Acylation-stimulating protein (ASP): structure–function determinants of cell surface binding and triacylglycerol synthetic activity

Ian MURRAY*, Jörg KÖHL† and Katherine CIANFLONE*[1]

*Mike Rosenbloom Laboratory for Cardiovascular Research, Royal Victoria Hospital, McGill University Health Center, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1, and †Institute of Medical Microbiology, Hannover Medical School, Hannover, Germany

Acylation-stimulating protein (ASP or C3adesArg) is a potent lipogenic factor in human and murine adipocytes and fibroblasts. The arginated form of ASP, i.e. complement C3a (C3a), stimulates immunological responses in human granulocytes, mast cells, guinea pig platelets and guinea pig macrophages; however, ASP is inactive in stimulating these responses. Thus both ASP and C3a are bioactive across species but are not functionally interchangeable. Tertiary structure of both proteins by X-ray crystallography and NMR spectroscopy predicts a tightly linked core region consisting of three α-helices linked via three disulphide bonds, with one of the α-helices extending out from the core and terminating in a flexible conformationally irregular carboxy-tail region. The present studies were undertaken in order to define the functionally active domains of ASP, distinctive from those of C3a, using chemical modifications, enzymic cleavage and synthetic peptide fragments. The results indicate that: (i) the N-terminal region (< 10 amino acids) plays little role in ASP receptor binding and triacylglycerol synthesis stimulation; (ii) the native C-terminal region had no activity, but modifications which increased hydrophobicity increased receptor binding, and led to some activation of triacylglycerol synthesis stimulation; (iii) an intact disulphide-linked core region is essential for triacylglycerol synthesis stimulation activity but not for receptor interaction. Finally, basic charges in the carboxy region (His) are essential for ASP triacylglycerol synthesis stimulation but not for receptor binding, whereas both functions are eliminated by the modification of Lys in the disulphide-linked core region. The present results suggest that there are two functional domains in ASP, one that is responsible for the initial binding to the cell surface receptor, and a second domain that activates and increases triacylglycerol synthesis stimulation. This contrasts markedly with the structure–function studies of C3a where both binding competency and function were dependent on the C-terminal Arg. Thus ASP demonstrates distinct bioactivity.

Key words: complement C3a, receptor, triacylglycerol synthesis.

INTRODUCTION

Acylation stimulating protein (ASP) has a unique function in lipid metabolism. ASP stimulates triacylglycerol synthesis in primary human adipocytes and to a lesser degree in preadipocytes and fibroblasts [1]. The effect of ASP is mediated through a coordinate effect on fatty acid esterification by increasing the activity of diacylglycerol acyltransferase, the final enzyme in triacylglycerol synthesis [2], and stimulating glucose transport. In adipocytes, smooth muscle cells, preadipocytes and fibroblasts, glucose transport increases due to translocation of the glucose transporters (GLUT1, GLUT3 and GLUT4) from intracellular sites to the plasma membrane surface [3,4]. These dual effects are mediated through specific cell surface binding [5] resulting in activation of a signalling pathway that includes activation of protein kinase C [6].

Sequence analysis of ASP indicated that it was identical to the desarginated form of complement C3a (C3a), i.e. C3adesArg [1]. C3a is generated when the complement system is activated via either the classical or alternative pathway. In adipose tissue, alternative pathway activation involving C3 (the precursor protein), factor B and adipsin (factor D) has been demonstrated [7]. The terminal Arg of C3a is cleaved by carboxypeptidase N to generate ASP, and in normal human plasma, only the ASP form of the protein is present [8]. C3a stimulates proinflammatory responses in different immunocompetent cells such as histamine release from interleukin-3 stimulated basophils [9], chemotaxis of eosinophils [10] and mast cells [11], release of reactive oxygen metabolites from polymorphonuclear leukocytes [12] and aggregation and degranulation of guinea pig platelets [13]. These bioactive effects are mediated by specific interaction with the C3a receptor, which belongs to the large group of G-protein-coupled receptors [14,15]. The desarginated form of the protein has no effect on the above mentioned cell populations and does not bind to the cloned C3a receptor [16,17]. These data are in contrast to its potent bioactivity in adipocyte lipogenesis [5]. Human ASP has been shown to stimulate triacylglycerol synthesis and glucose transport in human adipocytes [1,4,5], murine 3T3 cells [5], primate cynomologous and African green monkey adipocytes [5], rat L6 myotubes [18] and human fibroblasts [1]. Thus these two similar polypeptides have distinct metabolic properties.

The tertiary structures of both C3a and ASP have been analysed using X-ray crystallography and solution NMR spectroscopy [19,20]. No structural changes induced by the presence of the C-terminal Arg could be demonstrated [20], but this might be difficult due to the flexible irregular conformation of the carboxy-tail region. Nevertheless, the presence of this terminal Arg appears to be crucial for C3a bioactivity [16]. The physical analyses indicate that ASP is composed of a tightly linked core region consisting of three α-helices (residues 17–27, 35–41 and 47–73) held intact through three disulphide bonds with one of the

Abbreviations used: ASP, acylation-stimulating protein; DEPC, diethyl pyrocarbonate; C3a, complement C3a; DMEM, Dulbecco's modified Eagle's medium; HSF, human skin fibroblasts; 3T3, 3T3-L1 preadipocytes.

[1] To whom correspondence should be addressed (e-mail mdkc@musica.mcgill.ca).

