Identification of a Novel C5L2 Variant (S323I) in a French Canadian Family With Familial Combined Hyperlipemia

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Objective—A functional acylation-stimulating protein (ASP) receptor, C5L2, has been recently identified in ASP-responsive cells. Impaired ASP-mediated triglyceride synthesis has previously been described in a subset of hyperapolipoprotein B/familial combined hyperlipidemia subjects.

Methods and Results—DNA sequencing of C5L2 coding region in 61 unrelated probands identified a heterozygous variant (G968→T) in 1 subject, resulting in Ser323→Ile substitution in the carboxyl terminal region. This variant was not detected in 2176 additional chromosomes by restriction fragment length polymorphism or fluorescence polarization genotyping. Eight family members of the proband were identified with one altered (+/−)C5L2 allele. Nine other family members had the wild-type (+/+)/C5L2 sequence. The abnormal allele was associated with increased plasma triglyceride, plasma cholesterol, low-density lipoprotein (LDL) cholesterol, apolipoprotein B and ASP. Of 23 subjects tested in cell-based ASP bioactivity assays, those with C5L2(+/−) variant (n=2) had a 50% reduction in ASP-stimulated triglyceride synthesis, glucose transport and marked reduction in maximal binding (B_max). By contrast, a C5L2(+/+ ) family member responded normally, as did hyperapolipoprotein B normal ASP subjects compared with C5L2(+/+ ) controls (n=6).

Conclusion—The S323I variant may alter C5L2 function and might be one molecular basis contributing to familial combined hyperlipidemia. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: acylation-stimulating protein ■ adipose ■ C3adesArg ■ gene defect ■ G protein–coupled receptor ■ hyperapolipoprotein B ■ triglyceride synthesis

Increased plasma apolipoprotein B (apoB) is a common dyslipoproteinemia associated with coronary artery disease1 and is an excellent marker for diagnosis and treatment monitoring.2 The hallmark is increased low-density lipoprotein (LDL) particles with overproduction of hepatic apoB and increased small dense LDL particles.3 Within this group, familial combined hyperlipidemia is considered to be the most frequent lipoprotein disorder in premature coronary artery disease (CAD).4 The lipoprotein pattern varies in time and the level of penetrance may be incomplete, especially in premenopausal women. The pathogenesis of familial combined hyperlipidemia is complex and is very similar to that encountered in the metabolic syndrome.6 This has hampered research in the cause of the disorder. Studies from Genest et al7 and Castro-Cabezas et al8 showed that hyperapolipoprotein B (hyperapoB) subjects have delayed triglyceride (TG) clearance and increased fatty acid after an oral fat load.8 Peripheral resistance to fatty acid uptake in adipose tissue leads to increased hepatic flux, stimulating lipoprotein pro-
duction.10 We have proposed that, in a subset of patients, a defect in adipose tissue storage (particularly postprandially) might contribute to these abnormalities.

In support of this hypothesis, acylation stimulating protein (ASP) is a potential candidate. ASP binds to adipocytes and preadipocytes, promotes triglyceride synthesis through stimulation of both glucose transport and fatty acid esterification,11,12 indirectly enhancing the action of lipoprotein lipase.13 Patients with coronary heart disease have increased plasma ASP,11,12 and this is more frequent in those with dyslipidemia (hypertriglyceridemia and increased LDL), suggestive of ASP resistance. In vivo human studies demonstrate a correlation between increased plasma ASP and delayed postprandial TG clearance in men and women.14 Finally, previous in vitro studies support this hypothesis, because a subset of hyperapoB subjects with high plasma ASP demonstrated impaired ASP stimulation of TG synthesis.15 Recently, we have identified an ASP receptor, C5L2, a G protein-coupled 7-transmembrane domain complement 5α
receptor-like receptor. ASP initiates a cascade of events mediated by protein kinase C, leading to activation and phosphorylation of the receptor, stimulating glucose transport and fatty acid esterification. Recently, genome-wide scans of affected families strongly suggest that several genes may underlie specific characteristics of familial combined hyperlipidemia and the prediabetic state, and at least 1 gene has been identified, USP-1, on chromosome 16. Additional studies point to other regions, including chromosome 19q13, the region of the C5L2 receptor. In the present report, we examined whether variability at the C5L2 gene could be associated with hyperapoB hyperlipidemia phenotypes frequently observed in familial combined hyperlipidemia.

