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Role and Regulation of Adrenomedullin Signalling During Adipogenic Conversion of Mouse 3T3-L1 Preadipocytes

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By

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ABSTRACT.

Dramatic downregulation of Adrenomedullin (AM) mRNA and peptide secretion during the induction of differentiation in 3T3-L1 preadipocytes has been reported. This observation led us to hypothesize that increased levels of AM may have inhibitory effect on 3T3-L1 preadipocyte differentiation. We, therefore, examined the effect of application of exogenous AM and its specific receptor antagonist AM_{22-52} on adipogenic differentiation in this cell line. We also examined mRNA regulation of CRLR, which in combination with RAMP-1, and RAMP-2 or RAMP-3 proteins, forms CGRP and AM receptors respectively. Here we report that AM stimulation on day 2 of differentiation dose dependently inhibits mRNA expression of adipocyte markers PPARγ2, leptin, and adipsin on day 7 differentiated adipocytes, and, that this effect can be, at least partially, inhibited by pre-treatment with AM_{22-52}. We further report on the expression of CRLR mRNA and protein, and RAMP-1 and RAMP-2 mRNA regulation during 3T3-L1 differentiation. We also report striking upregulation of RAMP-3 mRNA expression during the first day of induction of differentiation.

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LIST OF ABBREVIATIONS.

Ab	Antibody
AC	Adenylate cyclases
ADD1	Adipocyte determination and differentiation factor 1
AM	Adrenomedullin
AM1	CRLR/RAMP-2 AM receptor 1
AM2	CRLR/RAMP-3 AM receptor 2
AT1aR	Angiotensin II 1a receptor
BAEC	Bovine aortic endothelial cells
bFGF	Basic fibroblast growth factor
C/EBP	CCAAT/enhancer binding proteins
CFTR	Cystic fibrosis transmembrane conductance regulator
CGRP	Calcitonin gene-related peptide
CP	Carboxypeptidase like activity
CPD	Carboxypeptidase D
CPE	Carboxypeptidase E
CREB	cAMP response element binding protein
CRLR	Calcitonin receptor-like receptor
cRNA	Complementary RNA
CS	Cell surface
CTX	Cholera toxin
DEX	Dexamethasone
DMEM	Dulbecco's modified Eagle's culture medium
EC	Endothelial cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
FACS	Fluorescence activated cell sorting
GAPs	GTPase-activating proteins
GH	Growth hormone
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
hRAMP-3	Human RAMP-3
IBMX	3-isobutyl-1-methylxanthine
IDI	Insulin/DEX/IBMX
IGF-1	Insulin-like growth factor 1
IGF-1R	IGF-1 receptor
IL	Interleukin
IP	Immunoprecipitation
IR-AM	Immunoreactive AM
MAPK	Mitogen activated protein kinases
MERM	Merlin and ezrin/radixin/moesin
MMP	Matrix metalloproteinase
Mr	Apparent molecular weight
NHERF-1	Na+/H+ exchanger regulatory factor 1

NSF	N-ethylmaleimide-sensitive factor
O.D.	Optical density
PAI-1	Plasminogen activator inhibitor 1
PAM	Peptidylglycine alpha-amidating monooxygenase
PAMP	Pro-adrenomedullin N-terminal 20 amino acids peptide
PC	Pro-protein convertase
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDZ	PSD-95/Discs-large/ZO-1 homology
PI3K	Phosphoinositide 3-kinases
PKA	cAMP dependent protein kinase
PKB	Protein kinase B
PLC	Phospholipase C
PNGase F	Peptide-N-glycosidase F
PPARγ2	Peroxisome proliferator-activated receptor γ 2
PTK	Protein tyrosine kinase
PTX	Pertussis toxin
RAMP-1	Receptor activity modifying protein 1
RCP	CGRP-receptor component protein
RIA	Radioimmunoassay
SREBP-1	Sterol regulatory element-binding protein 1
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor α
V_2R	Vasopressin 2 receptor
VSMC	Vascular smooth muscle cells
β_2AR	β_2-adrenergic receptor
βAR	β-Adrenergic receptor
βARK	βAR kinase

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1. INTRODUCTION.

1.1 *Preamble:*

Obesity is becoming increasingly prevalent in North America [1]. In light of this new epidemic, elucidating factors controlling adipogenesis and, more narrowly, adipogenic conversion of preadipocytes to adipocytes has become of ever-greater interest. The adipose tissue itself is becoming well recognized for its function beyond that of a simple storage depot. A number of bioactive signaling proteins are secreted by the adipose tissue. These proteins include, among others, leptin, tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), plasminogen activator inhibitor 1 (PAI-1), adipsin, adiponectin and resistin. At the same time, the adipose tissue is equipped to respond to a number of hormones and signaling peptides. For instance, receptors for insulin, glucagon, growth hormone (GH), angiotensin II and leptin are expressed by the adipose tissue [2]. Here, I investigated the effect of a bioactive signaling peptide Adrenomedullin (AM), and the regulation of its receptor expression on adipogenic conversion of pre-adipocytes to adipocytes using mouse 3T3-L1 pre-adipocyte cell line as a well established model of this process.

1.2 *3T3-L1 cell line as a model system for adipogenesis.*

3T3-L1 cell line was derived from disaggregated Swiss 3T3 mouse embryos [3] and is perhaps the most widely used cell line for studying molecular events underlying terminal differentiation of pre-adipocytes into adipocytes. Its functional validity as a model is underscored by its ability, upon injection into live nude mice, to give rise to fat pads that are

morphologically indistinguishable from native adipose tissue [4]. Extensive ultrastructural similarity of cultured 3T3-L1 cells to tissue adipocytes has also been documented [5].

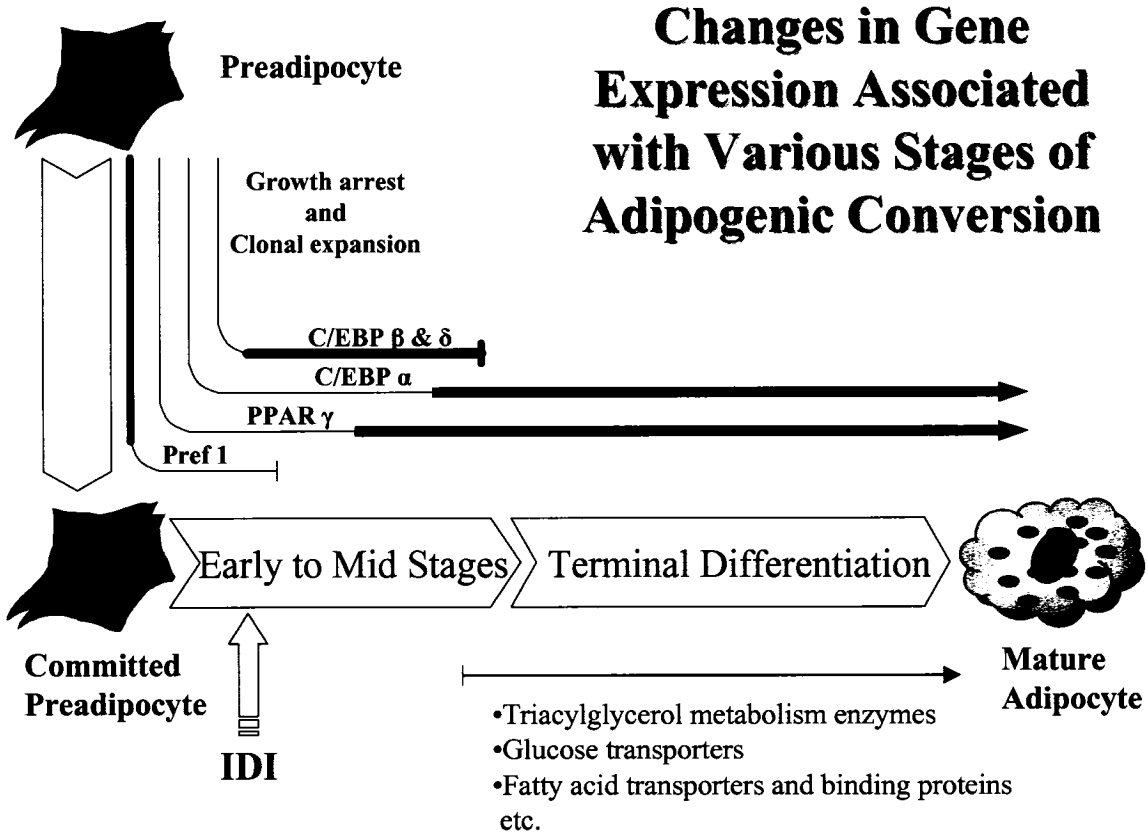
A well established protocol for induction of terminal differentiation in 3T3-L1 pre-adipocytes has been developed and minor variations thereof can be encountered in numerous publications. In essence, confluent 3T3-L1 pre-adipocytes are stimulated with a three component mixture containing an insulin-like growth factor 1 (IGF-1) receptor agonist [6], a glucocorticoid receptor agonist, and an agent increasing the intracellular cAMP levels [7]. Traditionally, pharmacological concentrations of insulin, synthetic glucocorticoid dexamethasone (DEX), and the cAMP-phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) are used to fulfill these roles, respectively [8].

A wealth of literature exists on the external determinants as well as intracellular events underlying the process of adipogenic differentiation [9-12]. The key changes in expression of transcriptional regulators (Fig. 1) begin with transient upregulation of CCAAT/enhancer binding proteins (C/EBP) β and δ detectable as early as 1 hour after stimulation with the insulin/DEX/IBMX (IDI) cocktail and persisting for about 2 and 8 days, in the case of C/EBP β and δ respectively, after IDI stimulation is withdrawn (reviewed in [11, 13]). This is followed by sequential and sustained upregulation of peroxisome proliferator-activated receptor γ 2 (PPARγ2) and C/EBP α transcription factors responsible for the activation of a vast number of adipocyte-specific genes expressed in mature adipocytes (Fig. 1) [9, 10]. Cross activation between PPARγ2 and C/EBP α [14] is thought to maintain elevated levels of expression of these transcription factors in the absence of C/EBP β and δ. Adipocyte determination and differentiation factor 1 (ADD1), also known as sterol regulatory element-binding protein 1 (SREBP-1) is another global transcription factor

Fig. 1 Changes in gene expression associated with various stages of adipogenic conversion.

Confluent 3T3-L1 preadipocytes undergo growth arrest followed by one or two rounds of mitotic division known as clonal expansion. Stimulation of resulting committed preadipocytes with the insulin/DEX/IBMX (IDI) cocktail initiates well defined changes in expression of transcriptional regulators of adipogenesis that begin with transient upregulation of CCAAT/enhancer binding proteins (C/EBP) β and δ detectable as early as 1 hour after stimulation. This is followed by sequential and sustained upregulation of peroxisome proliferator-activated receptor γ 2 (PPARγ2) and C/EBP α transcription factors responsible for the activation of a vast number of adipocyte-specific genes expressed in mature adipocytes and results in terminal differentiation of committed preadipocytes into mature adipocytes. This is accompanied by transcriptional downregulation of the inhibitor of differentiation, pref-1, to undetectable levels.

Fig. 1


Changes in Gene Expression Associated with Various Stages of Adipogenic Conversion
Preadipocyte
Growth arrest and Clonal expansion
C/EBP β & δ
C/EBP α
PPAR γ
Pref 1
Early to Mid Stages
Terminal Differentiation
Committed Preadipocyte
IDI
Mature Adipocyte
•Triacylglycerol metabolism enzymes
•Glucose transporters
•Fatty acid transporters and binding proteins
etc.

upregulated during preadipocyte differentiation [15]. ADD1/ SREBP-1 activates an array of genes involved in cholesterol and fatty acid synthesis. Importantly, its expression is required for the production of, an as yet unidentified, endogenous PPARγ ligand, that is lacking in 3T3-L1 preadipocytes, and enhanced PPARγ activity [16-18].

Several excellent reviews on regulation and molecular mechanisms of adipocyte differentiation have been published [9-11]. The information referred to in the rest of this section has been derived mostly from the reviews by Gregoire et al. [9] and Rosen et al. [10]. An extensive list of both pro- and anti- adipogenic factors has been assembled, mostly from in vitro studies [11]. Although their precise mechanism of action and relevance in vivo in many cases remain to be further investigated, a number of well established regulators have emerged.

Insulin-like growth factor 1 (IGF-1) and insulin can activate small GTPase ras (Fig. 2). Forced expression of constitutively active ras stimulates differentiation and eliminates the need for IGF-1 receptor stimulation in 3T3-L1 cells. This effect can be blocked by expression of a dominant negative form of the ras downstream effector, Raf-1. However, transfection of Raf-1 oncogene has only a partial effect suggesting Raf-1 independent signaling downstream of ras also takes place [9, 10].

Insulin mediated activation of protein kinase B (PKB)/Akt may also play a role in induction of differentiation. Transfection of constitutively active Akt in 3T3-L1 cells results in their spontaneous differentiation (Fig. 2) [9].

Signaling through epidermal growth factor (EGF) receptor by EGF or transforming growth factor (TGF) α has been shown to inhibit preadipocyte differentiation in a number of systems. TGFα inhibits differentiation of mouse 3T3-F442A and rat preadipocytes and

Fig. 2 Pathways implicated in IDI-induced adipogenesis.

Induction cocktail that was empirically established to stimulate maximal differentiation in 3T3-L1 cells and other model systems includes Insulin, dexamethasone (Dex) and IBMX. Blue yellow and green shaded boxes encompass pathways stimulated by the respective components of this induction cocktail.

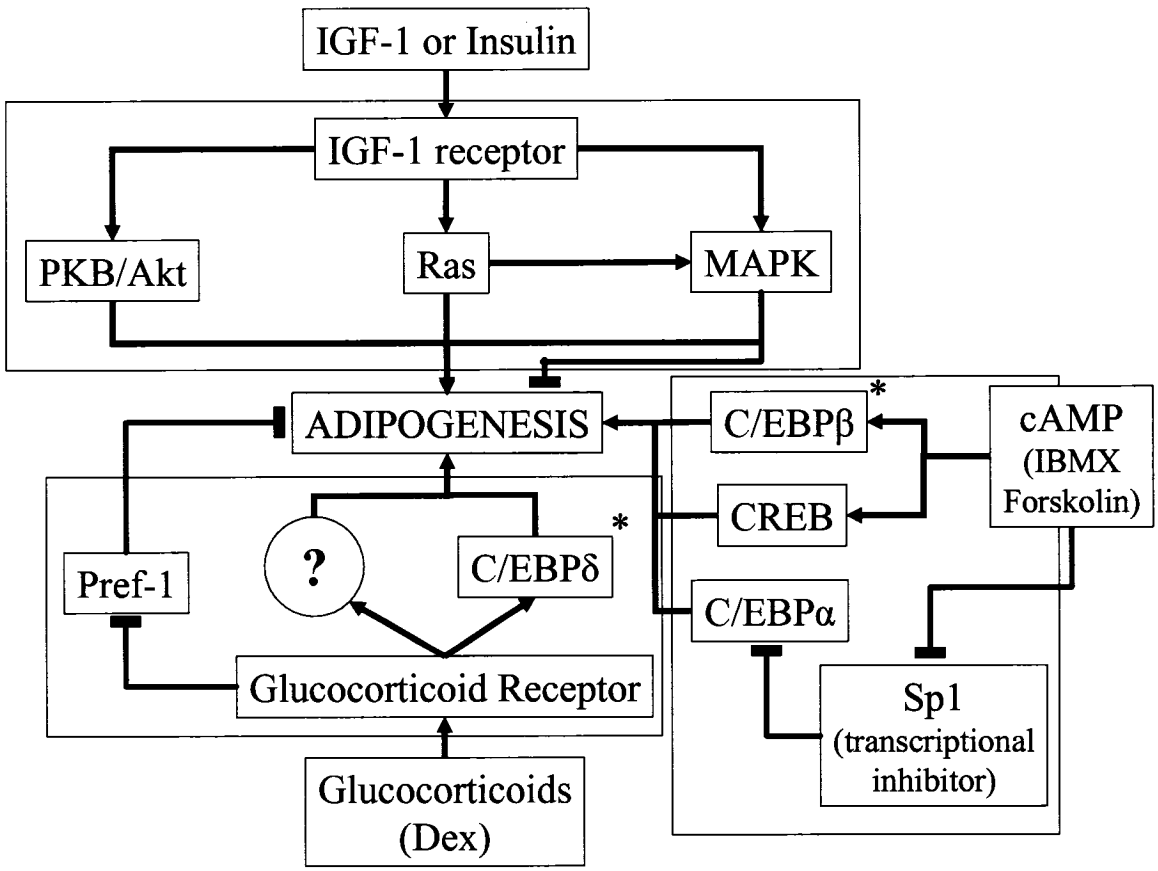
IGF-1 or pharmacological concentrations of Insulin act through IGF-1 receptor to activate small GTPase ras and protein kinase B (PKB)/Akt, to stimulate adipogenesis. Activation of IGF-1 receptor has also been shown to activate mitogen activated protein kinases (MAPK) erk1 and erk2, possibly through activation of ras. Both pro- and anti- differentiation effects have been reported for these kinases and timing of activation was suggested as a determinant.

