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HHS Public Access

Author manuscript

J Neuroendocrinol. Author manuscript; available in PMC 2015 November 18.

Published in final edited form as:

J Neuroendocrinol. 2013 March ; 25(3): 292–301. doi:10.1111/j.1365-2826.2012.02378.x.

A novel transcript is up-regulated by fasting in the hypothalamus and enhances insulin signaling

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Abstract

A transcript of unknown function, regulated by fasting and feeding, was identified by microarray analysis. The transcript is up-regulated in the fasting state. An 1168 base-pair cDNA was cloned from rat hypothalamus and sequenced. This sequence is consistent with adipogenesis downregulating transcript 3 (AGD3) (also known as human OCC-1) mRNA. A protein sequence identical to AGD3 was determined by mass spectrometry. In rat brain, AGD3 mRNA is distributed in arcuate nucleus, ventromedial hypothalamus, amygdaloid nuclei, paraventricular nucleus (PVN), hippocampus, and somatic cortex. Double in situ hybridization showed that AGD3 mRNA is co-localized with pro-opiomelanocortin and neuropeptide Y in arcuate nucleus neurons. AGD3 binds with insulin receptor substrate 4 and increases insulin-stimulated phospho-AKT and regulates AMPK and mTOR downstream target S6 kinase phosphorylation.

Keywords

adipogenesis downregulating transcript 3 (AGD3); insulin; proopiomelanocortin (POMC); neuropeptide Y (NPY)

Overweight and obesity represent major public health problems. These conditions affect morbidity and mortality as factors contributing to diabetes mellitus, cardiovascular disease, pulmonary disease, osteoarthritis and several types of cancers. Research has established the hypothalamus as a crucial site in controlling body weight. Peripherally produced hormones such as insulin, and nutrients such as glucose and fatty acids communicate energy status to hypothalamic centers [1–3]. The structure of the hypothalamic arcuate nucleus (ARC) is consistent with its role as an integrative center in energy homeostasis. Neuroanatomical studies have identified two distinct populations of ARC neurons [4]. The first population expresses the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AGRP). The second group of neurons expresses anorexigenic neuropeptides cocaine and amphetamine-related transcript (CART) and proopiomelanocortin (POMC) [4, 5].

Gene analysis using microarrays and high-throughput sequencing has been utilized extensively as a platform for biological exploration, including conditions associated with obesity. Using this approach, a previous report from our laboratory confirmed the

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differential expression of several neuropeptides associated with fasting and obesity [6, 7]. This study also suggested that intracellular regulatory genes are involved in energy homeostasis and are differentially regulated during fasting and feeding states. Over the past decade, microarray studies of cDNAs have revealed thousands of novel transcripts [8]. When the functional roles have been unclear, these have been designated as transcripts of unknown function (TUF). It is widely assumed that the majority of TUFs are non-coding RNAs [9, 10]. These non-coding RNAs may regulate gene function as microRNAs, as small nucleolar RNAs, or through non-coding repression of transcription factors [10]. We identified a TUF as strongly upregulated in the rat arcuate nucleus by fasting. In the current study, this TUF was identified as derived from an mRNA of 1.2 kbp identical to the previously described overexpressed in colon cancer-1 [11] and, recently, adipogenesis downregulating transcript 3 (AGD3) [12]. The AGD3 mRNA has an open reading frame that produces a 63-amino acid peptide. AGD3 is expressed in brain areas relevant to feeding behavior and is involved in cellular signaling pathways that relate to energy metabolism and may be involved in controlling feeding behavior.

Materials and Methods

Antibodies and reagents

Anti-Myc antibody-conjugated agarose was purchased from Bethyl Laboratories (Montgomery, TX). Anti-Myc monoclonal antibody was purchased from BD Bioscience (Palo Alto, CA). Protein A SepharoseTM CL-4B was from GE Healthcare (Sweden). Anti-human IRS4 antibody (EP907Y) was from Abcam (Cambridge MA). Phospho-AKT (Thr308) antibody, AKT antibody Phospho-AMPKa (Thr172) antibody, AMPKa antibody, Phospho-S6 (Ser235/236) antibody, and S6 Ribosomal Protein antibody were obtained from Cell Signaling Technologies (Beverly, MA).

Cell culture

Human embryonic kidney (HEK293) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) with 100 U / ml penicillin and 100 μg / ml streptomycin. Human neuroblastoma (SK-N-AS) cells were from ATCC (Manassas, VA) and were cultured in ATCC-formulated DMEM with 1% nonessential amino acids, 10% FBS, and 100 U / ml penicillin and 100 μg / ml streptomycin. Human colorectal adenocarcinoma (CACO-2) cells were obtained from Sigma-Aldrich (St. Louis, MO) and were cultured in EMEM with 2 mM glutamine, 1% nonessential amino acids, and 10% FBS. Mouse hypothalamus (GT1-1) cells were cultured in DMEM with 10% FBS containing 100 U / ml penicillin and 100 μg / ml streptomycin.

Animals and experimental protocol

Male Sprague-Dawley rats (Harlan Laboratories, Madison, WI) weighing 280–300 g were used to study AGD3 expression. Rats were housed in accordance with the University of Michigan Unit of Laboratory Animal Medicine guidelines. Five rats were assigned to the fed group and five rats to the fasted group. Animals in fed group were allowed free access to regular rat chow and water. Animals in fasted group were food-deprived 48 hours but were allowed free access to water. At the end of 48-h period animals were sequentially

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euthanized. Whole brain was removed and frozen. A 2-mm-thick section from Bregma −2 mm to −4 mm was sliced. ARC was punched out using a Stoelting brain punch set. Then total RNA was isolated from the samples.

Reverse Transcription Quantitative PCR (RT-QPCR) was used to examine changes in rat AGD3 expression. Reverse transcription was performed using the SuperScript OneStep RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen). Q-PCR primers were : AGD3: 5′-CCG ACA GGA GCA GTT AAA GAT G-3/5′-CTT CCA AAT TCA CGA GCA AGC/Probe: 5′-56-FAM/AGA TAA CGC AAT GGC TGG ATA CCC G/36-TAMSp/-3′, and GAPDH: 5′-ACC ACA GTC CAT GCC ATC AC/5′-CAG CTC TGG GAT GAC CTT GC/5′-/Probe: 56-FAM/CAC AGC CTT GGC AGC ACC AGT GGA/36-TAMTph/-3′. GAPDH primers were used as internal control.

For analyzing AGD3 protein expression in ARC, another set of ten rats, five fed and five fasted, were used. Animals were euthanized. Brain was removed and directly dissected a 2 × 2 × 2 mm cube in ARC area. The tissue was homogenized in lysis buffer and supernatant was subject to Western blot analysis.

