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Genetic Mapping and Functional Studies of a Natural Inhibitor of the Insulin Receptor Tyrosine Kinase: The Mouse Ortholog of Human α_2-HS Glycoprotein

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Fetuin/α_2-HS glycoprotein (α_2-HSG) homologs have been identified in several species including rat, sheep, pig, rabbit, guinea pig, cattle, mouse and human. Multiple physiological roles for these homologs have been suggested, including ability to bind to hydroxyapatite crystals and to specifically inhibit the tyrosine kinase (TK) activity of the insulin receptor (IR). In this study we report the identification, cloning, and characterization of the mouse Ahsg gene and its function as an IR-TK inhibitor. Genomic clones derived from a mouse Svj 129 genomic library were sequenced in order to characterize the intron–exon organization of the mouse Ahsg gene, including an 875 bp subclone containing 154 bp upstream from the transcription start site, the first exon, and part of the first intron. A second genomic subclone harboring a 3.45 kb Bgl II fragment contained exons 2, 3 and 4 in addition to two adjacent elements within the first intron-a repetitive element of the B1 family (92 bp) and a 271 bp tract of (T,C)*n* * (A,G)*n*. We have mapped mouse Ahsg at 16 cM adjacent to the Diacylglycerol kinase 3 (Dagk3) gene on chromosome 16 by genotyping interspecific backcross panels between C57BL/6J and *Mus spretus*. The position is syntenic with human chromosome 3q27, where the human AHSG gene resides. Using recombinant mouse α_2-HSG expressed from a recombinant baculovirus, we demonstrate that mouse α_2-HSG inhibits insulin-stimulated IR autophosphorylation and IR-TKA *in vitro*. In addition, mouse α_2-HSG (25 μg/ml) completely abolishes insulin-induced DNA synthesis in H-35 rat hepatoma cells. Based on the sequence data and functional analysis, we conclude that the mouse Ahsg gene is the true ortholog of the human AHSG gene.

Keywords: α_2-HS glycoprotein; Fetuin; Mapping; Insulin receptor; Tyrosine kinase inhibitor

Data Base Accession Numbers: AF025820, AF025821

INTRODUCTION

Heremans and Schmid described α_2-HSG for the first time in 1960,[1,2] as a 49–60 kD human plasma glycoprotein secreted into the circulation by the liver at a concentration of 0.4–0.85 g/L. Human α_2-HSG is a negative acute phase

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reactant protein [3, 4] and altered concentrations of α_2-HSG have been reported in several disease conditions including Paget's disease, osteogenesis imperfecta, lymphoma, leukemia and myelofibrosis. [5, 6] Several functions have been attributed to α_2-HSG, including its involvement in immune response, [7] the chemotactic response of macrophages, [8, 9] enhancement of phagocytic function of human monocytes, [10, 11] bone mineralization, [12, 13] bone accumulation, [14, 15] calcification [16, 17] and as an inhibitor of insulin receptor tyrosine kinase activity [IR-TKA; [18]]. Recently, Jahnen-Dechent *et al.* [19] demonstrated that mice deficient for α_2-HSG can develop ectopic microcalcifications in soft tissues, supporting the idea that α_2-HSG may operate as an inhibitor of apatite crystal growth *in vivo*.

Classified as a member of the cystatin superfamily, [20] human α_2-HSG is synthesized as a type I secreted glycoprotein with a signal sequence of 18 amino acids [21] and three major domains – two *N*-terminally located cystatin domains, D1 and D2 (116–118 amino acids), and a single, proline-rich domain D3[106–115 residues; [20, 22–24]]. Many key amino acid residues and the position of cysteine residues are perfectly conserved between human α_2-HSG and the fetuins of bovine, sheep, pig, rat and mouse origin, [25, 20, 26, 21, 27–29] suggesting that the fetuins may be the homologs of human α_2-HSG.

Rat fetuin, originally named pp63, [30, 31] secreted by rat hepatocytes in the phosphorylated state, [30] inhibits insulin-stimulated IR-TKA. [32] The gene for rat fetuin maps to chromosome 11 and spans approximately 8 kb, containing seven exons separated by six introns of different sizes. The rat fetuin cDNA sequence is similar to the cDNA sequences of both human α_2-HSG and bovine fetuin. [33]

When Yang *et al.* [34] first reported the deduced amino acid of the mouse fetuin cDNA, the question arose whether mouse fetuin was the true ortholog of human α_2-HSG. They suggested that the mouse protein take the name α_2-HSG instead of fetuin, because, unlike bovine fetuin, the mouse protein is not a major component synthesized by fetal liver. [34, 35] Alignment of human and mouse α_2-HSG reveals a 60% amino acid identity between the two proteins, with the majority of the identical residues found in the *N*-terminal two-thirds of the protein. Three *N*-linked glycosylation sites are present in mouse while only two are present in the human protein. Moreover, mouse α_2-HSG is 22 residues shorter than human α_2-HSG. [34]

In this study, we report the mouse Ahsg genomic structure derived from sequencing and restriction mapping of exons 1, 2, 3 and 4 contained in a contiguous 4.3 kb segment of the gene. We have also sequenced a 154 bp region upstream from the transcriptional start site. The chromosomal location of mouse Ahsg has been mapped to the proximal region of chromosome 16 at 16 centimorgans, adjacent to the gene Dagk3. Further, we demonstrate that recombinant mouse α_2-HSG inhibits insulin-stimulated IR autophosphorylation, IR-TKA and DNA synthesis, confirming that mouse α_2-HSG can play a role similar to the human homolog in modulating insulin action. Based on the structural features shared between the mouse and human genes, their syntenic chromosomal localization, and the IR-TK inhibitory activities shared between the two proteins, we suggest that the mouse Ahsg is not simply a family member, but the true ortholog of the human AHSG gene.