Glucocorticoids such as synthetic glucocorticoid Dex act through nuclear glucocorticoid receptor to downregulate the expression of inhibitory adipogenic factor pref-1, to alleviate pref-1 mediated inhibition of adipogenesis. At the same time activated glucocorticoid receptor stimulates expression of pro-adipogenic transcription factor C/EBPδ. It also acts to stimulate adipogenesis through some as yet unidentified, C/EBPδ independent, mechanism.

Treatment with IBMX or forskolin results in increased intracellular cAMP levels. Elevated cAMP in turn downregulates C/EBPα transcriptional inhibitor Sp1, and stimulates expression of C/EBPβ, thus upregulating activity of these two important pro-adipogenic transcription factors. cAMP response element binding protein (CREB) was also reported necessary and sufficient to induce differentiation in 3T3-L1 cells

* C/EBP β and δ exert their pro-adipogenic effect mostly through upregulation of PPARγ2 transcription factor.

Fig. 2 Pathways implicated in IDI induced adipogenesis.



overexpression of TGFα in transgenic mice results in 50% less total body fat. Rat, mouse, and human preadipocyte differentiation is inhibited by EGF, and subcutaneous injection of EGF in neonate rats significantly reduces fat pad weight. EGF, however, was reported to have no effect on porcine preadipocytes, and, differentiation of 3T3-L1 preadipocytes cultured in defined, serum free, medium was reportedly dependent on EGF. Effects of platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) are more ambiguous. PDGF was reported in two different studies of the same experimental system to either promote or have no effect on adipogenicity in the serum free medium cultured 3T3-L1 cells [19, 20]. Similarly, bFGF was reported to suppress adipogenesis in cell lines cultured in serum containing media, while having no effect in serum free media. TGFβ was reported to be a potent inhibitor of adipocyte differentiation in a number of cell culture models. TGFβ promoted increased synthesis of extracellular matrix (ECM) components which was proposed as a mechanism for TGFβ mediated inhibition of adipogenesis [9].

Cytokines typically exert inhibitory effects on adipogenesis. Thus, interleukin (IL) 11 is a dose dependent inhibitor of adipogenic conversion in 3T3-L1 and bone marrow stroma-derived H-1/A cells. Similar effect was observed for IL-1β and Interferon-γ in 3T3-derived preadipocytes and primary rodent preadipocytes. TNF-α is of particular interest as it not only inhibits preadipocyte differentiation but prolonged treatment with TNF-α can reverse the adipocyte phenotype of terminally differentiated cells. TNF-α treatment results in decreased mRNA and protein expression, and DNA binding activity of PPARγ2 and consequent downregulation of C/EBPα, the two transcription factors acting as master switch regulators of adipocyte-specific gene expression [9, 10].

Glucocorticoids have been shown to promote adipocyte differentiation in a variety of experimental systems. Differentiation of primary preadipocytes of porcine, rabbit and human origin in serum free medium has been reported to be strictly dependent on glucocorticoid treatment. In 3T3-L1 cells, synthetic glucocorticoid dexamethasone (DEX) induces expression of C/EBP-δ facilitating formation of C/EBP β/δ heterodimers and induction of PPARγ2 transcription [21]. In this study, direct effect of glucocorticoids on PPARγ2 transcription was not ruled out. Glucocorticoids also act to suppress expression of endogenous inhibitor of adipogenesis, Pref-1 protein. Overexpression of Pref-1 negates adipogenic effects of glucocorticoid, conversely, antisense Pref-1 knockdown reduces the dose of glucocorticoid required to promote adipogenesis (Fig. 2) [9, 10].

IBMX has been established empirically to promote differentiation as part of the induction cocktail in preadipocyte cell lines. It is thought to act through elevating intracellular cAMP levels by inhibiting phosphodiesterase, an enzyme responsible for clearance of cAMP generated by activated adenylate cyclase in response to extracellular signaling. In 3T3-L1 cells, cAMP stimulates expression of C/EBP-β required for transactivation of PPARγ2 transcription. Elevated cAMP also downregulates C/EBPα transcriptional inhibitor Sp1, and thus stimulates expression of C/EBPα. cAMP response element binding protein (CREB) was reported necessary and sufficient to induce differentiation in 3T3-L1 cells, although, surprisingly, CREB knockout mice do not show an aberrant adipose phenotype (Fig. 2) [9, 10].

Preadipocyte factor (Pref) 1 is an EGF repeat-containing protein exhibiting strong antiadipogenic properties [22]. It is initially expressed in 3T3-L1 preadipocytes and its expression drops dramatically during the differentiation. Preventing this downregulation also

prevents induction of differentiation [22], indicating a role for Pref-1 in regulation of adipogenesis. Furthermore, soluble forms of Pref-1 are generated by cells in culture by cleavage of its ectodomain and at least the largest form generated by the membrane proximal cleavage retains its inhibitory effect on differentiation [23, 24]. This inhibitory effect was reported to result from the inhibition of IGF-1 receptor mediated activation of erk1 and erk2 mitogen activated protein kinases (MAPK) and the suppression of clonal expansion (Fig. 2) [25].

1.3 Adrenomedullin System

1.3.1 Adrenomedullin peptide.

1.3.1.1 *Discovery and biological activity.*

Adrenomedullin was initially isolated from extracts of a rat pheochromocytoma based on its ability to raise the levels of cAMP in platelets [26]. In the same article, a potent vasodilator effect of AM was described. Consequently, its effect on the cardiovascular system was the main focus of study for a number of years. Vascular endothelial cells and vascular smooth muscle cells were discovered to be active secretors as well as targets of AM [27, 28]. In contrast to local vasodilator action of AM in the vascular beds, centrally administered AM was shown to have a vasopressor effect [29], illustrating the importance of site of action and hinting to autocrine/paracrine rather then endocrine mode of action. Since its discovery, AM was found to be almost ubiquitously expressed in different organs, tissues and cell types [30-32]. Accordingly, new functional effects of AM were described depending on the system under investigation. AM was reported to affect a wide range of

physiological processes including cell growth [33-35], apoptosis [36-38], hormone secretion [39-42], and angiogenesis [43-45], to name a few. While little had been reported on a role of AM in adipogenesis, there are indications for such a role. First, both AM and its receptor are expressed in adipose tissue [46, 47]. Second, although somewhat contradictory, there are reports of AM affecting the rate of lypolysis in cultured adipocytes [48, 49]. Finally, reports by Li et al. [50], Fukai et al. [47], as well as unpublished data from Dr. Mbikay's lab showed dramatic downregulation of AM message levels and peptide secretion during the induction of differentiation in 3T3-L1 confluent preadipocytes. This latter observation led us to hypothesize that increased levels of AM may have inhibitory effect on 3T3-L1 preadipocyte differentiation.

1.3.1.2 *Serum binding protein for AM.*

A specific serum AM binding protein was isolated and identified as complement factor H [51, 52]. Factor H is a crucial negative regulator of the alternative complement pathway [53]. The biological activity of both factor H and AM is enhanced by the binding [52]. Although the mechanism of enhancement of AM action by factor H is unknown, this factor was shown to protect AM from proteolytic digestion by matrix metalloproteinase (MMP) 2, thus increased half life of AM was proposed as a plausible mechanism for this enhancement [54].

1.3.1.3 *Biochemistry and biosynthesis.*

Rat and mouse AM is an amidated 50 amino acid-long peptide (52 in humans) containing an internal disulfide bond that forms a 6 member ring structure, characteristic of

the calcitonin gene-related peptide (CGRP) family of regulatory peptides [55]. From the open reading frame in its cDNA sequence, it was deduced that AM is initially produced as a larger precursor protein (Fig. 3) containing a signal peptide and the amino acid sequence of two known bioactive peptides: the pro-adrenomedullin N-terminal 20 amino acids peptide (PAMP) and the Adrenomedullin peptide. The signal peptide is cleaved co-translationally to produce pro-AM. Through a series of endo- and exo-proteolytic cleavages, pro-AM is further processed in the secretory compartment to yield inactive, glycine extended forms of PAMP and AM. Although the identity of the processing enzymes has not been examined, given the amino acid sequence of the cleavage sites, it is likely that furin/PCSK-3, a ubiquitously expressed member of a larger family of pro-protein convertase (PC) serine endoproteinases is responsible for the initial cleavage of pro-AM [56, 57]. Following the endoproteolytic cleavage, the removal of exposed carboxy-terminal basic residues is likely accomplished by exopeptidase activity of carboxypeptidase D (CPD) and/or E (CPE), as is the case for several other protein precursors matured by PCs [57-59]. The resulting glycine-extended, inactive peptides are then converted to bioactive forms by the removal of C-terminal glycine and amidation of the C-terminus by peptidylglycine alpha-amidating monooxygenase (PAM) (Fig. 3) [26, 60].

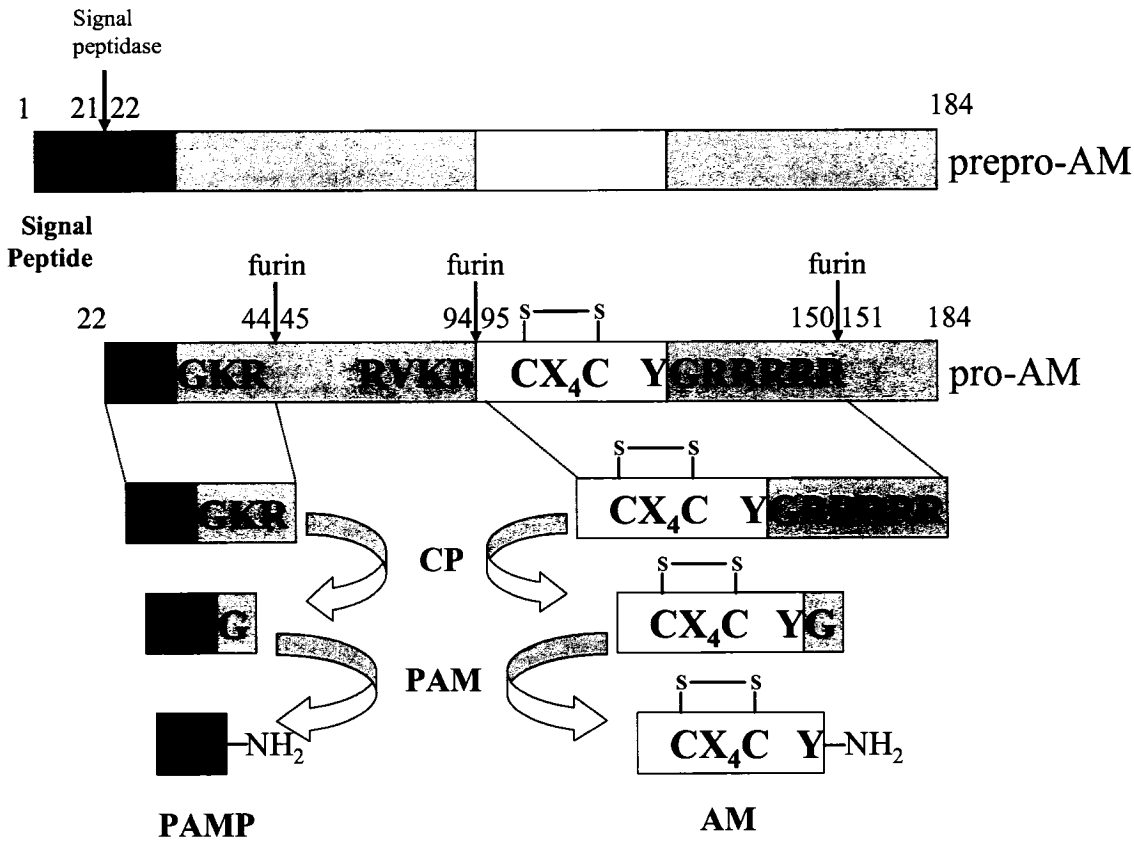
1.3.1.4 *Gene structure and alternative splicing.*

The message for AM prepro-polypeptide is encoded by 4 exon sequences separated by 3 short introns transcribed from a single gene locus on chromosome 11 in humans or chromosome 7 in mice [26, 60, 61]. An alternative splicing event, a failure to excise intron 3, introduces a stop codon resulting in the production of a truncated prepro-polypeptide

Fig. 3 Biosynthesis of AM and PAMP from prepro-AM precursor protein.

Mouse prepro-AM is initially synthesized as a 184 amino acid protein with N-terminal signal sequence. The signal peptide is cleaved upon translocation into the endoplasmic reticulum (ER). The resulting pro-AM precursor protein is further processed by furin/pcsk3 PC c-terminally of dibasic residues of the consensus furin/PCSK3 sequence R-X-(K)R-R. The numbers indicate location of amino acids flanking the cleavage sites according to mouse prepro-AM amino acid sequence. The basic residues are cleaved off by carboxypeptidase (CP) like activity to produce inactive, glycine-extended forms of AM and PAMP. The glycine residues are removed and exposed C-terminal residue is amidated by peptidylglycine alpha-amidating monooxygenase (PAM) to generate bioactive AM and PAMP peptides. S-S represents a disulfide bridge forming the six-membered ring characteristic of CGRP family of signaling proteins.

Fig. 3 **Biosynthesis of AM and PAMP from prepro-AM precursor protein.**



lacking AM sequence (Fig. 4). Interestingly, the ratio between the two splice isoforms not only varies from one tissue or cell type to another but can also be regulated within the same cell type by different extracellular stimuli such as tumor necrosis factor (TNF) α or hypoxia [62], conferring an additional level of regulation.

1.3.1.5 *AM knockout mice.*

Both pro-AM (AM and PAMP) [63] and specific AM knockout [64] mice are available. Both knockouts are embryonic lethal resulting from severe cardiovascular abnormalities with pro-AM knockout being more severe resulting in embryo mortality on E13.5-E14.5 as opposed to E14.5-E15.5 for AM specific knockout. Heterozygous knockouts are viable and fertile, expressing roughly half the wild type level of AM. With age, insulin resistance and increased body weight was reported in these mice [65].

1.3.2 Adrenomedullin Sensing System

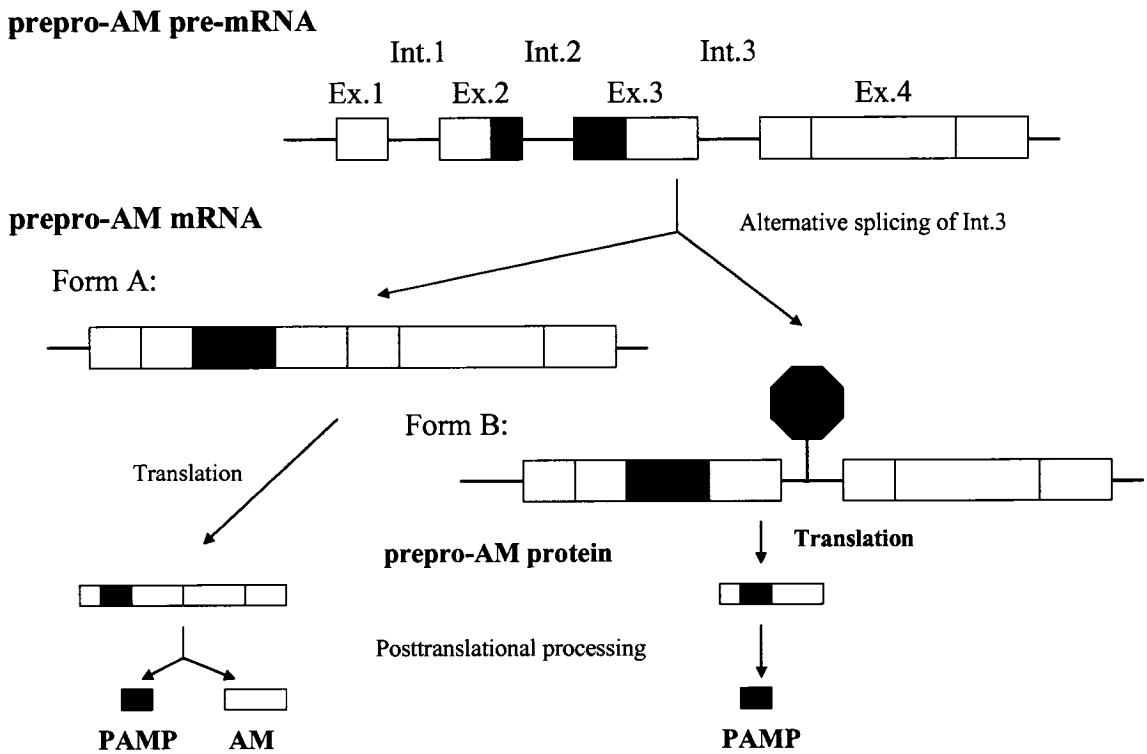
1.3.2.1 *General introduction of G-protein coupled receptors.*

AM signaling is mediated through activation of a subclass of the membrane receptor superfamily called G-protein coupled receptor (GPCR) [66]. All GPCRs share certain structural similarity. These are seven α-helical transmembrane domain proteins with extracellular N-terminus and cytoplasmic C-terminus. Although high resolution (2.6 Å) crystal structure of bovine rhodopsin is the only one available today that includes the transmembrane domain, structural predictions made from biochemical and biophysical studies of wild-type and mutated receptors correlate well with the available X-ray

Fig. 4 **Alternative splicing – regulated expression of AM gene products.**

Prepro-AM pre-mRNA is transcribed from a gene containing four exons (denoted as Ex. 1, 2, 3, and 4) interspaced by three short introns (Int. 1, 2, and 3). Form A of prepro-AM mRNA is generated by splicing of all three introns and translates into prepro-AM protein containing amino acid sequence for both PAMP and AM. Failure to splice out intron 3 generates form B of prepro-AM mRNA that contains a translation termination codon within Int. 3 sequence. The resulting prepro-AM protein is missing the AM amino acid sequence and its posttranslational processing generates PAMP peptide only.