Cloning of AGD3 cDNA

Our gene microarray study demonstrated that an EST (Expressed Sequence Tag) was up-regulated by fasting. The EST (Gene Bank accession number AA925734) was a 189 base segment. In order to identify this gene, the EST sequence was examined using the NCBI database. By aligning the EST sequences with overlapping cDNA segments, it was extended to a 1215 base cDNA sequence. Two pairs of PCR primers linked to EcoR I and Kpn I enzyme sites were used to clone two portions of the sequence from rat hypothalamus by using Reverse Transcription Quantitative PCR (RT-QPCR). One pair is 5′GGA ATT CCG GGT GGC GCG ATG GGT TGC/5′GGG GTA CCA GTC TCGT CAG CTT CCT TCT ATG, covering from 117–529; another pair is 5′GGA ATT CAC GCA ATG GCT GGA TAC CCG AGA G /5′ GGG GTA CCG TTG CTT ACA CTG CCG TCAT, covering from 329–1168. The PCR products were sequenced.

AGD3 antibody production

Polyclonal antibodies against the rat AGD3 sequence were generated and purified by affinity chromatography with custom service by Invitrogen (Carlsbad, CA). Specificity of the antibodies was characterized using myc-AGD3 fusion protein.

Construction of expressing AGD3 vector and its siRNAs

AGD3-pcDNA3.1—The sequence 117–529, which was cloned by RT-PCR from the rat hypothalamus and has an open-reading frame coding a 63 amino acids protein, was subcloned into the pcDNA3.1 using EcoR I and Kpn I sites. The Myc tag was fused to the N-terminus of AGD3. The correct frame of the construct was confirmed by DNA sequencing.

AGD3 siRNAs—Three pairs of double-stranded siRNAs targeting the human OCC-1 gene were used. Their sequences are: 1) 5′ CGA AAG ACA AGU UAA CAA ACU GUC A / 5′

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UGA CAG UUU GUU AAC UUG UCU UUC GUA. 2) 5′ AGC AGC CAA AGA UGU AAC AGA AGA A / 5′ UUC UUC UGU UAC AUC UUU GGC UGC UCC (IDT). 3) 5′ CCC UGG GUA CAU AUU GUU GTT / 5′ CAA CAA UAU GUA CCC AGG GTT. Lipofectamine RNAiMax was used to transfect siRNAs into cells according to the manufacturer′s protocol. After 72 hours of transfection, cells were lysed and supernatants were further analyzed.

Identification of the AGD3 transcript

Pierce Direct IP Kit (Pierce Biotechnology, Rockford) was used to pull down AGD3 protein from CACO-2 cells. Briefly, AGD3 antibody was linked to agarose beads and incubated with cell lysates. After overnight incubation at 4 °C, beads were rinsed thoroughly with wash buffer, and AGD3 was eluted. Eluates were resolved on 16.5 % Tris-Tricine gels (Bio-Rad). Gels were stained with Bio-SafeTM Coomassie Blue stain solution. Protein bands recognized by Western blotting directed against anti-AGD3 antibody at the 7 KD position were subjected to mass spectrometry analysis.

The protein identification was conducted at the Protein Structure Facility at the University of Michigan. Bands of interest were excised and the proteins digested *in situ* with trypsin (Promega). Digests were analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) on a Thermo Fisher LTQ Orbitrap Velos mass spectrometer fitted with a New Objective Digital PicoView 550 NanoESI source. On-line HPLC separation of the digests was accomplished with an Eksigent/AB Sciex NanoLC-Ultra 2-D HPLC system: column, PicoFrit™ (New Objective; 75 μm i.d.) packed to 15 cm with C18 adsorbent (Vydac; 218MS 5 μm, 300 Å). Precursor ions were acquired in the Orbitrap in centroid mode at 60,000 resolution (*m/z* 400); data-dependent collision-induced dissociation (CID) spectra of the six most intense ions in the precursor scan above a set threshold were acquired at the same time in the linear trap. Mascot (Matrix Science) was used to search the uninterpreted CID spectra against the SwissProt database (SwissProt_2011_03.fasta). Cross correlation of the Mascot results with X! Tandem and determination of protein and peptide identity probabilities were accomplished by Scaffold (Proteome Software). The thresholds for acceptance of peptide and protein assignments in Scaffold were 95% and 99.9%, respectively.

In situ hybridization

In situ hybridization was conducted using a modification of a previously described protocol [6]. Briefly, a 416-bp PCR generated fragment of the rat AGD3 was subcloned into pBluescript SK (Stratagene, La Jolla, CA, USA). The sense primer was: 5′-ACA AGA GGA GAA ACT ACG GAG GAG-3′. The antisense primer was 5′-TGG CCA GGA GGG AAA ACA C-3′. The NPY cDNA (AI045437) is in pT7T3D-PAC (Invitrogen). The rat POMC plasmid construct consists of an 833-bp insert in pGEM4Z (Promega, Madison, WI, USA).

The ^{35}S-labelled antisense and sense AGD3 RNA probes, and digoxigenin-labeled antisense and sense NPY and POMC RNA probes were generated using standard in vitro transcription methodology.

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For single label in situ hybridization, the sections were hybridized with antisense ^{35}S-labeled AGD3 riboprobe. For dual label in situ hybridization, the sections were hybridized with antisense digoxigenin (DIG)-labeled NPY or POMC and ^{35}S-labelled AGD3 riboprobes.

Male Sprague-Dawley rats were anaesthetized with ketamine/xylazine and perfused via the ascending aorta with 200 ml of phosphate buffered saline (PBS), followed by 200 ml of 4% paraformaldehyde in PBS. The brain was postfixed for 16 h then transferred to 20% sucrose (20% sucrose in PBS with 0.02% sodium azide) for 5 days at 4 °C. The brain was embedded with 20% sucrose and Tissue-Tek OCT (2: 1) and coronal sections of 14 μm were cut on a cryostat. The sections were dried overnight at room temperature and were stored at −80 °C until further processing.

Co-localization was considered if a cluster of more than 20 silver grains were above POMC or NYP positive cells.

Dual immunohistochemistry

The sections were rinsed in phosphate buffered saline, 0.5% triton X-100 and blocked in 10% serum, and then incubated with primary antibodies [rabbit anti-AGD3 (1:150) and goat anti-POMC (1:100)] overnight at 4°C. For AGD3 staining, biotinylated horse anti-rabbit secondary antibody was used and then visualized with FITC488 (green) -conjugated avidin. To visualize POMC, Texas Red-conjugated donkey anti-goat secondary antibody was used. Nuclei were stained with 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen).

