Functional bioactive recombinant acylation stimulating protein is distinct from C3a anaphylatoxin

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Abstract Acylation stimulating protein (ASP) acts upon adipose tissue to stimulate triglyceride synthesis and glucose transport. The aim of the present study was to produce recombinant ASP and to measure its bioactivity. The cDNA region of the parent complement C3 sequence coding for ASP (C3adesArg) was cloned and expressed in *E. coli*. Bioactivity of the purified recombinant material was tested by determining its effect on triglyceride synthesis, glucose transport, and competition binding assays. In standard assays, concentrations of 5.5 μM recombinant ASP (rASP) stimulated triglyceride synthesis comparably to plasma ASP (pASP): 228% versus 237%, respectively, in 3T3 preadipocytes and 568% versus 440% in human differentiated adipocytes. rASP also increased glucose transport in L6 myocytes (163% at 10 μM rASP) and in human differentiated adipocytes (334% rASP vs. 329% pASP at 5 μM). rASP competitively displaced radiolabeled plasma ASP from high affinity association with the cell surface in both human differentiated adipocytes and 3T3 preadipocyte fibroblasts. Furthermore, immunoprecipitation of rASP and pASP with a specific monoclonal antibody abolished stimulation of cellular triglyceride synthesis. Lastly, we contrasted the structure:function activities of the arginated (C3a) and desarginated (ASP) proteins. The lipogenic activity and the anaphylatoxic activity result from distinct structural domains of the polypeptides. Thus rASP retains full biologic ASP activity and may provide a tool to study structure–function relationships in this physiologic system.—**Murray, I., R. A. Parker, T. G. Kirchgessner, J. Tran, Z. J. Zhang, J. Westerlund, and K. Cianflone.** Functional bioactive recombinant acylation stimulating protein is distinct from C3a anaphylatoxin. *J. Lipid Res.* 1997. **38:** 2492–2501.

Supplementary key words recombinant • triglyceride synthesis • glucose transport • receptor • complement C3adesArg

Acylation stimulating protein (ASP) is a 76 amino acid, basic protein (pI 9.0) that stimulates triglyceride synthesis and glucose transport (1–3). ASP was initially isolated as a serum component that stimulated triglyceride synthesis in normal human fibroblasts (1). This effect on adipocytes, though, was much more pronounced than on fibroblasts (2). This serum fraction was purified to homogeneity and was found to be identical to C3adesArg (molecular weight 8932.5 Da), a biologic fragment of complement C3 produced through the alternate complement pathway (1). ASP is a nonglycosylated product of the N-terminal cleavage of the α chain of complement C3, mediated through the interaction of complement factors B and adipsin (complement factor D), followed by C-terminal arginine cleavage by serum carboxypeptidase N (4). Studies by Choy, Rosen, and Speigelman (5) and White et al. (6) demonstrated that mRNA of complement factors B, C3, and adipsin were produced in murine adipose tissue. We have shown in human adipocytes that C3, B, and adipsin increase with adipocyte differentiation and that the C3 cleavage product, ASP, also increases in the medium of differentiating cells (2, 7). The increased production of ASP correlates with the increased basal triglyceride synthetic capacity of human adipocytes as well as an increased response to ASP (2, 7). Furthermore, triglyceride synthesis, using in vitro complement-derived ASP (cdASP) (from factors B, C3, and adipsin) produced triglyceride synthetic activity comparable to that of plasma ASP (pASP) in human fibroblasts (2). ASP is thus the bioactive component of the adipsin pathway described in adipose tissue (5, 6).

It should be noted that although C3a has potent anaphylatoxic activity in immunologically relevant cells

Abbreviations: ASP, acylation stimulating protein; EDTA, ethylene diamine tetraacetate; HPLC, high pressure liquid chromatography; IPTG, isopropylthio-β-D galactoside; PCR, polymerase chain reaction; PMSF, phenylmethyl sulfonyl fluoride; SD, standard deviation; Tris, Tris-hydroxymethyl amino methane; TG, triglyceride; 2-DG, 2-deoxy glucose.

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such as U937 human monocytes (8) and other cells (review 9), this activity is entirely dependent on the presence of the carboxy terminal arginine (8, 9). C3adesArg (ASP) has no known immunologic function and does not appear to bind to the recently identified C3a receptor (10, 11).

In vitro expression of eukaryotic proteins has refined the process of structure–function analysis. In the present paper we report the production and purification of rASP and assess its biological activity. Similar to pASP, rASP was able *i*) to stimulate triglyceride synthesis, *ii*) to increase glucose transport, and *iii*) to competitively displace radiolabeled plasma ASP from association with apparent cell surface receptors. Immunoprecipitation abolished cellular triglyceride stimulation activity. Lastly, ASP activity was shown to be sensitive to solubilization conditions in contrast to C3a anaphylatoxic activity. Thus rASP has full biological activity and may be useful to study structure–function relationships of ASP in future research.

MATERIALS AND METHODS

All human tissue was obtained with informed consent and all experimental protocols were approved by the ethics committee of the Royal Victoria Hospital.

Recombinant ASP (rASP) and plasma (pASP) production

The cDNA sequence encoding human ASP (C3adesArg) was assembled in the pET22b expression vector (Novagen, Inc., Madison, WI) using synthetic oligonucleotides in which mammalian codons were modified to reflect *E. coli* codon bias as described previously for C3a (12). To our knowledge, this has never been done for ASP (C3adesArg).

Recombinant ASP (rASP) protein was purified as follows from BL21 (DE3) cells transformed with the above expression vector. Cells from the IPTG-induced cultures were pelleted and then lysed by sonication in 20 mM Tris-HCL, pH 7.4, 0.1 M NaCl, 1.0 mM EDTA, and 0.2 mM PMSF. Inclusion bodies, which contained rASP, were sedimented by centrifugation (2500 *g*, 4°C for 30 min). The inclusion bodies were resuspended in 50 mL of 1.0 N HCl, sonicated, neutralized with 10 N NaOH to pH 7.0, and the debris was removed by centrifugation. The rASP present in the supernatant was isolated by C-18 SepPak, followed by S-Sepharose chromatography, and lastly by reverse phase HPLC on a C-4 column as described in detail for plasma ASP (13). The yield of rASP was 0.5–1.0 mg per liter of starting culture. Human pASP was prepared as previously described (13). Protein concentrations were determined by a modification (14) of the Lowry protein assay (15) using a commercial albumin standard (Bio-Rad, Mississauga, Ontario, Canada). The pASP and rASP were stored at −80°C in siliconized glass vials.