α-helices extending out, terminating in a flexible irregular conformation carboxy-tail region of 5–6 amino acids that is free from interactions with the rest of the molecule [21]. The N-terminal segment does not remain docked to the Cys-stabilized core of the molecule but is in dynamic motion and is helical from residues 5–15.

It is striking that both ASP and C3a are bioactive across species but are not functionally interchangeable. Extensive functional studies have been performed on C3a (review [21]). Initial studies indicated that the disulphide-linked core region was not essential for C3a bioactivity because unfolding, reduction and refolding of the molecule did not affect activity [21]. However, it did appear that the native C-terminal region was sufficient for bioactivity to a greater [22] or lesser [23] degree. A number of C3a analogues were developed based on this information [24], and indeed such a bioactive peptide was instrumental in cloning and characterizing the C3a receptor [14,15]. However, since ASP and C3a share few biological effects, any modification to C3a may not be relevant to ASP. Although desargination of C3a destroyed proinflammatory responses, both the desarginated form of the protein and the arginated form were equally competent for triacylglycerol synthesis stimulation [5]. Preliminary studies indicated that the native C-terminal peptide of ASP had no triacylglycerol synthetic stimulatory bioactivity [5], although it has been reported to have from 10 % to full (100 %) C3a activity [22,25]. The aims of the present paper were two-fold: (i) to determine whether the modifications or peptides that have been shown to conserve C3a stimulatory activity previously (see above) also maintain ASP stimulatory activity, and (ii) to determine whether other modifications, which have not been examined with respect to C3a but have been shown to be important for a structurally similar molecule (C5a), might be important for the functional activity of ASP.

MATERIALS AND METHODS

ASP purification and peptides

Human plasma ASP was prepared as described previously [6]. Purity and molecular mass were verified by electrospray ionization–MS. The following C-terminal native and modified peptides were used: RASHLGLA [native ASP(69–76)], LRRQ-HARASHLGLA [native ASP(63–76)], LRRQAWRASALG-LAR (P117) and fluoren-9-ylmethoxycarbonyl-YAAALGLAR (P32). Peptides P32 and P117 were prepared by solid-phase synthesis and analysed by TLC, HPLC and amino acid analysis as described in [26]. The modified residues of P32 and P117 are underlined.

ASP digestion

ASP (500 μg) was digested with Asp-N endoproteinase (Boehringer Mannheim, Laval, Quebec, Canada) (Asp-N endoproteinase/ASP, 1:333, w/w) for 60 min at 37 °C. Chemical cleavage of methionines was performed using CNBr [27]. A crystal of CNBr (292 μg) was added to 1 ml of 88 % (v/v) formic acid (final reaction concentration = 70 %), followed by the addition of 500 μg of ASP (CNBr/total methionines, 300:1, molar ratio). The reaction was carried out in a fumehood in the dark for 4 h at room temperature. CNBr was removed under a stream of nitrogen in a fumehood and ASP was lyophilized and reconstituted in 500 μl of PBS. The fragments were separated by HPLC and the molecular mass of the isolated fragments was determined by electrospray ionization–MS. Protein concentration was measured using the Lowry assay [28].

ASP chemical modification

The disulphides were reduced and blocked to prevent dimerization of Cys residues [29]. Purifed ASP (500 μg) in PBS was incubated with an equal volume of a solution containing 8 M urea, 0.29 M Tris/HCl, pH 8.6, 0.4 % β-mercaptoethanol and 5.25 mM EDTA in a 2 ml siliconized tube at 37 °C for 1 h, and flushed with N_2 to prevent oxidation. The Cys residues were then blocked with the addition of iodoacetic acid (1.44 M) in 1 M NaOH (room temperature) or iodoacetamide (0.724 M) in PBS (4 °C) for 30 min in the dark. The reaction was terminated by re-purifying ASP by HPLC.

The basic Lys residues of ASP were modified as described by Turk and Macek [30]. Pyridoxal-5-phosphate (66 mM final concentration) was added to plasma ASP (500 μg) in 0.1 M phosphate buffer, pH 7.1, and incubated in the dark at 4 °C for 60 min. Ice-cold sodium borohydride was added (final concentration 250 nM) to reduce the imine bond and a drop of octanol was added to reduce foaming. The mixture was incubated at 4 °C for 50 min and then at room temperature for 10 min. The sample was degassed under vacuum then re-purified by HPLC.

Histidine residues were modified with diethyl pyrocarbonate (DEPC) [30]. DEPC concentration was determined in the solution prior to use [31]. To 500 μg of ASP in 0.1 M phosphate buffer, pH 6.0, DEPC [1 % (v/v) in anhydrous ethanol, 4 °C] was added to a final concentration of 1.4 mM and incubated for 60 min at room temperature. To prevent protein denaturation the alcohol concentration did not exceed 2 % (v/v).

As additional controls (for all of the chemical modifications), reagents were added to ASP and injected immediately on to HPLC columns for re-purification. The protein concentration of modified ASP was determined using the Lowry assay [28]. Changes in the overall charge of the molecule were assessed by native gel electrophoresis (16.7 % polyacrylamide) as described by Reisfield for basic proteins [32], as well as by CD (see below).

