C5L2 Is a Functional Receptor for Acylation-stimulating Protein*

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C5L2 binds acylation-stimulating protein (ASP) with high affinity and is expressed in ASP-responsive cells. Functionality of C5L2 has not yet been demonstrated. Here we show that C5L2 is expressed in human subcutaneous and omental adipose tissue in both preadipocytes and adipocytes. In mice, C5L2 is expressed in all adipose tissues, at levels comparable with other tissues. Stable transfection of human C5L2 cDNA into HEK293 cells results in ASP stimulation of triglyceride synthesis (TGS) (193 ± 33%, 5 μM ASP, p < 0.001, where basal = 100%) and glucose transport (168 ± 21%, 10 μM ASP, p < 0.001). C3a similarly stimulates TGS (163 ± 12%, p < 0.001), but C5a and C5a des-Arg have no effect. The ASP mechanism is to increase V_{max} of glucose transport (149%) and triglyceride (TG) synthesis activity (165%) through increased diacylglycerolacyltransferase activity (200%). Antisense oligonucleotide down-regulation of C5L2 in human skin fibroblasts decreases cell surface C5L2 (down to 54 ± 4% of control, p < 0.001, comparable with nonimmune background). ASP response is coordinately lost (basal TGS = 14.6 ± 1.6, with ASP = 21.0 ± 1.4 (144%), with ASP + oligonucleotides = 11.0 ± 0.8 pmol of TG/mg of cell protein, p < 0.001). In mouse 3T3-L1 preadipocytes, antisense oligonucleotides decrease C5L2 expression to 69.5 ± 0.5% of control, p < 0.001 (comparable with nonimmune) with a loss of ASP stimulation (basal TGS = 22.4 ± 2.9, with ASP = 39.6 ± 8.8 (177%), with ASP + oligonucleotides = 25.3 ± 3.0 pmol of TG/mg of cell protein, p < 0.001). C5L2 down-regulation and decreased ASP response correlate (r = 0.761, p < 0.0001 for HSF and r = 0.451, p < 0.05 for 3T3-L1). In HEK-hC5L2 expressing fluorescently tagged β-arrestin, ASP induced β-arrestin translocation to the plasma membrane and formation of endocytic complexes concurrently with increased phosphorylation of C5L2. This is the first demonstration that C5L2 is a functional receptor, mediating ASP triglyceride stimulation.

The function of acylation-stimulating protein (ASP,1 also known as C3a des-Arg) as a stimulator of triglyceride synthesis (TGS) has been well documented in human adipocytes, 3T3-L1 preadipocytes, and human skin fibroblasts (HSF) (2–5). The activity of ASP also is shared by C3a (6). In addition to stimulation of TGS, ASP stimulates glucose transport (7–9) and reduces triglyceride lipolysis (10), enhancing fat storage overall. Furthermore, high affinity binding of both ligands (ASP and C3a) to HSF and 3T3-L1 cells (1, 3, 11) and of ASP to adipose tissue plasma membranes (12) has been demonstrated. On the other hand, both ASP (C3a des-Arg) and C3a have other functions in common in addition to TGS stimulation (13). ASP and C3a influence secretion of a number of hormones, including interleukin-6 and tumor necrosis factor α (14, 15), as well as prolactin, growth hormone, and adrenocorticotropic in pituitary cells (16). ASP has also been shown to influence insulin secretion in cultured pancreatic islet cells and in vivo (17).

By contrast, no binding of ASP to the C3a receptor (C3aR) has been observed in cells that bind C3a, including U937 macrophage, polymorphonuclear monocytes, transfected RBL cells, or transfected HSF293 (11, 18, 19). Furthermore, ASP does not exhibit the anaphylatoxic functions of C3a: (i) stimulation of eosinophil chemotaxis (20), (ii) prostanoid production by guinea pig macrophages or rat Kupffer cells (21), or (iii) degranulation of U937 cells (18). The division between ASP and C3a, in binding and function, may be explained by the existence of two receptors: the C3aR, which binds only C3a, and another that binds both ligands.

We recently have reported that the orphan G protein-coupled receptor (GPCR) C5L2 binds ASP and C3a with high affinity in transfected RBL cells and exhibits saturable binding of ASP in transfected HEK293 cells (1). Furthermore, we demonstrated the expression of C5L2 mRNA in human adipose tissue, HSF, and 3T3-L1 by reverse transcription-PCR and expression of the protein on the cell surface of HSF using anti-C5L2 polyclonal antisemur. These data suggested that C5L2 might mediate the TGS-stimulating effect of ASP. Although C5L2 also binds C5a and C5a des-Arg, these ligands do not stimulate triglyceride synthesis in 3T3-L1 cells (1), and in fact, it has recently been demonstrated that C5L2 is a nonfunctional receptor for C5a (22).

