Expression and regulation of Mitogen Activated Protein Kinase (MAPK) Phosphatases (MKPs) in adipogenesis

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Thesis for the Master of Science Degree in Molecular Biology

Department of Molecular Biosciences

University of Oslo, December 2005

Contents

Acknowledgements

The present work was carried out from January 2005 to November 2005 in the laboratory of Professor Fahri Saatcioglu at the Department of Molecular Biosciences, University of Oslo.

First of all I want to thank my main supervisor Fahri Saatcioglu for giving me the opportunity to learn a lot about molecular cell biology in a great research environment. I thank him for always taking the time in his busy schedule to answer questions and for teaching me to think critically in the world of science.

I am especially grateful to Lene Malerød who started out as my lab supervisor and taught me so much about working in the lab. Thanks for answering my endless questions, and for always making me feel better when nothing seemed to work right. I also want to thank Judy Tsai for her patience, helpfulness, and adipocyte expertise. Thanks to all the other members of the FS lab and special credits to Piotr Kurys, Torstein Lindstad, and Tove Irene Klokk for technical support. Thanks to Yke Arnoldussen, a fellow master student in the lab, for good times and for listening to all my complaining throughout the year.

Finally I want to thank my friends and family, especially my parents for always believing in me. Last but not least, thanks to my beloved husband Amund for putting up with me and for always supporting and encouraging me.

Oslo, December 2005

Sunniva Stordal Bjørklund

Abbreviations

α-MSH	α-Melanocortin stimulating hormone
AgRP	Agouti related protein
aP2	Adipocyte-specific fatty acid binding protein 2
A^y	Agouti
BAT	Brown adipose tissue
BBB	Blood brain barrier
BMI	Body mass index
C/EBP	CCAAT/enhancer binding protein
CART	Cocaine and amphetamine related transcript
CCK	Cholecystokinin
DSP	Dual specificity phosphatase
ERK	Extracellular regulated kinase
GAPDH	Glycerophosphate dehydrogenase
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like protein 1
GPCR	G protein-coupled receptor
IBMX	Isobutylmethylxanthine
IGF-1	Insulin-like growth factor receptor 1
IL-6	Interleukin 6
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MC4R	Melanocortin 4 receptor
MCE	Mitotic clonal expansion
MCH	Melanin-concentrating hormone
MEK	MAPK/ERK kinase
MKK	MAP kinase kinase
MKP	MAP kinase phosphatase
NPY	Neuropeptide Y
Ob	Obese
ORF	Open reading frame
POMC	Proopiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PTP	Protein tyrosine phosphatase
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TNF-α	Tumor necrosis factor α
UCP	Uncoupling protein
WAT	White adipose tissue

General Introduction

Obesity

Obesity is a result of uncontrolled expansion of the adipose tissue and is associated with severe health risks (1). The most widely used index of obesity is the body mass index (BMI), which is calculated by dividing body weight in kilograms by the square of height in meters (kg/m^2). BMI between 18.5 and 25 is considered to be normal weight, BMI ranging between 25.0 and 29.9 is defined as overweight, and a score of 30 or above indicates obesity. Obesity has reached epidemic proportions in western industrialized countries. In the USA approximately 30% of all adults are estimated to be obese, up to 60% are considered overweight, and the prevalence in children is increasing dramatically (2, 3). It is also a rising problem in Norway where a recent report states that 14.7% of men and 12.5% of women are obese (4).

Obesity is directly linked to a number of different health risks. The most common obesity-related health condition is hypertension. The prevalence of type 2 diabetes has been shown to increase with increased body weight in both men and women and although coronary heart disease can not be directly linked to overweight it shows a significantly higher prevalence in obese individuals (5). There is also evidence that obesity increases the risk of certain types of cancer such as colon cancer and breast cancer (6).

Obesity is a consequence of a positive energy balance in the body, where consumed energy exceeds energy expenditure. This is due to both environmental and hereditary factors (7). Availability and composition of food as well as reduced requirement for physical activity are some environmental factors that have changed in recent years and contribute to a positive energy balance. Some of the genes involved have been characterized but a lot remains to fully understand how these components work in a physiological environment. Energy balance is composed of energy intake and energy expenditure. Energy intake is regulated by feeding and will be discussed below.

Energy expenditure can be divided into three categories: basal metabolism, physical activity, and adaptive thermogenesis (8). The first is the obligatory energy expended on basic cellular and physiological functions. The maintenance of energy balance requires

oxidization of the fuel that is ingested. Obese individuals who have lost weight are less effective in increasing fat oxidation in the presence of a high-fat diet than normal weight individuals (6). The energy expended in physical activity is directly related to body weight and it has been shown that exercise can accelerate the adaptation to a change from a low- to a higher-fat diet (9). Adaptive thermogenesis refers to the thermic effect of food and the ability to convert excess calories to heat. After food is ingested there is a rise in energy expenditure. This process is partly controlled by the sympathetic nervous system. Thermogenic activity of brown adipose tissue (BAT) is also under sympathetic nervous system control. This activity is primarily mediated in brown adipocytes by mitochondrial uncoupling protein 1 (UCP1) which allows protons to leak across the inner mitochondrial membrane instead of coupling these protons to ATP synthesis (10). This results in increased heat production. BAT is abundant in small animals and human infants but in adult humans the amount of BAT is minimal. It is therefore thought that the expression of UCP1 is not substantial to be physiologically meaningful in adults. A homologue of UCP1, UCP3 is expressed predominantly in skeletal muscles. A strong association between the expression of UCP3 and fat metabolism has been established but the nature of this association remains unknown (11).

Signaling of energy balance

Food intake in humans is influenced by emotional factors, social factors, and learned behavior (12). In addition to this, systems within the brain sense and integrate signals regarding energy stores and recent energy intake to maintain energy homeostasis. The hypothalamus integrates both peripheral and central signals and controls food intake, levels of physical activity, energy expenditure, and endocrine systems. Some of these signals are regarded as short-term and include signaling molecules from the gut in response to meals (13). More long-term signals include factors that are secreted from adipose tissue such as leptin, adiponectin, resistin, and visfatin, and insulin secreted from the pancreas (Fig. 1).

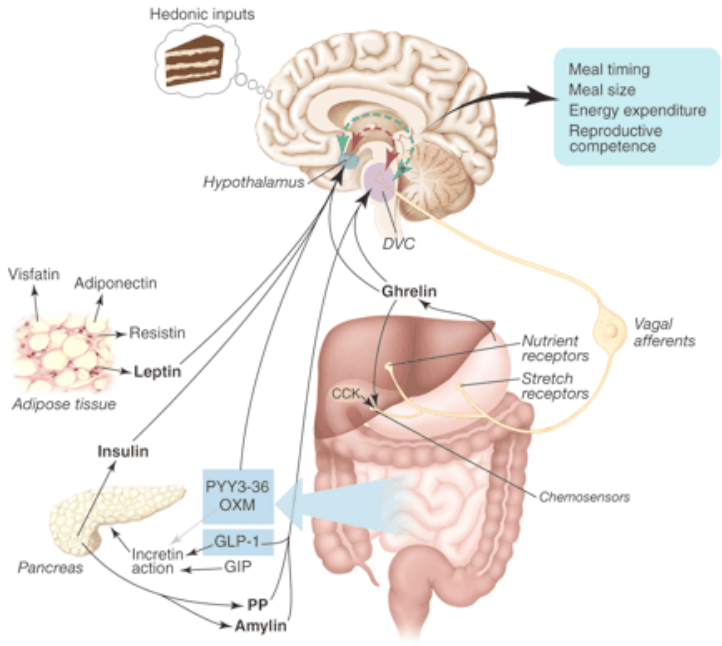

Figure 1. Integration of long-term and short-term signals of energy balance in the brain. *Adipose tissue and the pancreas produce peripheral signals that relate to long-term energy stores. Short-term signals include absorbed nutrients and peptides secreted from the gut. Figure from Badman and Flier 2005 (12).*

The gut is known as a source of signals that influence appetite. Stretch sensors in the gastro-intestinal tract send signals directly to the brain via afferent nerves and in addition there are a number of different endocrine signals secreted that can affect appetite. Cholecystokinin (CCK) is a prototypical satiety hormone that is produced in mucosal endocrine cells in the small intestine. It is secreted by these cells in response to the presence of food within the gut lumen. Sulfated CCK binds to CCK receptors on vagal afferent neurons, which transmit neural signals to the brainstem and results in a reduction in meal size (14). CCK receptors also inhibit gastric emptying, which may enhance the signals of satiety. Infusion of CCK into human subjects has been shown to suppress food intake and cause earlier meal termination (15). Glucagon-like peptide 1 (GLP-1) is another satiety peptide and is secreted from L-cells in response to nutrients in the form of free fatty acids and carbohydrates. GLP-1 inhibits gastric acid secretion and emptying and stimulates insulin release (16). Another peptide that stimulates insulin secretion is glucose-dependent insulinotropic polypeptide (GIP). GIP secretion from the small intestine is primarily induced by absorption of ingested fat. It has been shown that mice lacking the GIP receptor that were fed a high-fat diet were protected from obesity (17). Peptide YY (PYY) and pancreatic polypeptide (PP) belong to the same peptide family and are both 36 amino acid

long, tyrosine containing peptides (18). PYY is produced in endocrine cells in the ileum and colon and is secreted after meals to delay gastric emptying. It binds to Y2 receptors in the hypothalamus which inhibit neuropeptide Y (NPY) positive neurons and depresses feeding (19). Intravenous infusion of physiological levels of PYY reduces caloric intake (20, 21). PP is released from pancreatic islet cells and act on Y4 and Y5 receptors. It reduces both appetite and food intake without affecting gastric emptying (22). On the other hand, a peptide that stimulates hunger is ghrelin. This is a hormone secreted from cells located throughout the gastro-intestinal tract. It is known for appetite stimulating actions though activation of NPY expressing neurons (23). Intravenous injection of ghrelin to normal weight human subjects increases food intake (24). This might be useful in the treatment of anorexia.

The role of insulin in the adaptive response to peripheral changes in the energy balance is well known. Secretion of insulin by pancreatic β-cells after a meal leads to glucose transfer into cells followed by energy production. A receptor-mediated transport system of insulin across the blood brain barrier (BBB) was described in the 1980s (25) and led to the proposal that insulin also might play a role in more long-term regulation of the energy balance. It has been shown that administration of insulin within the central nervous system suppresses food intake in rodents and sub-human primates and regulates expression of hypothalamic neuropeptides that influence appetite (26). In addition, deletion of insulin receptors in neurons produces obesity in mice (27).

White adipose tissue (WAT) is a loose connective tissue that is dispersed throughout the body and positioned subcutaneously and surrounding every internal organ. WAT was long regarded only as a storage compartment for excess energy with triglycerols constituting more than 85% of the tissue weight (28). It is now known that the adipocytes secrete many different cytokines known as adipokines, and the adipose tissue is therefore regarded as an endocrine organ. The endocrine function of WAT may be best characterized by the secreted factor leptin. The *ob* gene, which codes for the 16-kDa protein leptin, was first cloned in 1994 by Zhang et al (29), the year after the leptin receptor was characterized (30). A frequently used model of obesity is the *ob/ob* mice which have a homozygous mutation in the *ob* gene (31). These mice are leptin deficient, which results in hyperphagia, morbid obesity, diabetes, and other health complications. Complete leptin deficiency is not

a frequent cause of human obesity, only very few individuals have homozygous loss-of-function mutants of leptin or its receptor (32), but a syndrome of partial leptin deficiency might be more common (33).

Leptin circulates as both a free and bound hormone. The levels of both adipose tissue and plasma leptin are dependent on the amount of energy stored as fat as well as the status of the energy balance. Leptin levels are elevated in obese individuals (34) and increase with high-energy intake, while lean individuals have lower leptin levels. Leptin receptors are mainly located in appetite-modulating neurons in the hypothalamus (35). Leptin enters the brain by a saturable transport mechanism, possibly by receptor-mediated transcytosis across the BBB (36). The long form of the leptin receptor activates Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling among other signal transduction pathways (37). The product of the *ob* gene was named leptin, from Greek leptos meaning thin, because it decreased bodyweight and fat mass when injected into mice (38). It is now understood that leptin also serves as an important signal of starvation when levels are low. This function is likely to be as important as its antiobesity role. The fact that leptin levels are elevated in most obese individuals is thought to indicate a state of leptin resistance. Two general mechanisms of leptin resistance have been proposed. The first may involve a defect in receptor-mediated leptin transport across the BBB to sites within the brain critical to regulation of energy balance (39). The other mechanism involves members of the suppressors of cytokine signaling (SOCS) family. SOCS3 is induced in hypothalamic neurons in a leptin dependent manner and is an antagonist of leptin signaling (40).

The adipose tissue secrets many other endocrine factors in addition to leptin (some of which are shown in Fig. 1). Adiponectin is an adipocyte-secreted collagen like protein that circulates at high concentrations (41). Levels of adiponectin are reduced in obesity, and the suppression correlates with insulin resistance (42). Levels can be induced by treatment with antidiabetic thiazoladinediones (TZDs). Resistin is another secreted protein and is induced in obesity. It might be in part responsible for systemic insulin resistance (43). The adipose tissue is known for its ability to metabolize sex steroids. The most important role of the sex steroids is in fat distribution. Estrogens stimulate adipogenesis in the breast and in subcutaneous tissue, while androgens promote central obesity (44). Central obesity has

been associated with insulin resistance, type 2 diabetes, hypertension, and coronary heart disease (44). Inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) are also produced and secreted by the adipose tissue. TNF-α regulates key components of fat metabolism, and has a net effect to prevent obesity through inhibition of lipogenesis, increased lipolysis, and facilitation of adipocyte death via apoptosis (45). IL-6 is an immune modulating cytokine which expression in adipocytes is increased in obesity. IL-6 deficient mice develop late-onset obesity that can be prevented by low-dose infusion of IL-6 into the brain (46, 47). The identity of upstream pathways responsible for this "inflammatory state" within adipose tissue remains an unanswered question.

