Abstract: Summary The present invention relates to G protein coupled receptors of the MAS related G protein coupled receptor family. The present invention furthermore relates to modulation of the activity of these receptors as a tool in treating 5 disorders such as obesity, and/or diabetes, and/or cardiovascular diseases.
DRUG TARGETS

Technical field of the invention

The present invention relates to G protein coupled receptors of the MAS related G protein coupled receptor family. In particular, the present invention relates to modulation of the activity of these receptors.

Background of the invention

G-Protein Coupled Receptors

MrgE is a seven transmembrane G protein coupled receptor (GPCR) of the MAS related G-protein coupled receptor family (Anderson et al. 2001; Dong et al. 2001; Golz et al. 2004; Wei et al. 2002). Many medically significant biological processes are mediated by signal transduction pathways that involve G-proteins (Lefkowitz 1991). The family of G-protein coupled receptors (GPCRs) includes receptors for hormones, neurotransmitters, growth factors, and viruses. Specific examples of GPCRs include receptors for such diverse agents as dopamine, calcitonin, adrenergic hormones, endothelins, cAMP, adenosine, acetylcholine, serotonin, histamine, thrombin, quinine, follicle stimulating hormone, opsins, endothelial differentiation gene-1, rhodopsins, odorants, cytomegalovirus, G-proteins themselves, effector proteins such as phospholipase C, adenyl cyclase, and phosphodiesterase, and actuator proteins such as protein kinase A and protein kinase C.

GPCRs possess seven conserved membrane-spanning domains connecting at least eight divergent hydrophilic loops. GPCRs, also known as seven transmembrane, 7TM, receptors, have been characterized as including these seven conserved hydrophobic stretches of about 20 to 30 amino acids, connecting at least eight divergent hydrophilic loops. Most GPCRs have
single conserved cysteine residues in each of the first two extracellular loops, which form disulfide bonds that are believed to stabilize functional protein structure. The seven transmembrane regions are designated as TM1, TM2, TM3, TM4, TM5, TM6, and TM7. TM3 is being implicated with signal transduction. Phosphorylation and lipidation (palmitylation or farnesylation) of cysteine residues can influence signal transduction of some GPCRs.

Most GPCRs contain potential phosphorylation sites within the third cytoplasmic loop and/or the carboxy terminus. For several GPCRs, such as the beta-adrenergic receptor, phosphorylation by protein kinase A and/or specific receptor kinases mediates receptor desensitization.

For some receptors, the ligand binding sites of GPCRs are believed to comprise hydrophilic sockets formed by several GPCR transmembrane domains. The hydrophilic sockets are surrounded by hydrophobic residues of the GPCRs. The hydrophilic side of each GPCR transmembrane helix is postulated to face inward and form a polar ligand binding site. TM3 is being implicated with several GPCRs as having a ligand binding site, such as the TM3 aspartat residue. TM5 serines, a TM6 asparagine, and TM6 or TM7 phenylalanines or tyrosines also are implicated in ligand binding.

GPCRs are coupled inside the cell by heterotrimeric G-proteins to various intracellular enzymes, ion channels, and transporters. Different G-protein alpha-subunits preferentially stimulate particular effectors to modulate various biological functions in a cell. Phosphorylation of cytoplasmic residues of GPCRs is an important mechanism for the regulation of some GPCRs. For example, in one form of signal transduction, the effect of hormone binding is the activation of the enzyme, adenylate cyclase, inside the cell. Enzyme activation by hormones is dependent on the presence of the nucleotide GTP. GTP also influences hormone binding. A G-protein connects the hormone receptor to adenylate cyclase. G-protein exchanges GTP for bound GDP
when activated by a hormone receptor. The GTP-carrying form then binds to
activated adenylate cyclase. Hydrolysis of GTP to GDP, catalyzed by the G-
protein itself, returns the G-protein to its basal, inactive form. Thus, the G-
protein serves a dual role, as an intermediate that relays the signal from
receptor to effector, and as a clock that controls the duration of the signal.

Over the past 15 years, nearly 350 therapeutic agents targeting 7TM
receptors have been successfully introduced into the market. This indicates
that these receptors have an established, proven history as therapeutic
targets. Clearly, there is a need for identification and characterization of
further receptors which can play a role in preventing, ameliorating, or
correcting metabolic dysfunctions or diseases including, but not limited to,
adiposity, insulin resistance, dyslipidemia, and arterial hypertension.

Combinations of these symptoms and diseases constitute what is defined as
the dysmetabolic syndrome by National Cholesterol Education Program
(NCEP)'s Adult Treatment Panel III (ATP-III). Patients suffering dysmetabolic
syndrome as defined by ATP-III criteria have significantly elevated risk of
cardiovascular disease (myocardial infarction, ischemic heart disease,
atherosclerosis, and stroke) and diabetes mellitus. Medical complications
accompanying monosymptomatic obesity such as osteoarthritis, cancers,
liver and gall bladder diseases, and certain sleep disorders are also in need
of substantially improved therapeutic means.

As it appears to be extremely difficult to change life style and loose weight
there is a massive need for an effective "obesity drug". There does not exist
any effective obesity drugs on the market having little or no side effects.
Much development has been focused on identifying targets for developing
drugs acting on the nerve system.
Mrg

A number of GCPR receptor members of the MAS related G-protein coupled receptor family are denoted Mrg. Members of this family include MrgA, B, C, D, E, and X.

Members of the MrgA family were originally cloned based on their specific expression in subsets of sensory neurons in dorsal root ganglia (Dong et al. 2001). The MrgD family also seems to be almost exclusively expressed in dorsal root ganglia (Dong et al. 2001). It has been suggested that the MrgA1 and A4 receptors bind neuropeptides, perhaps of the RFamide family (Dong et al. 2001). The fact that mrgA1 and A4, MAS1, and MrgC11 can be activated by the RFamide neuropeptides NPFF and NPAF, respectively, which have long-lasting analgesic effects when injected intraspinally as well as the specificity of expression of the Mrg families ((Dong et al. 2001; Han et al. 2002) has led to suggestions that ligands for most of these receptors include neuropeptides that modulate pain sensitivity. In addition, the MrgX2 receptor which has no identified rat or mouse homologues has been shown to bind cortistatin (Robas et al. 2003), a peptide previously shown to be involved in sleep regulation, locomotor activity, and cortical function, but which may also play a role in nociception.

Members of the Mrg gene family, named sensory neuron-specific GPCRs (SNSR) have also been cloned by Lembo and coauthors (Lembo et al. 2002) who found the same tissue specificity of expression and, in addition, found that the non-opioid part of the proenkephalin A gene product BAM22 was a high-affinity ligand for these receptors, substantiating a role of the Mrg/SNS receptors in pain modulation.

WO 0148015 discloses transcripts from G protein-coupled receptors (GPCRs). It is disclosed that MrgX2 transcripts are expressed in human liver
and adipose tissue and it is suggested that this protein is linked to metabolic disorders, inflammatory diseases and cancers. MrgX2 exhibits only sparse similarity (<35%) with MrgE. This document furthermore discloses MrgE transcripts (transcript 1002). This transcript is expressed in the brain, heart, kidney, liver, muscle, ovary, prostate, small intensive, spleen, testis, peripheral blood leukocytes and lung. These observations confirm previous assumptions about the linkage of MrgE with mental disorders.

