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Functional analysis of the Ala67Thr polymorphism in agouti related protein associated with anorexia nervosa and leanness

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Abstract

AgRP is a neuropeptide that stimulates food intake through inhibition of central melanocortin receptors (MCRs). In humans, the non-conservative amino acid substitution Alanine (Ala) 67 Threonine (Thr) has been associated with Anorexia Nervosa and with leanness. In the present study, the cellular distribution, processing and in vitro and in vivo activities of Ala67 and Thr67 AgRP were investigated. Western blots of media and lysates of BHK cells stably transfected with Ala67 or Thr67 expression constructs showed identical AgRP bands. Both Ala67 and Thr67 AgRP colocalised with the Golgi apparatus, but not with the ER or lysosomes when expressed in Att20 D16V cells. Also, no differences were observed between the potencies of bacterially expressed Ala67 and Thr67 AgRP to stimulate MC4R in a reporter gene assay or inhibit food intake in rats. Taken together, no evidence was found for a functional defect of Thr67 AgRP related to MC4R interactions.

Keywords: Agouti related protein; Melanocortin receptor; Polymorphism; Recombinant expression; Weight regulation; Anorexia

1. Introduction

AgRP is a potent orexigenic neuropeptide. In the brain, AgRP is expressed in the arcuate nucleus of the hypothalamus [1,2], which has a major role in the regulation of food intake and energy balance. Overexpression of AgRP in transgenic mice leads to increased food consumption, obesity and increased linear growth [1,3]. ICV injection of AgRP stimulates short term (h) and long term (days) food intake [4–7]. AgRP expression is suppressed by the satiety hormone leptin and is increased during fasting (for review see [8,9]).

AgRP is a 132 amino acid protein with a cysteine-rich C-terminal domain of known structure [10]. Both the full-length protein as well as several fragments containing the cysteine rich C-terminal part of the protein function as competitive antagonists at the melanocortin receptors (MCRs) 3 and 4 [1,11–15]. More recently, it has been demonstrated that the $AgRP_{83–132}$ fragment, and also the smaller $AgRP_{87–120}$, functions as an inverse agonist at the hMC3R and hMC4R, suppressing the constitutive activation of adenylate cyclase by these receptors [16–18]. It has been shown that the amino-terminal domain of AgRP interacts with syndecan 3, a cell surface heparan sulfate proteoglycan. Syndecan 3 may function as a co-receptor for AgRP. As such it may potentiate the inhibitory effects of AgRP on MCRs and may be involved in the regulation of AgRP signalling [19].

A single nucleotide polymorphism in the AgRP gene (G760A) was identified that was associated with Anorexia Nervosa [20]. This polymorphism leads to the non-conservative amino acid substitution of Alanine (Ala) 67 to Threonine (Thr). Screening for this polymorphism in healthy subjects showed that homozygosity for the Thr67 allele is associated with low body fat mass and low lean body mass [21], whereas homozygosity for the Ala67 (wild type) allele is associated with late-onset obesity [22].

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Ala67Thr is in the middle of the AgRP protein in front of the C-terminal cysteine rich part of the protein. This latter part has MCR binding activity. The non-conservative substitution of Ala67 to Thr could result in incorrect folding, leading to destabilisation and/or dysfunction of the protein. Furthermore, Ala67 is located only two amino acids from a proteolytic site at position 69 [1], and mutation of this amino acid might change proteolytic processing of the AgRP protein into smaller fragments. Algorithmic analysis of the secondary structure of the Thr67 mutant protein indeed showed that there is a probability that the conformation of the protein is changed [22]. In a first immunocytochemical investigation, however, no differences were observed in the apparent cellular localisation of Ala67 and Thr67 AgRP in stably transfected PC12 cells. Moreover, no differences were found in the mobility of the two forms of AgRP in Western blots of cellular extracts or media from cells expressing Ala67 and Thr67 AgRP [21].

In the present study, the cellular distribution, processing and in vitro and in vivo activities of Ala67 AgRP and Thr67 AgRP were investigated. Both forms of AgRP were stably expressed in BHK cells. AgRP in cell lysates and media from these cells was analysed on Western blot to detect possible differences in proteolytic processing between Ala67 and Thr67 AgRP. Furthermore, a detailed immunocytochemical analysis of the cellular localisation of Ala67 and Thr67 AgRP was performed in neuroendocrine Att20D16V cells. These cells contain a regulated secretory pathway and as such are a good model system for the synthesis, sorting and trafficking of AgRP in neurons. Misfolding of proteins may lead to accumulation in the endoplasmic reticulum (ER) or to delivery of these proteins to lysosomes for degradation (reviewed in [23] Therefore, co-localisation of both AgRP proteins with the ER, Golgi apparatus and lysosomes was analysed.

To study the activities of Ala67 and Thr67 AgRP, the two variant AgRP proteins were produced in a bacterial system. The potency of full-length proteins to inhibit activation of hMC4R by α-MSH was studied in a reporter gene assay in the absence and presence of co-transfected syndecan 3. In vivo activity of both AgRP forms was tested by measuring food intake following intracerebroventricular (ICV) injection in rats.