Co-immunoprecipitation

HEK293 cells were transfected with myc-AGD3 pcDNA3.1 using lipofectamine 2000 as described by the manufacturer (Invitrogen) and incubated for 48 h. Transfected cells were lysed with PK buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1mM Na_3VO_4, 50 mM NaF, 1% Triton X-100, 10% glycerol) and briefly sonicated. The supernatants of the cell lysates were incubated with protein A or anti-myc-conjugated beads for 24 h at 4°C. The beads were washed six times, and the immunoprecipitants were eluted with SDS sample buffer at 95 °C, resolved on an SDS-PAGE gel, and stained with the Coomassie Blue stain solution (Bio-Rad). Protein bands missing from the Protein A bands were excised from the gel. The gels were subject to mass spectrometry analysis.

In order to confirm the interactions between AGD3 and IRS4, which were revealed by the protein identification analysis, eluates were resolved on SDS-PAGE gel; the gels were used for Western blot analysis with anti-IRS4 antibody. For the co-immunoprecipitation of endogenous IRS4 from hypothalamus, rat hypothalamus tissue was homogenized in lysis buffer and the supernatant was used for immnoprecipitation with protein A and anti-AGD3 antibody.

IRS4 was detected with anti-IRS4 antibody. In the pull-down of AGD3 using IRS4, HEK293 cells were transfected p3xFlag-IRS4-CMV-10 plasmid DNA [13] along with myc-AGD3 pcDNA3.1 DNA. Forty hours after transfection, cells were disrupted by sonication in

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lysis buffer and Flag-IRS4 was immunoprecipitated using anti Flag-M2 antibody conjugated agarose (Sigma). After extensive washing, the beads were resuspended in SDS-PAGE loading buffer and heated at 85°C for 5 min. The protein was resolved with SDS-PAGE gel and transferred to polyvinylidene membranes. The membranes were blotted with either anti-myc, or anti Flag antibody.

Data analysis

For Western blots, analysis of densitometry was performed using Kodak 1D 3.6 software (Eastman Kodak, New Haven, CT). Data was analyzed using Graphpad Prism 4.0 (Graphpad Software, San Diego, CA), and expressed as mean ± SEM. Differences were analyzed by unpaired two-tailed Student's t test. A value of $p < 0.05$ was taken as significant.

Results

Up-regulation of AGD3 in rat arcuate nucleus by fasting and obesity

Gene profiles of the rat arcuate nucleus (ARC) were investigated in conditions of fasting and feeding by utilizing microarray analysis [6]. This analysis demonstrated 118 mRNA species that were increased by fasting relative to the fed state. An EST, gene bank number AA925743, was significantly increased in the fasted group. Quantitative PCR confirmed that this EST mRNA was increased two-fold in fasting animals relative to those in the fed group (Figure 1A).

We also examined the AGD3 transcript expression in the genetically obese Zucker rat (fa/fa) and the lean Zucker rat. The arcuate nucleus RNA was from our previous experiment [6]. Compared to the lean Zucker rat, AGD3 mRNA was up-regulated in the arcuate nucleus of the fa/fa rat (Figure 1B).

Cloning the open reading frame of rat AGD3

In order to identify this gene, the EST sequence was examined using the NCBI database. By aligning the EST sequence with overlapping DNA segments, it was extended to a 1215 base DNA sequence. Two pairs of PCR primers were used to clone two portions of the sequence and a segment of 1052 bases sequence was confirmed by DNA sequencing. This rat sequence has high homology with mouse and with the human overexpression in colon cancer-1 gene (OCC-1) [11]. This sequence also matches a transcript of unknown function, the adipogenesis downregulating transcript 3 (AGD3), reported by Kikuchi et al [12]. The 1052 base sequence contains several open reading frames. An open reading frame that can translate a 63 amino acid protein was proposed as a hypothetical protein. The rat hypothetical protein has 86% and 97% sequence identity with the human (NP_001138671) and mouse (NP_001138670), respectively.

Identifying the sequence of the hypothetical protein

A rat hypothetical protein was produced, derived from this putative sequence, and an antibody directed against the hypothetical protein was created. The antibody recognized a single band when AGD3-pcDNA3.1 was transfected into HEK293 cells, and recognized

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endogenous AGD3 protein in CACO-2 cells, SK-N-AS cells, and rat hypothalamus (Figure 2A). By using this antibody, we examined the protein expression of AGD3 in rat ARC, and the results showed that AGD3 protein significantly increased in fasted rats (Figure 2B).

Three anti AGD3 siRNAs were designed and transfected into CACO-2 cells. As shown in Figure 3A, AGD3 mRNA expression was inhibited by 90%. AGD3 protein expression was decreased to a similar degree (Figure 3B).

The antibody produced against the hypothetical protein was used to pull down AGD3 protein from CACO-1 cells. The bands that were recognized by the antibody on Western blot analysis were used to identify proteins (Figure 4A). Protein identification by mass spectrometry revealed the presence of the putative protein, over expressed in colon carcinoma (OCC-1_HUMAN; Uniprot accession number Q8TAD7), in samples 1 and 2 with 100% probability at the protein level. The unambiguous identification of AGD3 is based on the observation of 7 unique, well sequenced peptides (8 unique spectra and 11 total spectra) assigned to AGD3 in sample 1. In sample 2, three unique, well sequenced peptides (4 unique spectra and 7 total spectra) were assigned to AGD3. The sequence coverage for AGD3 in sample 1 is 86%, and in sample 2 is 56%. Figure 4B shows the identified sequence.

Distribution of AGD3 in rat brain

In situ hybridization—Figure 5 shows a photomicrograph of the neuroanatomical distribution of AGD3 mRNA. AGD3 mRNA was detected in several areas of the hypothalamus, including the arcuate nucleus (ARC), ventromedial nucleus (VMH), and paraventricular nucleus (PVN). AGD3 mRNA was also observed in the amygdala including the central and basolateral subnuclei of the amygdala, as well as in the hippocampus and the somatic cortex.

Figure 6 is a photomicrograph of double *in situ* hybridization in the arcuate nucleus. Eight brain sections that cover anterior and middle portions of the hypothalamus from Bregma −2.12 to −3.8 mm were hybridized and counted for each co-localization. Both POMC and NPY neurons have clusters of silver grains over them. Each slice has similar co-localization. Overall, 85 ± 12 % of POMC and 44 ± 9 % of NPY neurons express AGD3.

Immunohistochemistry—Immunohistochemistry revealed that AGD3 protein is present in the ARC, VMH, PVN, amygdala, hippocampus and somatic cortex, corresponding to its mRNA distribution. Double immunohistochemistry showed that AGD3 is present in POMC neurons from both the ARC and amygdala (Figure 7).

