglyceride synthesis closely parallels that induced by addition of an equivalent amount of C3a-desArg present in a partially purified plasma preparation of ASP. Both lines of evidence, i.e., the purification of the active principal as C3a-desArg and the *in vitro* generation studies, point to the same conclusion, namely, that C3a-desArg is the bioactive principle responsible for the increase in triglyceride synthesis observed in our previous studies (3–5, 7).

The adipsin-ASP system, therefore, does appear to be involved in the regulation of triglyceride metabolism in adipocytes but our data indicate that the function of this system is to increase the rate of triglyceride synthesis in adipocytes rather than, as was originally suggested, to increase the rate at which lipolysis occurs (10). If so, the decrease in adipsin mRNA reported in the obese mouse models would represent an adaptive rather than causal finding (10). Clearly, much further work is required to test these hypotheses. Nevertheless, it has been established that adipocytes contain message levels for the three proteins necessary to generate ASP (13), and the data taken together point to the existence of what would be a novel regulatory system in which the synthesis of a series of proteins then generates a product that, by acting on the cell surface, alters the essential metabolic property of the cell. Whether this system is operative in man, and whether it is involved in the pathogenesis of disorders such as HyperapoB and obesity, remains to be determined.

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