2. Materials and methods

2.1. Construction of Ala67 and Thr67 AgRP expression constructs

The long form of the human AgRP cDNA was amplified from a human hypothalamus cDNA library (Clontech, Palo Alto, CA) using primers AgRP3 (5′-CCTGTCCTGTGGAAATTTGTGG-3′) and AgRP4 (5′-TCGAGTTTCCTTGCCCCTACCC-3′) and a long template expand PCR system (Roche, Mannheim Germany). The amplified 0.7 kb fragment was ligated into PCR-Script Amp (Stratagene, La Jolla, CA) and verified by direct sequencing. A NotI-EcoRI restriction fragment of this construct was subsequently subcloned into the pcDNA3.1/*Myc*-His(-)A expression vector (Invitrogen, Carlsbad, CA). The Ala67Thr mutation was introduced in the expression construct using the quick change site directed mutagenesis kit (Stratagene, La Jolla, CA).

2.2. Cell culture and transfection

HEK293, BHK-fur [24] and Att20D16V cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK), supplemented with 10% heat inactivated fetal calf serum (Integro, Zaandam, The Netherlands), 2 mM glutamine, nonessential amino acids, 8.4 μg/ml penicillin and 200 μg/ml streptomycin. All cells were transfected using the calcium phosphate method. For the generation of stable clones expressing Ala67 and Thr67 AgRP, BHK-fur cells were co-transfected with either the Ala67 or the Thr67 AgRP expression construct and the pBabe Puro plasmid [25]. Stable transformants were selected at 0.5 μg/ml puromycin. For the harvest of AgRP proteins from the medium of stably transfected BHK cells, the foetal calf serum in the medium was replaced with 2% UltroserG (Invitrogen, Paisley, UK) for 2–3 days to reduce possible interference of serum proteins with subsequent analysis.

2.3. $AgRP_{83–132}$ and AgRP antibody

$AgRP_{83–132}$ was obtained from Phoenix Pharmaceuticals, Belmont, CA. The rabbit polyclonal antibody directed against AgRP [26] was kindly provided by Dr. G.S. Barsh (Stanford University, CA).

2.4. Radioimmunoassay

$AgRP_{83–132}$ was iodinated using iodo-gen iodination reagent (Pierce Rockford, IL) and [^{125}I]-Na (ICN, Aurora, OH) and subsequently HPLC purified on a C18 column (μBondapak 3.9 mm × 300 mm; Waters, Milford, MA) using a gradient of acetonitrile in 0.1% trifluoroacetic acid. Reacti-Bind 96-well polystyrene ELISA plates (Pierce, Rockford, IL) were coated (overnight at 4 °C or for 1 h at room temperature (RT)) with 100 μl of a 1:10,000 dilution of the polyclonal antibody directed against AgRP in PBS. Plates were washed with 0.05% Tween-20 in PBS and blocked with 10% heat inactivated foetal calf serum in PBS for 30 min at RT. Subsequently, in each well 50 μl of sample or standard was incubated with 50 μl of the tracer, [^{125}I]-labelled $AgRP_{83–132}$, for 1 h at RT. Samples were aspirated and wells were washed three times with 0.05% Tween-20 in PBS at RT. Bound tracer was dissolved in 100 μl 1M NaOH and counted in a γ-counter. Samples were measured in triplicate. A representative standard curve obtained using this assay is shown in Fig. 1.

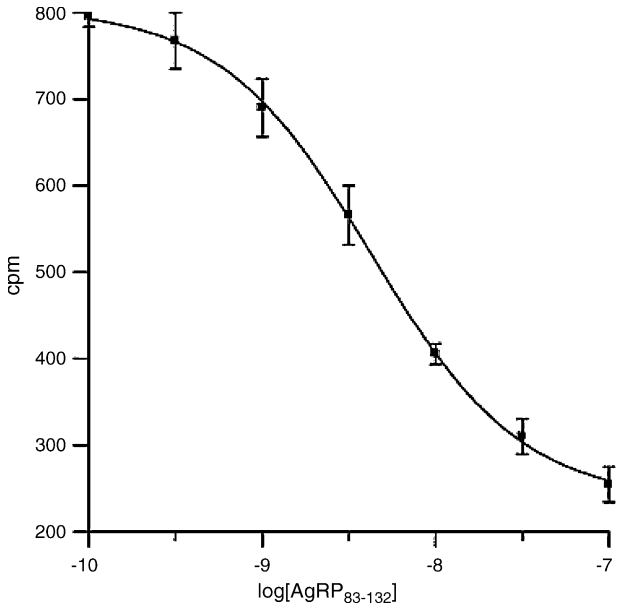

Fig. 1. Standard curve of $AgRP_{83–132}$ in radioimmunoassay. Amount of the tracer [^{125}I] $AgRP_{83–132}$ bound (cpm) in the presence of unlabeled $AgRP_{83–132}$. Data are shown as mean ± SEM.