**Methods**

**Subject Selection**

Complete sequencing of the C5L2 region that included a portion of the upstream region, 1 intron, and the exon with the complete coding region was initially conducted on 61 subjects. These subjects included 20 normolipidemic healthy adult subjects with no known disease (15 men and 5 women) with an average age of 41.8±13.0 years. All subjects had normal fasting plasma apoB levels (<1.2 g/L). In addition, 41 hyperapoB/familial combined hyperlipidemia subjects identified through cardiovascular and/or lipid clinics were chosen. All of these subjects were examined in the absence of lipid-lowering medication. Subjects were separated based on the presence (n=51) or absence (n=10) of cardiovascular disease. All probands examined had increased plasma TG and apoB levels (>1.20 g/L in the absence of any medication). Mean ages in these 2 groups were 53.2±8.0 years (24 men, 10 women) and 39.3±9.9 years (6 men and 4 women), respectively. Further evaluation of a specific C5L2 polymorphism was evaluated in 2 additional cohorts. A sample of 253 subjects (CAD cohort), with premature cardiovascular disease (mean age 49.8±7.5 years, 203 men and 50 women) with an apoB level >1.20 g/L, as previously described. A large sample of 835 subjects (Saguenay-Lac St Jean cohort) of French Canadian descent from Saguenay-Lac St Jean area in the Province of Quebec was also selected. These subjects were from ongoing diabetes and CAD studies. Finally, subjects belonging to the family identified with the mutation were also evaluated. All of these family members were examined in the absence of any lipid-lowering medication. All study subjects signed informed consent for analysis of plasma and DNA and, in selected patients, skin biopsies for fibroblast culture and in vitro assays (when appropriate). The research protocol was reviewed and approved by the Research Ethics Board of the McGill University Health Centre.

**Lipid and Protein Analyses**

Total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol and glucose were measured according to standardized clinical biochemistry laboratory techniques. LDL cholesterol was calculated with the Friedewald formula for plasma triglyceride levels <4.5 mmol/L. Nonesterified fatty acid was measured by commercial colorimetric assay (Wako Chemicals, Tex). ApoB was measured by nephelometry and insulin by radioimmunoassay according to standardized clinical chemistry methodology. ASP was measured by enzyme-linked immunosorbent assay.

**DNA Sequencing**

The structural organization and complete nucleotide sequence of human C5L2 gene (alias GPR77) were retrieved from the NCBI Reference Sequence, accession NM_018485, and the homo sapiens chromosome 19 genomic contig, accession NT_011109, from GenBank using Entrez databases. Direct sequencing of the human C5L2 gene was performed on genomic DNA from the study subjects using the dye termination method on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, Calif).

**Detection of the Variant**

Genotyping for the S323I C5L2 gene variant in the CAD cohort was performed by polymerase chain reaction amplification of 431 bp in exon 2 followed by restriction digestion with Hpy188III (New England BioLabs). The forward primer is 5'-GGCTGAACCCCTCTATCGT-3'; and the reverse primer is 5'-TCCACAGAAGAATATCAGCTCCC-3'; annealing temperature is 55°C.

The genotyping of the Sagueneay-Lac St Jean samples was performed by HEFP. After initial polymerase chain reactions, single-base extension detection primers of 16 bp length and melting temperature >55°C were designed in both orientations for each SNP using Primer3. The plates were analyzed using an Analyst HT 96 to 384 reader (Molecular Devices, Sunnyvale, Calif). A known heterozygote was included on every plate as a positive control. For detailed methods please see http://atvb.aha.journals.org.

**ASP Production, Cell Culture, Triglyceride Synthesis, Glucose Transport, and 125I-ASP Competition Receptor Binding Assays**

Human skin fibroblasts (HSF) were obtained and purified from human plasma, and TGS and glucose transport assays were performed. ASP was iodinated using commercial Iodogen as described by the manufacturer (Pierce Chemical Co) Competition binding assays for ASP binding to HSF were performed using 1 nmol 125I-ASP on adherent cells in 48 well microtiter plates.