As mentioned earlier, the hypothalamus is the main center for integrating signals that influence energy balance. Many signaling pathways involved in feeding and energy expenditure in the brain are activated and the best characterized of these is the melanocortin pathway (Fig. 2). This pathway involves two populations of neurons within the arcuate nucleus. One population expresses the orexigens (feeding-inducing) NPY and agouti related protein (AgRP), while the other population coexpresses mRNAs encoding the anorexigenic peptides cocaine and amphetamine related transcript (CART) and proopiomelanocortin (POMC) which is cleaved to α-melanocortin stimulating hormone (α-MSH) (26). AgRP and α-MSH are antagonistic ligands of the G protein-coupled receptor melanocortin 4 receptor (MC4R) (48). The activation of MC4R by α-MSH reduces food intake, while suppression of MC4R by the antagonist AgRP increases feeding (49). The dominant mutation causing the obese phenotype of the A^y mouse, another rodent obesity model, is due to ubiquitous expression of the coat color protein agouti (50). Agouti causes obesity by antagonizing the action of α-MSH on MC4Rs within the brain (51). There are different models on how these melanocortin signals produce downstream effects on appetite, energy expenditure, and neuroendocrine function. One of these models involves direct projection of the arcute melanocortinergic neurons (AgRP and POMC) onto neurons located within the lateral hypothalamus that express the orexigenic peptides melanin-concentrating hormone (MCH) and orexin (52). MCH stimulates food intake (53). Some of the functions of leptin can be understood through the actions of the melanocortin pathway. Leptin signaling induces the expression of CART and α-MSH, while the action

of leptin reduces the expression of NPY and AgRP (52), explaining the reduction of food intake when leptin levels rise.

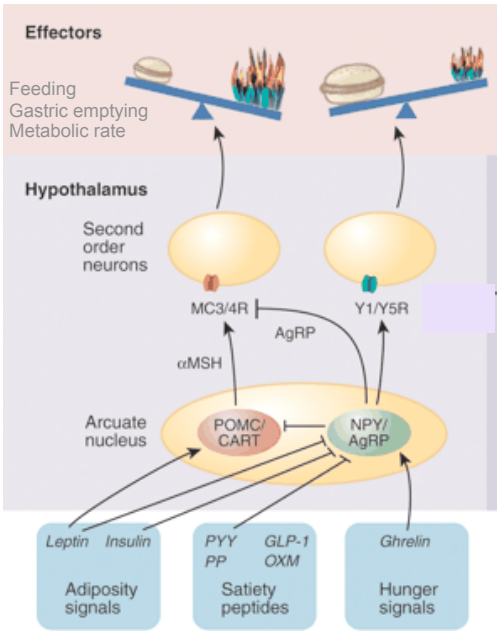

Figure 2. The melanocortin pathway. *Simplified representation of potential action of gut peptides and adipose factors on the hypothalamus. Circulating agents enter into the arcuate nucleus of the hypothalamus through the blood brain barrier. Integration of peripheral signals within the brain involves an interplay between the hypothalamus and hindbrain structures. Red - appetite-inhibiting neurons, green - appetite-stimulating neurons, → direct stimulatory, ⊥. – direct inhibitory. Figure from Badman and Flier 2005 (12).*

Adipogenesis

The adipose tissue is comprised of lipid-filled cells (adipocytes) surrounded by a matrix of collagen fibers, blood vessels, lymph nodes, fibroblasts, nerves and immune cells. It is an important component of the body's system of energy balance, and the major energy reserve in higher eukaryotes. Adipocytes are derived from mesenchymal stem cells which can also differentiate into muscle cells and chondrocytes under the right conditions (54). Adipogenesis, the differentiation of preadipocytes into mature adipocytes, is highly regulated in a temporal manner and is a well-characterized process *in vitro* (55). The cell systems most widely used for studying adipogenesis *in vitro* are the preadipocyte cell lines 3T3-F442A and 3T3-L1 that are already committed to the adipocyte lineage (56). More recently, studies have also been performed using pluripotent mesenchymal stem cells that can be induced to differentiate into adipocytes, as well as cells of other lineages (57-59).

Mouse models, where important adipose factors are over-expressed or knocked out, also present new knowledge about adipocytes and adipogenesis (31, 60).

3T3-L1 preadipocytes are morphologically similar to fibroblasts. After induction of differentiation the cells convert to a spherical shape, start to accumulate lipid droplets, and acquire morphological and biochemical characteristics of mature white adipocytes. Initially, preadipocytes enter growth arrest when they reach confluence, although cell-cell contact is not strictly required for growth arrest to occur (61, 62). The initial phase of growth arrest is followed by one or two additional rounds of mitosis, known as mitotic clonal expansion (MCE). MCE is a prerequisite for differentiation of 3T3-L1 preadipocytes into adipocytes (63).

Growth arrest and MCE are accompanied by complex changes in gene expression. Many different transcription factors are critical to adipocyte differentiation (Fig.3); the most important factors will be discussed here. Expression of CCAAT/enhancer-binding protein-β (C/EBPβ) and C/EBPδ has a temporal rise during MCE (64), and it has been established that C/EBPβ is required for MCE to take place (65). C/EBPβ and C/EBPδ are two of the members of the C/EBP family of transcription factors. These proteins contain a highly conserved basic leucine zipper domain which mediate homo- and hetero-dimerization, and are expressed in a variety of tissues (66). Recent gain- and loss-of function studies indicate that these proteins have a profound effect on fat cell development (67). Expression of C/EBPα in adipogenesis is thought to be transcriptionally activated by C/EBPβ (68). C/EBPα activates the expression of many adipocyte genes, including adipocyte-specific fatty acid binding protein (aP2), lipoprotein lipase (LPL), glycerophosphate dehydrogenase (GAPDH), and fatty acid synthase, which give rise to the adipocyte phenotype. In addition to activating the expression of C/EBPα, C/EBPβ also activates transcription of the peroxisome proliferator-activated receptor-γ (PPARγ) (68). A positive feedback loop exists that mutually reinforces the expression of C/EBPα and PPARγ. PPARγ is a member of the nuclear receptor protein subfamily that forms heterodimers with the retinoid X receptor (RXR). It regulates gene transcription through the binding to response elements as a heterodimer complex with RXRs. Use of high-affinity selective PPARγ agonists TZDs and *PPARγ* null mice has been important in establishing PPARγ as a critical factor for adipogenesis (67).

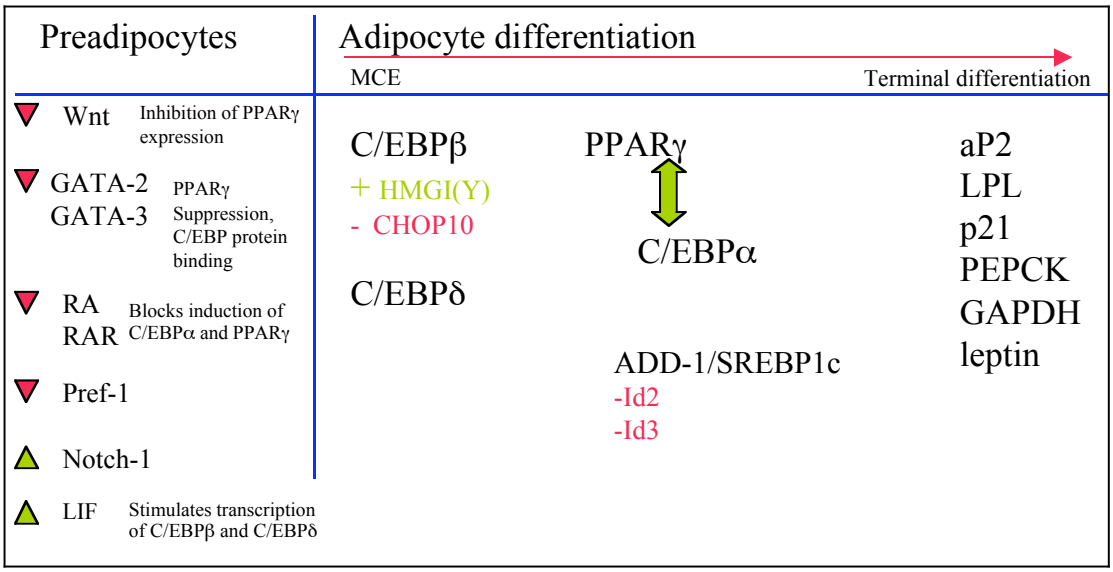

Figure 3- Transcription factors involved in adipogenesis. *The figure summarizes some of the transcription factors that are most critical to adipogenesis and some of the factors critical to the adipocyte phenotype. Some factors expressed in preadipocytes must be down-regulated in order to initiate adipogenesis. Retinoic acid (RA), retinoic acid receptor (RAR), preadipocyte factor-1 (Pref-1), leukemia inhibitory factor (LIF), high-mobility group I (Y) (HMGI(Y)), C/EBP homologous protein-10 (CHOP10), adipocyte determination and differentiation factor 1 (ADD-1), sterol regulatory element binding protein-1c (SREBP-1c), inhibitor of DNA binding (Id), phosphoenolpyruvate carboxykinase (PEPCK).*

Many intracellular and extracellular signals influence the growth of preadipocytes and terminal differentiation. Only some of the most important will be included here. Insulin is produced in pancreatic β cells and is involved in short-term regulation of energy homeostasis. It increases the percentage of cells that differentiate into mature adipocytes *in vitro* and also increases the amount of lipid accumulated in each fat cell (69) although it is not required for the differentiation of all preadipose cell lines (70). Preadipocytes express few insulin receptors and the effect of insulin on differentiation has been shown to occur through cross activation of the insulin-like growth factor receptor-1 (IGF-1) (56). Downstream signaling of insulin and IGF-1 include stimulation of Ras. Ectopic expression of activated Ras has been reported to induce adipogenesis without the need for hormonal stimulation (71). Ras is an activator of the mitogen-activated protein kinase (MAPK) pathway. The role of the MAPK pathway in adipogenesis will be discussed below. Akt is another downstream effector of insulin action. Expression of a constitutively activated allele of Akt in 3T3-L1 cells induces their spontaneous differentiation (72).

Glucocorticoids have been used for many years to optimize differentiation of cultured preadipose cell lines. The synthetic glucocorticoid dexamethasone is the most widely used glucocorticoid. It is thought to operate through the activation of the glucocorticoid receptor, which is a member of the nuclear receptor superfamily. Dexamethasone induces expression of C/EBPδ, which is involved in the early stages of differentiation (73). It has also been reported that dexamethasone reduces the expression of preadipocyte factor-1 (Pref-1), a negative regulator of adipogenesis (74). cAMP is also known to promote differentiation by inducing the transcription factor C/EBPβ (64). Isobutylmethylxanthine (IBMX) is a phoshodiesterase inhibitor that increases the half-life of cAMP and thereby potentiating agents that act by stimulating adenylyl cyclase, increasing the levels of cAMP in the cell. In addition, a variety of cytokines and growth factors have been found to suppress fat cell differentiation. The most studied proteins include TNF-α, IL-1, epidermal growth factor, and platelet-derived growth factor. The mechanisms underlying the effects of these factors are still unknown, though suppression of expression of key adipogenic factors have been observed (75).

The MAPK signaling pathway

MAPKs are important mediators of signal transduction and play a key role in regulation of many cellular processes, such as cell growth and proliferation, differentiation, and apoptosis as well as more short-term changes required for homeostasis and acute hormonal responses (76). Mammalian MAPK pathways can be activated by a wide variety of different stimuli acting through diverse receptor families, including hormones and growth factors that act through receptor tyrosine kinases, peptides acting through G protein-coupled receptors (GPCRs), transforming growth factor (TGF)-β-related proteins acting through Ser-Thr kinase receptors, and environmental stresses such as osmotic shock and ionizing radiation (77). Three groups of MAPKs have been well characterized; extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), and p38 MAPKs (Fig. 4). The last two families of MAPKs are often referred to as stress-activated protein kinases (SAPKs) which also includes the recently described ERK5 sub-family (78). The MAPKs are serine/threonine kinases regulated by phosphorylation cascades organized in specific modules. All modules include two additional protein kinases that are activated in series and lead to activation of a specific MAPK. MAP kinase kinases (MAPKKs) activate the MAPKs through phosphorylation on tyrosine and threonine

residues within a conserved Thr-X-Tyr motif in the kinase activation loop. Both the tyrosine and the threonine residue must be phosphorylated for the MAPKs to be active; as a consequence the MAPKs can be deactivated by all three major groups of protein phosphatases, serine/threonine phosphatases, tyrosine phosphatases, or dual specificity phosphatases (discussed below) (79). The MAPKKs are regulated by Ser/Thr phosphorylation, catalyzed by MAPK kinase kinases (MAPKKKs). The MAPK pathways are organized in complexes by scaffolding or linker proteins and although specific MAPK pathways are activated through specific signaling, several MAPKs may phosphorylate the same substrates and could also affect each other through cross-talk reactions and feedback mechanisms (78).