Fig. 4 **Alternative splicing – regulated expression of AM gene products.**



crystallographic structure and suggest similar transmembrain domain topography for other members of GPCR superfamily [67]. Beyond structural similarities, GPCRs share a common paradigm of signal transduction. As the name implies, these receptors typically exert their action through coupling to a large class of proteins known as G-proteins [68]. The agonist induced transition from ground state to activated GPCR allows for activation of the heterotrimeric G-protein complex consisting of Gα, Gβ and Gγ subunits. This activation promotes GDP to GTP exchange on the Gα subunit of the inactive heterotrimeric complex leading to complex dissociation and liberating Gα/GTP monomer and Gβγ heterodimer to activate a variety of effector molecules. The duration of activation depends on the intrinsic Gα GTPase activity and the presence of GTPase-activating proteins (GAPs) such as cGMP phosphodiesterases (PDEs) and phospholipase C (PLC) β's, which, incidentally, can be effector molecules for some Gα and/or Gβγ proteins [68]. Once GTP hydrolysis takes place, inactivated Gα/GDP sequesters Gβγ heterodimer into an inactive heterotrimeric complex, thus completing the activation cycle [68].

As of 1999, 16 genes encoding Gα's, 5 genes for Gβ, and 12 genes for Gγ are known in mammals [69]. Although not all combinations are possible, considerable signaling diversity and specificity can be generated. A great number of proteins have been identified as the downstream targets of G-proteins and hence GPCR mediated signaling. The list includes, but is not limited to, adenylate cyclases (AC) 1 through 9, phospholipase C (PLC), phosphoinositide 3-kinases (PI3K) β and γ, phosphodiesterases (PDE), ion channels, tyrosine kinases, and more [67].

Several classifications of GPCRs exist. The most widely used one seems to be the one proposed by Kolakowski (1994) [70]. This system distributes all known GPCRs into six

families A, B, C, D, E, and F, with subclasses in each family denoted by Roman numerals. Recently, a new five-family classification system based on the analysis of some 800 human GPCR sequences has been proposed. The five main families named after a representative family member are glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin, forming the GRAFS classification system. The authors claim this system to be more fitting for classifying the mammalian GPCRs as well as suited for GPCR classification in all species [71]. The GPCR involved in AM signal transduction would classify as a member of the B family or secretin family GPCR according to Kolakowski or GRAFS classifications respectively.

1.3.2.2 *Discovery of the molecular identity of AM receptor.*

Pharmacological studies of AM quickly revealed its close relationship to another member of the calcitonin gene related peptide family of regulatory peptides, the calcitonin gene related peptide (CGRP) itself [72]. A number of studies reported that effects of AM can be antagonized by the $CGRP_{8-37}$ fragment, a specific antagonist for CGRP type 1 ($CGRP_1$) receptor, and that AM competes with ^{125}I-CGRP for CGRP specific binding sites. In these studies, however, the reported affinity for AM was about 10 fold lower than for CGRP [31]. At the same time AM binding to functional receptors that could not be antagonized by neither CGRP nor $CGRP_{8-37}$ even at concentrations as high as 10 μM was reported in vascular smooth muscle cells (VSMC) [73], indicating a presence of specific AM receptors distinct from $CGRP_1$ receptors. Specific AM receptors were also reported in Rat-2 fibroblasts that lack CGRP receptors all together [74]. Finally, specific AM receptors with negligible binding of CGRP, amylin, or calcitonin (all members of the same CGRP family of

regulatory peptides as AM) were reported in numerous tissues: heart, lung, spleen, liver, vas deference, kidney glomeruli, skeletal muscle, hypothalamus, spinal cord; and cell lines: mouse astrocytes, bovine endothelial cells, oral and skin keratinocytes [31].

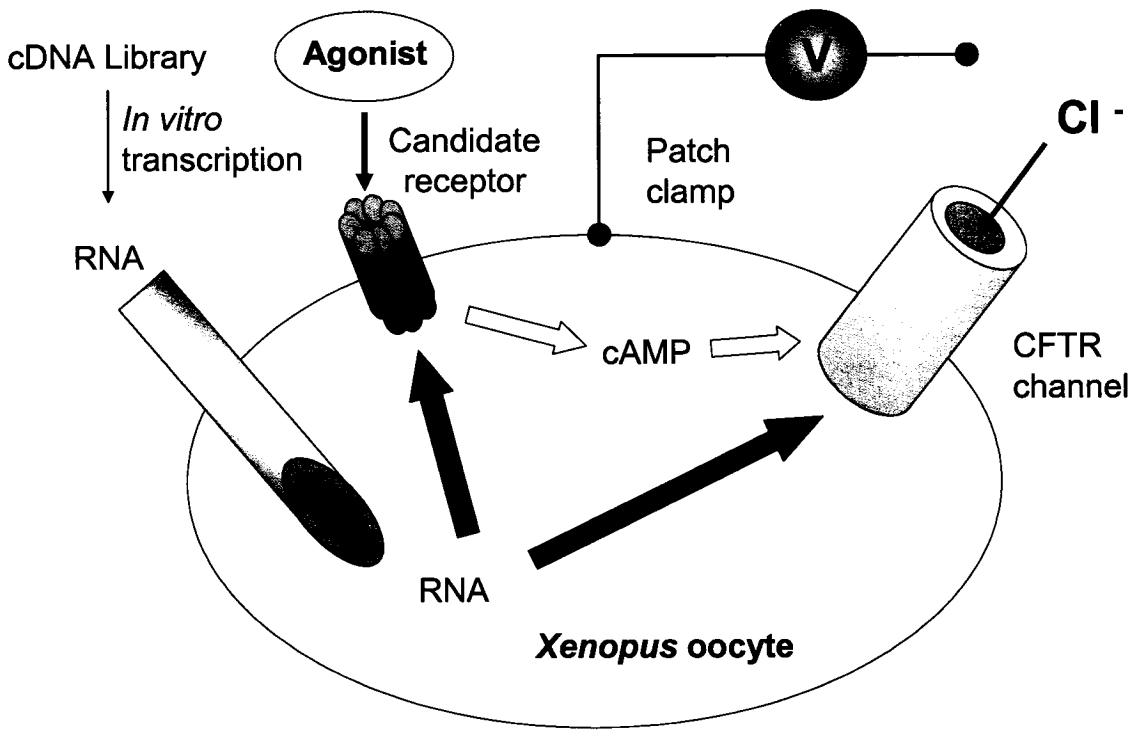
Identification of the molecular form of specific AM receptor was first reported by Kapas et al., 1995. They cited an orphan receptor known as L1 or G10d [75, 76] as being able to mediate the AM-induced cAMP response (with low nM ED_{50}) that could be antagonized with only high concentrations of $CGRP_{8-37}$, when transfected into COS-7 cells [77]. Subsequent attempts to reproduce these results for L1/G10d and its human homolog in COS-7 cells failed to detect any AM binding above the control levels and no cAMP response to AM could be detected [78]. In parallel to these events, another orphan GPCR was cloned in the same year as L1/G10d and was termed calcitonin receptor-like receptor (CRLR) [79]. It was recognized for its sequence similarity to known calcitonin receptor, yet it had broad tissue distribution, with particularly high levels in the lung, uncharacteristic of calcitonin receptor. The authors hypothesized it to be a possible CGRP or amylin receptor that could be important for regulation of vascular tone [79]. However, when transfected into COS-7 cells, the receptor failed to bind either calcitonin or CGRP and no cAMP response to a variety of stimulants, including all of the members of CGRP family of proteins, was detected [80]. In contrast, when independently cloned cDNA for the same receptor was transfected into HEK-293 cells, a $CGRP_1$ receptor-like pharmacology was observed [81], indicating one or more factors may be absent from COS-7 cells but present in HEK293 cells allowing for the expression of a functional receptor. The idea that CGRP and AM receptors may actually be multi-protein complexes was also supported by crosslinking to ^{125}I labeled agonist studies [82]. Consistent with this notion, first, CGRP-receptor component protein (RCP) [83], and

later, receptor activity modifying protein 1 (RAMP-1) [66] were functionally cloned, using *Xenopus* oocyte/cystic fibrosis transmembrane conductance regulator (CFTR) reporter system (Fig. 5). The system used in these studies was based on the method developed by Uezono et al. [84]. It utilizes a property of the CFTR chloride channel to be activated by cAMP dependent protein kinase (PKA) in response to the elevated intracellular cAMP levels. CFTR activation results in a transmembrane current that can be recorded and, in this assay, serves as a sensitive indicator of adenylate cyclases (AC) activity. Oocytes are co-injected with complementary RNA (cRNA) encoding CFTR channel and a putative AC-coupled receptor. After a certain incubation period to allow for the cell surface expression of CFTR and the putative receptor, oocytes are clamped for membrane current read-out and challenged with the appropriate agonist. *Xenopus* oocytes express endogenous CGRP receptor, therefore, candidate receptor cRNA had to augment endogenous CGRP induced cAMP response over that of the oocytes injected with CFTR cRNA alone (Fig. 5). Both RCP [83] and RAMP-1 [66] cRNA had matched this criterion. RCP was found to be a cytoplasmic protein and so could qualify as a cofactor for the receptor but not the receptor itself. Hydrophobicity plot of RAMP-1 indicated it was a single transmembrane (1TM) domain protein. That meant RAMP-1 was still in the race as a receptor candidate. This finding, however, was contrary to the expected 7TM domain GPCR protein. Working from the assumption that RAMP-1 was a novel receptor cofactor, two previously reported putative CGRP/AM GPCR sequences: CRLR and canine homolog of L1/G10d, RDC-1, were expressed in *Xenopus* oocyte/CFTR reporter system either alone or together with RAMP-1. The authors were able to show that, while expression of neither CRLR nor RDC-1 alone had

Fig. 5 ***Xenopus*** **oocyte / cystic fibrosis transmembrane conductance regulator (CFTR) reporter system.**

Candidate receptor cDNA from a cDNA library is in vitro transcribed and resulting RNA product is injected in combination with CFTR RNA into *Xenopus* oocyte. The RNA is used by oocyte's endogenous translational machinery resulting in cell surface expression of CFTR chloride channel and the putative Gs-coupled receptor. cAMP accumulation, resulting from stimulation with the appropriate agonist, activates CFTR chloride channel. Resulting influx of chloride ions is detected by a patch clamp.

Fig. 5 ***Xenopus*** **oocyte / cystic fibrosis transmembrane conductance regulator (CFTR) reporter system.**



any effect on response to CGRP, co-expression of CRLR, but not RDC-1, together with RAMP-1 resulted in an increased CGRP sensitivity that was over and above that observed from previous expression of RAMP-1 alone. They went on further, to show CRLR, the true GPCR component of CGRP receptor, depended on RAMP-1 for its function and cell surface expression. Co-expression of CRLR with RAMP-1 homologs RAMP-2 or RAMP-3 resulted in functional receptors with altered ligand specificity. While CRLR/RAMP-1 complex presented with typical CGRP receptor pharmacology, CRLR/RAMP-2 and CRLR/RAMP-3 turned out to be specific AM receptors [66]. The discovery amounted to a significant new concept in receptor biology that the same receptor molecule could serve as a specific signaling receptor for distinct extracellular stimuli and have distinct pharmacological properties depending on the repertoire and the relative expression level of the receptor cofactors in a given cell type.

Since RAMP-1 and RCP were functionally cloned, considerable attention has been given to the RAMP family of proteins while RCP remained on the sidelines. One reason for this could be that, co-transfection of RCP with CRLR and RAMP in COS-7 cells was not required to produce functional CGRP or AM receptors [66]. Nevertheless, depletion of endogenous RCP from cells expressing endogenous AM and CGRP receptors has shown that while RCP had no effect on cell surface receptor density, agonist affinity or specificity, its expression was crucial for the signal transduction by both AM and CGRP receptors. The precise mode of action of RCP is not known but it was shown to co-immunoprecipitate with CRLR and was hypothesized to be involved in coupling to G-proteins [83, 85, 86].

1.3.2.3 *AM receptor pharmacology.*

Consistent with the pharmacological data suggesting the presence of two distinct specific binding sites for AM in the rat brain and vas deferens [87], the two molecular forms of AM receptor, CRLR/RAMP-2 (AM receptor 1, AM1) and CRLR/RAMP-3 (AM receptor 2, AM2) exhibit distinct pharmacological properties [88]. AM receptor pharmacology was studied in both tissue preparations containing endogenous specific AM binding sites and in reconstitution experiments looking at cell lines transfected with various combinations of individual receptor protein components. Although integration of the pharmacological data available from reconstitution experiments with the AM1 and AM2 receptors is complicated by the differences stemming from studies done on receptor components derived from different species, expression in different host cell lines, or the presence of endogenous binding sites, several generalizations could be made. AM1 receptor is more selective for AM than AM2. In transfected cell lines AM1 receptor affinity for AM is roughly 100 to 1000 fold higher than for CGRP, while AM2 is less selective and binds AM and CGRP proteins with only 10 to 100 fold difference in affinity. AM1 and AM2 also exhibit differential sensitivity to peptide antagonists hAM_{22-52} and $hCGRP_{8-37}$ fragments. AM1 receptors are more readily antagonized by hAM_{22-52} than $hCGRP_{8-37}$. AM2 receptors seem to be equally or even more sensitive to $CGRP_{8-37}$ than to AM_{22-52} in transfected cell lines [72]. Although pharmacology of endogenous AM receptor sites is in general agreement with data from reconstituted receptors certain inconsistencies have been observed. In fact, rat AM2 receptor reconstituted in COS7 cells could not be antagonized by AM_{22-52} even at 10 μM concentration [88]. At the same time endogenous AM2 receptors were effectively inhibited by AM_{22-52} in rat mesanglial cells at 1 μM concentration [89]. Endogenous AM binding

sites, with high affinity for AM (low nM range), that are fairly insensitive (effective concentrations in μM range) to hAM_{22-52} [32, 90, 91] (possibly AM2 sites) have been reported. Similarly, endogenous high affinity AM binding sites insensitive to $CGRP_{8-37}$ (possibly AM1 sites) have also been reported [32, 92]. Additional receptor cofactors and other possible determinants overlooked in receptor reconstitution experiments and tissue or cell type specific differences inherent in endogenous binding site experiments certainly play a role in determining model cell specific aspects of AM receptor pharmacology.

1.3.2.4 *AM Receptor signaling pathways.*

Activation of adenylate cyclases, presumably through receptor coupling to Gs, rise in the levels of intracellular cAMP and subsequent activation of PKA is the most common and well established mode of signaling by AM and all members of CGRP peptide family. cAMP was shown to mediate the proliferative effect of AM on Swiss 3T3 cells [90]. An opposite effect on platelet-derived growth factor (PDGF) stimulated proliferation of Rat-2 fibroblasts was also mediated by cAMP [74]. Most of the effects of AM on vascular endothelial and smooth muscle cells are associated with elevated intracellular cAMP [32]. In fact, the initial discovery of AM was based on its ability to raise cAMP levels in platelets [26].