**Calculations and Statistical Analysis**

Body mass index (BMI) was measured as weight (kg)/height (m²). Results are reported as average ± standard deviation or standard error of the mean as indicated. Statistical differences were evaluated by ANOVA; correlations were evaluated by linear regression. Analysis was conducted with GraphPad Prism or Sigma Stat computer program analysis. P<0.05 was taken as significant.

**Results**

Sequencing of the C5L2 region, including all transcribed regions (2 exons) and 260 bp of the promoter was initially conducted on 61 subjects (20 normolipidemic and 41 hyperlipidemic subjects) as described in Table 1 and Methods. As seen in Table 2, 1 proband (hyperapoB CAD positive) was identified with an amino acid substitution (S323I) because of an exonic G968T variation in the C5L2 gene. This amino acid substitution was within a serine/threonine-rich region of the carboxy terminus (as indicated in Table 2). In all cases, except this one, the C5L2 protein coding sequence was identical to that previously reported (NCBI Reference Sequence, NM_018485).

Two additional cohorts were analyzed for the S323I variant, as also described in Table 1. The first cohort of 253 unrelated people consisted of subjects with premature CAD recruited within the greater Montreal area. There were 50 women and 203 men, with an average age of 49.8±7.5 years, of whom 19% had diabetes. No subjects were identified with the S323I variant. Similarly, 835 subjects derived from a large French Canadian cohort from the Saquenay-Lac St Jean region of Quebec were also analyzed, of whom 71% had cardiovascular disease. The incidence of diabetes, average BMI, and plasma lipids (triglycerides, total cholesterol, LDL and HDL cholesterol) were similar to the CAD cohort (Table 1). Again, no subjects with the S323I variant were identified. Further, no evidence of any known C5L2 variant could be obtained in the online databases searched. To our knowledge, this is the first C5L2 variant identified.
The family members of the proband were further examined for the presence of the variant. As shown in Figure 1, 7 additional family members in the kindred (1 man and 7 women in total) shared the same genotype [indicated as (+/-)], which appeared to be transmitted in a Mendelian fashion. Nine other family members (5 men and 4 women) had the wild-type genotype only (indicated as (++/+). Family characteristics and the plasma lipid and lipoprotein profiles of the 2 groups within the family are shown in Table 3. No subjects were diabetic. There was no difference in average age or BMI (Table 3), prevalence of smoking [66% in (++/+ group and 50% in (+/-) group] or blood pressure (not shown). By contrast, total cholesterol and glucose were significantly increased in the heterozygote group as compared with the nonaffected members of the family, although the differences were especially marked for the plasma triglyceride values (average increase of almost 2-fold). When evaluated according to age- and gender-matched values,27 5 of 8 (+/-) subjects (63%) had plasma TG or LDL cholesterol >90th percentile. Six of 8 (75%) had plasma total cholesterol >90th percentile, and 5 of 8 (63%) had HDL cholesterol <10th percentile. By contrast, only 1 of 9 of the unaffected (++/) group (11%) had values that were within these ranges. As shown in Figure 2, in the affected (+/-) group, plasma triglyceride correlates significantly with BMI (r=0.975, P<0.0001, slope=0.364). However, in the unaffected (++/) group, there is no significant correlation. Note that even with exclusion of the severely hypertriglyceridemic obese subject, the correlation between BMI and plasma TG remains highly significant in the (+/-) subjects (r=0.944, P=0.0014, slope=0.302). As also shown in Figure 2, plasma ASP, the functional ligand for C5L2, is substantially increased in the affected (+/-) subjects by 78% (P=0.004).