CD of ASP

CD spectra of ASP preparations in PBS, pH 7.4, were measured in the far-UV range (200–250 nm) using a Jasco 710 spectropolarimeter. Spectra represent the accumulation of 10 scans per sample at a scan speed of 10 nm/min, bandwidth 1.0 nm and resolution 0.2 nm at a sensitivity setting of 100 millidegrees. Dynode voltage did not exceed 600 V and the sample concentration was from 0.3 to 1.1 mg/ml in cuvettes of 0.1 cm path length. Protein concentrations of all preparations were determined by the Lowry assay [28] and the spectra adjusted accordingly by computer-assisted analysis (J-700 for Windows Standard Analysis, Ver 1.10.00; Jasco Inc., Easton, MD, U.S.A.) and expressed as molar mean residue ellipticity.

Lipid synthesis in cultured cells

Human skin fibroblasts (HSF) were obtained from forearm biopsies of normolipidemic subjects and cultured as described previously [1]. 3T3-L1 preadipocytes (3T3; obtained from A.T.C.C., Manassas, VA, U.S.A.) were cultured in the same fashion as the human fibroblasts. Both types of cells were stimulated by ASP to the same extent and the cell type used is indicated in the Figure legends [5]. Cells were subcultured, plated out at 3×10^3 cells/cm^2, and grown in 10 % (v/v) fetal calf serum in Dulbecco's modified Eagle's medium (DMEM)/F12 medium. Cells were used for experiments at 80 % confluency. 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. Triacylglycerol synthesis was measured as the incor-

poration of [^{3}H]oleate (specific radioactivity 10.0 Ci/mmol; Du Pont-New England Nuclear, Mississauga, Ontario, Canada) into triacylglycerol. Cells were incubated for 18 h in 100 μM oleate complexed to BSA (5:1, molar ratio; average final specific radioactivity = 100 d.p.m./pmol) in serum-free DMEM/F12 medium as described previously [5]. Results were expressed as nmol of [^{3}H]oleate incorporated into triacylglycerol per mg of cell protein.

Radiolabelled competition binding assay

ASP was radiolabelled using Iodogen (Pierce Chemicals, Rockford, IL, U.S.A.) and the specific radioactivity was measured as d.p.m. per μg of trichloroacetic acid [10% (w/v)] precipitable protein (average specific radioactivity = 20 d.p.m./fmol). Competitive binding was performed on HSF cultured in 96-well plates. Cells were preincubated with serum-free DMEM/F12 medium overnight. Cells were pre-chilled on ice for 15 min followed by an incubation for 1 h with 1 or 50 nM ^{125}I-ASP in 100 μl of 1% (w/v) BSA in PBS and increasing concentrations of unlabelled ASP. After a 1 h incubation, 5 μl aliquots (in triplicate) of medium were counted for the calculation of free ASP. Cells were washed three times with ice-cold PBS and the soluble cell protein was dissolved in 100 μl of 0.1 M NaOH. Aliquots were taken for counting bound ASP, and cell protein determination was by the method of Bradford [33] using a commercial assay (Bio-Rad, Mississauga, Ontario, Canada). The results were calculated as nmol of ASP bound per mg of soluble cell protein and expressed as % competition where 100% = amount of ^{125}I-ASP bound in the absence of competitor. Calculation of competition was performed by iterative four-parameter logistic function analysis (Sigma Plot, Jandel Scientific, San Rafael, CA, U.S.A.) in order to calculate the specific binding, non-specific binding, and IC_{50}.

Statistics

All results are expressed as the mean ± S.E.M. Significant differences were calculated by one-way or two-way ANOVA, or Student's *t*-test (as indicated in the text).

RESULTS

Phylogenetic comparison of the primary sequence of ASP from a number of species including human (accession no. M65080) [34], rat (accession no. P01026) [35], mouse (accession no. P01027) [36], guinea pig (accession no. P12387) [37], chicken (accession no. V16848) [38], cobra (accession no. L02365) [39], trout (accession no. P98093) [40], lamprey (accession no. Q00685) [41], hagfish (accession no. P98094) [41] and pig (accession no. P01025) [42] was performed by computer-assisted analysis. Overall there was 67 to 72% primary sequence homology between the mammals. The C-terminal region (LGLA) was highly conserved with 100% identity between mammals for the last four amino acids. Similarly, the N-terminal region (amino acids 1–15) was 73 to 80% identical between the mammals. For all sequences, there were six Cys residues, with three disulphide bonds in the motif $CC(X)_{12}$-$C(X)_{12}$-$C(X)_6$-CC. This was not only characteristic of ASP sequences but also of C5a, C4a, fibulin and sex-linked protein [43]. Finally, human ASP is a very basic molecule with a pI of 9.1 [1]. A basic pI (9.05–9.99) was also predicted from the other mammalian sequences. The positively charged amino acids in human ASP are primarily Arg ($n = 10$), Lys ($n = 7$) and His ($n = 2$), with the Lys and His located mainly in the N-terminal core 'head' region and C-terminal 'tail' regions respectively.