MATERIALS AND METHODS

Cloning of Mouse Ahsg cDNA into a Recombinant Baculovirus

A full-length cDNA encoding the entire 1035 nt open reading frame (ORF) was cloned from liver poly(A+) RNA using reverse transcriptase (RT)-polymerase chain reaction (PCR). The cDNA was amplified in two segments, a 533

bp *N*-terminal segment (using primers SSF1, 5′-GGATCCTGACATTTGCCCATTTTCC-3′OH, and SSB3, 5′-CGGTGTGGACCACGTTGGTATC-3′OH) and a 1095 bp *C*-terminal segment (using primers VIV1, 5′-CTGCCAATCCGCTCCACAAGG-TA-3′OH and VIV2, 5′-TGTGGTATTGCTTTGT-CAGTGGA-3′OH). A continuous cDNA of 1182 bp was then amplified from the two segments using PCR and short overlap extension (primers SSF1 + VIV2) and the PCR product cloned into plasmid pCR (Invitrogen, Carlsbad, CA); the plasmid was sequenced from a combination of dye primer and dye-terminator automated sequencing reactions. The Ahsg ORF was shuttled as a 1.23 kb BamHI-Xbal fragment into the baculovirus transfer vector pBlueBacIII (Invitrogen, Carlsbad, CA) after blunting both the Xbal end of the Ahsg ORF and the Hind3 site of pBlueBacIII with the Klenow fragment of *E. coli* DNA polymerase I (Pharmacia, Piscataway, NJ); resulting in a transfer vector, pMusBacα3 (11,440 bp) in which the Xbal site is restored. This transfer vector (2 μg) was transfected into Sf9 cells (Gibco Life Technologies, Gaithersburg, MD) in the presence of 5 μg of the wild-type baculoviral DNA, and blue plaques selected from 2% agarose plugs stained in Bluo-Gal (Gibco Life Technologies, Gaithersburg, MD). Twice-purified viral plaques were expanded at 27°C, and recombinant viral stocks checked for homogeneity using PCR (baculoviral primers −44, 5′-TTTACTGTTTTCGTAACAGTTTTG-3′OH; and +778, 5′-CAACAACGCACAGAATCTAGC-3′OH).

Expanded recombinant baculoviral stocks were used to transfect cabbage looper High-Five™ cells (Invitrogen, Carlsbad, CA). Supernatants collected after 72 h were purified using affinity chromatography on jacalin lectin columns (Sigma, St. Louis, MO), washed with 100 mM Tris-pH 7.4 and eluted with 25 μM melibiose (Sigma, St. Louis, MO). Proteins separated on 12% SDS-PAGE were electroblotted to nitrocellulose and probed with a polyclonal rabbit antibody specific for rat fetuin.

Screening of Genomic Clones Harboring the Mouse Ahsg Gene

An Svj 129 library, constructed in λDASH2 (Stratagene, LaJolla, CA) using spleen genomic DNA from male Svj mice, was screened for the mouse Ahsg gene (kind gift of Dr. Roger Askew, University of Cincinnati, OH). After infection of PLK-17 cells, plaques were generated at 50,000 plaques per plate, and lifted onto 137 mm Ø Nytran filters (0.45 μ, Schleicher and Schüll, Keene, NH). Filters were hybridized at high stringency (50% formamide, 43°C) with a [^{32} P]-labeled cDNA probe (1.2 kb) derived representing the mouse Ahsg cDNA.

Chromosomal Mapping of Mouse Ahsg

Using the 3′-UTR of the mouse Ahsg cDNA as a target, a primer pair [sense 5′-CTTCAAAATC-TAGGCTTGATTCGG-3′OH and antisense 5′-GCTTTATGCCTTTCATCAAATTTGACCATT-3′ OH] was selected using the Primer Detective program.[36] Mouse genomic DNA (25 ng) was amplified using the above primers in a Thermal Cycler Model 9600 (Perkin-Elmer-Cetus, USA). The samples were heated at 95°C for 1 min and amplified by 35 cycles of denaturation (94°C for 30 sec), annealing (at the optimized temperature of 58°C for 30 sec), and extension (at 70°C for 1 min), followed by 3 min of extension (70°C) after the last cycle.[37] The Jackson Laboratory BSS interspecific backcross mouse panels were used to determine the mouse Ahsg chromosomal location.[38] A customized polyacrylamide gel electrophoresis apparatus (Nihin Eido, Japan) was used to obtained high resolution of the mapping results. A 28-well comb was specially designed to accommodate two interdigitated sample loadings with a 12-channel micropipetter. A total of 24 samples were loaded per 10% gel. The gels were run at 250 volts for 1 h, stained with 0.5 μg/ml of ethidium bromide and photographed by UV transillumination. Allele types, C57BL/6J or *M. spretus*, were scored by visual

inspection of the gels and analyzed with the computer program, Map Manager.[39] The localization of the markers were determined according to the composite map of backcross panels.[38] The composite map of the BSS panel data contains 451 markers, including 49 MIT markers. In these composite maps, the average centimorgan length of the 95% confidence interval for these markers is 7.6 cM (BSS).

Functional Studies of Recombinant Mouse α_2-HSG with Respect to the Insulin Receptor

Insulin receptors were partially purified from the H-35 rat hepatoma cell line (ATCC, Rockville, MD) as described earlier.[40] Autophosphorylation of crude insulin receptors was assessed by preincubating various concentrations of α_2-HSG in the presence of insulin (100 nM) for 30 min at 20°C. The phosphorylation reaction was initiated in the presence of [^{32} P]-ATP (3000 Ci/mmol), $MnCl_2$ (8 mM), ATP (10 μM), PNPP (10 mM), and Na-orthovanadate (100 μM). Reactions were stopped after 10 min by boiling in the presence of 3% SDS and 100 mM DTT. Proteins were separated by SDS-PAGE 10% gel and the ^{32}P incorporated was detected by autoradiography of the dried gel. An exogenous substrate, poly ($Glu^{80}Tyr^{20}$), was used to assess the IR-TK activity.

Insulin-induced DNA synthesis was monitored by incorporation of [^{3}H]-thymidine into H-35 cells. The cells were grown to 30% confluence and incubated in serum-free DMEM, containing 0.1% insulin-free BSA for 36 h and reincubated for 14 h with 100 nM insulin, in the presence of various concentrations of mouse α_2-HSG. Cells were pulsed with 1 μCi/ml of [^{3}H]-thymidine (NEN-Dupont, Wilmington, DE) for 1 h, and then washed twice with ice-cold PBS. The cells were solubilized and the DNA precipitated with ice-cold TCA. The precipitates were collected on glass filters and washed twice with ice-cold 5% TCA. The radioactivity incorporated was quantitated in a scintillation counter.