2.5. Western blotting

Media from untransfected BHK cells and BHK cells expressing Ala67 and Thr67 AgRP were harvested, aliquotted and stored at −20 °C until further use. For the preparation of cell lysates, cells were washed once with PBS and incubated with M-PER protein extraction reagent (Pierce, Rockford, IL) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) for 5 min at RT. Lysates were cleared by short centrifugation, aliquotted and stored at −20 °C until further use.

Samples and standards (kaleidoscope polypeptide standards, Bio-Rad laboratories, Hercules, CA) were run on 12% acrylamide gels using the tris tricine buffer system [27]. Separated proteins were transferred to nitrocellulose membranes (Hybond C, Amersham biosciences, Freiburg, Germany). Blots were blocked with 10% normal goat serum (BioTrading, Mijdrecht, The Netherlands) in tris buffered saline (TBS)/0.05% Tween-20 and probed with 1:500 diluted anti-AgRP antibody. Blots were analysed using a peroxidase-conjugated secondary antibody and Super Signal West Dura Extended ECL-substrate (Pierce, Rockford, IL) on a Fluor S MultiImager (Bio-Rad, Tokyo, Japan).

2.6. Immunocytochemistry

Att20D16V cells were transiently transfected with the Ala67 or Thr67 transfection construct. After 48 h transfection cells were fixed in 4% paraformaldehyde. Following fixation double immunocytochemistry was performed as follows; cells were washed with 50 mM TBS, pH 7.6, and incubated at 4 °C for 60 h with the primary anti-bodies, polyclonal anti-AgRP (1:1000) with either monoclonal anti-ID3 (1:5, ER marker) or monoclonal anti-CTR433 (1:20, Golgi marker) in 50 mM TBS supplemented with 0.025% gelatine and 0.5% Triton TX-100. The anti-AgRP staining was completed using fluorescent DAR-FITC (1:200, Jackson ImmunoResearch, West Grove, PA). Amplification of the primary antibodies ID3 and CTR433 was performed utilising a GAM-biotinylated secondary antibody (1:400, Vector Laboratories, Burlingame, CA) which was incubated for 1 h at RT and subsequently washed to remove residual non-bound antibody. Cy3 conjugated streptavidin (1:1000, RT for 1 h, Jackson ImmunoResearch, West Grove, PA) was used to visualise the biotinylated secondary antibody. For visualisation of lysosomes transfected cells were incubated with 75 nM Lysotracker Red DND-99 (Molecular Probes, Leiden, The Netherlands) for 30 min before fixation. Cover slips were mounted onto glass slides with 1,4-diaza-bicyclo(2,2,2)octane/mowiol and viewed with a TCS NT confocal laser-scanning microscope (Leica, Heidelberg, Germany). The ID3 and CTR433 antibodies were a kind gift of Dr. P. van der Sluijs (UMCU, Utrecht, The Netherlands).

2.7. Bacterial expression, purification and folding of Ala67 and Thr67 AgRP

AgRP missing the 20 amino acid signal sequence as well as the first five amino acids of the mature protein and including Met-Lys at the N-terminus (MKd5-AgRP [14]) was created from AgRP (1–132) plasmid in the bluescript vector (kindly provided by Dr. G.S. Barsh) with a pair of synthetic oligonucleotides (5′-ACTGTAAGCTCATATGAAAGCCCCCATGGAGGGC-3′ and 5′-CGCTCTAGAACTAGTGGATCCTGTTGG-3′) encoding a BamHI and NdeI cleavage site and Met and Lys residues on the N-terminus. To include a 10× His tag that could be removed by either a factor Xa or enterokinase protease cleavage site, the MKd5-AgRP cDNA was ligated into either the Pet16B or Pet 19a cloning vectors respectively (Invitrogen, San Diego, CA). The G to A mutation in the cDNA of 10× His-MKd5-AgRP pet16b was introduced using a quick-change kit and verified by DNA sequencing. The resulting Ala67Thr mutation was verified with mass spectrometry. The expression vectors were used to transform BL21 *E. Coli*. Cells were grown to an optical density of 0.6 in LB media with Ampicillin at 37 °C and then induced with 50 mM IPTG. The cell pellet was collected by centrifugation and suspended in lysis buffer (20 mM Tris pH 7.5, 500 mM NaCl, 0.1% triton, 10% glycerol) and sonicated for 4 min. The suspension was centrifuged at 15,000 g and both the soluble and insoluble fractions were tested for the presence of AgRP using PAGE gels. 10×His-MKd5-AgRP was found exclusively in inclusion bodies. Inclusion bodies were solubilised in 6 M guanidine hydrochloride, 10 mM Tris, 1 mM DTT and 15 mM imidazole, pH 8.0. Proteins were purified using a Ni-NTA superflow column, washed with 50 mM imidazole and