C5L2 belongs to the 7 transmembrane family of G protein-coupled receptors. The S323I variant is located in the carboxyl terminal intracellular tail, in a serine/threonine-rich region. This region has been shown to contain important phosphorylation sites associated with activation, desensitiza-

TABLE 2. Sequence of C5L2 Variant in Exon 2

<table>
<thead>
<tr>
<th>943</th>
<th>968</th>
<th>990</th>
</tr>
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<tr>
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<tr>
<td>Proband (S323I) cag gcc cag gac gaa agr gtg gac ATC I aag — — aaa acc acc acc cat Gac</td>
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</table>

**Serine/Threonine Rich Region**

Sequences are indicated for the region from 943–990 bp of the human sequence with corresponding amino acids, as well as the corresponding regions for the mouse and chimpanzee C5L2 sequences (alignment based on amino acid sequence). The variant in the proband is indicated as an AGC to ATC variant resulting in a S323 to I substitution.
tion, and downregulation of this class of GPCR.28 Accordingly, we evaluated the effects of this variant on cellular response to ASP in HSF obtained from three family members (301, 303, and 305 as indicated in Figure 1 and Table 3). ASP has previously been shown to directly stimulate triglyceride synthesis (via fatty acid uptake and esterification) and glucose transport in adipocytes, preadipocytes,11,12 as well as in HSF.15,25 This effect on fatty acid esterification occurs downstream of lipoprotein lipase action on chylomicrons/very-low-density lipoprotein and is independent of lipoprotein lipase.13 These ASP functional effects are dependent on the presence of C5L2 as demonstrated recently.18 In the present study, the cellular response to ASP of the three family members was compared with that of cells derived from a subset of the study subjects described in Table 1, which included: (1) control subjects (n=11005/6) who had normal apoB and normal ASP plasma levels and (2) hyperapoB subjects with normal (n=11005/6) or increased (n=11005/8) plasma ASP levels. Complete C5L2 sequencing of these subjects (included in Table 1) demonstrated a normal C5L2 sequence.

Results for ASP stimulation of triglyceride synthesis and glucose transport are shown in Figure 3. Increasing concentration of ASP increased triglyceride synthesis up to 200% in responsive control cells (Figure 3, top panel). Subject 303, Figure 1. Identification of C5L2 S323I variant in family 21513. Indications are: original proband 301 (arrow), women (circles), men (squares), and unavailable [•]. Restriction fragment length polymorphism (RFLP) analysis with Hpy188III indicate 2 wild-type alleles (+/+), single band) or heterozygous C5L2-S323I variant (+/-, 3 bands).

**TABLE 3. Subject Characteristics and Lipid and Lipoprotein Profile of Family Members With (+/-) and without (+/+ ) C5L2 Variant**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Type</th>
<th>Age</th>
<th>Sex</th>
<th>BMI</th>
<th>ApoB</th>
<th>TG</th>
<th>TCHOL</th>
<th>HDL-C</th>
<th>LDL-C</th>
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<td>240</td>
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<td>8.18*</td>
<td>0.74*</td>
<td>5.14*</td>
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<td>3.73*</td>
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<td>1.00</td>
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<td>0.23</td>
<td>0.65</td>
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Values are provided as average ± SD. F indicates female; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; M, male; TCHOL, total plasma cholesterol; TG, plasma triglyceride; N/A, not available. *Values >90th percentile (for TG, TCHOL, and LDL-C) or <10th percentile (for HDL-C) for age- and gender-matched subjects based on Lipid Research Clinic data.27
with the wild-type (+/+) genotype, responded normally to ASP stimulation (maximal stimulation = 184%). By contrast, subjects 301 and 305 (both possessing the variant (+/-) genotype) had a reduced response to ASP (on average 50% less response). For comparison, cells from hyperapoB--normal ASP responded normally to ASP, whereas cells derived from hyperapoB--hyperASP subjects respond to a lesser extent (Figure 3, top panel, inset).

The ASP stimulation of glucose transport is shown in Figure 3 (bottom panel). As with triglyceride synthesis, ASP stimulated glucose transport twofold in control cells. A similar response was seen with subject 303 (Figure 3, bottom panel). ASP stimulation was again blunted in cells from subjects 301 and 305 by 72% and 35%, respectively. Cells derived from hyperapoB normal ASP subjects possessing a wild-type C5L2 sequence responded normally to ASP with hyperapoB hyperASP to a slightly lesser extent (Figure 3, bottom panel, inset). By contrast, the stimulation by insulin, which is a potent stimulator of TG synthesis and glucose transport,16 and PMA, an activator of protein kinase C,17 was normal in subjects 301 and 305 as compared with control cells and hyperapoB cells (results not shown).