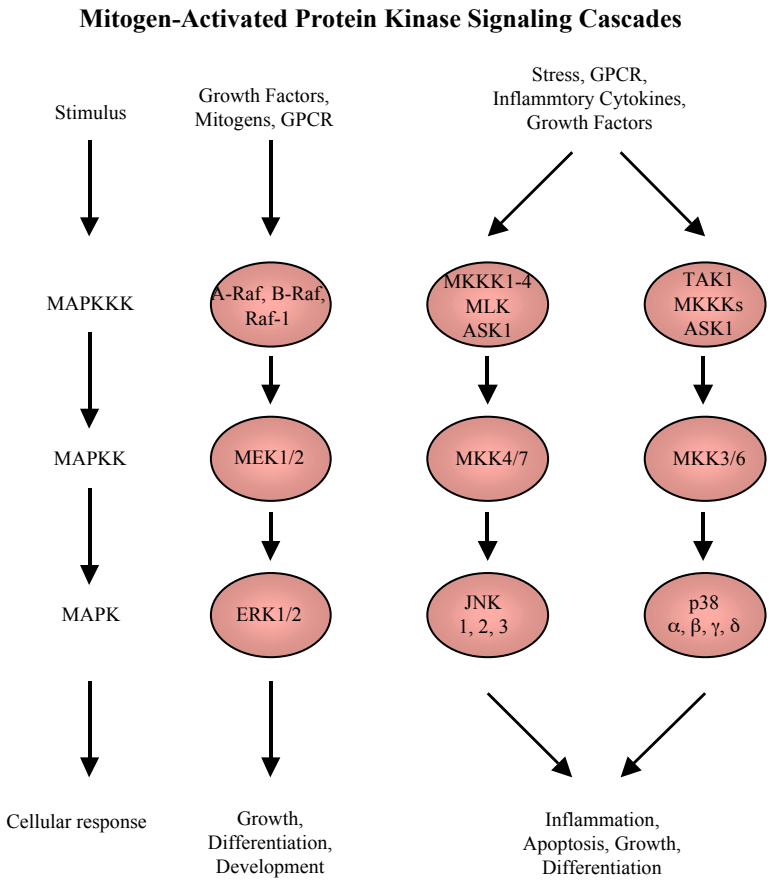

Figure 4. Overview of the MAPK signaling cascades. *The classic MAPK cascade consists of three sequential intracellular protein kinase activation steps and is initiated when a MAPKKK is activated and phosphorylates a MAPKK, which subsequently activates a MAPK. The activated MAPKs translocate to the nucleus to activate numerous proteins including transcription factors.*

The ERK pathway is preferentially activated by mitogens such as serum or growth factors and is an important regulator of cell cycle and proliferation (80). Most cell surface receptors can activate the Ras GTPases. Ras GTPases comprise a large family of mostly membrane-resident proteins that shuttle between an inactive GDP-bound and an active GTP-bound conformation (81). Activated Ras can bind to a number of different effector molecules, including the serine/threonine kinase Raf. All three Raf family members, A-Raf, B-Raf, and Raf-1 can bind to Ras. After binding to Ras, Raf can be activated by members of the Rho GTPase family through phosphorylation mediated by p21-activated kinase as well as by other kinases including Src and protein kinase C (82). Ras independent activation of Raf has also been reported (79). Raf in turn activates the dual-specificity kinases MAPK/ERK kinase 1 (MEK1) and MEK2 by phosphorylating two serines in the MEK activation loop (83, 84). MEK1/MEK2 are localized to the cytoplasm where they bind ERK1/ERK2 which are the only known substrates of MEK (85). Phosphorylation of ERK leads to dissociation of ERK from MEK and translocation from the cytosol to the nucleus. Within the nucleus ERK phosphorylates many transcription factors including nuclear factor-κB, c-Myc, cyclic AMP response element binding protein, and activating protein 1 (86).

Cytokines, different ligands for GPCRs, agents that interfere with DNA and protein synthesis, and many types of stress including UV and γ-irradiation activate the JNK pathways (87). There are three known mammalian isoforms of JNK; JNK1, JNK2, and JNK3 (79). The dual-specificity kinases MKK4 and MKK7 are known to directly activate JNK (88, 89). MKK4 and MKK7 transmit signals from many upstream activators such as apoptosis signal-regulating kinase-1 (ASK-1), mixed lineage kinases (MLKs), and MKKK1-4 (90). JNK inhibitory protein (JIP) is known to bind kinases at each level of the JNK pathway and is thought to act as a scaffolding protein (87). When activated, JNK activates different transcription factors such as c-Jun, activating transcription factor 2 (ATF-2), p53 and c-Myc (90). In addition, JNK also phosphorylates non-transcription factors such as Bcl-2 and Bcl-x_L, which is known to inhibit the anti-apoptotic activity of these proteins (91).

Members of the p38 MAPK family were first identified as kinases that were strongly activated by cytokines and were involved in pro-inflammatory activity (78). Four isoforms

of p38 MAPK have been identified (α, β, γ, and δ) and in addition to being activated by cytokines these kinases are also activated by different forms of cellular stress such as UV irradiation and osmotic shock (76). MKK3 and MKK6 are thought to be the major kinases responsible for p38 MAPK activation (79). Similar to the JNK pathway, MKK3 and MKK6 are also activated by the upstream protein kinases ASK1, MKKKs, and transforming growth factor-beta-activated kinase 1 (TAK1). In addition, Rho family GTPases take part in the regulation of p38 MAPK activity (90). p38 MAPKs control the function of transcription factors, kinases, and phosphatases such as ATF-2, myocyte-specific enhancer factor 2, and cell division cycle protein 25 (cdc25) (90). p38 MAPK pathways are involved in a variety of cellular responses including cell death (92), cell growth (93), and differentiation (94).

The role of MAPKs in adipogenesis

The development of obesity and expansion of the adipose tissue is a result of both hypertrophy and hyperplasia of adipocytes. Several studies have analyzed the role of MAPKs in differentiation of established preadipocyte cell lines *in vitro*. Because of its essential role in cell proliferation and the fact that adipogenic stimuli, such as insulin, activate the ERK pathway, the role of this pathway in adipogenesis has been extensively investigated (95-102). Initial studies reported that ERK is required for differentiation of 3T3-L1 cells. It was shown that the expression of transfected Ras oncogenes led to differentiation of 3T3-L1 cells into adipocytes in the absence of insulin, while transfection of a dominant inhibitory Ras mutant resulted in inhibition of differentiation (71). Since the oncogenic form of Ras protein is a strong activator of the ERK pathway, this suggests a positive role for ERK in adipogenesis. Another study confirmed these results by the use of an antisense strategy to decrease expression of ERK in 3T3-L1 cells. Knock-down of ERK blocked DNA synthesis by ~90% and prevented the differentiation of 3T3-L1 fibroblasts into adipocytes (101). Later, the discovery that ERK phosphorylates PPARγ and that this reduces the transcriptional activity of this adipogenic transcription factor and inhibits adipocyte differentiation (97, 99) made the picture more complicated. A specific MEK inhibitor, U0126, that was administered to 3T3-L1 cells during MCE, was shown to block differentiation (63). Other studies again found a positive role for ERK in adipocyte differentiation. The phosphorylation of C/EBPβ by ERK enhanced the DNA-binding activity of this transcription factor (102) and the activation of MEK/ERK signaling has

also been linked to enhanced expression of C/EBPα and PPARγ (100). These results indicate the importance of ERK activity early in adipogenesis. PPARγ expression is not detected during MCE and increases during terminal differentiation. On the other hand, ERK activity has been shown to decrease during later stages of differentiation (98), which could be necessary to avoid negative PPARγ phosphorylation (80). Preadipocyte cell lines have been extensively used to investigate the role of ERK in adipogenesis, but other model systems have also provided useful information. Activation of the ERK pathway has been shown to be required during early stages of adipocyte differentiation in embryonic stem cells (57). The use of *ERK1*$^{-/-}$ mice has also linked ERK to the regulation of adipocyte differentiation *in vivo*. *ERK1*$^{-/-}$ mice have decreased adiposity and fewer adipocytes than wild-type animals and are also resistant to high-fat diet induced obesity (96).

p38 MAPK is active in preadipocytes and early stages in adipogenesis. This activity decreases dramatically during later stages of differentiation. Treatment of 3T3-L1 cells with two different p38 MAPK inhibitors prevented the differentiation of these cells into adipocytes (103). It was shown in the same study that C/EBPβ bears a consensus site for p38 MAPK phosphorylation and serves as a substrate for p38 MAPK *in vitro*. The same p38 MAPK inhibitors also impaired transcriptional induction of PPARγ. Later it was shown that the induction of a constitutively active form of MKK6, an upstream activator of p38 MAPK, was sufficient to stimulate 3T3-L1 cells to differentiate into adipocytes (98). However, prolonged activation of p38 MAPK leads to cell death.

JNK has also been reported to be active at early stages of differentiation. Studies addressing JNK and its role in adipogenesis and obesity include evidence that PPARγ is phosphorylated by JNK and that this phosphorylation decreases PPARγ-dependent transcriptional activity (104). Studies using different rodent obesity models show that the absence of JNK1 results in decreased adiposity and that JNK activity is abnormally elevated in obesity (60).

MAPK phosphatases (MKPs)

Protein phosphorylation is a critical posttranslational modification that is involved in the regulation of many cellular activities. MAPK activation requires phosphorylation on a threonine and tyrosine residue within the activation loop of the kinase domain. Both

duration and magnitude of activation is crucial in determining the physiological outcome in the cell (105). Dephosphorylation is vital for the control of MAPKs and is carried out by protein phosphatases. Dephosphorylation of either the tyrosine or the threonine residue can result in enzymatic inactivation (106). Both protein serine/threonine phosphatases and protein tyrosine phosphatases have been reported to dephosphorylate MAPKs (107). Another family of phosphatases that has been recognized as key players for inactivating different MAPK isoforms is the MAP kinase phosphatases (MKPs). MKPs are dual specificity phosphatases (DSPs) that can dephosphorylate both the tyrosine and the threonine residue within the kinase activation domain. Activation of MAPKs can result in immediate gene transcription of important cellular proteins and cytokines as well as the transcription of MKPs. The transcription of MKPs provides a negative feedback mechanism for MAPK activity (108).

All MKPs share amino acid sequence identity, in particular in two domains. The dual specificity phosphatase catalytic domain contains the highly conserved consensus sequence -HCXXXXXR-, where X represents any amino acid, localized within the carboxyl-terminal half of these enzymes. The active site cleft in the DSP domain is able to accommodate both the phosphorylated tyrosine and threonine residues within the MAPK kinase domain (108, 109). The cysteine and arginine residues within the signature motif in the active site, and an additional highly conserved aspartate residue are essential for catalyzing the dephosphorylation reaction (106). In addition to the conserved motif in the active site, the MKPs also share sequence identity in two short regions in the N-terminal that are homologous to sequences in the cdc25 phosphatase. These motifs are catalytically inactive and their function is at present unknown.

In 1992 MKP-1 was identified as the first member of the MKP family (110). Sequence comparisons indicated strong similarity to the protein tyrosine phosphatase VH1 that was identified the previous year in vaccinia virus (111). Later, several other members of the MKP family have been characterized. To date there are 14 identified members. The MKPs are structurally and functionally distinct and can be grouped into four categories as listed below (108) (see Table 1 and Fig. 5).

Table 1. Overview of the members of the MKP family. *The table summarizes presently known features of the MKP proteins and includes names of the mouse and human orthologues, structure, substrate preference, and subcellular localization. Recently, a new addition to the MKP family was made, MKP-8 (112). This table is modified from Farooq et al. 2004 (108).*

	Name Mouse	Human orthologue	MAPK specificity	Subcellular localization	Accession No
Type I	MKP-6	DUSP-14	ERK ~ JNK >> p38	-	NM_019819
	DSP2	DUSP-22	p38 ~ JNK >> ERK	Nuclear/ Cytosolic	NM_134068
	VHR	DUSP-3	ERK >> JNK ~ p38	Nuclear	NM_028207
Type II	MKP-1	DUSP-1	p38 ~ JNK >> ERK	Nuclear	NM_013642
	MKP-2	DUSP-4	ERK ~ JNK ~ p38	Nuclear	AK080964
	MKP-3	DUSP-6	ERK >> JNK ~ p38	Cytosolic	NM_026268
	MKP-4	DUSP-9	ERK ~ JNK ~ p38	Nuclear/ Cytosolic	AY_316312
	MKP-X	DUSP-7	-	Cytosolic	NM_153459
	PAC-1	DUSP-2	ERK >> p38 ~ JNK	Nuclear	U09268
	VH3	DUSP-5	-	Nuclear	XM_140740
Type III	MKP-5	DUSP-10	p38 ~ JNK > ERK	Nuclear/ Cytosolic	NM_022019
Type IV	MKP-7	DUSP-16	JNK ~ p38 >> ERK	Cytosolic	NM_130447
	VH5	DUSP-8	JNK ~ p38 >> ERK	Nuclear/ Cytosolic	BC052705

Type I MKPs

Type I MKPs are approximately 200 amino acid residues in length and contain only the DSP domain. The three members that have been identified to date are MKP-6 (113), DSP2 (114), and VHR (115).