MrgE

The nucleotide sequence of MrgE is accessible in public databases by the accession number NM_001002288 and is given in SEQ ID NO 1. The predicted amino acid sequence of MrgE is depicted in SEQ ID NO 2. Human MrgE (accession number XM-171536) is given in SEQ ID NO 3 (nucleotide sequence) and SEQ ID NO 4 (amino acid sequence), respectively. MrgE is expressed in humans and in mice (Dong et al. 2001; Vassilatis et al. 2003). The MrgE receptor (Anderson et al. 2001; Dong et al. 2001; Lind et al. 2001; Miwa et al. 2002; Vassilatis et al. 2003; Vogeli et al. 2001a; Vogeli et al. 2001b; Wang et al. 2001; Wei et al. 2002) and has a high degree of homology to other receptors of the Mrg-Receptor family. MrgE is expressed in the brain (in particular in the hypothalamic sub areas denoted "PVN" and "DMH"), placenta and thyroid (Wang et al. 2001) as well as in blood cells and testis (Wei et al. 2002). It has furthermore been suggested that MrgE is implicated in pain signalling (Anderson et al. 2001) and in development of mental disorders (Lind et al. 2001; Vogeli et al. 2001a; Vogeli et al. 2001b).

There is a sequence identity between human and rat MrgE of about 73.3%. The ligand-binding part of the molecule is thought to be most highly conserved.
MrgE molecules are rather distantly related to other Mrg molecules. Mrg molecules most closely related to MrgE are as follows: sequence identity of about 40.7% between human MrgE and MrgX3 (also denoted SNSR1); sequence identity of about 40.2% between human MrgE and MrgX2; sequence identity of about 38.4% between human MrgE and MrgX1; sequence identity of about 37.6% between human MrgE and MrgX4; sequence identity of about 36.8% between human MrgE and SNSR2; sequence identity of about 38.4% between human MrgE and SNSR3; and sequence identity of about 37.1% between human MrgE and MrgG.

Diabetes

Diabetes is characterized by a decreased ability to regulate blood glucose concentrations. Diabetes can be grouped in two major classes: diabetes type I, wherein the insulin-producing cells are partly or fully destroyed by an autoimmune reaction and diabetes type II, wherein the insulin-producing cells are intact or essentially intact and wherein the decreased ability to regulate blood glucose is thought to be caused by decreased insulin-sensitivity. Diabetes type II is often accompanying obesity. In the present invention, the term diabetes is thus equivalent to diabetes type II. A number of pathological conditions furthermore accompany obesity and/or diabetes such as hypertension, and cardiovascular diseases.

It thus follows that there is a long felt need in the art for novel obesity drugs and drug targets. There is also a need in the art for identifying drugs and drug targets that may affect the fat tissue directly rather than affecting nerve tissues. Such drugs and drug targets will furthermore be useful in connection with diabetes treatment as well as treatment of conditions that accompany obesity and/or diabetes.
Summary of the invention

The present invention lies in the unexpected findings that:

- MrgE transcripts are relatively abundant in preadipocytes.
- MrgE expression is down-regulated during differentiation of preadipocytes to adipocytes.
- MrgE is expressed in fat tissue.
- MrgE expression is up-regulated as a response to starving and down-regulated as a response to over-feeding/obesity.

These findings together form basis for therapeutical approaches in connection with obesity and/or diabetes for modulating MrgE activity as well as using MrgE as a tool for identifying obesity/diabetes drugs.

In a first aspect, the present invention thus relates to a composition for use as a medicament, said composition comprising at least one compound selected from the group consisting of:

- a polynucleotide encoding an MrgE polypeptide, said polynucleotide comprising the sequence of SEQ ID NO 1, or SEQ ID NO 3, a fragment, or variant thereof.
- a polynucleotide encoding an MrgE polypeptide, wherein said polypeptide comprises the amino acid sequence according to SEQ ID NO 2, or SEQ ID NO 4, or a fragment, or variant thereof,
- an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
- an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
- a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
• a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof.

5 In a second aspect, the present invention relates to use of one of the compounds according to the invention for manufacturing a pharmaceutical for modulating expression of MrgE in fat tissue for treating, alleviating, or preventing obesity, and/or diabetes, and/or cardiovascular diseases.

10 In a third aspect, the present invention relates to a method of screening an MrgE polypeptide for an interaction partner said method comprising the following steps:

(i) contacting an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof with a compound or an array of test compounds; and

(ii) detecting binding between MrgE and a compound capable of binding MrgE.

In a fourth aspect, the present invention relates to a method of screening for therapeutic agents useful in the treatment of obesity and/or diabetes and/or cardiovascular diseases, said method comprising the following steps:

(i) determining the activity of an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment or variant thereof, in the presence and optionally also in the absence of a test compound,

(ii) determining the activity of an MrgE polypeptide in the presence and optionally also in the absence of a compound known to be a regulator of a MrgE polypeptide.

(iii) identifying compounds capable of modulating MrgE activity.
In a sixth aspect, the present invention relates to a method of treating obesity and/or diabetes, wherein at least one compound selected from:

- a polynucleotide encoding an MrgE polypeptide, said polynucleotide comprising the sequence of SEQ ID NO 1, a fragment, or variant thereof.
- a polynucleotide encoding an MrgE polypeptide, wherein said polypeptide comprises the amino acid sequence according to SEQ ID NO 2, or SEQ ID NO 4, or a fragment, or variant thereof,
- an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
- an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
- a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and
- a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

is administered to the patient in an amount sufficient to modulate MrgE activity in fat tissue.

**Brief Description of the Drawings**

**Fig. 1** shows a multiplex quantitative RT-PCR experiment. RNA was isolated from various tissues and RT-PCR was performed with primers specific for Tata box binding protein (TBP) and MrgE. The signals were quantified and the MrgE signal was normalized to the TBP signal.

**Fig. 2** shows a multiplex quantitative RT-PCR experiment. RNA was isolated from 3T3 L1 cells at different days during the differentiation to adipocytes and
RT-PCR was performed with two sets of primers specific for Tata box binding protein (TBP) and MrgE or for TBP and PPARγ. The signals were quantitated and the MrgE and PPARγ signals, respectively were normalized to the TBP signals.

Fig. 3 shows the physiological regulation of an MrgE receptor polynucleotide in mesenteric adipose tissue for rats exposed to different feeding regimes. One group of rats was given chow ad libitum (ad lib chow), one group of rats was given high energy chow mixed with chocolate spread (Nutella + HE), one group was given chow ad libitum and food was withdrawn 48 hours before the termination of the experiment (chow + 48 hr fast), and one group was kept at 70% of the amount of food consumed by the ad lib chow group (70% chow). A. Bodyweight curve. B. Multiplex quantitative RT-PCR experiment. Mesenteric white adipose tissue was isolated from the rats and the expression of MrgE and TBP was determined by Multiplex quantitative RT-PCR. Error bars show Standard Error of the Mean (SEM).


Ligand binding domains are expected to be placed in the extracellular domains.