Finally, 125I ASP competition equilibrium binding to cells was measured to evaluate receptor affinity (Kd) and maximal binding sites (Bmax). Note, all binding curves were evaluated for 1-site versus 2-site binding, and in all cases 1-site was the preferred mathematical model. There was no difference in average Kd between control cells (133 ±24 nmol±SEM, n=6), hyperapoB normal ASP cells (195 ±36 nmol±SEM, n=6), hyperapoB hyperASP cells (170 ±44 nmol±SEM, n=8), and subjects 301 (+/-), 303 (+/+), and 305 (+/+)) (135, 98, and 169 nmol, respectively). By contrast, although Bmax for subject 303 (+/+) was normal (184 pmol/mg cell protein), Bmax for subjects 301 and 305 were reduced by more than 50% (63 and 31 pmol/mg cell protein, respectively) compared with the control (165±35, n=6) and hyperapoB normal ASP cells (205±61, n=6) and, in fact, were <25th percentile of the control cells (91 pmol/mg cell protein). In hyperapoB hyperASP cells, Bmax tended to be reduced as compared with control cells (116±25 pmol/mg cell protein, n=8).

**Discussion**

The results in the present study demonstrate that a mutation in the C5L2 receptor leads to altered ASP bioactivity. Not only do these results confirm the identity of C5L2 as a functional ASP receptor, they also reinforce the link between ASP and hyperapoB and, more broadly, the link between adipose tissue dysfunction and dyslipidemia such as familial combined hyperlipidemia. Whereas linkage disequilibrium should always be considered to explain genetic association results, we do not believe that it is a likely explanation for the following reasons: (1) the mutation results in an amino acid change (S323H) that is predicted to eliminate a serine phosphorylation site and (2) sequencing of all transcribed regions (coding and noncoding), as well as 260 bp of the promoter, did not reveal any other variants in the proband for this family. Whereas we cannot formally exclude the possibility that the amino acid change is in linkage disequilibrium with another variant in a nearby gene, we believe that the most parsimonious explanation is that the amino acid changing variant is responsible for the observed data.

C5L2 belongs to the family of 7 transmembrane G protein-coupled receptors. We have previously demonstrated that ASP binds to C5L2, initiating a cascade of events that includes phosphorylation, β-arrestin translocation, and receptor internalization.18 Activation of the receptor initiates a signaling pathway that includes protein kinase C activation and translocation and glucose transporter translocation.11,12,20 This leads to increased glucose transport and fatty acid...
esterification, resulting in a net accumulation of adipose TG stores.\textsuperscript{11,12,18} In the present kindred, a number of family members were identified that express 1 normal C5L2 and 1 modified S323I C5L2 variant. In vitro testing demonstrates a reduced ASP binding and a reduced ASP stimulation of both glucose transport and TG synthesis, although the response to insulin and PMA both appear to be normal. These experimental data suggest that this variant leads to a functional disruption in signaling consequent to a serine to isoleucine substitution in the carboxyl terminal tail. Serine–threonine clusters in the carboxyl terminal tail of G protein-coupled receptors have been demonstrated to be common phosphorylation sites\textsuperscript{28} and, indeed, computer analysis of the C5L2 sequence via Net-Phos (www.cbs.dtu.dk) strongly predicts this serine (S323) as the highest (score 0.997) potential serine phosphorylation site. G protein-coupled receptor phosphorylation can be crucial, not only for initiation of G protein signaling but also in desensitization, internalization, down-regulation, and cross-talk with other receptors.\textsuperscript{28} This remains to be evaluated directly in vitro with this specific mutation. Nonetheless, the cellular and physiological data in this family support the hypothesis that ASP-C5L2 receptor dysfunction leads to altered adipose tissue and metabolic consequences. Increased plasma ASP may be a marker of “ASP resistance,” either genetically or environmentally induced.