Preparation of in vitro complement-derived ASP (cdASP)

Five hundred μg of human factor C3 and B (Calbiochem, LaJolla, CA) in 50 mM magnesium chloride were incubated in a glass test tube for 3 min at 37°C to activate C3. To this, 50 μg of human factor D (Calbiochem) was added and the mixture was incubated for a further 15 min at 37°C. From this mixture 25 μg (15 μg/mL) of cdASP was obtained, based on 100% conversion as assessed by SDS-PAGE (1, 2). Preparations of C3a were treated with carboxypeptidase B (1 unit/mL for 5 min at 37°C) to generate in vitro ASP.

Cell culture

Human fibroblasts were obtained from forearm biopsies of normolipidemic subjects and cultured as previously described (1). 3T3-L1 preadipocytes (obtained from ATCC, Rockville, MD) were cultured in the same fashion as the human fibroblasts. Cells were subcultured, plated out at 3×10^3 cells/cm^2 and grown in 10% fetal calf serum in DMEM/F12 medium. Cells were used for experiments at 80% confluency. For experiments with 3T3 adipocytes, cells were differentiated as previously described (2). Primate adipose tissues (green monkey and cynomolgus) were obtained from omental sites. Human adipose (subcutaneous) tissue was obtained with informed consent at the time of elective laparotomy. After collagenase digestion and centrifugation, stromal vascular preadipocytes were isolated from the cell pellet and primary adipocytes were isolated from the floating layer. Human preadipocytes were differentiated to adipocytes in hormone-supplemented DMEM/F12 medium for 18 days as described previously (2, 7). U937 cells were obtained from ATCC and cultured as described by Klos et al. (8).

Triglyceride synthesis assay

Cultured cells in 24-well dishes (1.7 cm^2/well) were preincubated in serum-free DMEM/F12 medium overnight (18 h) prior to the experiments. Triglyceride synthesis was measured as incorporation of [^{3}H]oleate (sp act 10.0 Ci/mmol, DuPont-New England Nuclear, Mississauga, Ont, CA) into triglyceride. Cells were incubated for 4–18 h (as indicated) in 100 μM oleate complexed to BSA (5:1 molar ratio, average final specific activity = 100 dpm/pmol) in serum-free DMEM/F12 as previously described (13). Results are expressed as

nmol [^{3}H]oleate incorporated into triglyceride per mg of cell protein.

Glucose transport assay

Cells were cultured, as described above, in 35-mm tissue culture dishes and preincubated in serum-free DMEM/F12 medium 18 h prior to the experiments. Cells were stimulated with rASP or pASP for 1 h at 37°C and glucose transport was assessed by measurement of cellular uptake of [^{3}H]2-deoxy-glucose (^{3}H 2-DG, sp act 25–50 Ci/mmol, DuPont-New England Nuclear) as previously described (3). Results were corrected for background binding assessed as zero time counts. The results are expressed as nmol of [^{3}H]2-DG uptake per mg of cell protein.

Cellular Ca^{2+} flux

Ca^{2+} uptake into U937 cells was assayed by the Fura-2 method as described by Klos et al. (8).

^{125}I-labeled ASP competition assay

pASP was radiolabeled using Iodogen (Pierce Chemicals, Rockford, IL) and specific activity was measured as dpm per μg of trichloroacetic acid (10% TCA)-precipitable protein (average specific activity = 20 dpm/fmol). Competitive binding was performed on cells cultured in 96-well plates. Cells were preincubated with serum-free DMEM/F12 medium overnight. Cells were prechilled on ice for 15 min followed by incubation for 1 h with 50 or 500 nM ^{125}I-labeled ASP in 100 μL of 1% bovine serum albumin (BSA) in PBS and increasing concentrations of unlabeled ASP. After a 1-h incubation, 5-μL aliquots (in triplicate) of medium were counted for calculation of free ASP. Cells were washed three times with ice-cold PBS and soluble cell protein was dissolved in 100 μl of 0.1 N NaOH. Aliquots were taken for counting of bound ASP and cell protein determination by the method of Bradford (16) using a commercial assay (Bio-Rad, Mississauga, Ontario, Canada). The results are expressed as nmol of ASP bound per mg soluble protein. Calculation of competition (IC_{50}) was performed by iterative four parameter logistic function analysis (Sigma Plot, Jandel Scientific, San Rafael, CA).

ASP immunoprecipitation assay

Protein G agarose (Calbiochem) was prewashed three times with 20 volumes of PBS, with centrifugation (100 *g*) between the washes to allow for removal of the supernatant. For immunoprecipitation, 15 μg pASP, rASP, or cdASP was added to 316 μg of monoclonal anti-human C3a IgG (Quidel, San Diego, CA) or the same amount of non-immune mouse IgG (Sigma, St. Louis, MO) (molar ratio of IgG to ASP was 1:1). After incubation at 20°C for 20 min and then 4°C for 14 h, the entire mixture was then added to the protein G agarose pellet and incubated at 20°C for 20 min, followed by 4°C for 2 h, with gentle mixing. The sample was centrifuged (100 *g*), to pellet the protein G agarose–antibody–ASP complex and the supernatant was collected and tested for the ability to stimulate triglyceride synthesis.

SDS polyacrylamide gels

Proteins were separated on discontinuous 15% SDS-PAGE under denaturing conditions according to the method of Laemmli (17). Proteins were dissolved in 0.125 M Tris, glycerol (0.125 g/mL), 4% w/v SDS, and 0.11 g/mL dithiothreitol and heated (100°C, 5 min). Gels were stained with Coomassie blue R-250.

Western blot analysis

Proteins were separated on SDS polyacrylamide gels as described above. Proteins were transferred by electroblotting to a PVDF membrane. Western analysis was performed using a monoclonal murine anti-human C3a antibody (Quidel) as primary antibody and detected with an alkaline phosphatase-conjugated rabbit anti-mouse IgG secondary antibody using a commercial colorimetric kit (Bio-Rad).