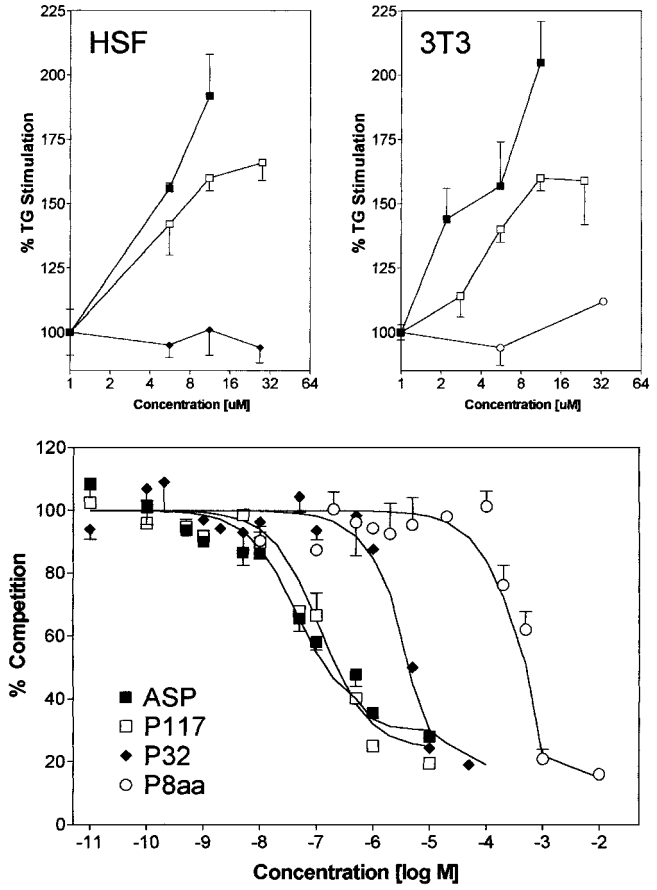

Figure 1 Effect of native and modified peptides on triacylglycerol synthesis stimulation and ASP competition binding

HSF and 3T3 cells (as indicated) were cultured as described in the Materials and methods section. Top: for triacylglycerol synthesis stimulation experiments, the indicated concentrations of ASP or peptides were added to serum-free medium containing 100 μM [^{3}H]oleate complexed to BSA. The cells were incubated for a further 6 h. Triacylglycerol synthesis stimulation was measured as the incorporation of [^{3}H]oleate into triacylglycerol. Results are expressed as % TG stimulation where basal triacylglycerol synthesis (nmol triacylglycerol/mg of cell protein) for HSF = 71.9 ± 7.0 ($n = 2$) and 3T3 = 78.3 ± 10.3 ($n = 4$). Bottom: for competition binding studies, HSF were incubated at 4 °C for 1 h with 1 nM ^{125}I-ASP and increasing concentrations of unlabelled ASP or peptides as competitors (15 different concentrations carried out in duplicate). Results are expressed as % competition, each competitor was tested in two or three separate experiments, where ASP binding at 100% (no competitor) = 140 ± 22 fmoles of ^{125}I-ASP/mg of cell protein ($n = 13$). Both the degree of inhibition (top − bottom plateau) and the IC_{50} were calculated by iterative four parameter logistic function analysis (IC_{50} 44 nM native ASP, 120 nM P117, 5.6 μM P32, 1.2 mM P8aa; $P < 0.01$ by two-way ANOVA, $P < 0.05$ for P32 and P8aa versus native ASP, not significant for P117 versus native ASP). uM, μM.

Conserved sequences which are common across species may represent important functional sites. Structure–function modifications were targeted to the following aspects of the molecule: the disulphide knot core region, basic charges, and the C- and N-termini. The modifications used were (i) chemical modifications of specific amino acids, (ii) enzymic and chemical cleavage of specific regions of the protein, and (iii) use of synthetic peptide fragments which have been previously documented to retain C3a activity [15,22,24]. In each case, the modifications were tested in two ways: first, for triacylglycerol synthesis stimulatory capacity, which was determined as the increase in [^{3}H]oleate incorporation into storage triacylglycerol in human fibroblasts or murine 3T3 cells, and secondly, for the capacity to competitively inhibit binding of native ^{125}I-ASP to cell-surface receptor-binding sites.

Table 1 Effect of N-terminal cleavage of ASP on triacylglycerol synthesis stimulating activity and competition binding

HSF and 3T3 cells were cultured as described in the Materials and methods section. ASP was treated with CNBr or Asp-N endoproteinase and re-purified by HPLC. Triacylglycerol synthetic stimulation was measured as [^{3}H]oleate incorporation into triacylglycerol for $n = 5$–7 (HSF) and $n = 2$ (3T3) experiments. Basal triacylglycerol synthesis is expressed as nmol of [^{3}H]oleate incorporated into triacylglycerol/mg of cell protein, where * $P < 0.05$ versus basal levels (results from Bonferroni test following ANOVA). For competition experiments, the effectiveness of the competitor was tested over 15 different concentrations (in duplicate). The degree of inhibition (top − bottom plateau) was compared with native ASP where binding at 1 nM ^{125}I-ASP = 173 ± 14 fmol of ^{125}I-ASP bound/mg of cell protein ($n = 11$). The IC_{50} was calculated using iterative four-parameter logistic function analysis where IC_{50} native ASP = 35 ± 5 nM and nd = not determined.

	Basal triglycerol synthesis (nmol/mg of cell protein)	Triacylglycerol synthetic stimulation (%)		ANOVA	IC_{50} (nM)
		Native ASP	Modified ASP		
CNBr–ASP					
HSF	127 ± 14	228 ± 17*	222 ± 25*	$P < 0.006$	113
3T3	56 ± 1	185 ± 2*	231 ± 11*	$P < 0.001$	nd
AspN endoproteinase–ASP					
HSF	144 ± 18	175 ± 28*	199 ± 2*	$P < 0.001$	123
3T3	56 ± 1	138 ± 3*	165 ± 2*	$P < 0.001$	nd

Since native and modified carboxy peptides of C3a have previously been shown to possess C3a biological activity [22, 23,44], we first examined the effects of native as well as modified C-terminal peptides on triacylglycerol synthesis. The results from the effects on triacylglycerol synthesis are shown in Figure 1 (top panels). The native eight residue C-terminal peptide of ASP (P8aa; Figure 1, top right panel) did not stimulate triacylglycerol synthesis even at molar concentrations 3-fold higher than that of ASP, which at 11.2 μM stimulated triacylglycerol synthesis in 3T3 cells by 205 % versus basal levels ($P < 0.002$ by ANOVA). P8aa was also ineffective at inhibiting ^{125}I-ASP binding to cell-surface receptors as assessed in a competition binding assay. Only at extremely high concentrations was the peptide capable of inhibiting ASP binding (IC_{50} 1.2 mM; Figure 1, bottom panel).