RESULTS

Isolation of Genomic Clones Harboring the Mouse Ahsg Gene

A screening of 350,000 clones of the λDASH2 Svj 129 genomic library resulted in 11 independent clones. The relatedness of these clones to the mouse Ahsg gene was confirmed by PCR amplification of crude phage DNA using mouse Ahsg primers KOAF1 (5′-GCCCTTCGGAGTGGTG-TATGAGATG-3′OH) and KOAB10 (5′-ACGTTGG-TATCGTTGAACGGAGTC-3′OH) designed from targets in the cDNA sequence. PCR amplification of the clones resulted in a fragment of 0.95 kb which could be cleaved into three pieces using BstEII digestion. One clone (λKOA-1B) was selected for further characterization, and 10 μg of phage DNA prepared from plate lysates for restriction enzyme analysis. Restriction analysis performed on this clone using digestion with EcoRI or Bgl II (Fig. 1) suggested an insert size of 18.6–23.0 kb. Bgl II fragments of 6.0, 5.65, 3.42, 2.62 and 0.9 kb representing the mouse genomic insert were found (in addition to the λ arms); likewise, digest of λKOA-1B revealed EcoRI fragments of 6.6, 6.0, 4.85, 2.45, 1.7 and 1.42 kb (in addition to the λ arms). Two Bgl II fragments excised from an agarose gel (3.45 and 0.9 kb) were chosen for subcloning into the BamHI site of pGEM4Z, resulting in clones D and delta; both subclones were completely sequenced.

Sequence analysis of these two clones reveals that the smaller clone (delta) harbors the first exon (290 nt) in addition to 5′-regulatory elements up to the-154 position, as well as 431 nt of intron downstream of the first exon (Figs. 2A and 4A). We suggest that position 155 in Figure 2A be taken as the transcriptional start site (cap site) based on the alignment of

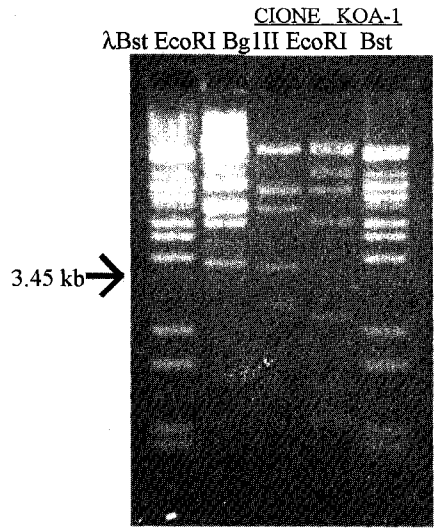

FIGURE 1 *Restriction analysis of Ahsg genomic clone λKOA-1B.* A 1.2 kb mouse Ahsg cDNA was used as a probe to screen a mouse genomic library. One of the eleven positive clones (λKOA-1B) was plaque-purified to homogeneity and 10 μg of DNA purified from plate lysates. DNA was cut with EcoRI or Bgl II and the DNA fragments separated on a 1% agarose gel. DNA in lanes 1 and 4 is a λBstE2 marker, lane 2 is λKOA-1B cut with Bgl II and lane 3 is λKOA-1B cut with EcoRI. Digestion with Bgl II generated bands ranging from 900 bp to about 8.5 kb. Two Bgl II fragments were selected for subcloning, a 3.45 kb Bgl II fragment (indicated by the arrow) and a 0.9 kb Bgl II band (not shown). Summation of the sizes of the non-vector fragments in lanes 2 and 3 imply that clone λKOA-1B, harbors a total of 18.6–23.0 kb of mouse DNA, more than twice the size of the known rat and human AHSG genes (7–8 kb; [49, 50]).

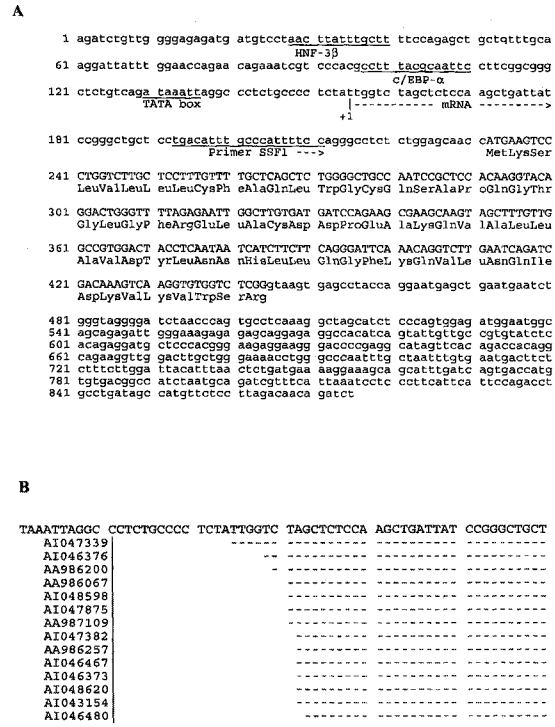

FIGURE 2 *The 875 bp mouse Ahsg subclone delta contains 154 nt of 5′-flanking DNA in addition to exon 1 and part of the first intron.* The DNA sequence analysis shown in **Panel A** reveals a 154 bp segment upstream from the transcriptional start site, the first exon and part of the first intron. Putative transcriptional motifs [hepatic nuclear factor (HNF)-3β and C/EBP-α] are indicated, as well as the transcriptional start site. One of the primers used to construct the baculoviral cDNA expression clone (SSF1) is indicated. The DNA in exon 1 is indicated in uppercase. This sequence is available from GenBank, accession number AF025820. The transcriptional start site is deduced from the alignment of 14 EST clones available in the public database (**Panel B**); the most 5′ EST clone (AI047339; Sugano mouse liver EST project) is taken to define the transcriptional start site.

expressed sequence tag (EST) clones available in public databases, especially those from the Sugano mouse liver EST project (Marra *et al.*, Washington University, St. Louis, MO). In Figure 2B, we show the alignment of 14 of the more than 50 EST sequences available; the most 5′ sequence (file identifier 1450748/ud65a11.y1, accession number AI047339) is taken to define the transcriptional start site. Upstream of the cap site (+1) can be found a TATA box (ATAAATT) at the -24/−18 position, and two motifs suggestive of sites for transcription factors C/EBP-α (−58 to −45, CCTTTACGCAATTC) and HNF-3β (−126 to −115, ACTTATTTGCTT). Both of these factors are abundant in liver, and associated with the expression of liver-specific genes. Alignment of the corresponding human AHSG and rat AHSG upstream sequences (Fig. 5) reveals that all of these putative transcriptional elements are conserved in the proximal upstream regions of mouse, rat, and human genes. Moreover, this alignment of the three sequences reveals that the splice donor (SD) at the 3′-end of the first exon is precisely conserved among the three species, strongly suggesting that the mouse Ahsg genomic segment in clone delta represents the true ortholog of human AHSG.