RESULTS

The purification of rASP produced in *E. coli* is shown in **Fig. 1A.** With IPTG induction, there is an increase in rASP as compared to the non-stimulated cells (lane 4 vs. lane 3) which is present in inclusion bodies (lane 5). After sonication of the inclusion bodies in 1 N HCl, the majority of the large globular proteins precipitate. However, ASP remains in the soluble fraction. The fractions from the SepPak and S-Sepharose purification steps are shown in lanes 7 and 8. Finally, the proteins were fractionated by C-4 HPLC and a typical profile obtained during the purification is shown in Fig. 1B. After HPLC fractionation of rASP, each of the protein fractions (numbered in Fig. 1B) was tested for triglyceride synthetic capacity on human fibroblasts (Fig. 1C). pASP was fractionated in parallel and the isolated fractions were also tested for activity (Fig. 1C). The pre-HPLC samples had activities of 233% ± 30 for pASP and 224% ± 32 for rASP where basal triglyceride synthesis was defined as 100%. The pASP elutes in a narrow band and rASP followed a similar profile. The fractions eluting at 55% to 65% acetonitrile (fraction #3) caused the greatest increase in triglyceride synthesis for both pASP (343% ± 48) and rASP (376% ± 39). This yielded a protein of identical molecular weight and immunogenicity as pASP as shown by SDS-PAGE and Western blot

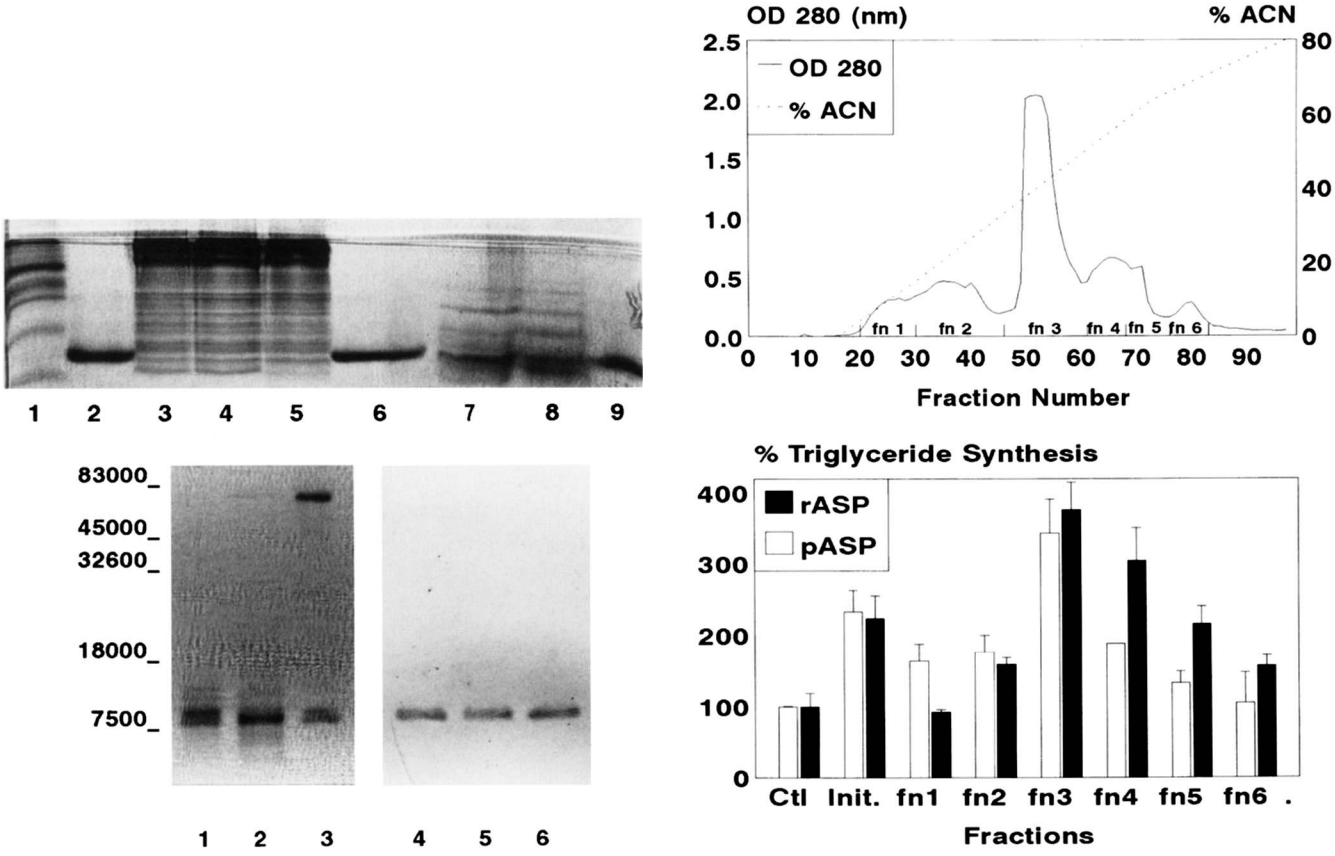

Fig. 1. Purification and activity of recombinant ASP. Top left: The fractions obtained during purification of rASP were separated on 15% SDS-PAGE and stained with 0.12% Coomassie R250 blue (10 μg protein/lane). Lane 1: molecular weight markers, lanes 2, 6, 9: control plasma ASP, lane 3: uninduced *E. coli* bacterial pellet, lane 4: 4-h induction with IPTG, lane 5: inclusion body pellet, lane 7: C-18 Sep-Pak eluant, lane 8: S-Sepharose eluant. Top right: rASP was purified from *E. coli* exclusion bodies by hydrophobic (C-18 SepPak), ion exchange (S-Sepharose) and C-4 HPLC separation (a sample profile is shown). Bottom right: Triglyceride synthetic capacity of rASP and pASP fractions from C-4 HPLC separation. Triglyceride synthesis was measured as [^{3}H]oleate incorporation into triglyceride in human skin fibroblasts over 6 h. The results are expressed as percent change (average ± SD) where basal triglyceride synthetic activity was 7.1 nmol/mg cell protein per 6 h and is shown as 100% (n = 3). Bottom left: 15% SDS-PAGE stained with Coomassie blue (lanes 1–3) and Western blot using monoclonal anti-ASP (lanes 4–6) of the final C-4 HPLC purified rASP from two different recombinant preparations (lanes 2, 3, 5, 6) and pASP (lanes 1 and 3) with 2 μg protein loaded per lane.