eluted with 500 mM imidazole, followed by RP-HPLC on a C18 column. To fold MKd5-AgRP, 1 mg of lyophilized material was dissolved in 100 μl DMSO followed by the addition of 10 ml of folding buffer. Folding buffer contained 2.5 M guanidine hydrochloride, 100 mM Tris, 0.2 mM oxidized glutathione and 1 mM reduced glutathione, pH 8.0. RP-HPLC showed the disappearance of the peak corresponding to the fully reduced protein, followed by the gradual emergence of a single HPLC peak that eluted at an earlier retention time than the reduced material, as expected for a folded protein. Mass spectrometry of the HPLC peak corresponding to folded MKd5-AgRP showed the expected loss of 10 Da relative to the fully reduced material, due to the formation of five disulfides bridges. To ensure that the single folded species corresponded to the correctly folded protein, MKd5-AgRP was folded followed by proteolytic cleavage between residues 82 and 83 liberating $AgRP_{83–132}$. The cleavage was carried out at pH 5 to avoid any disulfide exchange. $AgRP_{83–132}$ liberated in this manner, from folded full-length protein, exhibited antagonist activity at MC4R equivalent to fully active C-terminal $AgRP_{86–132}$ (data not shown), indicating that the folded full-length protein had the correct disulfide arrangement. MKd5-67Thr-AgRP was folded under identical conditions. HPLC of MKd5-67Thr-AgRP folding also exhibited the expected disappearance of the reduced peak and appearance of a single peak shifted to earlier retention time, and mass spectrometry of reduced versus folded MKd5-67Thr-AgRP showed the expected difference of 10 Da, indicating that the mutant protein was also correctly folded. His-tag removal was carried out with recombinant enterokinase or factor Xa (Invitrogen) in a modified cleavage buffer consisting of 20 mM morpholinoethanesulfonic acid, 50 mM NaCl and 2 mM $CaCl_2$ pH 6.4. Cleaved protein was purified by HPLC and then folded as above. Proper folding was monitored by peak shift on HPLC, loss of 10 Da measured by mass spectrometry and antagonist activity at MC4R. Either the His-tag was removed or the protein was folded directly.

2.8. LacZ reporter gene assay

HEK 293 cells seeded out in 10 cm diameter culture dishes were transfected with 10 μg LacZ reporter construct, 100 ng hMC4R expression construct and 1 μg of either empty pcDNA3 vector or a rat syndecan 3 expression construct (kind gift of Dr. O. Goldberger, Harvard Medical School, Boston, MA). Assays were performed as described before [28]. α-MSH was purchased from Bachem (Bubendorf, Switzerland).

2.9. ICV injection in rats

Wistar WU rats (Harlan, Horst, The Netherlands) were implanted with ICV cannulas and injected with 1 nmol of His-tagged full-length Ala67 or Thr67 AgRP or 1 nmol $AgRP_{83–132}$. Food intake was measured 24 and 48 h after injection. The described procedures were approved by the ethical committee on use and care of animals of the University of Utrecht, The Netherlands.

2.10. Data analysis

All data are shown as mean ± standard error of the mean (SEM). RIA and reporter gene assays were analysed using GraphPad Prism 2.0.1. Food intake data were analysed using a paired *t*-test in SPSS11.5. pIC_{50} values were considered statistically different if their 95% confidence intervals did not overlap. Food intake data were considered significantly different at $p < 0.05$.

3. Results

3.1. Heterologous expression of Ala67 and Thr67 AgRP in BHK cells

Media from stably transfected BHK cells were assayed for the presence of AgRP using a radioimmunoassay. Three cell lines expressing the highest levels of AgRP were selected for use in the Western blot experiments; cell line wt1 which expresses Ala67 AgRP (4.3 nM AgRP in medium) and cell lines mut 1 and 2 which express Thr67 AgRP (11 and 2.7 nM AgRP in media respectively). Fig. 2 shows Western blots of $AgRP_{83–132}$ and cell lysates and media from untransfected BHK cells and cells stably expressing Ala67 and Thr67 AgRP. Cell lysates of transfected cells contained a single band that ran at the same height as the 17.3 kDa molecular weight marker and was absent in the control lysate. Media contained the same band, but also a faint band of lower molecular weight. This smaller fragment was larger than $AgRP_{83–132}$, which ran at the same height as the 8.2 kDa marker. No AgRP was found in the medium from untransfected cells. As protein gels were run under non-denaturing and non-reducing conditions, the size of the polypeptide standards does not give an exact estimation of the size of the proteins on the blot. This is illustrated by the apparent molecular mass of 8.2 kDa of $AgRP_{83–132}$, which has an actual molecular mass of 5.7 kDa. The main AgRP band with apparent mass around 17.3 kDa as such corresponds with the expected full-length AgRP protein (13 kDa). The smaller, faint band found in the media probably corresponds with AgRP cleaved at one of the sites (residues 46/48/50 or residues 69/71) described by Ollmann et al. [1]. No differences were found between Ala67 and Thr67 AgRP.