This peptide sequence was modified as described by Gerardy-Schahn et al. [23] to increase the hydrophobicity of the peptide through the addition of a fluoren-9-ylmethoxycarbonyl group, which they proposed would enhance the interaction with biomembranes and alter the association with the receptor from a 3-dimensional to a 2-dimensional interaction to increase the rate of association. This peptide (P32) was fully bioactive for C3a activity [23]. The modification increased the capacity of P32 to inhibit native ASP binding, although the concentration of peptide required was still greater compared with ASP (IC_{50} of 5.6 μM compared with 44 nM for native ASP, $P < 0.05$). However, this modified peptide, P32, demonstrated no stimulation of triacylglycerol synthesis even at high molar concentrations (Figure 1, top left panel).

Longer peptides were also used. A 15-mer native peptide was incapable of stimulating triacylglycerol synthesis or inhibiting binding of ASP (results not shown). A modified 15-mer peptide (P117) that has been shown to possess full C3a activity [15] was also tested for triacylglycerol synthetic stimulatory activity. The modifications to the peptide from the native sequence were designed to increase its hydrophobicity and enhance membrane interaction (as with P32) as described by Crass et al. [15]. P117 was competent to inhibit ASP binding at higher concentrations (an IC_{50} of 120 nM was not significantly different compared with native ASP), and had a consistent effect on the stimulation of triacylglycerol synthesis in both HSF (Figure 1, top left panel; $P < 0.001$ by ANOVA versus basal levels) and 3T3 cells (Figure 1, top right panel; $P < 0.001$ by ANOVA versus basal levels), although at higher concentrations than ASP [166 % HSF and 159 % 3T3 at 25 μM; Figure 1]. Thus, the modified C-terminal region was not sufficient for complete ASP bioactivity but it did appear to mimic partial receptor binding.

We tested the involvement of the N-terminal region in binding and triacylglycerol synthesis stimulation. ASP was treated with CNBr or with Asp-N endoproteinase in order to remove the N-terminal region and the remaining ASP was re-isolated and tested. MS analysis indicated that with Asp-N endoproteinase treatment, the N-terminal peptide (1–9) was removed leaving a protein of 7860 Da. In the CNBr-treated ASP both the N-terminal peptide (1–9) and the peptide fragment (28–32) that forms part of a loop region in the core were removed (remaining protein = 7203 Da). In both cases, as shown in Table 1, the remaining ASP fragment was capable of stimulating triacylglycerol synthesis in both HSF and 3T3 cells (199 ± 2 % Asp-N endoproteinase, $P < 0.05$, and 222 ± 25 % CNBr, $P < 0.05$ versus basal levels for HSF and 165 ± 2 % ASP-N endoproteinase, $P < 0.05$ and 231 ± 11 % CNBr, $P < 0.05$ versus basal levels for 3T3). In addition, both proteins were capable of inhibiting ASP binding to the same extent as native, intact ASP (IC_{50} of 123 nM for Asp-N endoproteinase and 113 nM for CNBr, not significant by ANOVA versus ASP). Thus, the small N-terminal segment in the flexible amino region (less than 10 amino acids) appeared to play little role in ASP bioactivity.

The disulphide-linked core-region structure was modified to alter the secondary structure of ASP. The protein was unfolded with urea, and the disulphides cleaved and blocked with either iodoacetamide (Cys-NH), which does not alter the overall protein charge, or iodoacetic acid (Cys-COOH), which adds one new acidic group per Cys residue to the protein and renders the protein less basic. As a result, the migration on native basic gel electrophoresis was substantially retarded for Cys-COOH, but less so for Cys-NH (results not shown). The conformation of these modifications was analysed by CD in the far-UV region as shown in Figure 2 (left panel). Native ASP demonstrated a characteristic 'w' profile, indicating high α-helix content. Denaturation of ASP (with β-mercaptoethanol at 100 °C for 5 min) reduced the molar ellipticity dramatically (a similar profile was obtained with ASP boiled at 100 °C for 3 h; results not shown). These results are comparable with those reported previously for C3a [45]. Treatment of ASP with urea alone (without reduction of the Cys disulphide bonds) followed by the removal of urea has been shown to have no inhibitory effect on triacylglycerol stimulating activity or on competition binding [5], and as shown here, had no effect on molar ellipticity analysed by CD (Figure