In the present study, we provide evidence, through both gain-of-function and loss-of-function assays in physiologically relevant cells, that C5L2 not only binds ASP but also is a functional ASP receptor. This is further documented through demonstration of ligand-activated β-arrestin-green fluorescent protein (GFP) translocation (23). The β-arrestin-GFP translocation assay has been used effectively and extensively to study diverse GPCRs that are sensitive to different ligands (24–26). It is broadly applicable for GPCR deorphaning because mammalian blast(s); TG, triglyceride; TGS, triglyceride synthesis; HA, hemagglutinin; ANOVA, analysis of variance; hC5L2, human C5L2; mC5L2, mouse C5L2.
malian receptors that couple to different signaling pathways desensitize by using a common set of GPCR kinases and arrestin proteins.

**EXPERIMENTAL PROCEDURES**

**Analysis of Murine Receptor Expression by Real Time PCR**—Wild type C57BL/6 mice were sacrificed at 9–10 weeks of age and dissected, and ~50–100 mg of tissue was stored in 500 μl of RNAlater stabilization reagent (Qiagen, Mississauga, Canada) at −80 °C. Fatty tissues (adipose, heart) were homogenized in Qiazol lysis reagent (Qiagen), all other tissues were homogenized in Buffer RLT (Qiagen) with β-mercaptoethanol, and RNA was isolated using Qiagen minikit spin columns as per the protocol provided by the manufacturer. An additional DNAase treatment and RW1 wash was used for the fatty tissues. RNA was quantified using the 260/280 OD with ratios of 1.9–2.0 obtained. RNA (5 μg) was reverse transcribed and diluted to 100 μl. 

**TAQMAN real time PCR** was used for quantification of mouse C5L2 relative to glyceroldehyde-3-phosphate dehydrogenase with primers designed by Applied Biosystems (Toronto, Canada) for C5L2 (Mm 01267981_s1) and glyceroldehyde-3-phosphate dehydrogenase (number 4308313). Template cDNA (5 μl of a 1:10 dilution) with Universal Master Mix was used according to the Applied Biosystems protocol with detection on an ABI 7700 system. C5L2 is expressed relative to the Master Mix was used according to the Applied Biosystems protocol with TGS and glucose transport assays were performed in the presence of 0.5 mM CoA and 2.5 mM ATP as described previously in adipocyte (30) and HEK (31) particulate (membrane) fractions.

**Analysis of Human Receptor Expression by Reverse Transcript-PCR**—Total RNA was isolated by Trizol extraction from freshly isolated samples of tissues. For adipose tissue, mature floating adipocytes and stromal vascular cells (containing preadipocytes) were isolated as described previously (4, 5). For reverse transcription-PCR, cDNA was produced from 3 μg of RNA by reverse transcriptase, and 4% of the reaction was amplified by PCR with 1.5 μM MgCl2 and 0.01 μM tetramethylthiuram disulfide, using the following protocol: 95 °C, 1 min at 60 °C, 1 min at 72 °C for 35 cycles. Amplification was linear over the range used. Primers for the human C5L2 gene were as follows: sense, 5′-CTCCGTGGTGTCACGTTGAC-3′; antisense, 5′-GGGAGAAGTTGGTGTCAC-3′. Primers for mouse 18 S rRNA were used as an internal control (Ambion, Austin, TX). Reaction products were separated on a 7.5% polyacrylamide gel and detected by silver staining (Bio-Rad), and a 100-bp ladder (NEB, Pickering, Canada) was used as an internal control.

**Production of ASP and C5L2 Polyclonal Antiserum**—ASP and C3a were purified from human plasma as described previously (6). C5a and C5a des-Arg were prepared as described previously (1, 27). Antiserum to ASP and C5L2 polyclonal antiserum (2) was generated with the Primer3 program (available on the World Wide Web at http://www.bioinformatics.com). 