3.2. Immunocytochemistry

Fig. 3 shows immunofluorescence pictures of AtT20D16V cells transiently transfected with Ala67 and Thr67 AgRP expression constructs and double stained with antibodies directed against AgRP and antibodies directed

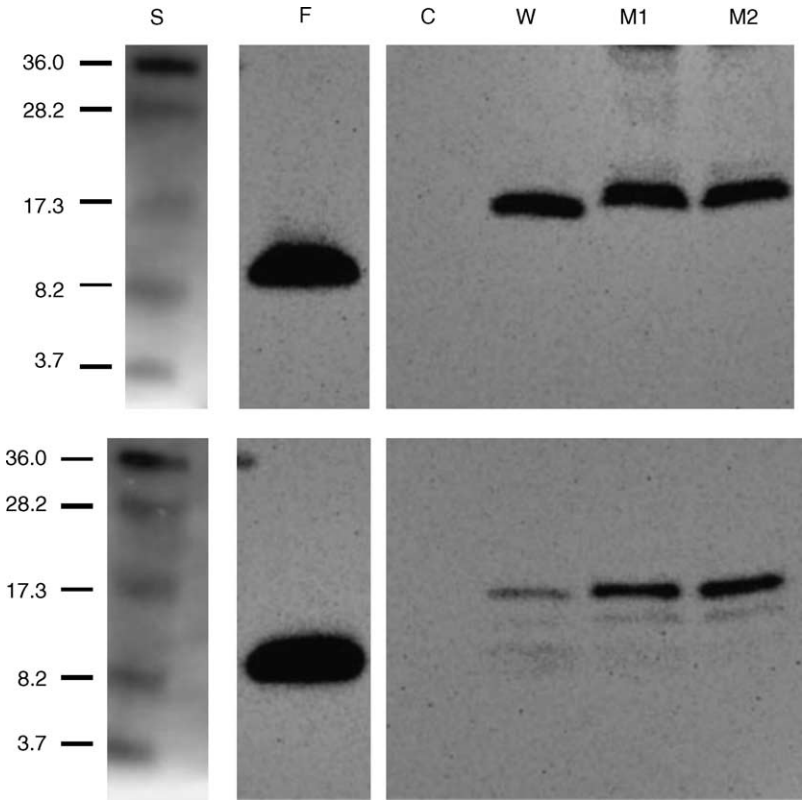

Fig. 2. Western blot of cell lysates (upper panel) and media (lower panel) of untransfected BHK cells (C) and BHK cells stably expressing Ala67 (W) and Thr67 AgRP (M1 and M2). $AgRP_{83–132}$ (F) and polypeptide standards (S) were photographed from the same blots; molecular weight is indicated in kDa.

against ER or Golgi markers or the lysosomal marker Lysotracker Red. Both forms of AgRP colocalised with the Golgi apparatus, but not with the ER or lysosomes. No differences were observed between Ala67 and Thr67 AgRP.

3.3. Inhibition of hMC4R in lacZ assays

The response of hMC4R to 50 nM α-MSH was near the maximal response both in cells transfected with hMC4R alone and in cells transfected with hMC4R and rat

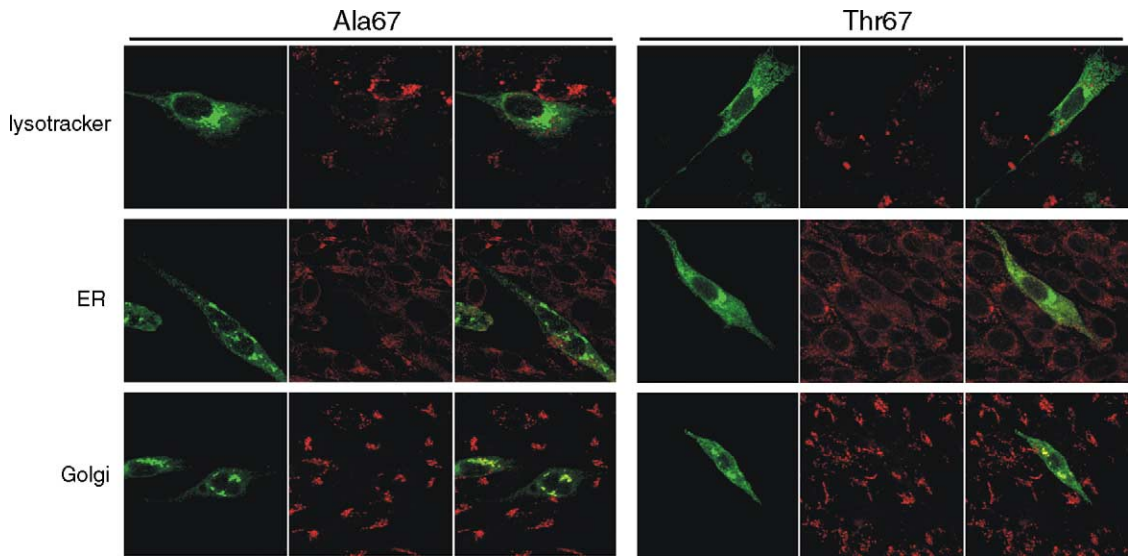

Fig. 3. Immunofluorescence pictures of AtT20D16V cells transiently tranfected with Ala67 (left block) and Thr67 (right block) AgRP expression constructs. Cells were double stained with antibodies directed against AgRP and the lysosomal marker Lysotracker Red (first row) or antibodies directed against ER (second row) or Golgi markers (third row). AgRP staining is shown in green (left columns), organel markers are stained in red (middle columns), co-localisation in overlay pictures (right columns) appears yellow. Both forms of AgRP co-localise with the Golgi apparatus.