Beyond the SD site, there is little conservation of sequence between the mouse and human first intron.

The larger clone (D, 3.45 kb Bgl II insert) harbors exons 2, 3 and 4 (Figs. 3 and 4B), in addition to two elements in the intron upstream

```
 1 gatctcatat gctaacagac tgaaactatg aaaccgccaa tacttgtctg cttttctttt
       PRIMER VIV3 --->                                  |-- (CT)n ----

 61 cctgtttctg tctgtctgtc tgtctttctt cctttctttc tcttctctct ttctttctct
    ------------ microsatellite (CT)n repeat -----------------------

121 tcctctttct ttcttcttcc ttcctttctc ttctttctct ttctttcttc ctttttcttc
    ------------ microsatellite (CT)n repeat -----------------------

181 tttctttctc tttgctcttt ctttcccttc cttccttcct ttctcttctt tctcttctct
    ------------ microsatellite (CT)n repeat -----------------------

241 ctttcttttc cttccttttct tccttccttc cttccttcct tccttccttc cttccttcct
    ------------ microsatellite (CT)n repeat -----------------------

301 tccttctttc cttcttcttt ttgagacaga aaggtttctc tttgtagccc tgactgtcct
    -- (CT)n repeat -------|         |---- B1 repetitive element ----

361 ggaacttact atgtagacca ggctggcctc aaacacacag agatccacct gcctctgcct
    Primer VIV7 -->
    ------------------------ B1 repetitive element ------------------

421 cctggctcct ggaatcatag gcatgtgtca ccgactattt gtctgctttt aacatgcaaa
    -|

481 gttggaaact ccatacggtt cagcttaaca taaagatgag aagaacaagt ttgtgtcact
541 agagacttag gatttaggag gaaaataagg taaacaccag gatgctcaga gtgaggattg
601 acaaccagct ttacaatggg acagctgatt tgaaaccacg gttttcctgg gtgagtttta
661 agggcagttg gcaaaagacg taatggccgg ctctctgcct agtttacatg ctgaagggaa
721 agccgtgagc gagcactgtg catgtgctac gtgctgattg tgagatgctc attatgggat
781 gcccgagtgg atcaagaant tccctgcaca taaacccagt gcatctaccc atggttagtt
841 ctgaggtctc cggagagtga aaatgcccag tgaactaaat tgggttgaga gttttcaaac
901 tttggggcat ttcaaggtgt gaacggggaa tacatagaca ggtgaaacac tgaactcctc
961 acagggtcct gcaagcttcc caaaatgctt ccatcctagt ggtgacagtt tcccagcctc
1021 agaatagaaa ggcggcaaac aggagatagg actctctgtg catccaggac ccaggaaggt
1081 agaagataaa gagccaaggg aggagcaaga gaaaccttta aggacacaaa cactcaaaga
1141 aagggagaaa agtgggcaac tagagagaag aaagaatgaa gcagaatgaa agatagcaaa
1201 gataataaac ctttcaagat aaaagctagc cctcagagtc acttctttgt aaagagagct
1261 cagaataagg agtatggctg ggacagctgt tggagcacgg ccccccctcc cgtccctttt
1321 ttccccctcc cccctcccca tttcacaccc gctccatcta tagtggaaga ctaaaaagcc
1381 aaaacaaaac aaaaagaaaa aaaaaaaaaa ccactgcagc actgcatagc tggaaggggt
1441 ggggctgaca tctccttagt cctcccaccc ctcagccaag ccccaccaca gggctagtgt
1501 tcacactagg tgccatgcat tggatcagcg gagcagggcg tttgctcacc tctcccttct
     <-- Primer VIV11

1561 gtccggctcc acagCGGCCC TTCGGAGTGG TGTATGAGAT GGAAGTTGAC ACACTGGAGA
                  SA      Primer KOAF1 -->              exon 2
                      <--- Primer VIV5 ----------
                   ArgPro PheGlyValV alTyrGluMe tGluValAsp ThrLeuGluT

1621 CCACTTGCCA TGCTTTGGAC CCCACCCCGC TGGCAAACTG TTCTGTGAGG CAGCTGACTG
     hrThrCysHi sAlaLeuAsp ProThrProL euAlaAsnCy sSerValArg GlnLeuThrG

1681 AGCACgtgag tgctgccttg tggttggttg gtgggtgggt gggtgggtgg gagctgccca
     luHis

1741 gccaccacag ttcagcaaag tgcaggtttg gctttctcca tctcccagca gccatcttgg
1801 ctagccagag agcaaagtct aaaacccgct gtgggataga tggtgccttc cccgaggttg
1861 attttcacaa cacttgggct tttcttcttg aagccctcgg gagagcagat tatgatgttt
1921 caataacacc cgtgaaggtt gccttgggca ggttacctcc cacaaccctg ccaagacgct
1981 ccctgaatga gcagccagag tatatatact gcttcagaat gccggcatct gatttcttta



2041 cccagGCGGT GGAGGGAGAC TGTGACTTCC ACATCCTGAA ACAAGACGGC CAGTTCAGGG