Type II MKPs

Type II MKPs are between 300 and 400 amino acid residues in length and contain an N-terminal MAP kinase-binding (MKB) domain in addition to the DSP domain. Members identified so far include MKP-1 (110, 116), MKP-2 (117), MKP-3 (118), MKP-4 (119), MKP-X (120), PAC-1 (121) and VH3 (122). These MKPs display different specificities towards MAPK substrates (see Table 1). Little is known about the specificity of MKP-X and VH3 towards MAPKs but both are known to dephosphorylate the ERK MAPK.

Type III MKPs

The only member identified in this subgroup so far is MKP-5. It is approximately 500 amino acid residues in length. In addition to the DSP domain and MKB domain characteristic of the type II MKP subgroup, MKP-5 also contains an N-terminal domain of unknown function (123, 124).

Type IV MKPs

Type IV MKPs are between 600 and 700 amino acids in length and contain both the DSP domain and MKB domain. In addition, members of this subgroup contain a sequence of approximately 300 residues C-terminal to the DSP domain. This sequence is rich in proline (P), glutamine (E), serine (S), and threonine (T) residues and is therefore referred to as a PEST sequence. It might be involved in degradation of type IV MKPs through ubiquitin-mediated proteolysis, which could be an important regulatory mechanism (125). The members of this subgroup include MKP-7 (126) and VH5 (127).

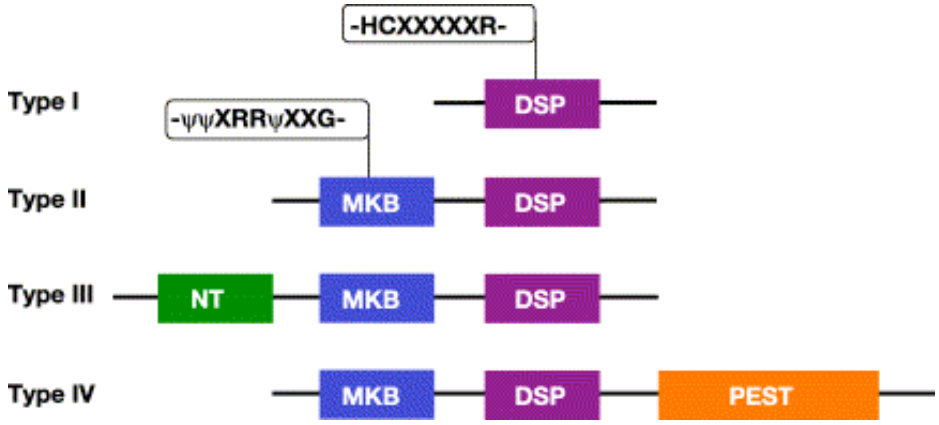

Figure 5. Subgrouping of the MKP family according to structure. *MKP family members and characteristics of each group are described in the text. Figure from Farooq et al. 2004 (108).*

Although much is known about the domains of the MKPs the structural basis of enzyme-substrate interactions and the mechanism of dephosphorylation of MAPKs are still not clear. The study of the crystal structure of an inactive mutant of VHR in complex with a biphosphorylated substrate has given some information to the mechanism of dephosphorylation (109), but more studies including three-dimensional structures of MKPs alone and in complex with MAPKs is necessary to complete the picture. What is already known is that many of the MKPs become catalytically activated through protein-protein interactions with different MAPKs. MKP-1 (128), MKP-2 (129), MKP-3 (130), MKP-4 (130), MKP-X (131), and PAC-1 (132) have all been reported to be catalytically activated upon substrate binding. In the cases of MKP-3 and PAC-1 it is known that this activation is due to a conformational change (132, 133). MKP-5 (123) is not reported to be activated upon substrate binding. VHR (109) and VH3 (134) are known to be in an optimal confirmation for catalysis.

Aim of the study

As documented in the introduction above, obesity is an increasing health problem in western industrialized countries. In short, obesity is a result of the expansion of the adipose tissue, both by hyperplasia and hypertrophy of the adipose cells. Based on present knowledge that MAPK activity is reduced during adipogenesis and that the MKPs are a family of phosphatases known to mediate the dephosphorylation of MAPKs, the goal of this study was to determine the expression and possible regulation of MKPs during adipogenesis.

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Manuscript

Expression and regulation of Mitogen Activated Protein Kinase (MAPK) Phosphatases (MKPs) in adipogenesis

Summary

Obesity has reached epidemic proportions in western industrialized countries and can be linked to a number of health risks such as hypertension, type 2 diabetes, coronary heart disease, and certain types of cancer. Obesity is due to a positive energy balance in the body that results in expansion of the adipose tissue caused by both hyperplasia and hypertrophy of adipocytes. Previous studies have found that MAPK activity is required in early stages of adipogenesis. It has also been reported that two of the MKPs, phosphatases known to dephosphorylate MAPKs, have a role in adipocyte differentiation. Based on this knowledge we carried out a systematic analysis of the expression and possible regulation of members of the MKP family during differentiation of 3T3-L1 preadipocytes. Results showed that most members of the MKP family are down-regulated during adipogenesis. We hypothesized that ectopic expression of one of the MKPs that was significantly down-regulated at early stages of differentiation, MKP-5, would have an effect on adipogenesis. Retroviral expression of MKP-5 in 3T3-L1 cells had a positive effect on adipocyte differentiation. In rodent obesity models *ob/ob* and A^y, most of the MKPs showed up-regulation in white adipose tissue, including MKP-5, with especially dramatic differences in subcutaneous fat depots. Although further studies are needed to verify these data, the results suggest that MKP-5, and possibly other MKPs, may have distinct roles during adipogenesis.

Introduction

Obesity has reached epidemic proportions in western industrialized countries. In the United States nearly 30% of the adult population are considered obese and up to 60% are considered overweight (1); in Norway the percentage of the population that is obese has increased drastically during the past 10 years and is now approximately 13% (2).

A number of severe health risks are directly linked to obesity, such as hypertension, coronary heart disease, type 2 diabetes, and certain types of cancer (3, 4). Obesity is a result of a positive energy balance in the body where the consumed energy exceeds the energy expended and is related to expansion of the adipose tissue. Energy homeostasis is highly regulated and the main centre for integrating signals regarding the body's energy state is the hypothalamus (5). Both long-term and short-term signals are integrated in this part of the brain and result in efferent outputs, such as regulation of appetite, energy expenditure, reproduction, and growth (6). Short-term peripheral signals are mainly meal related and are secreted by the gut while the adipose tissue secretes signals related to energy stores in a more long-term manner. Many systems have been used to study obesity and the role of adipose genes such as the established rodent obesity models *ob/ob* and A^{y}. These mouse models have mutations in genes involved in the regulation of the body's energy balance and have obese phenotypes, as well as other known characteristics (7).

The expansion of the adipose tissue related to obesity is due to both hyperplasia and hypertrophy of the adipose cells, which is the main component of this tissue. Given that mature adipocytes do not undergo cell division it is thought that increase in adipocyte number *in vivo* is a result of proliferation and differentiation of preadipocytes, also present in adipose tissue. This differentiation process, often referred to as adipogenesis, is especially well characterized in the established mouse preadipocyte cell lines 3T3-L1 and 3T3-F442A, where the expression and sequential induction of many different proteins and transcription factors has been described (8, 9). In short, the cells first undergo a limited number of cell divisions, known as mitotic clonal expansion (MCE) (10), then the cells become quiescent and start expressing adipocyte-specific genes, accumulate lipid droplets, and acquire biochemical and morphological characteristics of mature white adipocytes. Many intracellular and extracellular signals are known to influence the growth of

preadipocytes and induce terminal differentiation. These work through several different intracellular signaling pathways, one of which is the MAPK pathway.

MAPK signaling is activated by many different stimuli, such as mitogens, inflammatory cytokines, growth factors, and stress (11). Three main groups of MAPKs have been identified; ERK, JNK, and p38 MAPK, and these are known to be key regulators of diverse cellular processes, such as cell growth and proliferation, differentiation, and apoptosis (12). Several studies have analyzed the role of MAPKs in adipocyte differentiation. ERK has been shown to both induce and inhibit adipogenesis (13-16), something that at first seemed contradictory. Later, a hypothesis emerged stating that ERK activation is required for early stages of adipogenesis, but needs to be turned off for terminal differentiation (17). Both p38 MAPK and JNK have also been shown to be active early in adipogenesis (18). This activation is reduced towards the end of differentiation. In addition, JNK has been reported to affect adiposity in rodent obesity models (19).

MAPKs are activated by phosphorylation of both threonine and tyrosine residues in their kinase domain (20). The activation is mediated by upstream kinases and subsequently dephosphorylation by phosphatases is important in regulating their activity. A family of dual specificity phosphatases, also known as MAP kinase phosphatases (MKPs), has been recognized as key players for inactivating different MAPK isoforms (21, 22). To date, 14 different MKPs have been identified. They share some common features, such as a catalytic phosphatase domain, but they have also been reported to have distinct substrate specificity, different tissue distribution, and different subcellular localization (23).

One MKP of special interest in this study is MKP-5. Two different groups identified MKP-5 as a new member of the MKP family in 1999 (24, 25). MKP-5 is evenly distributed in both the cytoplasm and the nucleus of COS-7 and NIH3T3 cells. Studies of the ability of MKP-5 to dephosphorylate MAPKs indicate selectivity for p38 MAPK and JNK in COS-7 cells, NIH3T3 cells, and a human prostate carcinoma cell line (24-26), but it also showed some activity towards ERK in COS-7 cells (25).

Two members of the MKP family have previously been studied for their possible role in adipogenesis. One study found that MKP-1 was increased at both the mRNA and protein

levels during differentiation (27). Knock down of *MKP-1* reduced adipogenesis, an effect that was attributed to sustained activation of ERK throughout adipogenesis. Another study found that MKP-4 has an inhibitory effect on adipogenesis (28). The levels of MKP-4 are low in 3T3-F442A preadipocytes and are up-regulated during differentiation. MKP-4 has also been suggested to play a role in insulin resistance which is a fundamental aspect of type 2 diabetes, one of the most common health risks related to obesity (28). These previous results indicate a role for members of the MKP family in adipogenesis.

Since MAPKs have been implicated for adipogenesis and the MKPs are known to play a key role in their regulation we investigated the possible regulation of known members of the MKP family during differentiation of 3T3-L1 cells and in different tissues in two rodent obesity models.

Materials and methods

Materials

TRIzol reagent, glutaraldehyde, hygromycin, and SuperScript II reverse transcriptase were purchased from Invitrogen. Penicillin/Streptomycin, L-glutamine, Trypsin, and DMEM were purchased from Bio-Whittaker-Cambrex. Neomycin, Oil Red O, insulin, IBMX, dexamethasone, oligo(dT), fetal calf serum (FCS), Triton X-100, HePes, EDTA, $MgCl_2$, NaCl, dithiothreiol (DTT), β-glycerophosphate, sodiumortovanadate, phenylmethylsulphonyl-fluoride (PMSF), leupeptin, Tween 20, bovine serum albumin (BSA), horseradish peroxidase-conjugated anti-rabbit IgG antibody, horseradish peroxidase-conjugated anti-mouse IgG antibody, and anti-α-tubulin mouse monoclonal antibody were from Sigma-Aldrich. LightCycler instrumentation, capillaries, and LightCycler® FastStart DNA Master SYBR Green I were obtained from Roche. The phase contrast microscope was from Olympus. Bradford solution, Precision Plus Protein dual protein marker and PVDF membrane were obtained from Bio-Rad Laboratories. T4 DNA Ligase, BamHI and HindIII restriction enzymes were from New England Biolabs. The pGEM-T Easy vector was obtained from Promega. pREV-TRE and pRevTet-Off retroviral vectors were delivered by BD Biosciences. QIAquick Gel Extraction Kit was purchased from QIAGEN. Phusion™ High-Fidelity DNA Polymerase and DyNAzyme™ II DNA Polymerase were from Finnzymes. Anti-phospho-JNK polyclonal antibody, anti-total JNK

antibody, anti-phospho-ERK antibody, and anti-total ERK antibody were from Cell Signaling. MKP-5 antibodies were obtained from Abcam and Imgenex. Horseradish peroxidase-conjugated anti-goat IgG antibody was purchased from Santa Cruz Biotechnology. The aP2 antiserum was a generous gift from Gökhan Hotamisligil, Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, USA. Adipose tissue was a generous gift from Arne Klungland, Rikshospitalet University Hospital, Oslo, Norway. Hepa1c1c-7 cells were a generous gift from Trond Berg, Department of Molecular Biosciences, University of Oslo, Norway. J55 cells were a generous gift from Inger Sandlie, Department of Molecular Biosciences, University of Oslo, Norway.

Cell culture

3T3-L1 cells and BOSC cells (viral packaging cells) obtained from ATCC were grown in DMEM supplemented with 10% fetal calf serum (FCS), 50U/ml penicillin, 50µg/ml streptomycin and 2mM L-Glutamine. The cells were incubated at 37°C in a humidified 5% CO_2, 95% air incubator. Differentiation of 3T3-L1 cells was induced by adding 0.5mM isobutylmethylxanthine (IBMX), 1µM dexamethasone and 5µg/ml insulin to cells grown to confluence. After 48 hours the cells were re-fed with DMEM supplemented with 10% FCS and 5µg/ml insulin for 12 days. Cells were harvested at the following points: 50% confluence (day -2), 100% confluence (day 0), 2, 4, 6, 8, or 12 days after changing the medium and supplementation with 5µg/ml insulin. 3T3-L1 cells stably expressing MKP-5 and control cells infected with empty vector were kept in DMEM containing 20% fetal calf serum and differentiation was induced as described above, in the absence of selecting agent.