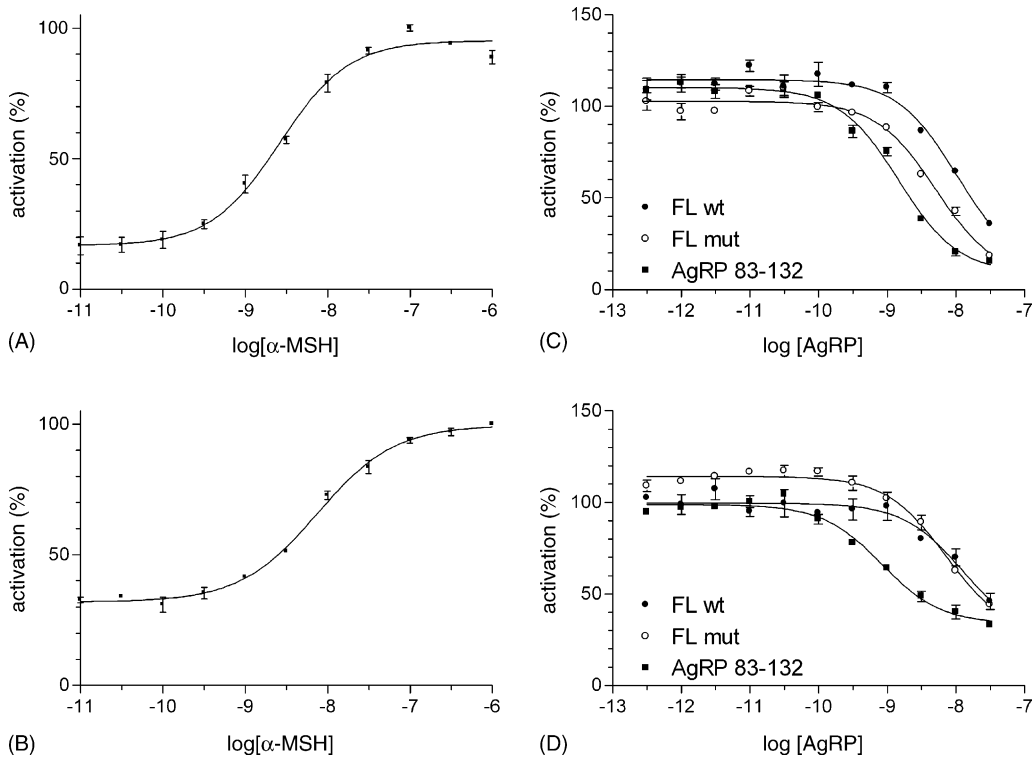

Fig. 4. Activation of hMC4R by α-MSH in HEK-293 cells in the absence (A) and presence (B) of syndecan 3, relative to maximal activation by α-MSH. Suppression of the response to 50 nM α-MSH by full-length (FL, squares) Ala67 (wt, filled circles) and Thr67 (mut, open circles) AgRP and $AgRP_{83–132}$ in the absence (C) and presence (D) of syndecan 3, relative to the activation by 50 nM α-MSH in the absence of AgRP. Representative experiment from a set of three independent experiments; data are shown as mean ± SEM of duplicate data points.

syndecan 3 (Fig. 4). Full-length bacterially expressed His-tagged Ala67 and Thr67 AgRP were equipotent in the suppression of the response of hMC4R to 50 nM α-MSH (Fig. 4 and Table 1). The $AgRP_{83–132}$ fragment was more potent than the full-length AgRP proteins. Co-expression of rat syndecan 3 did not potentiate the effects of either full-length AgRP protein. Repeating the experiment with Ala67 and Thr67 AgRP from which the His-tag had been removed, yielded similar results (data not shown). Antagonism of hMC4R by Ala67 an Thr67 AgRP was surmountable and identical for both forms of the protein as is shown by the parallel rightward shifts of the α-MSH dose response curve in the presence of 10 and 100 nM Ala67 and Thr67 AgRP (Fig. 5).