Interaction with IRS4

In order to find molecules that interact with AGD3, myc-AGD3 pcDNA3.1 was transfected into HEK293 cells. Cell lysates were incubated with anti-Myc agarose beads or protein A beads plus normal IgG. The gel bands in AGD3 lanes that were missing bands in control were sent to identify constituents by mass spectrometry analysis. Insulin receptor substrate 4 (IRS4) was pulled down by anti-AGD3 antibody. In order to confirm this interaction, we performed co-immunoprecipitation experiments using HEK293 cells that have endogenous

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IRS4 expression. Myc-AGD3 was expressed in HEK293 cells. The cell lysates were incubated with anti-Myc antibody conjugated to agarose. Anti-IRS4 antibody was used to detect endogenous IRS4. As shown in Figure 8A, endogenous IRS4 was co-precipitated with AGD3; IRS4 was pulled down by anti-AGD3 antibody from rat hypothalamus tissue (Figure 8B). In order to examine whether IRS4 can pull down AGD3, HEK293 cells were transfected Flag-IRS4-CMV plasmid along with myc-AGD3 pcDNA3.1 DNA. AGD3 was pulled down by IRS4 (Figure 8C).

Involvement of the ADG3 in insulin signaling

Because AGD3 interacts with IRS4, which is an element in the insulin signaling pathway, we investigated the role of AGD3 in AKT phosphorylation, a component of insulin signaling. SK-N-AS cells were transfected with AGD3 and AKT phosphorylation was examined. AGD3 expression in SK-N-AS cells significantly enhanced insulin-stimulated AKT phosphorylation (Thr308) (Figure 9A). When the SK-N-AS cells were transfected with AGD3 siRNA for 72 hours, insulin-stimulated AKT phosphorylation (Thr308) was significantly inhibited. The inhibition was 28.6 ± 6 % (Figure 9B).

Involvement in AMPK and mTOR signaling pathway

The AMPK kinase and mTOR signaling systems are responsive to cellular energy status and are involved in hypothalamic adaptation to fasting and feeding. An AGD3 siRNA approach was used to determine if AGD3 interacted with these pathways. As shown in Figure 10, transfection of GT1-1 cells with AGD3 siRNA affected these pathways differentially, decreasing AMPK phosphorylation and increasing expression of pP70 and pS6, which are the downstream signal molecules in the mTOR signal pathway [14]. This result indicates that AGD3 up-regulates phosphorylation of AMPK and inhibits mTOR pathway.

Discussion

The current studies have described a novel 63-amino acid peptide, AGD3, expressed in areas of the brain related to the control of ingestive behavior that interacts with energy signaling pathways. This conclusion is supported by the following distinct observations: 1) AGD3 mRNA is regulated by fasting and feeding in the hypothalamic arcuate nucleus; 2) AGD3 encodes a novel 63-amino acid protein; 3) the protein interacts specifically with insulin receptor substrate 4; 4) the protein is expressed in regions of the rat brain relevant to feeding behavior; there is a high degree of overlap with brain areas that also express insulin receptor substrate 4; 5) the protein affects AKT, AMPK and mTOR-dependent signaling *in vitro*.

The novel protein characterized here is derived from a gene of approximately 1.2 kbp that has a high degree of homology with the human overexpression in colon cancer-1 gene (OCC-1) firstly described by Pibouin et al [11]. These investigators identified a novel human cDNA overexpressed in a colon carcinoma cell line derived from a 29-year-old woman with hereditary nonpolyposis colorectal cancer. The gene is composed of 6 exons and is located in the q24.1 region of human chromosome 12. Elevated levels of *OCC-1* mRNA were observed in 3 of 8 colon carcinomas relative to normal mucosa from the affected patient. However, no specific *OCC-1* proteins were detected.

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The peptide characterized here has also been referred to as adipogenesis downregulating transcript 3 (ADG3) by Kikuchi et al [12]. These investigators used a panel of human bone marrow-derived mesenchymal stem cells (hMSC) and measured adipogenic and osteogenic induction in response to an array of transcripts of unknown function. AGD3 strongly inhibited adipogenic induction in response to a medium containing insulin, dexamethasone, indomethacin and isobutylmethylxanthine. Polyclonal antibody was prepared against the hypothetical human AGD3 protein. Western blotting confirmed that *ADG3* expression was reduced when hMSC were treated with AGD3 siRNAs, implying that an intracellular peptide was produced by this transcript.

The current study is the first to describe the amino acid sequence and the localization of this transcript in normal adult rat brain tissues. AGD3 mRNA was detected in several regions of the brain that are relevant to feeding behavior. Within the hypothalamus, these include the arcuate nucleus, ventromedial nucleus, and paraventricular nucleus. The arcuate nucleus (ARC) is a well-recognized center for integration of peripheral and central signals that regulate appetite and metabolism [5]. ARC neurons express receptors for a variety of circulating factors including insulin [15, 16]. AGD3 mRNA was also observed in the amygdala, the hippocampus and the somatic cortex. The amygdala is primarily involved in emotions such as fear, anger and pleasure, as well as memory [17, 18]. The amygdala may also be involved in motivation and reward associated with eating [5, 19]. The amygdala sends projections to the lateral hypothalamic area, and forms part of a circuit linked to initiation of feeding [20–22].

In both the hypothalamus and amygdala, AGD3 mRNA was detected in neurons that were positive for proopiomelanocortin (POMC) peptide. The best described anorexigenic neurons within the arcuate nucleus express POMC and cocaine-and amphetamine-regulated transcript (CART). POMC neurons in ARC play a key role in the regulation of energy homeostasis. Two cleavage products of the POMC precursor, α and β-melanocyte stimulating hormones, inhibit food intake and increase energy expenditure via melanocortin receptor subtypes 3 and 4 in the arcuate nucleus, paraventricular nucleus and lateral hypothalamus [4, 5]. Efferent projections from POMC/CART neurons to secondary energy homeostatic neurons in intra- and extra-hypothalamic sites are key features of energy homeostatic circuitry.

The AGD3 distribution in brain tissue showed substantial overlap with that of insulin receptor substrate 4 (IRS4). We have previously reported that IRS4 mRNA is expressed in the arcuate nucleus, ventromedial nucleus, dorsomedial nucleus, lateral hypothalamus and amygdala [13]. Insulin receptor substrate (IRS) proteins represent a family of adapter proteins that play central roles in signal transduction by insulin, insulin-like growth factor 2, and cytokines such as leptin [23–25]. Although the different IRS proteins vary in size, they share structural and functional characteristics. The NH_2 -terminal of each protein contains of a homologous pleckstrin homology domain and a phosphotyrosine binding region which mediate interactions with insulin receptors. The COOH-terminal portion of each protein contains tyrosine phosphorylation sites [26, 27]. Tyr-phosphorylated IRS proteins serve as signaling scaffolds that propagate hormone action through binding of Src homology 2 domain-containing proteins.