**Design and Transfection of Antisense Oligonucleotides**—One DNA oligonucleotide was made to the translation start site of each of the human and mouse C5L2 mRNAs. Two other oligonucleotides were designed with the Primer3 program (available on the World Wide Web at http://www.bioinformatics.com) using the same default criteria as that for PCR primers. These oligonucleotides target regions in the middle and the last 25% of the translated sequence. Oligonucleotides were all phosphorothioated for stability. Oligonucleotides for the human C5L2 mRNA were as follows: 5′-GCTGAGAAATGTGGTGCCCAT-3′ (oligonucleotide 1), 5′-CTGAACCTGACCCACAAGG-3′ (oligonucleotide 2), 5′-ACAGAAGCTTCGCGACG-3′ (oligonucleotide 3). Oligonucleotides for the mouse C5L2 mRNA were as follows: 5′-CTGGTGTTGTCCTGTTGAC-3′ (oligonucleotide 1), 5′-TAAGAAGGAGTGCCCTGCTG-3′ (oligonucleotide 2), and 5′-CAATAGAAAAACCCACAC-3′ (oligonucleotide 3). The reverse complement of oligonucleotide 2 was used as a control oligonucleotide for each C5L2 mRNA, with some manual alterations necessary for the human sequence so that it would not match any sequence in the human genome. The oligonucleotides were as follows: human, 5′-ATCTTTAAGCTTACCAAGCAGAT-3′ (control (CT) oligonucleotide) and mouse, 5′-TCAGCCGAGACCTTCTTCTCA-3′ (CT oligonucleotide). Parallel sets of either HSF or 3T3-L1 cells, at 40–60% confluence in 24-well plates, were transfected with Oligofectamine (Invitrogen) according to the manufacturer’s instructions, using 2.5 or 4 μl of a stock of 20 μM oligonucleotide combined with 1 or 2 μl of Oligofectamine in triplicate (A = 2.5/1, B = 4/1, C = 2.5/2, D = 4/2 μl of oligonucleotide/μl of Oligofectamine). Cells were assayed 2 days post-transfection in parallel for (i) quantitation of C5L2 by Protein A immunoassay and (ii) ASP stimulation of TGS.

**β-Arrestin Translocation Assay**—HEK-hc5L2 cells were seeded onto glass coverslips placed in 6-well tissue culture plates and transfected with a 35 pmol of fluorescent DNA for a fusion protein of GFP-β-arrestin (32). Alternately, HEK293 cells were simultaneously transiently transfected with hc5L2 and GFP-β-arrestin. Two days later, cells were preincubated in serum-free medium for 2 h and then incubated for the indicated times in serum-free medium with the indicated ligands. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, and images were collected using an LSM-510 META confocal microscope as described previously (32).

was evaporated (lyophilization), resolubilized in chloroform/methanol (2:1, v/v), and applied to a thin layer chromatography plate (Whatman LK5D silica gel 150A, Mandel Scientific, Toronto, Canada). Lipids were separated using a solvent system of chloroform/methanol/2.5 M HCl/0.88 M triolein and oleic acid standards were used to visualize the lipid following exposure to iodine (l2) vapor. The lipid spot was scraped into scintillation vials, scintillation fluid was added, and samples were counted in a scintillation counter. Aliquots of the serum-free radiolabeled medium were counted to determine specific activity. For glucose transport, following incubation with ASP and insulin in serum-free medium for 1 h, washed three times, cells were rinsed with warm serum-free, glucose-free medium and then incubated for 5 min with 14C-labeled 2-deoxyglucose (10–500 μM, final specific activity 60–120 dpm/pmol) in serum-free glucose-free medium at 37 °C. Following incubation, the reaction was stopped with rapid washing of the cells with cold phosphate-buffered saline, and cell protein was dissolved in 0.1 N NaOH. Aliquots were taken for scintillation counting. Results were calculated as dpm of [125I]protein A bound/mg of cell protein and expressed relative to basal levels in untreated cells (equal to 100%).
Phosphorylation of C5L2—HEK cells stably transfected with hC5L2 containing an HA tag were plated in 6-well plates and incubated in serum-free phosphate-free medium for 4 h. Cells were washed once with the same medium and then incubated for 2 h in the same medium with 70 μCi of $^{32}$P/Powell. The medium was then replaced with fresh medium supplemented with 20 μM ASP for the indicated times. Cells were placed on ice, and the medium was replaced with cold radioimmunoprecipitation buffer containing aprotinin (25 μg/ml), leupeptin (25 μg/ml), pepstatin (1 μM), phenylmethylsulfonyl fluoride (1 μM) and orthovanadate (0.1 μM). After lysing for 30 min at 4 °C, cell debris was pelleted at 90,000 × g for 20 min. C5L2 was immunoprecipitated overnight at 4 °C with 30 μl of anti-HA-agarose beads (Roche Applied Science). After three washes with cold radioimmunoprecipitation buffer, samples were incubated for 30 min at 65 °C in sample buffer (containing SDS and β-mercaptoethanol), electrophoresed on SDS-PAGE, and autoradiographed.