Table 1
pEC_{50} values of activation of hMC4R by α-MSH in HEK293 cells in the absence and presence of syndecan 3 and pIC_{50} values of suppression of the response to 50 nM α-MSH by full-length Ala67 and Thr67 AgRP and $AgRP_{83–132}$ in the absence and presence of syndecan 3

	pEC_{50}	pIC_{50}
No syndecan		
α-MSH	8.58 ± 0.06	
Ala67 AgRP		7.99 ± 0.12
Thr67 AgRP		8.29 ± 0.09
$AgRP_{83–132}$		8.83 ± 0.06[a]
Syndecan		
α-MSH	8.12 ± 0.04	
Ala67 AgRP		7.91 ± 0.21
Thr67 AgRP		8.15 ± 0.08
$AgRP_{83–132}$		9.08 ± 0.07[a]

Representative experiment from a set of three independent experiments (data are listed as mean ± S.E).
[a] Significantly different from full-length AgRPs.

3.4. ICV injection of Ala67 and Thr67 AgRP in rats

As is shown in Fig. 6, food intake was significantly increased during the first day (24 h) following ICV injection of Ala67 (140 ± 8% relative to basal, mean ± SEM) or Thr67 AgRP (139 ± 7% relative to basal, mean ± SEM). The $AgRP_{83–132}$ fragment significantly increased daily food intake at first and second day after injection. For Ala67 and Thr67 AgRP on day 2 after injection, food intake still tended to be increased relative to basal conditions (121 ± 9 and 121 ± 12% respectively). However, this increase did not reach significance. No difference was observed between the effects of Ala67 and Thr67 AgRP on food intake.

4. Discussion

No differences in proteolytic processing were found between Ala67 and Thr67 AgRP when heterologously expressed in BHK cells. Moreover, immunocytochemistry showed that both forms of AgRP were distributed in AtT20D16V cells in a similar fashion. Neither form of

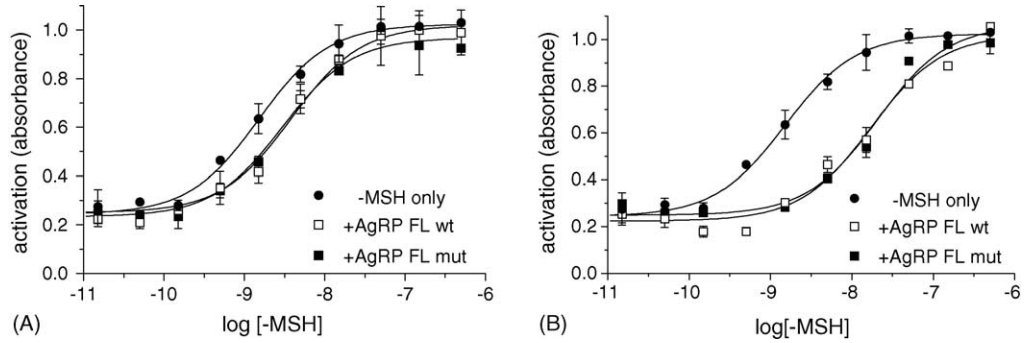

Fig. 5. Parallel rightward shift of the α-MSH dose response curve by His-tagged Ala67 and Thr67 AgRP. (A) dose response curves of α-MSH (filled circles; pEC_{50} 8.82 ± 0.07), α-MSH in presence of 10 nM Ala67 AgRP (wt; open squares; pEC_{50} 8.42 ± 0.11) and α-MSH in presence of 10 nM Thr67 AgRP (wt; closed squares; pEC_{50} 8.53 ± 0.09). (B) dose response curves of α-MSH (filled circles; pEC_{50} 8.82 ± 0.07), α-MSH in presence of 100 nM Ala67 AgRP (wt; open squares; pEC_{50} 7.774 ± 0.08) and α-MSH in presence of 100 nM Thr67 AgRP (wt; closed squares; pEC_{50} 7.69 ± 0.09). pEC_{50} values are shown as mean ± standard error. Datapoints are shown as mean ± SEM of duplicates.

AgRP was found in the ER or in lysosomes, which would be indicative of the formation of a misfolded protein [23]. Both Ala67 and Thr67 AgRP accumulated in the Golgi apparatus, from which the proteins were probably directed into the regulated secretory pathway. These data are in accordance with the findings of Marks et al. [21] and indicate that the mutant AgRP protein, Thr67, is processed and secreted normally. Subsequently, effects of the Ala67Thr substitution were determined at a functional level.