Oil Red O staining

3T3-L1 cells were fixed at the indicated time points using 0.5% glutaraldehyde/PBS for 10 minutes and stained with 0.15% of the triglyceride specific dye, Oil Red O, until aggregates started to form (approximately 15 min). Cells were then rinsed in PBS and 60% isopropanol prior to photography using a phase contrast microscope with the 20×objective.

RT-PCR

Total RNA was extracted from 3T3-L1 cells, mouse adipose tissue, Hepa1c1c-7 cells, and J55 cells using the TRIzol reagent, following the manufacturer's instructions. 2-5µg RNA was reverse transcribed using SuperScript II reverse transcriptase and oligo(dT) primers. Gene specific primers were designed by the web-based program Primer3 Output (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). In each set the 3´and 5´ primers were chosen from different exons to avoid genomic amplification. This was later confirmed by performing RT-PCR on cDNA samples synthesized in the presence or absence of SuperScript II reverse transcriptase. Samples that were not reverse transcribed did not give PCR products. Primer sequences and conditions are listed in table 1.

Each PCR reaction contained 3µl template, 0.2mM dNTP, 0.2µM of each primer, and 1U DyNAzyme™ II DNA Polymerase in a total volume of 25µl. PCR conditions included an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of 94°C denaturation for 30 seconds, 60-64°C annealing for 30 seconds (see Table 1 for the annealing temperature for different primer pairs), and 72°C extension for 90 seconds.

Quantitative PCR

The same primers as described above were used for quantitative PCR, which was carried out on LightCycler instrumentation. Each PCR reaction contained 1µl cDNA template, 0.5µM of each primer, 2-4mM $MgCl_2$, and 0.25µl LightCycler® FastStart DNA Master SYBR Green I in a total volume of 10µl added to LightCycler capillaries. $MgCl_2$ concentrations and temperature conditions were optimized for each primer pair. Cycling conditions include an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles of 95°C denaturation for 10 seconds, 60-64°C annealing for 5 seconds (see Table 1), and 72°C extension for 20 seconds. A negative control, containing only water as template, was included for each primer pair. Melting curve analysis was performed for each reaction to confirm specific PCR products. SYBR Green I, a double-stranded DNA specific dye, was used for continuous fluorescence monitoring. The crossing point (CP) values, defined as the cycle at which the fluorescence of the sample rises significantly above the background fluorescence, were used to determine relative expression of the specific gene. All relative concentrations were normalized to a housekeeping gene to compensate for variations in quality and quantity of different samples. The ribosomal protein 36B4 mRNA

was used as a housekeeping gene for 3T3-L1 cells (29). All data analysis was performed using Microsoft Excel.

Cloning and vectors

MKP-5 was amplified from cDNA made from RNA of 50% confluent 3T3-L1 cells using Phusion™ High-Fidelity DNA Polymerase and primers (MKP-5 open reading frame (ORF)) as specified in Table 1. The PCR product was run on a 1% agarose gel, and the fragment was isolated from the gel using QIAquick Gel Extraction Kit following the manufacturer's instructions. The isolated PCR product was A-tailed using DyNAzyme™ II DNA Polymerase before ligation into the pGEM-T easy vector using T4 DNA Ligase. The sequence of the insert was then verified by sequencing before further use. MKP-5 cDNA was cut out of the pGEM-T easy vector using BamHI and HindIII restriction enzymes and ligated into the same sites of pREV-TRE vector.

Table 1. *Conditions and sequences of primers used for quantitative PCR (Q-PCR) and RT-PCR*

Name	**Sense**	**Antisense**	Annealing-temperature (RT-PCR)	Annealing temperature (Q-PCR)	[$MgCl_2$] (Q-PCR)
MKP-1	catcaaggatgctggaggga	gaggtaagcaaggcagatggtg	64°C	64°C	4mM
MKP-2	ctgtacctcccagcaccaat	actcgatggcttccatgaac	60°C		
MKP-3	ccaacttggacgtgttggaa	aatgcaccaggacaccacag	62°C	62°C	2mM
MKP-4	ggagcaaggcaggaacagagt	ccaccagtaggcacgtgaaat	64°C	64°C	3mM
MKP-5	cttgcccttcctgttccttg	agcaactgccccatgaagtt	62°C	64°C	3mM
MKP-6	gccttagcaggtgcagtagg	tcccagggcacactaatttc	62°C	62°C	2mM
MKP-7	cagcgagatgtcctcaacaa	taagcacacagccattggag	60°C	60°C	2mM
MKP-X	ctgtccagatcctgccctac	ccccatgaagttgaagttgg	62°C	64°C	3mM
DSP-2	attcatgagtgccgactcca	cagcaccagcctgaactttg	60°C	60°C	2mM
VHR	cgtctgtggctcaggacatc	cattgagctggcagagttgg	60°C		
36B4	aagcgcgtcctggcattgtct	ccgcaggggcagcagtggt		64°C	3mM
aP2	gtcaccatccggtcagagag	tcgactttccatcccacttc		64°C	3mM
MKP-5 ORF	cgggatcccgatgcctccatct *BamHI*	cccaagcttgggtcacacaactg *HindIII*	66°C		

Retroviral expression assay

The use of retroviruses is an efficient approach to deliver a mammalian expression vector containing a gene of interest to a cell line (30). The viruses are modified so that they are no longer able to replicate autonomously, but after packaging by the help of a packaging cell line, the virus particles, containing recombinant DNA, can infect target cells. After infection of the target cells the DNA is integrated into the genome, making it possible to obtain cells that stably express the gene of interest. The expression vector contains a selection marker that is used to select the cells that have been successfully infected.

The protocol from BD Biosciences was followed. Briefly, 2×10^5 BOSC cells were plated out in $6cm^2$ dishes one day prior to transfection. Cells were transfected with 5µg pREV-TRE/pREV-TRE-MKP-5 or pRevTet-Off by adding 75mM $CaCl_2$, 100µM cloroquine and Hepes buffer saline (to final concentrations of 16.8mM NaCl, 3mM Hepes and 90µM Na_2HPO_4) to 4ml medium. 2×10^4 3T3-L1 cells were plated out in $6cm^2$ dishes one day prior to infection with viral supernatant. Viral supernatant was collected from BOSC cells 48 hours post transfection and added to target cells in 4ml medium containing 1µg/ml polybrene. Cells were grown to 70-80% confluence before selection with 200µg/ml hygromycin for 6 days (pREV-TRE/pREV-TRE-MKP-5) or 400µg/ml neomycin (pRevTet-Off) for 10 days. 3T3-L1 cells were sequentially infected and selected, first with pREV-TRE/ pREV-TRE-MKP-5, then pRevTet-Off.

Western Blot analysis

Whole cell protein extracts from 3T3-L1 cells were prepared by resuspending cells in 80-100µl of lysis buffer [0.1% Triton X-100, 20mM HePes (pH 7.7), 300mM NaCl, 0.2mM EDTA, 1.5mM $MgCl_2$, 0.5mM DTT, 20mM β-Glycerophosphate, 0.1mM sodiumortovanadate, 0.5mM PMSF, and 2µg/ml leupeptin]. After two hours of rotation at 4°C, suspensions were centrifuged at 13000 rpm for 10 minutes. The post-nuclear supernatant was collected and stored at -80°C. Protein concentrations were determined using the Bio-Rad protein assay. 50-100µg of proteins were reduced by adding 100mM DTT and heated to 100°C for 3 minutes prior to SDS-polyacrylamide gel electrophoresis on 12-15% separating and 6% stacking gel. A Precision Plus Protein dual protein marker was used as a molecular weight marker. The proteins were transferred to a PVDF membrane activated in methanol. The blotted membrane was blocked in 5% milk in TBS-

Tween for 1 hour prior to incubation with primary antibody overnight at 4°C. The antibodies used were aP2 polyclonal antibody (1:2000), anti-phospho-ERK (phERK) polyclonal antibody (1:1000), anti-total ERK polyclonal antibody (1:1000), anti-phospho-JNK (phJNK) polyclonal antibody (1:500), anti-total JNK polyclonal antibody (1:1000), and anti-α-Tubulin monoclonal antibody (1:4000). Two different anti-human MKP-5 antibodies were tested. The aP2 antiserum was IgG-purified before use, following the protocol described in Molecular Cloning (3.rd edition, Sambrode and Russell). Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:10000), horseradish peroxidase-conjugated anti-mouse IgG antibody (1:5000), and horseradish peroxidase-conjugated anti-goat IgG antibody (1:8000). The enhanced chemiluminescence kit was used for detection according to the manufacturer's instructions.

Statistics

Statistical analysis was performed using the Student's t-test. Values of $p<0.05$ were considered significant.

Results

Regulation of MKP expression during adipogenesis and in mouse models of obesity

The mouse preadipocyte cell line 3T3-L1 was differentiated and used as a model system to investigate expression and possible regulation of MKPs during adipogenesis. Differentiation of 3T3-L1 adipocytes was confirmed by lipid staining and gene expression analysis. Oil Red O staining of 3T3-L1 cells show a gradual accumulation of lipids during adipogenesis by red staining of triglycerides and cholesteryl oleate (31). There was robust Oil Red O staining after differentiation (Fig. 1A). Adipocyte-specific fatty acid binding protein (aP2) is induced early in adipogenesis by the transcription factor C/EBPα (32). Up-regulation of aP2 levels during adipogenesis was determined by quantitative PCR and Western Blot analysis. As shown in Fig 1, levels of aP2 mRNA (Fig. 1B) and protein (Fig. 1C) were increased starting at day 2 post induction, and remained elevated throughout terminal differentiation.

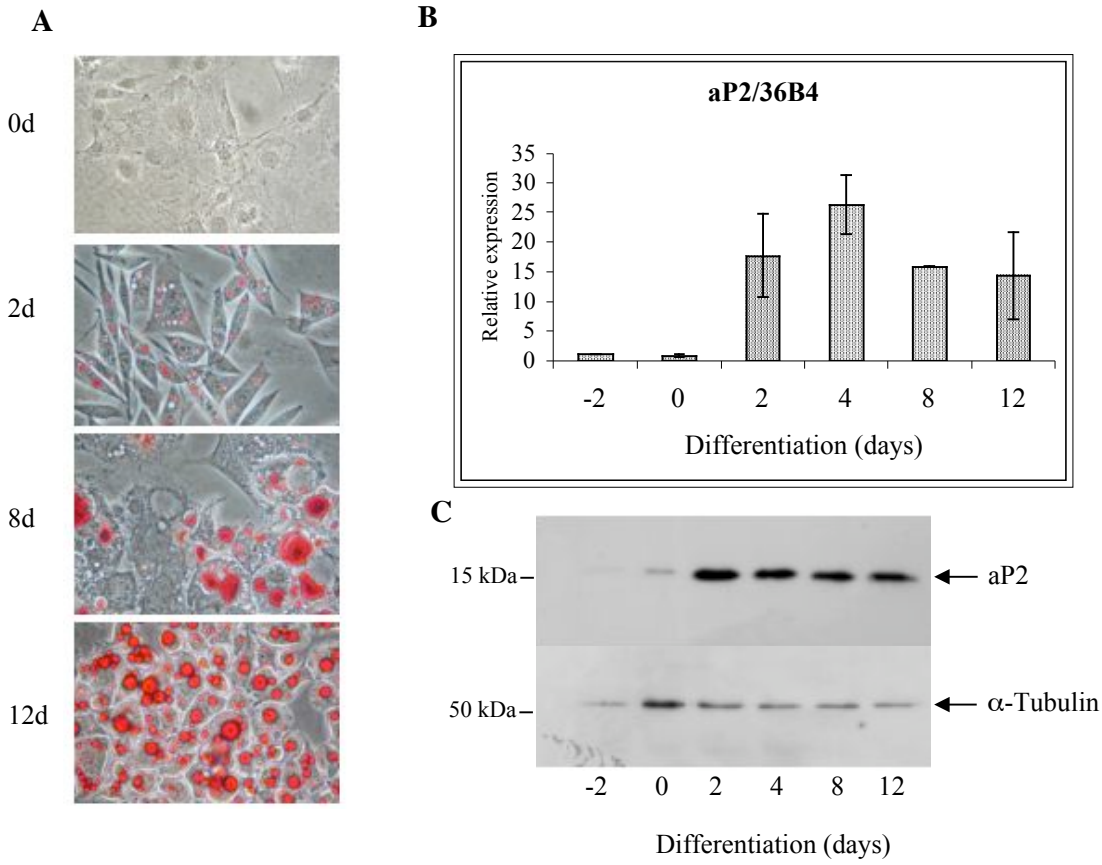


Figure 1 – Differentiation of 3T3-L1 cells. *Differentiation was induced in 100% confluent 3T3-L1 cells (day 0) using 0.5mM IBMX, 1µM dexamethasone and 5µg/ml insulin. After 48 hours in induction medium, the cells were continuously stimulated with 5µg/ml insulin and harvested after 2, 4, 8, and 12 days. Differentiation was verified by Oil Red O staining of lipids (panel A), and induced expression of aP2 mRNA (normalized to 36B4 mRNA expression, relative to day –2 samples) (panel B) and aP2 protein (panel C) analyzed by quantitative PCR and Western Blot analysis, respectively.*

First, RT-PCR was performed on mRNA obtained from preadipocytes (50-60% confluent 3T3-L1 cells), fully differentiated 3T3-L1 cells (day 12), adipose tissue from mice, and cell lines known to express MKPs (Hepa1c1c-7 and J55) to assess expression of different MKPs and confirm that the primer sets were functional. MKP-1, MKP-2, MKP-3, MKP-4, MKP-5, MKP-6, MKP-7, MKP-X, VHR, and DSP2 were all found to be expressed in preadipocytes as well as in mature adipocytes and mouse adipose tissue (Supplementary Fig. 1).