Statistical Analysis—Results are indicated as average ± S.E. Significance was determined by t test or by ANOVA (with Bonferroni post hoc test) where significance was set at p < 0.05.

RESULTS AND DISCUSSION

C5L2 mRNA Is Expressed in Tissues That Are Responsive to ASP—We have shown recently that C5L2 mRNA is expressed in human adipose tissue, HSF, and 3T3-L1 preadipocytes (1), which all respond to ASP with an increase in TGS (1) and glucose transport (7, 9). Previous reports have shown the expression of C5L2 in a number of tissues (22, 33, 34), but none have evaluated relative expression in adipose tissue. Using real time PCR, mC5L2 in mouse tissues is expressed relative to brain, from which it was first isolated (33). As shown in Fig. 1A, mC5L2 mRNA is expressed in all adipose tissues examined, with the highest levels in gonadal and inguinal fat depots and lower levels in brown adipose tissue. Whereas spleen clearly had the highest levels of all tissues examined, which may relate to other potential functions of C5L2, the levels in fat depots were similar to those in other tissues (liver and small intestine).

RNA was also isolated from human subcutaneous and omental adipose tissue. In addition, in some preparations, adipose tissue was separated into stromal vascular cells (containing preadipocytes) and mature floating adipocytes. Reverse transcription-PCR with primers for human C5L2 mRNA demonstrates expression of C5L2 mRNA in both preadipocytes and mature adipocytes (Fig. 1B), consistent with the ASP stimulation of TGS in both adipocytes and preadipocytes (5).

Gain of Function Assay: TGS Stimulation by ASP in HEK-hC5L2 Cells—We have reported previously that HEK293 cells are competent for TGS, but ASP does not stimulate TGS in these cells (1). HEK293 cells were stably transfected with hC5L2, sorted based on fluoresc-ASP binding/uptake, and were shown to highly express C5L2 (1). As shown in Fig. 2A, stimulation of HEK-hC5L2 cells with increasing concentrations of ASP stimulated TGS severalfold and was maximal at 5 μM ASP (193 ± 33% stimulation, p < 0.001). By contrast, TGS in non-transfected HEK293 was not stimulated even at 10 μM ASP (Fig. 2A). The effect in HEK-hC5L2 cells is comparable with the stimulation seen in HSF and 3T3 cells (1). On the other hand, insulin, another known stimulator of TGS, which increases TGS in HSF cells (1), has no effect on either HEK293 or HEK-hC5L2 even at a high concentration of 100 nM (Fig. 2A, inset). This is the first demonstration that C5L2 can mediate TGS stimulation by ASP. Since insulin does not stimulate TGS in HEK-hC5L2, the enhanced effect appears to be specific to ASP. The maximal effect of ASP is obtained at concentrations about 20× the $K_d$ (1). This is similar to C5a with the C5a receptor, where the $K_d$ is about 2 nM, and maximal stimulation is obtained at about 40 nM (35).

We have shown previously that C5a as well as C4a and their des-Arg counterparts are unable to stimulate TGS in 3T3-L1 cells as measured by fatty acid (oleate complexed to bovine serum albumin) incorporation into TG (1). Accordingly, the effect of C3a, C5a, and C5a-des-Arg on oleate incorporation into TG was also investigated in HEK-hC5L2 cells. As shown in Table I, although C3a stimulated TGS comparably with ASP (as shown previously in 3T3-L1 cells (1), neither C5a nor C5a-des-Arg had any effect on TGS. In addition, C3a stimulation of TGS was specific to the expression of C5L2 in HEK cells, since addition of C5a to HEK-C5aR stably transfected cells had no effect (Table I).