Fig. 5 shows a multiplex quantitative RT-PCR experiment. RNA from human white adipose tissue was used for RT-PCR with primers specific for TATA box binding protein (TBP) and MrgE. The signals were quantified and the MrgE signals were normalized to the TBP signals.
**Detailed description of the invention**

**Definitions**

5 An "oligonucleotide" is a strand of nucleotide residues which has a sufficient number of bases to be used as an oligomer, amplimer or probe in a polymerase chain reaction (PCR). Oligonucleotides are prepared from genomic or cDNA sequence and are used to amplify, reveal, or confirm the presence of a similar DNA or RNA in a particular cell or tissue.

10 Oligonucleotides or oligomers comprise portions of a DNA sequence having at least about 10 nucleotides and as many as about 35 nucleotides, preferably about 25 nucleotides.

"Reporter molecules" are radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents which associate with a particular nucleotide or amino acid sequence, thereby establishing the presence of a certain sequence, or allowing for the quantification of a certain sequence.

20 "Chimeric" molecules may be constructed by introducing all or part of the nucleotide sequence of this invention into a vector containing additional nucleic acid sequence which might be expected to change any one or several of the following MrgE characteristics: cellular location, distribution, ligand-binding affinities, interchain affinities, degradation/turnover rate, signaling, etc.

25 "Active", with respect to a MrgE polypeptide, refers to those forms, fragments, or domains of a MrgE polypeptide which retain the biological and/or antigenic activity of a MrgE polypeptide.

30 "Naturally occurring MrgE polypeptide" refers to a polypeptide produced by cells which have not been genetically engineered and specifically
contemplates various polypeptides arising from post-translational modifications of the polypeptide including but not limited to acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

"Conservative amino acid substitutions" result from replacing one amino acid with another having similar structural and/or chemical properties, such as the replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

"Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by producing the peptide synthetically while systematically making insertions, deletions, or substitutions of nucleotides in the sequence using recombinant DNA techniques.

"Inhibitor" is any substance which retards or prevents a chemical or physiological reaction or response. Common inhibitors include but are not limited to antisense molecules, antibodies, and antagonists.

"Standard expression" is a quantitative or qualitative measurement for comparison.

It is based on a statistically appropriate number of normal samples and is created to use as a basis of comparison when performing diagnostic assays, running clinical trials, or following patient treatment profiles.

"Animal" as used herein may be defined to include human, domestic (e.g., cats, dogs, etc.), agricultural (e.g., cows, horses, sheep, etc.) or test species (e.g., mouse, rat, rabbit, etc.).
A nucleotide sequence encoding an MrgE polypeptide having a sequence according to SEQ ID NO 2; accession number NM_001002288 or SEQ ID NO 4; accession number XM-171536; SEQ ID NOs 1 and 3 are examples of nucleotide sequences encoding MrgE. The skilled person knows that the nucleotide sequence of SEQ ID NO 1 or 3 can be varied to a large extent, due to the alternative codon usage, while still encoding a polypeptide having an amino acid sequence as given in SEQ ID NO 2 or SEQ ID NO 4. A nucleotide sequence encoding MrgE may furthermore be a fragment of a sequence as given in SEQ ID NO 1 or 3, or a variant thereof with a length of at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900 bases, and even up to 925 bases. It thus follows that a nucleotide sequence encoding MrgE encodes at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300 amino acids and even up to about 305 amino acids of the sequence identified as SEQ ID NO 2 or SEQ ID NO 4. A fragment of a nucleotide sequence encoding MrgE furthermore preferably comprises at least the fraction of the molecule that encodes the ligand-binding domain of the molecule, denoted as extracellular domains on figure 4.

A variant of SEQ ID NO 1 or 3 denotes all DNA sequences encoding an MrgE polypeptide having an amino acid sequence as given in SEQ ID NO 2 or SEQ ID NO 4, or a fragment or variant thereof. A variant should furthermore be understood as a DNA sequence with at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95%, and even most preferably at least 98% identity with SEQ ID NO 1 or 3. It is furthermore preferred that the ligand-binding domain of the molecule is the most highly conserved part of the molecule, preferably at least 95%, more preferably at least 98%, and even most preferably at least 99% conserved compared with SEQ ID NO 1 or 3.
A variant of MrgE denotes a polypeptide with an amino acid identity of at least 80%, preferably, at least 85%, more preferably at least 90%, even more preferably at least 95%, and most preferably at least 98% identity with the sequence as given in SEQ ID NO 2 or 4, or a fragment thereof. It is furthermore preferred that the ligand-binding encoding domain of the molecule is the most highly conserved part of the molecule, preferably at least 95%, more preferably at least 98%, and even most preferably at least 99% conserved compared with SEQ ID NO 2 or 4. Conservative amino acid substitutions are preferred, especially in the ligand binding domains. In one embodiment the variant has substantially the same biological function as the molecule from which it is derived.

An MrgE specific antibody denotes an antibody or a pool of antibodies (e.g. monoclonal or polyclonal antibodies) that has been raised against an MrgE polypeptide according to SEQ ID NO 2 or 4, or a fragment thereof and/or a variant thereof. Preparation of antibodies is well known in the art, see Howard and Bethell, 2000 for an example (Howard and Bethell 2000)

A nucleotide sequence that is antisense to an MrgE sequence denotes a sequence that has the capability of base pairing specifically with an MrgE transcript encoding an MrgE polypeptide having an amino acid sequence according to SEQ ID NO 2, or 4, or a variant, or a fragment thereof. The antisense sequence may be in the form of a single stranded DNA, RNA, PNA, or LNA molecule. The antisense sequence has a length of at least about 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 300, 400, 500, 600, or 700 bases. Upon base pairing of the antisense sequence with the MrgE transcript within a cell, expression of MrgE is significantly down regulated in said cell due to rapid degradation of double stranded mRNA/antisense complex.
siRNA Small interfering RNAs (siRNAs) were recently discovered in plants and in animals. Since their discovery in the nematode *Caenorhabditis elegans* (Fire et al. 1998) these 21-23-nucleotide double-stranded RNAs bearing 3' overhanging ends have shown a tremendous potential for the silencing of genes in experimental as well as in therapeutical settings. A vast amount of literature has been published on siRNA structure and mechanism of action as well as on the use of siRNA in gene silencing, see the following references for recent reviews: (Alisky and Davidson 2004; Gilmore et al. 2004; Jones et al. 2004; Karagiannis and El-Osta 2004; Wadhwa et al. 2004; Zhang and Hua 2004).

A sample derived from fat tissue denotes a sample comprising a number of components from fat tissue, e.g. transcripts expressed in fat tissue and/or proteins expressed in fat tissue. The proteins may of course have been subject to post-translational modification such as signal peptide cleavage, glycosylation, acylation, etc.

A regulator of a MrgE polypeptide denotes any compound which is known to have the ability of modulating the activity of MrgE. Examples of MrgE regulators comprises e.g. MrgE specific antibodies.

As used herein, the terms "specific binding" or "specifically binding" refer to the interaction between a protein or peptide and an agonist, an antibody, or an antagonist.

In a first aspect, the present invention thus relates to a composition for use as a medicament, said composition comprising at least one compound selected from the group consisting of:

- a polynucleotide encoding an MrgE polypeptide, said polynucleotide comprising the sequence of SEQ ID NO 1, or SEQ ID NO 3, a fragment, or variant thereof.
• a polynucleotide encoding an MrgE polypeptide, wherein said polypeptide comprises the amino acid sequence according to SEQ ID NO 2, or SEQ ID NO 4, or a fragment, or variant thereof,

• an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

• an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

• a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and

• a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof.