(Fig. 1D). This process yielded 0.5 to 1 mg of rASP per liter of starting culture. Both the rASP cDNA and recombinant protein sequences were verified by DNA and amino acid sequencing.

During the course of purification of the recombinant ASP, it became clear that the yield and activity were sensitive to isolation conditions. Equal amounts of ASP were aliquotted and treated as described in **Fig. 2.** As shown, losses could be kept to a minimum through the use of siliconized plastic tubes and by adding 1 mg/mL BSA to dilute preparations (condition F in Fig. 2). On the other hand, there were losses (both mass and activity) when ASP was concentrated by centrifugation (C and D) or lyophilized in non-siliconized tubes ("A and B"). Sticking to plastic and glass is characteristic of basic proteins.

The functional activity of the rASP was first assessed for stimulation of triglyceride synthetic activity. As shown in **Fig. 3** (left panel) the triglyceride synthetic activity of 3T3-L1 preadipocytes was stimulated equivalently by both rASP and pASP to a maximum of 237% ± 1% pASP versus 228% ± 20% at the highest ASP concentration (5.6 μM). The response of cultured human differentiated human adipocytes is shown in Fig. 3, right panel. It should be noted that the responses in the differentiated adipocytes were much higher, with a proportional increase to 568% ± 34% at 5.6 μM with rASP in human cultured adipocytes.

We have previously shown that pASP increases 2-deoxy glucose (2-DG) transport in rat L6 myotubes, adipocytes, and fibroblasts (3, 18, 19). Thus, rASP was tested for stimulation of 2-DG transport in human adi-

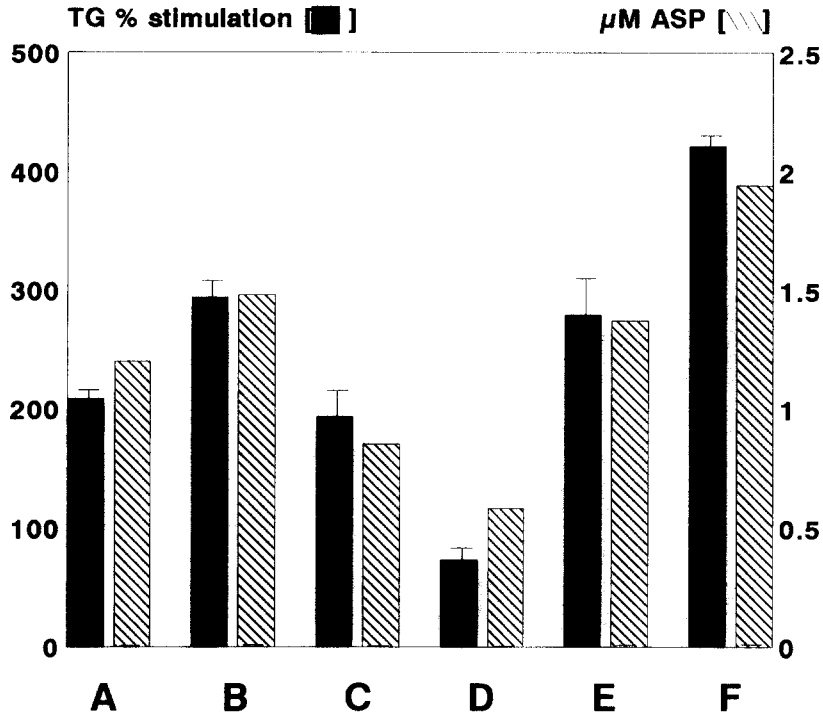

Fig. 2. Effect of isolation conditions on triglyceride synthetic activity of ASP. Cells were preincubated overnight in serum-free DMEM/F12 and then changed to serum-free DMEM/F12 supplemented with 100 μM [^{3}H]oleate complexed to BSA and 125 μL of ASP. Equivalent aliquots of ASP were prepared under the following conditions: (A) lyophilized, (B) lyophilized + 1 mg/mL BSA, (C) concentrated by membrane centrifugation, (D) as in C plus 1 mg/mL BSA, (E) lyophilized in plastic siliconized vials or (F) as in E plus 1 mg/mL BSA. Triglyceride (TG) synthetic capacity of equivalent aliquots was measured in human skin fibroblasts overnight as [^{3}H]oleate incorporation into triglyceride. Results are expressed as percent change (average ± SD), [solid bars] where basal triglyceride synthesis (shown as 100%) was 27.3 ± 4.7 nmol/mg cell protein per 18 h (n = 3). Corresponding ASP concentration is indicated in the hatched bars.

pocytes and L6 myotubes. rASP stimulated glucose transport in L6 differentiated myotubes (160% ± 20 at 10 μM ASP), an effect comparable to insulin as shown in **Fig. 4,** left panel. In human differentiated adipocytes, rASP and pASP also stimulated glucose transport equivalently (Fig. 4, right panel): 329% (pASP) versus 344% (rASP) at 5.6 μM (where basal glucose transport is shown as 100%). The percent response in adipocytes was greater than in L6 myotubes.

Further evidence of the functionality of rASP was obtained by assaying the ability of rASP to displace radiolabeled ^{125}I pASP (500 nM) binding to cell surface receptor binding sites in cultured human skin fibroblasts and human differentiated adipocytes (**Fig. 5**). In both cell types, rASP was as effective as pASP in displacement of receptor binding. The IC_{50} (500 nM ASP) for rASP (2.7×10^{-6} M and 4.2×10^{-7} M) was comparable to pASP (3.9×10^{-6} M and 8.8×10^{-7} M) for fibroblasts and adipocytes, respectively.

The effect of ASP across several species was also examined. As shown in **Table 1,** ASP was as potent as insulin in stimulating triglyceride synthesis at equimolar amounts (0.7 μM) in primary adipocytes from other primates (cynomolgus and African green monkey). In contrast, ASP did not have as great an effect on glucose transport stimulation as did insulin in murine cells (Table 1) although the effects of ASP and insulin on glucose transport are comparable in human differentiated adipocytes (18) and in L6 myotubes (19). Nonetheless, ASP was effective at stimulating adipocytes from all species tested regardless of the activity index used (triglyceride synthesis or glucose transport).