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FIGURE 3 *Internal exons 2–4 of mouse Ahsg gene.* The 3.45 kb Bgl II fragment cloned pGEM4Z (clone D) was sequenced using a combination of dye primers (SP6 and T7, at the 3′ and 5′-ends of the subclone, respectively), and dye terminator reactions. Sequencing primers are indicated beneath the DNA sequence. Exons are indicated in the DNA sequence as uppercase letters. The first intron contains 271 bp of the microsatellite $(C,T)n*(A,G)n$ adjacent to a middle repetitive element of 92 bp in the B1 family. This sequence is available from GenBank, accession number AF025821.

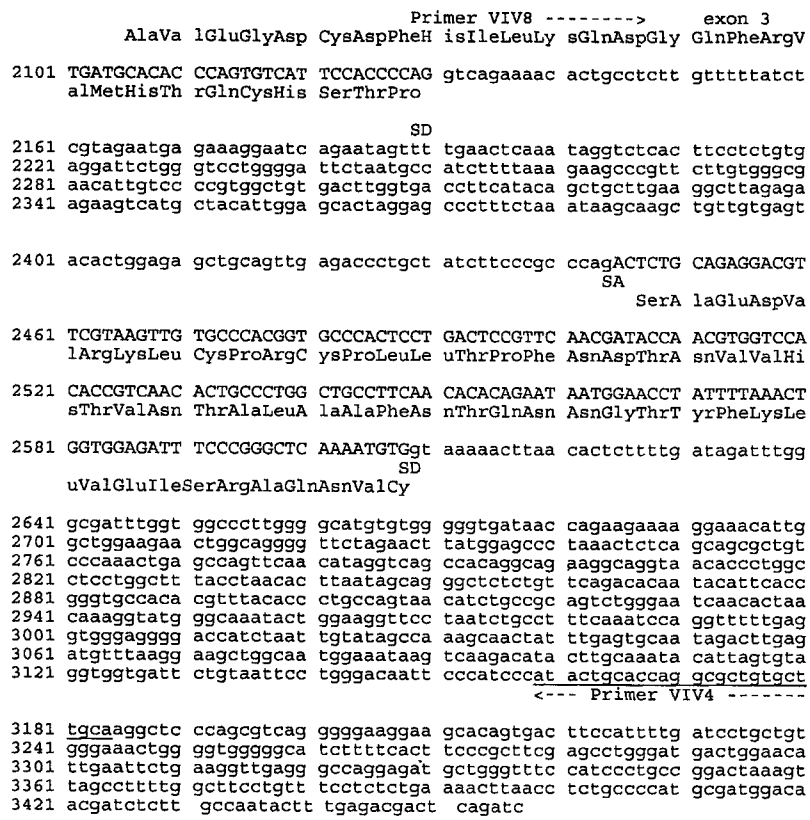

FIGURE 3 (Continued).

of exon 2. The first of these elements is a 271 nt long microsatellite (T,C)$n*$(A,G)n composed of 96% C or T in the coding strand. The second element found in this intron is a 92 bp sequence with high homology to the family of B1 middle repetitive elements characterisitic of mouse DNA.[41, 42]

Chromosomal Mapping of Mouse Ahsg

In order to map the mouse Ahsg gene to its chromosomal site, we targetted a PCR amplicon of 198 bp located in the 3′-UTR of the mouse Ahsg cDNA; using a site in the 3′-UTR was used to increase the possibility of finding sequence polymorphism between different mouse strains.[43, 44] The data indicate a localization of the Ahsg gene to chromosome 16 at approximately 16 cM, adjacent to Dagk3 (Diacylglycerol kinase 3) gene, a position syntenic with human chromosome 3q27, the location of the human AHSG gene.[21, 45] The mapping profile of the Ahsg gene, with respect to the genes already mapped on chromosome 16, and a composite map of the Jackson BSS panel Map, *versus* the MGD composite map are depicted in (Fig. 6).

Expression of Mouse Ahsg cDNA as a Recombinant Baculovirus and Assay for Insulin Receptor Tyrosine Kinase Activity

A baculoviral transfer vector, pMusBacα3 (11,440 bp) was created using a 1.2 kb BamHI-Xbal segment of the mouse Ahsg cDNA featuring an intact 1035 bp open reading frame, as described in Materials and Methods. This transfer vector (2 μg) was transfected into Sf 9 cells along with 5 μg of the wild-type baculoviral

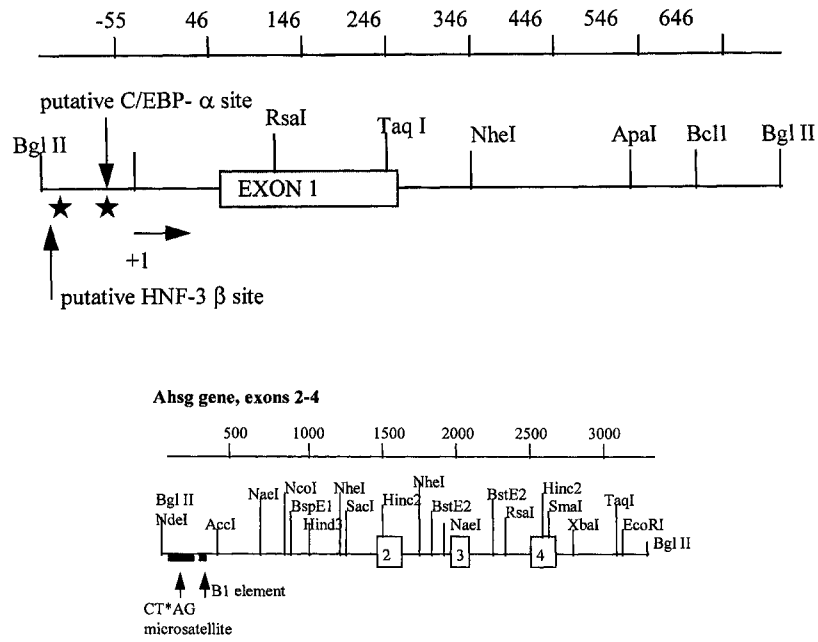

FIGURE 4 *Schematic structure of subclones delta and D.* Shown in **Panel A** is the schematic structure of the upstream subclone delta whose sequence is shown in Figure 2A. Putative transcriptional motifs (hepatic nuclear factor (HNF)-3β and C/EBP-α) are indicated, as well as the transcriptional start site. The segment of the first exon which encodes the first 85 amino acids of mouse α_2-HS-glycoprotein is shown in the shaded box. Shown in **Panel B** is the schematic structure of exons 2, 3 and 4, including part of the first intron and part of the intron downstream from exon 4. Shaded boxes indicate the exons. The 271 bp microsatellite (C,T)n*(A,G)n and the 92 bp middle repetitive (*B*1 family) element are indicated by bracketing.

DNA, and blue plaques selected from 2% agarose plugs stained in Bluo-Gal. Twice-purified viral plaques were expanded at 27°C, and recombinant viral stocks checked for homogeneity using PCR (baculoviral primers −44, 5′-TTTACTGTTTTCGTAACAGTTTTG-3′OH; and +778, 5′-CAACAACGCACAGAATCTAGC-3′OH). Expanded recombinant baculoviral stocks were used to transfect cabbage looper HighFive™ cells, and supernatants collected after 48–72 h. Supernatants were purified using affinity chromatography on jacalin lectin columns and proteins eluted with 25 μM melibiose were separated on 12% SDS-PAGE and electroblotted to nitrocellulose. Probing of this membrane with a polyclonal rabbit antibody specific for rat fetuin reveals two prominent bands of 60 and 66 kD (Fig. 7A) in addition to a fainter band of a slightly smaller apparent molecular weight.

Inhibition of Insulin Receptor Tyrosine Kinase Activity