To analyze possible regulation of different MKPs during adipogenesis, quantitative PCR was performed on mRNA isolated from 3T3-L1 cells harvested at different time-points

during differentiation. Regulation of the different MKPs at the mRNA level is shown in Fig. 2. MKP-1 and MKP-4 were down-regulated during adipogenesis starting after induction of differentiation (at day 0). MKP-3, MKP-7, and MKP-X were down-regulated at days 8 and 12 post induction. Most significant was the regulation of MKP-5 and MKP-6. MKP-5 was significantly down-regulated at day 2 post induction and mRNA levels increased slightly towards the end of adipogenesis. MKP-6 decreased at day 0, increased at day 2 and finally decreased again. DSP2 was relatively stably expressed during adipogenesis.

We then assessed the possible regulation of MKP expression in mouse models of obesity. The *ob/ob* mouse is a frequently used model to study obesity. *ob/ob* mice display early-onset obesity and insulin resistance, as well as hyperphagia, reduced energy expenditure and infertility (7). Different tissues from *ob/ob* mice were tested for regulation of MKP expression by quantitative PCR (Fig. 3A). MKP-1 was up-regulated in brown adipose tissue (BAT) and subcutaneous adipose tissue. MKP-4 was highly up-regulated in subcutaneous adipose tissue. MKP-5, MKP-6 and DSP2 were up-regulated in liver and subcutaneous adipose tissue. MKP-7 was up-regulated in subcutaneous adipose tissue. MKP-X was up-regulated in epididymal and subcutaneous adipose tissue. MKP-3 was not significantly regulated. The same MKPs were also analyzed for regulation in A^y mice, which is a different mouse model of obesity (33) (Fig. 3B). In general, the same pattern of regulation is observed in A^y mice, although the MKPs seem to be less extensively regulated. In contrast to what is observed in *ob/ob* mice, MKP-3 expression is reduced in A^y compared to wild-type (wt) mice.

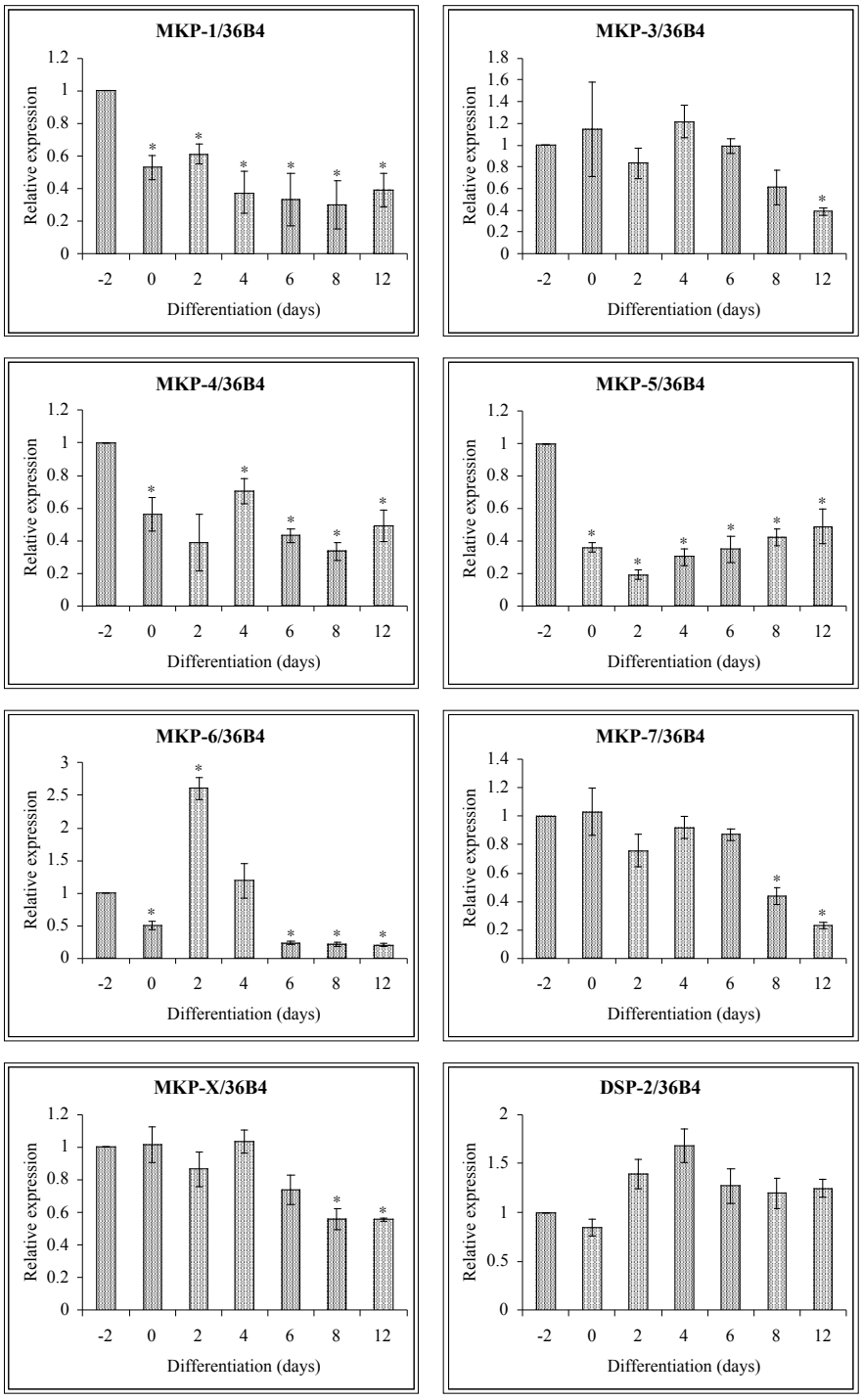
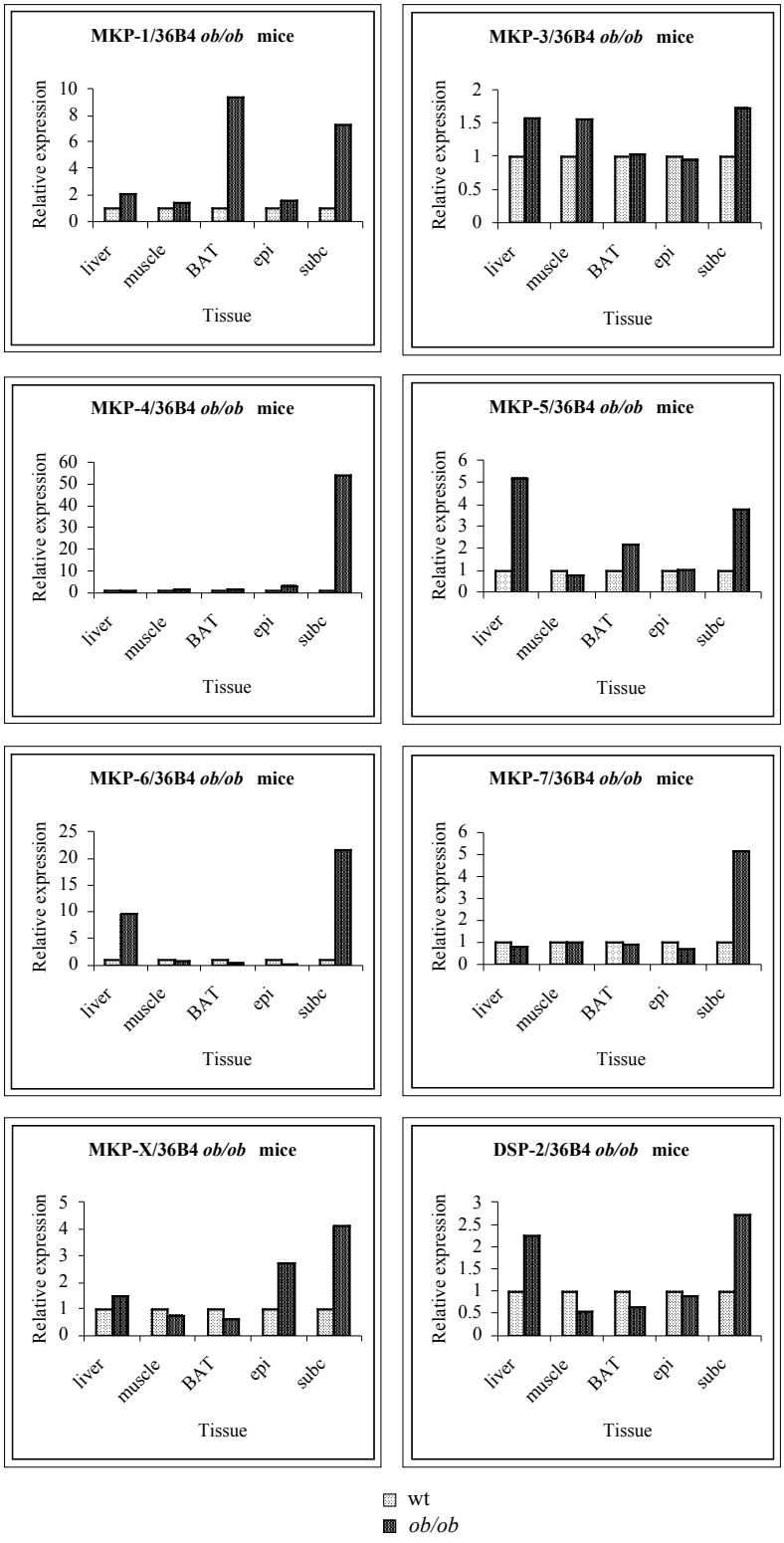


Figure 2- MKP mRNA levels during differentiation of 3T3-L1 cells. *3T3-L1 cells were induced to differentiate and were then harvested at the indicated time points. Total RNA was isolated and converted to cDNA and expression of MKP-1, MKP-3, MKP-4, MKP-5, MKP-6, MKP-7, MKP-X, and DSP2 was measured by quantitative PCR (normalized to 36B4 mRNA expression, relative to day –2 samples). The result presented is the average of three independent experiments +/-SE.* ****** *$p < 0.05$ indicates significant difference from –2 day samples.*

A


MKP-1/36B4 ob/ob mice
MKP-3/36B4 ob/ob mice
MKP-4/36B4 ob/ob mice
MKP-5/36B4 ob/ob mice
MKP-6/36B4 ob/ob mice
MKP-7/36B4 ob/ob mice
MKP-X/36B4 ob/ob mice
DSP-2/36B4 ob/ob mice
Relative expression
liver
muscle
BAT
epi
subc
Tissue
wt
ob/ob