To demonstrate the specificity of the effect on triglyceride synthesis, rASP, pASP, and cdASP were immunoprecipitated and the remaining supernatant was tested for the capacity to stimulate triglyceride synthesis in human skin fibroblasts (**Fig. 6**). Basal triglyceride synthesis (control) did not change significantly with the addition of either non-immune mouse IgG or monoclonal anti-C3a. The triglyceride stimulatory capacity of the positive controls for pASP (189% ± 23), cdASP (190% ± 14), and rASP (167% ± 18) were not significantly different from each other, and all stimulated triglyceride synthesis effectively. When non-immune mouse IgG was added, there was also no significant change in triglyceride synthesis stimulation of pASP (176% ± 18), cdASP (159% ± 1), or rASP (181% ± 19). Non-immune IgG did not immunoprecipitate ASP and all of the ASP remained in the supernatant as assessed by SDS-PAGE (not shown). However, triglyceride synthesis was significantly reduced by 126% ± 5 (pASP), 78% ± 14 (cdASP), and 120% ± 7 (rASP) when the monoclonal anti-C3a antibody was used for immunoprecipitation. Thus triglyceride synthesis stimulation is abolished when ASP is immunoprecipitated. SDS-PAGE analysis indicates that 88% of the ASP was present in the IgG-Protein G agarose immunoprecipitate and no ASP was detected in the supernatant (not shown). Thus the triglyceride synthetic stimulatory activity was attributable specifically to bioactive ASP derived from either

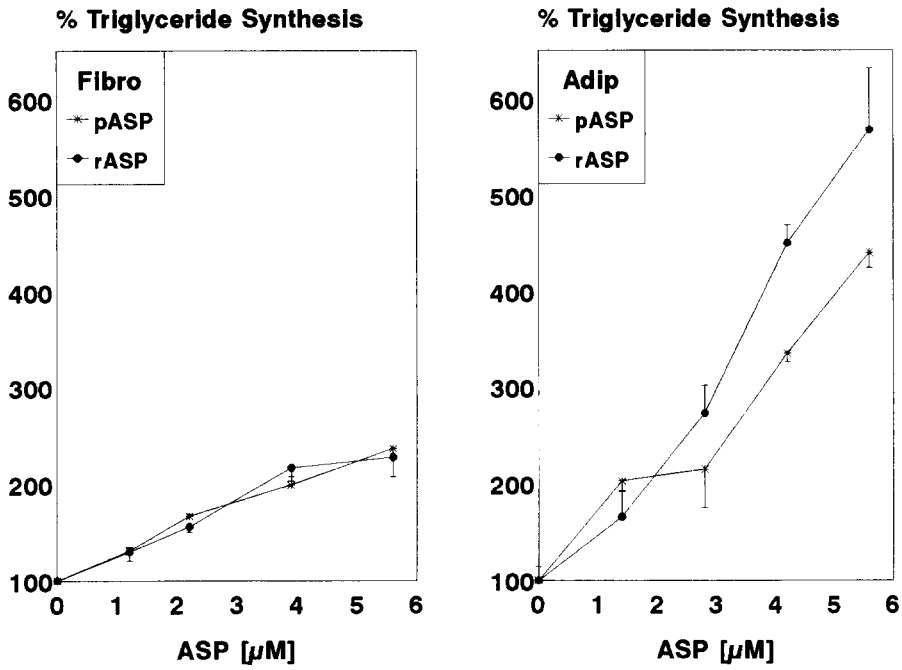

Fig. 3. Triglyceride synthetic capacity of purified rASP and pASP. Cells were preincubated overnight in serum-free DMEM/F12 and then changed to serum-free DMEM/F12 supplemented with 100 μM [^{3}H]oleate complexed to BSA and increasing concentrations of rASP or pASP. Triglyceride synthetic capacity was measured in 3T3 fibroblasts for 24 h (left panel) and in cultured human differentiated adipocytes for 2 h (right panel) as [^{3}H]oleate incorporation into triglyceride. Results are expressed as percent change (average ± SD) where basal triglyceride synthesis (shown as 100%) was 54.9 ± 1 nmol/mg cell protein per 24 h for 3T3 fibroblasts (n = 3) and 11.9 ± 1.3 nmol/mg cell protein per 2 h for human differentiated adipocytes (n = 3).

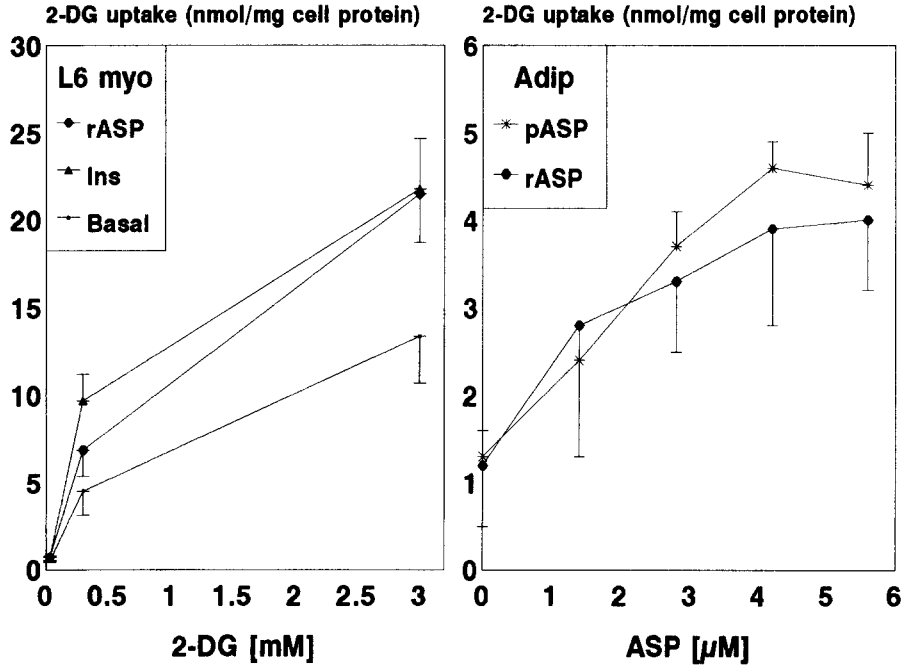

Fig. 4. Effect of rASP and pASP on glucose transport. Cells were preincubated in serum-free DMEM/F12 media and then stimulated with rASP, pASP or insulin for 1 h. Glucose transport was assessed as [^{3}H]2-deoxyglucose (2-DG) uptake over 10 min in differentiated L6 myotubes (rASP = 10 μM, insulin = 1 nM) at 3 concentrations of 2-DG (left panel, n = 4) and in differentiated human adipocytes with 0.05 mM [^{3}H]2-DG (right panel, n = 3). Results are expressed as average ± SD nmol 2-DG per mg cell protein.