Since incorporation of oleate into triglyceride could result from de novo synthesis of TG, and/or lipolysis of TG coupled to reesterification of diglyceride or monoglyceride, we also assessed glucose incorporation into TG. Glucose is taken up via glucose transporters and converted to two glycerol-3-phosphate molecules, both of which are used as the backbone for fatty acid esterification. As shown in Table II, both ASP and C3a increased $[^{14}C]$glucose incorporation into TG, whereas C5a and C5a-des-Arg had no effect. With de novo esterification of fatty acid, the theoretical ratio of $[^{14}C]$oleate/
[3H]glucose is 6:1, and a decrease in this ratio is indicative of partial lipolysis/re-esterification of TG. With all additions (ASP, C3a, C5a, and C5a des-Arg), the [14C]/[3H] ratio remained constant, as shown in Table II, indicating that the effect of ASP and C3a is on TG synthesis, not recycling, as shown previously for ASP (36). This is further supported by previous studies indicating that ASP inhibits lipolysis (10). Finally, we also assessed [14C]oleate and [3H]glucose incorporation into ASP and C3a is on TG synthesis, not recycling, as shown previously for ASP (36). This is further supported by previous studies indicating that ASP inhibits lipolysis (10). Finally, we also assessed [14C]oleate and [3H]glucose incorporation into
as before, insulin has no effect. This glucose transport-inde-
glucose transport stimulation. As shown in Table III, even in
significant). (equal to 100%). Basal 14C/3H ratio
incorporation into TG is measured as pmol of [14C]TG/mg of cell protein ± S.E. and expressed as percentage of basal (Bsl) triglyceride synthesis (where basal = 100%). Results are presented as average ± S.E. for n = 6 for each value. NS, not significant; ND, not determined.

| Cells                  | 0.1 μM | 1 μM  | 2.5 μM | 5 μM  | 10 μM | ANOVA |
|------------------------|--------|-------|--------|-------|-------|-------|
| ASP        | HEK-hC5L2 Bsl = 100 ± 6.3% | 97 ± 2.1 | 121 ± 8.7 | 125 ± 2.7 | 158 ± 10.8 | 185 ± 22.4 | p = 0.0009 |
| C3a        | HEK-hC5L2 Bsl = 100 ± 6.3% | 109 ± 4.0 | 121 ± 10.2 | 139 ± 10.0 | 141 ± 11.5 | 162 ± 12.0 | p = 0.007 |
| C5a        | HEK-hC5L2 Bsl = 100 ± 6.3% | 113 ± 4.2 | 118 ± 10.8 | 113 ± 10.9 | 105 ± 6.4 | 116 ± 5.0 | NS |
| C5adesArg  | HEK-hC5L2 Bsl = 100 ± 6.3% | 102 ± 1.7 | 109 ± 7.5 | 119 ± 9.6 | 117 ± 3.1 | 120 ± 1.0 | NS |
| C3a        | HEK-hC3aR Bsl = 100 ± 15% | ND  | 95 ± 15 | 95 ± 12 | 109 ± 12 | ND | NS |

Asp 0.01. *p < 0.001.
**p < 0.05.
**p < 0.01.

Non-TG lipids (phospholipids) with ASP, C3a, C5a, and C5a des-Arg. In all cases, there was no change in the amount of non-TG synthesis (data not shown).

We have previously demonstrated that the mechanism of ASP stimulation of TGS is mediated via increased glucose transport (6–9, 29) and increased acylation activity (5, 9, 30) in a time- and concentration-dependent manner (for reviews, see Refs. 2 and 37). Specifically, ASP increased V\text{max} but not K\text{m} of glucose transport, with increased translocation of Glut1, Glut3, and Glut4 (5–9, 29, 30). However, the effects of ASP are not solely dependent on the presence of glucose (9), and increased V\text{max} (not K\text{m}) of acylation enzymes also contribute (5, 9, 30), although fatty acid transport does not appear to be increased (9). Accordingly, we investigated each of these steps to determine the mechanism of ASP stimulation in the HEK-hC5L2 cells.

Whereas HEK cells do not express Glut3 or Glut4, they do express Glut1 (38–40). As shown in Fig. 2B, ASP stimulates glucose transport in a concentration-dependent (Fig. 2B) and time-dependent (Fig. 2C) manner. The effects appear to be maximal after just 1 h of stimulation with ASP. By contrast, although insulin mediates other effects in HEK cells, such as mitogenesis (41–43), it has no effect on glucose transport (Fig. 2C) as previously reported (41). The ASP effect on glucose transport is a consequence of increased V\text{max} (1697 ± 318 with ASP versus 1140 ± 188 basal, p < 0.001; Fig. 2D), with no change in K\text{m} (309 ± 117 with ASP versus 271 ± 94 basal, p not significant).