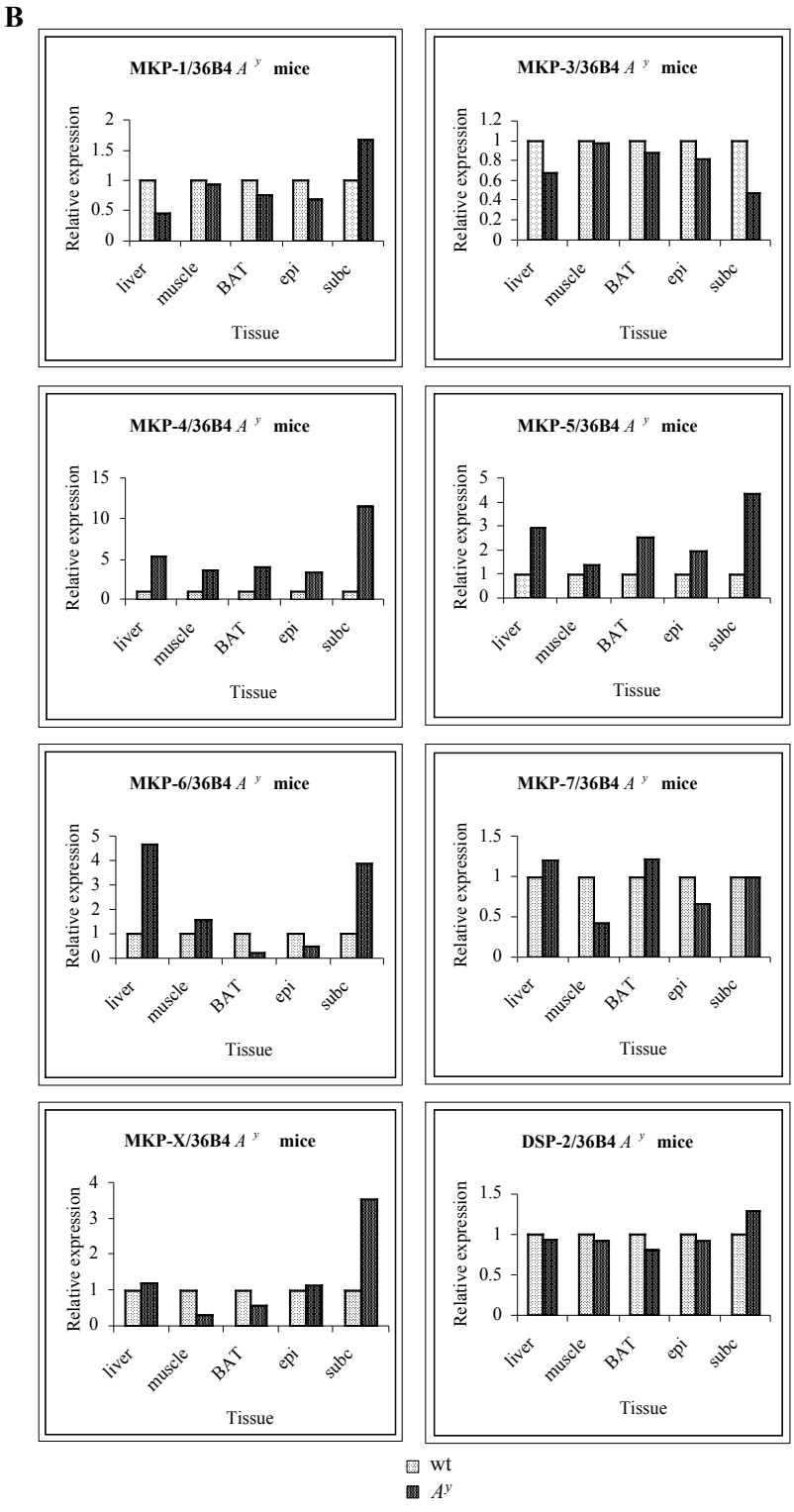


Figure 3- MKP mRNA levels in tissue samples from ob/ob, A^y, and wt mice. *RNA was extracted from liver tissue, muscle tissue, BAT, epididymal (epi) and subcutaneous (subc) white adipose tissues from ob/ob and wt mice (panel A) and A^y and wt mice (panel B) and converted to cDNA. Expression of MKP-1, MKP-3, MKP-4, MKP-5, MKP-6, MKP-7, MKP-X, and DSP2 was analyzed by quantitative PCR (normalized to 36B4 mRNA expression, relative to wt samples). Each sample was a pool from 2 mice.*

Ectopic expression of MKP-5 in 3T3-L1 cells enhances adipogenesis

MKP-5 expression was significantly regulated during adipogenesis (Fig. 2) as well as in subcutaneous adipose tissue of *ob/ob* and A^y mice (Fig. 3A and 3B); it was therefore chosen to study its possible effect on adipogenesis in 3T3-L1 cells by retroviral expression. The full length MKP-5 cDNA (1500bp) was cloned from 3T3-L1 preadipocytes and ligated into the expression vector pREV-TRE (Fig. 4A and 4B). Induced expression of MKP-5 (8-12 fold) was verified on the mRNA level by quantitative PCR analysis (Fig. 4C). Western blot analysis was performed using two different antibodies, but an MKP-5 signal was not detected probably due to the fact that they were raised against the human MKP-5 (data not shown). At present, there are no antisera available for mouse MKP-5.

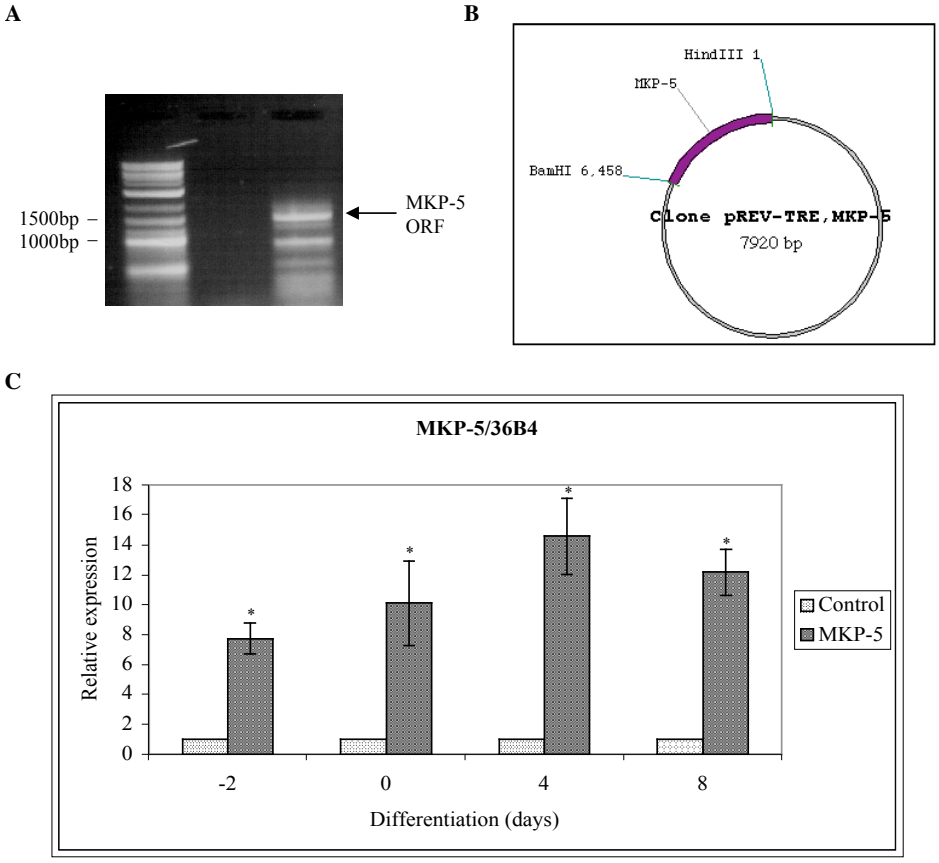


Figure 4- Ectopic expression of MKP-5 in 3T3-L1 cells*. The MKP-5 ORF was amplified from 3T3-L1 preadipocytes (panel B) and ligated into the pREV-TRE expression vector (panel A). 100% confluent cells infected with pREV-TRE-MKP-5 or empty pREV-TRE retroviruses were induced to differentiate using 0.5mM IBMX, 1µM dexamethasone and 5µg/ml insulin. After 48 hours in induction medium, the cells were continuously stimulated with 5µg/ml insulin and harvested after 2, 4, and 8 days. Total RNA was converted to cDNA and MKP-5 expression was analyzed by quantitative PCR (normalized to 36B4 mRNA expression, relative to control samples) (panel C). The results shown are the average of three independent experiments +/- SE. * $p<0.05$ indicates significant difference from control cells.*

3T3-L1 cells transfected with an empty vector (control) and cells ectopically expressing MKP-5 were induced to differentiate in parallel. Lipid staining and gene expression analysis was used to assess the extent of differentiation. Oil Red O staining indicated that MKP-5 over-expressing cells had enhanced adipogenesis compared to control cells (Fig. 5). In addition, aP2 mRNA and protein levels were significantly increased.

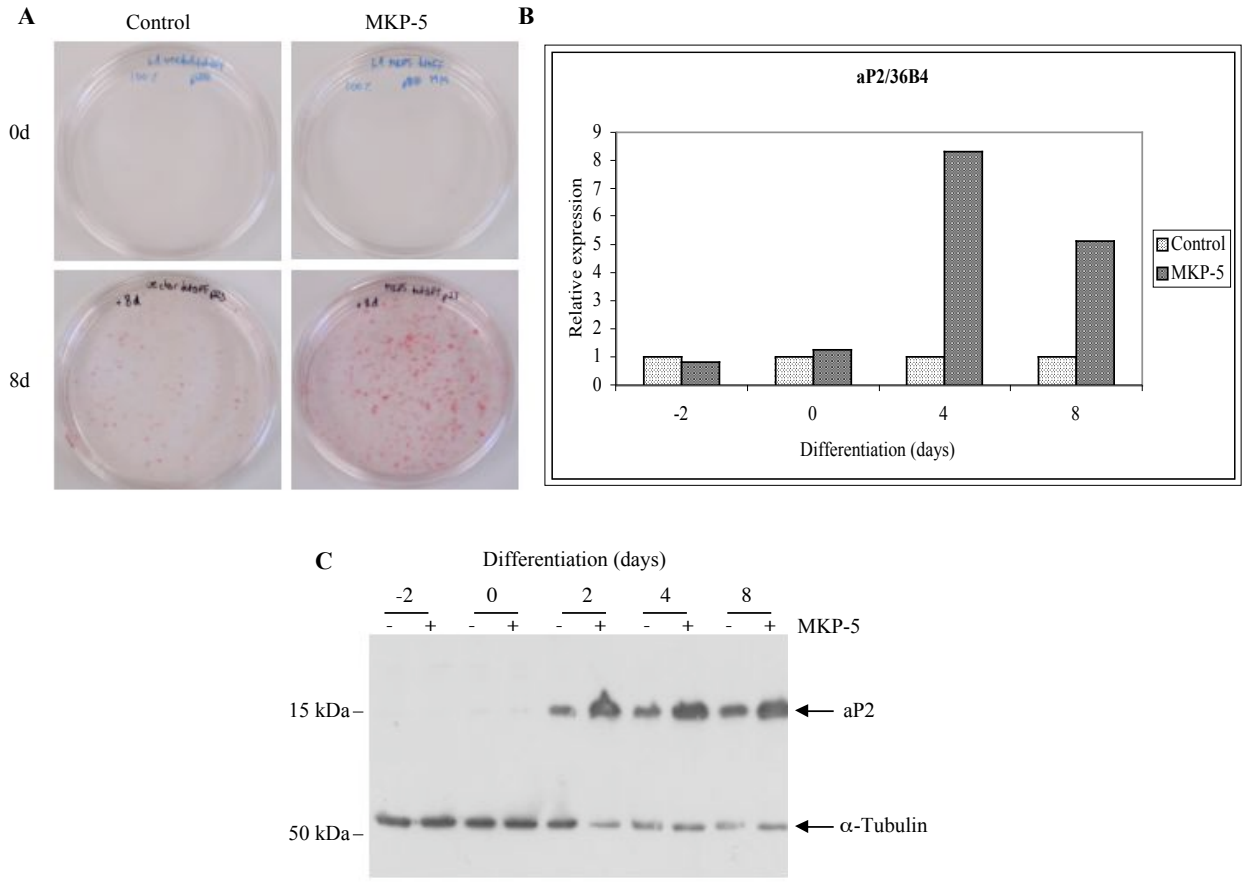


Figure 5- Effect of MKP-5 expression on adipogenesis. *3T3-L1 cells ectopically expressing MKP-5 and control cells were induced to differentiate using 0.5mM IBMX, 1µM dexamethasone and 5µg/ml insulin. After 48 hours in induction medium, the cells were continuously stimulated with 5µg/ml insulin and harvested after 2, 4, and 8 days. Differentiation was verified by Oil Red O staining of lipids (panel A), and induced expression of aP2 mRNA (normalized to 36B4 mRNA expression, relative to day –2 sample) (panel B) and aP2 protein (50µg) (panel C), analyzed by quantitative PCR and Western Blot analysis respectively. Results shown here are representative of three individual experiments.*

ERK, but not JNK activity is regulated by MKP-5 in 3T3-L1 cells

MKP-5 has earlier been reported to dephosphorylate the MAPKs JNK, p38, and ERK (25). To investigate whether MKP-5 regulates JNK and ERK activity in 3T3-L1 cells, levels of phERK, total ERK, phJNK, and total JNK were examined by Western analysis of 3T3-L1 cells ectopically expressing MKP-5 compared with cells containing an empty expression plasmid. In the absence of ectopic expression of MKP-5, phERK increased at day 0 and then rapidly declined to low levels by day 2. This is consistent with previous work (18). At day 4, phERK expression started to increase again. In the presence of MKP-5 expression, the pattern of phERK expression during adipogenesis was the same, but there was significantly more (60%) phERK at day -2 and less (30%) phERK at day 0 in cells expressing MKP-5 (Fig. 6A). At day 4 post induction, phERK levels increased and at day 8 were back to levels observed at day -2.

In contrast, JNK activity was not regulated by MKP-5 expression (Fig. 6B). PhJNK levels were also reduced during adipogenesis which is consistent with previously published data (18).