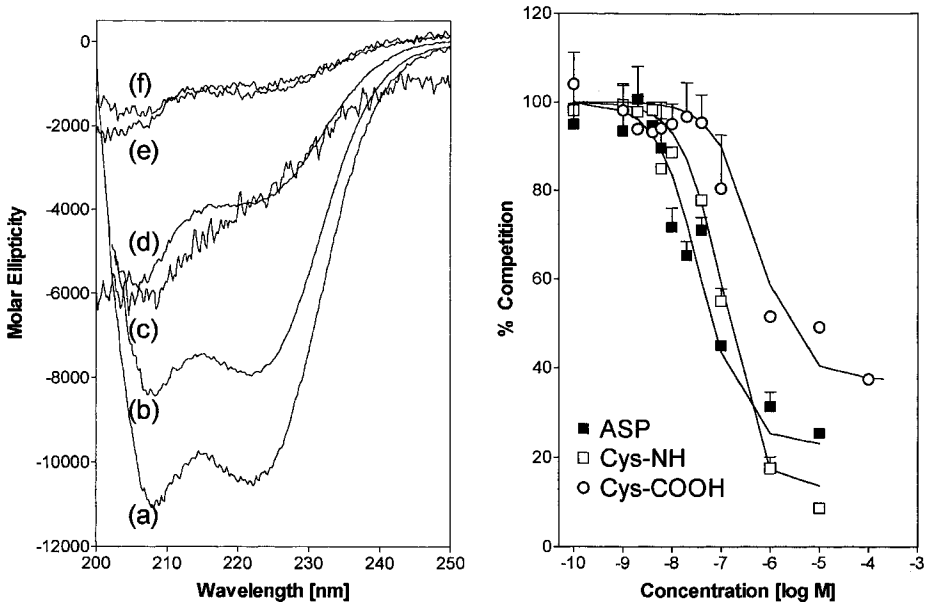

Figure 2 Effect of modified Cys of ASP on CD profile and competition binding

CD of native and modified ASP in PBS (left). a, native ASP; b, urea-treated ASP; c, boiled and β-mercaptoethanol treated ASP; d, ASP treated with urea and β-mercaptoethanol; e, Cys-NH ASP; f, Cys-COOH ASP. Units of molar ellipticity, θ. HSF were cultured as described in the Materials and methods section (right). ASP was modified following treatment with urea and iodoacetamide (Cys-NH) or iodoacetic acid (Cys-COOH) and was re-purified by HPLC following treatment. Results are expressed as % competition, where each competitor was tested in two or three separate experiments and where ASP binding at 1 nM ^{125}I-ASP (100% no competitor) = 202 $\pm$ 25 fmoles of ^{125} I-ASP/mg of cell protein ($n = 9$). Both the degree of inhibition (top — bottom plateau) and the IC_{50} were calculated by iterative four-parameter logistic function analysis (IC_{50} 37 nM native ASP, 120 nM Cys-NH and 510 nM Cys-COOH; $P < 0.001$ by two-way ANOVA, $P < 0.05$ for Cys-NH and Cys-COOH versus native ASP). In all cases, ASP was re-purified by HPLC, then tested for bioactivity and analysed by CD.

Table 2 Triacyglycerol synthetic activity of modified ASP

3T3 cells were prepared as described in the Materials and methods section. ASP was modified by treatment with urea and iodoacetamide (Cys-NH–ASP), urea and iodoacetic acid (Cys-COOH–ASP), DEPC (His-ASP), or pyridoxal-5-phosphate (Lys-ASP) and re-purified by HPLC following treatment. Activity was measured as nmol of [^{3}H]oleate incorporation into triacylglycerol/mg of cell protein (see legend to Figure 1), where * $P < 0.05$ and ** $P < 0.001$ for modified versus native (untreated) ASP.

Modification	Basal triglycerol synthesis (100%)	Native ASP (%)	Modified ASP (%)	Inhibition (%)
Cys-NH–ASP ($n = 8$)	109 $\pm$ 22	232 $\pm$ 34 (213)	128 $\pm$ 23* (117)	85
Cys-COOH–ASP ($n = 7$)	109 $\pm$ 22	232 $\pm$ 34 (213)	158 $\pm$ 30* (145)	60
His-ASP ($n = 4$)	94 $\pm$ 23	165 $\pm$ 56 (175)	104 $\pm$ 41* (111)	85
Lys-ASP ($n = 9$)	80 $\pm$ 12	177 $\pm$ 27 (221)	76 $\pm$ 15** (95)	100

2, left panel). In both cases, treatment to block Cys residues resulted in an increase in the IC_{50} from 3- to 14-fold compared with native ASP (IC_{50} 37 nM native ASP versus 120 nM Cys-NH and 510 nM Cys-COOH, $P < 0.001$ by ANOVA; Figure 2). However, the triacylglycerol-stimulating activity was lost (85 $\pm$ 6% inhibition Cys-NH, $P < 0.05$; 60 $\pm$ 2% inhibition Cys-COOH, $P < 0.05$ versus native ASP, $P < 0.003$ ANOVA; Table 2). As might be expected, there was a marked change in the structural conformation of the treated ASP, and for both Cys-NH–ASP and Cys-COOH–ASP, CD analysis demonstrated a marked reduction in the α-helicity of both molecules (Figure 2, left panel). Thus an intact core region appears to be essential for activity, although not necessarily for receptor interaction.