However, the ASP effect on TGS is not solely dependent on glucose transport stimulation. As shown in Table III, even in the absence of glucose, with or without pyruvate supplementation, ASP stimulates TGS to the same extent as with glucose. As before, insulin has no effect. This glucose transport-independent effect of ASP can be attributed to an effect on esterification enzymes. As shown in Fig. 2E, ASP increases V\text{max} of oleate incorporation into TG (2162 ± 211 with ASP versus 1310 ± 84 basal, p < 0.001), again with little effect on K\text{m} (313 ± 59 with ASP versus 199 ± 58 basal, p = 0.05). HEK cells express all enzymes required for TGS: glycerol-3-phosphate acyltransferase, phosphatidate phosphohydrolase, and diacylglycerol acyltransferase (31). Note, however, that both glycerol-3-phosphate acyltransferase and phosphatidate phosphohydrolase are common to both phospholipid and TG synthesis, and ASP does not stimulate PL synthesis (as mentioned above in HEK-hC5L2 and shown previously (36, 44, 45). Further, ASP does not stimulate fatty acid transport, acyl-CoA synthase, glycerol-3-phosphate acyltransferase, or phosphatidate phosphohydrolase activity (9, 30). However, ASP increases adipocyte diacylglycerol acyltransferase activity (30). Following incubation with ASP for 2 h, HEK-hC5L2 cells were collected, and cell homogenates were assayed for diacylglycerol acyltransferase activity. As shown in Fig. 2F, HEK-hC5L2 cells treated with ASP have increased diacylglycerol acyltransferase activity that is specific to the presence of hC5L2, since there is no effect on HEK cells (HEK-hC5L2 slope 0.14 ± 0.03 basal versus HEK-hC5L2 + ASP slope = 0.28 ± 0.03, 200 ± 21%, p < 0.013).

The TGS stimulation data are in contrast, but not contradictory, to those recently reported by Okinaga et al. showing that C5L2 is a nonfunctional receptor for C5a (22). On the other hand, it is difficult to explain the absence of C3a binding to C5L2 in their assays. It is possible that the differences are a consequence of differences in experimental conditions. The competition binding procedure used by Okinaga et al. (according to their published method) indicates that binding was conducted in cells in suspension, at 37 °C, using competitor concentrations that ranged from 0 to 300 nM. At 37 °C, there is both binding and internalization resulting in a lack of equilibrium conditions for K\text{d} evaluation. By contrast, our binding assays are conducted in adherent cells at subphysiological temperatures (4 °C or room temperature, no internalization) using competitor concentrations up to 10 μM. Previous studies indicate that C3a binding to C5L2 has a higher K\text{d} than either C3a to the C3a receptor or C5a to C5L2 (1). Thus, the limited range of competitor concentrations and the binding temperature used by Okinaga et al. (22) may have prevented a proper assessment of C3a binding to C5L2.
FIG. 3. Antisense oligonucleotides to human C5L2 mRNA diminish both C5L2 expression and TGS stimulation by ASP in HSF and 3T3 cells. A and C, antisense oligonucleotide 1, 2, or 3 alone, combined (All), or control oligonucleotide (CTL), at conditions A, B, C, or D (see “Experimental Procedures”) for human (HSF cells) or mouse (3T3-L1 cells) were transfected into HSF cells (A) or 3T3-L1 preadipocytes (C) seeded the day before in 24-well plates. Two days post-transfection, cells were assayed for cell surface C5L2 expression using an anti-C5L2 antibody (anti-human C5L2 for HSF, anti-mouse C5L2 for 3T3-L1) coupled to a [125I]protein A assay. Results for C5L2 cell surface protein expression (cpm of [125I]protein A bound/mg of cell protein) are shown for untransfected cells (no oligonucleotide, which are set at 100%), control (CTL), and antisense oligonucleotides (all conditions, A–D), and antisense oligonucleotides (only those that significantly decrease C5L2 expression). Nonimmune serum (NI) indicates background binding of [125I]protein A. Results are given as average ± S.E. for n = 3 for each value, where p < 0.0001 by two-way ANOVA for all results analyzed together.