Jacalin lectin affinity-purified recombinant mouse α_2-HSG (Fig. 7A) was used to test inhibitory activity of the insulin receptor in a TK assay. Mouse α_2-HSG was tested at concentrations ranging from 5 μg/ml to 20 μg/ml in the presence of insulin. Approximately 70% inhibition of IR-TK activity was observed at 15 μg/ml (Fig. 7B).

Inhibition of Insulin-stimulated IR Autophosphorylation and Insulin-stimulated DNA Synthesis

Multiple experiments were performed in duplicate in order to verify the ability of recombinant mouse α_2-HSG to inhibit autophosphorylation of the 95 kD β-subunit of the IR. The proteins were

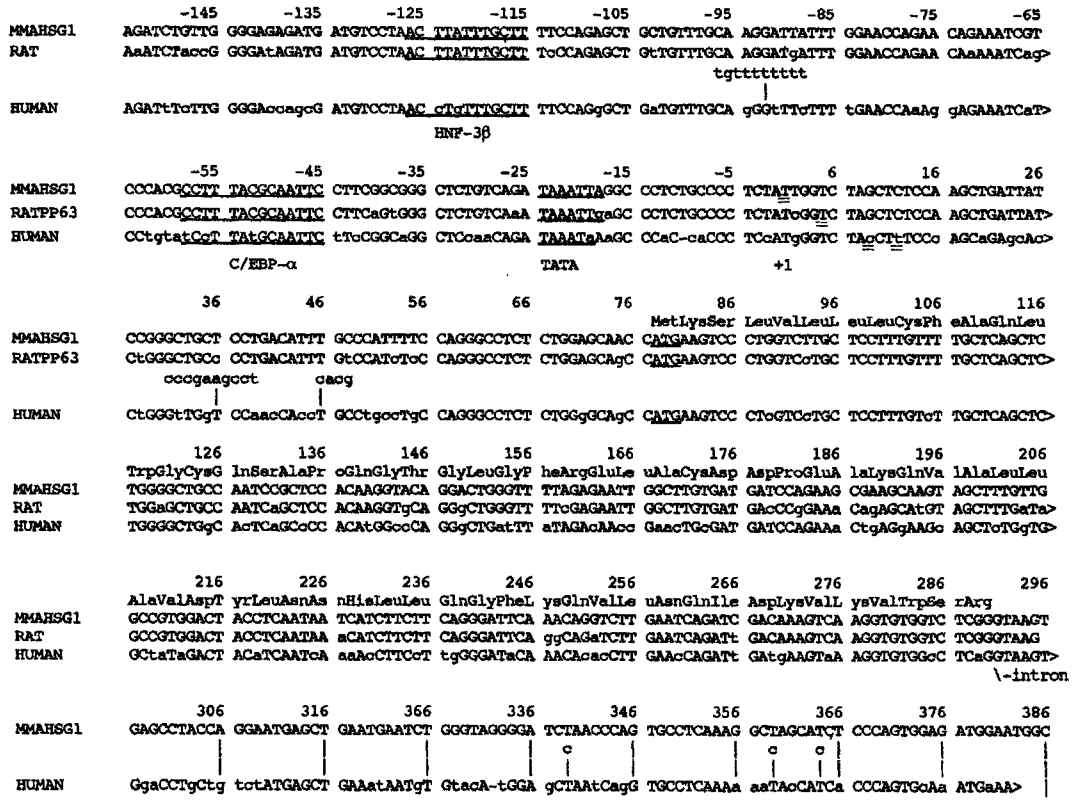

FIGURE 5 *Alignment of the proximal promoter sequences of mouse, rat, and human AHSG genes reveals an evolutionary conservation of putative transcription factor motifs, TATA box, and splice donor (SD) sequences.* The mouse sequence is taken from Figure 2A; the rat sequence is taken from GenBank files M36547 and X63446;[49, 26] the human sequence is taken from GenBank files Y09540 and M16961.[55, 21] Sequences were aligned using CLUSTAL. Transcriptional start site are indicated by double underlining. The C/EBP-α and HNF-3β motifs and the MET initiator codon are indicated by single underlining. The conceptual translation of the mouse Ahsg sequence is indicated above the line MMAHSG1. Where the rat or human sequence agrees with the nucleotide in the mouse sequence, it is capitalized; otherwise, mismatched bases are indicated in lowercase in the human and rat sequence lines. The human sequence has two insertions relative to the mouse sequence; the rat sequence has a single deletion relative to the mouse sequence, filling in the gap with a (-). The numbering above the mouse sequence line is relative to the mouse transcriptional start site defined in Figure 2.

FIGURE 6 *Chromosomal mapping of the mouse Ahsg gene obtained from typing patterns derived from PCR analysis using the Jackson Laboratories BSS interspecific backcross.* Locations of allele types of C57B1/6J or *M. spretus* were determined by the composite map of backcross panels. Mouse Ahsg is mapped to chromosome 16 at 16 centimorgans. Mapping profile of the Ahsg gene with respect to other genes mapped to chromosome 16 (**Panel A**). Demonstration of a composite map of the Jackson BSS panel Map *versus* the MGD composite map. The Ahsg gene is localized at approximately 16 cM closely to the Dagk3 gene (**Panel B**).

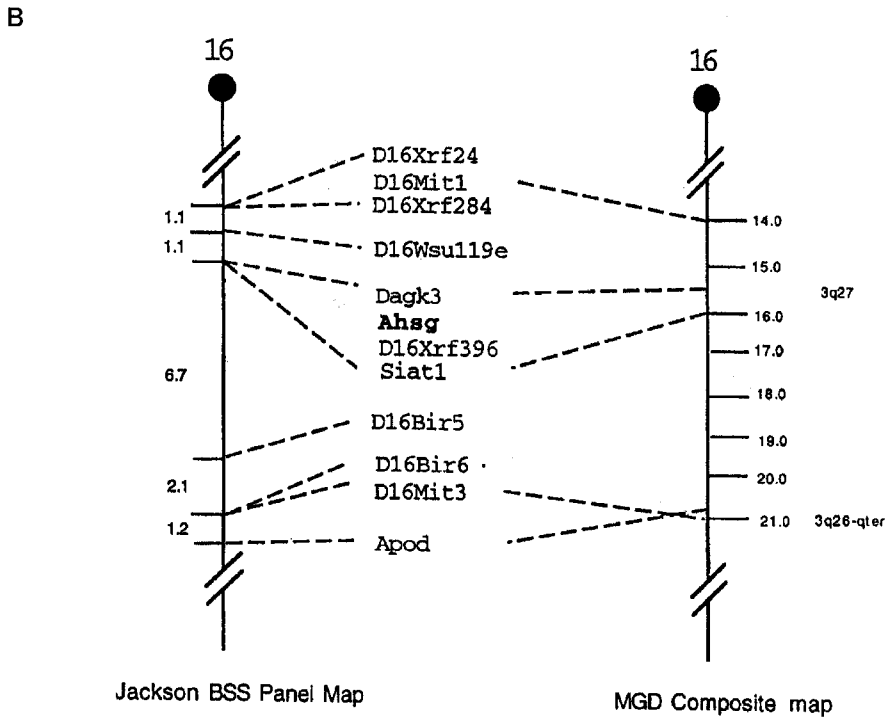

FIGURE 6 (Continued).

separated on a 10% SDS-PAGE and the ^{32}P incorporated was detected by autoradiography of the dried gel. Mouse α_2-HSG at a concentration of 1 μg/ml completely abolished insulin-induced autophosphorylation of the β-subunit of partially purified rat IR (Fig. 7C). Various concentrations of recombinant mouse α_2-HSG were tested for their ability to inhibit

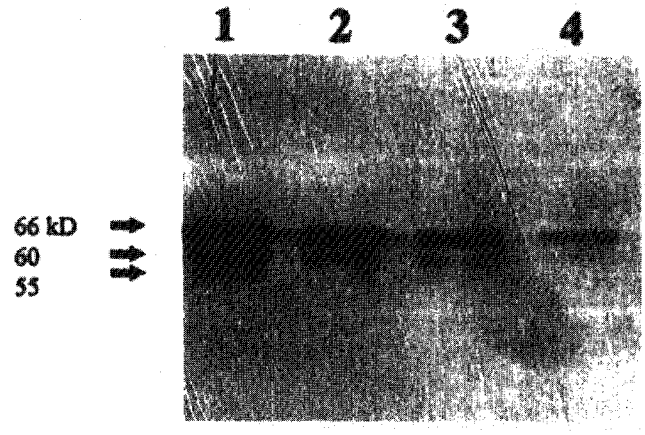

FIGURE 7A *Recombinant mouse* α_2-HS-glycoprotein expressed in a baculoviral system. Immunoblot of mouse α_2-HSG synthesized in insect cells, partially purified by lectin chromatography. Numbers below the lanes indicate fractions eluted from the jacalin affinity column. Two forms are prominent, with apparent molecular weights of 60–66 kD.

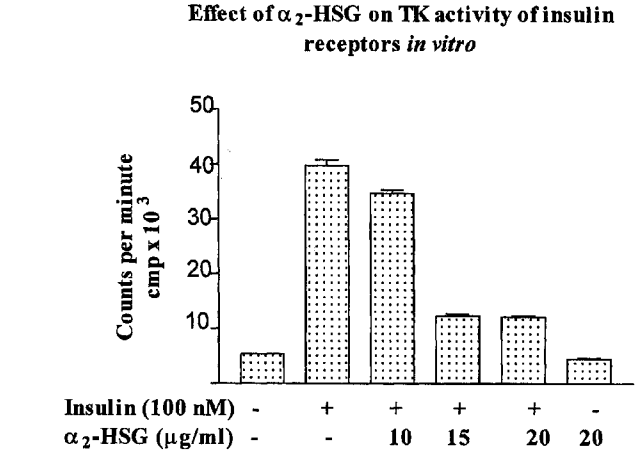

FIGURE 7B *Recombinant mouse* α_2-HSG is an inhibitor of the insulin receptor (IR) at the tyrosine kinase (TK) level. The bar graph indicates that the tyrosine kinase activity of the insulin receptor is inhibited by α_2-HSG. IRs purified from H-35 rat hepatoma cells were incubated for 30 min, 20°C with various concentrations of mouse α_2-HSG (10, 15 or 20 μg/ml) in the presence or absence of insulin (100 nM). Insulin-stimulated IR-TKA was assessed by its ability to transfer ^{32}P onto the synthetic substrate poly ($Glu^{80}Tyr^{20}$). Lane 1 represents stimulation of the insulin receptor with 100 nM insulin. Mouse α_2-HSG inhibits over 70% the insulin-stimulated receptors at a concentration of 15 μg/ml. The results shown are representative of three separate experiments.