Increase in cAMP and subsequent PKA activation, however, is not an exclusive mode of AM signaling. For instance, in rat endothelial cells (EC), serum deprivation induced apoptosis in a subpopulation of cells. AM exerted protective, antiapoptotic action on these cells. While cAMP levels were elevated in response to AM, other cAMP inducing agents, such as forskolin, failed to imitate AM action. Furthermore, cAMP antagonist, adenosine 3',5'-cyclicmonophosphothioate Rp-isomer, could not antagonize the antiapoptotic action of

AM, suggesting a cAMP independent mechanism [36]. Dual signaling was also reported in bovine aortic endothelial cells (BAEC). As in rat EC, AM stimulated cAMP response in BAEC. In addition, AM stimulated a biphasic increase in intracellular Ca^{2+} concentrations. The initial phase consisted of a peak within 30 sec. of AM stimulation and was thapsigargin sensitive, indicating Ca^{2+} efflux from intracellular stores. The second phase consisted of a prolonged plateau and was sensitive to receptor operated channel and L-type Ca^{2+} channel blockers, suggesting it was due to influx of extracellular Ca^{2+}. Both phases were inhibited by application of the PLC inhibitor, U-73122. Consistent with the activation of PLC, the levels of IP3 were elevated with a peak at 10 sec. after AM stimulation. PLC activation, IP3 formation and subsequent rise in intracellular Ca^{2+} would be typical of Gq coupled receptor signaling except that both Ca^{2+} and cAMP effects of AM in these cells turned out to be cholera toxin (CTX) sensitive and pertussis toxin (PTX) insensitive, suggesting both effects are mediated by Gs and not Gi coupling [93].

cAMP independent signaling is not limited to EC. The mitogenic effect of AM on vascular smooth muscle cells (VSMC) was also unaffected by either cAMP antagonist or selective PKA inhibitor. Further examination also eliminated the Ca2+/PKC pathway as no changes in IP3 levels or intracellular Ca2+ could be detected and the highly specific PKC inhibitor (GF109203X) had no effect on AM stimulated activation of mitogen activated protein kinase (MAPK), a downstream effector of mitogenic stimulation. On the other hand, involvement of protein tyrosine kinase (PTK) activity with subsequent recruitment of Shc and Grb2 adaptor proteins and downstream activation of p42/p44 MAPK in response to AM stimulation was demonstrated in these cells.

Another interesting and somewhat puzzling report of interplay between AM and Amylin-responsive GPCR and a PTK receptor was reported by Cornish et al. [94] in primary rat osteoblasts. While proliferative effects of Amylin or AM and transforming growth factor β (TGF-β) are additive, co-stimulation with amylin and IGF-1 does not result in stimulation greater than when using amylin or IGF-1 alone, suggesting convergence of amylin and IGF-1 signaling pathways in these cells. Interestingly, no proliferative effect of either IGF-1, amylin, or AM could be detected in IGF-1 receptor (IGF-1R) -/- derived osteoblasts. This could be explained if AM and amylin responsive GPCR induced tyrosine phosphorylation of IGF-1R receptor which could then serve as a scaffold for Shc/Grb2 complex recruitment at the membrane and subsequent downstream activation of p42/p44 MAPK. Similar MAPK activation cascade has been proposed for other GPCRs [95]. More puzzling was the observation that treatment with amylin or AM receptor antagonist abrogated IGF-1 induced proliferation. Amylin or AM receptor antagonists did not compete with IGF-1 for binding to IGF-1R and proliferative effects of TGF-β were unaffected by the antagonists. The authors suggested a physical interaction between the liganded amylin or AM receptor and IGF-1R that may be somehow responsible for this interdependency between these two receptors [94].

1.3.2.5 *AM receptor desensitization*

Most GPCRs studied to date are subject to a process of desensitization whereby the amplitude of the agonist induced response diminishes upon repeated stimulation. A classical, well studied example is desensitization of β-Adrenergic receptor (βAR). Upon prolonged or repeated stimulation with catecholamines, βAR is phosphorylated on specific serine/threonine residues in its cytoplasmic C-terminal tail and third cytoplasmic loop by

βAR kinase (βARK) or PKA leading to attenuated receptor signaling activity. Although AM receptor desensitization has been reported, there is a considerable degree of inconsistency between reports from different laboratories even in the same cell lines. For instance, in the human neuroblastoma cell line SK-N-MC expression of CRLR/RAMP1 CGRP receptors and CRLR/RAMP2 AM receptors has been studied in the landmark paper by McLatchie et al. postulating the RAMP hypothesis [66]. The hypothesis might predict that since AM and CGRP share the same GPCR molecule, CRLR, their respective receptor desensitization should follow the same pattern. To test this prediction Drake et al. [96] chose SK-N-MC cell line to test the effect of AM and CGRP pretreatment on cAMP response to agonist re-challenge. They found roughly 50% reduction in cAMP response to CGRP stimulation after pretreatment with either CGRP or AM. The observed desensitization was completely abolished by cAMP dependent PKA inhibitor, H-89. Contrary to the predicted results, no desensitization of the AM receptor by either AM or CGRP could be detected and H-89 had no effect on cAMP induction by AM [96]. Later, AM and CGRP receptor desensitization in the same cell line was revisited by Hay et al. [97]. In this study, AM signaling was significantly attenuated by pretreatment with CGRP, but not AM (although authors acknowledge a trend toward significance); pretreatment with either CGRP or AM did not affect CGRP stimulated cAMP production [97].

In rat aortic VSMC, Drake et al. obtained very similar results to what they observed in SK-N-MC cells [98]. As before, they reported effective, PKA inhibitor sensitive, desensitization of CGRP receptors by a variety of cAMP inducing, PKA activating agents, none of which, including AM and CGRP, had any affect on subsequent AM response [98]. These results contradicted a previous report by Iwasaki et al. in the same cells, where a 10

fold higher concentration of AM pretreatment for a comparable period of time (20 min. Drake et al. vs. 15 min. Iwasaki et al.) resulted in a 23% reduction in AM induced cAMP generation. Prolonged, 2 hour pretreatment resulted in a robust (80% reduction), long lasting (24 hour) desensitization of AM receptors [99]. Interestingly, desensitization was unaffected by inhibitors of PKA (KT5720), PKC (GF109203X), or PTK (genistein). This prolonged effect of AM pretreatment could be suggestive of a receptor endocytosis and degradation dependent mechanism of desensitization. Surprisingly, disruption of neither the actin microfilament assembly by cytochalasin D nor the microtubule assembly by colchicine had any effect on desensitization in these cells [99].

In contrast to the long lasting desensitization observed in rat aortic VSMC, receptor sensitivity was readily, within 2 hours, recovered in rat mesanglial cells [100]. Direct activation of adenylate cyclase by forskolin mimicked the effect of AM pretreatment, suggesting a PKA dependent mechanism of desensitization. This mechanism was further confirmed with PKA inhibitor H89. Consistent with a role of serine/threonine phosphorylation, inhibition of serine/threonine phosphatase activity by okadaic acid (125 nM) prevented the recovery of AM sensitivity. At this concentration okadaic acid results in complete inhibition of phosphatase 2A and partial inhibition of type-1 phosphatases making them the candidate enzymes mediating resensitization of AM receptors in these cells [100].

While there are reports of both PKA dependent and independent desensitization of AM receptors and AM receptor signaling through effectors other than the cAMP PKA cascade has been shown, no reports directly implicating effectors other than PKA are available to date. This is particularly curious since consensus phosphorylation sites for PKC but not PKA are present in both CRLR and RAMPs of human and rat origin [97]. Currently,

a possible role of PLC and PKC in AM receptor desensitization remains to be established. Agonist-dependent desensitization is typically regulated by a class of serine/threonine kinases known as G-protein coupled receptor kinases (GRKs). GRK6, but not GRK2 or GRK5, has been shown by antisense RNA experiments to play a crucial role in desensitization of transfected porcine CGRP receptor in HEK293 cells [101]. A possible role of GRKs in AM receptor desensitization awaits investigation.

1.3.2.6 *Intracellular trafficking and biochemistry of CRLR and RAMPs.*

Cell surface expression of CRLR was reported to be dependent on co-expression of RAMPs [66, 102, 103] in HEK cells. Fluorescence activated cell sorting (FACS) and immunofluorescence confocal microscopy experiments have shown that, when CRLR alone is overexpressed in HEK293 or HEK293T cells, it remains mostly intracellular. On the other hand co-expression with any of the RAMPs resulted in marked translocation of CRLR to the cell surface and acquisition of the CGRP or AM receptor phenotype [66, 102, 103]. A very different result was reported by Flahaut et al. in Xenopus oocytes. Cell surface expression of Flag tagged CRLR was quantified using radiolabeled anti-Flag antibody. In this study expression of CRLR alone resulted in cell surface translocation that was as effective as when co-expressed with any of the RAMPs [104]. Whether this discrepancy is host cell specific or is due to the effect of Flag tag on CRLR transport remains to be clarified. Peptide tags have been reported to affect efficiency of protein transport. Flag tagged RAMP2 and 3, in the same study, showed a considerable degree of cell surface expression in the absence of CRLR, although, cell surface expression was further improved by CRLR co-expression [104]. Similarly, considerable cell surface expression in the absence of CRLR was observed for N-

terminal Myc-tagged RAMP-2 and RAMP-3 in HEK293 cells. At the same time, N-terminal HA-tagged or C-terminal V5-tagged RAMP exhibited a very limited cell surface expression in the absence of CRLR [105] but translocated efficiently with CRLR co-expression. Hilairet et al. [106] also reported a profound effect of the choice of tag on CRLR maturation in HEK293T cells. When transfected alone, Myc- and HA-tagged CRLR showed significant cell surface expression by immunoprecipitation (IP)/western blot analysis, which the authors attributed to the expression of endogenous RAMP-2 in these cells. However, there was a striking difference in the glycosylation state of the cell surface (CS) Mic- and HA-tagged CRLR that could only be attributed to a difference in the tag molecule. While the core-glycosylated, endo H sensitive form of CRLR with apparent molecular weight (Mr) of 58 kDa was predominant in the total extract from Myc- or HA-CRLR transfected cells, as well as cell surface IP of HA-CRLR, a major band of Mr 90 kDa was detected in cell surface IP from Myc-CRLR cells [104]. This band is thought to represent a terminally glycosylated form of CRLR in an SDS resistant complex with RAMP. Mr 90 kDa and Mr 66 kDa bands were predominant at the surface of Myc-CRLR, RAMP-2 co-transfected cells. Biochemical investigation of AM binding sites began even before the molecular identity of AM receptor was established. Cross-linking studies of endogenous receptors using ^{125}I-AM and ^{125}I-CGRP reported, in most cases, two bands, corresponding to specific agonist binding sites, with apparent molecular weight ranging from 70 to 94 kDa, and 105 to 122 kDa for the major and minor bands respectively [32]. In this respect the Mr 90 kDa receptor form detected at the cell surface of Myc-CRLR or Myc-CRLR/RAMP-2 transfected HEK cells is more in line with the reported endogenous binding site sizes. Mr 66 kDa and Mr 64 kDa bands were detected in cells co-transfected with Myc-CRLR and RAMP-1 or RAMP-3 respectively. 90

kDa, 66 kDa, and 64 kDa forms are endo H resistant and likely represent mature products of golgi processing. Peptide-N-glycosidase F (PNGase F) digestion of each of the three forms yields a Mr 48 kDa band representative of the un-glycosylated form of CRLR. Thus confirming that all three bands contain a differentially glycosylated form of the same CRLR molecule and suggesting that formation (or at least stability) of SDS resistant CRLR/RAMP complex is dependent on terminal glycosylation of its constituent proteins. In addition, a Mr 58 kDa form appeared strongly in IP from the total extract and was much less abundant but still detectable in the IP of cell surface proteins from cells transfected with Myc-CRLR alone or in combination with RAMP-2 or RAMP-3. As mentioned above, the 58 kDa form is endo H sensitive and likely represents a core-glycosylated, pre-golgi processing form of CRLR [106].

Cell surface expression of RAMPs was also reported to depend on CRLR co-expression. This requirement is particularly stringent for RAMP-1, while attenuated cell surface expression, rescueable by co-transfection of CRLR, was reported in Xenopus oocytes transfected with RAMP-2 or 3 alone [104]. In HEK cells, immunofluorescence and FACS analysis show that RAMP remain mostly intracellular in the absence of CRLR [66, 103], although, the extent of cell surface expression was reported dependent on the kind of tag molecule attached [105]. A number of publications reported that intracellular RAMP-1 and RAMP-3 can exist as reducing agent, DTT, sensitive homodimers [107, 108]. Disassociation of these homodimers and heterodimerization with CRLR is believed to take place to allow translocation of CRLR/RAMP complex to cell surface.

Whether or not the individual components of AM receptor make it to the cell surface alone in these overexpression systems, radiolabeled ligand cross-linking and agonist induced

signaling experiments indicate that it is the terminally glycosylated, RAMP/CRLR complexed molecules that are involved in agonist binding and signal transduction [32, 104-106].

As was discussed above, AM and CGRP receptors undergo rapid desensitization following agonist stimulation. Both slow [99] and fast [100] recovery, indicating internalization followed by either lysosomal degradation (slow recovery) or endosomal recycling (fast recovery) have been reported in VSMC and mesanglial cells respectively [99, 100]. Kuwasako et al. [102] were the first group to specifically investigate the fate of AM and CGRP receptors following agonist induced internalization. They reported that in HEK cells, co-expressed CRLR and RAMP are rapidly and extensively internalized from the cell surface as a complex upon stimulation with the appropriate agonist. Internalization was potently inhibited by incubation in hypertonic solution indicating a clathrin-coated vesicle mediated endocytosis. Less than a 10% increase in the number of cell surface receptors was observed after 2 hours of incubation (in the presence of protein synthesis inhibitor cycloheximide) following 30 min. of agonist stimulation and agonist washout. Thus, no significant recycling of the receptor took place in HEK cells. Instead, both CRLR and RAMP were found to co-localize with the lysosomal marker LysoTracker Red, suggesting targeting to a degradation pathway [102].

A subsequent more detailed mechanistic investigation focusing on CRLR/RAMP-1 specifically, reported that CRLR, but not RAMP-1, is phosphorylated in response to CGRP binding [108]. Receptor phosphorylation in response to agonist binding is a common regulatory mechanism for GPCRs. It usually leads to association with a multifunctional adaptor protein β-arrestin capable of interacting with an array of different proteins. Binding

of β-arrestin to GPCR can prevent receptor re-association with the trimeric G-protein complex and its subsequent activation. This is thought to be a general mechanism responsible for rapid receptor desensitization of GPCRs such as β_2-adrenergic receptor (β_2AR) for instance. β-arrestin is known to bind clathrin adaptor protein AP-2 and clathrin directly to promote receptor endocytosis via clathrin-coated vesicles. Receptor-specific pattern of receptor phosphorylation determines the avidity of β-arrestin for the receptor. Weak binding observed in Class A GPCRs, such as β_2-adrenergic receptor (β_2AR), results in endocytosis followed by β-arrestin complex dissociation and receptor recycling. Class B GPCRs, such as vasopressin 2 receptor (V_2R) or angiotensin II 1a receptor (AT1aR), bind strongly to β-arrestin. The resulting complex may persist in the endosomal compartments for prolonged periods of time before being targeted to lysosomes for degradation or slowly recycled [109, 110].

Immunofluorescence analysis of subcellular distribution patterns of CRLR, RAMP-1 and β-arrestin2 in comparison to the pattern observed for β-arrestin2 and either β_2AR or V_2R before and after agonist stimulation revealed a striking similarity in the co-localization patterns observed for the two Class B receptors, V_2R and CRLR/RAMP-1, and a distinct pattern for the Class A receptor β_2AR. Considerable overlap between V_2R or CRLR/RAMP-1 localization with β-arrestin2 was observed in the endocytic compartment after agonist stimulation, consistent with the formation of stable ternary complex between CRLR, RAMP-1 and β-arrestin2. In contrast, β-arrestin2 distribution remained diffusely cytoplasmic and little co-localization with β_2AR could be detected after agonist stimulation, consistent with transient association of this receptor with β-arrestin2 [108]. While endocytosis followed by sorting to lysosomes for degradation is the fate of reconstituted CRLR/RAMP receptors in

HEK cells and likely other cell types such as VSMC where extensive resensitization times were reported [99], alternative mechanisms must exist in cells such as rat mesanglial cells where complete resensitization is achieved within 2 hours after agonist washout [100].