In one embodiment the present invention thus relates to a composition for use as a medicament, said composition comprising at least one compound selected from the group consisting of:

• an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

• a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and

• a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof.

In another embodiment the composition for use as a medicament comprises a polynucleotide encoding an MrgE polypeptide, said polynucleotide
comprising the sequence of SEQ ID NO 1, or SEQ ID NO 3, a fragment, or variant thereof.

In another embodiment the composition comprises a polynucleotide encoding an MrgE polypeptide, wherein said polypeptide comprises the amino acid sequence according to SEQ ID NO 2, or SEQ ID NO 4, or a fragment, or variant thereof.

In another embodiment the composition comprises a MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof.

In another embodiment the composition comprises an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof.

In another embodiment the composition comprises a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof.

In another embodiment the composition comprises a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof.

In a second aspect, the present invention relates to use of one of the compounds according to the invention for manufacturing a pharmaceutical for modulating expression of MrgE in fat tissue for treating, alleviating, or preventing obesity, and/or diabetes, and/or cardiovascular diseases.
In a third aspect, the present invention relates to a method of screening an MrgE polypeptide for an interaction partner said method comprising the following steps:

(i) contacting an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof with a compound or an array of test compounds; and

(ii) detecting binding between MrgE and a compound capable of binding MrgE.

In a preferred embodiment, said method is a method for screening fat tissue for an interaction partner of an MrgE polypeptide, said method comprising the following steps:

(i) contacting a sample derived from fat tissue with an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and

(ii) detecting binding between MrgE and a compound capable of binding MrgE.

In a fourth aspect, the present invention relates to a method of screening for therapeutic agents useful in the treatment of obesity and/or diabetes and/or cardiovascular diseases, said method comprising the following steps:

(i) determining the activity of an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment or variant thereof, in the presence and optionally also in the absence of a test compound,

(ii) determining the activity of an MrgE polypeptide in the presence and optionally also in the absence of a compound known to be a regulator of a MrgE polypeptide.

(iii) identifying compounds capable of modulating MrgE activity.
In a preferred embodiment according to the present invention, the MrgE polypeptide is expressed at the surface of a cell. The cell is preferably grown in vitro.

In another preferred embodiment, the test compounds and the MrgE polypeptide are contacted in a cell-free system.

In a third preferred embodiment, the polypeptide is coupled to a detectable label.

In a fourth preferred embodiment, the test compound is coupled to a detectable label.

In a fifth preferred embodiment, the test compound or test sample displaces a ligand which is first bound to the polypeptide.

In a sixth preferred embodiment, the polypeptide is attached to a solid support.

In a fifth aspect, the present invention relates to a composition according to the invention for the treatment of obesity and/or diabetes, and/or cardiovascular diseases.

In a sixth aspect, the present invention relates to a method of treating obesity and/or diabetes, wherein at least one compound selected from:

- a polynucleotide encoding an MrgE polypeptide, said polynucleotide comprising the sequence of SEQ ID NO 1, a fragment, or variant thereof.
- a polynucleotide encoding an MrgE polypeptide, wherein said polypeptide comprises the amino acid sequence according to SEQ ID NO 2, or SEQ ID NO 4, or a fragment, or variant thereof,
• an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

• an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

• a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and

• a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

is administered to the patient in an amount sufficient to modulate MrgE activity in fat tissue.

In one embodiment the present invention relates to a method of treating obesity and/or diabetes, wherein at least one compound selected from:

• an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

• a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and

• a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

is administered to the patient in an amount sufficient to modulate MrgE activity in fat tissue.
Examples

Example 1:

Search for homologous sequences in public sequence databases

The degree of homology can readily be calculated by known methods. Preferred methods to determine homology are designed to give the largest match between the sequences tested. Methods to determine homology are codified in publicly available computer programs such as BESTFIT, BLASTP, BLASTN, and FASTA. The BLAST programs are publicly available from NCBI and other sources in the internet.

For MrgE the following hits to known sequences were identified by using the BLASTP algorithm (Altschul et al. 1997) and the following set of parameters: Wordsize 11, low complexity masking. The following databases were searched: NCBI (non-redundant database) and Nucleotides from the Patent division of GenBank:

>gi|33325633|gb|AF518247.1| Rattus norvegicus MRGE G protein-coupled receptor (Mrge) gene, complete cds Length = 1171 Score = 1844 bits (930), Expect = 0.0 Identities = 930/930 (100%)

>gi|50511331|ref|NM_001002288.1| Rattus norvegicus MAS-related G-protein coupled receptor, member E (Mrgpre), mRNA Length = 930 Score = 1844 bits (930), Expect = 0.0 Identities = 930/930 (100%)

>gi|34861293|ref|XM_219509.2| Rattus norvegicus similar to MRGE G protein-coupled receptor (LOC293639), mRNA Length = 1332 Score = 1836 bits (926), expect = 0.0 Identities = 929/930 (99%)
g31342123|ref|NM_175534.2| Mus musculus MAS-related GPR, member E (Mrgpre), mRNA Length = 3575 Score = 1499 bits (756), Expect = 0.0
Identities = 885/928 (95%)

5

>g329469607|gb|AC109309.5| Homo sapiens chromosome 11, clone RP11-11A9, complete sequence Length = 119560 Score = 381 bits (192),
Expect = e-102 Identities = 600/736 (81%)

10

>g37541642|ref|XM_171536.4| Homo sapiens mas-related G protein-coupled MRGE (MRGE), mRNA Length = 936 Score = 381 bits (192),
Expect = e-102
Identities = 600/736 (81%)

15

>g329611545|gb|AY255572.1| Homo sapiens MAS-related G protein-coupled receptor mRNA, partial cds Length = 435 Score = 260 bits (131),
Expect = 5e-66
Identities = 356/431 (82%)

20 Expression profiling

The procedure is based on methods described previously by Jensen et al. (Jensen et al. 1996)

25 Fresh tissue samples from the following brain areas: Striatum, prefrontal cortex, cortex, colliculus superior, hypothalamus, hippocampus, amygdala, cerebellum, medulla spinalis, thalamus, brainstem (raphe), brainstem (NTS) as well as from the peripheral tissues: Lung, Kidney, Stomach, Ileum, Testes, Muscle, Thymus, and Epididymal white adipose tissue were isolated from

30 Sprague-Dawley rats and immediately submerged in RNALater (Ambion, Texas, U.S.A.).
Total RNA was then extracted from the tissue samples using RNeasy spin columns (QIAGEN Inc., California, USA), following the manufacturer's instructions.

First-strand cDNA was prepared using 1 µg total RNA, the Superscript II RT kit, and random hexamer primers (GIBCO BRL, Gaithersburg, Maryland, USA), according to the manufacturer's instructions. The cDNA was diluted 1:6 in distilled water. A PCR mixture was prepared. For 13.5 µl, 1.35 µl 10 x polymerase buffer with MgCl2, 0.20 µl dNTP (4 mM, 2 mM dCTP), 0.25 µl of each primer (10 mM), 0.125 µl Taq polymerase, 0.0625 µl 33P-α-dCTP (10 mCl/ml, Amersham), 1.5 µl cDNA solution, and finally water to 13.5 µl was used. Two primer sets were included in each reaction, one set specific for MrgE (5'- GATATGGCCAGTCAGGGG-3' and 5'-GTCATGGTGTTGGCAACTG-3', product length 262 bp), the second set specific for TBP (5'- ACCCTTCACAAATGACTCTATG-3' and 5'-TGACTGCAGCAATCGCTTGG-3', product length 186 bp) and used as an internal standard. All samples were subjected to 25 rounds of amplification in the following PCR program: An initial denaturation (2 min. 94 degrees), 25 rounds of denaturation (30 sec. 94 degrees), annealing (30 sec. 55 degrees) and elongation (30 sec. 72 degrees), and finally a long elongation period (5 min. 72 degrees).