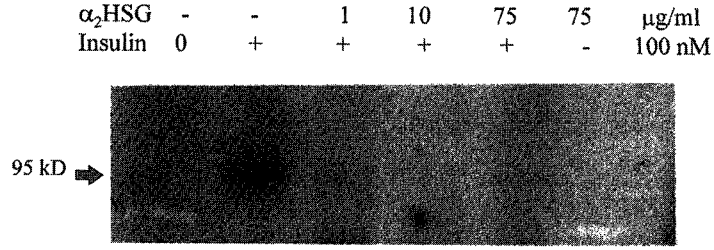

FIGURE 7C *Effect of* α_2-HSG on autophosphorylation of the IR *in vitro*. The autoradiograph reveals that recombinant mouse α_2-HSG inhibits the autophosphorylation of the insulin receptor. Partially purified insulin receptors from H-35 cells were pre-incubated with or without insulin (100 nM) in presence or absence of mouse α_2-HSG (1, 10, 75 μg/ml) respectively. Autophosphorylation was determined as described in Materials and Methods. The position of the 95 kDa β-subunit of the IR is indicated. Insulin-induced autophosphorylation of the β-subunit of the IR compared to basal (lanes 2 and 1, respectively). Purified mouse protein at concentrations of 1 μg/ml completely abolished insulin-induced autophosphorylation of the IR.

insulin-induced DNA synthesis. Mouse α_2-HSG at 25–35 μg/ml completely abolished insulin-induced DNA synthesis in H-35 hepatoma cells (Fig. 8).

DISCUSSION

The human glycoprotein α_2-HSG acts as a specific inhibitor of the human IR, at the level of the TK.[18, 46, 47] In this study, we have cloned the mouse homolog of human AHSG, determined its genomic organization, chromosomal location and demonstrated its inhibitory activity towards the IR-TK.

The mouse clone we have isolated by screening a mouse Svj/129 genomic library (λKOA-1B), harbors 18.6–23.0 kb of the mouse Ahsg gene. Sequence analysis shows that the mouse gene is organized with the same exon–intron organization as both the rat fetuin gene[48, 49] and the human AHSG gene.[50] Both the rat

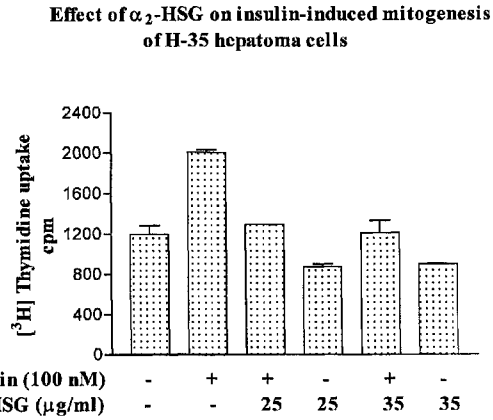

FIGURE 8 *Mouse* α_2-HSG inhibits insulin-induced mitogenesis on H-35 hepatoma cells. The data describe the effect of recombinant mouse α_2-HSG on insulin-induced mitogenesis of H-35 rat hepatoma cells. Subconfluent dishes were treated as described in Materials and Methods and the uptake of [^{3}H]-thymidine was measured. Insulin induced DNA synthesis (represented in the first bar). Insulin-stimulated mitogenesis is completely blocked at concentrations of 25–35 μg/ml of mouse α_2-HSG (bar 3 and 5). The p value was calculated by comparing the second bar, insulin-stimulated mitogenesis and the third bar, α_2-HSG (25 μg/ml) and insulin-treated cells (the $P < 0.05$, $p = 0.008$ is considered significant). A comparison between the second bar, insulin-stimulated cells and the fifth bar, α_2-HSG (35 μg/ml) and insulin-treated cells demonstrated $P < 0.01$, $p = 0.008$.

fetuin gene and the human AHSG gene feature 7 exons. This genomic subclones we have sequenced cover exon 1 (Figs. 2A and 4A) and exons 2–4 (Figs. 3 and 4B). We have not sequenced clone λKOA-1B downstream of exon 4, and we are unable to address the question of exon–intron organization downstream of exon 4. In all three species, intron 1 occurs at the same codon position and features the same splice acceptor (SA). Our sequence data demonstrate that intron 1 is 2000 bp in length in the mouse, slightly larger than the 1.7 kb reported for the corresponding intron in the rat,[49] and slightly smaller that the corresponding intron in the human gene (2.3 kb;[50]). Clone λKOA-1B also contains a 271 bp tract of (T,C)n*(A,G)n adjacent to a B1 element of 92 bp. Numerous copies of B1, B2 and D1 elements are found in rodent genomes.