An interesting insight into a possible mechanism of rapid AM receptor resensitization and RAMP mediated regulation of CRLR receptor function came from experiments where N-ethylmaleimide-sensitive factor (NSF) was co-expressed with CRLR and human RAMP-3 (hRAMP-3) in HEK cells. NSF is well established as a crucial factor universally involved in regulation of membrane fusion in eukaryotic cells as divergent as yeast and human neurons. NSF is a hexameric ATPase capable of binding (through a specific adaptor protein, SNAP) to SNARE proteins. SNARE proteins within a vesicle (v-SNARE) and target membrane (t-SNARE) form a complex responsible for driving the fusion vesicle and its target membrane. While the fusion process itself does not require energy, dissociation of the SNARE complex and its subsequent reentry into a new fusion cycle is dependent on NSF ATPase activity [111-114].

Additional roles for NSF, outside of SNARE mediated membrane fusion, have been proposed after it was found to interact directly with the GluR2 subunit of Glutamate receptor and to regulate its signaling properties [114]. More recently Cong et al. have shown direct binding and regulation of recycling of β_2AR by NSF [115]. A similar relationship with NSF was reported for CRLR/RAMP-3 receptor. As was discussed previously, agonist stimulation of CRLR/RAMP overexpressing HEK cells induced CRLR/RAMP complex endocytosis and degradation resulting in prolonged desensitization. In contrast, overexpression of NSF, CRLR and hRAMP-3, but not RAMP-1 or -2, resulted in rapid (complete in 4 hours) resensitization and recycling of the AM receptor. The role of NSF as an endosomal recycling

promoting factor was further confirmed in rat mesanglial cells, where AM sensitivity is normally recovered within 2 hours in naïve, untransfected cells. Treatment with NSF inhibitor, N-ethylmaleimide, abolished rapid resensitization of AM receptor in these cells [116]. Binding of NSF to β_2AR was shown to occur at the PSD-95/Discs-large/ZO-1 homology (PDZ) domain on the C-terminal end of the receptor [115]. It is the PDZ motif present also at the C-terminus of hRAMP-3, but not RAMP-1 or -2, that was shown to interact with NSF and to be both necessary and sufficient to confer rapid resensitization and CRLR/RAMP receptor recycling in NSF overexpressing HEK cells [116]. Interestingly, rodent RAMP-3 does not contain a classical PDZ domain sequence found in human RAMP-3 and β_2AR. Nevertheless, a series of experiments in rat mesanglial cells, including endogenous RAMP-3 knock down by RNA interference and treatment with NSF inhibitor, have shown that both rat RAMP-3 and NSF are required for recycling and resensitization of endogenous AM receptor in these cells [116]. A specific location of NSF binding site on the rat RAMP-3 molecule remains to be elucidated.

Na+/H+ exchanger regulatory factor 1 (NHERF-1) is a multifunctional adaptor protein reported to bind to PDZ domains of both hRAMP-3 and β_2AR [117, 118]. NHERF-1 contains two PDZ domains of its own, allowing for interactions with a multitude of cytosolic and membrane proteins. In addition, it contains merlin and ezrin/radixin/moesin (MERM) binding site that allows tethering of PDZ bound target proteins to the MERM family of cytoskeletal proteins [118]. Co-expression of NHERF-1 with CRLR/hRAMP-3 receptor in HEK cells prevented agonist induced receptor internalization without any effect on receptor desensitization. Even after prolonged exposure to agonist most of the receptor remained at the cell surface. Treatment with actin depolymerizing agent, cytochalasin D, or co-

expression of mutant NHERF-1, lacking the MERM binding site, rescued agonist induced receptor internalization. This suggested NHERF-1 adaptor mediated tethering of CRLR/hRAMP-3 complex to the actin cytoskeleton as a likely mechanism for the previously observed inhibition of agonist induced CRLR/hRAMP-3 endocytosis. The significance of this interaction was also confirmed in human proximal tubule epithelial (PTE) cells that express endogenous CRLR, RAMP-2, RAMP-3, and NHERF-1. As in the transfection experiments in HEK cells, agonist stimulation of human PTE cells resulted in receptor desensitization but not receptor internalization, even after prolonged exposure to AM. RNA interference knock down of either RAMP-3 or NHERF-1 resulted in marked reduction in the number of AM binding sites at the cell surface following stimulation with AM. Thus NHERF-1 mediated arrest of CRLR/RAMP-3 receptor at the cell surface in human PTE cells under physiological conditions [117]. NHERF-1 binding was shown to take place at the same amino acid sequence as was reported for NSF in both AM2 receptor and β_2AR [115, 116]. Given that the PDZ domain sequence found at this location in β_2AR and hRAMP-3 is not conserved in rodent RAMP-3, and that this sequence still likely mediates the binding of rat RAMP-3 to NSF, it would be interesting to examine whether regulation of CRLR/RAMP-3 receptor endocytosis by NHERF-1 also takes place in rodent cells. Unfortunately, NHERF-1 binding to rodent RAMP-3 has not been examined. Also given the importance of endocytosis and recycling for receptor resensitization, it would be interesting to know what effect, if any, does NHERF-1 mediated arrest of CRLR/RAMP-3 receptor at the cell surface have on resensitization.

1.4 Purpose and Approach.

The idea for this study came from the observation that expression of autocrine/paracrine signaling protein AM was strongly downregulated during the first two days of induction of adipogenic differentiation in 3T3-L1 preadipocyte cell line. Since, in addition to its well established role as a potent hypotensive peptide, multiple other, target cell specific, effects of AM were reported, we hypothesized that AM could play a role as a negative modulator of adipogenic conversion in the 3T3-L1 preadipocyte model system. A direct approach was chosen, whereby exogenous, mature AM peptide was supplemented into the culture medium of 3T3-L1 cells at various stages during differentiation and the extent of adipogenic conversion after a predefined time interval was examined. Measure of mRNA abundance of several marker proteins known to be upregulated during differentiation was taken as a sensitive indicator of adipogenesis.

Furthermore, detailed examination of mRNA expression of four proteins comprising the molecular forms of AM/CGRP receptor complexes was conducted throughout IDI induced differentiation process in 3T3-L1 cells.

2. MATERIALS AND METHODS

2.1 *Materials.*

Anti-CRLR affinity purified antibody was purchased from Alpha Diagnostic International and used at 1 μg/ml conc. Mature, amidated, disulfide bonded mouse AM_{1-50} synthetic peptide (cat. 010-31) and human AM_{22-52} peptide (cat. 010-04) were purchased from Phoenix Pharmaceuticals. Peptide was reconstituted in water and aliquots were lyophilized and stored at -80°C until used. Just before cell treatment AM was reconstituted in appropriate amount of culture medium and applied to the cells. Alternatively, for agonist/antagonist experiments, peptides were reconstituted in water containing 0.1 mg/ml BSA and added to cell culture media to a final concentration of 2 μg/ml BSA, 1 μM AM_{22-52}, and/or 100 nM AM_{1-50} .

2.2 *3T3-L1 cell culture and differentiation.*

3T3-L1 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's culture medium (DMEM) containing 10% FBS and 28 μg/ml Gentamycin. Before experiments, cells were maintained at less than 80% confluence. For induction of differentiation, cells from passage 12 or less were allowed to reach confluence and were further cultured for 2 to 4 days postconfluence. At this point, cells are referred to as day 0 preadipocytes (D0). Differentiation was induced by exposing D0 cells to DMEM containing 10% FBS and 28 μg/ml Gentamycin supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM Dexamethasone, and 1 μg/ml Insulin (IDI media) for 48 h (D1 and D2) or for 24 h (D1) followed by IDI medium replacement and additional 24 h

incubation (D2) for the experiments where differential AM peptide treatments were carried out on D1 and D2. Following induction cells were re-fed daily (day 3 to day 7, D3-D7) with DMEM containing 10% FBS and 28 μg/ml Gentamycin for the duration of the experiment.

2.3 *Real time quantitative RT-PCR.*

Total cellular RNA was isolated with RNeasy Qiagen kit. RNA concentrations were determined as measured optical density (OD) at 260 nm (in a 10 mm path length cuvette) multiplied by 40 μg/ml conversion factor, and ratio of OD at 260 nm to OD at 280 nm was determined to be between 1.6 and 1.9 by spectrophotometry on Ultrospec 3100*pro* UV/Vis spectrophotometer (Applied Biosystems Inc.). Total purified RNA was decontaminated of genomic DNA by digestion with HPLC purified DNaseI (Pharmacia Biotech. Inc.). cDNA was synthesized using oligo-dT_{18} oligonucleotide primers and SuperScriptII (Invitrogen) reverse transcriptase from 5-10 μg total RNA template in 40 μl total reaction volume. The resulting cDNA was diluted 1 in 3; equivalent of 1 μl of the diluted cDNA was used as template for real time quantitative PCR with SYBR Green I kit from Roche. Reactions were run in the Roche LightCycler and analyzed against a standard curve included in each run using the LightCycler 3.5 software. The standard curve for each run was constructed by the software from serial 10 fold dilutions of agarose gel-purified PCR product, specific for each set of primers, used as a standard. Ribosomal protein L30 mRNA was used as an internal standard. Reverse transcriptase negative reactions were set up and analyzed by real time quantitative PCR to control for genomic DNA contamination. The amount of target RNA was calculated relative to the amount of L30 mRNA. Table 1 lists the primer information.

Table 1. Quantitative real time RT-PCR primer sequences.

List of forward (F) and reverse (R) primer sequences. RAMP-3 F and R primers are located in the 3'-untranslated region. RAMP-3 F1 and R1 are the 5'-end primers for the RAMP-3 mRNA, that are separated by 18 kbp in the genomic sequence.

Table 1. Quantitative real time RT-PCR primer sequences.

Target prot.	Primer pair sequence	
Leptin	F	CTC ATG CCA GCA CTC AAA AA
	R	AGC ACC ACA AAA CCT GAT CC
PPARγ2	F	ATGGGTGAAACTCTGGGAGAT
	R	CATCCTTCACAAGCATGAACTC
Adipsin	F	CCTGAACCTTACAAGCGATG
	R	GGTTCCACTTCTTTGTCCTCG
AM	F	GCTACTGTCAGAAGGACTTCT
	R	GGAACTGCGAAGGAGTATCTG
CRLR	F	AGCTTCCTCCACCAACAAGA
	R	TGTGGCTCCCTTAGACATCA
RAMP-1	F	AACAGCATCAGGAGCAGGTC
	R	CCCACAGTGGTGATGAAGTG
RAMP-2	F	TCCCCTCCCTACCCACTTAC
	R	CTCAGCTGTGCTGCTCTACG
RAMP-3	F	CTAGCCGTTTCTTGGCAGAG
	R	GGTCACACGTGGCTCCTAAT
	F1	TGCATCTTAGTTGGCCATGA
	R1	CCTGTGGATTCCAGTGATGA
L30	F	AAGTGGGAAGTACGTGCTGG
	R	CACCAGTCTGTTCTGGCATG

Specificity of amplification was monitored by examining the melting curve of PCR products and by agarose gel electrophoresis.

2.4 *IR-AM measurement in spent cell culture media and cell lysates.*

Mouse AM-specific radioimmunoassay (RIA) kits were purchased from Phoenix Pharmaceuticals. Cells plated in 60 mm plates were grown and induced to differentiate as described above in 2 ml of media per plate. 24-hour spent media was collected at indicated time points. After addition of a Complete Mini protease inhibitor cocktail (Roche), the media was stored at -80°C until assayed. 100 µl of media were assayed directly, or 1/2 and 1/10 dilutions with the supplied RIA buffer were made and 100 µl were used for the assay according to manufacturer's protocol.

2.5 *Triglyceride assay.*

Cells were washed twice with PBS; care must be taken to remove all trace of PBS before proceeding with extraction. Triglycerides were extracted once for 10 min and two times for 30 min with 2 ml of 2:3 mixture of isopropanol to heptane. Extracts were combined mixed, and two 10 % (volume) aliquots or a 10 % and a 25 % aliquote from each sample were placed in individual tubes and evaporated on a SpeedVac. The following triglyceride assay is based on the method originally described by Van Handel [119]. For the triglyceride essay, 300 µl of isopropanol followed by 150 µl of saponification reagent (10 % w/v KOH, 25 % v/v isopropanol) was added to each tube, vortexed, and incubated for 10 min at room temp. Next, 300 µl of sodium metaperiodate reagent (3 M sodium metaperiodate, 1 M ammonium acetate, in 6 % v/v acetic acid) were added and vortexed; 300 µl of acetyl

acetone reagent (0.4 % v/v 2-4-pentadione in isopropanol) were added, vortexed and the mixture was incubated at 65°C for 10 to 15 min. Tubes were then cooled down to room temperature and optical density at 410 nm was measured. Linear relationship between optical density and triolein standard concentration in the OD_{410} 0 to 1.6 range was established (Fig. 6).

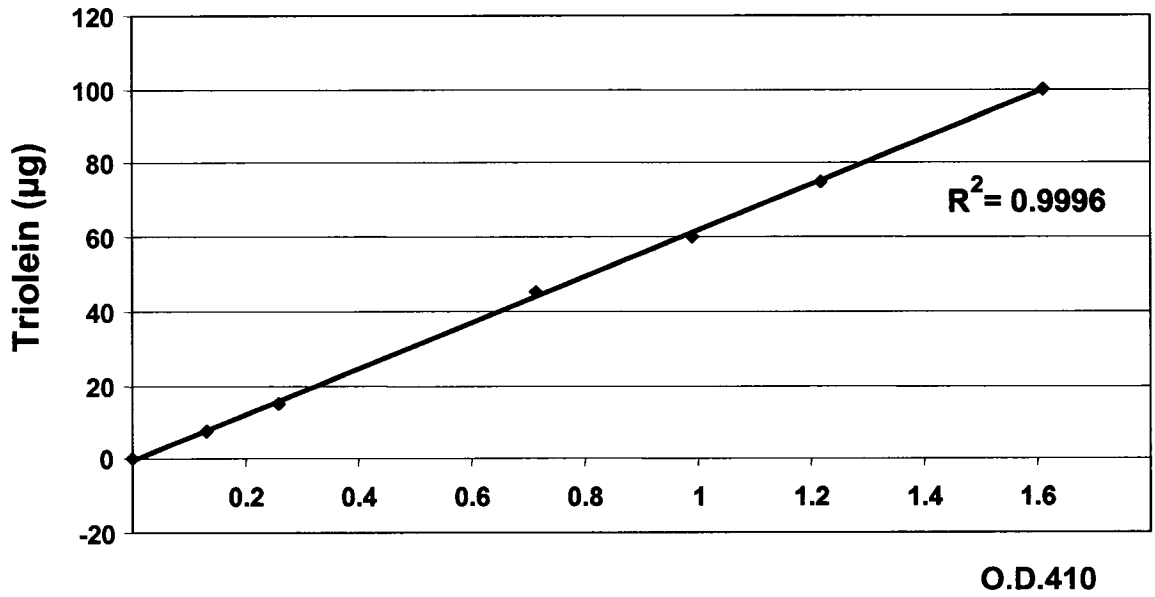
2.6 *Semi-quantitative immunoblotting.*

At indicated time points, cells were rinsed three times with PBS pH 7.2 at room temperature (RT) and lysed on dishes with ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 0.5% deoxycholate, 1% NP40, 0.1% SDS, and Complete Mini protease inhibitor cocktail (Roche). The dishes were incubated on ice for 10 min; cells were then scraped off the plates, transferred into microcentrifuge tubes and incubated on ice for an additional 10 min. Lysates were homogenized by passing them through a 26G needle; they were spun down for 10 min at 10,000g and 4°C. The supernatants were collected for Western blot analysis. Total protein concentration per sample was estimated by Bradford protein essay (Bio-Rad) against BSA standard. Samples were denatured by heating to 99°C for 5 min. in sample loading buffer (SLB) (containing final concentration of 50 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS, 100 mM DTT, 0.1% Bromophenol Blue) and 50 µg of total protein were loaded per lane for SDS-PAGE. Proteins were electrophoretically transferred onto PVDF membrane (Millipore). The membrane was blocked with 5% milk in TBS-Tween 20 (0.2%) solution for 1 h at RT and probed with primary antibody (Ab) at 4°C overnight. Blots were rinsed three times 15 min with TBS-Tween 20 and incubated with appropriate HRP-conjugated secondary Ab for 1 h at 4°C. The secondary Ab's were rinsed off three times for 15 min with

Fig. 6 Triglyceride assay: Linear relationship between optical density and triolein standard.