The number of cycles was chosen in the range where the limiting factor for the amount of product is the amount of input template cDNA. The final PCR reactions were mixed with 98% formamide denaturing loading buffer and loaded in duplicate and separated on a 6% (wt/vol) polyacrylamide gel, containing 7 M urea. The gel was subsequently dried, exposed to a phosphorimager screen, and the resulting scan analyzed using Quantity One (Biorad). Finally, the MrgE expression was normalized to the TBP expression (Fig. 1).
The analysis showed (Fig. 1) that the MRG E gene is expressed in relatively high levels in the brain and in the thymus, but that there also is a substantial expression in the epididymal white adipose tissue.

Example 3

Differential expression of MrgE in response to adipocyte differentiation

3T3-L1 cells (a mouse fibroblast/preadipocyte cell line) were grown to confluence and induced to differentiate as described (Student et al. 1980). Briefly, preadipocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum and 2 days after reaching confluence (day 0), differentiation was induced by culturing the cells in DMEM with 10% fetal calf serum (FCS), 167 nM insulin (INS), 0.5 mM methylisobutylxanthine (MIX), and 0.25 µM dexamethasone (DEX) for two days, DMEM with FCS and INS for two days and DMEM with FCS until RNA was isolated.

RNA was isolated on different days during the differentiation process and reverse transcribed, followed by multiplex PCR performed essentially as described above (Example 2) except that the mouse-specific MrgE primers (5’- CTGTCCAAGAACCCTGTCTTG -3’ and 5’- AGTCATGTCCACAAAGTCCCCC-3’, product length 228 bp) were used instead of the rat-specific primers mentioned above.

In parallel, separate multiplex reactions were performed with primers specific for the adipocyte differentiation marker PPARγ2, 5’- AGTGTGAATTACAGCAAATCTC-3’ and 5’- ATGGTAATTCTTTGTGAAGTGCC-3’, again using TBP as a reference (Figure 2).
The adipocyte marker PPARγ2 is upregulated during the adipocyte differentiation, showing that the adipocytes are indeed differentiating, and, importantly, the MrgE expression is regulated during the differentiation to adipocytes. Thus, the preadipocyte fibroblast cell line 3T3-L1 had the highest expression of MrgE before differentiation to adipocytes. The expression of MrgE was turned down to approximately half the preadipocyte level at the time when the expression of PPARγ2 was induced.

It is presently not known, whether stimulation or inhibition of MrgE receptor activity can influence the differentiation process, but it is believed that stimulation or inhibition of the MrgE receptor can affect preadipocyte and/or adipocyte function as well as the differentiation process leading from preadipocytes to adipocytes.

Example 4

Feeding influences the expression of MrgE in white adipose tissue

Thirty-three male Sprague-Dawley rats (Charles River, Sweden) of 300-350 g were housed in single cages and fed normal rat chow for one week, and then randomised by weight the day before starting the 32-day feeding paradigm. The rats were separated into four groups. Eight rats were given chow ad libitum, nine were given 70% of the amount eaten by the ad libitum chow group, eight were given Nutella-chow mix and HE chow ad libitum (Nutella is a chocolate spread containing 52.5% fat, 43.9% carbohydrate, 5.7% protein and 5.14 Kcal/g. Nutella diet is made by mixing one third powdered chow with two thirds Nutella by weight), and eight were given chow ad libitum for 30 days and fasted the last two days of the experiment.
The last day of the experiment the rats were sedated with CO₂ and decapitated. The mesenteric white adipose tissue was removed and stored in 5 volumes of RNAlater until RNA preparation.

RNA preparation, synthesis of cDNA, and multiplex PCR was performed as in Example 2 above.

Figure 3A shows the average body weight of each group of animals in the experiment; clearly demonstrating that the Nutella fed rats had a more rapid increase in body weight than the other rats. These rats became obese and standard parameters such as white adipose tissue expression of leptin was in agreement with these data (results not shown). The expression of MrgE was downregulated in Nutella fed rats in the mesenteric white adipose tissue (Figure 3B), well in agreement with the downregulation seen during adipose differentiation (example 3) of 3T3-L1 cells.

Preliminary results indicate that the regulation of MrgE is not identical in different white adipose tissue compartments (results not shown), indicating that pharmacologically induced changes in MrgE activity may have different effects on different white adipose tissue compartments.

Example 5

Antisense Analysis

Knowledge of the correct, complete cDNA sequence coding for MrgE enables its use as a tool for antisense or siRNA technology (short interfering RNA) in the investigation of gene function. Oligonucleotides, cDNA or genomic fragments comprising the antisense strand or a small double stranded portion of a polynucleotide coding for MrgE are used either in vitro or in vivo to inhibit translation and/or direct degradation of the mRNA. Such
technology is now well known in the art, and antisense and siRNA molecules can be designed at various locations along the nucleotide sequences. By treatment of cells or whole test animals with such sequences, the gene of interest is effectively turned off. Frequently, the function of the gene is ascertained by observing behavior at the intracellular, cellular, tissue or organismal level (e.g., lethality, loss of differentiated function, changes in morphology, etc.).

In addition to using sequences constructed to interrupt transcription of a particular open reading frame, modifications of gene expression is obtained by designing antisense sequences to intron regions, promoter/enhancer elements, or even to transacting regulatory genes.

Example 6

Expression of MrqE

Expression of MrqE is accomplished by subcloning the cDNAs into appropriate expression vectors and transfecting the vectors into expression hosts such as, e.g., E. coli. In a particular case, the vector is engineered such that it contains a promoter for β-galactosidase, upstream of the cloning site, followed by sequence containing the amino-terminal Methionin and the subsequent seven residues of β-galactosidase. Immediately following these eight residues is an engineered bacteriophage promoter useful for artificial priming and transcription and for providing a number of unique endonuclease restriction sites for cloning.

Induction of the isolated, transfected bacterial strain with isopropyl-P-D-thio-galactopyranoside (IPTG) using standard methods produces a fusion protein corresponding to the first seven residues of β-galactosidase, about 15 residues of "linker", and the peptide encoded within the cDNA. Since cDNA
clone inserts are generated by an essentially random process, there is a probability of 33% that the included CDNA will lie in the correct reading frame for proper translation. If the CDNA is not in the proper reading frame, it is obtained by deletion or insertion of the appropriate number of bases using well known methods including in vitro mutagenesis, digestion with exonuclease III or mung bean nuclease, or the inclusion of an oligonucleotide linker of appropriate length.