TABLE III

| Substrate | Basal | With ASP | p | With insulin | p |
|-----------|-------|----------|---|--------------|---|
| Glucose   | 741 ± 4.8 | 995 ± 26.6 | <0.001 | 842 ± 52.3 | NS |
| Pyruvate  | 718 ± 18.2 | 1161 ± 37.1 | <0.001 | 727 ± 8.7 | NS |
| Neither   | 816 ± 10.6 | 1038 ± 28.2 | <0.001 | 814 ± 2.3 | NS |

Loss of Function through C5L2 mRNA Down-regulation Diminishes Both C5L2 Expression and TGS Stimulation by ASP—Because the above results clearly show that ASP can function through C5L2 in transfected cells, we were interested in assessing the role of C5L2 in cells that normally respond to ASP. A gene-silencing technique was employed to specifically inhibit translation of the C5L2 mRNA in both human (HSF) and mouse (3T3-L1 preadipocyte) cells. The antisense technique involves the transfection of DNA oligomers that are complementary to the target mRNA and, upon binding, inhibit passage of the mRNA through the ribosome (46). Generally, oligonucleotides are designed to different regions of the mRNA of interest, and the most effective are determined empirically. Three antisense oligonucleotides (oligonucleotides 1–3) directed to human C5L2, either alone or in combination (All), were transfected into HSF cells using two concentrations of oligonucleotides and two amounts of transfection reagent (conditions A–D, as described under “Experimental Procedures”). A nonsilencing oligonucleotide was used as CTL. After 2 days, identical sets of cells were assayed in parallel for cell surface expression of C5L2 (using an anti-C5L2 antibody coupled to a [125I]protein A detection assay) and ASP stimulation of TGS. As shown in Fig. 3A, hC5L2 cell surface expression in HSF was decreased by the various oligonucleotide treatments to a maximal decrease of 54 ± 4% (p < 0.001) relative to the untreated cells (equal to 100% expression). This level of cell surface detection was comparable with the background binding obtained with nonimmune rabbit antiserum (not shown); thus, a large proportion of cell surface expression could be reduced. In untreated (no oligonucleotide) cells, ASP stimulated TGS by 144 ± 5% (31.0 ± 1.4 pmol of TG/mg of cell protein with ASP versus 14.6 ± 1.6 basal, p < 0.001). Control (nonsense) oligonucleotide treatment had no effect on ASP stimulation (18.8 ± 2.8 pmol of TG/mg of cell protein). However, treatment with antisense decreased the ASP stimulation to 64 ± 6%, p < 0.001 versus no treatment (decreased to 11.0 ± 0.8 pmol of TG/mg of cell protein). This suppression of ASP stimulation resulted in TGS at or lower than basal levels of TGS (complete inhibition of ASP stimulation). Overall, comparing cell treatments, there was a close correlation between the cell surface hC5L2 and ASP responsiveness, as shown in Fig. 3B (r = 0.761, p < 0.0001).

Antisense experiments were also carried out in mouse...
3T3-L1 preadipocytes with oligonucleotides directed to mC5L2. As shown in Fig. 3C, depending on the amount and combination of antisense oligonucleotides, there were decreases in cell surface mC5L2 expression down to 69.5 ± 0.5% of basal levels (p < 0.001 versus untreated cells). On average, nonimmune polyclonal serum background represented 55 ± 1.9% binding.

In the absence of treatment (no oligonucleotide), ASP stimulated TGS in 3T3-L1 by 177 ± 22.6% (39.6 ± 8.8 pmol of TG/mg of cell protein with ASP versus 22.4 ± 2.9 basal, p < 0.001). As with HSF, treatment with control oligonucleotide did not prevent the ASP stimulation of TGS in 3T3-L1 cells (45.7 ± 9.8 pmol of TG/mg of cell protein, 204% of basal). However, treatment with specific antisense oligonucleotides resulted in a decrease in ASP stimulation of TGS, down to 25.3 ± 3.0 pmol of TG/mg of cell protein, 113% of basal, where basal = 100%). Overall, then, the level of cell surface C5L2 again correlated with ASP responsiveness (Fig. 3D, r = 0.45, p < 0.05). The data of the same oligonucleotide conditions that lower C5L2 expression also lower the ability of ASP to stimulate TGS confirms the role of C5L2 as a mediator of ASP function in endogenous cells.

ASP Couples C5L2 to β-Arrestin—β-Arrestin is a cytosolic protein that mediates the desensitization and internalization of GPCRs. It has been found to be translocated from the cytosol to the plasma membrane and associate with GPCR only once the receptors have been bound by their ligands and phosphorylated by GPCR kinases (23, 47). In fact, this activation assay has been proposed as a general approach to ligand identification by GPCR kinases (23, 47). In fact, this activation assay has been proposed as a general approach to ligand identification by GPCR kinases (23, 47). In fact, this activation assay has been proposed as a general approach to ligand identification by GPCR kinases (23, 47).

Phosphorylation of C5L2 over the same time course is shown in Fig. 4B. Mock-transfected cells demonstrate no visible C5L2 phosphorylation (not shown). In the absence of ASP, there is little basal phosphorylation of C5L2 (lane 1), but following 5–15-min exposure to ASP, there is a time-dependent increase in phosphorylation (lanes 2–4).