[41,42] Some of these B1 elements are not neutral relics of evolutionary spread of the DNA element, but may play a functional role in the regulation of the genes in which they are present. For example, B1 elements can act as negative regulators of gene expression.[51,52] Moreover, androgen regulation of one gene seems to have been acquired during evolution through the insertion of a B1 repetitive element into the transcription unit.[53] Whether the B1 element in the first mouse Ahsg intron plays a role in the regulation of Ahsg transcription has not yet been addressed experimentally. The conservation of C/EBP-α and HNF-3β binding sites in the proximal 5′ upstream region of the mouse and human AHSG gene is likely to have a functional significance. Falquerho *et al.*[54] have shown these motifs to be functional in transient transfection of the rat AHSG gene. Moreover, Banine *et al.*[55] have analyzed the human AHSG promoter–enhancer in transient transfection assays as well, suggestive of a functional role for the C/EBP-α and HNF-3β sites in the human gene. The putative HNF-3β site in the mouse Ahsg gene is conserved between mouse, rat and human. Given the role of human,[18] rat,[48] and mouse (this study) proteins in the inhibition of insulin receptor function, it is intriguing to note that one form of maturity onset diabetes of the young (MODY), maps to the gene encoding HNF-3β in man.[56–58] Another site conserved between the rat, mouse, and human genes is the putative C/EBP-α site at −58 to −45 (CCTTTACGCAATTCC in mouse). This site has been implicated in the cytokine-induced down regulation of the rat fetuin gene associated with the acute phase.[59]

We have successfully employed the 3′ UTR of the mouse Ahsg cDNA as a target for interspecies polymorphism. The 3′ UTR region was chosen because it is not disrupted by introns in the human gene,[50] and, therefore, the primer pairs designed from the 3′ short end of the cDNA should amplify the same 198 bp fragment from the genomic DNA. The 3′ UTR sequences constitute a rich source of genetic markers for the mouse genome.[43] Using this PCR based

mapping technique, and panels between *C57BL/6J* and *Mus spretus*, the mouse Ahsg gene was localized to chromosome 16 at 16 cM. This location is syntenic with the position of the human AHSG gene on chromosome 3 band q27. It is interesting to note that the mouse Ahsg maps in the vicinity of other genes implicated in signal transduction and gene regulation such as Dagk3 (diacylglycerol kinase 3, gamma 3, 15.5 cM), Prkm1 (protein kinase, mitogen activated kinase 1, 14.5 cM), and EIF4β, eukaryotic translation initiation factor 4B, 14.2 cM). These genes have also been mapped at the syntenic position in man.

In the present study, we have used the baculoviral system to express mouse α_2-HSG as a recombinant protein. The baculoviral protein has an apparent molecular weight of 60–66 kD and has been shown to block insulin-induced IR-autophosphorylation at 1 μg/ml, IR-TKA at 15 μg/ml and insulin stimulated mitogenesis at 25 μg/ml *in vivo*. In the rat, it has been shown that pp63/fetuin can inhibit IR-TK and IR autophosphorylation with a half-maximal inhibition of 0.24 μg/ml.[48] These results demonstrate that the IR inhibitory activity of rat fetuin,[48] human α_2-HSG,[18,46] and bovine fetuin[60] now extends to mouse α_2-HSG. Together, these results suggest that the mouse Ahsg gene is the ortholog of the human AHSG gene. Additional studies will be necessary to determine whether the *in vitro* demonstration of IR inhibitory activity demonstrated now for four of these proteins (rat, bovine, human and mouse) has a physiological significance for glucose regulation in these species.

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