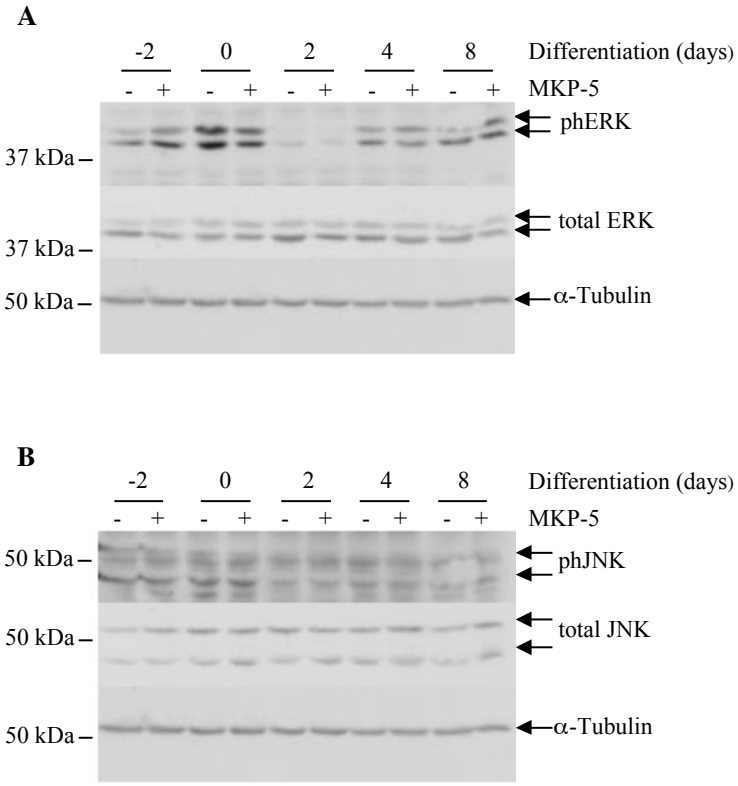


Figure 6- ERK and JNK activity in differentiating 3T3-L1 cells over-expressing MKP-5 and control cells. *MKP-5 expressing cells and control cells were induced to differentiate, protein extracts (100μg) were collected at indicated time-points and analyzed by Western blotting. Extracts were probed with phERK, total ERK, and α-tubulin antibodies (panel A) and phJNK, total JNK, and α-tubulin antibodies (panel B). The blots shown are representative of two individual experiments.*

Discussion

The MKP family of dual specificity phosphatases has 14 identified members. Although these proteins share common features, they are also known to differ in their function and expression patterns. The expression and possible function of these proteins has mainly been studied in specific human tissues, such as brain, heart, liver, and skeletal muscle, where some show restricted expression (24, 34-39). MKP-4 has previously been shown to be present in white adipose tissue and may contribute to its biology (28). In this study, we found that all members of the MKP family tested were present in adipose tissue from mouse, as well as in both preadipocytes and mature adipocytes of the 3T3-L1 cell line at the mRNA level (Supplementary Fig. 1). In addition we showed that most of the MKPs are

down-regulated during adipogenesis. We studied MKP-5 in more detail and found that 3T3-L1 cells differentiate more easily in response to ectopic expression of MKP-5.

Two MKPs have previously been investigated in relationship to adipogenesis, MKP-1 and MKP-4. There are conflicting results regarding MKP-1. One group has shown an up-regulation of MKP-1 during adipogenesis and that MKP-1 is the protein responsible for the down-regulation of ERK activity that is known to occur after the initial stages of differentiation (27). In contrast, another group has shown down-regulation of MKP-1 protein during differentiation of 3T3-L1 cells (18). In our work, we have shown that MKP-1 is down-regulated at the mRNA level during adipogenesis, which is consistent with the observations made in the latter report.

MKP-4 was previously reported to be up-regulated during adipogenesis in 3T3-F442A cells, a cell line similar in properties to 3T3-L1 cells (40, 41). In contrast, we have found that MKP-4 mRNA levels are down-regulated during differentiation of 3T3-L1 cells. Since the cell lines used are different, this might indicate that the L1 and F442A cells may have some different properties, which has also been previously reported (42, 43). Parallel experiments in both cell lines are necessary in the future.

In this study we also investigate regulation of many of the other members of the MKP family during differentiation of 3T3-L1 cells. In general they are all down-regulated to a certain extent in terminally differentiated cells, except DSP2, which does not show significant regulation during adipogenesis (Fig. 2). The time point at which the mRNA levels of the different MKPs were down-regulated varied. Even though MKP-3 levels have previously been reported to be unchanged during differentiation (up to day 8 post induction) (27) we show a reduction of MKP-3 mRNA at day 12 post induction. The fact that so many of the MKPs are regulated at the mRNA level during adipogenesis suggests that they have a functional role in differentiation of 3T3-L1 cells.

MKP-4 was previously reported to be expressed and up-regulated in adipose tissue of several rodent obesity models, including *ob/ob* mice (28). Consistent with these previously published data, we found that MKP-4 was highly up-regulated in subcutaneous white adipose tissue of *ob/ob* mice as well as in A^y mice (Fig. 3A and 3B). The same general

picture also applied for MKP-1, MKP-5, MKP-6, MKP-7, and MKP-X (Fig. 3). The previous study of MKP-4 established a link between this phosphatase and insulin signaling (28). MKP-4 was up-regulated specifically in insulin-responsive tissues including liver, muscle, white adipose tissue, and BAT. MKP-4 was also shown to reduce insulin-regulated glucose uptake in 3T3-L1 cells.

MKP-4 was the first dual-specificity phosphatase to be implicated in insulin-resistance. Before this the main focus was on protein-tyrosine phosphatases (PTPs). For instance, it is well established that PTP-1b is a negative regulator of insulin action and a mediator of insulin-resistance (44, 45). A study of *PTP-1b* deficient mice also showed that these animals had low adiposity, linking this phosphatase directly to obesity (46). Given that many of the MKPs are down-regulated in adipogenesis, yet show up-regulation in adipose and other tissues of obese mice indicate that aspects of obesity other than increased fat mass could influence the regulation of these proteins. Increased secretion of cytokines and insulin-resistance, which is related to the obese state, might be factors that could effect the expression and activity of MKPs.

Studies of MKP-1 and MKP-4 in adipocyte differentiation have established that members of the MKP family are directly relevant to adipogenesis. MKP-1 expression has been reported to be of physiological importance for adipocyte differentiation of 3T3-L1 cells, while cells stably expressing MKP-4 were shown to differentiate poorly (27, 28). ERK and p38 MAPK activity has been reported to be critical to initial phases of adipogenesis (15, 47-49). Previous studies have shown that MKP-5 can deactivate MAPKs (24, 25). Based on this knowledge we hypothesized that since MKP-5 is significantly down-regulated early in differentiation of 3T3-L1 cells (Fig. 2), ectopic expression of MKP-5 might have an effect on differentiation, possibly through dephosphorylation of MAPKs. JNK activity was not affected and the effect on p38 MAPK activity is unresolved, but an induction of ERK activity was observed at day -2 and a reduction was observed at day 0 of treatment of 3T3-L1 cells (Fig. 6). This indicates that in 3T3-L1 cells MKP-5 prefers ERK as a substrate to JNK, in contrast to what is previously published in NIH-3T3 cells and COS-7 cells (24, 25). Anyhow, ERK activity was induced in day -2 cells. The results correlate with the observation that a higher percentage of cells ectopically expressing MKP-5 differentiated into mature adipocytes compared to the control cells, assessed by up-regulation of aP2 and

increased lipid accumulation (Fig. 5). This might indicate a more complex role for MKP-5 than first assumed. Identification of other possible substrates and targets of MKP-5 in adipocytes would be important in defining a potential function for MKP-5 in adipogenesis. For instance a recent study reported that MKP-5 inhibits the TNF-α promoter in immune cells (50); TNF-α is a known inhibitor of adipogenesis (51). Although the MAPKs are the only identified substrates for the MKPs, other targets for dephosphorylation mediated by the MKPs is of course possible. This has been an issue earlier, for instance c-Jun N-terminal kinase, JNK, was first identified as a kinase that phosphorylates c-Jun, as the name indicates (52). Later many other JNK substrates have been identified (53, 54). The MKP family has mainly been studied regarding structure and ability to dephosphorylate MAPKs, but much remains to be elucidated concerning biochemical and functional aspects. Down-regulation of inhibitors of adipogenesis could give an explanation to the observations made in this study.

Previous results state that ERK is active during initial stages of adipogenesis, and that phosphorylated ERK is down-regulated around day 2-4 after induction of differentiation (18, 27). Results presented here are consistent with this previously shown data in that ERK is active in preadipocytes and confluent 3T3-L1 cells, but is down-regulated at day 2. However, in our experiment, ERK activity reappears at day 4. The percentage of cells differentiating into mature adipocytes in this study is somewhat lower than expected. It has been shown that ERK acts negatively on adipogenesis by phosphorylation of PPARγ (14, 55). This could be the basis for the lesser degree of differentiation observed here.

Since results obtained in this study suggest a positive role for MKP-5 in adipogenesis, it will be of interest to study this further. Establishing an inducible system where ectopic expression of MKP-5 in 3T3-L1 cells can be controlled could verify if the results obtained here are due to MKP-5 action as apposed to clonal differences between MKP-5 expressing cells and control cells. Inhibitors of known signaling pathways could be used to assess which factors that are involved in the down-regulation of MKP-5 during adipogenesis. Experiments involving the ERK specific inhibitor, PD98059, could indicate if ERK activity is required for the MKP-5 induced differentiation. Further studies of MKP-5 in mouse models could give insight into the function of MKP-5 on adipose tissue formation and adipogenesis *in vivo*. Knock-down studies of MKP-5 using RNA interference in 3T3-

L1 cells and knockout of MKP-5 in mouse models of obesity could provide information on the possible role of MKP-5 expression in adipogenesis, although it is known that many of the members of the MKP family share the same substrate specificity and a certain degree of redundancy seems likely (21). Studies in human mesenchymal stem cells and human adipose tissue samples would also be important in investigating the role of MKP-5 in human adipocyte differentiation and obesity. Studies in stem cells would give an indication of a possible function for MKP-5 in lineage determination as well as in terminal differentiation of adipocytes. Further work is necessary to assess these possibilities.

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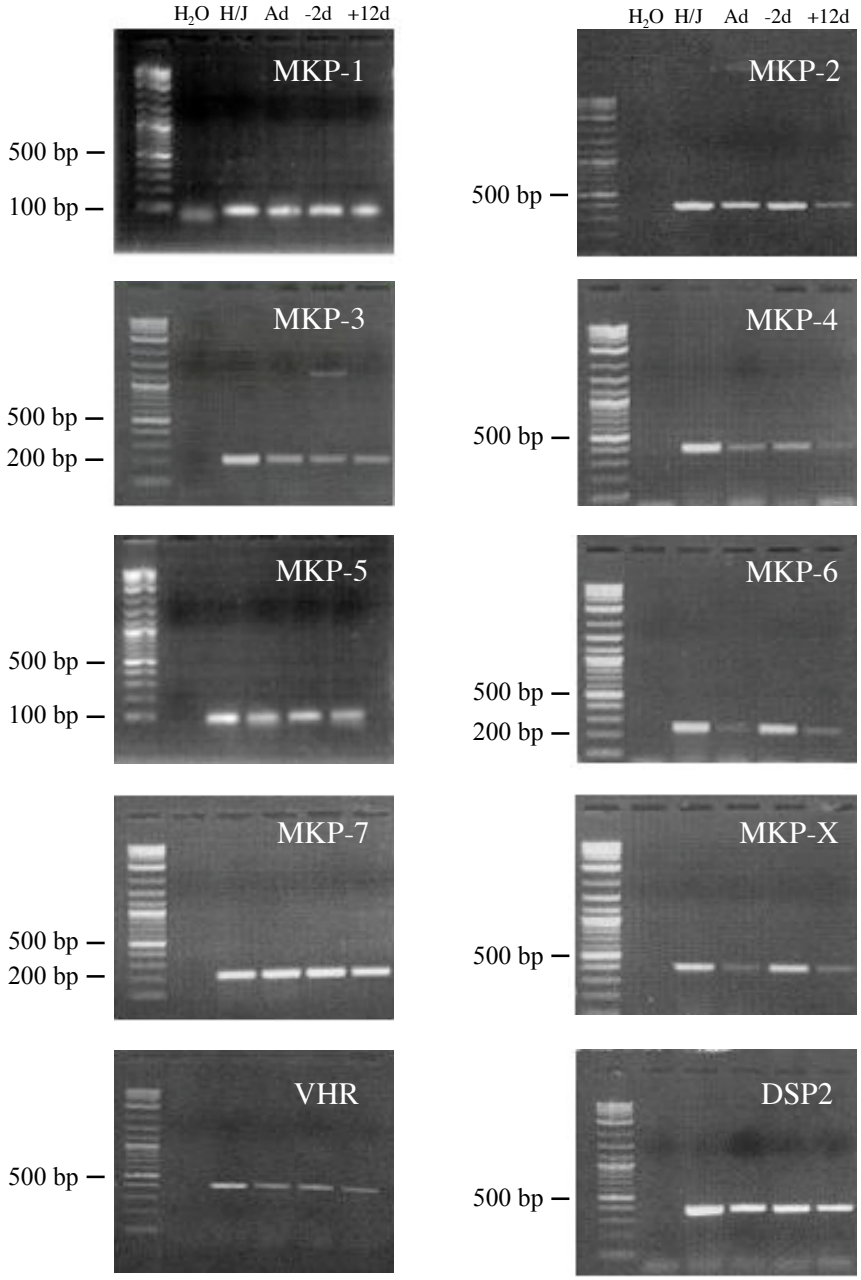
Supplement



Supplementary Figure 1 – Expression of MKPs in mouse adipose tissue and preadipocyte and fully differentiated 3T3-L1 cells*. Total RNA from Hepa1c1c-7 cells (H), J55 lymphocytes (J), mouse adipose tissue (Ad), preadipocyte 3T3-L1 cells (-2d), and fully differentiated 3T3-L1 cells (+12d) was converted to cDNA and expression of MKP-1, MKP-2, MKP-3, MKP-4, MKP-5, MKP-6, MKP-7, MKP-X, VHR, and DSP2 was analyzed by RT-PCR. Hepa1c1c-7 cells and J55 cells were used as a positive control.*