ASP function and interference with ASP action has been examined in a number of mouse models. ASP deficient mice were obtained through genetic knockout (KO) of complement C3, the precursor to ASP. Administration of ASP to wild-type mice enhances postprandial triglyceride and glucose clearance, and this is true even in obese mouse models (ob/ob and db/db).\textsuperscript{11,12} Conversely, ASP deficiency in C3 KO mice results in a delay in triglyceride and fatty acid clearance. This delay leads to a reduced efficiency of fat storage relative to food intake, resulting in a leaner mouse relative to wild-type. This is true in ASP-deficient C3 KO, as well as in several double KO models: (1) ASP-deficient C3 KO ob/ob mice and (2) ASP-deficient C3 KO crossed with apoE-LDL receptor KO mice.\textsuperscript{11,12,30} In all instances, the mice have a reduced adipose tissue mass (ranging from 25% to 70% reduction in adipose tissue mass).

The positive and negative consequences of this energy re-partitioning need to be considered. The downside is that ASP deficiency leads to increased food intake,\textsuperscript{11,12} which may be an attempt to compensate for reduced fat stores. This, in turn, leads to diversion from storage to muscle and liver, a diversion which can be corrected with a bolus injection of ASP at the time of the fat load. Increased delivery of substrate to the liver promotes lipogenesis and lipoprotein production, a phenotype markedly evident in the ASP-deficient apoE-LDL receptor KO mice, in which serum triglyceride levels and atherosclerotic lesions almost double, relative to the ASP positive apoE/LDL receptor KO mice.\textsuperscript{30}

However, interference with the ASP pathway and decreased partitioning into adipose tissue is not without beneficial effects. In high-fat diet and genetically obese models, ASP deficiency provides resistance to development of obesity and results in increased insulin sensitivity.\textsuperscript{11,12} Further, energy expenditure is increased at a number of levels: basal metabolic rate, food-induced thermogenesis, and physical activity. This is true even in ob/ob mice that already have reduced energy expenditure, and the effect is independent of leptin. Although the exact mechanism of increased energy expenditure is as yet unknown, we have demonstrated increased re-partitioning of fat to the muscle, increased fatty acid oxidation products, and increased cycling through uncoupling proteins. Clearly, additional studies in humans are required.

In the present study, the response of the ASP-receptor pathway in cellular assays correlated with the lipoprotein profile. The consequences of a partially deficient ASP-receptor pathway (a single mutated allele) in this family resulted in a decreased ASP binding and cellular response, which was associated with increased plasma lipoproteins relative to the unaffected family members. These differences are even more striking, considering that the affected members are almost exclusively women, who typically present with lower lipid levels than men of comparable age. It should be pointed out, however, that hyperapoB/familial combined hyperlipidemia is multifactorial, and not all subjects with a decreased response to ASP necessarily have a C5L2 mutation. Although this present mutation (S323I) appears to be relatively rare, there may be other as yet unrecognized mutations in the coding region. Further, alterations in the promoter region (not yet examined) may also influence C5L2 expression. This is further supported by the reduced response of hyperapoB hyperASP cells to ASP, even in the absence of any coding region mutations. Specific modifications in the promoter region or in factors that influence C5L2 expression (transcription factors, hormones etc) may play a role. Down-regulation of the C5L2 receptor (ASP resistance) may also contribute to the phenotype. Potentially, the negative consequences of interfering with the ASP-C5L2 pathway, increased food intake (in the mice) and increased hepatic lipoproteins (both mice and humans), may be overcome by coupling treatment to food restriction and increased exercise. Consequently the beneficial effects (decreased fat storage, increased muscle oxidation, increased energy expenditure) might be capitalized on.

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Disclosures
None.

References
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**Detailed Methods**

*Detection of the variant.* Genotyping for the S323I C5L2 gene variant in the CAD cohort was performed by PCR amplification of 431 bp in exon 2 followed by restriction digestion with *Hpy*188III (New England BioLabs). The forward primer is 5’-GGCTGAACCCCTCATCGT-3’; and the reverse primer is 5’-TCCACTAGAAGAATATCAGCTCCC-3’; annealing temperature is 55°C. The PCR product (15µL) was incubated with *Hpy*188III (5U) in a total volume of 25µL overnight at 37°C, and the resulting fragments were separated on a 1.5% agarose gel. The presence of the S323I variant creates an *Hpy*188III site producing two fragments of 273 and 158 bp. The variant was examined using this method in all available family members of the carrier proband and in the CAD cohort examined.