Triglyceride assay was performed on known amounts of triolein standard and O.D. readings at 410 nm were plotted against the μg amount of standard. Linear relationship was observed in the O.D.410 range from 0 to 1.6. (This figure is a courtesy of Betina Choo, a 4th year honors student in the laboratory).

Fig. 6 **Triglyceride assay: Linear relationship between optical density and triolein standard.**



TBS-Tween 20 and the signal was detected on Kodak Biomax Light autoradiography film from Western Lightning Chemiluminescence Reagent. The film was scanned on the Chemigenious 2 Bioimaging System and band densities quantified using GeneTools software from SynGene. Quantities were normalized to actin immunoblotting signal as an internal standard.

2.7 *Analysis of quantitative data.*

Data were presented as average value ± SEM. Significance was established by student's *t* test, $P < 0.05$ was considered significant.

3. RESULTS:

3.1 Effect of Exogenous Adrenomedullin on Adipogenic Conversion of Mouse 3T3-L1 pre-adipocytes.

3.1.1 *Expression of endogenous AM is regulated with differentiation in 3T3-L1 cells.*

When confluent 3T3-L1 preadipocytes are induced to differentiate into adipocytes with IDI induction cocktail, AM expression dramatically decreases on the first day of induction (Croissandeau & Mbikay, unpublished, [47, 50]). To confirm and extend these observations, we examined the level of AM mRNA expression and protein secretion into culture medium throughout differentiation, from D0 to D7. Cells were cultured and induced to differentiate as described in the methods section. For each experiment triplicate samples were collected at each time point from D0 (confluent preadipocytes just before the induction) to D7 (at roughly 24 hour intervals). Total RNA was extracted and cDNA was prepared for analysis by real time PCR. In agreement with previously reported results, the level of AM mRNA decreased during the first two days of differentiation (D1 and D2, Fig. 7A) and reached its lowest level of 2 ± 0.1% (percentage of D0 ± SEM $p<10^{-12}$, n=9) on D2. It was restored in subsequent days and increased to ~2.5 fold the D0 level in D6 and D7 mature adipocytes (Fig. 7A).

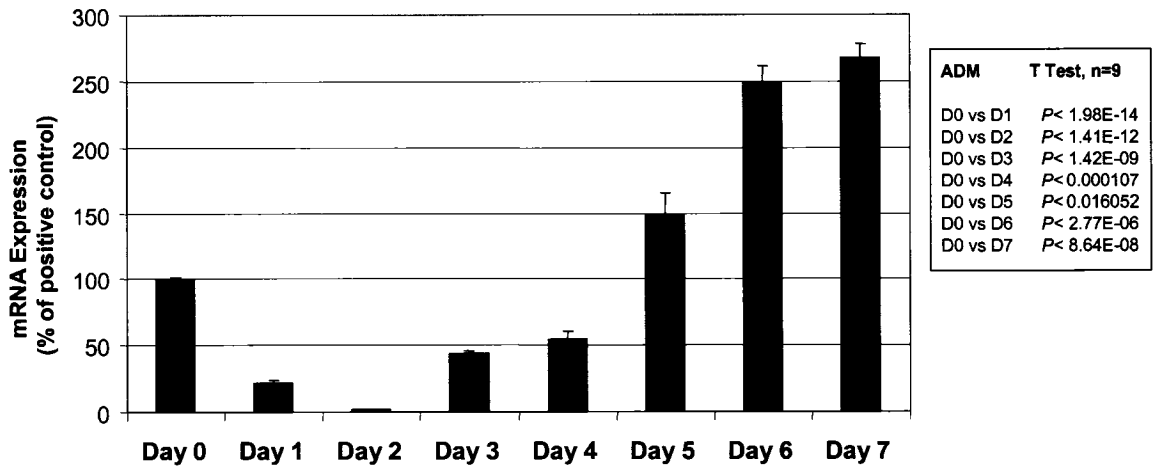
We have further examined secretion of AM peptide by measuring the amount of immunoreactive AM (IR-AM) accumulated in the cell culture media of both IDI induced and non-induced cells over a period of 24 hours throughout differentiation from D0 to D7. Consistent with the reduction in AM mRNA, the amount of IR-AM secreted by the cells in pg/60 mm plate/24 h was reduced from 473 ± 53 pg on D0 to 60 ± 9 pg on D2 ($p<0.005$, n=5) (Fig. 7B). The reduction was also significant when compared to time matched (D2)

Fig. 7 **Change in Adrenomedullin mRNA levels and protein secretion throughout IDI induced differentiation in 3T3-L1 cells.**

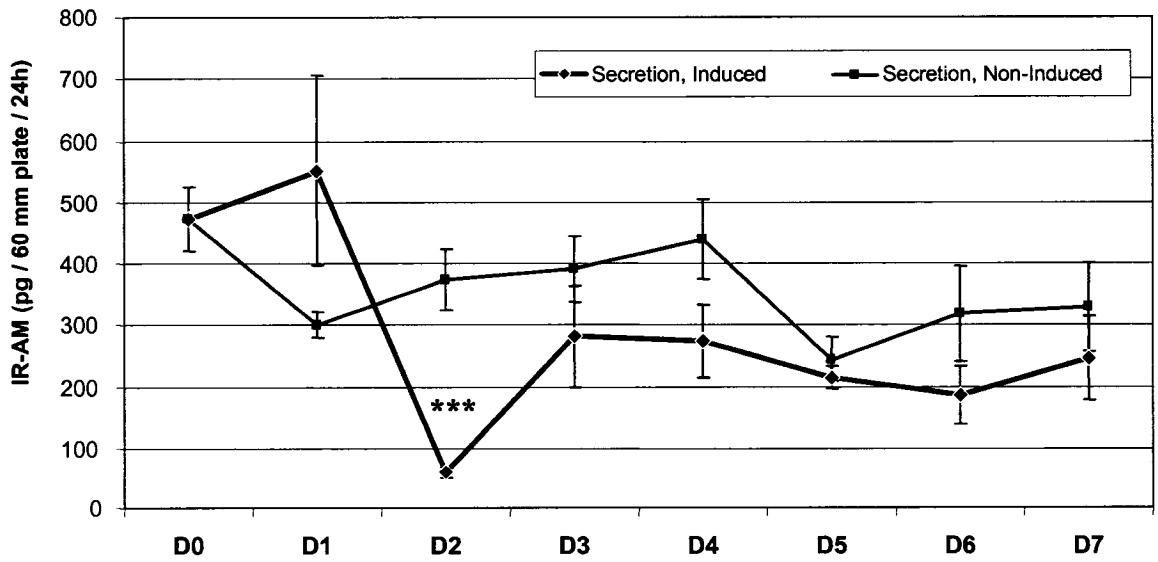
Confluent 3T3-L1 preadipocytes were induced to differentiate as described in Materials and Methods section. **A:** Samples were collected at indicated time points and processed for the total RNA isolation. Real time quantitative RT-PCR analysis of the abundance of AM message relative to that of housekeeping gene L30 were performed and the data presented as a percentage of D0 value. Significant changes are listed in the table on the right side of the graph. **B:** 24 hour spent media was collected throughout experiment from D0 to D7 from cells induced to differentiate and time matched non-induced controls. RIA assay for IR-AM was performed and expressed as pg total IR-AM secreted per plate over a 24 hour interval. *** indicates significant, $p<0.005$, change as compared to time matched non-induced control.

Fig. 7 Change in Adrenomedullin mRNA levels and protein secretion throughout IDI induced differentiation in 3T3-L1 cells.

A



B



non-induced control ($p<0.005$, n=5). Following D2, the secretion of IR-AM rose back to 281 ± 83 pg on D3 and did not change significantly on subsequent days (Fig. 7B).

3.1.2 *Effect of supplemented AM on 3T3-L1 cell expression of adipogenic markers.*

The regulation of AM expression and secretion during 3T3-L1 cell adipogenic differentiation suggested that this peptide might influence this process. We therefore assessed the effect of mature mouse AM_{1-50}, added at different time points, on IDI-induced differentiation by examining, at D7, the mRNA expression level of leptin and PPARγ2, two proteins whose mRNA levels are known to progressively increase during adipogenic conversion.

Cells were cultured in medium containing 100 nM AM_{1-50} for 24 h, either on D1, D2, D3, D4, or throughout the experiment from D1 to D7 (AM containing medium replaced every 24 h). Positive control cells were allowed to differentiate in the absence of AM treatment. The two negative controls consisted of cells not treated with IDI and incubated from D1 to D7 in medium with or without AM_{1-50}. When cells were cultured in fresh medium daily supplemented with 100 nM AM_{1-50} from D1 to D7, there was a significant reduction in the levels of transcripts for leptin (43 ± 9% of control, $p < 0.01$, n = 9) and PPARγ2 (18 ± 7% of control, $p<0.005$, n = 6) (Fig. 8A). Interestingly, when cells were incubated in AM-supplemented medium only during D2, a significant reduction was also observed in the levels of transcripts for leptin (47 ± 9% of control, $p<0.05p$, n = 9) and PPARγ2 (45 ± 10% of control), $p < 0.05$, n = 6) (Fig. 8A). There was no significant change in the level of these transcripts when AM was supplemented for D3 or D4 only. These results

Fig. 8 **Timing and concentration-dependent effect of supplemented AM on mRNA expression of adipocyte markers in terminally differentiated, D7 3T3-L1 adipocytes.**

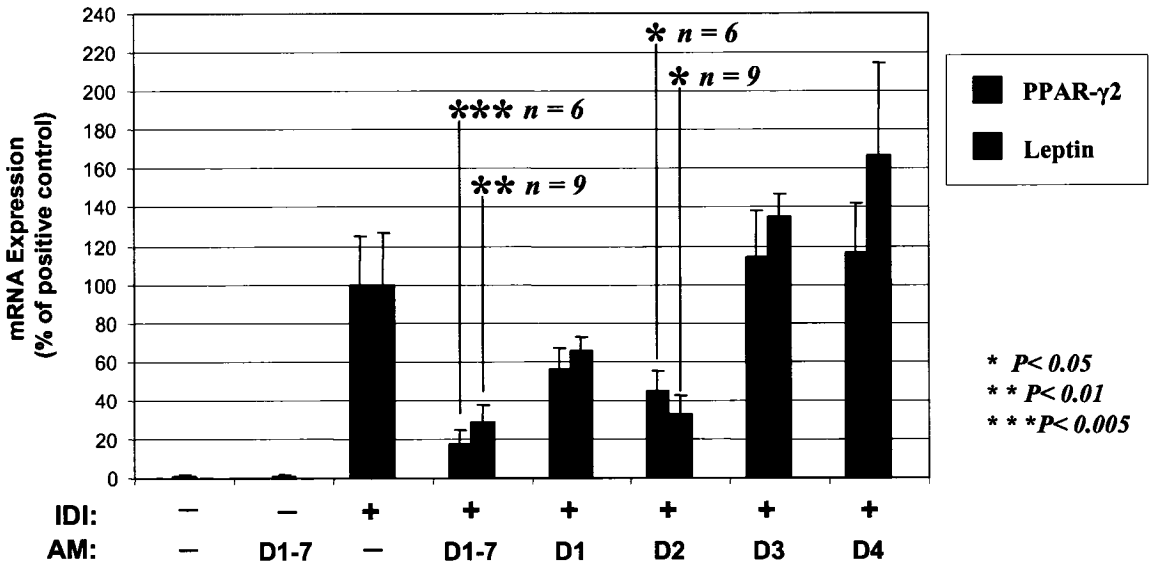
Confluent 3T3-L1 preadipocytes were induced to differentiate as described in Materials and Methods section. Samples were collected at D7 and processed for the total RNA isolation. Real time quantitative RT-PCR analysis of the abundance of PPAR-γ2, leptin and adipsin mRNA relative to that of housekeeping gene L30 were performed and the data presented as a percentage of IDI induced, untreated positive control value. Significant changes are indicated: * $p<0.05$; ** $p<0.01$; *** $p<0.005$. N indicates total number of experimental plates for each data point.

A: Induced, IDI (+), or non-induced, IDI (-) cells were treated for the indicated periods of time with 100 nM mAM1-50. The bars represent mRNA levels of PPARγ2 or leptin at D7.

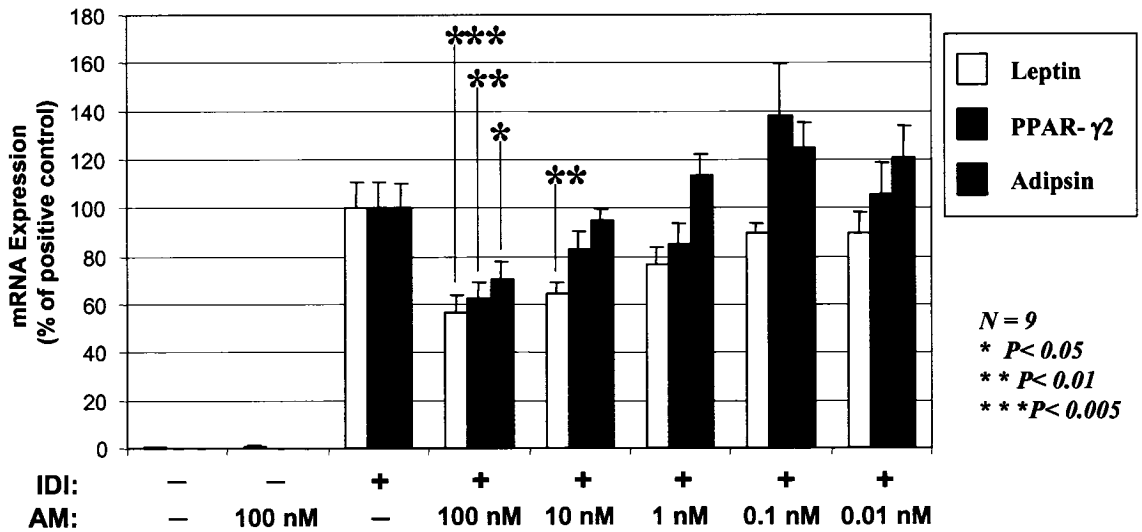
B: Induced, IDI (+), or non-induced, IDI (-) cells were treated for 24 h during the D2 only with 100 nM mAM1-50. The bars represent mRNA levels of leptin, PPARγ2 or adipsin at D7.

Fig. 8 **Timing and concentration dependent effect of supplemented AM on mRNA expression of adipocyte markers in terminally differentiated, D7 3T3-L1 adipocytes.**

A



B



suggest that AM negatively regulates expression of adipocyte markers in 3T3-L1 cells and that these cells are most sensitive to this inhibitory effect on D2 of IDI induction.

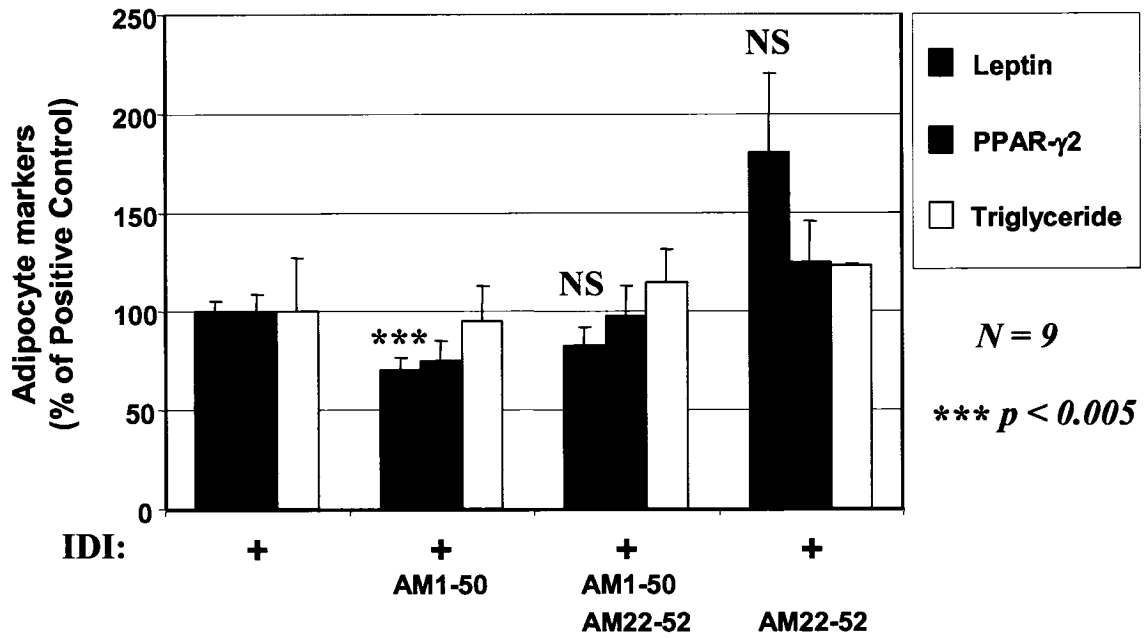
To examine the dose-dependence of this effect, 3T3-L1 preadipocytes were induced to differentiate; on D2, they were then incubated for 24 h in medium containing 100, 10, 1, 0.1, or 0.01 nM AM_{1-50}. On D7, cells were harvested for quantitative RT-PCR analysis of leptin, PPARγ2 and adipsin mRNAs. The level of leptin transcripts was significantly reduced by treatment with 10 nM and 100 nM mouse AM_{1-50} (65 ± 5% *p<0.01,* n=9 and 57 ± 7% *p<0.005,* n=9, respectively). A significant reduction of PPARγ2 and adipsin mRNA levels (63 ± 6% *p<0.01,* n=9 and 71 ± 7% *p<0.05,* n=9, respectively) was observed with 100 nM AM_{1-50} treatment and a dose-dependent trend was observed at lower concentrations of supplemented AM_{1-50} (Fig. 8B).