Incubation of HEK293 cells transiently transfected with hC5L2 and β-arrestin then treated with C3a, C5a, and C5a des-Arg is shown in Fig. 5. In the absence of C5L2 transfection in HEK293 cells, the β-arrestin pattern remains diffuse for all ligands (ASP, C3a, C5a, and C5a des-Arg) (shown only for ASP in Fig. 5). C3a, as with ASP, clearly results in redistribution of β-arrestin. On the other hand, C5a and C5a des-Arg also stimulate redistribution of β-arrestin but do not stimulate TGS. Thus, C5a may bind to C5L2, induce phosphorylation (as shown by Okinaga et al. (22)), and induce β-arrestin-mediated C5L2 internalization without activating the same signaling pathway as for ASP. This phenomenon has been previously described for other GPCRs. For instance, it was recently reported that ligands for the AT1R that do not promote the coupling of the receptor can still induce the formation of a β-arrestin-receptor complex and induce the internalization of the receptor (49). By contrast, both ASP and C5a appear to bind to the same site, activate β-arrestin-mediated internalization, and stimulate TGS.

In the C3aR and C5aR (CD88), both the carboxyl-terminal intracellular tails as well as additional intracellular regions are important in G protein activation and phosphorylation. Analysis of the carboxyl-terminal region of C5L2 (last 41 amino acids) with the same region in the C3a receptor and C5a receptor indicates 41% similarity of C5L2 with C3a receptor and 43% similarity of C5L2 with C5a receptor. Both receptors employ the same signaling pathway for G protein coupling and activation and are not yet known. The apparent binding of C5a to sites independent of ASP/C3a bind-
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Whereas there is much left to learn about the physiological role of ASP in humans, the information gained to date suggests that ASP is not only an important regulator of energy storage and adipose tissue metabolism but also may play a role in obesity and related diseases. The demonstration of a functional ASP receptor, through gain-of-function and loss-of-function assays, is a critical step in understanding the mechanisms of ASP action. Further investigation of C5L2 activation and signaling, particularly ligand binding, may aid in finding C5L2 antagonists and exogenous agonists, which could be used to modulate the ASP pathway. Together these studies will place the ASP pathway, including C5L2, in its proper physiological context.

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22. Okinaga et al. (22) to conclude that C5L2 is obligately uncoupled from G proteins and must, therefore, be a nonsignaling receptor. Not only do our data clearly show C5L2 function and suggest G protein signaling; there are also reported exceptions to the DRY motif paradigm. Mutation of the arginine in the α2α motif (51) and β2α motif (52) adrenergic receptors shows that the arginine is not required for G protein activation by these GPCRs. In the CB2 cannabinoid receptor, mutation of the homologous arginine only partially reduced activity (53), whereas arginine mutation in the type 1A angiotensin II receptor only abolished coupling to some G proteins but not others (54).

Interestingly, the secretin family of GPCRs have a YL motif predicted to be in the same position as the first two amino acids of the rhodopsin family DRY motif. Mutation of the leucine in the vasoactive intestinal peptide 1 receptor led to a pronounced impairment of G protein activation (55). Perhaps the existence of a leucine in C5L2 (as with vasoactive intestinal peptide 1 receptor) indicates a specific functionality. Determining the role of the DLC sequence of C5L2 is another area that may yield understanding of the mechanism of C5L2 function and signaling.

Together these studies demonstrate clearly that C5L2 is a functional receptor for ASP. It should be pointed out that these studies were conducted with stable cell lines that had been sorted based on fluorescent ASP binding and uptake, thereby selecting for a functional receptor population. Overall, these results contrast with, but do not contradict those of Okinaga et al. (22), which demonstrated that C5a is not a functional ligand for C5L2.

Fig. 5. C5a, C5b, and C5a des-Arg stimulate β-arrestin translocation in HEK-hC5L2 cells. HEK cells were transiently co-transfected with plasmids containing hC5L2 and GFP-β-arrestin. Two days later, the cells were incubated with the indicated ligands (1 μM) for 15 min and then fixed and assayed for fluorescent distribution. In native HEK293 cells with no C5L2 transfection, the β-arrestin pattern remains diffuse for all ligands (ASP, C5a, and C5a des-Arg). A representative scan with ASP is shown. C5a, C5a, and C5a des-Arg produce a similar pattern as for ASP at 15 min.
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