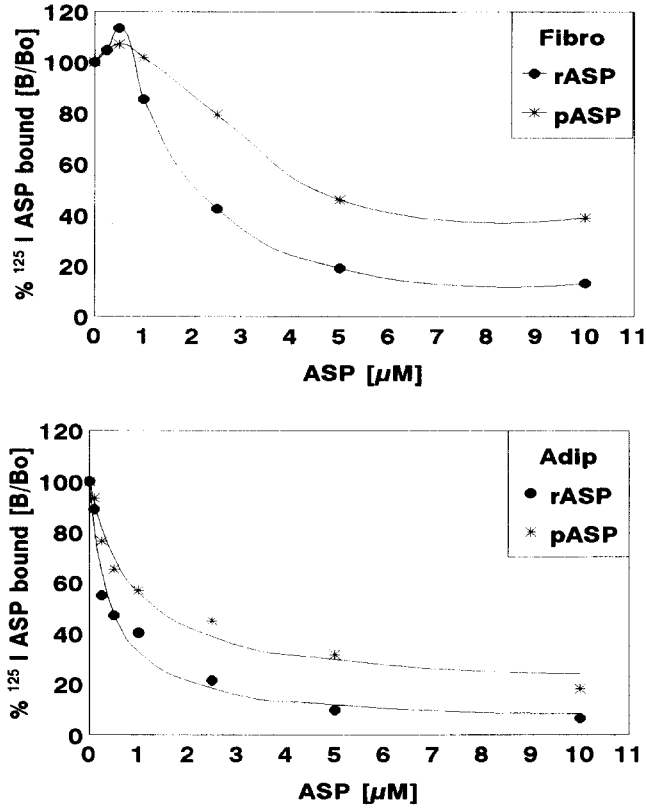

Fig. 5. Competition binding of rASP and pASP. 3T3 fibroblasts (top panel) and human differentiated adipocytes (bottom panel) were preincubated in serum-free DMEM/F12 overnight. ^{125}I-labeled ASP (500 nM, sp act 2.0 dpm/fmol) in 1% BSA in PBS was added with increasing concentrations of unlabeled ASP (up to 10 μM) and incubated for 1 h on ice. Aliquots of solubilized cells were taken for counting and protein determination. Results are expressed as percent ASP bound (average n = 2) where 100% is the amount of ^{125}I-labeled ASP bound in the absence of competitor (0.290 ± 0.045 and 0.488 ± 0.066 nmol/mg cell protein for 3T3 fibroblasts and human adipocytes, respectively).

pASP, cdASP, or produced by recombinant *E. coli* (rASP).

Functional bioactivity of the rASP appeared to be sensitive to solubilization conditions. It has previously been shown that treatment of C3a with urea under reducing conditions has no effect on its anaphylatoxic bioactivity (9, 20). However, solubilization of the inclusion bodies containing rASP with either urea or guanidium isothiocyanate plus dithiothreitol, followed by purification, resulted in isolation of an inactive protein. This structure:function effect was investigated in more detail. The effect of various solubilization conditions used in purification of recombinant proteins was assessed. ASP was treated for 1 h as indicated in **Table 2,** and then ASP was repurified by HPLC and TGS activity was measured. As shown in Table 2, high salt (1 M NaCl) or acid (1 M HCl) did not affect the ASP activity.

TABLE 1. Effect of ASP and insulin on adipocytes

		Triglyceride Synthesis		Glucose Uptake	
Adipocytes	n	% of Basal	*P*	% of Basal	*P*
Cynomologous omental primary	6				
ASP, 0.7 μM		190 ± 16	<0.005		
Insulin, 0.7 μM		221 ± 16	<0.0005		
African green, omental primary	6				
ASP, 0.7 μM		194 ± 44	<0.05		
Insulin, 0.7 μM		155 ± 38	=0.065		
3T3 differentiated cultured	3				
ASP, 10 μM				168 ± 19	<0.005
Insulin, 2 nM				347 ± 156	<0.001

Primary isolated adipocytes from primate omental adipose tissue were prepared by collagenase digestion. Cells were then incubated for 2.5 h with 100 μM [^{3}H]oleate and the indicated concentrations of ASP or insulin to measure triglyceride synthesis. 3T3 murine cells were differentiated to adipocytes for 5 days. Cells were preincubated with ASP or insulin for 1 h, then glucose transport (glucose uptake) was measured as [^{3}H]2-deoxy glucose transport (0.3 mM) uptake over 10 min. Results are expressed as % change compared to basal levels of triglyceride synthesis or glucose transport (mean ± SD).

As well, urea alone had little negative effect. In contrast, the combination of urea and β-mercaptoethanol caused a loss of 75% of the activity of ASP. This treatment of ASP resulted in a marked change in the charge/shape of the molecule as demonstrated by reduced migration on native gel electrophoresis (data not shown). This loss of bioactivity was paralleled by a decrease in the affinity of the modified ASP for the receptor as shown in competition binding studies (**Fig. 7,** 50 nM ASP) where the IC_{50} concentrations were 4.1×10^{-8} M (unmodified ASP), 1.7×10^{-7} M (urea-treated ASP), and 3.39×10^{-7} M (urea + β-mercaptoethanol treated ASP, $P < 0.0001$ by ANOVA).