To further examine whether the observed inhibitory effect of AM_{1-50} is mediated through specific AM_{1-50} receptors and to test whether endogenously produced and secreted AM_{1-50} has an effect on differentiation of IDI induced 3T3-L1 cells, I have measured the relative levels of Leptin and PPARγ2 transcripts at D7 in IDI induced cells treated for 24h during D1 and D2 with either AM_{1-50} alone; AM receptor antagonist (1 μM hAM_{22-52}) alone; or, pretreated with 1 μM hAM_{22-52} alone for 20 min followed by addition of AM_{1-50} and incubation for the remainder of 24h. Leptin and PPARγ2 expression of IDI induced cells treated with peptide reconstitution buffer containing no AM peptides was set at 100 % (Fig. 9). As before, significant reduction in Leptin expression was observed in AM_{1-50} treated cells (70 ± 6% *p<0.005,* n=9*).* Preincubation with a specific AM receptor antagonist hAM_{22-52}, partially relieved the inhibitory effect of AM as leptin mRNA expression was no longer significantly different from that of positive control. However, relief of agonist effect was

Fig. 9 **Effect of AM receptor antagonist on endogenous and supplemented AM mediated modulation of adipocyte markers in terminally differentiated, D7 3T3-L1 adipocytes.**

Confluent 3T3-L1 preadipocytes were induced to differentiate as described in Materials and Methods section. On D1 and D2 cells were treated for 24 h with either: 100 nM mAM1-50; 1 μM hAM22-52; pretreated with 1 μM hAM22-52 for 20 min followed by addition of 100 nM mAM1-50; or vehicle alone. Samples were collected at D7 and processed for either total triglyceride measurement or the total RNA isolation. Real time quantitative RT-PCR analysis of the abundance of PPAR-γ2 and leptin message relative to that of housekeeping gene L30 were performed. The accumulation of total triglyceride and mRNA abundance data were presented as a percentage of IDI induced, vehicle treated positive control value. Significant change in leptin mRNA relative to IDI induced, vehicle treated positive control is indicated - *** $p<0.005$, n=9; NS – not significant.

Fig. 9 **Effect of AM receptor antagonist on endogenous and supplemented AM mediated modulation of adipocyte markers in terminally differentiated, D7 3T3-L1 adipocytes.**



only partial, since significance was not attained when compared to treatment with agonist (AM_{1-50}) alone (82 ± 9% *p<0.309, n=9).* In spite of an apparent increase in Leptin expression in cells treated with antagonist (hAM_{22-52}) alone, it failed to reach statistical significance when compared to untreated, IDI induced cells (180 ± 40% *p<0.085, n=9).* The increase, however, was significant when compared to agonist (AM_{1-50}) treated samples (*p<0.05, n=9*). While the expression profile for PPARγ2 correlated well with that of Leptin, the changes observed were not found to be statistically significant (Fig. 9).

In parallel experiments the effect of AM and its receptor antagonist on triglyceride accumulation was investigated. The amount of triglycerides per plate was measured against trioleate standard after cells were treated as described above. No significant effect on triglyceride accumulation at D7 was observed (Fig. 9).

3.2 Regulation of adrenomedullin signaling system during IDI induced 3T3-L1 preadipocyte differentiation.

3.2.1 *mRNA expression of AM receptor complex components during differentiation.*

The response of the cells to exogenous or endogenous AM depends on the presence of a functional CRLR-RAMP receptor complex. We therefore examined whether the mRNA expression levels of the AM receptor components were modulated during IDI induced adipogenic differentiation of 3T3-L1 preadipocytes. Cells were cultured and induced to differentiate as described in the methods section. The level of CRLR, RAMP-1, RAMP-2, and RAMP-3 mRNA expression was examined at 24 hour intervals from D0 to D7. For each

experiment triplicate samples were collected at each time point. Total RNA was extracted and cDNA was prepared for analysis by real time PCR (Fig. 10).

The level of CRLR mRNA was slightly but significantly increased on D2 (163 ± 19% of D0, $p<0.05$, n=9) as well as D5 and D6 (135 ± 14% and 127 ± 11% respectively, $p<0.05$, n=9) (Fig. 10A).

The levels of RAMP-1 transcripts decreased on D1 and D2 to 13 ± 2% and 11 ± 2% respectively ($p<7x10^{-10}$, n=9), then rose on subsequent days to a plateau corresponding to 6-fold the starting D0 level (Fig. 10B).

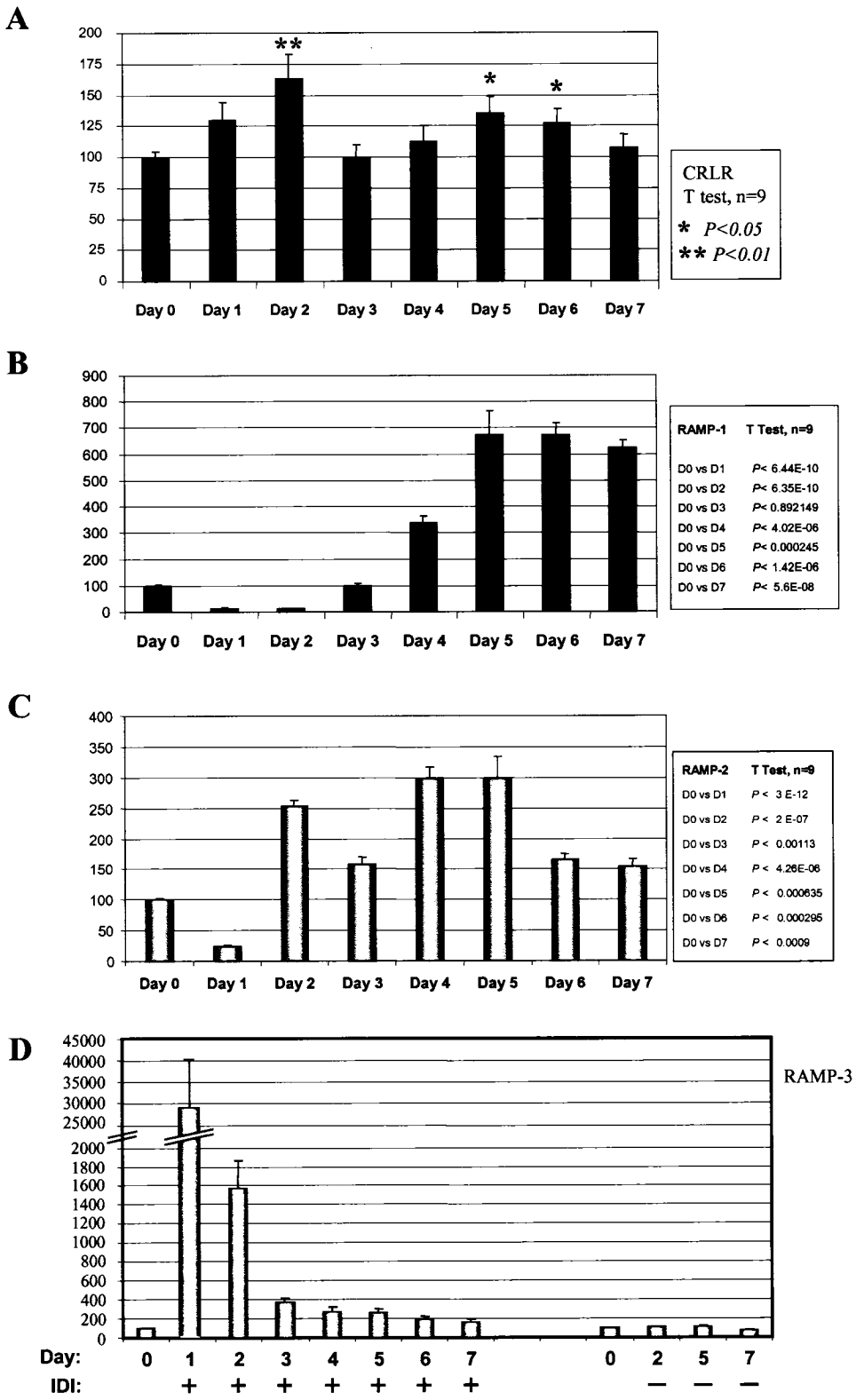
The level of RAMP-2 mRNA level fluctuated somewhat during the differentiation. Relative to D0, it decreased to 23 ± 3% ($p<3x10^{-12}$, n=9) on D1, and then increased to 253 ± 10% ($p<2x10^{-7}$, n=9) on D2, 158 ± 12% ($p<0.005$, n=9) on D3, just under 300% on D4 and D5 (298 ± 19%, $p<5x10^{-6}$ and 297 ± 37%, $p<0.001$ respectively, n=9), and finally dropped to 164 ± 11%, ($p<0.005$ n=9) and 155 ± 11%, ($p<0.001$, n=9) on D6 and D7 respectively (Fig. 10C).

Relative to D0 level, RAMP-3 mRNA level increased 300 fold on D1, decreased sharply to 16 fold on D2 and then gradually subsided to 1.7 fold ($p<0.05$, n=9) of D0 level by D7 (Fig. 10D). This increase was consistently detected in three independent experiments each performed in triplicate. Relative to average D0 value, the smallest and the largest increase measured was 93 fold and 1134 fold respectively, with a median increase of 194 fold (n=9). Given the remarkable amplitude of RAMP-3 upregulation we decided to confirm the target specificity by other methods in addition to PCR product melting curve and agarose gel electrophoresis. The PCR primers used in these experiments were directed to the 3'-untranslated region of the mouse RAMP-3 mRNA sequence. The PCR product was

Fig. 10 Regulation of CRLR, RAMP-1, RAMP-2 and RAMP-3 mRNA throughout IDI induced differentiation in 3T3-L1 cells.

Confluent 3T3-L1 preadipocytes were induced to differentiate as described in Materials and Methods section. Samples were collected at indicated time points and processed for the total RNA isolation. Real time quantitative RT-PCR analysis of **A:** CRLR; **B:** RAMP-1; **C:** RAMP-2; and **D:** RAMP-3 mRNA levels relative to that of housekeeping gene L30 were performed and the data presented as a percentage of D0 value. Significant changes are listed in the table on the right side of the graph.

Fig. 10 **Regulation of CRLR, RAMP-1, RAMP-2 and RAMP-3 mRNA throughout IDI induced differentiation in 3T3-L1 cells.**



sequenced with either 5' or 3' PCR primer, overlapping sequences were obtained and both sequences matched the expected target sequence. We further designed an additional pair of PCR primers that amplified a 306 bp fragment in the 5' end of RAMP-3 mRNA but were interspaced by 18 kbp in the genomic sequence. An additional triplicate experiment was performed and RAMP-3 mRNA levels were measured using this new set of primers. Consistent with the previous results, 745 fold increase in RAMP-3 mRNA was observed on D1 compared to D0 in IDI induced cells.

3.2.2 *Expression of AM receptor complex proteins during differentiation.*

Protein expression of CRLR during adipogenic conversion was also examined. A major band with apparent molecular weight of 54 kDa was consistently observed in the total cell lysates and its density was measured relative to that of actin by semi-quantitative immunoblotting. When comparing average densitometry results from three independent experiments, the levels of this protein appeared to mirror those of CRLR mRNA; however, the changes failed to reach statistical significance (Fig. 11 A and B).

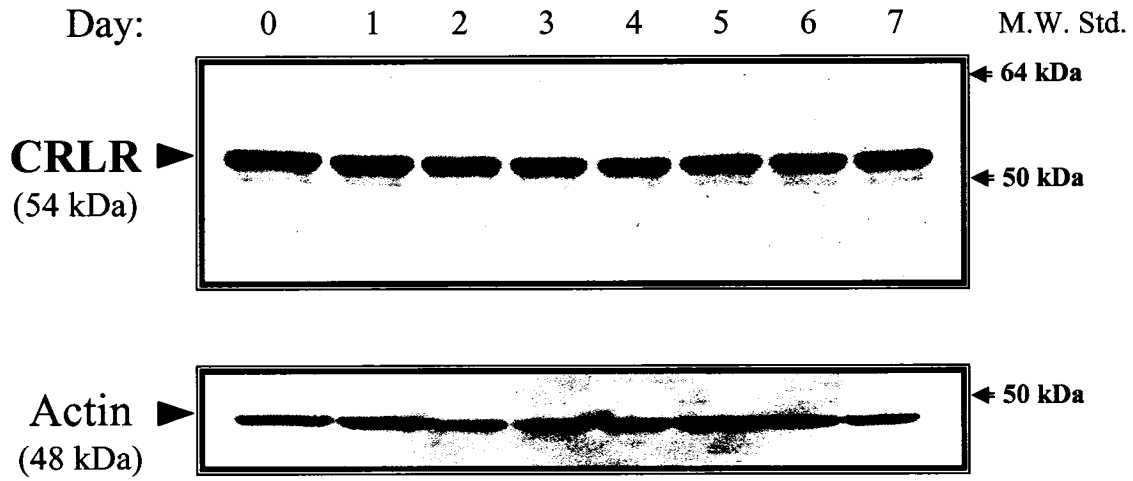
Attempts were made to examine protein levels of RAMP-2 by western blotting and RAMP-3 using two different commercially available antibodies for western blotting, metabolic labeling and pulse/chase experiments followed by anti-RAMP-3 immunoprecipitation of radiolabeled protein-antibody complexes, SDS-PAGE and autoradiography, and by cell surface protein biotinylation-pull down and N-Glycosidase-F digest. The results of these experiments did not allow for definitive identification of RAMP-2 or RAMP-3 specific immunoreactivity (data not shown).

Fig. 11 Expression of CRLR throughout IDI induced differentiation in 3T3-L1 cells.

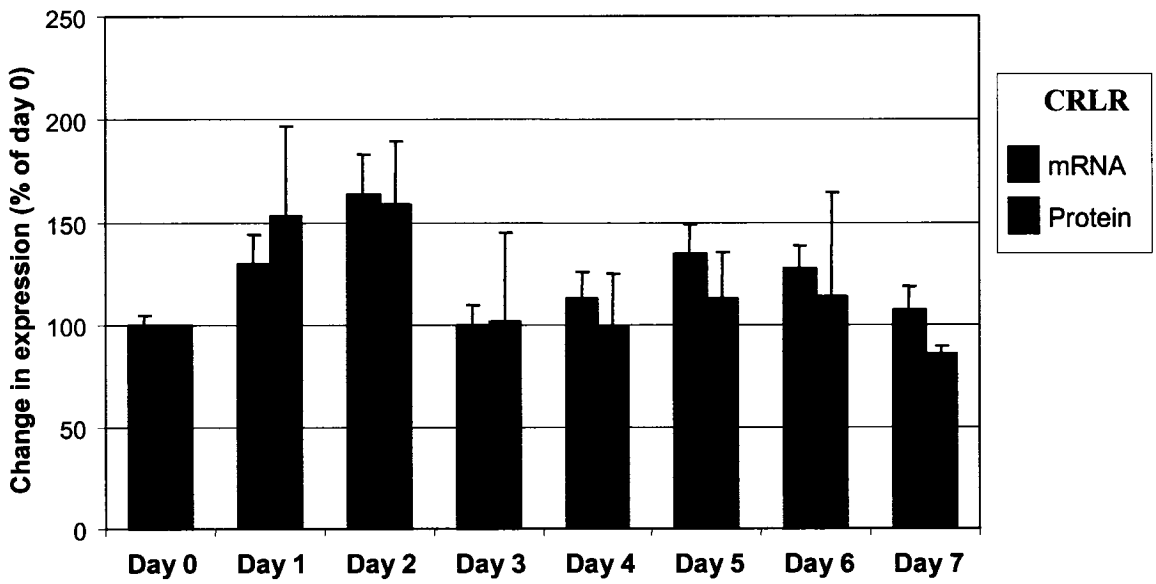
Confluent 3T3-L1 preadipocytes were induced to differentiate as described in Materials and Methods section. At indicated time points total cell lysates were collected. Total cell proteins were sepsrated by SDS-PAGE and blots probed with anti-CRLR anibodies. **A:** Western blot representative of three independent experiments. **B:** Densitometry analysis were performed. Measurments were normalized against actin as an internal control. Values were expressed as a percentage of D0 and averages of three independent experiments were plotted side by side with those of CRLR mRNA for comparison.