The genotyping of the Saguenay–Lac St. Jean samples was carried out by HEFP™ as follows: The initial PCR reactions were performed in a GeneAmp™ PCR System 9700 (Applied Biosystems), on 6.0ng of genomic DNA in a 8.0µL reaction mixture containing 2.5mM MgCl₂, 25mM dNTPs, 0.2Unit HotstartTaq DNA polymerase (Qiagen) and 100nM each primer. PCR was initiated by denaturing the samples at 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 10 seconds, annealing at 54°C for 30 seconds and a 72°C extension for 30 seconds with the final extension at 72°C for 6 minutes. PCR products were treated with Exonuclease I and Shrimp Alkaline Phosphatase as recommended (AcycloPrime-FP SNP Detection Kit, Perkin Elmer, Wellesley,
Single-base extension (SBE) detection primers of 16bp length and melting temperature above 55°C were designed in both orientations for each SNP using Primer3. The FP-SBE reaction was performed in both orientations (AcycloPrime-FP SNP Detection Kit). After addition of reading buffer, the plates were analyzed using an Analyst HT 96-384 reader (Molecular Devices, Sunnyvale, CA). A known heterozygote was included on every plate as a positive control.

**Cells and Culture Conditions:** Human skin fibroblasts (HSF) were obtained as previously described. HSF cells were cultured in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% (v/v) fetal bovine serum at 37°C, 5% CO₂. For all functional assays (triglyceride synthesis, glucose transport and ASP competition binding assays) cells were used at 80-95% confluency, and were transferred to serum-free medium two hours prior to the assay.

**ASP Production, Triglyceride Synthesis and Glucose Transport:** ASP was purified from human plasma as described previously. TGS and glucose transport assays were performed as previously described. Cells were preincubated in serum free medium for 2 hours. For triglyceride synthesis, cells were incubated with vehicle (phosphate buffered saline, PBS), ASP, insulin (Sigma Chemicals, St Louis, MO) or phorbol myristic acetate (PMA, Sigma Chemicals, St Louis, MO) at the indicated concentrations, in the presence of 100 µM ³H oleate complexed to albumin (molar ratio 5:1, specific activity 50-70 dpm/pmol oleate) diluted in serum-free medium, for 4 hours at 37°C. At the end of the experimental incubation, cells were rinsed with cold PBS, and lipids extracted with isopropanol:heptane (2:3 v/v). The organic extract 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, ON). Lipids were separated using a solvent system of hexane:ether:acetic acid (75:25:1 v/v/v). Triolein and oleic acid standards were used to visualize the lipid following exposure to iodine (I₂) vapour. 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 radiolabelled media were counted to determine specific activity. TGS was calculated as pmol $^3$H oleate incorporated into triglyceride (TG) per mg soluble cell protein.

*For glucose transport:* following incubation in serum free media for 2 hours, then with ASP, insulin or PMA in serum free medium for 2 hours, cells were rinsed with warm serum free, glucose free media, then incubated for 10 minutes with $^3$H 2-deoxyglucose (50 uM, final specific activity 60-120 dpm/pmol) in serum free glucose free media at 37°C. The reaction was stopped with rapid washing of the cells with cold PBS, and cell proteins were dissolved in 0.1 N NaOH. Aliquots were taken for scintillation counting for 2-deoxy-glucose uptake. Cell protein was measured by Bradford assay (BioRad, Mississauga, ON). Glucose transport was measured as pmol $^3$H 2-deoxyglucose uptake per mg soluble cell protein.

*Radiolabelled Ligand Competition Receptor Binding Assays:* ASP was iodinated using commercial Iodogen as described by the manufacturer (Pierce Chemical Co.) Competition binding assays for ASP binding to HSF were performed using 1 nM $^{125}$I ASP on adherent cells in 48 well microtiter plates as
described previously in detail 1,4,5. Competition curves were generated by incubating adherent cells with increasing concentrations of unlabelled ASP (1 x 10^{-12} to 1 x 10^{-6} M), followed by addition of 1 nM 125I ASP for 1 hour at room temperature. Cells were then rinsed rapidly with cold PBS, and dissolved in 0.1 N NaOH. Aliquots were taken for gamma counting and protein analysis (as described above). The IC_{50}, B_{max} and K_d were obtained by regression analyses using GraphPad Prism 4.00 software.

References