This contrast in structural stability between ASP and C3a was further investigated. C3a was prepared by in vitro cleavage of C3, B, and adipsin and treated with carboxypeptidase B to produce in vitro generated ASP. The activity of these two proteins, as well as carboxy terminal peptides, was tested. Lee et al. (21) and Ember, Johanson, and Hugli (22) have previously demonstrated that the 21 carboxy terminal peptide (C57–77) is sufficient for C3a anaphylatoxic activity. As shown in **Fig. 8** (top panel), C3a increased Ca^{2+} flux into U937 cells by 160%. The 21-residue peptide (C57–77) also increased Ca^{2+} flux to the same extent (Fig. 8, bottom panel), 200%. Relative activities are shown in **Table 3.** Neither ASP (C3adesArg) nor the 20 amino acid residue desArg peptide (C57–76) had any effect on Ca^{2+} flux (Fig. 8). Amino terminal peptides (N 1–19, N 1–30, and N 10–29) used as negative controls, also had no effect on Ca^{2+} flux. By contrast, although ASP and C3a

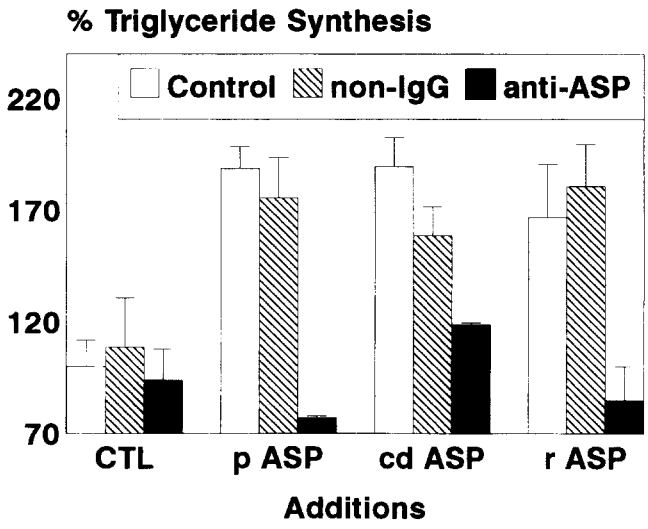

Fig. 6. Immunoprecipitation of rASP, pASP and cdASP. Basal triglyceride synthetic activity is shown as 100% (control, open bars) measured as [^{3}H]oleate incorporation into triglyceride over 4 h (19.9 ± 6.5 nmol/mg cell protein). The stimulatory effect of pASP, cdASP, and rASP (1.1 μM) is shown in the open bars. Comparable amounts of rASP, pASP, and cdASP were immunoprecipitated with monoclonal anti-C3a/ASP antibody (solid bars) or non-immune mouse IgG (hatched bars) and the supernatant was then tested for triglyceride synthetic capacity in human fibroblasts. Results are shown as average ± SD for n = 4 experiments with duplicate dishes in each experiment.

had comparable effects on triglyceride synthesis assayed in parallel in fibroblasts, neither the 21 or the 20 residue carboxy peptides had any effect on triglyceride synthesis even at concentrations 50 times higher (100 μM) than the holo-proteins (Table 3).

DISCUSSION

To our knowledge, this is the first report detailing functional expression of bioactive ASP (C3adesArg). Although previous expression of partially bioactive C3a has been described (12), it is important to note that these two polypeptides (ASP and C3a) appear to be functionally distinct. The results demonstrate that rASP is functionally active, whether assessed by triglyceride synthesis or glucose transport. As well, in competitive binding studies, the recombinant ASP was able to inhibit the binding of radiolabeled ASP to the same extent as native plasma ASP. Finally, immunoprecipitation removed the rASP bioactivity as well as plasma ASP. The activity of rASP confirms that ASP (C3adesArg) is indeed the bioactive plasma component that acts in an autocrine/paracrine manner to stimulate TG synthesis in adipocytes in vivo.

Clearly, although ASP (C3adesArg) is bioactive for triglyceride synthesis, it is not competent to stimulate anaphylatoxic activity in U937 cells and other cells (8, 9) whereas C3a (with the carboxy terminal arginine intact) is a potent activator of this pathway. As well, C3a activity is not sensitive to denaturing conditions that change the structural conformation of the protein (9, 20), while ASP certainly is. Thus ASP may be sensitive to refolding conditions, a finding which would not be unexpected considering the presence of 6 cysteines (3 disulphide bridges) within a 36 amino acid region. On the other hand, C3a bioactivity is clearly dependent on the presence of the carboxy terminal arginine as shown here and in many other studies (8, 9, 20–22). By contrast, the ASP triglyceride stimulatory capacity is obviously not dependent on the presence of the terminal arginine. Thus, the lipogenic activity and the anaphylatoxic activity appear to reside in specific and distinct structural domains of the polypeptide. It is important to note that purification techniques that maintain the anaphylatoxic activity of C3a may abrogate the lipogenic activity of ASP as shown in the present study. The present study also demonstrates that human ASP can stimulate TGS not only in human cells, but across several species (primates, mice, rats).

TABLE 2. Effect of solubilization conditions on ASP activity

Treatment	Basal	+ ASP	% Stimulation	+ Treated ASP	% Inhibition	*P*
NaCl, 1 M	83.8 ± 5.3 (8)	251 ± 7.3 (12)	300 ± 9	239 ± 11 (12)	8	ns
HCl, 1 M	90 ± 5.4 (11)	243 ± 8.2 (15)	279 ± 13	223 ± 12.6 (15)	13	ns
Urea, 4 M	109 ± 22 (10)	232 ± 34 (14)	234 ± 12	257 ± 32 (8)	0	ns
Urea, 4 M + β-Me, 8.8 mM	164 ± 28 (5)	373 ± 22 (6)	234 ± 27	215 ± 34 (6)	75	<0.001

ASP was treated with the indicated conditions for 1 h, then repurified by reverse phase HPLC. Cells were preincubated overnight in serum-free DMEM/F12 and then changed to serum-free DMEM/F12 supplemented with 100 μM [^{3}H]oleate complexed to BSA and 5.6 μM ASP. Triglyceride synthetic capacity was measured in human skin fibroblasts overnight as [^{3}H]oleate incorporation into triglyceride over 18 h. Results are expressed as nmol triglyceride/mg cell protein (mean ± SD) with the number of assays in parentheses; ns, not significantly different.