The MrgE cDNA is shuttled into other vectors known to be useful for expression of proteins in specific hosts. Oligonucleotide primers containing cloning sites as well as a segment of DNA (about 25 bases) sufficient to hybridize to stretches at both ends of the target CDNA is synthesized chemically by standard methods. These primers are then used to amplify the desired gene segment by PCR. The resulting gene segment is digested with appropriate restriction enzymes under standard conditions and isolated by gel electrophoresis. Alternatively, similar gene segments are produced by digestion of the cDNA with appropriate restriction enzymes. Using appropriate primers, segments of coding sequence from more than one gene are ligated together and cloned in appropriate vectors. It is possible to optimize expression by construction of such chimeric sequences.

Suitable expression hosts for such chimeric molecules include, but are not limited to, mammalian cells such as Chinese Hamster Ovary (CHO) and human 293 cells, insect cells such as Sf9 cells, yeast cells such as Saccharomyces cerevisiae and bacterial cells such as E. coli. For each of these cell systems, a useful expression vector also includes an origin of replication to allow propagation in bacteria, and a selectable marker such as the β-lactamase antibiotic resistance gene to allow plasmid selection in bacteria. In addition, the vector may include a second selectable marker such as the neomycin phosphotransferase gene to allow selection in transfected eukaryotic host cells. Vectors for use in eukaryotic expression hosts require
RNA processing elements such as 3'polyadenylation sequences if such are not part of the cDNA of interest.

Additionally, the vector contains promoters or enhancers which increase gene expression. Such promoters are host specific and include MMTV, SV40, and metallothionine promoters for CHO cells; trp, lac, tac and T7 promoters for bacterial hosts; and alpha factor, alcohol oxidase and PGH promoters for yeast. Transcription enhancers, such as the rous sarcoma virus enhancer, are used in mammalian host cells. Once homogeneous cultures of recombinant cells are obtained through standard culture methods, large quantities of recombinantly produced MrgE are recovered from the conditioned medium and analyzed using chromatographic methods known in the art. For example, MrgE can be cloned into the expression vector pcDNA3, as exemplified herein. This product can be used to transform, for example, HEK293 or COS by methodology standard in the art. Specifically, for example, using Lipofectamine (Gibco BRL catalog no. 18324-020) mediated gene transfer.

Example 7

Isolation of Recombinant MrgE

MrgE is expressed as a chimeric protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals and the domain utilized in the FLAGS extension/affinity purification system (IMMUNEX Corp., Seattle, Washington). The inclusion of a cleavable linker sequence such as Factor Xa or enterokinase (Invitrogen, Groningen, The Netherlands) between the purification domain and the MrgE sequence is useful to facilitate expression and purification of MrgE.
Purified recombinant MrgE or alternatively cells expressing MrgE are useful in screening for MrgE binding partners. Purified recombinant MrgE as well as an expression vector encoding MrgE may furthermore form part of a pharmaceutical formulation.

Native or recombinant MrgE may also be purified by immunoaffinity chromatography using antibodies specific for MrgE. In general, an immunoaffinity column is constructed by covalently coupling the anti-MrgE antibody to an activated chromatographic resin.

Example 8

Establishing a functional assay for MrgE

A number of reporter gene assays have been developed for high-throughput studies of GPCRs. These include β-galactosidase, receptor selection and amplification technology (R-SAT), β-lactamase, luciferase, Fluorescence imaging plate reader (FLIPR), and aequorin.

In addition, universal G-protein adaptors such as Gα16 or the even more promiscuous Gα16/z chimeras 16z25 and 16z44 (Liu et al. 2003) have been identified that are able to translate GPCR activation into Ca²⁺ mobilization, making it possible to use the FLIPR and aequorin methods that are highly amenable to automation and high-throughput identification and/or analysis of ligands.

FLIPR assay

COS-7 cells are maintained in Dulbecco’s modified Eagles’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/ml
penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO₂ and 95% air at 37 °C. The cells are seeded at a density of 20,000 cells per well in 96-well plates designed for FLIPR assays using Opti-MEM with 10% FBS in a volume of 100 μl/well. Transfection is carried out using Lipofectamine 2000® reagent. For each well, 0.2 μg of GPCR and G protein cDNAs are diluted with 25 μl of Opti-MEM and 0.2 μl of Lipofectamine 2000® is diluted with another 25 μl of Opti-MEM. They are added to the appropriate well after the 2 components are mixed for 20 min.

After 48 h of incubation post transfection, 50 μl of transfection medium is removed from each well followed by the labelling of transfected cells with 100 μl of 2 μM Fluo-4 in calcium containing HBSS (Hank’s balanced salt solution) with 20 mM HEPES, pH 7.5 and 2.5 mM probenecid for 1 h at 37 °C. Then, 70 μl of 3X drugs are prepared and aliquotted into the corresponding wells in the V-well drug plate. Changes in fluorescence are detected in the FLIPR96 with an excitation wavelength of 48 nm. The background fluorescence is adjusted to the range of 8000 to 12000 units by altering the laser power and exposure time for the FLIPR setup. Then, 50 μl of each agonist solution is added to the corresponding wells, and the fluorescent emission (between 510 and 560 nm) is monitored for 3 min. Results are expressed as changes in fluorescent intensity units.

Example 9

Testing of Chimeric GPCRs

An alternative to coupling the GPCR to a promiscuous G-protein is to perform tests on chimeric GPCRs. Functional chimeric GPCRs are constructed by combining the extracellular receptive sequences of a new isoform with the transmembrane and intracellular segments of a known isoform for test purposes. This concept was demonstrated by Kobilka et al.
(Kobilka et al. 1988) who created a series of chimeric α2-β2 adrenergic receptors (AR) by inserting progressively greater amounts of α2-AR transmembrane sequence into β2-AR. The binding activity of known agonists changed as the molecule shifted from having more α2 than β2 conformation, and intermediate constructs demonstrated mixed specificity. The specificity for binding antagonists, however, correlated with the source of the domain VII. The importance of T7G domain VII for ligand recognition was also found in chimeras utilizing two yeast α-factor receptors and is significant because the yeast receptors are classified as miscellaneous receptors. Thus, the functional role of specific domains appears to be preserved throughout the GPCR family regardless of category.

In parallel fashion, internal segments or cytoplasmic domains from a particular isoform are exchanged with the analogous domains of known GPCRs and used to identify the structural determinants responsible for coupling the receptors to trimeric G-proteins. A chimeric receptor in which domains V, VI, and the intracellular connecting loop from β2-AR were substituted into α2-AR was shown to bind ligands with α2-AR specificity, but to stimulate adenylate cyclase in the manner of β2-AR.

This demonstrates that for adrenergic-type receptors, G-protein recognition is present in domains V and VI and their connecting loop. The opposite situation was predicted and observed for a chimera in which the V->VI loop from α1-AR replaced the corresponding domain on β2-AR and the resulting receptor bound ligands with β2-AR specificity and activated G-protein-mediated phosphatidylinositol turnover in the α1-AR manner. Finally, chimeras constructed from muscarinic receptors also demonstrated that V->VI loop is the major determinant for specificity of G-protein activity.
Chimeric or modified GPCRs containing substitutions in the extracellular and transmembrane regions have shown that these portions of the receptor determine ligand binding specificity. For example, two Serine residues conserved in domain V of all adrenergic and D catecholamine GPCRs are necessary for potent agonist activity. These serines are believed to form hydrogen bonds with the catechol moiety of the agonists within the GPCR binding site. Similarly, an Asp residue present in domain M of all GPCRs which bind biogenic amines is believed to form an ion pair with the ligand amine group in the GPCR binding site.