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Although IRS4 is thought to have a role in signal transduction similar to other member of the IRS family, the tissue distribution of IRS4 is substantially different than that of other insulin receptor substrates. Expression of IRS4 mRNA is restricted to the hypothalamus in the brain, suggesting that IRS4 might be involved in the regulation of energy homeostasis [28]. In a previous study, we demonstrated that IRS4 is expressed in POMC neurons of the arcuate nucleus. The expression of IRS4 in POMC neurons implies that IRS4 may function to mediate the action of insulin in these neurons.

Because IRS4 is a component of insulin signaling, the effect of AGD3 expression on insulin signaling was examined. We investigated the role of AGD3 in AKT phosphorylation, using SK-N-AS cells which were transfected with AGD3. AGD3 expression in SK-N-AS cells significantly enhanced insulin-stimulated AKT phosphorylation (Thr308).

The AMPK kinase and mTOR signaling systems are responsive to changes in organism and cellular energy status and have been studied extensively in hypothalamic control of feeding. An AGD siRNA approach was used to determine if AGD3 interacted with these pathways. In GT1-1 cells treated with AGD3 siRNA AMPK phosphorylation was suppressed while phosphorylation of P70 and S6, molecules in the mTOR signal pathway, were increased.

In summary, AGD3 functions within the hypothalamic arcuate nucleus as a component of the insulin signaling pathway. The localization of AGD3 within both POMC-positive neurons and NPY-expressing neurons, which have generally opposing actions in regulation of ingestive behavior, further suggests that AGD3 may have a central role in sensing organismal energy states. The regulation of the AMPK and mTOR pathways by AGD3 is consistent with this possibility and is an area for further investigation.

Acknowledgments

The work was supported by NIH grants 2R01DK054032 and 5R37DK043225-20.

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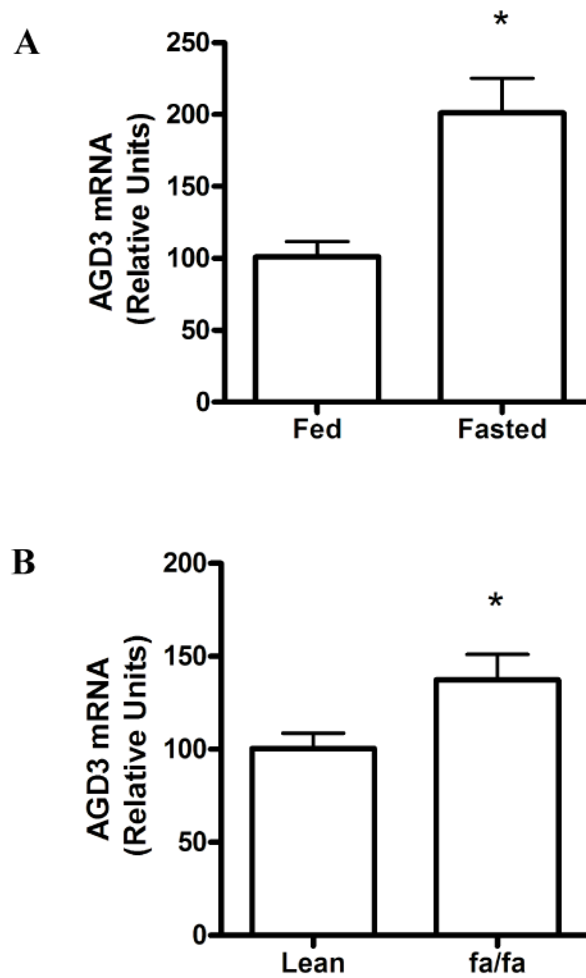

Figure 1.

Up-regulation of AGD3 mRNA in ARC neurons by fasting and obesity. A) AGD3 mRNA was significantly increased in fasted rats when compared with fed rats (n=5). B) Compared with lean Zucker rats, AGD3 mRNA was significantly increased in obese Zucker rats (fa/fa) (n=5). AGD3 mRNA levels were measured by qPCR. *$p < 0.05$.

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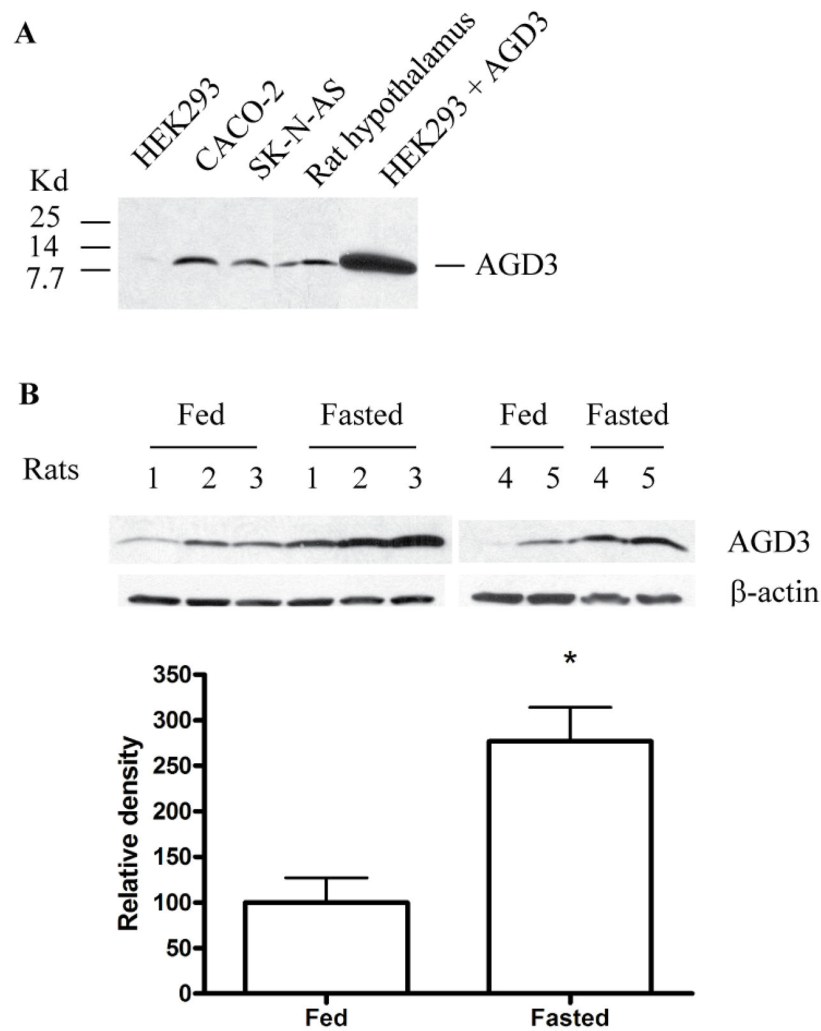

Figure 2.