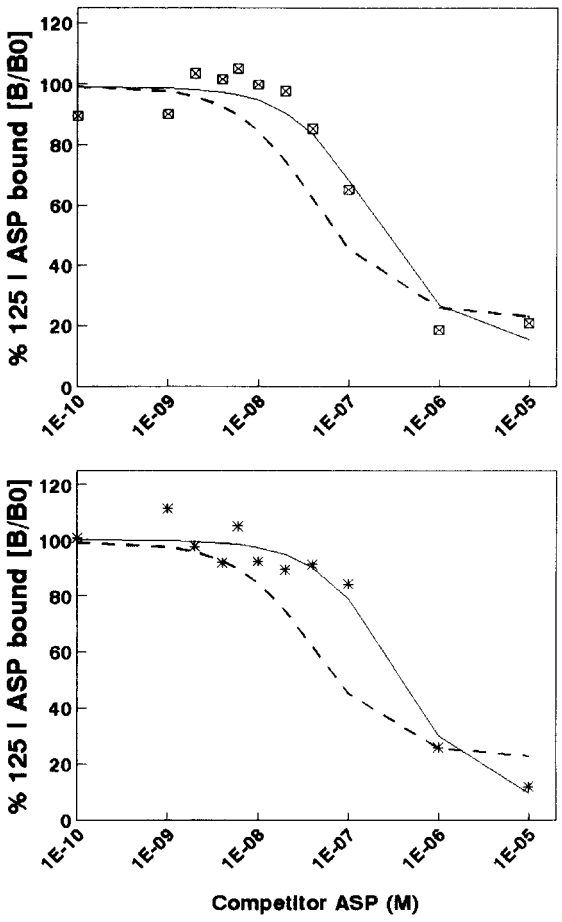

Fig. 7. Competition binding of modified ASP. 3T3 fibroblasts (top panel) were preincubated in serum-free DMEM/F12 overnight. ^{125}I-labeled ASP (50 nM, sp act 20 dpm/fmol) in 1% BSA in PBS was added with increasing concentrations of unlabeled native ASP (dotted line) or modified ASP. Top panel: urea-treated ASP (solid line), bottom panel: urea + β-mercaptoethanol-treated ASP (solid line) and incubated for 1 h on ice. Aliquots of solubilized cells were taken for counting and protein determination. Results are expressed as percent ASP bound (average n = 2) where 100% is the amount of ^{125}I-labeled ASP bound in the absence of competitor.

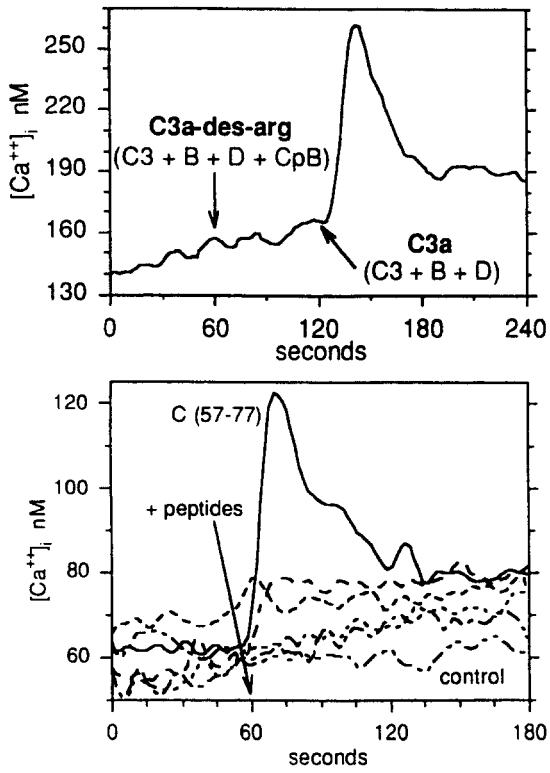

Fig. 8. Activity of ASP, C3a, and carboxy terminal peptides on Ca^{2+} flux and TGS. Ca^{2+} flux was measured in human macrophage U937 cells by the Fura-2 method for C3a and ASP (C3adesArg) in the top panel, and for the 21 (solid line, C57–77) and 20 (C57–76) carboxy terminal amino acid peptides as well as several amino terminal peptides (N1–19, N1–30, and N10–29) in the bottom panel. Relative activities are given in Table 3.

It is interesting to note that, although the primary amino acid sequence can vary from species to species, there appears to be phylogenetic conservation of both the carboxy terminal region as well as the cysteine bridges (4). This latter structural domain is important in ASP lipogenic activity, although not for C3a anaphylatoxic activity.

Why do both ASP and C3a stimulate triglyceride synthesis in adipocytes, yet only C3a is bioactive in macrophage and other immunologic cells? The differences may be due to distinct cell surface receptors or to differences in cell signalling and links to downstream cell-specific pathways triggering alternate events: lipogenesis or immunologic response. A human C3a receptor has been cloned and characterized (10, 11) although the reports did not examine whether it is present in human adipocytes. In the literature, there are examples of specific receptors that can differentiate between the desArginated form versus arginated polypeptides. For example, bradykinin and bradykinin desArg bind specifically and with different affinities to two distinct receptors (BK1 and BK2) that can be present in the same cell type to generate differential responses (for review, see 23–25). This may also be true of ASP (C3adesArg) and C3a. Future studies using site-directed mutagenesis and deletion analysis to investigate the structure–function relationships of ASP in a recombinant model, as well as receptor interactions will allow these possibilities to be explored.

This work was supported by a grant from the Heart & Stroke Foundation of Canada awarded to K. Cianflone who is the recipient of a Scholarship from the Heart and Stroke Foundation of Canada and les Fonds de la Recherche en Santé du

TABLE 3. Comparison of lipogenic and anaphylatoxic activities of C3a, ASP, and carboxy terminal peptides in human skin fibroblasts and differentiated U937 cells

Addition	Concentration	TGS	Ca^{2+} Flux
	μM	*(% of control)*	
C3a holo form	1.7	181 ± 8	160
C57–77 peptide	100	110 ± 10	200
ASP (C3adesArg) holo form	1.7	158 ± 11	nc
C57–76 peptide	100	105 ± 15	nc

Human skin fibroblasts were preincubated overnight in serum-free DMEM/F12 and then changed to serum-free DMEM/F12 supplemented with 100 μM [^{3}H]oleate complexed to BSA and the indicated concentrations of ASP, C3a, or peptides. Triglyceride synthetic capacity (TGS) was measured for 6 h as [^{3}H]oleate incorporation into triglyceride. Results are expressed as percent change (mean ± SD) for n = 4. U937 cells were differentiated with dibutryl cAMP for 3 days and Ca^{2+} flux was measured by the Fura-2 method for C3a, ASP, and the 21 and 20 carboxy terminal amino acid peptides as shown in Fig. 8; nc, no change from basal values.

Québec. Ian Murray is the recipient of a fellowship from the Royal Victoria Hospital Research Institute.

Manuscript received 10 December 1996 and in revised form 31 July 1997.

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