ASP is a very basic polypeptide (pI 9.1) [1]. These basic charges are due principally to His, Lys and Arg residues. The Arg residues [10] are distributed along the 'backbone' of the protein, while the His residues [2] are found in the carboxy-tail region. Of the seven Lys residues, five are found in the N-terminal region (between residues 7–21) and two at residues 50 and 51. These residues cluster as a 'crown' in the 'head' region of the α-helix–disulphide-linked core. We targeted Lys and His for chemical modification. In each case, modification resulted in changes in mobility as assessed by native basic gel electrophoresis (results not shown). The results for competition binding are shown in Figure 3 (right panel). His modification reduced the capacity to inhibit specific binding (IC_{50} = 707 nM versus 44 nM for native ASP), whereas with Lys modification there was a complete loss of competitive binding. CD analysis of the chemically modified ASP, His-ASP, demonstrated a 'w' profile comparable to native ASP, whereas the molar ellipticity for Lys-ASP was substantially diminished, suggesting major changes in the structure of the molecule. In each case, the capacity to stimulate triacylglycerol synthesis was markedly reduced (85 $\pm$ 11% inhibition His-ASP, $P < 0.05$, and 100 $\pm$ 8% inhibition Lys-ASP, $P < 0.001$ versus positive ASP control which stimulated up to 2-fold; Table 2). Thus modifications that

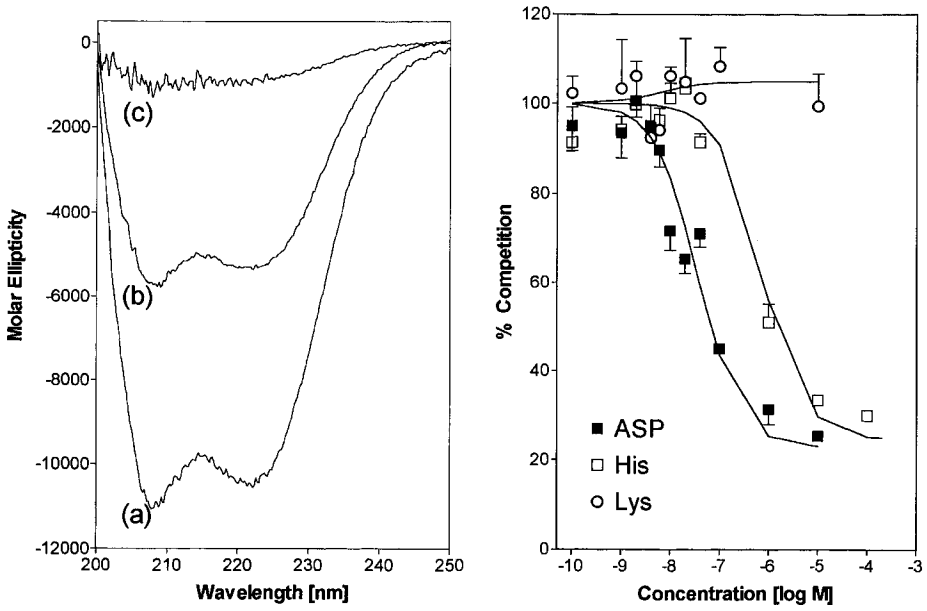

Figure 3 Effect of modifying basic residues of ASP on competition binding

HSF were cultured as described in the Materials and methods section. ASP was modified following treatment with DEPC (for His modification), or pyridoxal-5-phosphate (for Lys modification) and re-purified by HPLC following treatment. Left: CD of native ASP (a), His-ASP (b), and Lys-ASP (c). Conditions are as described in the legend to Figure 2. Units of molar ellipticity, θ. Right: results are expressed as % competition for the curve calculated from duplicate points at each of 15 concentrations; each competitor was tested in two or three separate experiments where ASP binding at 100% (no competitor) $= 142 \pm 84$ fmol of ^{125}I-ASP/mg of cell protein ($n = 2$). Both the degree of inhibition (top $-$ bottom plateau) and the IC_{50} were calculated by iterative four-parameter logistic function analysis (IC_{50} 44 nM native ASP, 707 nM His-ASP, and cannot be calculated for Lys-ASP; $P < 0.003$ by two-way ANOVA, $P < 0.05$ for His-ASP and Lys-ASP versus native ASP).

interfered with the receptor binding capacity also resulted in a loss of activity and the results indicated that the basic residues appeared to be important for both receptor interaction and subsequent activity.

DISCUSSION

The results suggest that ASP function is mediated through initial binding to a cell surface receptor, and then cellular activation resulting in increased triacylglycerol synthesis. Binding can occur without stimulation of triacylglycerol synthesis but triacylglycerol synthesis stimulation cannot occur in the absence of ASP binding. Modifications which altered the Cys-bound core region had little effect on specific binding. Similarly, removal of the N-terminal nine amino acid portion of ASP had no effect on binding. In constrast, modification of the Lys residues, five of which are present in the N-terminal region of the core (between residues 7–21), did affect binding greatly. In addition, the C-terminal region appeared to be involved directly in receptor binding since alteration of the two C-terminal His residues decreased binding affinity substantially. Although the native eight or fifteen C-terminal amino acid peptide could not inhibit ASP binding, modifications which resulted in increased hydrophobicity (and presumably increased cell surface contact) did possess partial inhibitory capabilities, suggesting that this region participates but is not sufficient for complete receptor binding competency.