Fig. 11 **Expression of CRLR throughout IDI induced differentiation in 3T3-L1 cells.**

A



B



4. DISCUSSION.

4.1 AM peptide and its effect on IDI induced 3T3-L1 differentiation.

4.1.1 *AM expression is regulated during the differentiation program.*

In agreement with the previously published results [47, 50], we have observed a significant reduction in the level of AM mRNA and of secreted AM on D2 of induction (Fig. 7), suggesting that AM signaling could have a regulatory role in this process. At the mRNA level, this reduction was reversed by day 5 and there was a 2.5 fold increase on D6 and D7 relative to D0 level (Fig. 7A). However, this mRNA increase was not accompanied by a comparable increase in secreted IR-AM (Fig. 7B) or accumulation of intracellular IR-AM (data not shown). The reason for this discrepancy is unclear. Several explanations can be invoked to explain it: (i) the excess AM mRNA may not be translated; (ii) the excess mRNA could be due to the alternate AM gene transcript splicing isoform, which contains intron 3 sequence and translates into a precursor protein carrying the PAMP amino acid sequence but not the AM amino acid sequence due to the interruption of the open reading frame within intron 3 sequence (Fig. 4). This form of regulation of AM secretion has been reported in the literature [62]; (iii) the excess AM produced could be rapidly degraded by various peptidases [54, 120].

There are two reports on AM secretion by 3T3-L1 cells during adipogenic differentiation. Consistent with our results, Li et al [50] reported a 3-fold decrease in secreted IR-AM accumulated over 24 h on D3 and D4. They also observed a corresponding decrease of AM mRNA detectable by Northern blot analysis on D2 to D4 of induction. They

did not examine other time points. Fukai et al [47] examined AM peptide secretion and AM mRNA on D0, D2, D6, and D9 of differentiation. They also observed an initial decrease in the levels of both the pro-AM mRNA and of the secreted peptide on D2. For both the mRNA and the peptide, the reduction was largely reversed by D6 and the level was significantly increased on D9. Although the time points examined in our study extended only to D7, our results indicate that the levels of secreted AM remain unchanged and are not significantly different between time-matched induced and non-induced cells from D3 to D7 (Fig. 7B). A similar phenomenon was observed by Harmanvey et al in the 3T3-L1 sister cell line 3T3-F442A [121]. Both cell lines were derived as subclones of Swiss 3T3 fibroblasts locked into adipogenic lineage and capable of undergoing IDI stimulated adipogenic conversion [11]. These authors data indicate a 3 to 4 day lag time between AM mRNA upregulation and a corresponding increase in the rate of IR-AM secretion. The apparent inconsistency between our results and those reported by Fukai et al [47] remains to be resolved, but could reflect a phenomenon similar to that observed in 3T3-F442A cells.

4.1.2. *Expression of adipogenic markers on D7 is dose-dependently inhibited by stimulation with exogenous AM.*

We have demonstrated that supplementing exogenous AM to differentiating 3T3-L1 preadipocytes throughout the differentiation protocol or only during the D2 of induction significantly reduced mRNA levels of PPARγ2, adipsin, and leptin (Fig.8 A and B), three well-established markers of adipocyte phenotype [9, 10]. Supplementing AM on D2 only, we observed a clear trend of AM concentration-dependence in the reduction of the levels of mRNA for these adipogenic markers in the 0.1 to 100 nM range (Fig. 8B). The reduction

was significant for leptin mRNA from 10 nM AM, and at 100 nM AM for the transcripts of other adipocyte markers.

4.1.3. *Effect of AM receptor antagonist hAM_{22-52}.*

Our data also suggest that the inhibitory effect of AM can be partially antagonized by pretreatment with a specific AM receptor antagonist, human AM_{22-52} peptide. Assuming specific receptor-mediated action of an agonist, an incomplete effect of an antagonist would be consistent with either an insufficient concentration of the antagonist or a presence of more than one species of specific receptors exhibiting different sensitivity to the antagonist (or both). In fact, as was discussed in detail in the introduction (sections 1.3.2.2 and 1.3.2.3), two specific AM receptors with differential affinity for AM and sensitivity to the antagonist used in this study (hAM_{22-52}) have been described [88] and necessary components of both receptors are expressed in 3T3-L1 cells. In terms of antagonist concentration, the general rule of thumb when working with competitive binding mechanism of inhibition is to use a 100 fold or greater concentration of antagonist compared to that of the agonist. Only a 10 fold greater antagonist concentration could be achieved. Therefore, a non-competitive mode of action approach was attempted through stimulating endocytosis of AM receptor [102] by pretreatment with AM peptide fragment (hAM_{22-52}) that binds to the receptor but lacks the amino acid sequence necessary to induce downstream signaling [88]. Whether or not endocytosis was the mechanism, this approach worked at least in rat mesanglial cells where stimulation of adenylate cyclase activity by 100 nM AM was strongly inhibited by 10 min preincubation with 1 μM hAM_{22-52} [89].

4.1.4. *Other relevant comments and observations.*

Early stages in the execution of the differentiation program have been shown to be particularly sensitive to the actions of negative modulators of adipogenesis. Previously, our laboratory has reported that adipogenesis in 3T3-L1 cells could be inhibited by early (D0-D2) treatment with general inhibitors of proprotein convertases (PCs) and that same inhibitor treatment had no effect after D2 [122]. Similarly, application of either general matrix metalloproteinase (MMP) inhibitors or specific MMP-2 inhibitor during D0-1 but not D2-7 could inhibit adipogenesis [123]. Interestingly, AM was shown to be a specific substrate for MMP-2 (but not MMP-9). Six MMP-2 cleavage products of AM were identified: two of them retaining full AM activity, three inactive products, and one having an opposite physiological effect [54]. This suggests that MMP-2 could be an important modulator of AM signaling and indicates a partial mechanism by which inhibition of MMP-2 could translate into inhibition of adipogenesis.

Surprisingly, despite a significant decrease in the adipogenic marker mRNA levels (Fig. 8), little difference in the apparent frequency of differentiated, triglyceride filled cells was apparent upon gross microscopic examination, and measurements of total triglyceride accumulated by D7 did not reveal significant differences between IDI induced AM treated vs. untreated 3T3-L1 adipocytes (Fig. 9). One possible explanation for this observation could stem from the fact that several transcription factors are involved in determining adipocyte phenotype. While expression of PPARγ is absolutely crucial for adipocyte differentiation, [124, 125] expression of C/EBPα is crucial for triglyceride accumulation since retroviral mediated expression of PPARγ in C/EBPα (-/-) mouse-derived fibroblasts still induces differentiation but resulting adipocytes are defective in lipid accumulation [126].

In this study we did not examine C/EBPα or ADD-1/SREBP-1c expression but it is possible these factors may not be sufficiently affected to produce significant, detectable reduction in triglyceride accumulation. Therefore, sensitivity of detection could be one issue. Another issue is inherent 3T3-L1 cell line heterogeneity and possible differences between different batches of these cells used for independent experiments, complicating interpretation of individual experiments and reducing overall significance. For instance, I have also performed two independent triplicate experiments, where rate of triglyceride accumulation from D1 to D7 was compared between AM stimulated and non-stimulated IDI induced cells, with conflicting results: while, based on values obtained from three different plates per treatment per time point, in one experiment significantly less triglycerides ($p< 0.01$, $n=3$) was accumulated in AM treated cells on the last three days; in the second experiment - an almost identical rate of accumulation was observed (data not shown).

4.2 Regulation of AM receptor system during adipogenic differentiation of 3T3-L1 cells.

4.2.1 *The limiting CRLR expression model.*

We have observed that expression of adipogenic markers in differentiated 3T3-L1 adipocytes can be modulated by stimulation with AM and that timing of AM stimulation is crucial in eliciting inhibitory response in differentiating 3T3-L1 cells (Fig. 8). It was interesting to see whether the cellular machinery involved in sensing AM mediated signals is also regulated during this process. The fact that cell surface expression of AM receptors and the specific type of receptor expressed depend on the relative abundance of individual protein

components of CRLR/RAMP complex receptor, offers a new mode of regulation for AM signaling to its target cells. Indeed, a model could be envisioned where limiting expression of the core 7TM domain GPCR protein, CRLR, forces a competition between the three RAMP isoforms for binding to CRLR and subsequent cell surface expression of a particular receptor subtype. Thus binding of RAMP-2 will result in cell surface expression of AM1 receptor, RAMP-3 – AM2 receptor, and RAMP-1 will alter the specificity of the receptor to result in cell surface expression of CGRP specific receptor, with a low affinity for AM. The repertoire of these receptors at the cell surface will depend, at least in part, on the intrinsic affinity of CRLR for the specific RAMP isoform and on the relative abundance of these isoforms in the early secretory compartment.

4.2.2 *Dynamic regulation of AM receptor component expression during IDI induced differentiation in 3T3-L1 cells.*

We therefore investigated the changes in expression of CRLR and the three RAMP isoforms throughout IDI induced differentiation in 3T3-L1 cells. Coincidental with AM exerting most of its effect during the first two days of induction, drastic changes in mRNA expression of all three RAMPs were observed during D1 and D2 of differentiation (Fig. 10). On the other hand, only a modest increase in CRLR expression was observed (Fig. 10A and 11), suggesting that it was the type rather than the abundance of the CRLR/RAMP complexes that was being regulated. Consistent with the importance of AM signaling during D1 and D2, mRNA expression of the CGRP receptor forming RAMP isoform, RAMP-1, was strongly downregulated on these days. Its expression recovered on D3 and was maintained at roughly 6 fold D0 level for the last three days of the experiment (Fig. 10B). Interestingly,

RAMP-2 mRNA was also substantially downregulated on D1 but rebound sharply to 2.5 fold starting levels on D2 (Fig. 10C). In contrast to RAMP-1 and RAMP-2, whose message was strongly downregulated on D1, a dramatic, 300 fold, increase in RAMP-3 mRNA was consistently observed on the first day of induction of differentiation (Fig. 10D). Whether this increase is mediated through transcriptional induction or increased stability of RAMP-3 mRNA (or both) was not investigated in this study. However, there is at least one report of RAMP-3 mRNA upregulation mediated entirely through the latter mechanism. Induction of RAMP-3 mRNA by PDGF stimulation in rat mesanglial cells was shown to be independent of de novo RNA synthesis as it was not at all impaired by either actinomycin D or α-amanitin [89]. In these experiments RAMP-3 mRNA half-life was found to be extended from 66.5 min to 331.6 min upon stimulation with PDGF [89]. An interesting parallel between the above mentioned study and our data is that in both systems, RAMP-3 seems to be upregulated as part of a negative feed back mechanism. In the rat mesanglial cells, PDGF has a proliferative effect while action of AM is anti-proliferative [33]. Stimulation with PDGF induces cell proliferation and at the same time stimulates RAMP-3 expression and a consequent increased sensitivity to PDGF-antagonizing action of AM. Analogously, in 3T3-L1 cells, IDI induced differentiation is accompanied by increased RAMP-3 mRNA abundance and, presumably, increased sensitivity to negative modulation of adipogenic markers by AM in these cells.

Assuming a corresponding increase in RAMP-3 protein synthesis, and taken together with the 90% and 80% reduction of RAMP-1 and RAMP-2 mRNA levels, respectively, it is tempting to hypothesize that high levels of RAMP-3 are being built up to effectively compete

out any ER resident RAMP-1 or RAMP-2 proteins from forming a complex with CRLR and their subsequent translocation to the cell surface.

Unfortunately, our attempts to identify RAMP-3 immunoreactivity using several different approaches and two different commercial antibodies against RAMP-3 did not succeed. High level of crossreactivity and a lack of positive control played a role. However, substantial protein increase corresponding to such a robust rise in mRNA levels was expected to facilitate identification of RAMP-3 specific immunoreactivity. Although several bands that appeared to be regulated with induction at different time points were observed, the apparent molecular weight and the modest amplitude of observed changes did not merit a positive identification of RAMP-3 immunoreactivity. This failure to detect robust increase in an anti-RAMP-3 antibody immunoreactive species could mean that increased RAMP-3 message did not translate into increased RAMP-3 biosynthesis. Alternatively, effective RAMP-3 antigen recognition by the antibodies used in these experiments could be dependent on the golgi-mediated terminal glycosylation state of RAMP-3 protein. Taking into account the limiting CRLR expression model and seeing how little change in CRLR mRNA and protein expression could be observed, a comparable, modest increase in RAMP-3 being trafficked through the golgi to the cell surface could be expected. In this case, the excess RAMP-3 accumulated in the ER and possibly early golgi would not be detected by these antibodies.

Compared to D1, the abundance of RAMP-3 mRNA drops rapidly on D2 but still remains high, 16 fold greater than on D0 (Fig. 10D). At this point, RAMP-1 expression is still suppressed (Fig. 10B). In contrast to RAMP-1, after being suppressed on D1, RAMP-2

message is sharply upregulated on D2 (Fig. 10C), suggesting that in addition to AM2 type receptor (CRLR/RAMP-3), AM1 receptors (CRLR/RAMP-2) are now being phased in.

Taken together, these data suggest that AM signaling may be differentially regulated through AM1 and AM2 receptor signaling throughout the differentiation: the latter being preferentially expressed at the cell surface during the initial stages of differentiation, whereas mixed expression of both AM1 and AM2 receptors could be expected during the later stages as differentiation progresses.

4.2.3 *Suggested special role for RAMP-3 containing AM receptor subtype.*

The final composition of cell surface CRLR/RAMP complex-type receptors is likely determined by at least two intracellular processes whose rates can be regulated independently of each other. These are the rate of receptor biosynthesis and trafficking to the cell surface, as well as the rate of receptor internalization from the cell surface. The third determinant common for many GPCR receptors, and playing a major role in cell surface expression of β2AR for example, is the rate of endosomal sorting and recycling of the endocytosed receptors back to the cell surface [127]. Both CGRP and AM1 receptors were shown to be targeted for lysosomal degradation following agonist induced endocytosis [102, 108], and, therefore, their cell surface expression is likely to be determined primarily by the rates of biosynthesis and internalization. On the other hand, unique adapter protein binding features of RAMP-3 [116, 117], open a possibility of an added level of regulation for the AM2 receptor through endosomal recycling [116] or through retardation of AM2 receptor endocytosis by tethering the receptor to actin cytoskeletal elements at the cell surface [117],

depending on the endogenous expression of NSF and NHERF-1 (and possibly other factors) in 3T3-L1 cells.

5. Summary statement of the significance of conducted research.

In summary, my studies demonstrate, for the first time, that reduction of AM levels during adipogenic induction in 3T3-L1 cells contributes to normal expression of mRNA for adipocyte markers PPARγ2, Leptin, and, to a lesser extent, Adipsin during differentiation. Surprisingly, AM treatment did not appear to effect triglyceride accumulation by the mature adipocytes. The effect of AM was shown to be dose dependent and be mediated through specific AM receptors.

I also provide, for the first time, a comprehensive description of the regulation of AM receptor components CRLR (mRNA and protein), RAMP-2 and RAMP-3, as well as, CGRP receptor forming, RAMP-1 (at mRNA level) during the process of IDI induced differentiation in 3T3-L1 cells. In particular, dramatic upregulation of RAMP-3 mRNA was observed in response to IDI stimulation, suggesting a special role for RAMP-3 containing AM receptor subtype in mediating AM signals during this process.

The observed mRNA changes point to the likely involvement of these proteins in either differentiation process or subsequent functioning of mature adipocytes. The precise role and mechanism of their action, however remains to be further investigated. Specifically, regulation of expression at the protein level and protein expression knockdown experiments would seem a logical next step toward understanding the role and mechanism of AM signaling in adipocyte differentiation and function.

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