Functional, cloned GPCRs are expressed in heterologous expression systems and their biological activity assessed. One heterologous system introduces genes for a mammalian GPCR and a mammalian G-protein into yeast cells. The GPCR is shown to have appropriate ligand specificity and affinity and trigger appropriate biological activation (growth arrest and morphological changes) of the yeast cells.

An alternate procedure for testing chimeric receptors is based on the procedure utilizing the purinergic receptor (P2u). Function is easily tested in cultured K562 human leukemia cells because these cells lack P2u receptors. K562 cells are transfected with expression vectors containing either normal or chimeric P2u and loaded with fura-a, fluorescent probe for Ca++. Activation of properly assembled and functional P2U receptors with extracellular UTP or ATP mobilizes intracellular Ca++ which reacts with fura-a and is measured spectrofluorometrically.

As with the GPCRs above, chimeric genes are created by combining sequences for extracellular receptive segments of any new GPCR polypeptide with the nucleotides for the transmembrane and intracellular segments of the known P2u molecule.
Bathing the transfected K562 cells in microwells containing appropriate ligands triggers binding and fluorescent activity defining effectors of the GPCR molecule.

Once ligand and function are established, the P2u system is useful for defining antagonists or inhibitors which block binding and prevent such fluorescent reactions.

**Example 10**

**Drug Screening**

MrgE can be used for screening therapeutic compounds by using MrgE or binding fragments thereof in any of a variety of drug screening techniques.

As MrgE is a G protein coupled receptor any of the methods commonly used in the art may potentially be used to identify MrgE ligands. For example, the activity of a G protein coupled receptor such as MrgE can be measured using any of a variety of appropriate functional assays in which activation of the receptor results in an observable change in the level of some second messenger system, such as adenylate cyclase, guanylylcyclase, calcium mobilization, or inositol phospholipid hydrolysis.

Alternatively, the polypeptide or fragment employed in such a test is either free in solution, affixed to a solid support, borne on a cell surface or located intracellularly.

One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant nucleic acids expressing the polypeptide or fragment. Drugs are screened against such transformed cells in competitive binding assays. Such cells, either in viable or fixed form, are
used for standard binding assays. Test compounds might be compounds or a pool of compounds commonly used for screening 7TM receptors for interaction partners.

Measured, for example, is the formation of complexes between MrgE and the agent being tested. Alternatively, one examines the diminution in complex formation between MrgE and a ligand caused by the agent being tested.

Thus, the present invention provides methods of screening for drug candidates. These methods, well known in the art, comprise contacting such an agent with MrgE polypeptide or a fragment thereof and assaying (i) for the presence of a complex between the agent and MrgE polypeptide or fragment, or (ii) for the presence of a complex between MrgE polypeptide or fragment and the cell. In such competitive binding assays, the MrgE polypeptide or fragment is typically labeled. After suitable incubation, free MrgE polypeptide or fragment is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular agent to bind to MrgE or to interfere with the MrgE-agent complex.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to MrgE polypeptides. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with MrgE polypeptide and washed. Bound MrgE polypeptide is then detected by methods well known in the art. Purified MrgE are also coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies are used to capture the peptide and immobilize it on the solid support.
This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding MrgE specifically compete with a test compound for binding to MrgE polypeptides or fragments thereof. In this manner, the antibodies are used to detect the presence of any peptide which shares one or more antigenic determinants with MrgE.

Example 11

Identification of Other Members of the Signal Transduction Complex

The inventive purified MrgE is a research tool for identification, characterization and purification of interacting G or other signal transduction pathway proteins.

Radioactive labels are incorporated into a selected MrgE domain by various methods known in the art and used in vitro to capture interacting molecules. A preferred method involves labelling the primary amino groups in MrgE with $^{125}$I, Bolton-Hunter reagent. This reagent has been used to label various molecules without concomitant loss of biological activity.

Labelled MrgE is useful as a reagent for the purification of molecules with which it interacts. In one embodiment of affinity purification, membrane-bound MrgE is covalently coupled to a chromatography column. Cell-free extract derived from synovial cells or putative target cells is passed over the column, and molecules with appropriate affinity bind to MrgE. MrgE-complex is recovered from the column, and the MrgE-binding ligand disassociated and subjected to N-terminal protein sequencing. The amino acid sequence information is then used to identify the captured molecule or to design degenerate oligonucleotide probes for cloning the relevant gene from an appropriate cDNA library.
In an alternate method, antibodies are raised against MrgE, specifically monoclonal antibodies. The monoclonal antibodies are screened to identify those which inhibit the binding of labeled MrgE. These monoclonal antibodies are then used therapeutically.

Example 12

Use and Administration of Antibodies, Inhibitors, or Antagonists

Antibodies, inhibitors, or antagonists of MrgE or other treatments and compounds that are limiters of signal transduction (LSTs), provide different effects when administered therapeutically. LSTs are formulated in a nontoxic, inert, pharmaceutically acceptable aqueous carrier medium preferably at a pH of about 5 to 8, more preferably 6 to 8, although pH may vary according to the characteristics of the antibody, inhibitor, or antagonist being formulated and the condition to be treated.

Characteristics of LSTs include solubility of the molecule, its half-life and antigenicity/immunogenicity. These and other characteristics aid in defining an effective carrier. Native human proteins are preferred as LSTs, but organic or synthetic molecules resulting from drug screens are equally effective in particular situations.

LSTs are delivered by known routes of administration including but not limited to topical creams and gels; transmucosal spray and aerosol; transdermal patch and bandage; injectable, intravenous and lavage formulations; and orally administered liquids and pills particularly formulated to resist stomach acid and enzymes. The particular formulation, exact dosage, and route of administration is determined by the attending physician and varies according to each specific situation.
Such determinations are made by considering multiple variables such as the condition to be treated, the LST to be administered, and the pharmacokinetic profile of a particular LST. Additional factors which are taken into account include severity of the disease state, patient's age, weight, gender and diet, time and frequency of LST administration, possible combination with other drugs, reaction sensitivities, and tolerance/response to therapy. Long acting LST formulations might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular LST.

Normal dosage amounts vary from 0.1 to 105 µg, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature (Katre et al. 1993; Kung and Goldstein 1987; Martin et al. 1993). Those skilled in the art employ different formulations for different LSTs.

It is contemplated that abnormal signal transduction, trauma, or diseases which trigger MrgE activity are treatable with LSTs. These conditions or diseases are specifically diagnosed by the tests discussed above, and such testing should be performed in suspected cases where MrgE expression could be an issue.

**Example 13**

**Production of Non-human Transgenic Animals**

Animal model systems which elucidate the physiological and behavioral roles of the MrgE are produced by creating nonhuman transgenic animals in which the activity of the MrgE is either increased or decreased, or the amino acid sequence of the expressed MrgE is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of
normal or mutant versions of DNA encoding a MrgE, by microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriately fertilized embryos in order to produce a transgenic animal or 2) homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these MrgE sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and hence is useful for producing an animal that cannot express native MrgE'S but does express, for example, an inserted mutant MrgE, which has replaced the native MrgE in the animal's genome by recombination, resulting in underexpression of MrgE.