A) Specificity of AGD3 antibody. Various cell lines and rat hypothalamus were lysed with lysis buffer and supernatants were detected by Western blot analysis using the anti-AGD3 antibody. This antibody detected a strong band in HEK cells transfected with AGD3-pcDNA3.1, whereas no detectable signal was found in HEK cells without transfection. AGD3 is also present in CACO-2 cells and SK-N-AS cells and rat hypothalamus. B) Up-regulation of AGD3 protein expression in ARC by fasting. AGD3 was measured by Western blot analysis by using the anti-AGD3 antibody. Each group has five animals, $*p < 0.05$.

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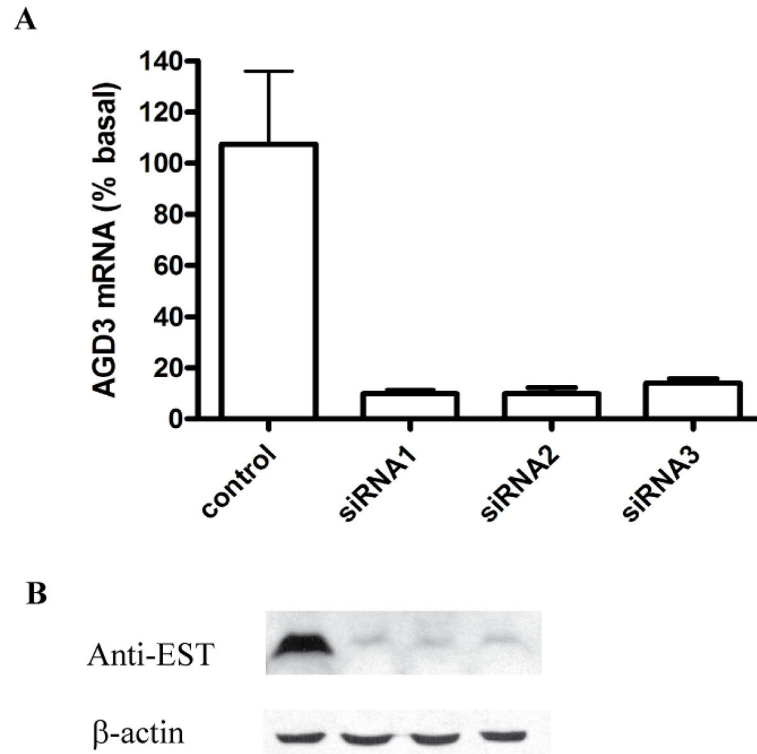

Figure 3.

Three siRNAs against different domains of AGD3 were transfected into CACO-2 cells. These siRNAs inhibited AGD3 mRNA (A), resulting in decreased AGD3 protein expression (B).

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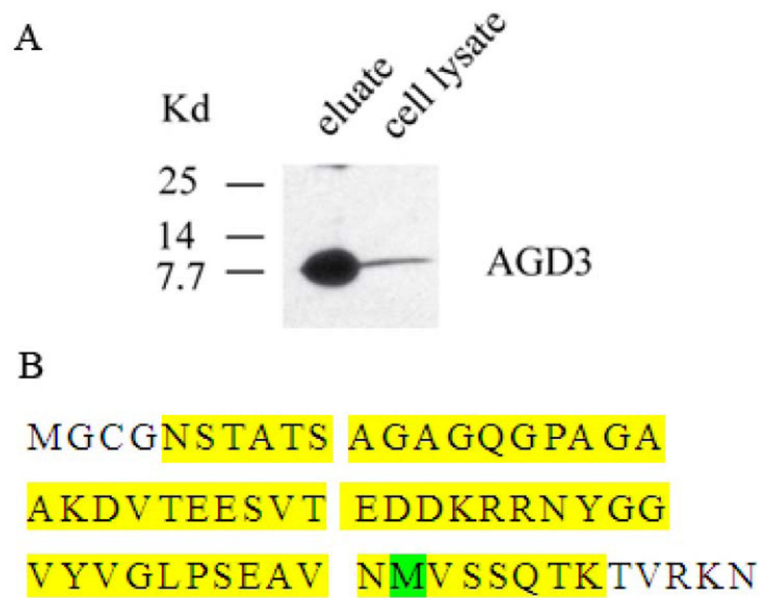

Figure 4.

Identification of human AGD3 protein sequence. A. Anti-AGD3 antibody was linked to agarose beads to pull down AGD3 protein. Eluates were separated by electrophoresis and the bands, which were confirmed by Western blot analysis, were analyzed by mass spectrometry. B. Mass spectrometry identified the presence of AGD3 protein. The sequence depicted is human hypothetical protein sequence, with highlighted portions confirmed by mass spectrometry.

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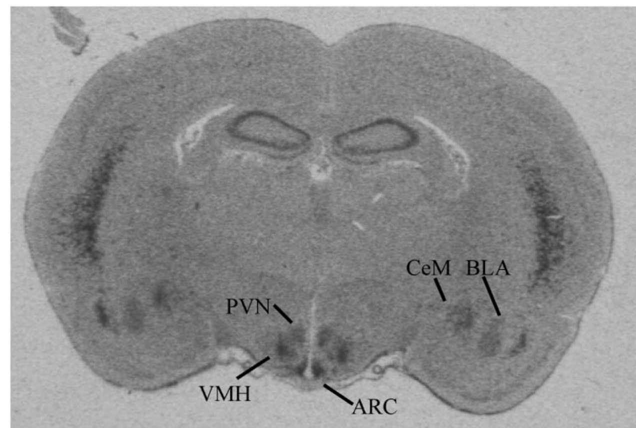
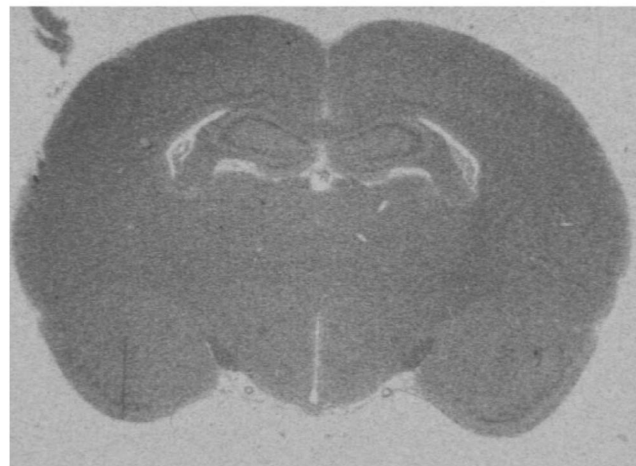


Anti-sense probe



Sense probe

Figure 5.

In situ hybridization of AGD3 in rat brain. AGD3 mRNA is distributed in arcuate nucleus (ARC), ventromedial hypothalamus (VMH), amygdaloid nuclei (CeM and BLA), paraventricular nucleus (PVN), hippocampus, and somatic cortex.