Triacylglycerol synthesis stimulatory activity of ASP was dependent on binding. In the absence of binding to the cell surface there was no triacylglycerol stimulatory activity remaining. This was clearly demonstrated by the modifications that targeted the basic residues (His and Lys), which were poor competitive inhibitors and had no significant triacylglycerol stimulatory activity. However, the distinct nature of a postulated 'binding domain' compared with an 'active domain' could be seen in some modifications where the molecule was clearly competent to bind, but had little bioactivity. For example, the C-terminal peptide, P117, had an IC_{50} that was 3-fold that of native ASP, yet it could only partially stimulate triacylglycerol synthesis. The P32 peptide was even less efficient at inhibiting radiolabelled ASP binding, and stimulating triacylglycerol synthesis. It has been suggested that hydrophobic elements enhance the structural conformation of the peptide to stabilize receptor interaction and increase activation [44], or that the local concentration of the peptides is increased by the hydrophobic interaction of the fluoren-9-ylmethoxycarbonyl moiety and/or the modified amino acid residues with the cell membrane [46]. An even more striking example is the two treatments that modified the Cys residues within the globular core. In both cases, the polypeptides were effective binding inhibitors, but had no significant residual triacylglycerol stimulatory activity. This also points towards the importance of structures in the tightly folded core region in the activation of triacylglycerol synthesis.

The results in the present study on ASP binding and activity contrast markedly with the structure–function studies on C3a carried out by several groups [22,23,47]. In those studies, it was clearly demonstrated that both the binding competency and the functional domain, as assessed in differentiated U937 cells as well as other immunocompetent cells, were dependent on the C-terminal Arg (which is not present in ASP). Thus a native C-terminal peptide was sufficient to demonstrate activity, and modified C-terminal peptides of eight to fifteen residues [22,23] retained complete binding capacity and degranulation activity, although peptides lacking the terminal Arg, as well as ASP, did not demonstrate any degranulation activity [5,22,23,47]. These same modified peptides only contained partial inhibitory effects on ASP binding and some triacylglycerol stimulatory activity, highlighting the contrasts in the two different biological systems.

Other examples of peptides which exist in both the desarginated, as well as the arginated form have been documented. For example C5a, which has a structure very similar to that of ASP, with a tightly folded Cys-linked core region and a flexible C-terminal tail region, also exists in a desarginated form. In this case, the two forms of C5a differ in their binding affinities by a factor of 10–30 [48,49], while their activity level is either decreased from nM to μM (as demonstrated for smooth muscle cells) or not affected at all (enzyme release from differentiated U937 cells) [49]. Structure–function analysis through site-directed mutagenesis suggests that domains other than the C-terminal Arg, such as basic residues located in the core region, are responsible not only for C5a activity, but also for binding, and these studies were used as the basis for the ASP modifications examined in the present paper. A two-site model is proposed for the C5a receptor accommodating a 'binding region' of the anaphylatoxin composed of the N-terminal region knotted by disulphides which presents an 'activating domain' containing the C-terminal positions 67–74 [50], and we hypothesize that this may also be so for ASP.

Bradykinin and bradykinindesArg exhibit characteristics similar to those of C3a and ASP. Bradykinin is a nine residue peptide that has potent dilatory and motility effects through various second messenger systems, including effects on intracellular calcium, cAMP and cGMP, in aortic mesangial and endothelial cells, to name but a few. BradykinindesArg has similar effects (for reviews see [51,52]). The effects of these two peptides, however, are mediated through the interaction with two distinct receptors (B1 and B2) derived from two different genes. At the protein level, these receptors are similar in secondary structure (both are G-protein-linked receptors), but share only 54% sequence identity in humans. Thus B2 binds bradykinin with high affinity, whereas B1 binds bradykinindesArg to a similar extent, although different affinity forms of the receptor also exist. The difference in structure between the two peptides is sufficient such that agonists/antagonists have been developed which can selectively block the activity of one peptide without affecting the activity of the other. Some cells express only B1 receptors (rabbit aorta) or only B2 receptors (rabbit jugular vein), whereas other cells express both (human umbilical vein endothelial cells and fibroblasts).

Thus we hypothesize that the situation, with respect to ASP and C3a, would appear to be similar to that of bradykinindesArg and bradykinin. In human adipocytes and fibroblasts, both ASP and C3a are able to stimulate triacylglycerol synthesis [5], whereas in human granulocytes, mast cells and differentiated U937 cells, only C3a produces a degranulation response [5,9–12,16]. Differential competitive binding characteristics are also demonstrated. Both ASP and C3a can bind with high affinity to adipocytes and fibroblasts (M. Maslowska, D. Kalant, A. D. Sniderman and K. Cianflone, unpublished work), but only C3a can bind to basophils, polymorphonuclear leukocytes, monocytes, mast cells, differentiated U937 cells or guinea pig platelets [16,53–57]. These results suggest that there may be two distinct receptors, one that binds ASP and C3a, and a second one that only binds C3a and is dependent on the presence of the terminal Arg of C3a. A C3a receptor has been cloned from a U937 expression library [15] and from a neutrophil cDNA library using expression tag analysis [14], and this receptor does not bind ASP [16,17]. An ASP receptor remains yet to be identified.

We have demonstrated that ASP plays a role in regulating adipose tissue metabolism both *in vitro* and *in vivo* [8,58]. A number of disorders such as obesity, diabetes and cardiovascular disease have been associated with abnormal adipose tissue metabolism and understanding adipose tissue metabolism and regulation has become a major research area in recent years [59]. ASP dysfunction is present in specific metabolic disorders [58] with increased ASP levels in obesity [60], and ASP dysfunction in a subgroup of hyperapoB patients [61–63]. Thus, one of the implications of defining regions that are important in ASP activity, but have no effect on C3a bioactivity, is that this may permit the identification of peptides or other organic compounds that may be used as selective ASP agonists or antagonists. These would be valuable tools in elucidating further the physiological role of ASP, or defining future pharmacological treatments for use in modifying adipose tissue function.

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