Microinjection adds genes to the genome, but does not remove them, and the technique is useful for producing an animal which expresses its own and added MrgE, resulting in over expression of the MrgE.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as cesium-chloride M2 medium. DNA or cDNA encoding MrgE is purified from a vector by methods well known to the one skilled in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the transgene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the transgene.

The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a piper puller) and the egg to be injected is put in a depression slide. The needle is inserted into
the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudo pregnant mouse which is a mouse stimulated by the appropriate hormones in order to maintain false pregnancy, where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg but is used here only for exemplary purposes.

In the case of MrgE it can be extremely useful for the elucidation of the function to make tissue-specific knock-outs, particularly in adipose tissue. This will allow for the elucidation of the particular function of MrgE in the adipose tissue without disturbing brain-specific pathways including MrgE. One methodology for inducing tissue-specific knockouts of MrgE is to replace the native MrgE gene with an MrgE gene flanked by loxP sites in one mouse strain and then cross mice from this strain with mice expressing the cre recombinase under control of an adipocyte-specific promoter.

**Example 14**

**MRG E is expressed in human adipose tissue**

20

Total RNA from human adipose tissue was purchased from Biochain Inc., (Hayward, CA). The RNA had been isolated from the following donors: Great Omentum (a, male 32 years; b, male, 24 years; c, male, 21 years; d, female, 30 years), Mesenterium (e, male, 23 years; f, male, 25 years), and Subcutis (g, male, 44 years).

First-strand cDNA was prepared using 1 μg total RNA, the Superscript II RT kit, and random hexamer primers (GIBCO BRL, Gaithersburg, Maryland, USA), according to the manufacturer’s instructions. The cDNA was diluted 1:6 in distilled water. A PCR mixture was prepared. For 13.5 μl, 1.35 μl 10 x polymerase buffer with MgCl2, 0.20 μl dNTP (4 mM, 2 mM dCTP), 0.25 μl of each primer (10 mM), 0.125 μl Taq polymerase, 0.0625 μl 33P-α-dCTP (10
mCi/ml, Amersham), 1.5 μl cDNA solution, and finally water to 13.5 μl was used. Two primer sets were included in each reaction, one set specific for human MrgE (5'-ATGATGGAGCCAGAGAAGCTG-3' and 5'-AAGGAAGATGAGATCCGCGAG-3', product length 219 bp), the second set specific for human TBP (5'-TGGCTCTCATGTACCCTGCG-3' and 5'-TGCACAATAATGCCCCCTTC-3', product length 187 bp) and used as an internal standard. All samples were subjected to 25 rounds of amplification in the following PCR program: An initial denaturation (2 min. 94 degrees), 25 rounds of denaturation (30 sec. 94 degrees), annealing (30 sec. 55 degrees) and elongation (30 sec. 72 degrees), and finally a long elongation period (5 min. 72 degrees).

The number of cycles was chosen in the range where the limiting factor for the amount of product is the amount of input template cDNA. The final PCR reactions were mixed with 98% formamide denaturing loading buffer and loaded in duplicate and separated on a 6% (wt/vol) polyacrylamide gel, containing 7 M urea. The gel was subsequently dried, exposed to a phosphorimager screen, and the resulting scan analyzed using Quantity One (Biorad). Finally, the MrgE expression was normalized to the TBP expression.

The analysis showed (Fig. 5) that the MRG E gene is expressed in human adipose tissues, but at very variable expression levels.
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Claims

1. A method of screening an MrgE polypeptide for an interaction partner said method comprising the following steps:

(i) contacting an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof with a compound or an array of test compounds; and
(ii) detecting binding between MrgE and a compound capable of binding MrgE.

2. A method according to claim 1, wherein said method is a method for screening fat tissue for an interaction partner of an MrgE polypeptide, said method comprising the following steps:

(i) contacting a sample derived from fat tissue with an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and
(ii) detecting binding between MrgE and a compound capable of binding MrgE.

3. A method of screening for therapeutic agents useful in the treatment of obesity and/or diabetes and/or cardiovascular diseases, said method comprising the following steps:

(i) determining the activity of an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment or variant thereof, in the presence and optionally also in the absence of a test compound,
(ii) determining the activity of an MrgE polypeptide in the presence and optionally also in the absence of a compound known to be a regulator of a MrgE polypeptide,
(iii) identifying compounds capable of modulating MrgE activity.
4. A method according to any one of claims 1-3, wherein the MrgE polypeptide is expressed at the surface of a cell.

5. A method according to any one of claims 4, wherein the cell is in vitro.

6. A method according to any one of claims 1-3, wherein the step of contacting is in a cell-free system.

7. A method according to any one of claims 1-6, wherein the polypeptide is coupled to a detectable label.

8. A method according to any one of claims 1-7, wherein the test compound or test sample displaces a ligand which is first bound to the polypeptide.

9. A method according to any one of claims 1-3, and 6-8, wherein the polypeptide is attached to a solid support.

10. A composition for use as a medicament, said composition comprising at least one compound selected from the group consisting of:

- a polynucleotide encoding an MrgE polypeptide, said polynucleotide comprising the sequence of SEQ ID NO 1, or SEQ ID NO 3, a fragment, or variant thereof.
- a polynucleotide encoding an MrgE polypeptide, wherein said polypeptide comprises the amino acid sequence according to SEQ ID NO 2, or SEQ ID NO 4, or a fragment, or variant thereof,
- an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
- an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
• a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and
• a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof.

11. Use of a compound selected from the group consisting of:
• a polynucleotide encoding an MrgE polypeptide, said polynucleotide comprising the sequence of SEQ ID NO 1, or SEQ ID NO 3, a fragment, or variant thereof.
• a polynucleotide encoding an MrgE polypeptide, wherein said polypeptide comprises the amino acid sequence according to SEQ ID NO 2, or SEQ ID NO 4, or a fragment, or variant thereof,
• an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
• an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
• a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and
• a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,

for manufacturing a pharmaceutical for modulating expression of MrgE in fat tissue for treating, alleviating, or preventing obesity, and/or diabetes, and/or cardiovascular diseases.

12. A composition according to claim 10 for the treatment of obesity and/or diabetes, and/or cardiovascular diseases.
13. A method of treating obesity and/or diabetes, wherein at least one compound selected from:

- a polynucleotide encoding an MrgE polypeptide, said polynucleotide comprising the sequence of SEQ ID NO 1, a fragment, or variant thereof.
- a polynucleotide encoding an MrgE polypeptide, wherein said polypeptide comprises the amino acid sequence according to SEQ ID NO 2, or SEQ ID NO 4, or a fragment, or variant thereof,
- an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
- an MrgE specific antibody raised against an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
- a nucleotide sequence that is antisense to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof, and a siRNA molecule that is specific to a transcript encoding an MrgE polypeptide comprising the amino acid sequence according to SEQ ID NO 2 or SEQ ID NO 4, or a fragment, or variant thereof,
- is administered to the patient in an amount sufficient to modulate MrgE activity in fat tissue.
Figure 1
Figure 2
Figure 3
Expression of MRG E in adipose tissues

Adipose tissue

- Subcutaneous g
- Mesenteric f
- Mesenteric e
- Great Omentum d
- Great Omentum c
- Great Omentum b
- Great Omentum a

Expression (relative to TBP)

Figure 5