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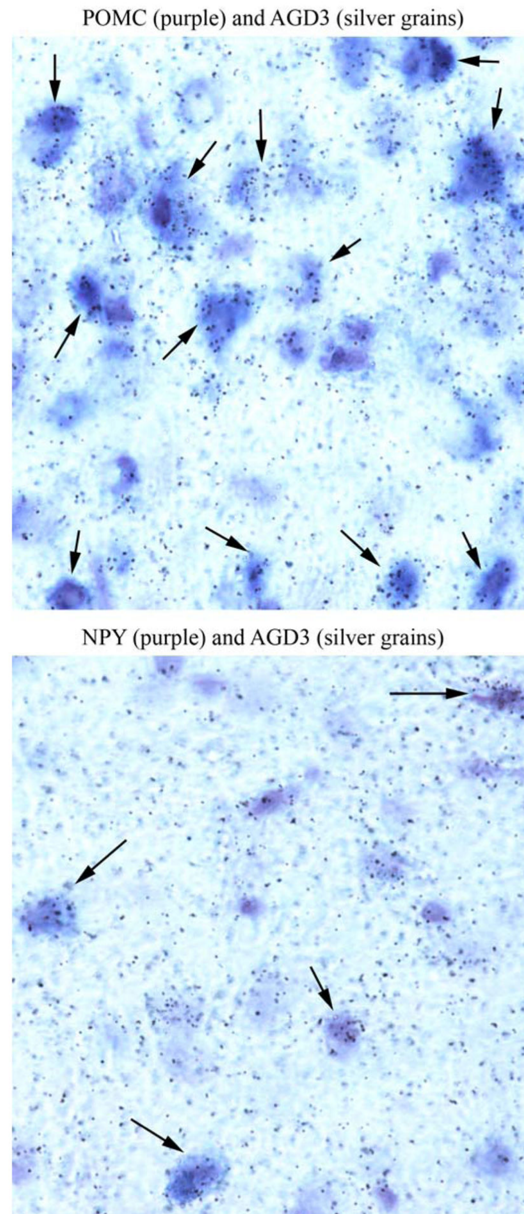

Figure 6.

Double in situ hybridization of AGD3 in rat ARC. AGD3 mRNA is co-localized with pro-opiomelanocortin (POMC) (upper panel) and neuropeptide Y (NPY) neurons (lower panel). Blue represents digoxigenin labeled POMC and NPY neurons. Silver grains indicate AGD3 mRNA.

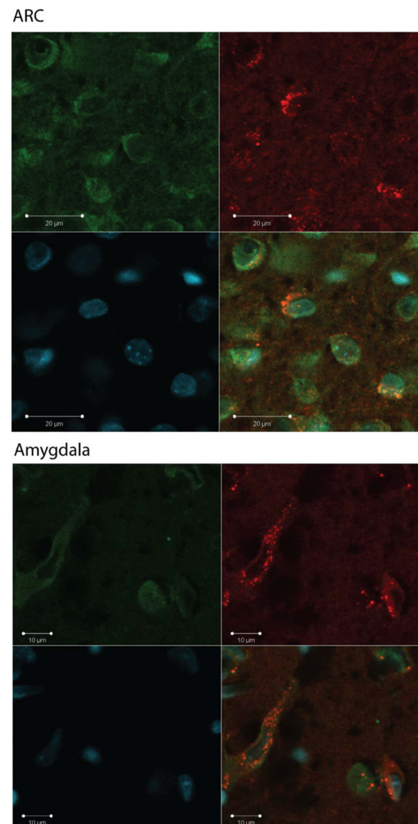

Figure 7.

Co-localization of AGD3 with POMC neurons in ARC and amygdala. Confocal photomicrographs of dual staining of AGD3 (green) and POMC (red) shows the co-localization of AGD3 with POMC. Nuclei are stained with DAPI.

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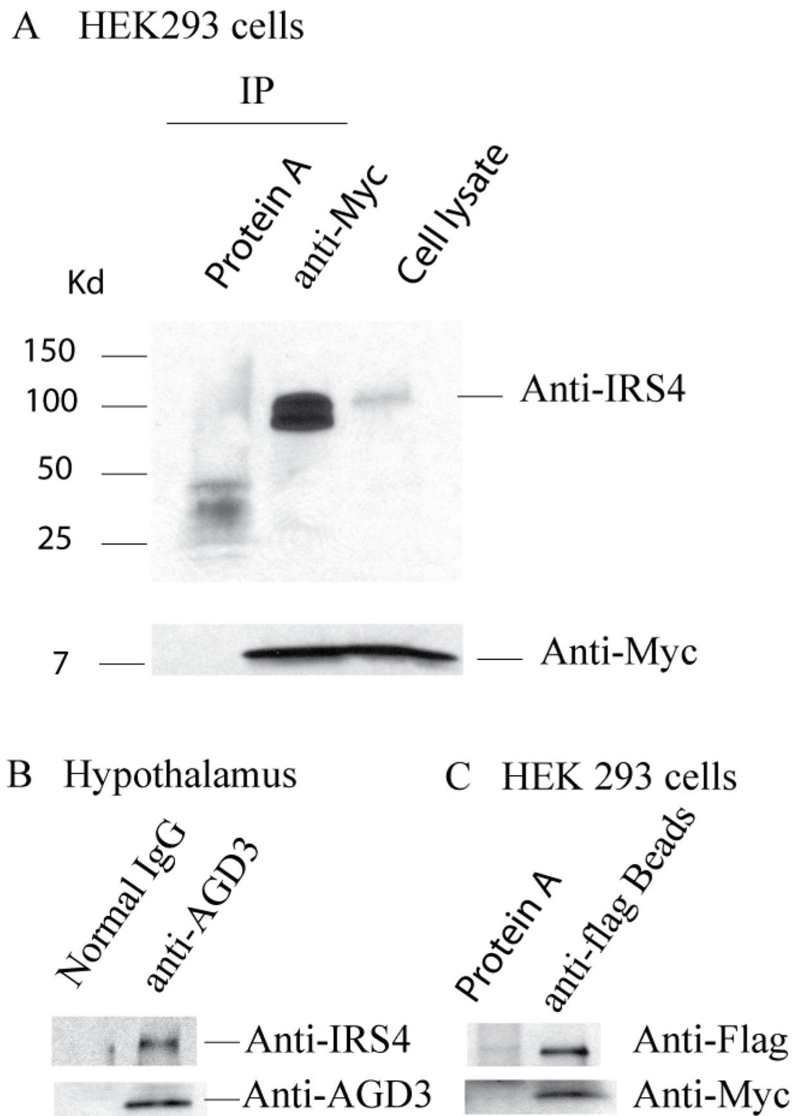

Figure 8.