In reporter gene assays, Ala67 and Thr67 AgRP were equally potent in inhibiting the response of hMC4R to α-MSH. Moreover, antagonism by Ala67 and Thr67 AgRP was surmountable. $AgRP_{83–132}$ was a more potent antagonist than the full-length AgRP proteins. This is consistent with the report of Ollmann et al. [1] in which the shorter form C of recombinant AgRP was more potent in a melanophore assay than the longer forms A + B, but not with the report of Rosenfeld et al. [14] in which recombinant $AgRP_{86–132}$ and MKd5-AgRP were equally potent at inhibiting hMC4R in a reporter gene assay in CHO cells. Thus, the effects of AgRP are dependent on the type of assay and cells used. It is possible that there are differences in the activities of Ala67 and Thr67 AgRP at hMC4R, but that the assay that was used did not have sufficient sensitivity to detect such differences. Syndecan 3 has been shown to bind to the amino-terminal part of the full-length AgRP protein and to potentiate the antagonistic effect of AgRP on hMC4R [19]. As the Ala67Thr mutation is in the amino-terminal part in AgRP, it might disturb binding to syndecan 3, resulting in less activity at hMC4R. However, in this study, cotransfection of rat syndecan 3 did not potentiate the inhibitory activity of full-length AgRP relative to $AgRP_{83–132}$. As such, the proposed function of syndecan 3 acting as a co-receptor for AgRP by binding to the amino-terminal part of the protein could not be demonstrated in this system. Also, cotransfection of syndecan 3 did not lead to differences between the effects of Ala67 and Thr67 AgRP. Cells transfected with syndecan 3 expression construct had a changed morphology as compared to empty vector transfected cells as has been described before [29], indicating that the protein is functionally expressed. However, we cannot exclude that expression levels at the cell surface were insufficient for syndecan 3 to function as co-receptor.

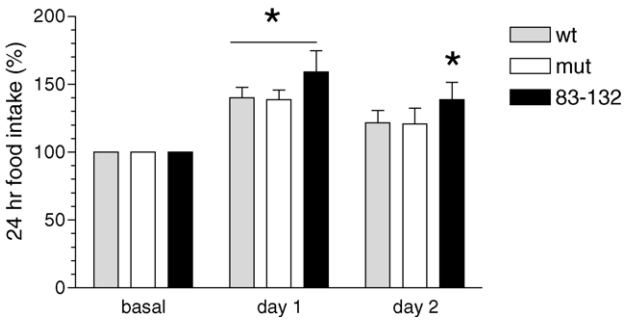

Fig. 6. Daily food intake of rats prior to and on two days following ICV injection of 1 nmol His-tagged Ala67 (wt; gray bar, $n = 3$) or Thr67 (mut; white bar, $n = 4$) full-length AgRP or $AgRP_{83–132}$ (83–132, black bar, $n = 8$) (mean ± SEM; (*) significantly different from basal food intake; paired t-test; $p < 0.05$).

Food intake of rats was significantly increased in the 24 h period following ICV injection of Ala67 and Thr67 AgRP as well as of $AgRP_{83–132}$. Ala67 and Thr67 AgRP were equally effective in stimulating food intake. In the period 24–48 h after injection, food intake still was significantly increased for the $AgRP_{83–132}$ fragment and tended to be increased for the full-length forms of AgRP, but this latter effect was not significant, probably due to the small group sizes. Since in the in vitro assays the $AgRP_{83–132}$ fragment was more potent in inhibiting MC4R than the full-length AgRP proteins, the longer term effects of the fragment could be caused by its higher potency relative to Ala67 and Thr67 AgRP.

In conclusion, in these data, the Ala67Thr mutation did not lead to disfunction of AgRP in inhibition of MC4R or stimulation of food intake for 2 days after ICV injection. However, the Ala67Thr mutation may alter AgRP function in alternative ways.

Firstly, the Ala67Thr mutation may influence other effects of AgRP than direct stimulation of food intake. Chronic administration of AgRP results in increased fat mass and reduced energy expenditure, independent of increased food intake [30]. Furthermore, AgRP affects the selection of food and will favour the intake of food with a high fat content or high palatability [31,32]. Disruption of these processes could result in decreased intake of food of high caloric content and in reduced fat storage, which would account for a lean phenotype or symptoms of AN.

Secondly, it cannot be excluded that AgRP also acts through another mechanism than inhibition of MC4R, and that this signalling is impaired in Thr67 AgRP. The observation that MC4R deficient mice increase food intake after injection with $AgRP_{83–132}$ [33] supports the possible existence of an alternative AgRP signalling pathway.

Thirdly, the Ala67Thr mutation may disrupt the function of biologically active amino-terminal fragments of AgRP. There are a few indications for the existence of biologically active amino-terminal fragments of AgRP. Chromatographic analysis comparing AgRP from hypothalamus and serum with recombinant forms of AgRP suggest that the protein is processed in vivo [34]. This processing could result in the formation of peptides comprising the amino-terminal part of AgRP. Recently, biological activity of synthetic $AgRP_{25–51}$ and $AgRP_{54–82}$ has been reported. ICV injection of these synthetic peptides resulted in decreased energy expenditure and increased fat mass in rats [35]. The mechanism of action of amino-terminal AgRP fragments may involve binding to syndecan 3, providing an alternative explanation for the involvement of syndecan 3 in the regulation of energy balance.

Finally, the G760A polymorphism leading to the Ala67Thr mutation could be in linkage disequilibrium with a polymorphism in the 5′ upstream region of the AgRP gene. In this case the Ala67Thr mutation in itself would have no effect on AgRP function, but AgRP expression could for instance be reduced due to a promoter defect.

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