Co-immunoprecipitation of AGD3 with IRS4. A) Myc-AGD3 vector was transfected into HEK293 cells, which have endogenous IRS-4 expression. Cell lysates were immunoprecipitated with anti-Myc antibody-conjugated agarose and blotted with anti IRS4 antibody. B) Normal rabbit IgG or anti-AGD3 antibody were incubated with Protein A beads and rat hypothalamus tissue lysate. IRS4 was detected by anti-IRS4. C) Flag-IRS4 and Myc-AGD3 vectors were co-transfected into HEK293 cells. Cell lysates were precipitated with anti-Flag M2 beads and blotted with anti-Myc antibody. The membranes then were stripped and re-blotted with anti-flag antibody. Protein A beads were used as negative control.

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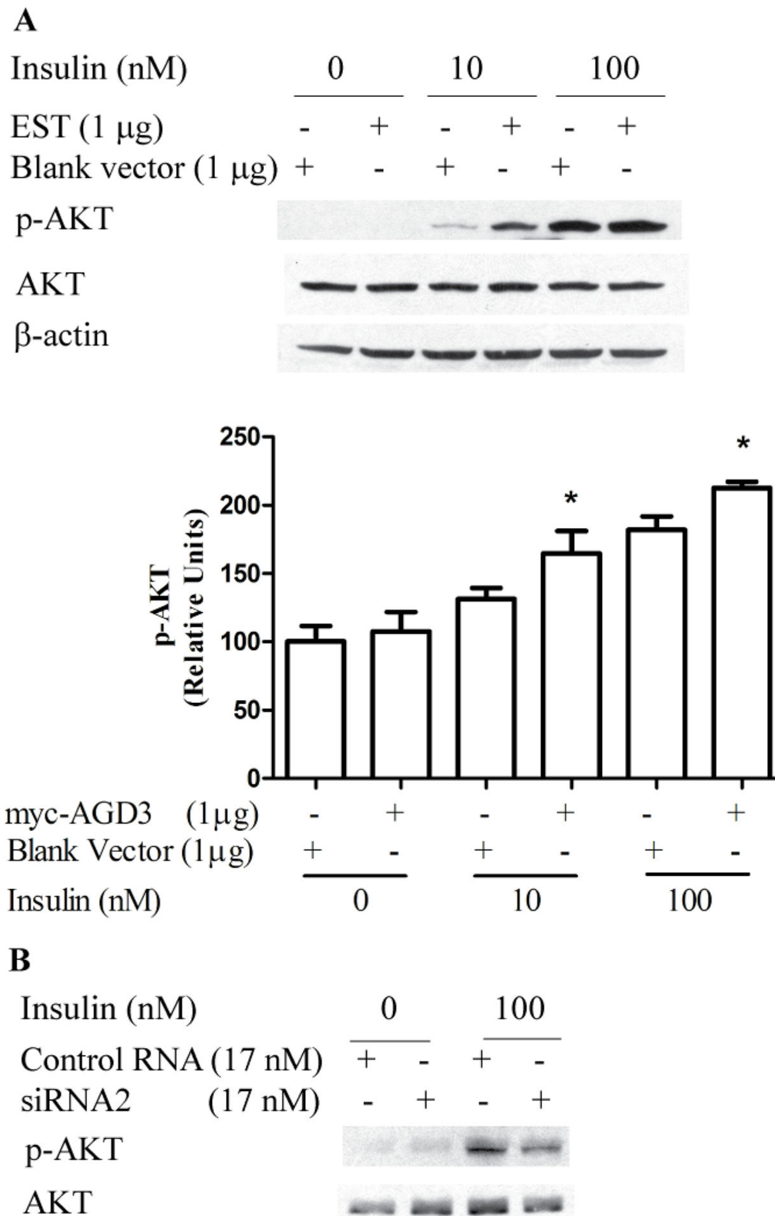

Figure 9.

Enhancement of insulin-stimulated AKT phosphorylation by AGD3. A) SK-N-AS cells were transfected with myc-AGD3 pcDNA3.1. After 48 h of transfection, 10% serum DMEM medium was replaced with serum free DMEM medium containing 0.1 % BSA and incubated for another 2 hours. Then, cells received insulin treatment for 20 minutes. B) SK-N-AS cells were transfected with AGD3 siRNA2 for 72 hours. Cells were incubated with 0.1 % BSA DMEM medium for 4 hours, then, treated with insulin for 20 minutes. Phosphorylation of AKT was detected by phospho-AKT antibody (Thr308).

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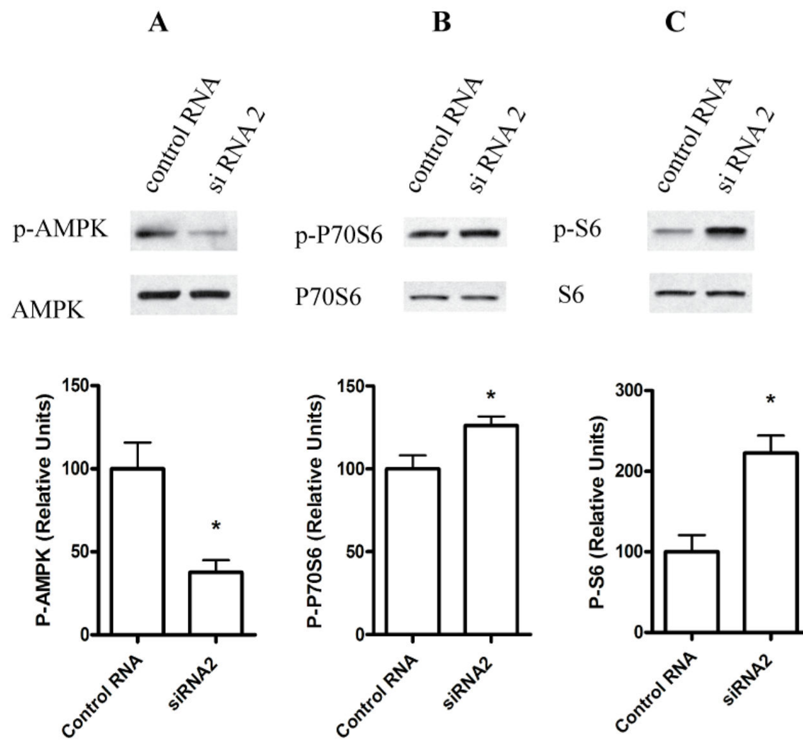

Figure 10.

The role of AGD3 in AMPK and mTOR signaling. siRNA against AGD3 mRNA were transfected in GT1-1 cells for 72 hours, then starved for four hours in serum-free DMEM medium containing 1 mM glucose. Cell lysates were lysed with lysis buffer, and supernatants were used to detect phosphorylation of AMPK (A), P70 S6 kinase (B) and S6 (C) by Western blot analysis.NC1 negative control RNA was used as control.